Binding of heparin to human platelet factor 4

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Platelet factor 4 is a small protein (M_r 7756) from the α -granules of blood platelets which binds strongly to and neutralizes the anticoagulant properties of heparin. From an analysis of X-ray crystallographic data a model for the binding of platelet factor 4 to heparin is proposed.

INTRODUCTION

Platelet factor 4 (PF4) is a potent heparin-neutralizing protein released from α -granules when platelets aggregate (Niewiarowski *et al.*, 1968). The mechanism by which PF4 neutralizes the anticoagulant properties of heparin is not fully understood. Heparin is a mucopolysaccharide which acts as an anticoagulant by binding tightly to antithrombin III and accelerating the rate at which this proteinase inhibitor can neutralize thrombin and other serine proteinases involved in the coagulation cascade (Rosenberg & Damus, 1973). It has been shown that the smallest fragment of heparin that will bind to antithrombin III is an octasaccharide (Thunberg *et al.*, 1980) containing a unique pentasaccharide sequence as the binding region (Lindahl *et al.*, 1980, 1983; Casu *et al.*, 1981; Thunberg *et al.*, 1982; Bjork & Lindahl, 1982). As heparin can bind both PF4 and antithrombin III simultaneously (Piepkorn, 1981) and as PF4 binds to heparins with either high or low affinity for antithrombin III (Niewiarowski *et al.*, 1979), it has been proposed (Lane *et al.*, 1984) that PF4 exhibits its neutralizing activity not through a direct competition for the antithrombin III binding site but by preventing the formation of the stable heparin–antithrombin III–serine-proteinase ternary complex proposed by Laurent *et al.* (1978).

Experiments on the binding of tetrameric PF4 to heparin oligosaccharides of various sizes (Denton *et al.*, 1983) indicate that, for an efficient interaction, oligosaccharides containing upwards of about 16 sugar units are required. In the present paper we show that the symmetry of the PF4 tetramer, as deduced from X-ray diffraction data, imposes limitations on the size and composition of a heparin oligosaccharide to which all four monomers will bind.

MATERIALS AND METHODS

Human PF4, prepared as described previously (Morgan *et al.*, 1977), was provided by Dr. F. J. Morgan.

Crystals of PF4 were grown by using either the vapour diffusion method with 2-methylpentan-2,4-diol as precipitant (Machin & Isaacs, 1984) or a vapour dilution method as used to crystallize bovine PF4 (St. Charles *et al.*, 1984). Both methods produced isomorphous crystal forms belonging to the orthorhombic system with cell dimensions $a = 8.63$ nm, $b = 7.83$ nm, $c = 4.35$ nm. The space group is $P2_12_12_1$ and for an assumed four molecules

in the asymmetric unit the ratio of unit cell volume to molecular mass of protein in the cell is 2.38×10^{-3} nm³/Da, which is in the range of values reported by Matthews (1968) for protein crystals. X-ray diffraction data were recorded photographically using the rotation method with a Rigaku RU-12 rotating anode generator (0.2 mm focus at 50 kV/40 mA) as a source of Ni-filtered Cu radiation. Diffraction data were recorded in steps of 0.01° through a rotation of 66° about the c^* axis. The film packs, containing two films, were changed after every 3° of rotation and still photographs were recorded for alignment purposes at the beginning and end of each exposure. The 22 films recorded from one crystal were scanned on an Optronics P-1000 film scanner and these data were converted to intensities and corrected for Lorentz and polarization effects; 10504 data were recorded and merged to 4397 unique reflections with a merging R , where:

$$R = \frac{\sum_h \sum_i |\bar{I}(h) - I(h)_i|}{\sum_h \sum_i I(h)_i}$$

of 4.5%. All computations were performed using the PROTEIN suite of programs written by Dr. W. Steigemann, Munich.

RESULTS AND DISCUSSION

A rotation function (Rossmann & Blow, 1962) calculated with diffraction data to a resolution of 0.6 nm showed the four molecules of PF4 are arranged with 222 symmetry; i.e. the four molecules are arranged about three mutually perpendicular two-fold rotation axes (Fig. 1). Assuming the proportion of the unit cell volume occupied by solvent is 43% (Matthews, 1968) and that each PF4 molecule is approximately spherical, then the radius of each molecule is 1.6 nm, the distance from the centre of the tetrahedrally arranged molecules to the outside surface is 3.6 nm and the distance from the centre to the point where the molecules are touching is 1.1 nm. These figures are very approximate, but they do indicate the size of the PF4 tetramer.

There are important implications that follow from the symmetrical 222 arrangement of the four PF4 monomers. If it is assumed that the heparin binding site is the same on each PF4 molecule then the symmetrical arrangement imposes the following conditions.

Abbreviation used: PF4, platelet factor 4.

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Fig. 1. Tetrahedral arrangement of the PF4 monomers

The mutually perpendicular two-fold axes are shown by arrows which are directed out of the page. The possible binding sites are indicated by letters.

1. All four binding sites are related by the two-fold axes. Definition of one binding site locates the other three binding sites.

2. The distance from each binding site to the centre of the tetramer is the same.

3. The distance from the binding site on the first PF4 molecule (site A) to the site on the second molecule (site B) is the same as the distance between sites on the third (site C) and fourth (site D) molecules.

4. The distance between sites B and C will probably differ from the distance between A and B (thus C and D).

5. If a line is drawn through each of the binding sites in turn it is seen that the sites occur on alternate sides of the line and also that the direction of the binding site reverses from one site to another.

It follows that for heparin to bind tightly to each monomer of the PF4 tetramer it must possess complementary binding sites with properties which fulfill these conditions.

Structural investigations of heparin (Atkins & Nieduszynski, 1976) favour a two-fold helical model for a linear polydisaccharide consisting mainly of alternating 1,4-linked 2-deoxy-2-sulphamino- α -D-glucose-6-sulphate and 1,4-linked 2-sulphate- α -L-idopyranosyluronic acid residues. The 2-deoxy-2-sulphamino- α -D-glucose-6-sulphate exists in the 4C_1 chair conformation with equatorially directed side chains, whereas the 2-sulphate- α -L-idopyranosyluronic acid residue has the 1C_4 chair conformation, with both glycosidic linkages axial at the 1 and 4 positions, the carboxyl group equatorially directed and the sulphate in the 2 position axially directed. The maximum extension of a disaccharide with alternating 1C_4 - 4C_1 conformation is 0.89 nm and two of these disaccharide units will form a two-fold helical structure with a helical repeat distance of 1.65 nm (Fig. 2).

This two-fold helical structure of heparin will complement the two-fold symmetry of the PF4 tetramer and the conditions for binding as listed previously will be satisfied if the heparin binds with binding sites at intervals of an odd number of disaccharide units. As there are upwards of eight disaccharide (16 monosaccharide) units of heparin required for binding to the PF4 tetramer

Fig. 2. Stereodiagram of two disaccharide units illustrating the two-fold helix of heparin (Atkins & Nieduszynski, 1976)

The first residue (top) is 2-deoxy-2-sulphamino- α -D-glucose-6-sulphate which exists in a 4C_1 conformation. The second residue is 2-sulphate- α -L-idopyranosyluronic acid and exists in the 1C_4 conformation; the side chains of all residues are in arbitrary orientations.

(Denton *et al.*, 1983) then the possible binding sites would occur at intervals of either 1, 5, 1 or 3, 1, 3 disaccharide units, i.e. binding at the disaccharides 1, 2, 7, 8 or 1, 4, 5, 8 for an interaction with eight disaccharides. To a first approximation, the least energy conformation of the heparin chain is the one with the smallest distortion from linearity. Also, the chain will be of less energy if it follows the surface of a sphere where the deviation from linearity will be inversely proportional to the radius r of the sphere (Fig. 3). It is possible to relate the symmetry requirements for the binding sites (all a distance r from the centre of the tetramer), length of the heparin chain and positions of binding sites on the chain to the radius of the sphere. For a heparin chain of eight disaccharide units with binding sites at disaccharides 1, 2, 7, 8 or 1, 4, 5, 8 it can be shown that the radius of the heparin sphere would be 1.7 nm or 1.1 nm respectively. Hence, the most likely binding sites are 1, 2, 7 and 8.

For a heparin chain of ten disaccharide units, intervals of 1, 7, 1 or 3, 3, 3 disaccharide units are required, i.e. binding at disaccharides 1, 2, 9, 10 or 1, 4, 7, 10. The minimum distortion requirement suggests the most likely binding sites are 1, 2, 9, 10, as shown in Fig. 4, since the radius of the heparin sphere would be 2.3 nm compared with 1.7 nm for sites 1, 4, 7, 10.

The binding of PF4 to heparin has been shown to involve lysine residues (Handin & Cohen, 1976). There are eight lysines in PF4 and four of these are clustered at C-terminal end of the molecule (sequence Lys-Lys-Ile-Ile-Lys-Lys-) where the molecule is predicted to have an α -helical secondary structure (Lawler, 1981). An α -helical structure would position the lysine residues on the same side of the helix with an ability to interact with negatively charged groups on the heparin chain. This type of interaction is similar to that proposed for the binding of heparin to antithrombin III (Villanueva, 1984). Because of the symmetry constraints imposed by the PF4 tetramer, the heparin must have a two-fold helical structure formed from alternating 1C_4 , 4C_1 chair forms for the 2-sulphate- α -L-idopyranosyluronic acid and the

Fig. 4. Proposed binding of a ten disaccharide heparin chain with tetrameric PF4

The small spheres represent disaccharide units, the large spheres PF4 monomers. The shaded spheres represent disaccharides binding to PF4 monomers. The centres of the disaccharide units are based on the co-ordinates of the glycosidic oxygen atoms (from Fig. 3); hence the chain has the same arrangement as that in Fig. 3.

2-deoxy-2-sulphamino- α -D-glucose-6-sulphate residues. The unique heparin octasaccharide sequence which binds to antithrombin III has α -L-iduronic acid, *N*-acetyl-D-glycosamino-6-sulphate and α -D-glucuronic acid for the first three residues (Lindahl *et al.*, 1980). Each of these residues prefers a 4C_1 conformation and thus the octasaccharide would not form the two-fold helical structure required to bind tetrameric PF4.

The sequence of lysine residues at the C-terminal end

Fig. 3. Stereodiagram of the ten disaccharide unit heparin chain with a curvature of radius 2.3 nm

This conformation was arrived at by rotating bonds for each monosaccharide about the glycosidic bond. The average rotation was 6° . For clarity, the side chains are omitted.

of the PF4 chain (Lys-Lys-Ile-Ile-Lys-Lys-) is palindromic, so has a region of localized two-fold symmetry on the presumed α -helix. Hence, although the two-fold symmetry between adjacent binding sites reverses the direction of the binding site from one monomer to another, this is compensated for by the localized symmetry in the α -helix. It is interesting to note that although β -thromboglobulin has 50% sequence homology with PF4 and also exists as a tetramer, it binds only weakly with heparin. The weak binding of β -thromboglobulin to heparin may be explained by the fact that the sequence of lysine residues at the C-terminal end of β -thromboglobulin, although similar to that in PF4, is not palindromic (Lys-Lys-Ile-Val-Gly-Lys-Lys-) and the orientation relative to the heparin chain would differ in adjacent binding sites.

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