Molecular determinants of the mechanism underlying acceleration of the interaction between antithrombin and factor Xa by heparin pentasaccharide

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Running title:- Interaction between factor Xa and antithrombin
Summary

The control of coagulation enzymes by antithrombin is vital for maintenance of normal haemostasis. Antithrombin requires the co-factor, heparin, in order to efficiently inhibit target proteinases. A specific pentasaccharide sequence (H5) in high-affinity heparin induces a conformational change in antithrombin that is particularly important for factor Xa (fXa) inhibition. Thus synthetic H5 accelerates the interaction between antithrombin and fXa 100-fold as compared to only 2-fold versus thrombin. We built molecular models and identified residues unique to the active site of fXa that we predicted were important for interacting with the Reactive Center Loop (RCL) of H5-activated antithrombin. To test our predictions, we generated the mutants E37A, E37Q, E39A, E39Q, Q61A, S173A and F174A in human fXa and examined the rate of association of these mutants with antithrombin in the presence and absence of H5. fXa^{Q61A} interacts with antithrombin alone with a near-normal $k_{ass}$, however, we observe only a 4-fold increase in $k_{ass}$ in the presence of H5. The X-ray crystal structure of fXa reveals that Gln^{61} forms part of the S1' and S3' pocket, suggesting that the P' region of the RCL of antithrombin is crucial for mediating the acceleration in the rate of inhibition of fXa by H5-activated antithrombin.
Introduction

The serine protease, factor Xa (fXa), is a central enzyme in the coagulation cascade. The extrinsic and intrinsic pathways converge at the point of the prothrombinase complex, of which fXa is a key component along with factor Va and phospholipids (1). The serpin antithrombin (ATIII), controls a number of important coagulation enzymes including fXa and thrombin with the aid of the co-factor, heparin (physiologically represented by heparan sulfate chains) (2). In the absence of heparin, ATIII is a relatively ineffective inhibitor of fXa and thrombin (k_{ass} [fXa] - 2.6 x 10^{3}; k_{ass} [thrombin] 1 x 10^{4}) (3). Heparin accelerates the interaction between ATIII and target proteinases by two distinct mechanisms. Firstly, long chain heparin is able to act as a "template", to which both ATIII and the proteinase bind; bringing inhibitor and proteinase into close proximity (2, 3, 4). Secondly, a specific pentasaccharide sequence present in high affinity heparin (5) is able to induce a unique conformational change throughout ATIII, culminating in exposure of the Reactive Centre Loop (RCL), the region of the serpin responsible for primary interaction with the target proteinase (6, 7, 8, 9). The "template" mechanism has been shown to be important for accelerating the interaction between ATIII and both thrombin and fXa (2, 5). In contrast, the conformational change induced by heparin pentasaccharide (H5) results in a 100-fold increase in the rate of interaction between ATIII and fXa, compared to only a 2-fold increase in the rate of interaction versus thrombin (2). Thus synthetic H5 is able to "target" ATIII to fXa, and therefore this molecule is an important potential therapeutic that has just successfully completed phase II clinical trials for treatment of deep vein thrombosis (10).

In order to understand why H5 is able to substantially accelerate the interaction between ATIII / fXa but not ATIII / thrombin, we built molecular models of these complexes and identified proteinase / serpin interactions in the fXa / ATIII complex that were absent in thrombin / ATIII complex. Our modelling studies suggested that five residues in the active
site of fXa made interactions specific to fXa / ATIII – Glu$^{37}$, Glu$^{39}$, Gln$^{61}$, Ser$^{173}$ and Phe$^{174}$ (enzyme residues numbered according to equivalent positions in chymotrypsin). Site-directed mutants of these residues in human fXa were generated and tested for their susceptibility to inhibition by ATIII in the presence and absence of H5. The results demonstrate that mutation of one residue, Gln$^{61}$, which forms part of the S1' and S3' pockets$^2$, is sufficient to almost completely abolish the H5-induced acceleration of the association rate observed between fXa and ATIII. Thus, in addition to providing a molecular explanation of the acceleration effect, our data provides the first evidence that residues C-terminal to the reactive centre of the serpin are involved in mediating the acceleration of inhibition.
Experimental Procedures

Materials

The fX activator protein (RVV-X) was isolated from Russell viper venom as described previously (11). Plasma ATIII was purified from time-expired plasma as described by Mackay et al. (12) and Nordenman et al. (13). The Boc-Ile-Glu-Gly-Arg-amidomethylcoumarin (IEGR-amc), Glu-Gly-Arg-amidomethylcoumarin (EGR-amc) and biotinylated Glu-Gly-Arg-chloromethyl ketone were supplied by Calbiochem-Novabiochem Inc (Melbourne, Vic., Australia). The monoclonal anti-human fXa antibody, methotrexate, avidin-horse radish peroxidase conjugate, horse radish peroxidase substrate and alkaline phosphatase substrate were supplied by Sigma (Sydney, NSW, Australia). The anti-human ATIII antibody was raised against purified ATIII in chickens and purified from the egg yolk as previously described for other proteins (14). Heparin pentasaccharide was from Sanofi Recherche (Toulouse, France). The HiTrap™ Q column was from Amersham-Pharmacia (Sydney, NSW, Australia).

Modelling

The structures of human fXa in complex with the inhibitor Rpr208815 (1F0R; 15), ATIII in complex with H5 (1AZX; 6), thrombin in complex with D-Phe-Pro-Arg-chloromethylketone (1ABJ; 16), trypsin in complex with ecotin (1SLU; 17) and trypsin in complex with Pancreatic Trypsin Inhibitor (2PTC; 18) were obtained from the protein data bank (19, 20). Modelling was performed using similar methods to those previously described (21, 22). Specifically, in order to model the P9-P7' of the RCL of ATIII into the active site of fXa, we first superposed the X-ray crystal structures of fXa and the trypsin / ecotin complex using a program by Arthur Lesk (23 and references contained therein). These two structures share 34% sequence identity over the proteinase domain and superpose over 208 Cα atoms.
with a root mean square (r.m.s.) deviation of 1.02 Å/ATOM. The "mutate" facility within Quanta (Accelrys Inc., San Diego, CA, USA) was used to change the sequence of the P7-P5' of ecotin (SSPVSTMMHCPV) to that of the P7-P5' region of the RCL of ATIII (AVVIAGRSLNPN). The structure of trypsin, all of the mutated ecotin apart from the P7-P5' region and the inhibitor Rpr208815 were then deleted, to leave the structure of fXa with the P7-P5' sequence of ATIII positioned in the active site. The structure of trypsin in complex with Pancreatic Trypsin Inhibitor was superposed onto the model, and this structure was used as a template to correctly position the P1 Arg of ATIII into the S1 subsite of fXa. The edit sequence facility within Quanta was also used to add additional residues (P8T, P9S, P6'R and P7'V) to the P and P' ends of our peptide. The entire model of fXa in complex with the P9-P7' sequence of the RCL of ATIII was then subjected to CHARMM minimization, first with the proteinase and the backbone atoms of the peptide constrained, then later with no constraints. Minimization was performed to convergence. A Ramachandran plot revealed all residues in our modeled RCL peptide were in allowed conformations. Similar methods were used to build the model of thrombin in complex with the P9-P7' region of the RCL of ATIII.

Subsequent to the completion of the modeling and experimental studies described, the X-ray crystal structure of the Michaelis serpin-protease complex was determined by Ye et al. (1199; 24). We therefore decided to build a complete model of the complex between H5-activated antithrombin and human factor Xa using the structure of serpin 1K in complex with rat trypsin as a template. The structure of H5-activated antithrombin was first superposed onto the serpin 1K molecule in the Michaelis serpin-protease complex using the sequence alignment and superposition facilities available in Quanta (MSI Inc, San Diego). The two structures superposed with an r.m.s. deviation of 1.3 Å over 333 Ca atoms. We then used the structure of serpin 1K as a template to build a model of the RCL of antithrombin using the homology modelling tools available in Quanta. The RCL of antithrombin is one residue
longer than that of serpin1K, and hence one residue was inserted with respect to the template after P6’. In order to accommodate this extra residue we performed a local refinement (using CHARMM) upon the region from P4’-P8’. In this way we obtained a model of antithrombin with the loop in a similar conformation to that seen in the X-ray crystal structure of the Michaelis serpin-protease complex.

We superposed the X-ray crystal structure of human factor Xa (pdb identifier 1F0R) onto the structure of rat trypsin portion of the Michaelis serpin-protease complex. The two structures superposed with an r.m.s. deviation of 0.42 Å over 183 Cα atoms. The structure of serpin1K in complex with rat trypsin was then removed to leave a model of the Michaelis complex between antithrombin and factor Xa. Initial inspection of the preliminary model revealed two minor steric clashes (the first between the sidechain of P9 and Glu 147 in factor Xa and the second between Ser 230 and Lys 148 of factor Xa). No other steric clashes between the proteinase and the body of the serpin were observed. The final model was refined (and the two clashes resolved) using CHARMM, first with the backbone residues constrained and later with no constraints.

*Mutagenesis, transfection and cell culture*

The construction of the expression system for fX using the pNut vector will be described elsewhere. The site-directed mutagenesis of the fX sequence in the pNut vector was accomplished by using the “Quik change mutagenesis kit” (Stratagene) using a PCR-based strategy. Several clones of each mutation in the pNut-fX vector were sequenced and analysed for protein expression.

Baby hamster kidney cells (BHK) were transfected with the wild type and mutant pNut-fX vectors. Stable cell lines were selected by the addition of 100 µM methotrexate to Dulbecco’s modified Eagle media (DMEM), supplemented with 5 IU/ml penicillin, 5 µg/ml
of streptomycin and 10% (v/v) fetal bovine serum. Isolated colonies were screened for fX expression by immunoassay and activity measurements. Serum free medium containing the recombinant fX protein was applied to a nitrocellulose membrane and was probed using the monoclonal anti-human fX antibody (4 µg/ml). Clones that expressed the enzyme were detected by alkaline phosphatase color development. For large scale expression of the recombinant proteins, selected stable cell lines described above were allowed to reach 80% confluence before the media type was changed to a phenol red free Dulbecco’s modified Eagle media (serum-free) which was supplemented with 5 IU/ml penicillin, 5 µg/ml of streptomycin, 100 µM methotrexate, 10 µg/ml of vitamin K1 and 50 µM zinc sulfate. The media from the cells was collected every 48 hrs for 5 collections and was stored at -20°C.

Purification of recombinant Factor X proteins.

Factor X was partially purified from the pooled media using the barium citrate purification method previously described (25). Briefly, the fX protein was precipitated by the addition of 60 mM barium chloride, followed by the addition 32 mM citrate. The resulting pellet was resuspended in 15 mM sodium citrate and was dialyzed overnight in 10 mM HEPES, 150 mM NaCl, pH 7.4. The solution was clarified by centrifugation at 5,000 x g and loaded onto a Hi-Trap™ Q column. The fX was eluted from the column using a linear gradient from 0.15 M to 1 M NaCl in 10 mM HEPES, pH 7.4 over 50 ml. Sterile glycerol (final concentration of 10% [v/v]) was added, the samples were aliquoted, snap frozen and stored at -70°C. SDS-PAGE was performed by the method of Laemmli (26). Proteins were visualized by silver staining or transferred to 0.45µm nitrocellulose, probed with a monoclonal anti-human fX antibody (4 µg/ml), and visualized with alkaline phosphatase detection.
Zymogen activation and enzyme assays

Factor X was activated by incubating with 100 nM RVV-X for 30 min at 37°C in 10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, 0.1% (w/v) PEG 8000 (25). All enzyme assays described henceforth were also performed in this assay buffer at 37°C.

The concentration of Factor Xa was determined by an active-site specific immunoassay using biotinylated Glu-Gly-Arg-chloromethyl ketone as described previously (27, 28).

The activity of fXa was routinely measured by determining the rate of hydrolysis of the substrate IEGR-amc using wavelengths of 370 nm and 460 nm for excitation and emission, respectively, over 10 min using a BMG Fluorostar plate reader. For each of the fXa mutants, the $K_m$ values for the IEGR-amc substrate were determined by monitoring the change in fluorescence over a range of substrate concentrations (usually 1-200 µM). The $K_m$ values for the EGR-amc substrate for fXa$^{S173A}$, fXa$^{F174A}$ and wild type fXa were determined by monitoring the change in fluorescence over a range of substrate concentrations (usually 1-500 µM). The initial rate of hydrolysis (ν) was plotted against the concentration of substrate and the data was fitted to the equation: $\nu = V_{\text{max}} [S] / (K_m + [S])$, where ν is the rate of change for a given concentration of substrate and [S].

Second order rate constants for the association between fXa and ATIII were measured using discontinuous assays as described by Olson et al. (29) using plasma-derived ATIII. A fixed concentration of fXa (1 nM) was incubated with 0.01 µM to 1.5 µM ATIII over various time periods. At the end of the incubation, the reaction was quenched by the addition of 80 µM IEGR-amc substrate and the residual amount of fXa activity was measured. In those reactions that contained H5, it was present at a concentrations of at least 500 nM or in 5-fold excess over the ATIII concentration, whichever was the greater value. The natural log of the residual fXa activity was plotted versus time for each concentration of ATIII, the slope of the line yielding an observed rate of inhibition, $k_{\text{obs}}$. The $k_{\text{obs}}$ values were plotted against their
corresponding ATIII concentration, and linear regression of the plots yielded the slope from which the $k_{\text{ass}}$ for the reaction was obtained.

**Stoichiometry of inhibition and analysis of the complex formation.**

The stoichiometry of inhibition (SI) was determined with a fixed concentration of fXa (10-200 nM) with increasing molar ratios of ATIII to enzyme in the presence and absence of H5. These reactions were allowed to incubate to completion and the residual fXa activity measured, following which residual activity was plotted against the molar ratio of inhibitor to enzyme. Linear regression analysis of the plotted values yields an intercept with the x-axis which in turn equates to the SI. The complexes formed between fXa and ATIII were analysed by separation on a 10% SDS-PAGE and were transferred to 0.45µm nitrocellulose, probed with a chicken anti-human ATIII antibody (7 µg/ml), and visualized by alkaline phosphatase detection.
Results

Modelling:

The hypothesis investigated in this study was that differences in the active site of thrombin and fXa render the latter enzyme more susceptible to inhibition by H5-activated ATIII. Thus, the objective of our modelling studies was to identify putative interactions between the RCL of ATIII and fXa that were absent in the thrombin / RCL model. In order to identify such residues, the two models were superposed and the RCL / active site interactions in each compared. It is important to note that residues that formed interactions with the modeled peptide, but were conserved between the two enzymes, were not targeted for site-directed mutagenesis. In addition, we did not investigate the position Gln^{192}, which has been the subject of extensive investigation by others (30). In this section, we describe the putative interactions that were identified and the residues in fXa that were targeted for subsequent site-directed mutagenesis (Fig. 1; Fig. 2A and B).

Figure 1 shows a structure-based alignment of thrombin and fXa, highlighting residues in the active site that differ between the two enzymes. Figure 2 shows stereo views of the putative fXa-specific interactions between active site residues of fXa and the RCL of ATIII revealed by the model. Thrombin contains two major insertions with respect to fXa. The 149 loop is located at the far end of the S1 specificity pocket and comprises a six residue insertion with respect to fXa (see Fig. 1). No additional interactions between this region and the modeled peptide in our fXa / RCL model were found when compared to the thrombin / RCL model. The other major insertion, the "60 loop" is replaced by a short α-helix in fXa (see Fig. 2B). Comparison of the X-ray crystal structures of thrombin and fXa reveals that Lys^{60F} protrudes into the active site in thrombin, whereas in fXa, Gln^{61} occupies an almost equivalent position in 3-dimensional space (see Fig. 2B). Furthermore, our modelling studies revealed that Gln^{61} is able to make a hydrogen bond to both the sidechains of P1'Ser and P3'Asn,
"bridging" these two residues (Fig. 2B). We predict that the Lys$^{60F}$ present in thrombin, even if optimally placed, would only be able to make one such interaction. Using the rotomer libraries available in Quanta, we also investigated whether Gln$^{61}$ was capable of making other interactions to those described above. In an alternative conformation, the ε oxygen of the sidechain of Gln$^{61}$ could form a hydrogen bond with the backbone nitrogen of the peptide bond between P1' and P2'. However, this interaction would only be predicted to result in one hydrogen bond. Again, the Lys$^{60F}$ residue present in thrombin would be unable to form such an interaction. These data suggest that Gln$^{61}$ represents a major difference in the active site of fXa as compared to thrombin. We predicted that this residue may confer an enhanced ability to interact with the RCL of ATIII and in order to test this prediction we generated the mutant, fXa$^{Q61A}$.

Our model revealed a putative salt bridge between Glu$^{37}$ of fXa and the P6' Arg (Fig. 2A). A two residue insertion in thrombin maps to this region and comparison of the structures reveal that no equivalent residue to Glu$^{37}$ exists in thrombin. Furthermore, we noted that Glu$^{39}$ of fXa, whilst not directly interacting with residues in the RCL of ATIII in our model, was only 6Å from the P4'-P5' peptide bond nitrogen with which it could potentially interact. We therefore decided to test, via site-directed mutagenesis, whether either Glu$^{37}$ or Glu$^{39}$ affected the interaction between fXa and H5-activated ATIII.

The S-side specificity pockets of both fXa and thrombin have been extensively characterised using both biochemical and structural studies. In our model, the P1-P3 residues made numerous contacts with the active site of fXa and thrombin that were conserved between both enzymes. In addition, the specificity of the S2 pocket of both thrombin and fXa has been characterized in some detail (31-33). The model indicated that the P4 Ile nestled in a deep hydrophobic pocket formed by Tyr$^{99}$, Trp$^{215}$ and Phe$^{174}$ (Fig. 2A). In contrast, the X-ray crystal structure of thrombin reveals that substitution of Tyr$^{99}$ with Leu and Phe$^{174}$ with Ile
results in a shallower and less well defined S4 pocket in this enzyme (17). Several recent crystal structures of FXa in complex with inhibitors (16, 34-37) also highlight the importance of this pocket in binding the P4 position of substrates. Previous studies by Rezaie (33) have shown that Tyr99 is critical for determining specificity at the S2 subsite of FXa. Thus in order to investigate whether the S4 pocket is important for recognizing the RCL of heparin activated ATIII, Phe174 was targeted for site-directed mutagenesis. In particular, a mutation in this position would be expected to affect specificity in the S4 pocket without altering S2 specificity.

Finally, we observed that the sidechain of Ser173 formed a hydrogen bond with the sidechain of P8 Thr of the ATIII RCL (Fig. 2A). Again, this putative interaction was absent in thrombin, Ser173 being substituted by an Arg in this enzyme (Fig. 1). In order to test whether this residue was important for the interaction between FXa and ATIII, the FXaS173A mutant was constructed.

Our model of the Michaelis complex between antithrombin and factor Xa revealed similar results to that from the model between factor Xa and the RCL peptide. Three of the four predicted interactions were present in the model of the Michaelis complex (P6’ salt bridging to Glu37, Gln61 forming a hydrogen bond to the peptide bond of P1’-P2’ and the P4 Ile buried in the pocket formed by Phe174, Tyr99 and Trp215). The exception, the interaction between Ser173 and P8 Thr, was absent in the new model, since in the serpin1K Michaelis complex the P6-P13 region forms an unusual “snake-like” conformation that forms van der Waals contacts with the 146-149 loop of trypsin (24). We did not predict this conformation in our initial modeling studies and the determination of additional X-ray crystal structures is required to support the hypothesis that this feature is common to all Michaelis serpin-proteinase complexes.
The structure of the Michaelis serpin-proteinase complex revealed few interactions between serpin and proteinase outside the RCL. Indeed the structure reveals that the proteinase is held relatively distant from the body of the serpin and intramolecular hydrogen bonds outside the RCL loop are limited to one hydrogen bond between Ser\textsuperscript{147} (trypsin) and Glu\textsuperscript{191} (serpin 1K). Similarly in our model of the Michaelis complex between antithrombin and factor Xa we see no extensive serpin / proteinase interactions outside the RCL and note only one putative hydrogen bond outside the RCL proteinase interface – an interaction between Asn\textsuperscript{233} (antithrombin) and the peptide backbone of Lys\textsuperscript{148} (factor Xa).

**Characterisation of mutant and wild type recombinant fXa**

The mutant fXa molecules were successfully expressed along with the wild type molecule in the expression system, yielding 1.6 mg/l of purified fX. The fX molecules were activated to fXa using purified Russell’s viper venom as described previously (25). The activated molecules were firstly assessed in terms of their interaction with the well characterised fXa substrate, IEGR-amc. It was established that the fXa\textsuperscript{S173A} and fXa\textsuperscript{Q61A} mutants were similar to wild type in terms of their $K_m$ and $k_{cat}$ values for the kinetics of hydrolysis of the substrate (Table 1). The fXa\textsuperscript{F174A} mutant displayed a nearly 3-fold increase in $K_m$ for the substrate and an increase in the $k_{cat}$ value. For fXa\textsuperscript{F174A}, the increase in the $K_m$ value for interaction with the IEGR-amc substrate might be expected since this residue would be predicted to interact with the P4 Ile of the substrate. The substrate EGR-amc, with the removal of the Ile residue being the only difference, was used to determine whether this was the case. As expected, the $K_m$ and $k_{cat}$ values of fXa\textsuperscript{F174A} were normal for this substrate. It should be noted that fXa\textsuperscript{S173A} was normal for the interaction with either substrate.

The initial mutants of fXa made at positions 37 and 39 (fXa\textsuperscript{E37A} and fXa\textsuperscript{E39A}) displayed up to 2- to 3-fold decreases in their $K_m$ for the IEGR-amc substrate. Values for $k_{cat}$ for this
substrate were essentially normal for fXa\textsuperscript{E37A}, while fXa\textsuperscript{E39A} had an increased $k_{cat}$ value. The fact that fXa\textsuperscript{E37A} and fXa\textsuperscript{E39A} were changed with respect to their interaction with the substrate representing P4-P1 residues made us concerned that these mutations had caused changes to the fXa active site distant from the site of the actual mutations. Glu\textsuperscript{37} and Glu\textsuperscript{39} would be anticipated to interact directly only with substrate residues C-terminal to the cleavage sites, most likely P5’ and P6’. Thus the fact that these mutations had changed interactions at P4-P1 sites led us to conclude that the mutations had caused larger scale changes to the active site of fXa than intended. We therefore also mutated these Glu residues to Gln residues. The mutants produced by this more conservative mutagenesis strategy had $K_m$ values for IEGR-amc which were still decreased in much the same way as the Ala residue mutants, fXa\textsuperscript{E37Q} had a normal $k_{cat}$ value and fXa\textsuperscript{E39Q} had a decreased $k_{cat}$ value.

**Stoichiometry of interaction between factor Xa molecules and antithrombin**

We investigated whether any of the mutant fXa molecules were altered in their interaction with ATIII with respect to the SI. In the absence of H5, all serpins displayed SI values of close to 1 for the interaction between serpin and enzyme. In the presence of H5, the SI of the wild type enzyme interacting with ATIII increased to 1.4 as has been found by others (3). The fXa\textsuperscript{S173A} and fXa\textsuperscript{F174A} mutants had very similar SI values to wild type for the interaction in the presence of H5. The fXa\textsuperscript{Q61A} mutant showed no increase in SI in the presence of H5, fXa\textsuperscript{E39A} was only slightly increased compared to when no H5 was present, fXa\textsuperscript{E37A} and fXa\textsuperscript{E39Q} had increased SI values in the presence of H5, although still below that found for wild type, while fXa\textsuperscript{E37Q} had a higher SI than wild type in the presence of H5. Western blotting of complexes between ATIII and the mutant or wild type fXa molecules showed no significant increase in the appearance of cleaved ATIII when the serpin interacted with the mutant molecules in the presence of H5, compared to the wild type enzyme (results not shown).
Second order association rates with H5-activated ATIII

Determination of the interaction between ATIII, with and without H5 activation, was undertaken to examine whether any of the mutants did indeed have lowered acceleration of their interaction rates. Most of the mutant fXa molecules had slightly lowered $k_{ass}$ values with ATIII alone (Table 2), indicating that all of the residues might make minor interactions with the corresponding residues in the RCL of ATIII alone. Upon activation with H5, it was evident that fXa$^{S173A}$ and fXa$^{F174A}$ mutants were essentially normal in terms of the increase in $k_{ass}$ with H5-activated ATIII, with both mutants having close to a 100-fold increase in the $k_{ass}$ values. The fXa$^{E37A}$ and fXa$^{E39A}$ mutants were both significantly decreased in terms of the acceleration induced by H5 activation, fXa$^{E37A}$ being decreased approximately 2-fold in terms of the acceleration achieved, while fXa$^{E39A}$ was 2.7-fold decreased. The more conservative Gln mutants at these positions were actually increased in terms of the acceleration achieved, particularly fXa$^{E39Q}$, which was 1.7-fold increased in terms of the acceleration achieved. However, the most effective mutation made was the Q61A substitution. The increase in $k_{ass}$ induced by H5 activation of ATIII was only 4.5 fold for this mutant, which indicates that a very important additional interaction mediated by this residue with the RCL of H5-activated ATIII was all but abolished.
Discussion

The structural basis underlying the well characterized allosterically-mediated increase in interaction between H5-activated ATIII and fXa has proved somewhat elusive. The crystal structures of ATIII alone and in complex with the H5 have been solved (38, 39, 6), but in each case the serpin forms a dimer with another ATIII molecule, in the so-called latent conformation, in which the RCL of the native ATIII is “docked” into the Cβ-sheet of the latent molecule. The RCL of ATIII clearly changes conformation upon binding H5 from a partially inserted form in the absence of heparin to a fully expelled position, but the consequences of this to the rest of the RCL are not evident due to the constraints imposed on the RCL of the native molecule by its interaction in the dimer form. It has been postulated that the RCL of ATIII changes conformation upon binding H5 to a "canonical" conformation similar to that seen in the crystal structure of the related serpin, α1-antitrypsin (8, 7, 40).

Recent evidence suggests that while P1 Arg of ATIII may indeed re-orientate upon binding H5, this alone does not explain the increased rate of interaction between the serpin and fXa (41, 42).

Here we have modeled the interaction between the RCL of ATIII and fXa and compared these data to a thrombin / RCL model. In particular, we aimed to identify residues in the active site of fXa that made specific interactions not present in the thrombin / RCL model. We reasoned that such interactions might mediate the unique acceleration in interaction seen between H5-activated ATIII and fXa, which is not observed with thrombin. Using this approach, we were able to identify a number of putative interactions that were unique to the model of fXa in complex with the RCL of ATIII. Our predictions were tested by mutating the active site residues of fXa to alanine residues to abolish such interactions. Support for a putative interaction would be provided by the mutant enzyme no longer displaying a marked increase in the second order rate constant for association with ATIII upon H5 activation. This
should also be further supported by evidence that the mutation has not caused a widespread change to the active site of the protease which may lead to “false positives”, where the increase in the association rate constant is no longer seen for structural reasons, rather than the loss of a specific interaction. This was tested by carefully examining the interaction of the enzyme with peptide substrates. Our expectation at the outset of the studies was that the increase in association would be mediated by a number of interactions, which would have a cumulative effect.

Our data reveal that much of the increase in association appeared to be mediated by a localised interaction, namely that between the Gln^{61} residue of fXa and the RCL of ATIII. Thus fXa^{Q61A} had a 22-fold lower increase in $k_{ass}$ for the interaction with ATIII in the presence of H5. While the SI for inhibition of the mutant by ATIII was normal in the absence of H5, it showed no increase in the presence of H5 as seen for wild type enzyme. An increase in the SI for the interaction between ATIII and fXa in the presence of H5 has been noted previously by other investigators (3) and was postulated to be related to the extra interactions mediated by H5 activation retarding insertion of the RCL into the A-sheet of the serpin. This would then shift the balance between the inhibitory and substrate pathways of the serpin slightly towards the substrate pathway and thus yield the increase in SI. The lack of an increase in SI for the fXa^{Q61A} mutant might therefore be attributed to the abolition of the major extra interaction mediated by H5 activation and thus this meshes well with the lack of acceleration in the association rate constant seen with this mutant. The mutant enzyme appeared to be normal in other respects, as judged by a normal affinity of interaction with the IEGR-amc substrate, ruling out possible contribution by gross structural changes to the active site. Analysis of the X-ray crystal structure of fXa, and our modelling data, revealed that Gln^{61} forms part of the S1’ and S3’ subsites. In particular, we predict that this residue is capable of forming hydrogen bonds to the sidechains of P1’ Ser and P3’ Asn, or alternatively
forming a hydrogen bond to the backbone nitrogen atom of the peptide bond between P1' and P2'. These data strongly indicate that allosteric activation of ATIII by H5 has the major effect of bringing the P' region of the RCL of antithrombin into contact with the Gln\textsuperscript{61} residue in the active site of the enzyme.

Less dramatic changes were observed for the other mutants of fXa. The fXa\textsuperscript{S173A} and fXa\textsuperscript{F174A} mutants were close to normal in terms of the acceleration mediated by H5 binding. The fXa\textsuperscript{F174A} mutant did have decreased affinity for the IEGR-amc substrate, but this was most likely due to the loss of the specific interaction with the P4 Ile residue, as demonstrated by the normal affinity of the enzyme for the EGR-amc substrate. The mutants at the Glu\textsuperscript{37} and Glu\textsuperscript{39} were more complicated to analyse, since substitution of an alanine or glutamine residue at these positions appeared to mediate an increase in the affinity of these enzymes for the IEGR-amc substrate and mixed effects on the rate of hydrolysis. This finding was unexpected, since these residues are on the opposite side of the active site to the subsites that interact with the P4-P1 positions of a substrate. Thus, the small decrease or increases in acceleration of association mediated by the H5 for these mutants should be viewed with caution, since it might be reflective of changes to the active site architecture, rather than affecting a specific interaction. Overall, the data for mutants at these positions suggest that there might be some interactions being mediated by these residues in the active site of fXa, but these appear limited compared to the interactions being mediated by Gln\textsuperscript{61}, where a 22-fold effect was observed, compared to 1-3-fold effects for the Glu\textsuperscript{37} and Glu\textsuperscript{39} mutants. However, these data lend support to our hypothesis that the P' region of antithrombin is at least in part responsible for mediating the enhanced interaction seen between fXa and H5-activated ATIII.

The results from this study were not only surprising in terms of the localization of the change in interaction between fXa and ATIII activated by H5, they are also somewhat
contradictory to previous studies on this topic. Theunissen et al. (43) showed that substitutions at the P1’ and P3’ positions of the RCL of ATIII markedly decreased the interaction of ATIII with thrombin in the presence of heparin, but had only moderate effects on fXa interactions with ATIII in the presence of heparin. A more recent study by Chuang et al. (44) found that the RCL residues P6-P3’ of ATIII did not contribute substantially to the increase in association seen with H5-activated ATIII and fXa, although it must be noted that no substitutions were made at P1’ position. Rezaie (45) recently showed that a mutant of ATIII which had substituted the P4-P4’ region of ATIII with the prothrombin activation sequence (IEGR-IVEG) still had normal, if not increased acceleration of its interaction with human fXa in the presence of H5, while its interaction with human thrombin was markedly decreased. The modelling study described here suggests that Gln^{61} in fXa is capable of making hydrogen bonds to the sidechains of the P1’ Ser and P3’ Asn residues in the RCL of ATIII. This would be difficult to explain in the light of the results obtained in the above studies, however, leading us to favor the hypothesis that Gln^{61} might in fact be making a hydrogen bond to the peptide bond between P1’ and P2'. This would mean that mutagenesis studies conducted on the RCL of ATIII would not have any effect on the interactions seen, as found in the above three studies. Therefore the only way to examine the interaction would be to make mutants of fXa, as carried out here.

Most recently, Rezaie (46) has conducted a study in which it was found that a mutant of antithrombin with a two residue deletion in the P’ portion of the RCL of the molecule was activated for inhibition of fXa by about 12-fold without the aid of H5. Conversely, this antithrombin mutant was 12-fold decreased in its inhibition of thrombin. Together, this lead the author to speculate that the mutant antithrombin molecule no longer had a fully loop-inserted conformation in the absence of H5 and that the resulting change in the conformation of the RCL played a large role in activating the mutant towards fXa inhibition. This study
tends to substantiate our hypothesis that it is the conformation of the RCL of antithrombin which plays the dominant role in determining whether the serpin interacts efficiently with fXa.

Recent studies of the interaction between H5-activated antithrombin and fXa (44) have suggested the possibility that an exosite on antithrombin is exposed upon binding H5 and that it is the exosite which provides the additional interaction mediating the acceleration of the interaction mediated by H5. The X-ray crystal structure of the Michaelis complex between serpin1K and rat trypsin (24) revealed that the majority of serpin/proteinase interactions occur between the RCL of the serpin and the proteinase. From our modeling studies we predict a similar paucity of extra-RCL interactions in the complex between fXa and antithrombin. Analysis of the model of the Michaelis complex between antithrombin and factor Xa reveal that the nearest non-RCL sidechain atom is over 8 Å away, suggesting that it is unlikely that Gln\textsuperscript{61} itself is acting as an exosite. Thus we suggest that the simplest explanation of our data is that Gln\textsuperscript{61} plays an crucial role in mediating the interaction with H5-activated antithrombin by forming hydrogen bonds with the P’ region of the RCL. However, we cannot exclude the possibility that an antithrombin specific exosite on factor Xa does exist – for example the complex between factor Xa and antithrombin may adopt a strikingly different conformation to that seen in the complex between trypsin and serpin 1K. Alternatively, an exosite may come into play after initial docking during the conformational change to the final complexed state.

Heparin pentasaccharide has just progressed successfully through phase II clinical trials for use as an antithrombotic compound, thus understanding the mechanism of action of this compound would seem to be vital in this context and also in furthering our knowledge of this complex system. The H5 is thus far thought to act mostly through its ability to mediate the increased association of ATIII with fXa. In light of this, the illustration here of potential interactions underlying the mechanism of action whereby H5-activated ATIII has a strongly
increased association with fXa is of great interest. Further work involving mutation of putative complementary residues in the ATIII RCL and closer examination of the encounter complex between serpin and enzyme, using appropriate mutants, will further augment our growing understanding of this vital control system.

Acknowledgements

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   3226-3232


Footnotes

1 Abbreviations used: fXa, factor Xa; fX, factor X; ATIII, antithrombin; H5, heparin pentasaccharide; RCL, reactive center loop; RVV-X, Russell’s viper venom factor Xa activator; IEGR-amc, Boc-Ile-Glu-Gly-Arg-amidomethylcoumarin; EGR-amc, Glu-Gly-Arg-amidomethylcoumarin; r.m.s., root mean square; DMEM, Dulbecco’s modified Eagle medium; $k_{ass}$, second order rate constant for association; $k_{obs}$, observed rate value; SI, stoichiometry of inhibition.

2 Subsites and RCL residues are numbered according to the convention of Schechter and Berger (47); a protease cleaves at the P1-P1' peptide bond in a substrate; residues N-terminal to the cleavage point are labeled P1, P2, P3 etc. counting outwards from the cleavage point, while residues in the substrate C-terminal to the cleavage point are labeled P1', P2', P3' etc. Corresponding subsites in the enzyme are labeled S3-S3'.

3 Bianchini et al., manuscript in preparation.
Figure Legends

Fig. 1. **Structure-based sequence alignment of factor Xa and thrombin.**

The sequence alignment between factor Xa and thrombin is based upon a structural comparison of the two enzymes performed using a program by Lesk (23). Thrombin contains several insertions with respect to factor Xa that are not structurally equivalent - these amino acids are contained in parenthesis in the sequence of factor Xa. Identical residues are shaded pink. Amino acids that differ between the two enzymes are shaded in green, or cyan if they were mutated in this study. The "60-loop" and "149-loop" are underlined and labeled. The conventional numbering scheme for factor Xa is based upon chymotrypsin numbering and is shown above the sequences. Insertions with respect to chymotrypsin are marked "-1" and deletions "+1".
Fig. 2. Interactions between residues in the active site of factor Xa and the reactive center loop of antithrombin as predicted by computer modeling studies.

A, Model of the complex between human factor Xa (grey) and the RCL of antithrombin (red). The sidechain atoms of the P8, P4, P1, P3' and P6' residues are in green ball and stick and are labeled. The P1 residue is shown in yellow ball and stick. Residues that were targeted for site-directed mutagenesis in this study are in cyan ball and stick and are labeled. Hydrogen bonds are represented by dashed black lines. The two residues (Tyr<sup>99</sup> and Trp<sup>215</sup>) that together with Phe<sup>174</sup> form the S4 specificity pocket are shown in magenta ball and stick.

B, Stereo diagram showing the predicted interaction between residue Gln<sup>61</sup> and the sidechain of P1' and P3'. Residues P1-P4' of the RCL of antithrombin are in green stick. The P1, P1' and P3' residues are labeled. Shown in red is the "60 loop" of factor Xa, with Gln<sup>61</sup> in red stick. The two predicted hydrogen bonds to the sidechain of P1' and P3' are represented by black dashed lines. We predict that Gln<sup>61</sup> may be able to form an alternative conformation which is shown in magenta. In this conformation we predict that Gln<sup>61</sup> is able to form a hydrogen bond to the backbone N of P1' (represented by the blue dashed line). The "60-loop" of thrombin is shown in grey in the position that it occupies when thrombin and factor Xa are superposed. The 60F lysine residue can be seen snaking into the active site, occupying a similar position to the sidechain Gln<sup>61</sup> in factor Xa. The figure was prepared using Molscript (48).
Table 1: The kinetics of the wild type and mutant Factor Xa proteins with peptide substrates.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IEGR-amc</td>
<td>IEGR-amc</td>
<td>EGR-amc</td>
<td>EGR-amc</td>
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<tr>
<td>Wild type</td>
<td>39 ± 4</td>
<td>1.4 ± 0.13</td>
<td>324 ± 42</td>
<td>2.7 ± 0.2</td>
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<tr>
<td>Q61A</td>
<td>38.7 ± 4.5</td>
<td>1.3 ± 0.07</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>S173A</td>
<td>50 ± 8</td>
<td>1.0 ± 0.09</td>
<td>345 ± 58</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>F174A</td>
<td>113 ± 16</td>
<td>2.3 ± 0.25</td>
<td>309 ± 49</td>
<td>2.3 ± 0.16</td>
</tr>
<tr>
<td>E37A</td>
<td>11.3 ± 3</td>
<td>1.6 ± 0.05</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>E37Q</td>
<td>15 ± 1.3</td>
<td>1.5 ± 0.05</td>
<td>n/d</td>
<td>n/d</td>
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<tr>
<td>E39A</td>
<td>18 ± 3</td>
<td>1.9 ± 0.09</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>E39Q</td>
<td>16 ± 2</td>
<td>1.1 ± 0.03</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

n/d = not determined.
Table 2: Second order rate constants for the interaction between ATIII and mutant Factor Xa in the presence or absence of H5.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>SI</th>
<th>$k_{\text{ass}}$ (x 10^3 M^{-1}.s^{-1})</th>
<th>SI with H5</th>
<th>$k_{\text{ass}}$ with H5 (x 10^3 M^{-1}.s^{-1})</th>
<th>Increase in $k_{\text{ass}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.0</td>
<td>2.6</td>
<td>1.4</td>
<td>255</td>
<td>100</td>
</tr>
<tr>
<td>Q61A</td>
<td>1.05</td>
<td>1.1</td>
<td>1.0</td>
<td>4.9</td>
<td>4.5</td>
</tr>
<tr>
<td>S173A</td>
<td>1.0</td>
<td>1.5</td>
<td>1.4</td>
<td>125</td>
<td>83</td>
</tr>
<tr>
<td>F174A</td>
<td>1.0</td>
<td>1.3</td>
<td>1.5</td>
<td>145</td>
<td>112</td>
</tr>
<tr>
<td>E37A</td>
<td>1.03</td>
<td>1.0</td>
<td>1.2</td>
<td>54</td>
<td>54</td>
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<tr>
<td>E37Q</td>
<td>1.02</td>
<td>0.5</td>
<td>1.6</td>
<td>85</td>
<td>170</td>
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<tr>
<td>E39A</td>
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<td>3.2</td>
<td>1.1</td>
<td>119</td>
<td>37</td>
</tr>
<tr>
<td>E39Q</td>
<td>1.01</td>
<td>1.9</td>
<td>1.3</td>
<td>230</td>
<td>121</td>
</tr>
</tbody>
</table>

All values shown had standard errors of less than 10%.
**Fig. 1**

| Factor Xa | 16 | I V G G Q E C K D G E C P W Q A L L I N E ( E N E ) G F C G G T |
| Thrombin | 40 | I V E G S D A E I G M S P W Q V M L F R K S P Q E L L C G A S |
| Factor Xa | 60 | I L S E F Y I L T A A H C L ( Y Q A K ) R F K V R |
| Factor Xa | 80 | Y G D R N T E Q E E L G O E A V H E V E V V I K H N R F T K |
| Thrombin | 100 | I G K H S R T R Y R E R N I E K I S M L E K I Y I H P R Y N W R |
| Factor Xa | 120 | E T Y D F D I A V L R L K T P I T R M N V A P A C L P E R D |
| Thrombin | 140 loop | E N L D R D I A L M K L K K P V A F S D Y H P V C L P D R E |
| Factor Xa | 140 | W A E S T L M T Q K T G I V S G F G R T H E K ( G ) R |
| Factor Xa | 160 | Q S T R L K M L E V P Y V D R N S C K L S S S F I I T Q N M F |
| Thrombin | 180 | Q P S V L Q V V N L P I V E R P V C K D S T R I R I T D N M F |
| Factor Xa | 200 | C A G Y D ( T K Q ) E D A C Q G D S G G P H V T R F K D |
| Factor Xa | 240 | T Y F V T G I V S W G E O C A R K G K Y G I Y T K V T A F L K |
| Thrombin | | R W Y Q M G I V S W G E G C D R D G K Y G F Y T H V F R L K K |

| Factor Xa | 240 | W I D R S M K T |
| Thrombin | | W I Q K V I D Q |
Fig. 2A
Molecular determinants of the mechanism underlying acceleration of the interaction between antithrombin and factor Xa by heparin pentasaccharide
Noelene S. Quinsey, James C. Whisstock, Bernard F. Le Bonniec, Virginie Louvain, Stephen P. Bottomley and Robert N. Pike

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