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A Model of the Platelet Factor 4 Complex With Heparin

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ABSTRACT **A model of heparin bound to bovine platelet factor 4 (BPF4) was completed using a graphically designed heparin molecule and the crystallographic coordinates of the native bovine platelet factor 4 tetramer. The oligosaccharides had a chain length of at least eight disaccharide units with the major repeating disaccharide unit consisting of (1→4)-O-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2-deoxy-2-sulfamino-2-D-glucopyranosyl 6-sulfate). Each disaccharide unit carried a −4.0 charge. The structure of BPF4 was solved to 2.6 Å resolution with R = 0.237. Each monomer of BPF4 contains an α-helix lying across 3 strands of antiparallel β-sheet. Each helix has four lysines, which have been implicated in heparin binding. These lysine residues are predominantly on one side of the helix and are solvent accessible. Electrostatic calculations performed on the BPF4 tetramer show a ring of strong, positive charge which runs perpendicularly across the helices. Included in this ring of density is His-38, which has been shown by NMR to have a large pK_a shift when heparin binds to BPF4. Our model of heparin bound to PF4 has the anionic polysaccharide perpendicular to the α-helices, wrapped about the tetramer along the ring of positive charge, and salt linked to all four lysines on the helix of each monomer.**

Key words: α-helix, lysine residues, disaccharide units

INTRODUCTION

Platelet factor 4, which is released from the α-granules of activated platelets during coagulation, binds and neutralizes heparin,[1,2] a heavily sulfated polysaccharide that catalyzes the inhibition of Factor Xa and thrombin by antithrombin III.[3] This paper describes a model of the complex between platelet factor 4 and heparin. We have used a refined, three-dimensional structure of bovine platelet factor 4 derived from the structure reported by St. Charles et al.,[4] and a model of heparin constructed from X-ray scattering data[5] and chemical analyses.[6]

Platelet factor 4 is a tetramer in solution.[7] The bovine protein contains 88 amino acids in each monomer (M_r = 9,505) and is 73% homologous with the human protein.[8] In the α-granules, platelet factor 4 is bound to a proteoglycan carrier that contains chondroitin-4-sulfate.[7,9] Since platelet factor 4 binds to glycosaminoglycans roughly in order of their negative charge density, that is, heparin >> heparan sulfate > dermatan sulfate > chondroitin sulfates > hyaluronic acid,[10] the tetramer transfers easily to more sulfated polysaccharides such as heparin when the carrier complex is released from the platelets.

Platelet factor 4, which is a member of a superfamily of cytokines,[11] has other physiological effects besides neutralization of heparin. PF4 is chemotactic for white blood cells and may be one of the signals that initiates an inflammatory response.[12] It inhibits megakaryocytopoiesis,[13,14] angiogenesis,[15] solid tumor growth,[16] and the migration of vascular endothelial cells.[17] It also reverses concanavalin-induced immunosuppression.[18]

The structure and activity relationships of heparin have been reviewed by Casu.[6] Heparin is a heterogeneous glycosaminoglycan that is comprised mostly of L-iduronic acid and 2-amino-2-deoxy-D-glucose disaccharide units. The heparin chains, which range in molecular weight from 3,000 to 35,000, are very acidic, due to three sulfate groups and one carboxyl group on each disaccharide unit. Platelet factor 4 binds nonspecifically to heparin,[19] unlike antithrombin III, which requires a specific pentasaccharide site for maximum activation.[20,21] However, the strength and stoichiometry of the binding of platelet factor 4 to heparin does vary with the length of the polysaccharide chain. Chains that are 8 sugars long bind very weakly,[22,23] chains that are 10, 12, and 14 sugars long bind increasingly tighter,[24] and chains that are 16 to 18 sugar units long bind almost as well as unfractionated heparin.[22–24]

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Abbreviations used: PF4, platelet factor 4; BPF4, bovine platelet factor 4; HPF4, human platelet factor 4; RPF4, rat platelet factor 4; PF4var1, a human gene variant of platelet factor 4; PBP, human platelet basic protein; HGRO, human gro gene product; CGRO, Chinese hamster gro gene product; IL-8, interleukin 8.

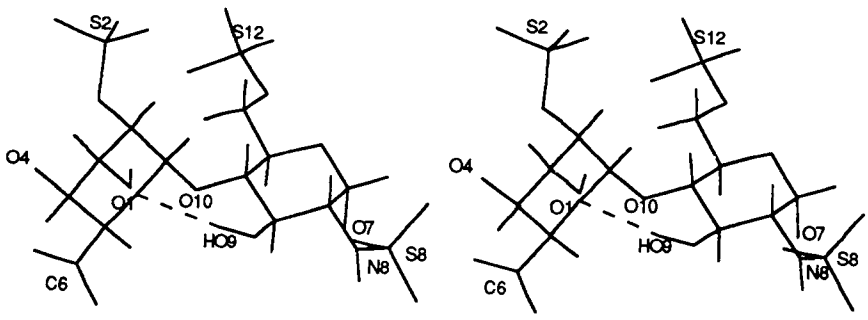

Fig. 1. Stereo diagram of the major repeating unit of heparin (1→4)-O-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-(2-deoxy-2-sulfamino-α-D-glucopyransyl 6-sulfate). The length of the disaccharide unit is 0.87 nm. Oxygen atoms labeled O1 through O6 belong to the idopyranosyluronic acid while those labeled O7 through O12 belong to the hexosamine. The dotted line depicts a hydrogen bond from a hydroxyl group on the hexosamine monomer to the ring oxygen of the iduronic acid monomer, which may provide stability to the 2-fold helical conformation of the heparin chain.

When heparin chains containing 16 sugars are in excess, platelet factor 4 binds two chains. When the heparin chain is approximately twice as long and in excess, then platelet factor 4 binds only one chain.[22] In other words, the tetramer of platelet factor 4 behaves as though it has two binding sites for heparin. A hexadecasaccharide can fill one heparin binding site while a heparin chain with 34 sugar units can wrap around the platelet factor 4 tetramer and fill both sites. In the latter case, free heparin competes poorly with heparin that is already bound to the first site because the free oligosaccharides lose more entropy when binding to the open, second site.[22]

Chemical modification has identified the four lysine residues near the carboxyl-terminus of platelet factor 4 as essential components of the heparin binding site. Guanidation of these lysines[10] on human PF4 or their removal by carboxypeptidase-Y[25] abolishes heparin binding. Conversely, modification of the arginine residues in human platelet factor 4 with 1,2-cyclohexanedione had no effect on the binding of heparin.[10] The four lysines, which are in the sequence -X-K-K-X-X-K-K-X-, match one of the two patterns common to heparin-binding sites in other proteins.[26] When arranged in an α-helix, which is the predicted conformation for the carboxyl-terminal residues of human platelet factor 4, this pattern places the lysine residues on one side of the helix and the hydrophobic residues (X) on the other side.[27]

The crystal structure of bovine platelet factor 4 showed that the four essential lysines of each monomer were arranged in the predicted helix.[4] Each helix lies across three strands of antiparallel β-sheet with its hydrophobic side against the sheet and its basic side facing outward. Moreover, the four monomers are arranged in two pairs, which St. Charles et al.[4] have named the AB and CD dimers. Each dimer has an extended antiparallel β-sheet of six strands and a helix bundle, in which, the two α-helices run antiparallel to one another with a separation of approximately 10 Å between their backbone atoms.

Previously, Cowan and colleagues[28] used the 222 symmetry of the human PF4 tetramer and the observation that heparin wrapped around the outside of the tetramer[29] to construct an approximate model of the PF4 complex with heparin in which the PF4 monomers were represented as 32 Å spheres. In this paper we have used the actual crystal structure of bovine platelet factor 4 to analyze the possible interactions between the tetramer and heparin molecules of various lengths.

MATERIALS AND METHODS

Construction of the Heparin Model

CHEMNOTE (Polygen Corporation) was used to build the disaccharide unit of heparin (Fig. 1). The conformation of the monomers was designed to coincide with the structural information obtained from the fiber diffraction pattern of heparin.[5,30] The α-L-uronic acid monomer has a ring conformation of $^1C_4$ with an equatorial carboxyl group at position 5, an axial sulfate group at position 2, and axial glycosidic linkages at positions 1 and 4.[30] The hexosamine monomer has a $^4C_1$ ring conformation with all large substituents equatorially directed. The ring structures were derived from the β-D-glucuronic acid and β-D-glucose residues currently defined in the polysaccharide library of CHARMM21. They were modified to α-L-iduronic acid and α-D-glucose, respectively, and linked together with an α(1→4) bond in QUANTA (Polygen Corporation). The sulfate and sulfamino groups were added to the rings in CHEMNOTE to complete the heparin disaccharide unit. The partial charge distribution of the atoms in the disaccharide unit was calculated using the GAST-HUCK algorithm in SYBYL (Tripos Corporation). The net charge on the disaccharide unit is −4.0. The combined parameters were used in CHARMM21 to create and energy minimize a hexadecasaccharide in which the disaccharide units were arranged in a 2-fold helix. The average length of a tetrasaccharide in the heparin chain is 1.67 nm, which is consistent with the layer line

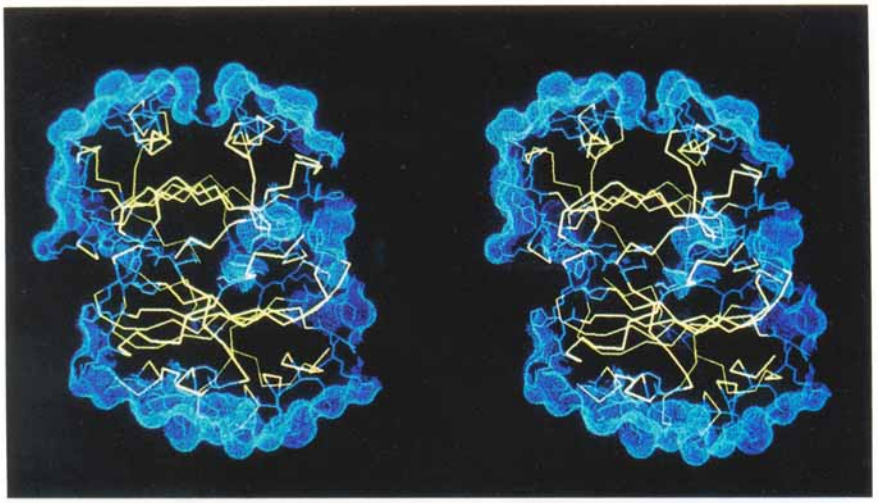

Fig. 2. Stereo view of the BPF4 tetramer (yellow) with the calculated positive electrostatic potentials (blue). The side chains of the basic residues, in blue, have been superimposed on the Cα-tracing. The positive density is shown to lie equatorially about the tetramer, perpendicular to both of the helix bundles on BPF4.

spacings (1.65–1.73 nm) of the sodium salts of heparin.[5]

Construction of the PF4 Model

The three-dimensional structure of native bovine PF4 that was used to model the complex with heparin was determined to 2.6 Å from data collected on a Siemens X100 X-ray area detector. The native PF4 structure, which is very similar to that of nickel-labeled PF4,[4] contains only residues 24 to 85. Residues 1–13 were removed by proteolysis to induce crystallization, and residues 14–23 and 86–88 are disordered in the electron density maps. The actual bovine PF4 structure used for these calculations had been refined to an R factor of 0.237 with an rms deviation of the bond lengths of 0.027 and no solvent atoms. Subsequently, the structure of native bovine PF4 has been refined to an R factor of 0.195 with a 0.025 rms deviation of bond lengths and 171 water molecules. The details of the solution and refinement will be published elsewhere. There were no significant changes in the overall structure or in the heparin binding residues of the solvated structure relative to the structure that was used in modeling. Electrostatic calculations were completed on the tetramer in QUANTA using a 1.8 Å probe (Fig. 2). Solvent-accessible surface areas were calculated in QUANTA with a probe radius of 1.4 Å.

Docking the Heparin and PF4 Models

The oligosaccharides were docked to the BPF4 tetramer with only the torsion angles of the glycosidic linkages being changed to maximize charge interactions. A single starting configuration was used for the individual ring groups and torsion angles of the oligosaccharides. The heparin chains were docked with their ring units sideways to BPF4, to reduce the amount of torsion strain produced when the molecule was wrapped about the BPF4 structure. The starting configuration of the sugar rings for the octatridecasaccharide at the AB helix bundle was identical to that of the hexadecasaccharide but it was 180° out of phase at the CD dimer because the two sites are separated by an odd number of 2-fold related disaccharide units. At the AB site, the interactions after minimization are essentially identical with those present in the hexadecasaccharide model; at the CD site, the 12, symmetry-equivalent PBF4 residues all form salt links, but to heparin acidic groups that are on the sides of the sugar rings opposite to those seen in the AB site. We also tried a model of the hexadecasaccharide complex in which the ring units of heparin were flat with respect to the protein surface. It generated severe atom contacts, major bond angle distortions, and fewer electrostatic interactions and was therefore not pursued further.

Keeping only the protein backbone constrained, the modeled complexes were then energy minimized by steepest descent and conjugate gradient methods in CHARMM21. Calculations were done with and without a 8.0 Å limit on nonbonded interactions. Glackin et al.[31] have shown that electrostatic interactions can occur between atoms up to 20 Å apart. Both approaches displaced the protein side chains by equal amounts. The rms difference was 0.02 Å between the two methods for the side chains in the minimized protein structures. The rms difference between the two minimized heparin structures was 0.20 Å. The calculations utilizing a nonbonded interaction limit of 20 Å allowed for more charge interactions between the heparin chain and the protein structure, and moved heparin farther away from the starting model, therefore the minimized

TABLE I. Heparin-PF4 Interactions for a Hexadecasaccharide Bound in the Parallel Orientation to the Helix Bundle of the AB Dimer

Heparin*		PF4 dimer		Distance
Residue	Atom	Residue	Atom	(Å)
3	O8C†	Lys-76	NZ	2.58
4	O12D‡	Lys-180	NZ	2.51
5	O8B	Lys-80	NZ	2.41
6	O2D	Lys-176	NZ	2.63
6	O12D	Lys-176	NZ	2.50

*The heparin residues (disaccharide units) start with the first heparin oxygen atom at the top of Figure 3 (middle). Oxygen atoms labeled O1 through O6 belong to the iduronic acid unit in a heparin residue while those labeled O7 through O12 belong to the hexosamine unit.
†The sulfate group oxygens are listed as A, B, C, and D.
‡The sulfylamine group oxygens are listed as A, B, and C.

complexes discussed in this paper were calculated using this approach.

The dielectric constant of the protein was also varied to check its effect on the model. Although the distance between oppositely charged groups lengthened as the dielectric constant was increased from 2 to 10, the BPF4 residues involved in the salt links did not change in the calculations. In the final model, we used a distance dependent dielectric constant ranging from 2 at the protein surface to 80 at the nonbonded limit of 20 Å.

RESULTS AND DISCUSSION

Loscalzo et al.[25] showed that the four lysine residues near the carboxyl terminus of human PF4 are an essential part of the heparin binding site. In the crystal structure of bovine PF4 labeled with a Ni $(CN)_4^{2-}$ group,[4] these residues are part of the single, carboxyl-terminal α-helix present in each subunit of the BPF4 tetramer. In the tetramer, the four helices form two helix bundles on opposite sides of the oligomer, in which helices from adjacent subunits run antiparallel to one another (Fig. 3, top).

The parallel arrangement, which puts the heparin polymer between the two helices of the bundle, is aesthetically pleasing but is shown here to be unlikely. As evident in Figure 3 (top), the heparin molecule, which has a strong negative charge, must pass through or near the negatively charged poles of the BPF4 tetramer, that is, within 4 Å of Asp-69 and Asp-84. A heparin hexadecasaccharide, in the parallel orientation, when bound symmetrically about one helix bundle, interacts with only four basic residues on the two subunits that form the helix bundle (Fig. 3, middle; Table I). As a consequence, the heparin chain in the energy minimized, parallel model is loosely bound, with only 185 $Å^2$ of the solvent accessible area of the free components being buried in the complex between one helix bundle and a symmetrically arranged hexadecasaccharide. Also, when heparin is bound to BPF4 in the parallel arrangement, an oligosaccharide chain of 18 or more monosaccharides is needed to bridge the gap between both sets of helices and form at least one electrostatic interaction per helix bundle (figure not shown); a hexadecasaccharide cannot interact with both helix bundles simultaneously.

In contrast, the heparin hexadecasaccharide in the symmetric, perpendicular orientation can interact with 12 basic residues on the two subunits that form one helix bundle, namely, eight bonds to the lysines in the two helices and four bonds to His-38, Arg-37, and their symmetry mates (Fig. 3, bottom; Fig. 4, top; Table II). These latter four residues lie in the belt of positive charge that runs around the BPF4 tetramer (Fig. 2). Moreover, in the perpendicular orientation, the heparin polymer avoids the acidic groups on BPF4 (Fig. 3, bottom). Consequently, the heparin chain in the energy minimized, perpendicular complex is more tightly bound than in the parallel model, as evidenced by the fact that 760 $Å^2$ of the solvent accessible area of the free components is buried in the perpendicular complex between one helix bundle and a symmetrically arranged hexadecasaccharide.

To be useful, a model must rationalize prior data and predict new experiments. The perpendicular model of the heparin:BPF4 complex explains why 16 or more sugar units are needed to achieve strong binding to BPF4, as opposed to the case of antithrombin III, for instance, where only a pentasaccharide is needed.[20,21] If the polysaccharide is centered on the helix bundle of the AB dimer, then Figure 4 (top) shows that 16 sugar units are needed to interact with all 12 positive charges on the dimer. If the polysaccharide, in the perpendicular orientation, bridges the gap between helices of the BC dimer, then 16 sugar units can interact with four lysines on one bundle and two lysines on the other bundle (Fig. 4, bottom; Table III). The next smaller heparin chain, which contains 14 sugar units, binds a maximum of two lysines on each helix bundle (Fig. 4 bottom), while lengths of 20 or 34 sugar units allow interaction with 4 or all 8 lysines, respectively, on each helix bundle (Fig. 5). A total of 38 sugar units are needed to wrap heparin completely around platelet factor 4 so that it may interact with all four His-38 residues.

A large number of charge interactions are needed to bind heparin to PF4 because it is on the exposed surface, as opposed to binding in a cleft. In our model the shorter oligosaccharides have more salt links with the AB dimer (Fig. 4, top) than with the BC dimer (Fig. 4, bottom) and are more loosely bound to the latter. An octadecasaccharide was needed for Fig. 4 (bottom) and Table III to ensure that at least 16 sugar units would form salt links with the protein because the sugar group on both ends of the oligosaccharide were too mobile to interact with pro-

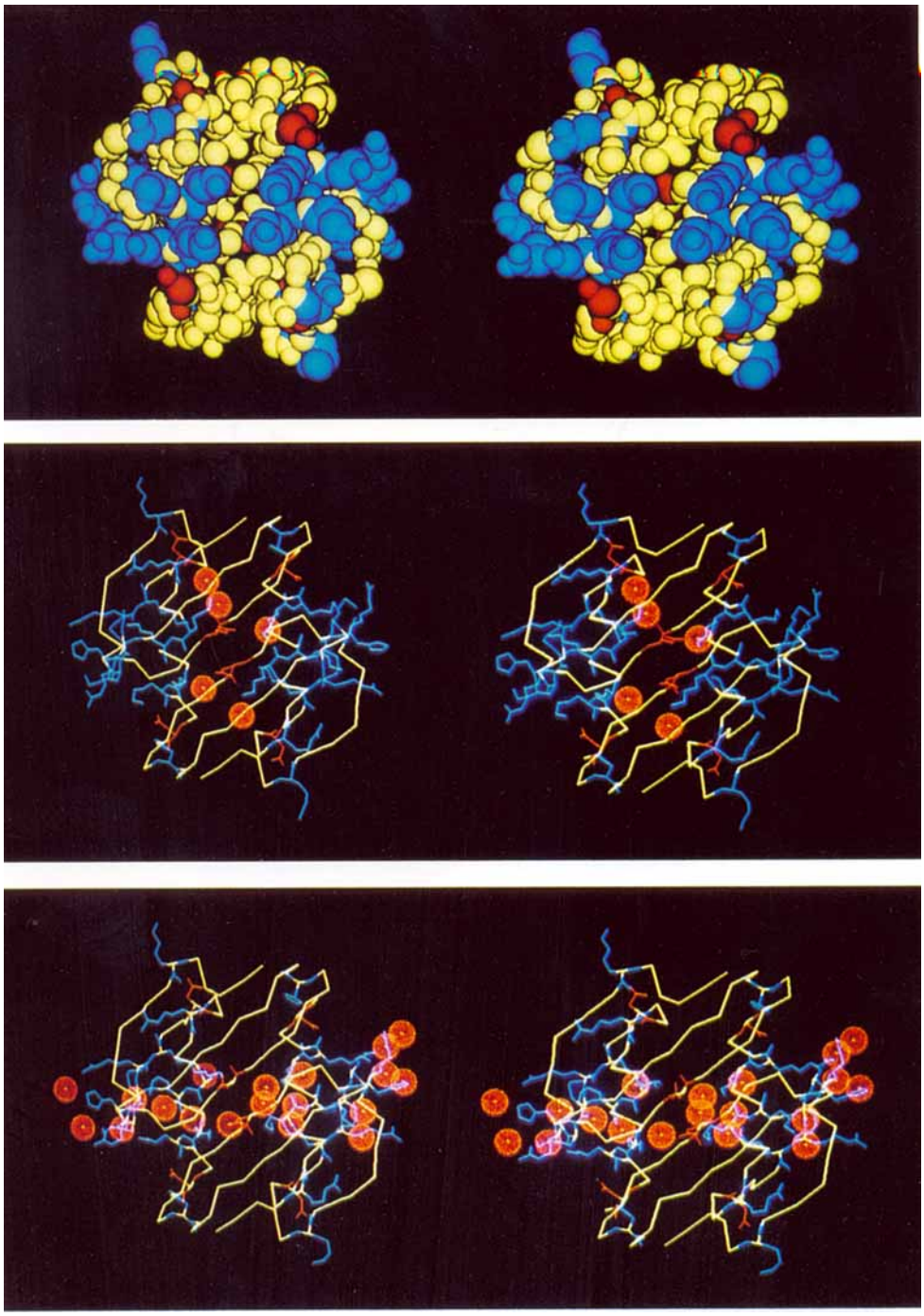

Fig. 3. Stereo views of a single heparin-binding site. The BPF4 dimer in each picture is rotated approximately 90° from Figure 2, so that the view is perpendicular to the β-sheet and helix bundle. **Top:** The distribution of charged residues on the AB dimer of BPF4 is shown as a space filling model. The protein backbone is shown in yellow, with the side chains of the basic residues shown in blue, and the acidic side chains shown in red. There are 13 basic residues and 3 acidic residues shown on each monomer. Each helix has one acidic residue at its amino-terminus and three acidic residues at its carboxyl-terminus of which only one is shown. The other two residues are disordered in the crystal. **Middle:** A hexadecasaccharide of heparin bound in the parallel orientation, symmetrically about one helix bundle. The oxygen atoms of heparin, which interact with the basic residues on BPF4, are shown as red van der Waals spheres. The five possible interactions between heparin and BPF4 shown above are listed in Table I. **Bottom:** A hexadecasaccharide of heparin bound symmetrically about the dimer in the perpendicular orientation. The oxygen atoms of heparin, which interact with the basic residues on BPF4, are depicted as red van der Waals spheres. The 19 possible interactions are listed in Table II.

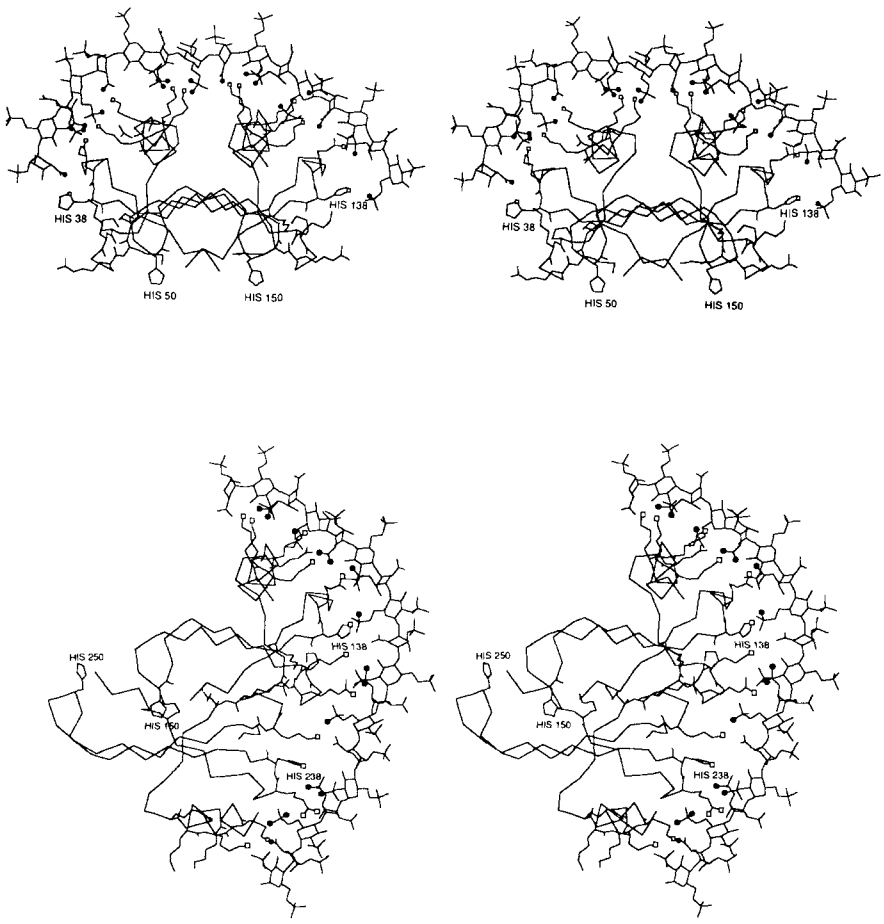

Fig. 4. Stereo view of the AB and BC dimers of BPF4 complexed with heparin. **Top:** A hexadecasaccharide of heparin bound symmetrically and perpendicularly across the helix bundle of the AB dimer is shown in stereo. This drawing is in the same orientation as the view in Figure 2. The heparin chain stretches from His-38 on monomer A to its symmetry mate on monomer B, interacting with all eight lysines on the helix bundle. The 19 possible interactions are listed in Table II. Each of the 12 basic residues on the dimer that contribute to the ring of positive electrostatic charge in Figure 2 has one or more charged groups from heparin within 3.0 Å. **Bottom:** An octadecasaccharide of heparin bound to the BC dimer of BPF4 is shown in stereo. The heparin chain, which starts with residue 1 at the top, follows the ring of positive density about the tetramer to form interactions with both helix bundles. Only 16 of the 18 sugar units interact with BPF4 residues (Table III). The first sugar ring in heparin residue 1 and the second sugar ring in heparin residue 9 are loosely bound and do not interact with the protein.

tein residues. This mobility of the end groups was not seen in calculations with the AB dimer (Fig. 4, top; Table II).

The perpendicular model also agrees with the observations of Bock et al.[22] that platelet factor 4 binds two hexadecamers of heparin when the heparin fragments are in excess but only one molecule of larger heparins even at very high concentrations relative to the platelet factor 4. A heparin molecule of 16 saccharide units can bind one helix bundle without interfering with the other site; larger heparin molecules wrap around platelet factor 4 and occupy both sites. Free heparin molecules cannot compete for the second site because they have a greater loss of entropy.[22]

The perpendicular model also predicts that HIS-38, which is approximately 3.0 Å from the nearest heparin sulfate group, will be perturbed more by heparin binding than His-50, which is greater than 10.0 Å away. Lee and co-workers have assigned the NMR resonances of the two histidines and measured their pK values in the presence and absence of heparin.[32] The change in the pK of His-38 was an order of magnitude larger than that of His-50. This observation further discounts the parallel orientation for heparin, which places heparin more than 14 Å from both histidine residues.

Our models predict that mutation of Arg-37, His-38, Lys-61, Lys-77, and Lys-81 in BPF4 will preferentially perturb heparin binding in the perpendicular orientation while mutation of Lys-29 will preferentially affect binding in the parallel orientation. Residues Lys-76 and Lys-80 interact with heparin in both models. The only other BPF4 residues

TABLE II. Heparin-PF4 Interactions for a Hexadecasaccharide Bound in the Perpendicular Orientation to the Helix Bundle of the AB Dimer

Heparin*		PF4 dimer		Distance
Residue	Atom	Residue	Atom	(Å)
1	O6A†	His-38	ND1	2.77
1	O8B‡	Arg-37	NH1	2.56
1	O8C	Lys-81	NZ	2.58
2	O2D§	Lys-81	NZ	2.52
2	O12B	Lys-77	NZ	2.59
3	O6B	Lys-77	NZ	2.48
3	O8C	Lys-80	NZ	2.61
4	O2D	Lys-80	NZ	2.60
4	O2C	Lys-76	NZ	2.61
4	O12C	Lys-80	NZ	2.62
4	O12D	Lys-76	NZ	2.59
5	O6A	Lys-180	NZ	2.60
5	O8B	Lys-176	NZ	2.60
6	O2D	Lys-180	NZ	2.54
6	O12C	Lys-177	NZ	2.56
7	O2B	Lys-177	NZ	2.58
7	O6B	Lys-181	NZ	2.49
8	O2D	Arg-137	NH2	2.72
8	O12C	His-138	NE2	2.95

*The heparin residues (disaccharide units) start with the first heparin residue on the left-hand side of Figure 4 (top). Oxygen atoms labeled O1 through O6 belong to the iduronic acid unit in a heparin residue while those labeled O7 through O12 belong to the hexosamine unit.
†The carboxyl oxygens are listed as A and B.
‡The sulfylamine group oxygens are listed as A, B, and C.
§The sulfate group oxygens are listed as A, B, C, and D.

TABLE III. Heparin-PF4 Interactions for an Octadecasaccharide Bound in the Perpendicular Orientation to the Two Separate Helices of the BC Dimer

Heparin*		PF4 dimer		Distance
Residue	Atom	Residue	Atom	(Å)
1	O8B†	Lys-176	NZ	2.56
2	O2D‡	Lys-180	NZ	2.56
2	O12C	Lys-177	NZ	2.58
3	O6B§	Lys-181	NZ	2.43
3	O6A	Arg-137	NH2	2.72
4	O2B	Arg-137	NH2	2.69
4	O12C	His-138	NE2	2.91
5	O8A	Arg-164	NH2	2.58
6	O2B	Lys-161	NZ	3.63
6	O12B	Lys-261	NZ	2.49
7	O8C	Arg-237	NH1	2.57
8	O2D	His-238	NE2	3.07
8	O12C	Arg-237	NH2	2.64
8	O12B	Lys-281	NZ	2.53
9	O6A	Lys-281	NZ	2.55
9	O6A	Lys-277	NZ	3.34

*The heparin residues (disaccharide units) start with the first heparin residue at the top of Figure 4 (bottom). Oxygen atoms labeled O1 through O6 belong to the iduronic acid unit in a heparin residue while those labeled O7 through O12 belong to the hexosamine unit.
†The sulfylamine group oxygens are listed as A, B, and C.
‡The sulfate group oxygens are listed as A, B, C, and D.
§The carboxyl oxygens are listed as A and B.

that interact with specific heparin atoms are Asn-35 in the perpendicular model and Gln-70 in the parallel model. Both amide groups are within hydrogen bonding distance of a sulfate oxygen.

Most of the basic residues that interact with heparin in the perpendicular model are conserved in other members of the PF4 superfamily that are known to bind heparin (Fig. 6), namely human PF4, platelet basic protein, IL-8, and HGRO. Moreover, the strength of the interaction generally correlates with the degree of conservation of the basic residues outlined in Figure 6. The strongest binding proteins such as bovine and human PF4 require over 1 M NaCl to elute from heparin affinity columns. The N35R replacement accounts for the slightly stronger binding of the human protein (L. Lee, personal communication). Human and rat PF4 have conserved the other basic residues that interact with heparin with the exception of two conservative replacements in the latter (R37K and H38R). Human PF4var1, which binds less tightly to heparin than human PF4 (P. Johnson, personal communication), has a mutation at position 81 (K81E), which interacts with heparin only in the perpendicular orientation. Platelet basic protein, which elutes from a heparin-sepharose column with 0.6–0.7 M NaCl,[45] has lost His-38 (H38N), which interacts with heparin only in the perpendicular model and Lys-80 (K80Q), which is common to both models. The new basic residue in the carboxyl-terminal helix (L82K) is unable to interact with heparin in our model. IL-8 elutes from heparin columns at approximately 0.5 M NaCl.[46] The lower affinity is most likely due to the mutations K61S, which binds heparin in the perpendicular model only, and K76Q and K80E, which function in both models. HGRO or human melanoma growth stimulatory activity has been found to bind to a heparin-sepharose column and elute with 0.3 M NaCl.[47] Again, the reduced heparin affinity is probably due to the mutations H38N and K80E.

No information is presently available on the heparin-binding properties of CGRO, KC3, 9E3, or IP-10. They have all retained some of the basic residues that interact with heparin in our model and probably bind it but less tightly than does BPF4. CGRO has the mutations K76Q and K80Q although K85 may be able to bind heparin. KC3, which may be the murine equivalent of HGRO, may have the same heparin binding capabilities as CGRO due to the conservation of the basic residues in the carboxyl-terminus. Even though 9E-3 has lost three of the four lysines in the carboxyl-terminus, K76Q, K77L, and K81A, it may still bind heparin but with a much lower affinity than BPF4 due to the mutation G85K. IP-10 would bind heparin with lower affinity than

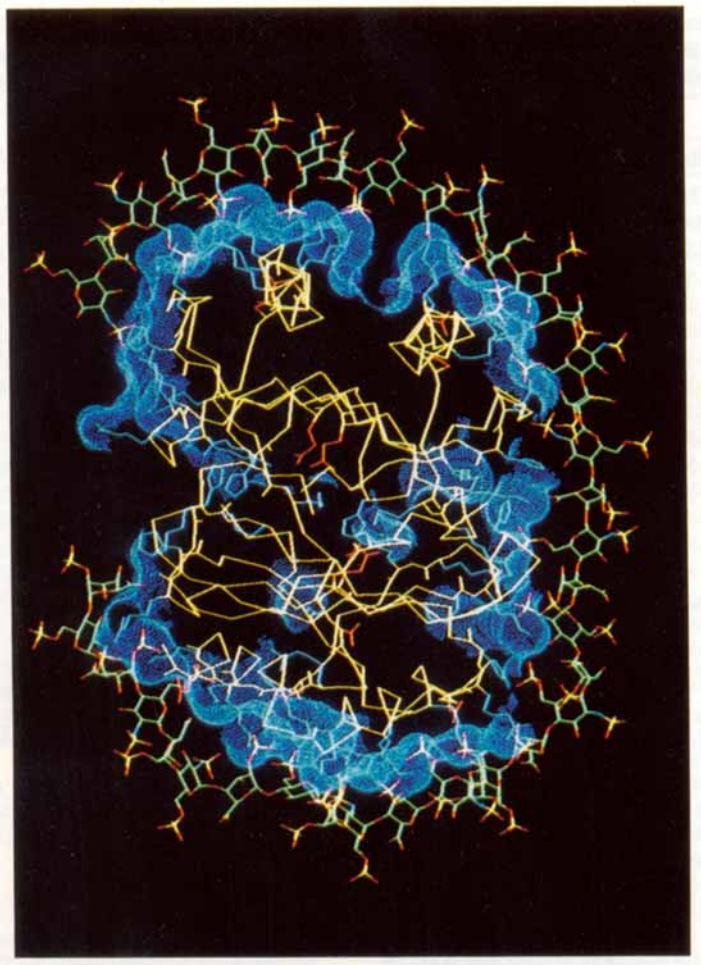

Fig. 5. Heparin bound to the PF4 tetramer. A 38 monosaccharide chain of heparin has been modeled equatorially about the PF4 tetramer following the calculated positive electrostatic density of PF4. The oligosaccharide binds perpendicular to all 4 helices of PF4. For clarity, only the side chains of the charged residues have been superimposed on the Cα tracing of PF4. Basic residues are shown in blue, acidic residues are shown in red, Cα atoms are shown in yellow, and the electrostatic density is shown in blue. The heparin atoms are colored green for carbon, yellow for sulfur, blue for nitrogen, and red for oxygen.

PF4 with the following residues at the COOH-terminus interacting with heparin: Lys-72 and Lys-76, or Lys-76 and Lys-80, or Lys-80 and Lys-84.

Interleukin-8 is the only other member of the PF4 superfamily whose three-dimensional structure is known.[48] Upon review of the X-ray structure of IL-8, His-35 appears to be in the path of heparin binding, which opens up the possibility for His-35 binding heparin in PBP, CGRO, HGRO, KC3, and 9-E3. The α-helices are arranged in a similar fashion to those in PF4 but in contrast to PF4 they contain a negative charge (E80) midway along their length. Inspection of our model shows that Arg-77 and Lys-81 in IL-8 could interact with heparin in the same way that Lys-77 and Lys-81 do in BPF4. Also, in our model, it appears that Lys-84 is in the proper position to form an interaction with heparin. If Glu-80 perturbs this arrangement, heparin could shift along the helix and interact with Lys-81, Lys-84, and Arg-85 on one helix and Arg-77 on the other helix instead. In the first case, IL-8 would have the same number of charge interactions with heparin as platelet basic protein.

The presence of the glutamate residues positioned between the two helices of the IL-8 dimer lends further support to the perpendicular model. The glutamate residues are smaller than the surrounding lysines and arginines, therefore the heparin chain can remain farther away from them and still form ~10 electrostatic interactions with IL-8. In the parallel model, heparin can form only up to 6 very weak interactions with BPF4, due to the presence of Glu-80, Glu-72, and Asp-69.

Variations among the PF4 homologs in the strength of heparin binding are also probably due to differences in their oligomeric state. Mayo[49] has shown by NMR that low-affinity PF4 (connective tissue activating protein-III) is primarily monomeric above 0.2 M NaCl at neutral pH. Under these conditions low affinity PF4 binds about one-third as

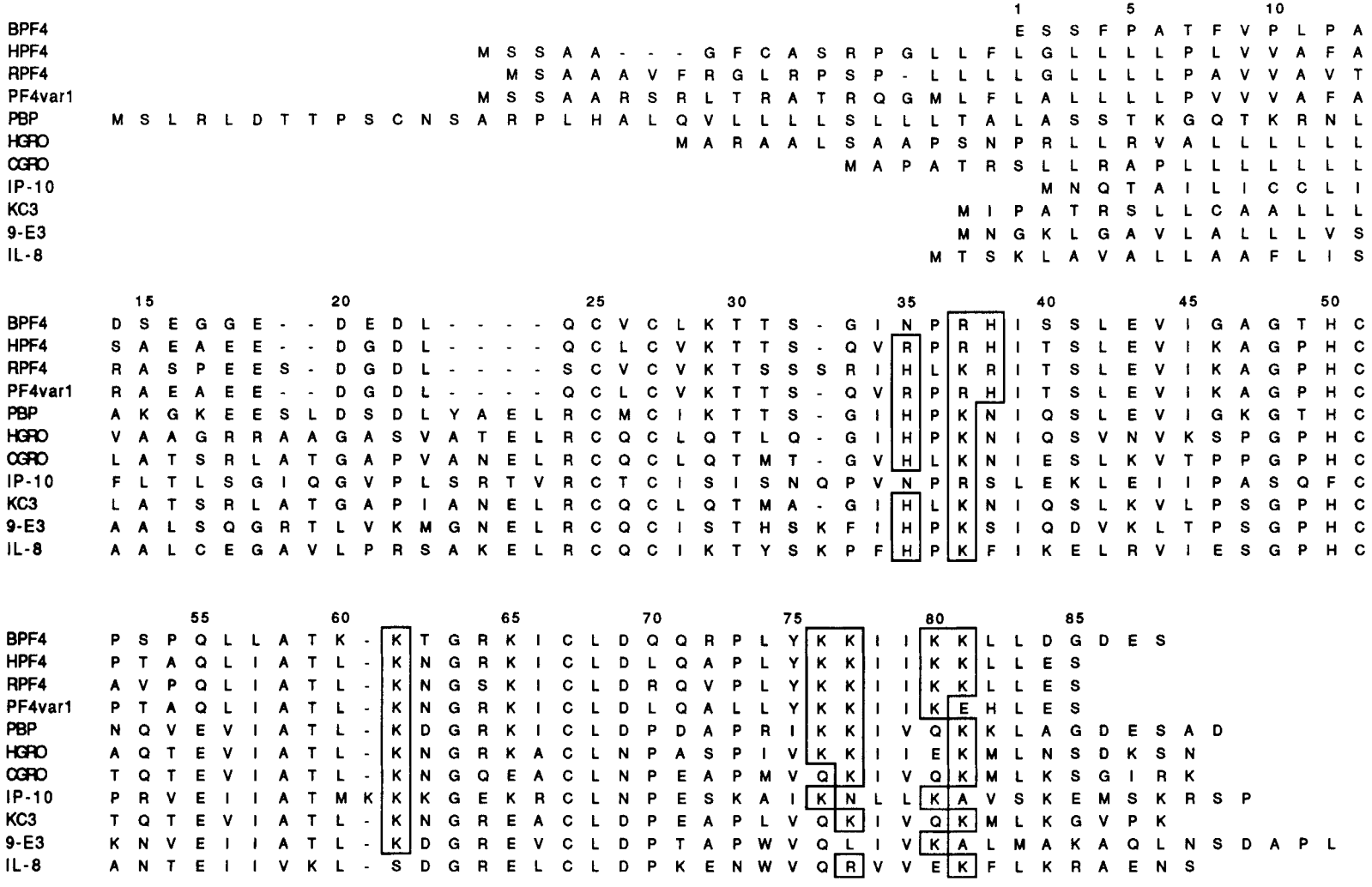

Fig. 6. Proteins in the PF4 superfamily that show homology to the heparin-binding regions of PF4. The sequences have been aligned to show the conservation of the four cysteine residues and maximum homology with possible heparin binding residues on PF4. The seven residues of PF4, that interact with heparin in our models, have been boxed. The proteins are identified as follows: PF4, bovine platelet factor 4;[8] HPF4, human platelet factor 4;[33] RPF4, rat platelet factor 4;[34] PF4var1, a human gene variant of platelet factor 4;[35] PBP, human platelet basic protein;[36] HGRO, human gro gene product;[37,38] CGRO, Chinese hamster gro gene product;[38] IP-10, a gamma interferon induced gene product;[39] KC3, a murine equivalent to HGRO;[40] 9E3, a Rous sarcoma virus induced gene product in chick embryo fibroblasts;[41] and IL-8, interleukin 8.[42–44]

tightly to heparin-agarose as does BPF4, suggesting that the weaker affinity for heparin is partly due to the low concentration of tetramers. Similarly, IL-8, which is bound to heparin-agarose as tightly as β-thromboglobulin, has been shown to form AB-type dimers at concentrations of 1.8 mM under acidic conditions,[50] but it has not been demonstrated whether this form persists at lower protein concentrations or neutral pH. Finally, the two cytokines, macrophage inflammatory proteins 1 and 2, both of which bind to heparin-agarose with affinities greater than that of β-thromboglobulin,[51,52] are also known to assemble into higher oligomers. A more detailed analysis of the differences in heparin binding by these PF4 homologs awaits a thorough understanding of their oligomeric states.

In this paper we have considered only a subset of the possible models for the heparin and BPF4 complex. First, the heparin molecule could bind to the helix bundle at some angle between the two limiting orientations—parallel or perpendicular—that we have examined in detail. Second, the results from energy minimization are highly dependent on the starting conditions and the precise details of the interactions will vary with the initial orientation of the BPF4 side chains and the heparin saccharides. We have investigated three of the possible starting configurations for the perpendicular heparin and BPF4 complex, namely sugar rings edgewise to the BPF4 surface, sugar rings edgewise and rotated by 180° (in the octatridecasaccharide), and sugar rings flat with respect to the BPF4 surface. Moreover, in solution there is probably a distribution of slightly different complexes with BPF4 due to the heterogeneity of heparin. Although the exact details of the interactions are indeterminate, the evidence presented herein, namely the model studies, the NMR data, and the homologies, all strongly support an approximately perpendicular arrangement in the complex.

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