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Platelet factor 4 neutralizes heparan sulfate-enhanced antithrombin inactivation of factor Xa by preventing interaction(s) of enzyme with polysaccharide☆

Martine M. Fiore* and Vijay V. Kakkar

Thrombosis Research Institute, Manresa Road, E. Kaye building, London SW3 6LR, UK

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Abstract

Platelet factor 4 (PF4) is a heparin-binding protein which exhibits anti-heparin activities through the inhibition of antithrombin (AT)-dependent reactions with the serine proteases thrombin and factor Xa. PF4 also neutralizes heparan sulfate (HS), a glycosaminoglycan (GAG) present on the surface of endothelial cells, thereby possibly modulating an anticoagulant response. Previous models of PF4 mechanism did not distinguish whether PF4 causes steric hindrance of AT binding to fXa or of AT binding to the surface of the GAG chain. To shed light on the mechanism of PF4, studies of HS/heparin-catalyzed fXa inactivation by AT were undertaken. The results were consistent with PF4 directly interfering with AT binding to fXa rather than AT binding to the GAG chain, since PF4 did not prevent the heparin-dependent increase in AT intrinsic fluorescence. In fact, PF4 mechanism was competitive with respect to AT and non-competitive with respect to fXa, suggesting inhibition of important regulatory/catalytic interactions of fXa with the polysaccharide. Altogether, the results suggested a model by which PF4 bound to proximal (but distinct) sites to AT, resulting in steric interference of fXa binding to both polysaccharide and AT. It is proposed that PF4 inhibited the sequence of events recapitulated in the template mechanism describing heparin-dependent inhibition of fXa.

Keywords: Heparin; Factor Xa; Antithrombin; Platelet factor 4; Anticoagulation

Heparan sulfate proteoglycans (HSPGs) provide large capacity storage sites on the vascular wall for coagulation inhibitors such as antithrombin (AT). For instance, endothelium-derived HSPGs contain in small proportion a pentasaccharide motif which is found in heparin and specifically interacts with high affinity with AT [1–3], thereby modulating a local anticoagulant response. At sites of vascular injury, activated platelets release from their α-granules; platelet factor-4 (PF4) [4,5], a glycosaminoglycan (GAG)-binding protein which neutralizes the anticoagulant function of AT towards thrombin and factor Xa [6–8]. The PF4 protein is secreted as a tetramer of identical subunits ($M \sim 7800$) which upon release transfers from its chondroitin-4 sulfate proteoglycan carrier to more highly sulfated polysaccharides, such as heparin [4,9,10]. Since PF4 is capable of binding to endothelial HSPGs [11] and decreases in situ formation of thrombin–AT complexes [12], HSPGs have been proposed to serve as an affinity matrix responsible for the rapid plasma clearance of heparin-releasable PF4 [7,13,14]. An important function of PF4 could thus be to interfere with the heparin-like activity of the endothelium.

There is little evidence to suggest that the mechanism of neutralization of heparin involves direct competition of PF4 with AT for the same binding sites on the GAG chain [15]. First, AT and PF4 may not compete for identical sites on heparin because PF4 does not show selectivity for heparin binding—on the contrary to AT—[16,17], heparin can bind both PF4 and AT simultaneously [18], and while PF4 prevents the binding of growth-promoting factor FGF-2 to cell surface HSPGs, AT does not [19]. Second, the neutralization capacity of

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* Corresponding author. Fax: +44-20-7351-8324.
E-mail address: mfiore@tri-london.ac.uk (M.M. Fiore).

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PF4 on heparin fractions with high-affinity for AT correlates with the ability to bind to immobilized thrombin, raising the interesting possibility that PF4 and the serine protease bind to similar domains on heparin [17].

The aim of the present study was to determine the mechanism of PF4 towards HS and to verify the hypothesis that PF4 can prevent an interaction of the enzyme with the polysaccharide. HS has been previously shown to exhibit anti-fXa activity and is also neutralized by PF4 [7]. In view of the heparin-like activity of HSPGs, it is relevant to address the possibility that neutralization of HS by PF4 shares common features as already described for the antagonism of heparin.

Materials and methods

Materials. Unfractionated heparin (MW ~ 22,000), LMW heparin (MW ~3000), and HS were from Sigma (Poole, UK). Bemiparin (MW range 3000–4200) was from Rovi (Spain). Human plasma proteins fXa, AT, and PF4 were obtained from Cambridge Biosciences (Cambridge, UK). Chromogenic substrate S2765 was purchased from Quadratech (Epsom, UK).

Kinetic methods. The rate of fXa inactivation by AT catalyzed by HS or heparins was measured by way of a discontinuous assay under pseudo-first-order conditions both in the presence and absence of the neutralizing agent PF4. Reactions were carried out in HBSA buffer (20 mM Hepes–NaOH, 0.15 M NaCl, pH 7.4, plus 0.1% BSA) and contained 5 mM $CaCl_2$ in all reactions [20]. AT (130 or 500 nM) and PF4 (100 nM or else as indicated in the legend) were added into calcium-containing buffer, followed by addition of polysaccharide (at the indicated concentrations) and 5 nM fXa in 200 μl total reaction volume. At various time intervals, 20 μl aliquot was taken out and dispensed into a 96-well microplate containing 25 μl quenching buffer per well (HBSA buffer supplemented with 10 mM EDTA and 1 mg/ml polybrene) plus 50 μl S2765 chromogenic substrate at a final concentration of 0.5 mM. Residual fXa amidolytic activity was monitored using a kinetics microplate reader (model 550 from Bio-Rad) with readings taken every 30 s. Pseudo-first-order rate constants were obtained by fitting the data to single exponential decay by non-linear regression analysis. The extent of neutralization by PF4 was calculated as described [8]. Experiments were carried to determine the inhibition constant (K_i) of PF4 and used varying concentrations of AT (8.5–200 nM) in the presence of HS (0.2 μg/ml) and PF4 at a constant concentration (5–50 nM). Reciprocal plots were indicative of competitive inhibition [21]. Inhibition constant for the competition of AT by PF4 was determined from the slope of reciprocal plots versus inhibitor concentration [21]. Kinetic parameters for the fXa substrate reaction catalyzed by HS were determined using varying concentrations of fXa (6–100 nM) in the presence of HS (1 μg/ml) and AT (0.5 μM) in the presence and absence of PF4 (200 nM). $K_{M(app)}$ and $V_{max(app)}$ were determined from plots of initial reaction velocity as a function of fXa according to [22].

Fluorescence spectroscopy. The effect of PF4 on the ability of heparin to enhance AT intrinsic fluorescence was monitored as previously described [23]. Emission intensities were recorded at 340 nm with excitation set at 290 nm to measure the contribution of tryptophan residues. Measurements were made on a Perkin–Elmer LS-5 spectrofluorometer with a 5 nm excitation and 2.5 nm emission band-pass. Each data set included five readings within a time interval of 30 s.

Results

Ability of PF4 to neutralize various GAGs

In order to characterize PF4 activity, the kinetics of fXa inactivation by AT on the surface of various glycosaminoglycans (GAGs) were monitored. PF4 neutralized HS almost to completion, yielding similar rate constant as for solution inactivation of fXa, indicating an effect of PF4 on the assembly of the fXa–AT complex on the GAG chain (data not shown). The activity of PF4 towards the LMW bemiparin was less efficient than in the presence of HS or full-length heparin (Table 1), consistent with previous report showing the requirement for a chain size with molecular weight above 5700 (18 dp) [6,17]. However, although PF4 was capable of abolishing HS activity, PF4 had no effect in the presence of 10 μg/ml HS. Accordingly, the PF4 concentration dependency which yielded a $K_{(app)} = 13 \pm 3.4$ nM in the presence of HS at 0.2 μg/ml was right-shifted in the presence of higher concentrations of HS (data not shown), indicating that PF4 effectiveness depended on a relatively low concentration of GAG.

Competitive mechanism of PF4 with AT on the polysaccharide surface

The hypothesis that PF4 interfered with AT was suggested previously [6,17]. However, it was not determined whether the target of PF4 was the active site of fXa or the polysaccharide surface. This question was addressed in experiments measuring AT-dependent saturation kinetics. The data (Fig. 1) showed that PF4 mechanism towards AT in the presence of HS was competitive, yielding a $K_i = 15$ nM, close to the value of $K_{(app)}$ determined above. To establish whether PF4

Table 1
Ability of low concentrations of PF4 to neutralize the activity of various heparans

Polysaccharide type	Neutralization (%)	
	PF4 5 nM	PF4 20 nM
Heparan sulfate	46 ± 7.0	75 ± 3.4
LMWH (bemiparin)	21 ± 3.2	33 ± 2.0
LMWH	63 ± 7.0	83 ± 4.1
UFH	96 ± 3.3	97 ± 3.5

The inhibition kinetics of fXa (5 nM) by AT (130 nM) were monitored in the presence of the following GAGs: HS (0.15 μg/ml), LMWH bemiparin (0.1 μg/ml), LMWH (0.1 μg/ml), or UFH (0.01 μg/ml). The concentration of each GAG was chosen as to allow the same value of rate to be observed in the control, in order to compare each GAG in its ability to be neutralized by PF4. In the absence of PF4, the value was on average; $k = 0.055\ \text{min}^{-1}$ in each reaction. Neutralization of the fXa–AT reaction catalyzed by various GAGs in the presence of PF4 (at the indicated concentrations) was calculated according to [8]. The observed rate constant k was equal to $0.015\ \text{min}^{-1}$ for solution inhibition of fXa by AT.

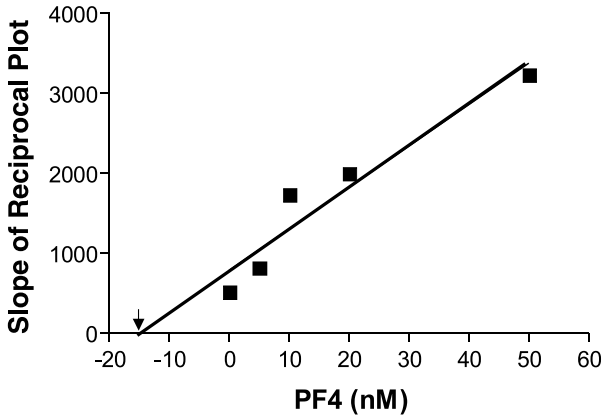

Fig. 1. Competitive mechanism of PF4 toward AT on the HS surface. Saturation kinetics of fXa (5 nM) by varying concentrations of AT (8.5–200 nM) and HS (0.2 μg/ml) were first measured for reactions in the absence or presence of a constant concentration of PF4 at 5, 10, 20 or 50 nM. These saturation curves were used to derive reciprocal plots of velocity versus AT concentration [21]. The data represent the slopes of such reciprocal plots (in ordinate) plotted versus inhibitor (PF4) concentration [21]. The value of K_i is indicated on the graph by an arrow.

prevented AT binding to the GAG surface, a direct method was employed which measured the increase in AT intrinsic fluorescence in the presence of varying concentrations of GAG. The results (Fig. 2) indicated that PF4 did not interfere with the AT–GAG interaction, in the conditions assayed which employed limiting concentration of heparin relative to AT and PF4. Instead, PF4 prevented AT access to the active site of fXa. Interestingly, addition of non-fluorescent PF4 to AT in the absence of heparin caused a transient increase (2.2-fold) of AT intrinsic fluorescence, suggesting the formation of a low-affinity complex between PF4 and AT which dissociated upon interaction of each molecule to heparin (data not shown).

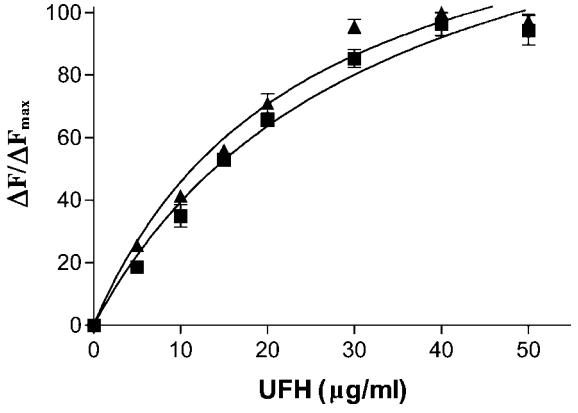

Fig. 2. Effect of PF4 on the heparin enhancement of AT intrinsic fluorescence. Titration curves of AT (1.3 μM) by varying concentrations of UF heparin (up to 50 μg/ml) are represented for reactions in the absence (squares) or presence of PF4 (1 μM) (triangles). Data (average of five readings) are plotted as the fractional increase in tryptophan fluorescence ($\Delta F/\Delta F_{max}$) versus heparin concentration.

PF4 prevents interaction of fXa with the polysaccharide surface

The mechanism of fXa inhibition by AT/heparin was previously shown to occur via a template mechanism, in which the enzyme bound to the polysaccharide surface [20]. This mechanism was ionic strength-dependent on full-length heparin, but ionic strength-independent in the reaction catalyzed by the pentasaccharide, because the latter was not large enough to support enzyme interaction [24]. We then sought to determine whether PF4 might interfere with the salt-dependent increase of fXa inactivation rates catalyzed by various GAGs (Fig. 3). As expected, fXa inactivation was enhanced at low-ionic strength (NaCl = 15 mM), both by HS and heparins, albeit to a different extent by the LMWH bemiparin. The increase in fXa inhibitory activity was abolished by 100 nM PF4 in all reactions assayed. We interpret this observation by PF4 preventing electrostatic interactions of fXa with the polysaccharide surface.

The mode of interaction of fXa with HS in the presence and absence of PF4 was explored further, using an active-site inhibited fXa (fXa-EGR) as competitor of fXa for the HS surface. It was reasoned that since PF4 appeared to have an effect on fXa interaction with GAGs, the contribution of the competitor fXa-EGR should also be altered, leading to some recovery of fXa inhibitory activity. As shown in Table 2, the addition of fXa-EGR caused a 4.4-fold decrease in fXa inhibition by AT, but only a 1.7-fold decrease in the presence of 200 nM PF4, indicating that PF4 restricted the effect of

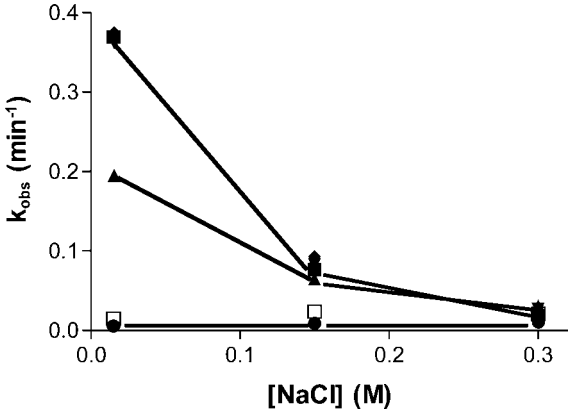

Fig. 3. NaCl-dependencies of fXa inhibition by AT in the presence and absence of PF4. The kinetics of fXa inhibition by AT were monitored in the presence of various GAGs at NaCl (0.015–0.3 M). Reactions of fXa (5 nM) with AT (130 nM) catalyzed by HS (0.15 μg/ml; filled squares); LMWH bemiparin (0.1 μg/ml; triangles); LMWH (0.1 μg/ml; inverted triangles) or UFH (0.01 μg/ml; diamonds) were neutralized by PF4 at 100 nM. For clarity purpose, only the neutralized reactions catalyzed by HS (circles) or bemiparin (blank squares) are represented. In the presence of PF4, the k values were similar for all reactions and ranged between 0.010 and 0.013 min^{-1}.

Table 2
Inhibition by PF4 of the competition by fXa-EGR of fXa interaction with HS

	k (min^{-1})		Activity (%)
	Control	fXa–GR	
Absence of PF4	0.35 ± 0.01	0.08 ± 0.02	23 ± 4.5
PF4 200 nM	0.07 ± 0.01	0.04 ± 0.00	57 ± 7.0

FXa (5 nM) was added to a mixture containing AT (130 nM), HS (1 μg/ml), and fXa–EGR (1.8 μM) preincubated for 10 min in the presence and absence of PF4. Data are represented as pseudo-first-order rate constants and activity remaining after incubation with fXa–EGR. Addition of PF4 resulted in less inhibition by fXa–EGR of the fXa–AT reaction. Also, the effect of PF4 was diminished by the addition of fXa–EGR. The contributions of PF4 and fXa–EGR appeared to be additive.

Table 3
Kinetic parameters of the HS-catalyzed fXa–AT reaction: saturation by fXa

Absence of PF4		PF4 200 nM	
K_M (nM)	V_{max} (nM/min)	K_M (nM)	V_{max} (nM/min)
151 ± 32	61 ± 18	123 ± 50	17 ± 6.0

FXa inactivation by AT (0.5 μM) catalyzed by HS (1 μg/ml) was assayed as described above at varying concentrations of fXa (6–100 nM) in the presence or absence of PF4. Kinetic parameters were determined from plots of initial reaction velocity as a function of initial fXa concentration according to [21].

the competitor, by preventing its full association with the GAG chain. Another approach was used to confirm the findings (Fig. 3 and Table 2), by considering the GAG surface as catalyst of the fXa–AT reaction, using fXa as substrate [21,25]. The kinetic parameters K_M and V_{max} are shown in Table 3. Surprisingly, the addition of PF4 had no effect on the apparent affinity of fXa for HS, as indicated by a similar K_M in the control and in the presence of PF4. However, the addition of PF4 resulted in an approximately 3.6-fold decrease in the maximum rate of fXa inhibition, suggesting that PF4 had an influence on the ability of HS to modulate the kinetics of the fXa–AT reaction. This finding is discussed below.

Discussion

In this report, we sought to provide new insight into the mechanism of PF4 towards HS as a model polysaccharide of the interaction of this procoagulant molecule with the endothelial surface. Since more PF4 was required to achieve efficient neutralization of the fXa–AT reaction when the concentration of HS was raised (from 0.2 to 1 μg/ml), the effective concentration of PF4 depended on the concentration of GAG. This suggested a requirement for PF4 binding on the GAG chain to sites located in the proximity of AT, because more HS would decrease the surface density of PF4. In fact, the mechanism of HS neutralization by PF4 was competitive towards AT only in the presence of low concentration of the polysaccharide (Fig. 1). Nevertheless, PF4 and AT likely bound to distinct sites, because PF4 did not prevent AT from binding to heparin (Fig. 2). The results altogether suggested that the effect of PF4 towards AT could only be evidenced in the presence of fXa.

We tested the hypothesis that PF4 interfered with the previously characterized association of fXa to polysaccharide [26–29]. The rationale was that if PF4 prevented fXa association with GAGs, this would lead to impaired access of AT to the active site of fXa. Several lines of evidence were consistent with this hypothesis. First, PF4 required the active site of fXa and competed with AT on the GAG chain. Second, PF4 prevented the electrostatic interactions between fXa and GAGs. Third, PF4 prevented fXa–EGR binding to HS. Altogether the data were consistent with the proposal that ternary assembly occurred via binding of protease to heparin.

The increasing rates of fXa inhibition observed with heparin fractions of increasing M_r have been explained in terms of an increased affinity between fXa and the heparin–AT complex [6,24,29]. It was previously shown that in the template mechanism describing the thrombin–AT reaction, the molar specific activity of heparin increased with molecular weight and correlated with both the number of enzymes and AT bound [30]. Hence, PF4 may have lowered the affinity of fXa for heparin-bound AT, not only by interfering with AT binding to fXa, but also by decreasing the number of HS-associated fXa molecules accessible to AT, thereby affecting the concentration of active sites (related to the occupancy of HS by fXa) and the V_{max} of the reaction (Table 3). The large ionic strength-dependent enhancement of the rate of fXa inactivation was consistent with the interaction of HS/heparin with positively charged residues on the catalytic domain of fXa [29] which were abolished by PF4 (Fig. 3). The interaction of UFH and HS with fXa involved more electrostatic contacts than by LMWH bemiparin. The lower ability of bemiparin to promote fXa binding correlated with the lower neutralization of this heparin by PF4 compared with the other GAGs tested, consistent with the proposal that fXa and PF4 shared common sites on the GAG chain.

Altogether, the data suggested a mechanism of PF4 relying on competition with AT for the active site of fXa rather than for binding sites on the GAG chain. The non-competitive nature of PF4 neutralization of the HS-catalyzed substrate reaction (with respect to fXa) suggested interference by PF4 of HS/heparin contacts on fXa which governed the rate of the fXa–AT reaction [29]. As shown in the previous studies [20,28], fXa binding to polysaccharide contributed to the acceleration of fXa inactivation by AT via a template mechanism, in which calcium enhanced a heparin-fXa bridging

interaction that was followed by ternary complex formation. In this view, PF4 may have reduced the maximum rate of fXa inactivation by AT by sterically interfering with the bridging of fXa with HS/heparin or by precluding a conformation of fXa competent for bridging. Further kinetic studies are required to decipher the in-depth mechanism of PF4. Thus, according to our data, a model of PF4 mechanism includes the following steps: (1) PF4 and AT form a transient complex which re-equilibrates on the HS/heparin template via high-affinity binding of PF4 and AT to adjacent but independent classes of binding sites. (2) PF4 occludes a space near the active site of fXa which interacts with GAGs, thereby preventing interaction of AT with fXa. That PF4 interfered with fXa–polysaccharide interaction(s) involved in catalysis may have an influence on the effect of heparin towards fXa during coagulation, supposedly by promoting the assembly of 'free' fXa into a prothrombinase complex, by allowing fVa and prothrombin to interact with fXa. Finally, in view of the other roles of PF4 as an antiangiogenic agent inhibiting FGF-2-dependent endothelial cell proliferation and migration, our model agrees with the proposal that interaction of FGF-2 with PF4 impairs association of the FGF-2 ligand with its cell surface receptor [31]. It also conforms to a general scheme in which PF4 precludes stabilization of receptor–ligand complexes by HS.

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