Hypothesis

Proposed cation-π mediated binding by factor Xa: a novel enzymatic mechanism for molecular recognition

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Abstract Factor Xa (FXa) is an important serine protease in the blood coagulation cascade. Small synthetic competitive inhibitors of FXa are under development as potential anticoagulants. To better understand FXa structural features and molecular recognition mechanisms, we have constructed three dimensional models of FXa–inhibitor complex structures via a new search approach that samples conformational space and binding space simultaneously for DABE and DX-9065a, two bis amidinoaryl derivatives that are among the most potent and selective FXa inhibitors reported to date. We find the most probable binding modes for the two inhibitors to be a folded conformation, with one distal amidino group extending into the S1 pocket, forming a salt-bridge with FXa Asp-189, and the other positively charged group fitting into the S4 subsite, and stabilized by a cation-π interaction. We propose as a hypothesis that the cavity-like S4 subsite formed by the three π-faces of the aromatic residues Tyr-99, Phe-174 and Trp-215 is sufficiently rich in π electrons that it is not only a hydrophobic pocket, but also forms a cation recognition site. This proposed cation-π binding mechanism is one of the first proposed for enzymatic molecular recognition, and for which experimental verification can be obtained without any complicating charge compensation mechanism. Our models provide plausible explanations of the structure–activity relationships observed for these inhibitors, and suggest that cation-π interactions may provide a novel mechanism for molecular recognition.

Key words: Anticoagulant; Factor Xa inhibitor; Cation-π interaction; Binding mode; Conformational search; Molecular recognition

1. Introduction

Factor Xa (FXa) is a key enzyme involved in the blood coagulation process, whose major function is to convert prothrombin to thrombin via specific proteolysis. Due to its central position linking the intrinsic and extrinsic activation mechanisms in the cascade-like coagulation pathway, inhibition of FXa should be highly effective in controlling the clotting process [1–6]. A series of synthetic FXa inhibitors has been investigated [1,7]. However, none of them have been developed as a therapeutic anticoagulant. The lack of a detailed structural understanding of FXa molecular recognition may hinder further progress on FXa inhibitor design. Recently the crystal structure of FXa has been determined at 2 Å resolution, providing the three dimensional structure at an atomic level, but crystallization of its complex with the inhibitor DEGR (dansyl-Glu-Gly-Arg chloromethyl ketone) was unsuccessful due to the intermolecular association between the C terminal of one FXa molecule and the active site of a crystallographically neighboring molecule [8]. Thus, computer modeling of inhibitor binding to FXa would be a useful alternative approach to provide insight into the detailed interactions between FXa and its inhibitors and the structure–function relationship of the FXa active site. To address this issue, we have constructed three dimensional models of the inhibited FXa complex with DABE [1] (inhibitor '2' in Table 1) and DX-9065a [7] (inhibitor '4' in Table 1), two of the most potent and selective FXa inhibitors in the bis amidinoaryl compound series. The proposed model substantially explains the structure–activity relationships and specificity of these inhibitors.

2. Methods

Calculations were carried out using the MacroModel/BatchMin program, Version 3.1 [9] employing the MM2 force field on truncated DABE and DX-9065a models with the distal amidino groups removed due to their limited influence on the overall conformation and to their low quality parameters in the force field. 5000 systematic pseudo Monte Carlo searches followed by 500 iterations of conjugate gradient energy minimization were conducted both in vacuo and in a pseudo-aqueous environment using the volume based continuum solvation model [10]. The conformers generated within a 10 kcal/mol energetic window from that of the lowest energy conformer were collected with the amidino groups added back. About 30 conformers were found for DABE, and docking into the FXa active site was attempted for each. About 1000 conformers were collected for DX-9065a; further energy minimization removed some duplicates, but still resulted in more than 100 conformers within a 2.5 kcal/mol energy window, which is too many for manually docking into the FXa active site. To solve this problem, we developed a new search method for flexible molecules, i.e. systematic conformational search within the active site, which is described below.

For this approach, the starting structures of DABE and DX-9065a were energy minimized with standard bond lengths and angles. Systematic searches were carried out using the

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Abbreviations: FXa, Factor Xa; DABE, 1,2-di-(5-amidino-2-benzofuranyl) ethane; DX-9065a, (2*S*)-2-[4-[[(3*S*)-1-acetimidoyl-3-pyrrolidinyl]oxy]phenyl]-3-(7-amidino-2-naphthyl) propanoic acid; DEGR, dansyl-Glu-Gly-Arg chloromethyl ketone; dansyl, 1,5-*N*-dimethylaminonaphtylsulfonyl; BPTI, bovine pancreatic trypsin inhibitor.

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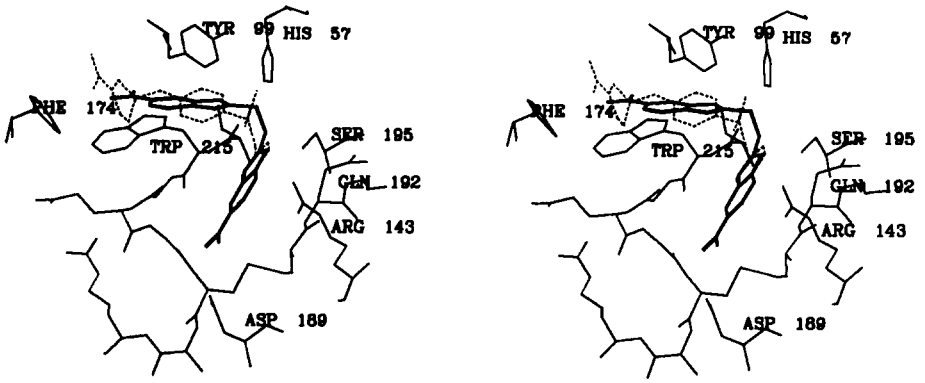


Fig. 1. Superposition of representative binding conformers of DABE (thick line) and DX-9065a (dotted line) in the FXa active site.

Biosym program *Search_Compare*. Since the S1 pocket of the arginine- and lysine-directed serine proteases is well known to be responsible for the recognition of the cationic group, we rationalized that one of the positively charged amidino groups in these inhibitors extends into the S1 pocket, forming a salt-bridge with the FXa Asp-189. The X-ray crystal structure of benzamidine complexed with thrombin [11] was thus used as a template to position the distal amidino group of DABE and DX-9065a in the S1 site of FXa by superimposing the active site residues of the two homologous enzymes first and then overlaying the amidinophenyl ring of the inhibitors to the benzamidine ring. Systematic conformational searches were first performed on the freely rotatable bonds of the inhibitors, with each torsion angle incremented in steps of 60°, and the van der Waals radii scaled at 0.95. The conformers generated within a 10.0 kcal/mol energetic window from the lowest energy minimum for both inhibitors were collected within the active site, and examined for their steric fit by applying the 'bump_check' utility and intermolecular energy evaluation in INSIGHT II (Biosym Technologies Inc.). Conformers with the second amidinoaryl group out of protein surface or having collisions with FXa were considered less probable and discarded, while those having the second amidinoaryl group complementary to the FXa surface cleft with much lower intermolecular energies were saved. The geometrical distance maps and torsional angles were calculated for the saved conformers, and used as constraints for searches with smaller torsional increments of 30°, and then the constraints obtained from the 30° increment searches were used for further 15° increment torsional searches. About 300 lower energy complexes have been explored. The final conformers, with optimal steric fit in FXa, and within a 5.0 kcal/mol energy window from the lowest internal energy conformer, were subjected to energy minimization within the FXa active site using the DISCOVER simulation program and the corresponding generalized CVFF force field parameters [12].

The intermolecular energies, including van der Waals and electrostatic energies between FXa and each potentially bound conformer of the inhibitors, were calculated using the EVALUATE facility in the INSIGHT II program.

3. Results

FXa is a trypsin-like serine protease, which recognizes a basic residue in position P1 of its substrates by forming a salt-bridge between a basic residue of the substrates and the acidic residue Asp-189 at the bottom of the S1 pocket (Fig. 1). The residues Trp-215, Phe-174 and Tyr-99 form an open pocket, corresponding to the 'aryl binding site' in thrombin [13]. Here we refer to it as the S4 pocket, as it was modeled to accommodate the fourth residue, Ile (P4) from the cleavage site of its natural substrate, prothrombin [14]. The dansyl ring of the FXa inhibitor DEGR has also been modeled to fit into this subsite by [8].

Systematic searches of the free torsional angles of the two inhibitors within the FXA acitve site optimized the location of the other amidino group of DABE and the pyrrolidinyl group of DX-9065a into the surface cleft, the S4 pocket, due to the lower intermolecular energy (Fig. 1). The other conformers with the second amidino group or the pyrrolidinyl group colliding with FXa residues or pointing into the solvent are less energetically favored. Table 1 shows the structures of the amidinoaryl derivatives and their inhibitory activities for FXa. Removal of the second amidinobenzofuran from DABE results in a remarkable reduction in potency [1], clearly supporting a model in which the second amidinoaryl moiety interacts with FXa instead of going into the solvent. DX-9065a has two basic moieties, amidinoaryl and pyrrolidinyl groups, that can both potentially interact with Asp-189 of FXa, but the planar shape of the S1 pocket of FXa has a preference for binding to amidinoaryl groups with a flat structure over the pyrrolidine [15]. After the potential binding region of the second amidino group or the pyrrolidinyl group was located, subsequent systematic searches with smaller torsional increments were carried out to fine tune the orientation of these groups in that binding region. The criteria for determining the potential binding modes from the saved conformers were first, that the complexes should have lower intermolecular energies, and then that the internal conformational energies for the inhibitors themselves should be lower, since the inhibitors are quite flexible, and the effect of inhibitor internal energy on the binding conformation is much less than that of intermolecular energy.

Fig. 1 displays the most probable binding mode of DABE and DX-9065a in the FXa active site. The proposed conformation of DABE, shown in thick line in Fig. 1, compared with other possible binding modes (not shown in Fig. 1) which differ slightly in the orientations of the second amidino group, has more favorable interactions with FXa as suggested by the intermolecular energy calculations. It also has lower intramolecular strain as determined by the Monte Carlo conformation search.

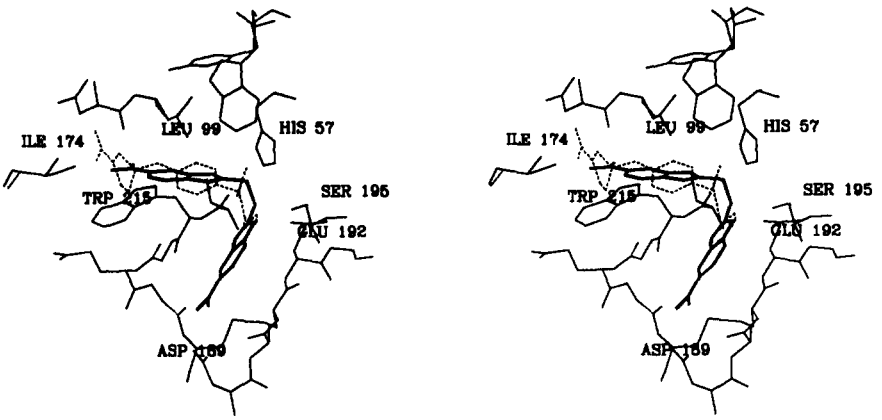


Fig. 2. Docking the proposed FXa binding conformers of DABE (thick line) and DX-9065a (dotted line) within the thrombin active site.

Besides the obvious salt-bridge formation in the S1 pocket, which is an important feature common to all benzamidine-based serine protease inhibitors, DABE might exhibit additional hydrogen bonding between the furan oxygen and the Ser-195 OH of FXa. This hydrogen bond would account for the observed reduction in the inhibitory activity of inhibitor '3' (Table 1), in which the benzimidazole has replaced the benzofuran of DABE [11]. This replacement should not significantly affect its conformational properties but would affect the ring charge distribution. Since either of the imidazole nitrogens can bond a hydrogen due to the tautomeric feature of the imidazole, neither of them would be as good as the oxygen in the furan as a hydrogen acceptor to interact with Ser-195 OH, assuming that inhibitor '3' binds to FXa in the same mode as DABE. In Fig. 1, the second benzofuranyl group of DABE crosses over the extended FXa backbone segment Trp-215–Gly-216, directing the distal amidino group into the S4 pocket formed by the three aromatic residues. The positively charged amidino group makes close contacts with the aromatic ring faces, with closest distances of 3.0 Å, 3.1 Å and 3.4 Å to the Trp-215, Phe-174 and Tyr-99 rings, respectively. The absence of any anionic residues around the S4 subsite rules out possible charge–charge stabilization of the amidino group by FXa. These findings led us to postulate that the basic amidino group is stabilized via a specific 'cation-π interaction' [16], which is essentially an attraction of a positive charge to the π-electrons of an aromatic ring, also called an 'ion-quadrupole' attraction [17,18]. The cation-π interaction is particularly effective when the aromatic system contains electron-rich residues like Tyr and Trp [16], especially when several such aromatic rings are spatially arranged in a pocket-like subsite, with all the π-faces of the aromatic rings facing toward the center of the pocket [19]. Since the S4 subsite of FXa possesses both of these features, the effects of the cation-π interaction should be substantial. Although at present there is no molecular mechanics function formulated to evaluate the magnitude of this interaction, its contribution to binding affinity to the synthetic host receptors has been demonstrated to be significant on the basis of experimental findings [20–22] and theoretical calculations [16,23–26]. If the cation-π interaction is taken into consideration, this conformation of DABE should have the most favorable intermolecular interactions with FXa as a result of the optimal position of the amidino group in the S4 pocket with the amidino hydrogens pointing directly to the π-face of Phe-174. The close face-to-face contact of the amidino group and the Trp-215 indole ring would also give rise to so called 'π-stacking' interactions.

Compared to DABE, DX-9065a is more flexible due to two additional freely rotatable bonds. Although the orientation of the DX-9065a pyrrolidine ring is less defined, all possible orientations confine a volume that fills the S4 subsite and generates favorable hydrophobic interactions with the three aromatic residues in the S4 subsite (not shown in Fig. 1). Of the possible binding conformers, the one shown in the dotted line in Fig. 1 was chosen as representative, since it has the most favorable VDW interactions with FXa, as well as the strongest cation-π interaction due to the closest contact of the pyrrolidine moiety to the ring faces of Phe-174 and Trp-215 in the S4. It is also the least conformationally strained structure among all the potentially bound conformations, and was found in the Monte Carlo searches to be a relatively stable local minimum structure. Besides the salt-bridge interaction in the S1 site, the carboxylate group of DX-9065a has close contact with the guanidinyl group of the FXa Arg-143. The positively charged acetimidoyl moiety surrounded by the three aromatic walls is strongly stabilized via the cation-π interactions mentioned above. The cation-π interaction provides an explanation of the activity difference between inhibitors '4' and '5' listed in Table 1. The modification of the pyrrolidine in '4' to an amidino type moiety in DX-9065a leads to an increased basicity of the cation due to electron delocalization, and thus enhances the cation-π interaction in the S4 subsite. Furthermore, it has been rationalized that weaker binding of protonated amines like NH_4^+ to the aromatic host over alkylated amines is due to their larger desolvation penalty upon binding than the alkylated amines [27]. Compared to the pyrrolidine group, the additional methyl group of the acetimidoyl moiety not only increases hydrophobic interactions with the FXa S4 subsite, but also decreases the desolvation energy cost.

Superposition of the proposed DABE and DX-9065a bound mode, as shown in Fig. 1, demonstrated essentially similar folded conformations for the two inhibitors. The major forces for binding are common to both, i.e. the salt-bridge formation in the S1 subsite and the cation-π interaction in the S4 subsite. Both inhibitors, especially DX-9065a, are selective for FXa compared to thrombin (Table 1). We docked the proposed bound structures of the two inhibitors into the active site of thrombin by superimposing the active sites of the two enzymes (Fig. 2). However, binding to thrombin would be energetically

disfavored due to strong interference between the amidino or acetimidoyl group of the inhibitors and thrombin Ile-174. These two inhibitors are sufficiently flexible that we cannot rule out possible conformational adjustments to avoid collision with Ile-174. However, the cation-π interaction is definitely absent in the corresponding thrombin 'aryl binding site' since it is formed by only one aromatic residue, Trp-215, and the two aliphatic residues Leu-99 and Ile-174, which cannot provide sufficient π electrons to stabilize the cation. The particularly poor affinity of DX-9065a with thrombin is probably due to additional charge–charge repulsion between the carboxylate group in DX-9065a and the Glu-192 side chain in thrombin [15] (see Fig. 2), while in FXa, position 192 has a neutral Gln residue, and the DX-9065a carboxylate group is stabilized by the nearby Arg-143 (Fig. 1).

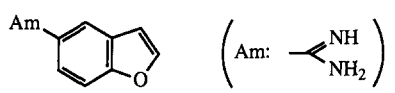
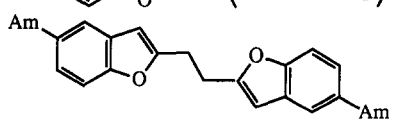
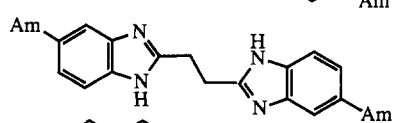
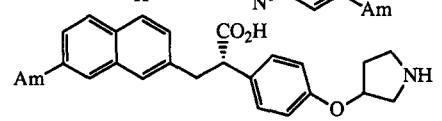
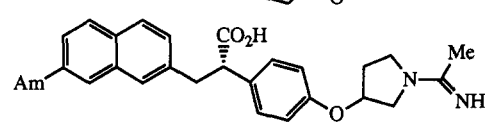
4. Discussion and conclusions

A complete conformational space search of flexible molecules, as well as their docking into a specific binding region is always complicated by the large number of possible conformers. To reduce the size of this problem, we developed a new search approach to construct the possible binding modes. Based on the rationale that one of the positively charged amidino groups in these inhibitors extends into the S1 pocket, since the S1 pocket of the arginine- and lysine-directed serine proteases is well known to be responsible for recognition of the cationic group, we fixed one of the amidino aryl groups in the S1 pocket, and started from a search of the entire conformational space of the inhibitors inside the FXa active site with relatively coarse torsional increments to locate the potential bound conformational region, and then conducted further intensive searches focused on this region. Our strategy was thus to combine conformational searching and binding mode searching into one process to eliminate unnecessary efforts in conformational space other than the potential bound conformation region, thus also eliminating a complex docking analysis.

The resultant modes show that both inhibitors DABE and DX-9065a adopt a folded conformation, with one amidino group extending into the S1 subsite and the other fitting into the S4 pocket. Although conformational searches of both inhibitors revealed that the extended conformation is most energetically favorable, consistent with the X-ray crystal structures [7], the flexibility of these inhibitors allows diverse conformations within a small energy difference. Moreover, induced fit effects from the enzyme should be large enough to compensate for the conformational energy loss. The internal energy of our proposed bound conformation was only about 2.0 kcal/mol higher than that of the lowest energy conformers found in the searches (not including binding interactions). Although the precise atomic location of the inhibitors inside the FXa need to be refined by crystallographic studies, the general binding interactions with S1 and S4 sites proposed by modeling should be plausible.

The most important findings of this study are first, the identification of a potential cation-π recognition site formed by the three aromatic residues Trp-215, Tyr-99 and Phe-173 within the FXa active site, and second, the clarification of the special molecular recognition mechanism of FXa toward the bis amidinoaryl derivatives. Recently the cation-π interaction has received considerable attention as a new type of binding interaction. Besides the extensive investigations on artificial host-guest systems [16,18,27–30], computational studies of several biolog-

Table 1. Structures of synthetic inhibitors and their inhibitory activities toward FXa and thrombin

Inhibitors		K_i, μm[a]		IC_{50}, μm[b]	
No.	Structure	FXa	Thrombin	FXa	Thrombin
1		169	123	ND[d]	ND
2		0.573	14.9	0.10	5
3		2.68	19.0	ND	ND
4		ND	ND	0.31	> 600
5		ND	ND	0.07	> 2000

[a] K_i: dissociation constants of the enzyme-inhibitor complexes from Tidwell et al.(1980).

[b] IC_{50}: concentrations needed for inhibiting enzymes by 50% from Nagahara et al.(1994).

[c] ND: no experimental data

ical systems have postulated an important role in molecular recognition for such systems as ion selectivity of the potassium channel [31], binding of acetylcholine to acetylcholinesterase [16,32], and binding of choline derivatives to phospholipases A_2 [33]. The most direct evidence for biological importance has been the X-ray crystallographic determination of such an interaction in the immunoglobulin Fab McPC603–phosphocholine complex [34]. However, in most cases, the presence of ionic residues near the cation binding site complicates interpretation of the role of cation-π interactions, since charge–charge interactions are long range electrostatic effects [16,32,35]. Our conformational analysis of FXa complexes with amidinoaryl derivatives provides a clear and verifiable hypothesis for the importance of cation-π interactions in molecular recognition. The FXa S4 subsite shares very similar architectural features with the Fab McPC603 antibody binding site, i.e. three aromatic rings arranged with two of them facing each other, edge to face with the third one. This forms a box-like cavity perfect to accommodate a cation like an amidino group. The absence of any acidic residue around the S4 subsite in FXa should establish the exclusive role of the cation-π interaction in molecular recognition. It is well known that the S4 subsite is quite hydrophobic and prefers to bind hydrophobic groups, such as the naphthyl ring of DEGR [8], the IleP4 of prothrombin [14] or the Pro of BPTI [8]. This does not contradict its cation recognition function. Actually, the S4 pocket of FXa should possess dual functionality, with its selectivity depending on the incoming ligand. This also explains its preference for a hydrophobic cation with high basicity, but low desolvation energy. We believe that this model is a better mechanism for molecular recognition than the model proposed by Katakura et al. [15] in which the acetimidoyl of DX-9065a is oriented out of the S4 pocket. That model cannot provide a plausible explanation of the effect of the basicity of a second charged group on inhibitory affinity. Our model proposes the involvement of the cation-π interaction in FXa molecular recognition, identifies the active mode of the structural elements with the FXa active site, and provides a sound explanation of FXa's selectivity toward the bis amidinoaryl compounds. This cation recognition should be unique to FXa. In thrombin, the corresponding pocket, known as the 'aryl binding site' is also hydrophobic, but would not be a cation recognition site, as it is formed by one aromatic Trp-215 and two aliphatic residues, Leu-99 and Ile-174, and it is not π electron rich enough to attract a cation. Trypsin and chymotrypsin also would not accept a cation in their corresponding sites for the same reason.

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