# Substrate-Dependent Modulation of the Mechanism of Factor XIa Inhibition

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ABSTRACT: Factor XIa is a serine protease which participates in both the extrinsic and intrinsic pathways of blood coagulation. In this work we used active site directed inhibitors to study the mechanism of factor IX activation by factor XIa. To this end, we developed a new sensitive method for the detection of factor IXa based on its affinity to antithrombin III. Using this assay, we found that the peptidic inhibitors, leupeptin and aprotinin, exhibited similar potencies in inhibiting factor IX activation and the cleavage of a tripeptidic chromogenic substrate by factor XIa. As expected, leupeptin and aprotinin were competitive with respect to the tripeptidic chromogenic substrate. However, the inhibition of factor IX activation was best described by mixed-type inhibition with the affinity of leupeptin and aprotinin to the factor XIa−factor IX complex only ∼10-fold lower than their affinity toward factor XIa. These results, consistent with previous factor XI domain analyses, suggest that the active site of factor XIa does not contribute significantly to the affinity of factor XIa toward factor IX. The competitive component of the inhibition of factor IX activation suggests that binding of factor IX to factor XIa heavy chain affects the interactions of leupeptin and aprotinin with the active site.

Factor XI (FXI)[1] was traditionally viewed as part of the contact activation system of blood coagulation. More recently, however, it has been shown that FXI can serve as part of an amplification process following the activation of the more physiological extrinsic pathway (see refs *1*−*3* for recent reviews). The activation of FXI by thrombin enables the formation of additional thrombin even after the factor VIIa pathway is shut down by the tissue factor pathway inhibitor (*4*−*6*). The result of this extra thrombin formation is a more rapid clot formation as well as an increase in clot resistance to fibrinolysis. The latter is believed to be mediated by the thrombin-activatable fibrinolysis inhibitor (*7*).

FXI is a 160 kDa homodimer which is cleaved, upon activation, to form a heavy and a light chain. The heavy chain contains four apple domains which mediate FXIa interactions with other coagulation proteins as well as with negatively charged surfaces and platelets (*8*−*11*). The light chain contains the serine protease domain of FXI. Factor IX (FIX) is apparently the physiologically important substrate of FXIa. The binding of FIX to FXIa is mediated by the second or third apple domain (or both) (*8*, *11*). The light chain in the absence of the apple domains has a very weak affinity toward FIX (*12*, *13*). FXIa can also cleave, albeit at a much slower rate, FXI and high molecular weight kininogen (*14*, *15*). The physiological importance of these reactions, however, is not clear. The activation of FIX by FXIa is believed to be restricted to the site of clot formation because of the high affinity of FXIa to activated platelet surface and the enhanced rate of FXI activation by thrombin on the activated platelet surface (*16*−*18*).

FXIa is unique among other coagulation serine proteases because it lacks a gla domain and it forms a dimer, which is capable of efficient activation of FIX in the absence of negatively charged surface. To gain a better understanding of the mechanism of FIX activation by FXIa, we chose to use active site directed inhibitors. To obtain high-quality data on the effect of FXIa inhibitors on FIX activation, we developed an ELISA-based assay to quantitatively measure the activation of FIX by FXIa. Using this assay, we analyzed the effect of the active site directed peptides, leupeptin and aprotinin, on FIX activation and compared it to their effect on FXIa amidolytic activity with tripeptidic chromogenic substrates. We found that both peptide inhibitors were equally potent in inhibiting FIX activation and amidolytic activity. Interestingly, while leupeptin and aprotinin displayed competitive inhibition of FXIa amidolytic activity, the inhibition of FIX activation was best described by a mixed-type mechanism. This observation suggests that while the active site of FXIa does not contribute significantly to the affinity of FIX to FXIa, binding of FIX to the heavy chain affects the binding of inhibitors to the active site.

## EXPERIMENTAL PROCEDURES

*FIX Activation by FXIa.* FIX activation by FXIa was carried out at room temperature in a buffer containing 50 mM HEPES, pH = 7.4, 145 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, PEG 8000 (0.1%), and BSA (0.1%). The reaction (50 $\mu$L) was carried out in a 96-well polypropylene plate for 15−60 min. FIX and FXIa (Haematologic Technologies Inc.) concentrations varied from 20 nM to 2 $\mu$M and from

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[1] Abbreviations: FXI, factor XI; FXIa, factor XIa; FX, factor X; FXa, factor Xa; FIX, factor IX; FIXa, factor IXa; ATIII, antithrombin III; PBS, phosphate-buffered saline; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HK, high molecular weight kininogen.

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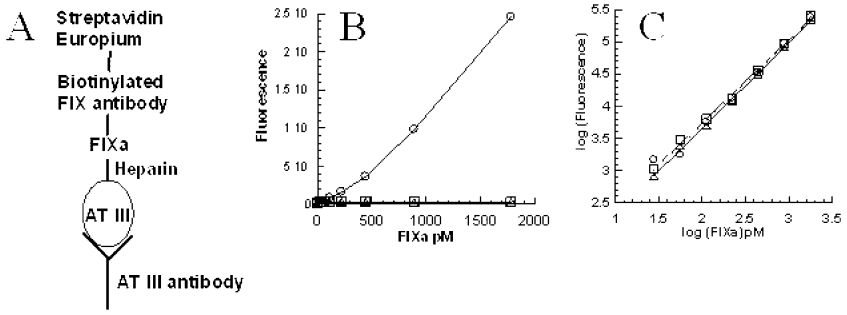



FIGURE 1: FIXa ELISA. (A) Schematic depiction of the FIXa ELISA. (B) FIXa dose−response curve: circles, in the presence of all assay components; squares, without ATIII; triangles, without heparin. (C) Effects of FIX and FXIa on the FIXa ELISA: circles, FIXa alone; squares, FIXa and 4 nM FIX; triangles, FIXa and 15 pM FXIa (the highest concentration present in the ELISA assay after quenching and dilution of the FIX activation assay).

15 to 500 pM, respectively. Under these conditions, less than 20% of FIX was converted to FIXa during the experiments, ensuring constant reaction velocity throughout the experiment. After incubation, the reactions were stopped by a 5-fold dilution into quenching buffer: phosphate-buffered saline (PBS) containing 0.05% Tween 20, 0.1% BSA, 1 unit/mL heparin, and 2 mM EDTA. The samples were then further diluted in quenching buffer to give a FIX concentration of 4 nM (based on the initial concentration) and assayed by ELISA.

*FIXa ELISA.* A high-binding Costar 9018 plate was coated overnight at 4 °C with 100 $\mu$L of anti-antithrombin III antibody (Enzyme Research Laboratories; 10 $\mu$g/mL in PBS). The plate was then blocked for 2 h at room temperature with 200 $\mu$L of 5% BSA (ICN) in PBS. After blocking, the plate was incubated for 1 h at room temperature with 100 $\mu$L of antithrombin III (ATIII, 10 $\mu$g/mL; Haematologic Technologies Inc.). One hundred microliters of sample in quenching buffer was added to each well. A standard curve of FIXa in the presence of 4 nM FIX was generated on each plate. Samples were incubated for 2 h at room temperature to allow capture of FIXa by the immobilized ATIII. The bound FIXa was then detected by incubating with biotinylated FIX antibody (5041, Haematologic Technologies Inc.; 0.4 $\mu$g/mL, biotinylated for 3 h at room temperature using 60-fold molar excess of aminohexanoyl-biotin *N*-hydroxysuccinimide from Zymed) for 1 h at room temperature, followed by incubation with Eu-labeled streptavidin (Perkin-Elmer) according to instructions supplied by the manufacturer. Plates were read using a Wallac 1420 VICTOR$^2$ plate reader.

*Amidolytic Activity of FXIa.* Amidolytic activity was assayed using S-2366 (pyroGlu-Pro-Arg-pNA; Chromogenix). Reactions were performed in a 3474 Costar assay plate using the assay buffer used for the FIX activation assay but without $CaCl_2$. S-2366 concentration was 31 $\mu$M to 2 mM. Reactions were initiated with the addition of FXIa (200 pM) and followed for 30 min at room temperature using a spectrophotometer plate reader (spectraMAX plus; Molecular Devices) at OD = 405 nm.

*Kinetic Analysis.* Reaction velocity was calculated by dividing the concentration of FIXa by the reaction time. Since less than 20% of FIX is converted to FIXa and time course analysis revealed linear reaction progression (Figure 2A), reaction velocity is constant throughout the experiment. Michaelis−Menten analysis of FIX dose−response data at fixed inhibitor concentration was performed using the equation $v = V_{max}[S]/(K_m + [S])$. Global fitting of all inhibitor concentrations was done using the Grafit program (Erithacus) assuming mixed-type inhibition with floating Hill coefficient as follows: $v = V_{max}[S]/(K_m(1 + ([I]/K_i)^n) + [S](1 + ([I]/K_{i'})^n))$, where $v$ = reaction velocity, $V_{max}$ = maximal reaction velocity, [S] = substrate concentration, $K_m$ = Michaelis−Menten constant, [I] = inhibitor concentration, $K_i$ = inhibitor−enzyme dissociation constant, $K_{i'}$ = inhibitor−enzyme substrate complex dissociation constant, and $n$ = Hill coefficient.

## RESULTS

*Development of a FIXa-Sensitive ELISA Assay.* To analyze the activation of FIX by FXIa, we needed a sensitive detection system that can process multiple samples simultaneously. The two most commonly used assays for the detection of FIX activation use radiolabeled activation peptide (*19*) or coupled tenase assay (*20*). Each of these assays has a number of significant drawbacks. The most important ones are the incompatibility of the activation peptide assay with processing multiple samples and the inability to distinguish between factor Xa and FXIa inhibition with the coupled tenase. To overcome these limitations, we decided to exploit the high affinity of FIXa for ATIII to develop a sensitive detection system for FIXa which discriminates between FIX and FIXa (Figure 1A). Briefly, ATIII is immobilized on a multiwell plate using ATIII-specific antibody. A sample containing a mixture of FIX and FIXa (resulting from activation by FXIa) is then added. In the presence of heparin, which is required for efficient interaction of FIXa with ATIII, FIXa is captured while FIX is washed away. The captured FIXa is quantified using a biotin-labeled FIX/FIXa specific antibody and streptavidin−europium fluorescence detection. This assay can detect FIXa with high sensitivity (Figure 1B). No signal is generated in the absence of ATIII or heparin, which is required for efficient interaction between FIXa and ATIII (Figure 1B). As has been commonly observed in ELISA assays, the signal grows exponentially with FIXa concentration. This probably results from the binding and enzymatic amplification steps following the binding of FIXa to ATIII. Plotting the fluorescence signal versus FIXa concentration on a logarithmic scale reveals a linear concentration response from 20 pM to 2 nM FIXa (Figure 1C). This sensitivity is much better than the previously described activation peptide assay (*21*; see Discussion). FXIa which, theoretically, can compete with FIXa for ATIII binding does not interfere with FIXa detection (Figure 1C). Because excess FIX generates some

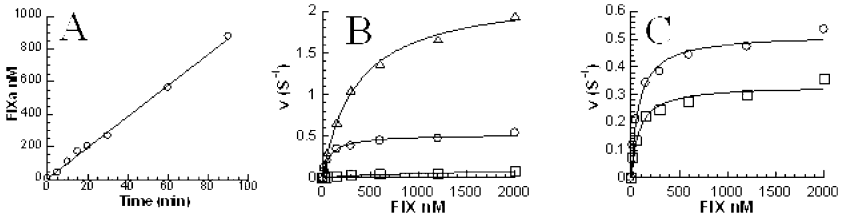
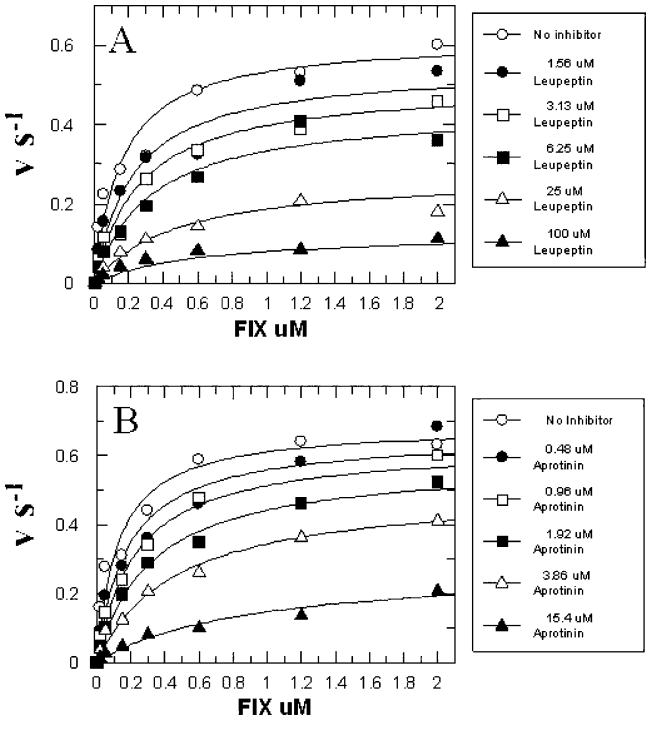



FIGURE 2: Kinetic analysis of FIX activation. (A) Time course. FXIa and FIX concentrations are 250 pM and 2 $\mu$M, respectively. The reaction was carried out at 22 °C. (B) Effect of temperature and $Ca^{2+}$: circles, 22 °C; squares, 22 °C without $Ca^{2+}$; triangles, 37 °C. (C) Effect of HK on FIX activation (22 °C): circles, no HK; squares, in the presence of 0.3 $\mu$M HK.

background, samples are adjusted to a final FIX concentration of 4 nM or less at which the background effect is minimal (Figure 1C). Overall, the FIXa detection assay yielded consistent results with small variations between similar independent experiments (see, for example, the kinetic parameters of FIX activation by FXIa below).

*Characterization of FIX Activation Using the FIXa ELISA.* As the developed FIXa ELISA is not a continuous assay, we had to establish that a single time point can reflect the steady-state reaction velocity. To this end, we mixed FXIa and FIX and sampled the reaction at multiple time points up to 90 min (Figure 2A). Indeed, the reaction rate appeared to be constant throughout the experiment, suggesting no loss of enzyme activity and lack of significant product inhibition. We then studied the kinetics of FIX activation by FXIa. A FIX concentration−response experiment (Figure 2B) showed a saturatable formation of FIXa at room temperature (22 °C). Michaelis−Menten analysis of the data gave a $K_m$ value of 151 nM ± 42 and $V_{max}$ value of 0.66 $s^{-1}$ ± 0.11 (average and standard deviation of six independent experiments). In the absence of calcium ions, FIX activation was barely detectable (Figure 2B), consistent with previous observations (*12*, *13*) and indicating that efficient quenching of the reaction is achieved by the addition of EDTA. When the reaction was performed at 37 °C, a 5−6-fold increase of both $K_m$ and $V_{max}$ was observed (Figure 2B). Overall, the kinetic parameters observed were well within the range of values reported earlier using radiolabeled activation peptide or coupled assays (*13*, *21*−*23*). Since lower FIX concentrations were required for saturating FXIa activity at room temperature, we decided to perform our mechanistic analyses at room temperature rather than at 37 °C. As FXI (and probably FXIa) is circulating bound to high molecular weight kininogen (HK), we tested the effect of HK on FIX activation (Figure 2C). We consistently observed a small but reproducible decrease in $V_{max}$ when HK was present. The average decrease in $V_{max}$ was 33% (four experiments). The presence of HK, however, did not change inhibitor potency (not shown), and further analyses were performed in the absence of HK.

*Inhibition of FIX Activation by Leupeptin and Aprotinin.* To gain a first insight into the inhibition of FXIa by reversible active site directed inhibitors, we initially tested a number of commercially available serine protease inhibitors. Two of them, leupeptin and aprotinin, were found to be potent inhibitors of FXIa without interfering with the FIXa ELISA even at their highest tested assay concentrations (20 and 3 $\mu$M, respectively; not shown). Leupeptin is a small, tripeptide aldehyde, serine protease inhibitor. Aprotinin is a larger peptide of 6.5 kDa which also occupies the active site of the target protease but may interact with FXIa outside the active site. We studied the effect of several concentrations of both inhibitors on the activation of FIX.

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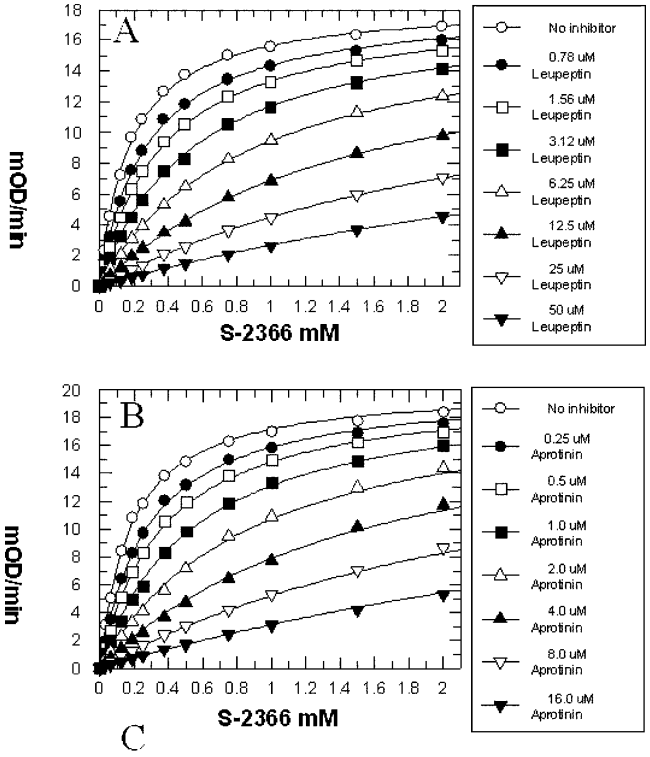
| | Leupeptin | Aprotinin |
|---|---|---|
| $V_{max}$ $S^{-1}$ | 0.62 ± 0.029 | 0.69 ± 0.023 |
| $K_m$ nM | 157 ± 28 | 125 ± 18 |
| $K_i$ $\mu$M | 2.91 ± 1.13 | 0.96 ± 0.23 |
| $K_{i'}$ $\mu$M | 19.12 ± 6.33 | 10.42 ± 3.18 |
| Hill coefficient | 0.81 ± 0.073 | 1.01 ± 0.084 |

FIGURE 3: Global fit Michaelis−Menten analysis of the effect of leupeptin and aprotinin on FIX activation. (A) Leupeptin. (B) Aprotinin. (C) The global fit kinetic parameters $K_m$ and $V_{max}$ are given for the uninhibited state of FXIa. The data are an average of two independent experiments.

The data from all inhibitor concentrations were then globally fitted to noncompetitive, competitive, and mixed-type inhibition equations, allowing the Hill coefficient to float. The global fit analysis results (Figure 3), for both inhibitors, are most consistent with mixed-type inhibition with a 7−11-fold lower inhibitor affinity to the FXIa−FIX complex ($K_{i'}$) than to free FXIa ($K_i$). Head to head *F*-test comparison of the mixed-type model versus purely noncompetitive and competitive models yielded *P* values of 0.0254 and 0.0066, respectively, for leupeptin and 0.0001 and 0.0134 for aprotinin. The uninhibited $K_m$ and $V_{max}$ values obtained from the global fits for each inhibitor are similar to the values observed in the absence of inhibitors (Figure 3C and above).

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| | Leupeptin | Aprotinin |
|---|---|---|
| $V_{max}$ mOD/min | 18.44 ± 0.07 | 20.17 ± 0.01 |
| $K_m$ μM | 179 ± 2.6 | 181 ± 3.7 |
| $K_i$ μM | 1.43 ± 0.03 | 0.52 ± 0.01 |
| $K_{i'}$ μM | 209 ± 56 | ∞ |
| Hill coefficient | 0.96 ± 0.01 | 0.96 ± 0.01 |

FIGURE 4: Global fit Michaelis−Menten analysis of the effect of leupeptin and aprotinin on FXIa amidolytic activity. (A) Leupeptin. (B) Aprotinin. (C) The global fit kinetic parameters $K_m$ and $V_{max}$ are given for the uninhibited state of FXIa. The data are an average of two independent experiments.

The Hill coefficient for each inhibitor was close to 1, consistent with lack of cooperativity between FXIa subunits and reversible inhibition. When we fitted the data from each inhibitor concentration, individually, an increase in $K_m$ and a decrease in $V_{max}$ were observed for each inhibitor as its concentration was increased (not shown), again consistent with a mixed-type inhibition mechanism.

*Inhibition of FXIa Amidolytic Activity by Leupeptin and Aprotinin.* Active site directed inhibitors, such as leupeptin and aprotinin, are expected to inhibit in a purely competitive manner. The observation that this was not the case for FIX activation prompted us to test the effect of these inhibitors on a small chromogenic substrate whose binding to FXIa is likely entirely mediated by interactions within the FXIa active site. To this end, we monitored FXIa amidolytic activity in the presence of varying concentrations of leupeptin and aprotinin and the well-characterized peptidic substrate pyroGlu-Pro-Arg-pNA (S-2366). Reaction velocity was calculated from a linear regression using the data points measured every 15 s for 30 min (no curvature was observed in the presence or absence of the inhibitors). As done for the FIX activation assay, the entire data set was fitted to the different inhibition models, allowing the Hill coefficient to float (Figure 4). As expected, the global fit analysis results revealed that leupeptin and aprotinin act as purely competitive inhibitors with small peptidic substrate. $K_{i'}$ was more than 100-fold higher than $K_i$ in the case of leupeptin and infinite in the case of aprotinin (Figure 4C). Moreover, the potency of leupeptin and aprotinin appeared to be only marginally affected by the substrate as $K_i$ was only 2-fold lower with the chromogenic substrate as compared to the FIX substrate (compare Figure 3C and Figure 4C). We have also fitted the data from each inhibitor concentration individually (not shown). The individual fits showed a large increase in $K_m$ as inhibitor concentration was increased but no change in $V_{max}$, consistent with competitive inhibition.

## DISCUSSION

In this paper we describe for the first time a detailed mechanistic analysis of the inhibition of FXIa by active site directed peptides. This analysis was performed using a novel sensitive assay for the detection of FIXa. The implications of our findings are discussed below.

The new FIXa detection system developed in this study affords a considerable advantage over the existing assays. It is at least 10 times more sensitive than the activation peptide release assay (based on a detection threshold of ~0.5 nM calculated from ref *21*). Furthermore, it eliminates the need to work with radiolabeled materials and the potential effects of modifying FIX and its activity or interactions with FXIa as a result of the chemical modifications caused by radiolabeling (*21*). The assay described in this paper is not more sensitive than the coupled assays, using the activity of factor Xa (FXa) as a readout after the formed FIXa is reconstituted to form a tenase complex by adding factor VIIIa, phospholipids, and FX (*20*). However, it does solve a number of significant issues. First the coupled assay involves quenching of the FIX activation reaction by calcium depletion, dilution into a calcium-containing solution to assemble the tenase complex, and then a second quenching step prior to the addition of the chromogenic substrate. The multiple dilutions, amplification steps, and the inherent instability of factor VIIIa introduce many potential sources for error which are absent from the assay developed in this study. In addition, after recalcification FIX activation can also be resumed in parallel to the FX activation reaction. Another significant caveat of the coupled assay is that FXIa inhibition cannot be distinguished from the inhibition of FXa. While inhibitors that inhibit both FIXa and FXIa can be problematic in both the assay reported in this study and the coupled assay, it appears that FIXa, especially when not part of the tenase complex, is relatively resistant to inhibition by FXIa inhibitors (e.g., leupeptin and aprotinin). Furthermore, the irreversible nature of the interaction of FIXa with ATIII can overcome moderate levels of FIXa inhibition, given that enough time is allowed for FIXa−ATIII binding in the assay plate.

Another important observation is that while the inhibition of FIX activation by leupeptin and aprotinin is best described by a mixed-type mechanism, the inhibition of amidolytic activity appears to be purely competitive. While the latter was expected due to the ability of the peptidic substrate to block the access of the inhibitor to the active site, the relatively high affinity of the inhibitors to the FIX−FXIa complex (only ~10-fold lower than the affinity toward free FXIa) suggests that the active site is still accessible to inhibitors even when FIX is bound. This idea is consistent with the implication of the apple 2 and apple 3 domains of

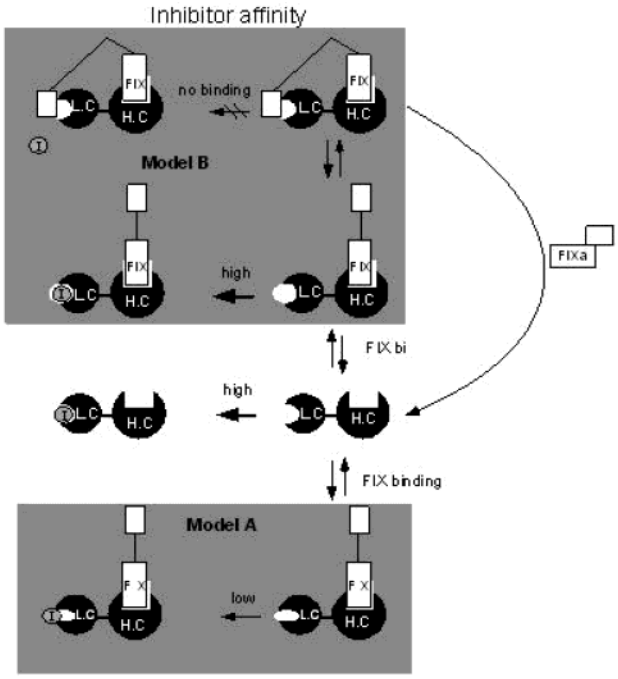


FIGURE 5: Models that explain the mode of FXIa inhibition by leupeptin and aprotinin. I = inhibitor, LC = FXIa light chain, and HC = FXIa heavy chain.

the noncatalytic heavy chain as the primary binding sites for FIX on FXIa (*8*, *23*). Accordingly, the $K_m$ of FIX activation by the isolated FXIa light chain is almost 3 orders of magnitude higher than the $K_m$ of the reaction with FXIa holoenzyme (*12*, *13*). Studies of the inhibition of the tenase and the prothrombinase complex by 4-aminobenzamidine revealed similar mechanisms of inhibition (*24*, *25*). In both cases the inhibitor was competitive when the processing of the small chromogenic substrate was studied but noncompetitive with respect to the physiological substrates. As in the case of FXIa, these patterns of inhibition were attributed to the existence of exosites that mediate the binding of factor X and thrombin to the tenase and prothrombinase complexes (*24*, *25*).

Despite the binding of FIX to FXIa being mediated by an exosite(s) on the heavy chain, both leupeptin and aprotinin retain a distinct competitive component evident from the ~10-fold reduction in inhibitor affinity to the FXIa−FIX complex. Interestingly, this competitive component was not observed for the inhibition of the tenase and the prothrombinase complex by 4-aminobenzamidine (*24*, *25*). At least two different mechanisms could account for the residual competitive nature of these inhibitors (Figure 5). First, it is possible that the binding of FIX to an exosite causes, allosterically, a small change in the active site conformation which reduces the affinity of the inhibitor (Figure 5, model A). A second possibility (Figure 5, model B) is that the initial FXIa−FIX complex allows access to the active site but a conformational change in FIX, following the initial binding event, brings the FIX cleavage site into the active site. This later state does not allow the inhibitor to access the active site. As the FXIa−FIX complex, at steady state, partitions between these two states, the overall affinity of the inhibitor to the FXIa−FIX complex will be reduced. The two models make different predictions with respect to the $K_i'/K_i$ ratio of different active site inhibitors. In the first model $K_i'/K_i$ ratio would vary between different inhibitors because each of them, depending on its exact interactions with the active site, will be affected differently by the conformational change elicited by FIX binding. The second model predicts that the $K_i'/K_i$ ratio will be similar among different active site inhibitors as it is the function of the relative time FIX−FXIa complex spent in the active site blocked conformation. As only two inhibitors analyzed in this study, a distinction between these possible mechanisms awaits a more extensive analysis of a larger set of inhibitors.

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