Research Article
The Modification of Xa-ATIII-Heparin Dynamics by Protamine Sulfate

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Heparin promotes the formation of 1° FXaα-ATIII and FXaβ-ATIII complexes from the free enzymes and antithrombin III. It further stimulates the transformations of the 1° complexes into the corresponding 3° complexes. Additionally, it stimulates the degradation of the 1° FXaα-ATIII and FXaβ-ATIII complexes into free FXaα, FXaβ, and ATIII. Protamine sulfate (PS) stimulates the transformation of FXaα into FXaβ and hence to FXay. It also stimulates the transformation of the 1°, 2°, and 3° FXa-ATIII complexes into their corresponding β-complexes. It further promotes the degradation of the 1° FXaα- and FXaβ-ATIII complexes into their corresponding 3° FXaα- and FXaβ-ATIII complexes, with the concomitant release of FXay. The addition of PS to FXa/ATIII/H mixtures results in a reduction in free FXa, ATIII, and 1° Xa-ATIII complex formation, together with the concomitant increase in 3° FXa-ATIII complex formation and release of FXay. The likeliest explanation of these results resides in the removal of the effective free heparin as a consequence of the generation of a stable heparin/PS salt upon the addition of the PS to the FXa/ATIII/H mixtures, thereby effectively lowering clotting times.

1. Introduction

Factor X is located at the juncture of the intrinsic and extrinsic coagulation cascade pathways. Factor X has two active forms (FXaα and FXaβ) that differ in molecular weight by about 4000 daltons. Factor Xaβ can be degradatively inactivated (autolyzed) to form FXay upon cleavage at the –NH₂ terminal of the active site [1–3].

The serpin antithrombin III (ATIII) is the primary naturally occurring inhibitor of Factor Xa. Factor Xa and antithrombin III form a 1 : 1 molar complex via covalent bonding [3–5]. Upon hydrolysis of this bond, a modified form of ATIII is produced (ATIII₅). ATIII₅ runs at a slightly higher apparent molecular weight than native ATIII in SDS-PAGE [3, 5, 6].

The polysulfonated glycosaminoglycan heparin (H) is administered clinically as an anticoagulant. H acts to catalyze interactions of ATIII with factors Xa and IIa, among other coagulation factors. Heparin fractions have varying affinity for ATIII, with the smallest fraction of high affinity being the pentasaccharide. Administration of H increases the FXa-ATIII complex formation by at least an order of magnitude [7] or several hundredfold when also in the presence of physiological levels of Ca²⁺[8–10]. Further, ATⅢ₅ increases 20–30% in the presence of FXa and high-affinity heparin [6, 11].

Positively charged protamine sulfate (PS) has long been used clinically to neutralize heparin in a variety of situations, for example, cardiopulmonary surgery and heparin overdose [12]. Protamine sulfate has a strong positive charge, which allows it to bind to the sulfuric acid groups of heparin to inhibit heparin activity [13, 14]. In addition to binding free H, PS dissociates heparin from the heparin-antithrombin III complex [15].

Interestingly, the ability of protamine sulfate to neutralize heparin varies with heparin chain length [13]. Related to this, PS can neutralize heparin-induced anti-IIa prolongation of clotting more quickly than heparin-induced anti-Xa prolongation of clotting [16, 17].
There is some disagreement over proper protamine dosing for heparin neutralization [12, 14, 17, 18]. However, it appears that effective protamine dosing for heparin neutralization is about 1 mg/100U of unfractionated H, but should not be lower [17]. Yet, PS overdose has a number of side effects including hypotension and platelet dysfunction [19]. Further, PS has been demonstrated to prolong clotting time [12, 20, 21].

This study was initiated to evaluate the effect of protamine sulfate on the individual and collective components of the Xa/ATIII/H interrelationship. H promotes formation of ATIII, possibly as a result of increased complex formation and its subsequent degradation. H also promotes conversion of 1′ FXaβ-ATIII to 3′ FXaβ-ATIII and/or 3′ FXaa-ATIII to 3′-FXaβ-ATIII. It has been shown elsewhere that PS encourages a transformation from FXaa to FXaβ and hence to inactive FXay [22]. Further, PS reduces FXaa-ATIII complexes, increases FXaβ-ATIII complexes, and increases transformation from 1′ to 3′ complexes. Thus, PS alone acts to reduce total complex in the presence of FXa and ATIII, and degrade the complex that is formed.

It is reported herein that successive addition of FXa, ATIII, H, and PS gives a combination of the individual H and PS effects; namely, there is reduced total (1′+2′+3′) FXa complexes, a transformation from 1′ to 3′ complexes, an increase in 3′ FXaβ-ATIII, and an increase in ATIII. However, generation of increased FXa from FXay by H alone is not observed.

2. Materials and Methods

Trizma base, sodium lauryl sulfate, acrylamide, N,N-methylene-bis-acrylamide, 3′,3′′,5′,5′′ tetrabromophenol-sulphonephthalein, and Coomassie Brilliant Blue were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, NaCl (enzyme grade), glyceraldehyde (enzyme grade), HCl (certified A.C.S. PLUS), and acetic acid (glacial) were purchased from Fisher Scientific (Pittsburgh, PA). Human factor Xa and human antithrombin III were purchased from Haematologic Technologies, Inc (Essex Junction, VT). Human Factor Xa and human antithrombin III were each diluted to 0.5 mg/mL with 18.5 mM Tris/56 mM NaCl buffer, pH 8.0. Heparin, purchased from Sigma Chemical Co. of St. Louis, MO (grade 1-A sodium salt 190 USP units/mg), was reconstituted to 1 mg/3 mL with the 18.5 mM Tris/56 mM NaCl buffer, pH 8.0. Protamine sulfate, purchased from Sigma Chemical Co. (Grade X, from Salmon, (Salmine)), was reconstituted to 5 mg/mL with the 18.5 mM Tris/56 mM NaCl buffer, pH 8.0. SDS-PAGE molecular weight standards, broad range, and transfer blot transfer membrane were purchased from Bio-Rad (Hercules, CA).

Methods: Preparation of Reactants for SDS-PAGE. For FXa or ATIII control preparations, 9 μL Tris/NaCl buffer was added to either 1.5 μg FXa or 1.5 μg ATIII in 3 μL of the Tris/NaCl buffer and incubated for 15′ at room temperature. In FXa/ATIII mixtures, 1.5 μg FXa in 3 μL Tris/NaCl buffer, 1.5 μg ATIII in 3 μL Tris/NaCl buffer, and 6 μL Tris/NaCl buffer were added at one-minute intervals (for an incubation volume of 12 μL) and incubated for 15′ at room temperature.

ATIII (1.5 μg in 3 μL Tris/NaCl buffer) and H (1 μg in 3 μL Tris/NaCl buffer) mixtures were incubated for 15′ at room temperature prior to addition of 1.5 μg FXa in 3 μL Tris/NaCl buffer and addition of 3 μL of Tris/NaCl buffer to a final volume of 12 μL, which was incubated for a further 15′ at room temperature. Alternatively, FXa (1.5 μg in 3 μL Tris/NaCl buffer) and H (1 μg in 3 μL Tris/NaCl buffer) mixtures were incubated for 15′ at room temperature prior to addition of 1.5 μg ATIII in 3 μL Tris/NaCl buffer and addition of 3 μL Tris/NaCl buffer to a final volume of 12 μL, which was incubated for a further 15′ at room temperature. FXa/ATIII/H mixtures were prepared as follows: 1.5 μg FXa in 3 μL Tris/NaCl buffer, 1.5 μg ATIII in 3 μL Tris/NaCl buffer, 1 μg H in 3 μL Tris/NaCl buffer, and 5 μL PS in 3 μL Tris/NaCl buffer were added at one-minute intervals and incubated for 15′ at room temperature. Mixtures of FXa, ATIII, H, and PS were prepared as follows: 1.5 μg FXa in 3 μL Tris/NaCl buffer, 1.5 μg ATIII in 3 μL Tris/NaCl buffer, 1 μg H in 3 μL Tris/NaCl buffer, and 5 μL PS in 3 μL Tris/NaCl buffer were added at one-minute intervals and incubated for 15′ at room temperature. Each lane had a constant FXa:ATIII contact time of 15 minutes and contact volume of 12 μL and was subsequently raised to a final volume of 20 μL by addition of 4 μL Tris/NaCl buffer and 4 μL stock sample buffer (53% v:v glycerol, 0.1% bromophenol blue, 0.2 M Tris/HC1, pH 6.8) before being boiled at 100°C for 2 minutes and loaded into the gel wells. Molecular weight markers were prepared by the addition of 2 μL β-mercaptoethanol to a 0.5 mL Eppendorf tube containing 2 μL molecular weight markers and 57.6 μL molecular weight marker stock solution and boiled for 5 minutes at 100°C before 12 μL was added to the gel well.

SDS-PAGE. A modified Laemmli discontinuous gel method for SDS-PAGE was employed [23]. Electrophoresis was carried out using the Bio-Rad Mini-Protein 3 system (Bio-Rad, Hercules, CA). The resolving gel (7.5% acrylamide/bisacrylamide, 0.377 M Tris, 0.035 M SDS, pH 8.8) was allowed to polymerize for approximately 30 minutes. Mixtures of FXa, ATIII, H, and PS were added at one-minute intervals and incubated for 15′ at room temperature. Mixtures of FXa, ATIII, H, and PS were prepared as follows: 1.5 μg FXa in 3 μL Tris/NaCl buffer, 1.5 μg ATIII in 3 μL Tris/NaCl buffer, 1 μg H in 3 μL Tris/NaCl buffer, and 5 μL PS in 3 μL Tris/NaCl buffer were added at one-minute intervals and incubated for 15′ at room temperature. Each lane had a constant FXa:ATIII contact time of 15 minutes and contact volume of 12 μL and was subsequently raised to a final volume of 20 μL by addition of 4 μL Tris/NaCl buffer and 4 μL stock sample buffer (53% v:v glycerol, 0.1% bromophenol blue, 0.2 M Tris/HC1, pH 6.8) before being boiled at 100°C for 2 minutes and loaded into the gel apparatus. The gels were electrophoresed at a voltage gradient from 120 to 140 volts at room temperature for approximately 75 minutes. Following electrophoresis, the gels were stained in 0.1% Coomassie Brilliant Blue, 50% methanol, 12.5% acetic acid for approximately 4 hours and subsequently destained in a solution containing 10% (v:v) methanol and 10% (v:v) acetic acid, for approximately 6 hours. Gels were photographed using Scion NIH Image Capture software. Bands were quantitated using GelPro Analyzer software from Media Cybernetics (Des Moines, IA). Statistical analysis was performed using a Student’s t-test, and significance was assigned as P < 0.05. N ranged from 4 to 14. Band composition was determined by Western blot analysis using either human factor X polyclonal antibody or human antithrombin III polyclonal antibody (data not shown).
3. Results

The Effect of Heparin on the Interaction between FXa and ATIII. Interaction of FXa with ATIII, as followed by SDS-PAGE, led to the observation of 1° FXaα- and FXaβ-ATIII complexes at 109 and 104 kDa, slight 3° FXaα- and FXaβ-ATIII complexes at 66 and 62 kDa, some ATIIIγM at 58 kDa, and slight FXay at 35 kDa (Figure 1). Additionally, 2° FXaα- and FXaβ-ATIII complexes (99 and 95 kDa) were observed sporadically in small and variable quantities, which may be short-lived degradative intermediates between the 1° and 3° complexes.

Addition of 1 μg H to FXa and ATIII reduced the 1° complexes, increased the 3° complexes and ATIIIγM, and reduced native ATIII, FXaa, and FXaβ (Figure 1). Visually, the distinct presence of FXay appeared elevated in the presence of H, although significant increases in FXay were not seen in the presence of H. The decrease in the 1° complex coupled with increases in the 3° complexes amounted to an overall slight decrease in total (1° + 2° + 3°) complexes for each incubation run. Additionally, a slight decrease in the ratio of the beta to alpha bands of the 1° complex and an increase in the beta to alpha ratio of the 3° complex were observed, with a concomitant decrease in the ratio of free FXαβ to FXaa. The data are expressed statistically in Figures 2, 3(a), 4(a), 8(a), and 9(a).

The Combined Effect of Heparin and Protamine Sulfate on the Interaction between FXa and ATIII (Figure 5). Reactions were carried out with 1.5 μg Xa, 1.5 μg ATIII, 1 μg H, and 5 μg PS and incubated for 15 minutes at room temperature. Addition of PS to an FXa/ATIII mixture leads to an increase in the appearance of 3° FXaα-ATIII and 3° FXaβ-ATIII bands with a concomitant drop in the level of the 1° complex bands. A marked increase in FXay is also observed. Addition of H caused an increase in the 1° and 3° bands. However, addition of PS to an FXa/ATIII/H mixture results in a striking drop in visual protein complexes (in lane 9). There is very little 1° complex formation observed and a significant increase in 3° complexes, with an especially larger amount of the 3° FXaαβ-ATIII complex relative to the 3° FXaα-ATIII complex. Decreases in ATIII and ATIIIγM are seen, and a notable increase in FXay is discerned, which is a consequence of the degradation of the free β-FXa and the 1° FXa-ATIII complexes in the presence of PS.

The individual Effects of Heparin on FXa (Figure 7). Heparin (1 or 3 μg) did not affect autolysis of FXa.

4. Discussion

ATIII is a weak inhibitor of coagulation proteases unless it first binds with heparin-like glycosaminoglycans [11]. In the case of FXa, the acceleration of FXa-ATIII complex formation is traditionally thought to be due to a heparin-induced conformational change [24–26] in the reactive site loop of ATIII [7, 23, 27]. Heparin activates ATIII by binding to the serpin through a specific pentasaccharide region [28, 29]. A ternary FXa/ATIII/H complex is then formed, and FXa and ATIII subsequently form an irreversible covalent bond [25, 28].

Moreover, FXa contains a heparin-binding exosite located in the same 3D region as the heparin-binding exosite of FIIa [15]. The FXa/ATIII/H reaction is several hundred-fold faster when also in the presence of physiological levels of Ca2+ [8–10]. The Ca2+ binds to the Gla region of FXa and allows the heparin to bind to FXa [18].

This calcium effect is dependent on chain length, increasing 3–5 x for short-chain heparins and 50 x for long-chain
Figure 2: Continued.
**Figure 2:** Band composition as a % of the total protein in each lane for FXa/ATIII/H mixtures. (a) 1° complex, α-band; (b) 1° complex, β-band; (c) 2° complex, α-band; (d) 2° complex, β-band; (e) 3° complex, α-band; (f) 3° complex, β-band; (g) modified ATIII; (h) free, native ATIII; (i) free FXaα; (j) free FXaβ; K: FXaγ (FXa degradation product). All panels contain 1.5 μg FXa, 1.5 μg ATIII, ±1 μg H. *P < 0.1, **P < 0.05.

**Figure 3:** Proportions of total (α + β) FXa/ATIII complexes in lanes. (a) FXa/ATIII/PS mixtures. (b) FXa/ATIII/H mixtures. (c) FXa/ATIII/H/PS mixtures.
high-affinity heparins [10]. These studies indicate a reaction mechanism of calcium-enhanced long-chain heparin bridging an intermediate FXa-ATIII encounter complex [10, 27].

This investigation was designed to examine the effect of H upon the interaction of FXa with ATIII and the formation of degradation products of the 1° complexes. It further examined the impact of PS thereon. Initially, the data do not appear to conform to the expected heparin-induced increase in FXa-ATIII complexes. Upon addition of low levels of H, a slightly decreased total (1°+2°+3°) complex formation was observed (Figure 3(a)). Additionally, a more even distribution between FXαATIII and FXβ-ATIII complexes is observed (Figure 8(a)). Both of these results are due to a decrease in alpha complexes, while the beta complexes display little change (Figure 8(a)). However, increases in ATIII_M of 50–80% are seen (Figures 1, 2(g)). If ATIII_M is produced by hydrolysis at the active site [5, 30], then it is probable that the complex is being formed and subsequently cleaved, liberating FXa together with the ATIII_M. Furthermore, H appears to promote the transformation of the 1° FXa-ATIII complex to the 3° FXa-ATIII complex, especially in the beta bands (Figure 9(a)). This process may occur by either 1° FXαATIII → 3° FXαβ-ATIII or 3° FXαATIII → 3° FXαβ-ATIII (Scheme 1). The degradation product, FXaγ, is visualized.
FXa/ATIII/PS mixtures
Primary complex, α-band

FXa/ATIII/PS mixtures
Secondary complex, α-band

FXa/ATIII/H/PS mixtures
Tertiary complex, α-band

FXa/ATIII/PS mixtures
ATIII, native

FXa/ATIII/PS mixtures
ATIII, modified

FXa/ATIII/PS mixtures
Primary complex, β-band

FXa/ATIII/H/PS mixtures
Secondary complex, β-band

FXa/ATIII/H/PS mixtures
Tertiary complex, lower band

Figure 6: Continued.
FXa/ATIII/H/PS mixtures

FXaα

FXaβ

FXaγ

Figure 6: Band composition as a % of the total protein in each lane for FXa/ATIII/PS/H mixtures. (a) 1° complex, α-band; (b) 1° complex, β-band; (c) 2° complex, α-band; (d) 2° complex, β-band; (e) 3° complex, α-band; (f) 3° complex, β-band; (g) modified ATIII; (h) free, native ATIII; (i) free FXaα; (j) free FXaβ; (k) FXaγ (FXa degradation product). All panels contain 1.5 μg FXa, 1.5 μg ATIII, ±5 μg PS ±1 μg H. *P < 0.1, **P < 0.05.

Figure 7: The individual effects of heparin on FXa. Lane 1: MWM. Lane 2: human factor FXa(1.5 μg)15′, at R.T. Lane 3: [FXa(1.5 μg) + H(1 μg)]15′, at R.T. Lane 4: [FXa(1.5 μg)+H(3 μg)]15′, at R.T.

in the gel (Figure 1) although no statistical differences are observed (Figure 2(k)). In summation, H affects an increase in the formation of the 1° FXa-ATIII complexes and their transformation into the 3° FXa-ATIII complexes, with the concomitant generation FXaα and FXaβ, as well as ATIIIβ.

Protamine sulfate has been shown to neutralize heparin by binding to heparin's sulfuric acid groups and thus forming a stable complex that lacks anticoagulant activity [15, 18]. PS can also dissociate the ATIII/H complex, without itself binding to ATIII, and allow the ATIII to retain its activity [18]. Interestingly, PS has also been shown to prolong coagulation time [20, 21].

Elsewhere, it has been shown that PS encourages an obvious shift from FXaα to FXaβ and subsequently to FXaγ [22], which is analogous to reports of FXaα transforming to FXaβ in the presence of lipids [1, 31]. This is likely the major source of FXaγ as seen in the gels (Figure 5). Further, addition of PS results in a decrease in total complexes, and a transformation of complexes from the FXaα-ATIII to the FXaβ-ATIII forms and from the 1° to 3° forms.

The successive addition of FXa, ATIII, H, and PS results in a combination of the H and PS effects. Mixtures of FXa/ATIII/H show a reduction in total complexes, while incubation of FXa/ATIII/PS shows a much greater reduction (Figure 3). Mixtures of FXa/ATIII/H/PS results in a reduction about half-way between the H or PS mixtures, that is,
not as much reduction as with PS alone, but greater than H alone. This may occur by heparin and protamine sulfate forming a salt, each essentially neutralizing the individual effects. The total complex formed during FXa/ATIII/H/PS incubation is much less than the FXa/ATIII control leading to the speculation that there may be some inhibition of FXa-ATIII complex formation.

There is evidence of proteolysis in the changing ratio of 1° to 3° complexes (Figure 9). In this instance, heparin and protamine sulfate appear to have an additive effect. In all incubations, the alpha and beta complexes are both greatly shifted from 1° to 3° complexes, indicating degradation; yet, this shift to the degradation products is most pronounced in the FXa/ATIII/H/PS mixtures (Figure 9). The transformation of 1° to 3° complexes is an additional source of the FXay as the FXa moiety on the 1° complex is degraded to form the 3° complex.

While in all mixtures there is a shift from alpha to beta complexes (Figures 4 and 8), the FXa/ATIII/H/PS incubation again appears to be an averaging of the individual heparin and protamine sulfate effects. For FXa/ATIII/H/PS, the shift is less pronounced than in FXa/ATIII/PS but more pronounced than FXa/ATIII/H mixtures. Uniquely, however, for FXa/ATIII/H/PS, the shift occurs primarily in the 2° and 3° complexes (Figure 4(b)). In fact, in the 1° complex, there is actually a reduction in the beta-to-alpha ratio.
It is possible that, in light of the degradative transformations of α-complexes to β-complexes (Figures 4 and 8) and 1° complexes to 3° complexes (Figure 9), the active sites of the enzymes are involved with autolysis and proteolysis in a manner akin to competitive inhibition of ATIII. It is certain that mixtures of FXa/ATIII/H/PS promote formation of ATIIIH (Figure 6(g)), reduce apparent FXa complex levels, and promote degradation of FXaα to FXaβ and FXay, as well as degradation of 1° complexes to 3° complexes. The generation of FXay may be primarily a function of the autolytic degradation of FXaβ in the presence of PS, and secondarily due to transformations of 1° to 3° complexes with concomitant appearance of the FXay fragment.

In summation, heparin accelerates the conversion of free FXaα and FXaβ into the 1° FXaα-ATIII and 1° FXaβ-ATIII complexes and their degradation to their corresponding 3° FXa-ATIII complexes. Additionally, it degrades the 1° complexes into ATIIIH and the corresponding free FXaα and FXaβ. PS which expedites the transformations of free FXaα into free FXaβ, and hence to FXay, also accelerates the transformations of the 1°, 2°, and 3° FXaα-ATIII complexes into the corresponding FXaβ-ATIII complexes and furthermore stimulates the degradation of the 1° complexes into the 3° complexes with the concurrent generation of FXay. Addition of PS to an FXa/ATIII/H mixture, however, affects a major drop in free Xa, ATIII, and 1° FXa-ATIII complex formation, together with a significant increase in the 3° FXa-ATIII complexes and FXay. The likeliest explanation of these results resides in the removal of the effective free H as a consequence of the generation of a stable H/PS salt upon addition of the PS to the FXa/ATIII/H mixture, thereby effectively reducing clotting times.

In conclusion, it is reported that H stimulates the interaction of FXaα and FXaβ with ATIII to form the covalent 1° FXaα-ATIII and 1° FXaβ-ATIII complexes, the degradation of which to the free enzymes and ATIIIH is also stimulated. Additionally, H promotes the degradation of the 1° complexes into the 3° FXaα-ATIII and 3° FXaβ-ATIII complexes together with FXy, which is also an autolytic product of the degradation of FXaβ. PS exhibits a twofold function, as seen in Figure 5, Lane 9. It lowers the free H level by forming a stable salt complex with the glycosaminoglycan, the consequence of which is a major reduction in the complex formation between FXa and ATIII, particularly the 1° complexes. The residual complexes reside as 3° complexes with concomitant FXay and ATIIIH being elevated. Hence, anticoagulant activity of H is neutralized.

References


