X-ray Structure of Active Site-inhibited Clotting Factor Xa

IMPLICATIONS FOR DRUG DESIGN AND SUBSTRATE RECOGNITION

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The 3.0-Å resolution x-ray structure of human des-Gla-coagulation factor Xa (fXa) has been determined in complex with the synthetic inhibitor DX-9065a. The binding geometry is characterized primarily by two interaction sites: the naphthamidine group is fixed in the S1 pocket by a typical salt bridge to Asp-189, while the pyrrolidine ring binds in the unique aryl-binding site (S4) of fXa. Unlike the large majority of inhibitor complexes with serine proteinases, Gly-216 (S3) does not contribute to hydrogen bond formation. In contrast to typical thrombin binding modes, the S2 site of fXa cannot be used by DX-9065a since it is blocked by Tyr-99, and the aryl-binding site (S4) of fXa is lined by carbonyl oxygen atoms that can accommodate positive charges. This has implications for natural substrate recognition as well as for drug design.

EXPERIMENTAL PROCEDURES

fXa was isolated from human plasma; des-Gla-fX was produced via chymotryptic cleavage (removing amino acids L1–L4); chymotrypsinogen numbering is used for the catalytic domain; the sequential fX numbering is used for the light chain, will be indicated by the prefix "L". Subsequently, fX was activated with the purified factor X activator from Russell's viper venom, resulting in des-Gla-fXa.

The inhibitor DX-9065a (Fig. 1) was prepared as described previously (12). Initial crystals were obtained by a fine pH screen (0.1 M MES/OH, pH 5.8, 10 mM CaCl₂, 18% polyethylene glycol 6000) after 2 weeks. These first rod-like crystals were orthorhombic, with cell constants a = 56.93 Å, b = 73.17 Å, and c = 79.08 Å and α = β = γ = 90.0, but diffracted weakly. Seeding techniques improved both size (0.1 × 0.1 × 0.4 mm³) and intrinsic order. Data to 2.8 Å were collected on a Siemens multiwire area detector and processed with SAINT data reduction software (16). The final data set used in refinement included reflections to 3.0-Å resolution with completeness of 85% and Rmerge of 31% in the outer shell. The crystals belong to the space group P2₁2₁2₁ with one molecule in the asymmetric unit. Patterson search techniques (17–19), model building, electron density map calculation, and model refinement was done within MAIN (20) and X-FLOR (21). The model (without addition of water molecules) has been refined to a crystallographic R-value of 19.7% (Rmerge = 26.1%) with good stereochemistry (root mean square deviation of bonds = 0.009 Å and angles = 2.2° from ideality (22)).

RESULTS

Overall fXa Structure—We describe here the structure of the complex of Daiichi inhibitor DX-9065a in des-Gla-fXa, i.e. the catalytic serine protease domain and both the EGF2 (proximate to the catalytic domain) and the EGF1 domains. The protein is thus identical to that of the tetragonal fXa crystal structure previously described (23, 24). Differences between the structures thus arise from the different crystal packing interactions or different ligand interactions (calcium and inhibitor) and can usually be attributed to the most local cause; the extent to which apparent ligand-induced structural changes determine crystal packing interactions and vice versa remains ambiguous. In the case of the tetragonal fXa crystal structure, the crystal packing interaction between the S1 specificity pocket and the EGF2 C-terminal Arg residue of a symmetry-related fXa molecule is simultaneously an enzyme-ligand interaction. This arginine binding interaction is eliminated in the fXa-DX-9065a structure. Similarly, the presence of calcium in the orthorhombic fXa-DX-9065a structure is associated simultaneously with differences in structure and with different crys-
The EGF1 domain, disordered in the tetragonal, arginine-bound crystal form, was suggested to become ordered in the presence of calcium (24). However, the orthorhombic crystals of fXa-DX-9065a grown at 10 mM calcium also show apparent disorder of the EGF1 domain. Furthermore, the crystal packing of the two crystal forms is not compatible with a unique EGF1 orientation (relative to EGF2). The tetragonal crystal packing of arginine-bound fXa suggested an extended EGF2-EGF1 arrangement similar to the one observed in fXa (25). In contrast, the orthorhombic crystal structure of fXa-DX-9065a allows only two alternate orientations, both of which result in a compact, globular quaternary fold with EGF1 in contact with the catalytic domain. This arrangement can be excluded for the tetragonal crystal. This is evidence against a rigid factor X module arrangement in solution or physiologically at the membrane surface. Rather, several conformations seem possible that may be physiologically important (possibly distinguishing, for example, fX as substrate and fXa as an enzyme). This is consistent with fluorescence energy transfer measurements that indicate that the distance of the active site to the membrane changes significantly upon cofactor (fVa) binding (26). Such a change is not observed in the analogous experiment with fXa and cofactor VIIIa (27).

The calcium-binding site in the catalytic domain is conserved in factor X, and studies based on synthetic factor X peptides suggest that the calcium-binding site participates in the prothrombinase complex (28). This site is well characterized in trypsin and other serine proteinases (29): calcium binding induces an ordering transition in the ligating residues, i.e. the side chains of Asp-70 and Glu-80 as well as the carbonyl groups of Asn-72 and Ala-75. In the fXa-DX-9065a structure, all these residues are ordered, and there is density at the expected calcium position, indicating at least partial occupation of the calcium-binding site. The tetragonal, arginine-bound fXa crystal structure differs somewhat in the calcium loop, but the orthorhombic crystal structure of arginine-bound fXa structure (23); twin-twin geometry has been observed for naphthamidine binding in thrombin (31) and trypsin (15). Surprisingly, however, the aspartic acid side chain appears to move, mainly by a χ^2 rotation of almost 90°, away from its original position, resulting in a single Asp O_4^\(-\)-twinn (naphthamidine) arrangement (Fig. 3). O_2^\(-\) of Asp-189 is directed toward the amides of Gly-184 (3.8 Å) and Gly-226 (3.5 Å). This new binding geometry involving a ligand-induced structural change of Asp-189 has only recently been observed in thrombin (24). The carbonyl oxygen of Gly-219 forms a hydrogen bond with the amidino group (2.6 Å). No water molecules were apparent in the electron density map function to as a hydrogen-bonding ligand to the other oxygen of Asp-189. This may be a consequence of the medium resolution of the diffraction data.

A second and perhaps more surprising ligand-induced structural change is the movement of the main chain of Gln-192–Gly-193 of almost 2 Å toward the naphthamidine group (Fig. 3). This change results from the accumulation of several small dihedral changes of Ala-190–Ser-195. This constrains the S1 pocket, perhaps counter-intuitively due to the large size of naphthamidine compared, for example, with benzamidine. However, the larger and more rigid ring system would leave a cavity at the entrance to the S1 pocket (Gln-192), unsuitable for a water molecule without such a movement. Since this conformational rearrangement involves a part of the oxyanion hole that has been observed in a single rigid conformation, a significant quantity of energy must be supplied by naphthamidine binding. This may be derived from the hydrophobic interaction of Gln-192 with the naphthamidine itself. Additionally, the binding energy of the aryl-binding site interactions may contribute due to overall stiffness of DX-9065a. In any case, the movement is apparent characteristic of naphthamidine binding in trypsin-like serine proteinases since a corresponding structure was also observed in naphthamidine binding in thrombin (31) and trypsin (15). This supports the hypothesis that the energy required derives from the naphthamidine interaction itself.

The second major anchoring interaction of DX-9065a involves the substituted pyrrolidine ring, which ideally fills the deep "aryl-binding site." The pyrrolidine ring is almost completely buried by a perpendicular "two-sandwich" arrangement: the aromatic ring systems of Phe-174 and Tyr-99 are both parallel to the pyrrolidine, while the aliphatic part of the Gln-97 side chain and Trp-215 additionally cover the pyrrolidine group from perpendicular orientations. The side chains of Phe-174, Tyr-99, and Gln-97 differ from those in the tetragonal, arginine-bound crystal structure (Fig. 3); however, the side chain of Gln-97 is not perfectly defined in the electron density map and probably occupies more than one conformation. The χ^2 rotation of Tyr-99 and Phe-174 apparently optimizes the hydrophobic stacking arrangement with the pyrrolidine.
to naphthamidine binding in the S1 pocket, the interaction in the aryl-binding site is not solely hydrophobic in character. The carbonyl oxygen atoms of Lys-96 and Glu-97 together with the Glu-97 side chain form a “nitrogen-cation hole” or simply “cation hole,” analogous to the oxyanion hole of the catalytic site. The cation hole interacts with the delocalized positive charge of the pyrrolidine ring and the charged acetimidoyl group pointing toward Glu-97. The electron density map indicates that the chiral center of the pyrrolidine is in the S-configuration (Fig. 2), as expected from the synthesis of the enantiomerically pure compound (Fig. 1).

It is clear from the electron density map that the enantiomer with an S-configuration of the propionic acid binds the enzyme. The charge of the propionic acid is not involved in a direct ionic interaction, but is exposed to solvent, reminiscent of the sulfonyl group of the NAPAP/TAPAP inhibitor series. N\textsuperscript{2} of Gln-192 forms a weak hydrogen bond with propionic acid (4.1 Å), while O\textsuperscript{1} interacts with the guanidinium group of Arg-143, i.e. the side chain of Gln-192 forms an ionic bridge between the propionic acid and Arg-143.

The center of the phenyl ring almost coincides with C\textsuperscript{2} of the D-Phe residue of a superimposed D-Phe-Pro-Arg chloromethyl ketone-thrombin complex structure. An aromatic ring system is advantageous entropically as a spacer between S1 and the aryl-binding site (S4) since it restricts the conformational degrees of freedom of the compound. This structure demonstrates the feasibility of ignoring hydrogen bonding at Gly-216. Thus, the geometric restraints of binding in the S1 and aryl-binding pockets should allow considerable variation of this spacer in inhibitor design.

**DISCUSSION**

**Implications for Natural Substrate Interactions**—The physiological role of fXa within the blood coagulation cascade consists of activation of prothrombin to thrombin and, as a form of positive feedback within the extrinsic pathway, activation of factor VII. There is, however, an ongoing debate about the physiologically relevant VIIa activator since VIIa, fXa, and thrombin can also activate VIIa to VIIa in vitro (1). Prothrombin activation occurs by two cleavages. The more rapid cleavage takes place at Arg-223–Ile-224 (designated as prothrombin species 2 in Table 1), so meizothrombin accumulates as the principal intermediate of the activation reaction under physiological conditions (35). As the resulting two chains remain
disulfide-linked, the proteolytically fully active meizothrombin remains membrane-bound (analogous to fVIIa, fXa, fXa, or activated protein C) and is released only with the second cleavage (Arg-274–Thr-275).

The two ligand-induced motions of the S1 site, namely the aspartic acid 189 rotamer transition and a rigid body motion of residues of the oxyanion hole, have also been observed in recent thrombin-inhibitor complexes (24, 31). The oxyanion hole remains intact, so motions of the main chain including residues 190–195 might also be induced by attractive polar interactions with the carbonyl group of natural substrates. Alternatively, ligand or cofactor binding might induce motions of this segment and alter substrate recognition. Thus, this motion may have physiological relevance as well.

The S2 site is hardly accessible to bulky substrate residues. The 60-loop is in direct contact with the 99-loop, partly via the polar interaction of the system of Tyr-60 with Lys-96 (3 Å) and partly via a hydrogen bond between the hydroxyl group of Tyr-60 and the amine of Lys-90. In its low energy conformation, Tyr-99 then blocks access to the S2 site for any side chain, which is consistent with the natural substrate specificity of fXa (Table I). The S2 site of fXa, and in particular Tyr-99, should not, however, be considered absolutely rigid; a strong binding ligand may provide the energy necessary to move Tyr-99 and alter substrate recognition. Thus, this motion may have physiological relevance as well.

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The clear preference for glycine at P2 for fXa substrates, the clear preferences for other residues at P2 for thrombin and fIIa substrates, and the conservation of Gly at this position in the limited number of fVII sequences available are evidence for a physiological role of fXa as activator of fVII. While the identities of the primed site residues at the activation site of serine proteases are restricted by structural requirements, the conservation of unprimed site residues, if real, most likely reflects requirements for substrate recognition. Alternatively, conservation of a sequence less optimal for thrombin or fXa cleavage might arise from a need to limit cleavage rates for these enzymes if required by the dynamics of the coagulation reactions.

The P3 residue typically forms a two-rung antiparallel 3-ladder with Gly-216 and by this constraint leaves the side chain of an L-amino acid exposed to solvent. Therefore, we expect a preference for hydrophilic residues at P3. The propionic acid in DX-9065a mimics P3 in this respect. It should be emphasized that conservation observed in cleavage sites may not necessarily imply a special binding interaction, but may reflect other requirements of the substrate; this is certainly the case with the P1', P2', and P4' residues of prothrombin species 2 and fVII cleavage sites as they are critical for the activation mechanism (37, 38).

The deep aryl-binding site (S4) is also distinctive with its surrounding carbonyl groups (from Lys-96 and Glu-97) together with the side chain of Glu-97. The importance of the cation hole for the recognition of physiological substrates has not been established. This structure suggests that besides hydrophobic residues (such as Ile), histidine in particular should be an ideal candidate for binding at the S4 site, although the sequential position on a substrate might not correspond to P4, considering the structure of thrombin-fibrinopeptide A (39): this site can also be occupied by substrate residue Pn, where n may be different from 4 (n > 4).

**Autolysis Loop: A Model for Substrate Recognition?**—The entire main chain of the autolysis loop (Arg-143–Arg-154) can be traced clearly in the electron density map; Arg-150 is the only side chain in this region with weak density. This segment contains two Gly-Arg motifs (positions 142–143 and 149–150) and was obviously autodigested in the inhibitor-free crystals, possibly accounting for the 3 months required for crystal growth. The main chain conformation of the loop is similar to the one in fIIa, which is identical in length. However, Lys-148 is not oriented toward the active site, but is exposed to the solvent. The guanidinium group of Arg-143 bridges O1 of Glu-192 (2.7 Å) and the carbonyl oxygen of Gly-149 (2.9 Å). The autolysis loop is further strengthened by a hydrogen bond of Gly-151 Nε2 with the carbonyl oxygen of Gly-40. This leaves only Arg-150–Ser-151 accessible as a scissile bond for initial autoproteolysis. From a comparison of the sequences in Table I, one should expect that Glu-147, which is found at position P4, will bind in the S4 site. This is in conflict with our analysis of the S4 recognition site and a preference for hydrophobic or basic residues. The apparent contradiction may be resolved by a docking experiment of the autolysis loop in the active site (Fig. 4). Interestingly, considering the discussion of residue

| P6  | P5  | P4  | P3  | P2  | P1  | P1' | P2' | P3' | P4' | P5' | P6' |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| pt human 1* | R  | A  | I  | E  | G  | R  | T  | A  | T  | S  | E  | Y  |
| pt bovine 1 | A  | A  | I  | E  | G  | R  | T  | S  | E  | D  | H  | F  |
| pt mouse 1 | D  | C  | L  | E  | G  | R  | C  | A  | M  | D  | L  | G  |
| pt rat 1  | D  | C  | L  | E  | G  | R  | C  | A  | M  | D  | L  | G  |
| pt chicken 1 | E  | E  | I  | A  | G  | R  | T  | I  | F  | Q  | E  | F  |
| pt hagfish 1 | V  | Q  | L  | S  | G  | R  | S  | E  | G  | A  | E  | A  |
| pt human 2  | S  | Y  | I  | D  | G  | R  | I  | V  | E  | G  | S  | D  |
| pt bovine 2 | S  | Y  | I  | D  | G  | R  | I  | V  | E  | G  | Q  | D  |
| pt mouse 2  | S  | Y  | I  | D  | G  | R  | I  | V  | E  | G  | W  | D  |
| pt rat 2   | S  | Y  | I  | D  | G  | R  | I  | V  | E  | G  | W  | D  |
| pt chicken 2 | S  | Y  | M  | G  | G  | R  | V  | V  | H  | G  | N  | D  |
| p hagfish 2 | R  | S  | Y  | D  | G  | R  | V  | V  | H  | G  | D  | N  |
| fVII human | S  | K  | P  | Q  | G  | R  | I  | V  | G  | G  | K  | V  |
| fVII bovine | S  | K  | P  | Q  | G  | R  | I  | V  | G  | G  | H  | V  |
| fVII rabbit | S  | N  | P  | Q  | G  | R  | I  | V  | G  | G  | K  | V  |
| fX human auto | T  | H  | E  | K  | G  | R  | Q  | S  | T  | R  | L  | K  |
| fX bovine auto | T  | H  | E  | K  | G  | R  | L  | S  | T  | L  | K  | |
| fX chicken auto | E  | F  | E  | A  | G  | R  | L  | S  | K  | R  | L  | K  |
* pt, prothrombin.
specificity above, we find His-145 (P5) in a rotamer conformation nearly perfectly adapted for binding to the aryl-binding site in an autocatalytic cleavage event. But since several examples are known where the substrate undergoes a significant rearrangement upon binding to the active site ("induced fit"), interpretation of this observation requires caution.

Implication for Future Drug Design—The structure of the complex of Xa with the specific and tightly binding DX-9065a inhibitor shows (consistent with the lack of any detectable water in the S1 pocket) the important hydrophobic contribution to binding. In addition, the reorientation of the Asp-189 side chain apparently does not optimize the electrostatic interaction. Conversely, the binding at the aryl-binding site shows significant electrostatic interaction in addition to the hydrophobic binding. In this way, a kind of symmetry between the two binding sites emerges. This is consistent with the success of Xa inhibitors such as the bisbenzamidine derivatives (40). Furthermore, this structure serves as a general paradigm in that it demonstrates simultaneously the potential and the limitations of studying inhibitor binding in model enzymes.

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