Drug Discovery

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Structure of Active Coagulation Factor XIII Triggered by Calcium Binding: Basis for the Design of Next-Generation Anticoagulants**

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Remarkable effort has been devoted to develop drugs that target coagulation factors or platelet activation to prevent and treat thrombosis, pulmonary embolism, and acute coronary syndromes, or for reducing the risk of stroke in patients with atrial fibrillation. For decades, vitamin K antagonists were the only available anticoagulants that could be administered orally. Only recently, a new generation of direct-acting oral anticoagulants that block thrombin directly or indirectly via upstream factor Xa has become available.[1] However, depending on its activation state, thrombin can either promote or prevent blood clotting. Interference with thrombin activity by the currently available anticoagulants is characterized by an enhanced bleeding risk, thus excluding many patients from beneficial treatment.[2]

The currently targeted enzymes of the blood-clotting cascade, namely factor Xa and thrombin, belong to the family of serine proteases. Remarkably, the final enzyme in the blood clotting cascade, coagulation factor XIII, shows a markedly different mode-of-action (see Figure S1 in the Supporting Information). It is a member of the transglutaminase family, and catalyzes the covalent cross-linking of protein chains bearing susceptible glutamine and lysine residues through isopeptide bonds.[3–6] In a blood clotting event, FXIII recognizes fibrin as the substrate and triggers clot maturation and accretion. In the case of congenital FXIII deficiency, delayed bleeding is reported as a clinical manifestation. Taking these considerations into account, FXIII is regarded as a prospective target to achieve potentially safer and more efficient thrombolysis at a lower dosage of clot-dissolving agents.[7] In fact, a FXIII inhibitor may even prevent thrombus formation altogether.[8]

FXIII acts downstream of thrombin, effectively determining the mechanical stability, half-life, and lysis rate of clots. Although discussed as an ideal target to interfere with coagulation, no suitable drug candidates are available to explore the pharmacological potential of FXIII inhibition. This situation might also result from the fact that a protein structure representing a relevant active state of FXIII was previously unknown, but is necessary to embark on structure-based drug design. Here we report the first high-resolution crystal structure (1.98 Å) of FXIII in an active state (termed FXIIIa° in the following, as suggested by Muszbek et al.[9] for Ca^{2+} activation without proteolysis) in complex with an irreversibly bound inhibitor. Only crystal structures of inactive, homodimeric FXIII have been reported so far, where the active site is completely buried and any access to the catalytic center is obscured. Such structures are unsuitable for drug design. In the structure described herein, three calcium ions recruit polar functional groups of the protein and establish local metal ion coordination sites, which induce the rearrangement of two domains along with local adaptations of the catalytic domain to expose the enzyme in an active state. The observed transformations establish the substrate and cosubstrate binding sites for the formation of an isopeptide bond and suggest involvement of a catalytic triad and a newly identified diad in the enzyme mechanism which is considered valid for the entire transglutaminase family.

Until now, very few FXIII inhibitors have been described. Finney et al.[10] reported that the 66 amino acid peptide tridegin from the salivary gland of the giant Amazon leech *Haementeria ghilianii* is a potent inhibitor. In the late 1980s, a series of small molecules that irreversibly inhibit FXIII were explored in animal models of thrombosis. As a result of the lack of selectivity along with the short plasma half-lives of only a few minutes, these inhibitors were solely considered as pharmacological tools to elucidate the physiological role of FXIII and not as prospective drug candidates.[11] For preclinical development of direct FXIII blockers it is important to have potent and selective compounds at hand to study the benefit-to-risk ratio relative to existing anticoagulants.

Factor XIII is composed, as are all human transglutaminases, of a β-sandwich domain, a catalytic domain, and two β-barrel domains. In several features, however, it differs remarkably from all other members of this enzyme family. The enzymatically inactive pro-transglutaminase carries a unique 37-residue-long activation peptide which is excised by thrombin in the final phase of a coagulation event. Moreover, FXIII is the only dimeric transglutaminase com-

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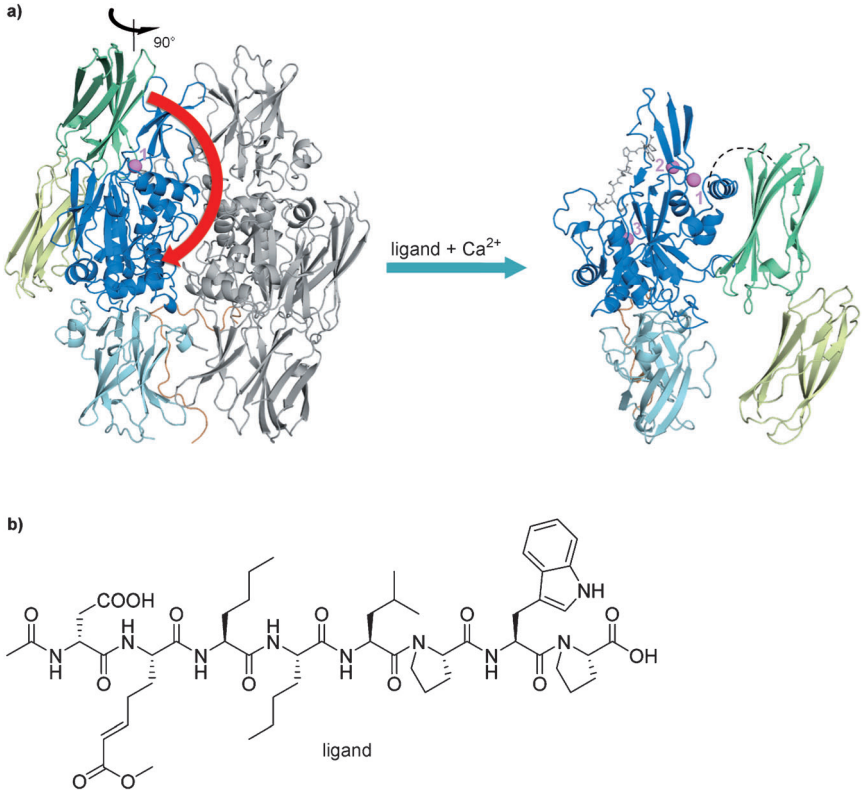

Figure 1. a) In the inactive state FXIII (PDB ID: 1GGU) exists as a dimer (one monomer colored by domain, second in gray). Upon substrate binding, FXIII dissociates and the positions of the β-sandwich (turquoise) and catalytic domain (blue) remain unchanged, whereas β-barrel 1 (green) and β-barrel 2 (yellow) undergo a remarkably large movement (red arrow). The activation peptide (orange) stabilizes the dimer contact in the inactive state and is still bound to the protein and partly exposed to solvent. The active site is located in the upper left part of the catalytic domain, where the inhibitor ZED1301 (gray, b) is covalently attached to the sulfur atom of Cys 314. The catalytic domain of the active form (PDB ID: 4KTY) contains three calcium ions (pink) instead of only one calcium ion in the case of the inactive form. The loop connecting the β-sandwich domain and β-barrel 1 (dashed line) is not defined by electron density.

posed of two zymogen A subunits (cellular FXIII-A_2 found, for example, in platelets and megakaryocytes) or complexed in plasma with two B-carrier subunits, thereby forming a heterotetramer (plasma FXIII-A_2B_2).

Recombinant human cellular factor XIII (FXIII-A_2) was activated using a high concentration of calcium ions rather than by applying proteolytic activation with thrombin.[12] Activated FXIIIa° was subsequently inhibited by the irreversible peptide inhibitor ZED1301, which possesses a Michael acceptor that mimics the substrate's glutamine residue (Figure 1). The complex crystallized in the space group *P*1, with two independent FXIIIa°-ZED1301 molecules per asymmetric unit.

In the previously determined inactive state, the catalytic site is covered by β-barrel 1, where Tyr 560OH forms a hydrogen bond to Sγ of Cys 314. Here we show that binding of ZED1301 in the presence of calcium ions induces a large conformational transition, whereby the two β-barrel domains swing aside to expose the catalytic center (Figure 1). The complex crystallizes as a monomer, in agreement with our biochemical data determined from size-exclusion chromatography. We propose that the adopted conformation represents an active FXIIIa° structure, relevant for drug design, as functionally important and previously hidden sites are exposed. These findings are in agreement with mass spectrometry data obtained for hydrogen/deuterium exchange in solution.[13,14] Structural rearrangements upon activation occur only within the catalytic domain, while the β-sandwich and the two β-barrel domains conserve their overall fold. The electron density of the loop connecting the catalytic and β-barrel 1 domain (residues 502–515) is not visible, possibly because of the pronounced flexibility of this portion. This observation agrees with biochemical evidence[15] that the Lys 513–Ser 514 peptide bond is easily accessible in this flexible loop and can be cleaved by thrombin. Furthermore, in our structure, the uncleaved pro-peptide remains at the N terminus of the β-sandwich domain. We cannot fully exclude an effect of the flexible and distant pro-peptide on the conformation of the catalytic subunit; however, we presume its relevance for drug design should be negligible. Nonetheless, most of the contacts formed in the inactive state are lost due to rearrangements upon activation and dissociation of the dimer in the monomeric state. Twenty-three amino acids of the N-terminal peptide chain are visible in the electron density.

There has been much speculation concerning the driving forces that induce rearrangement upon activation. Since the discovery of FXIII by Laki and Lorand in the 1940s, the importance of calcium ions for FXIII activation, as for all transglutaminases, has now become well established.[16] Thus, it was surprising that crystals grown with inactive FXIII at high calcium concentrations, or crystals of inactive FXIII exposed to solutions containing large amounts of calcium ions, indicated only a single calcium-binding site per monomer (Figure 1 a).[17,18] In our FXIIIa° structure, we observe three calcium ion sites (Figure 1 a) that are essential for driving the enzyme into an active state. Presumably, this form can only be captured when a peptide inhibitor is bound simultaneously with the three calcium ions and stabilizes the protein in this state.

From a comparison of the structures of active and inactive FXIII, the following activation mechanism can be proposed. Calcium binding site 1 is already populated in the inactive state. However, only Ala 457CO coordinates directly to the calcium ion and the remaining coordination sites are filled with water molecules. Upon activation, the calcium ion recruits, besides Ala 457CO, the side chains of Asn 436, Glu 485, and Glu 490 to accomplish its coordination polyhedron. This induces movement of a loop and adjacent α helix

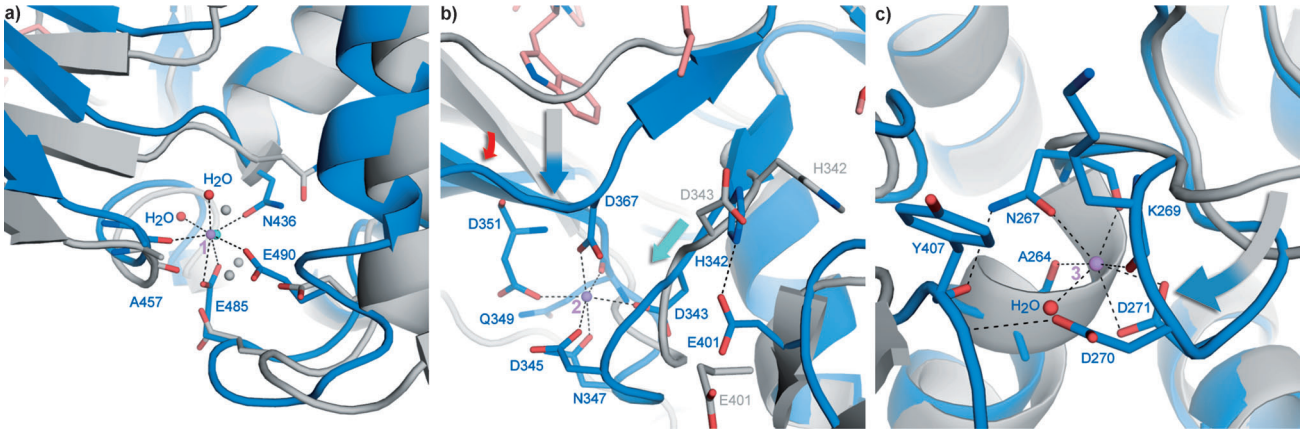

Figure 2. In the active state, three calcium ions (pink) bind to the catalytic domain (blue). They induce structural changes from the inactive to the active form (gray, site 1: 1GGU; sites 2 and 3: 1F13). Dashed lines indicate coordinative, hydrogen bonds or salt bridges. ZED1301 is colored in orange. a) At site 1, Ca^{2+} binding results in a slight shift of an α helix in the active state of FXIII. The inactive form also binds calcium (turquoise sphere), but only with one direct interaction to Ala 457CO. The remaining coordination sphere is completed by water molecules (gray spheres). b) At site 2, the calcium ion binding drags the loop containing Asp 367 downwards and supports rotational movement of the three-stranded β sheet by calcium coordination of Asp 351 (red arrow). Both interactions lead to the formation of the hydrophobic pocket next to the active site (gray-blue arrow), which is important for substrate accommodation. Site 2 is also involved in the formation of the catalytic diad. Coordination of Asp 343 to Ca^{2+} drags a loop downwards (turquoise arrow) and enables the spatial proximity of His 342 and Glu 401 required for activity. c) Population of site 3 results in a loop rearrangement that contains residues which are part of the K-substrate binding region such as Lys 269 and Tyr 407.

to form a more compact calcium environment (Figure 2a). The development of the second calcium binding site translocates part of the catalytic domain into a geometry that enables formation of a hydrophobic pocket next to the catalytic center (Figure 2b). This movement is initiated by the coordination of Asp 367 to Ca^{2+}, and the adjacent β strand is dragged towards the central ion. Importantly, the pocket thus created accommodates, and is stabilized by, the C-terminal Trp residue of our inhibitor. Furthermore, the β strand comprises Trp 390, whose indole moiety is flipped by almost 90° to form a hydrophobic tunnel next to the catalytic site together with the side chain of Trp 279. This tunnel can subsequently host the putative K substrate to form an isopeptide bond. Such a tunnel has similarly been observed for TG2 in the crystal structure assumed to correspond to an active state.[18–20]

Moreover, population of the second Ca^{2+} site affects the orientation of a three-stranded antiparallel β sheet in the upper part of the catalytic domain that is also involved in the formation of the above-mentioned hydrophobic pocket. The involvement of Asp 351 in Ca^{2+} coordination moves the adjacent β strand downwards. In addition, on the following loop, the side chain and the backbone carbonyl oxygen atom of Gln 349 together with Asn 347, Asp 345, and Asp 343 shape the second Ca^{2+} site by wrapping around the metal ion. This site has a significant impact on the structural establishment of a catalytic diad needed for activation of the K substrate (Figure 2b). In the inactive conformation, the N_ε imidazole nitrogen atom of His 342 and the carboxylate oxygen atom of Glu 401 are too distant for any productive interaction (about 10 Å, Figure 2b). The coordination of Asp 343 to Ca^{2+} drags the loop towards the calcium ion, thereby resulting in a shift of His 342 into a position now capable of forming a hydrogen bond with Glu 401, which is located on a flexible loop and can therefore easily be shifted towards His 342.

The third calcium binding site further affects the binding of the lysine-containing substrate that attacks the thioester intermediate from the opposite side of the above-mentioned hydrophobic tunnel (Figure 2c). The interaction of Asp 271 with Ca^{2+} results in a reorientation of a loop near the protein surface. Moreover, Asn 267 and Asp 270 are part of this loop and interact with Tyr 407. The incorporated calcium ions, particularly at sites 2 and 3, act as a template and induce, through the involvement of polar side-chain functions in Ca^{2+} coordination, structural changes essential for the enzyme to shift from its active-site-shielded, inactive state to a functionally active state.

The proposed mechanism is further supported by structural data from related human transglutaminases.[17,18,20,21] For example, three calcium binding sites are populated in TG3, which is enzymatically activated and cleaved between the catalytic domain and β-barrel 1.[18,20] The activated human transglutaminases show high structural similarity to that of the activated FXIII structure deduced in the present study. Nevertheless, there is an important difference at site 2. In the case of FXIII, the side chain of Asp 351 interacts with the calcium ion in this site, whereas in the TG3 structure a water molecule occupies this site. As a consequence, the adjacent three-stranded β sheet is only shifted, and not rotated, in TG3. In FXIII, complete rotation is essential to fully establish the substrate binding pocket. We, therefore, presume that the TG3 structure does not represent the fully active state. Remarkably, an Asp residue at this pivotal position is highly conserved across all transglutaminases except TG2, which contains a Glu residue in this position (see the Supporting Information).

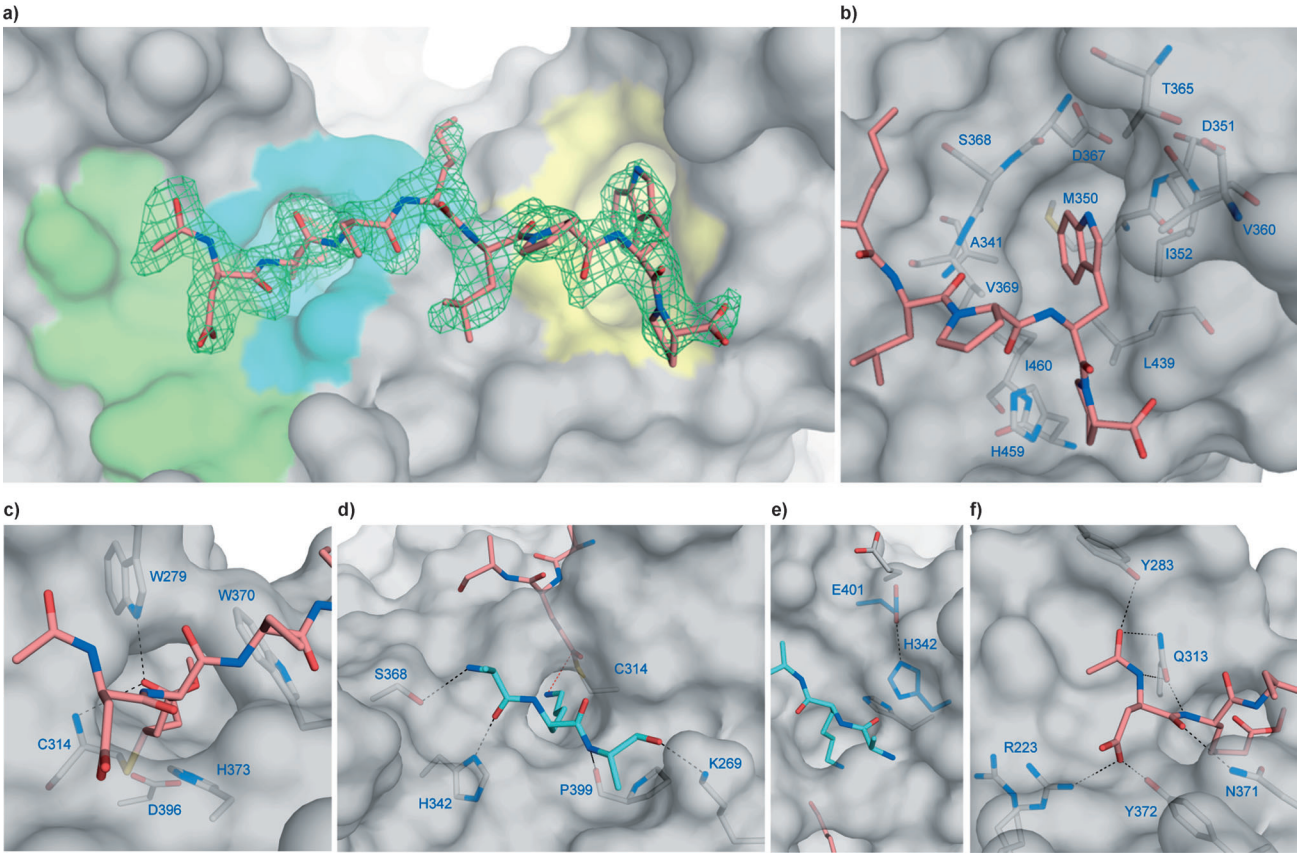

Figure 3. a) The ligand ZED1301 (orange, F_o-F_c difference electron density contoured at 3σ) binds covalently to Cys 314 in the catalytic site. Next to the latter site, the interaction region, called A space, is formed (green). At its far end, the ligand exposes an indole moiety to the hydrophobic pocket (yellow). The surface of FXIIIa° is depicted in gray. b) Enlargement of the hydrophobic pocket which is predominantly comprised of hydrophobic residues. c) The ligand forms a covalent bond to Cys 314 in the catalytic site and interacts through its Michael acceptor carbonyl oxygen atom with the NH groups of Trp 279 and Cys 314, which comprise the oxyanion hole. A hydrophobic tunnel is formed, flanked by Trp 279 and Trp 370, and prevents hydrolysis of the intermediately formed thioester. d) The K substrate modeled as an Ala-Lys-Ala tripeptide (cyan) fits perfectly into the back side of the hydrophobic pocket. Hydrogen bonds of the minimized tripeptide are shown as dashed lines. The original ligand was remodeled into a Q substrate (Ala-Gln-Ala tripeptide, orange) to simulate the binding of the two substrates. e) The newly established catalytic diad is located next to the entrance of the hydrophobic tunnel at the binding site of the K substrate. f) Hydrogen bonds formed between the ligand and protein in the A space.

Inhibitor ZED1301 binds at the surface of the catalytic domain of FXIIIa°. This region can be split into three main interaction sites, the catalytic site, the adjacent proximal "A space", and the more distant hydrophobic pocket (Figure 3a). The shape of the surface, which hosts the inhibitor, differs significantly from the geometry adopted in the inactive state, particularly with respect to the hydrophobic pocket induced upon calcium binding (Figure 3b).

Within the active site of FXIIIa°, the catalytic triad most likely adopts a geometry similar to that required for substrate binding and subsequent transformation into the thioester form. The Michael acceptor β-carbon atom of ZED1301 forms a covalent bond with Cys 314 (Figure 3c). Moreover, the carbonyl oxygen atom of the terminal methyl ester establishes hydrogen bonds to the oxyanion hole formed by the indole nitrogen atom of Trp 279 and the backbone NH group of Cys 314. The adjacent hydrophobic tunnel is formed from Trp 279 and Trp 370 and prevents hydrolysis of the intermediately formed thioester. The tunnel adopts a shape similar to that in TG2,[19] but in the active state of FXIIIa° the terminal "exit" that serves as an entrance port for the side chain of the reacting lysine residue of the K substrate is much better established. This helps to orient the K substrate in the correct geometry required to react with the thioester intermediate. This assumption is corroborated by docking an artificial Ala-Lys-Ala tripeptide that mimics the orientation of the K substrate in this site (Figure 3d). The lysine side chain fits perfectly into the hydrophobic tunnel and places the lone pair of electrons on its terminal ε-amino group at an appropriate distance to the thioester carbon atom. This location is compatible with the requirements of a nucleophilic attack of Lys on the thioester–enzyme intermediate. Furthermore, potential hydrogen bonds are readily identifiable between the tripeptide and protein, firmly fixing the substrate in position (Figure 3d).

In addition to the well-accepted catalytic triad, we propose the existence of a catalytic diad also exposed to the catalytic center to facilitate nucleophilic attack of the K substrate in FXIIIa°. This diad is composed of His 342 and Glu 401 and attains a productive arrangement upon

calcium activation (Figure 3e). Both His 342 and Glu 401 are conserved across all human transglutaminases except TG7, which contains Gln instead of Glu. About 20 years ago, Yee et al. attributed a catalytic function to His 342 and suggested assistance by Glu 434 and not Glu 401.[18] Mutation of His 342 to Ala resulted in a strikingly reduced activity with respect to the formation of the isopeptide bond, but only a minor change in the affinity of the K substrate towards the enzyme.[22] In the recently determined TG2 structure, assumed to represent the active state, the corresponding His 305 residue is oriented towards the glutamine-containing substrate-binding region.[19] Thus, one could argue that the latter structure does not yet correspond to the fully active state of TG2. Admittedly, our structure also represents only a different frozen situation in the solid state, reflecting a geometry the protein is, in principle, able to adopt. Nonetheless, we propose that this structure approaches the situation in the active state more closely as it can easily accommodate both substrates along with full population of all three calcium binding sites, establishment of the hydrophobic tunnel, and formation of the additional catalytic dyad.

The change in the protonation states of the substrates during formation of the isopeptide bond appears puzzling. Under physiological conditions, the lysine residue of the K substrate should bear a charged ammonium group. Yet, entering the protein binding site could induce a substantial pK_a shift towards lower basicity, thus resulting in an uncharged state of lysine. This effect might be supported by the adjacent His 373 of the catalytic triad, which could carry a positive charge. Upon formation of the amide bond, the catalytic diad could operate reversibly, similar to the well-known mechanism of the catalytic triad in serine proteases, and pick up the second proton from the amino head group of the reacting lysine residue.

Regarding the much-sought-after selectivity discrimination of drug candidates across the various human transglutaminases, it is interesting to note the marked differences in the architecture of the A space (Figure 3f) between FXIIIa° and TG2. We believe the selectivity issue is in particular relevant for this tissue transglutaminase, as TG2 is the most abundant member of the entire transglutaminase family. A tight network of strong hydrogen bonds between the inhibitor and FXIIIa° surface residues Tyr 283, Gln 313, Asn 371, Tyr 372, Arg 223, and Val 369 can be identified. In addition, FXIIIa° features some unique residues, such as Arg 223, and a negatively charged pocket at the lower A space formed by Glu 216, Asp 219, as well as Tyr 372. These differences should be exploited in tailoring inhibitors with sufficient affinity and selectivity for FXIIIa° in comparison to TG2. For experimental details of the structure determination see the Supporting Information.

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