Crystal structure of prethrombin-1

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Edited by Robert M. Stroud, University of California, San Francisco, CA, and approved September 24, 2010 (received for review July 14, 2010)

Prothrombin is the zymogen precursor of the clotting enzyme thrombin, which is generated by two sequential cleavages at R271 and R320 by the prothrombinase complex. The structure of prothrombin is currently unknown. Prethrombin-1 differs from prothrombin for the absence of 155 residues in the N-terminal domain and is composed of a single polypeptide chain containing fragment 2 (residues 156–271), A chain (residues 272–320), and B chain (residues 321–579). The X-ray crystal structure of prethrombin-1 solved at 2.2-Å resolution shows an overall conformation significantly different (rmsd = 3.6 Å) from that of its active form meizothrombin desF1 carrying a cleavage at R320. Fragment 2 is rotated around the y axis by 29° and makes only few contacts with the B chain. In the B chain, the oxyanion hole is disrupted due to absence of the I16-D194 ion pair and the Na⁺ binding site and adjacent primary specificity pocket are highly perturbed. A remarkable feature of the structure is that the autolysis loop assumes a helical conformation enabling W148 and W215, located 17 Å apart in meizothrombin desF1, to come within 3.3 Å of each other and completely occlude access to the active site. These findings suggest that the zymogen form of thrombin possesses conformational plasticity comparable to that of the mature enzyme and have significant implications for the mechanism of prothrombin activation and the zymogen → protease conversion in trypsin-like proteases.

X-ray crystallography | blood coagulation | E* form

Activation of trypsin-like proteases requires proteolytic processing of an inactive zymogen precursor (1) that occurs at the identical position in all known members of the family, i.e., between residues 15 and 16 (chymotrypsin numbering). The nascent N terminus induces formation of an ion pair with the highly conserved D194 that organizes both the oxyanion hole and primary specificity pocket for substrate binding and catalysis (2, 3). Through this mechanism, the zymogen irreversibly converts into the mature enzyme and affords regulation of catalytic activity. Importance of this paradigm is particularly evident in the activation, progression, and amplification of enzyme cascades, where each component acts as a substrate in the zymogen form in one step and as an enzyme in the subsequent step (4). Yet, recent findings suggest that this paradigm needs revision to account for the conformational plasticity of the protein acting as an enzyme or substrate (5, 6).

In the blood coagulation cascade, the protease thrombin is generated from the zymogen prothrombin by the prothrombinase complex, composed of factors Va and Xa, phospholipid membranes, and Ca²⁺ (7). Prothrombin is composed of a Gla domain, two kringle domains and the trypsin-like catalytic domain (Fig. 1). The prothrombinase complex converts prothrombin to thrombin along two pathways by cleaving sequentially at R271 and R320 (prothrombin numbering). Initial cleavage at R320 (R15 in the chymotrypsin numbering) between the A and B chains is the preferred pathway under physiological conditions and generates the active intermediate meizothrombin by triggering formation of the I16-D194 ion pair in the catalytic B chain, structuring the oxyanion hole and primary specificity pocket. The alternative initial cleavage at R271 sheds the Gla domain and the two kringles and generates the inactive precursor prethrombin-2. The physiological role of meizothrombin and prethrombin-2 is unclear. For the inactive precursors, there is currently no structure of prothrombin

Fig. 1. Schematic representation of prothrombin activation. Prothrombin contains a Gla domain, two kringles (K1 and K2), and a protease domain composed of the A and B chains. Cleavage at R320 separates the A and B chains and generates an active protease. Meizothrombin and prethrombin-2 are generated by a single cleavage at R320 or R271, respectively. Cleavage at both sites generates thrombin. The zymogen prethrombin-1 differs from prothrombin for the lack of the Gla domain and first kringle. The active form of prethrombin-1 is meizothrombin desF1.

and only two structures of prethrombin-2 (8, 9). Two available structures of the active intermediate meizothrombin refer to meizothrombin desF1 (10, 11), a version devoid of fragment 1 (residues 1–155 of prothrombin). The zymogen form of meizothrombin desF1 is prethrombin-1 and differs from prothrombin for the lack of the Gla domain and the first kringle domain (Fig. 1). Here we report the X-ray crystal structure of prethrombin-1 at 2.2-Å resolution that reveals an unprecedented conformation with occlusion of the active site similar to that observed in the inactive E* form of thrombin (11–15). Several independent lines of evidence suggest that trypsin-like proteases undergo an equilibrium between the inactive E* form and the active E form (5, 6) and that the distribution of these forms dictates the catalytic activity of the enzyme. The structure of prethrombin-1 validates this paradigm and extends it to the zymogen form.

Results

In the description below, we will use the prothrombin numbering for fragment 2 (residues 156–271 of prothrombin) and the A chain (residues 272–320 of prothrombin) and the chymotrypsin numbering for the B chain (residues 321–579 of prothrombin). The structure of prethrombin-1 was solved in the free form at 2.2-Å resolution with a final $R_{\text{free}} = 0.225$ (Table 1). The muta-

Author contributions: Z.C., L.A.P., and E.D.C. designed research; Z.C. and L.A.P. performed research; L.A.P. contributed new reagents/analytic tools; Z.C. and E.D.C. analyzed data; and E.D.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: Atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3NXP).

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tions R271A and R284A were introduced in the prethrombin-1 construct to avoid conversion to prethrombin-2 and further proteolytic processing of the A chain (11). Fragment 2 was resolved from Q169 to E254 missing a total of 13 residues on the N terminus and 17 residues on the C terminus connecting to the A chain (Fig. 2). Biochemical analysis revealed an intact construct, consistent with the absence of sites of autoproteolytic cleavage in the missing segment. The A chain was traced in the electron density map in almost its entirety, from T274 to the site of cleavage at R320, missing only T272 and A273. The B chain was well resolved from I16 to E247, with only V149c, G149d, K149e, and G150 missing in the typically disordered autolysis loop.

Fragment 2 assumes the expected fold for a kringle domain but is significantly (>5 Å) moved upward and rotated 29° around the y axis relative to the position in meizothrombin desF1 (11) (Fig. 2) or even the structure of thrombin bound to kringle 2 (16). The rotation and upward shift bring about changes in the contacts made with the B chain (Fig. 3) and contribute to the large rmsd = 3.6 Å (calculated from 292 common C α atoms) between prethrombin-1 and its active form meizothrombin desF1. H187 contacts the backbone O atoms of Y94 and P92 via its Ne2 and N δ 1 atoms, respectively. A patch of negatively charged residues of fragment 2 composed of D223, D225, E226, and E227 forms a Lys-binding kringle analogous to that present in plasminogen and tissue-type plasminogen activator (16) but binding to this patch is unlikely due to the conformation of K204 pointing out into the solvent as observed in the structure of thrombin bound to kringle 2 (16). The anionic patch engages the side chains of R93, R101, and R175 in meizothrombin desF1 (11). In prethrombin-1, E226 contacts a symmetry related molecule in the lattice. D223 makes strong ionic interactions with K240, which also engages D225, and a short H bond with the N ζ atom of K236 via its backbone O atom. The carboxylate of E227 inter-

acts with the guanidinium group of R101 and the Ne2 atom of H91. E249 makes strong H-bonding interactions with the N ζ atom of K169 and the guanidinium group of R165, which are contacts not present in the structure of meizothrombin desF1. Finally, the Oe2 atom of E254, a residue not resolved in the structure of meizothrombin desF1, H bonds to the guanidinium group of R126. Three disulfide bonds involving the Cys pairs 170–248, 219–243 and 191–231 are fully resolved in fragment 2, and so are W194 and W230 that are stacked. W230 is too distant for the cation- π interaction with R93 seen in the structure of meizothrombin desF1 and instead interacts with the N ζ atom of K240 via its Ne1 atom.

Removal of the site of proteolytic processing at R284 with the R284A substitution reveals how the A chain docks on the B chain in almost its entirety without making any contacts with fragment 2. Clear electron density detects 13 additional residues of the A chain for prethrombin-1 compared to the structure of meizothrombin desF1. Among these residues, the site of mutation at A284 is clearly visible and so is an aromatic trident formed by F280, F281, and F286 that penetrates a deep crevice of the B chain paved by I47, W51, I238, and part of the aliphatic side chains of K235 and K236 (Fig. 3). Residues 272–284 are not present in the structure of prethrombin-2 (8) and the segment T285–E290 is oriented differently compared to prethrombin-1. The last residue visible in the A chain of prethrombin-1 is T274, which is only three residues downstream of the cleavage site at R271 that separates fragment 2 from the A chain and generates prethrombin-2 (Fig. 1). T274 is positioned 27 Å away from the last traceable residue E254 in fragment 2 and 35 Å away from R320, which is fully exposed to solvent for proteolytic attack. Hence, substantial translation is necessary for factor Xa in the prothrombinase complex to access sequentially the two sites of cleavage at R271 and R320, as also implied by modeling studies (17).

The B chain of prethrombin-1 carries most of the changes of interest when compared to the active intermediate meizothrombin desF1. The activation domain shows an intact R15–I16 peptide bond (Fig. 4) with a conformation similar to that seen in the inactive precursor prethrombin-2 (8) and the zymogen forms of trypsin (18, 19), chymotrypsin (20), and chymase (21). Because of this intact bond, no N terminus is present in the B chain ready to engage the carboxylate of D194. Yet residues of the catalytic triad assume a correct orientation with the O γ atom of S195 within 2.6 Å of the Ne2 atom of H57 and the O δ 2 atom of D102 within 2.4 Å of the N δ 1 atom of H57, as seen in the structures of other zymogens (18–22). The side chain of D194 repositions and finds new H-bonding partners in the backbone N atoms of W141, G142, and N143 (Fig. 4). As a result, the entire 141–144 peptide segment is pulled upward toward the adjacent segment carrying E192 and G193, the highly conserved H-bonding interaction between the backbone N atom of N143 and the backbone O atom of E192 is disrupted and a new H bond forms between the backbone O atom of G193 and the backbone N atom of L144. The H bond between residues 143 and 192 is of utmost importance in trypsin-like proteases because it locks the 192–193 peptide bond in an orientation that enables the backbone N atoms of G193 and S195 to point toward the interior of the tight β -turn within the active site that defines the oxyanion hole (23–25). When the 143–192 H bond is broken, as illustrated directly by the N143P mutant of thrombin (15), the 192–193 peptide bond flips from a type II to a type I β -turn, the backbone O atom of residue 192 points inward toward the active site S195, and the 192–194 segment adopts a 3_{10} -helix conformation that disrupts the oxyanion hole. Examples of this perturbed conformation of the oxyanion hole have been reported for the inactive E* form of thrombin (12–15), complement factor B (26), the arterivirus nsp4 (27), the epidermolytic toxin A (28, 29), and clotting factor VIIa (30). Finally, the O δ atom of N143 engages the backbone N

Table 1. Crystallographic data for human prethrombin-1

Buffer	0.1 M Tris, pH 8.5
PEG	3,350 (20%)
PDB ID	3NXP
Data collection	
Wavelength, Å	0.979
Space group	$P2_12_12_1$
Unit cell dimensions, Å	$a = 67.9$ $b = 81.0$ $c = 88.5$
Molecules/asymmetric unit	1
Resolution range, Å	40–2.2
Observations	72,746
Unique observations	24,257
Completeness (%)	95.5 (94.5)
R_{sym} (%)	10.9 (39.0)
$I/\sigma(I)$	9.5 (2.4)
Refinement	
Resolution, Å	40–2.2
$R_{\text{cryst}}, R_{\text{free}}$	0.199, 0.225
Reflections (working/test)	21,751/1,244
Protein atoms	3,126
Solvent molecules	197
rmsd bond lengths,* Å	0.011
rmsd angles,* °	1.3
rmsd ΔB , Å ² (mm/ms/ss)	4.74/1.43/2.29
$\langle B \rangle$ protein, Å ²	41.5
$\langle B \rangle$ solvent, Å ²	40.6
Ramachandran plot	
Most favored, %	99.6
Generously allowed, %	0.4
Disallowed, %	0.0

PDB, Protein Data Bank; mm, main chain–main chain; ms, main chain–side chain; ss, side chain–side chain.

*Root-mean-squared deviation from ideal bond lengths and angles and rmsd in B factors of bonded atoms.

Fig. 2. Crystal structure of prethrombin-1 at 2.2-Å resolution (*Upper*) compared to the structure of meizothrombin desF1 (*Lower*) (11). Fragment 2 (gold) docking on the B chain (green) and making no contacts with the A chain (red) is moved >5 Å upward and rotated 29° around the y axis relative to the position seen in the active meizothrombin desF1 bound to PPACK (yellow sticks). Nineteen residues, from E255 to A273, connecting fragment 2 to the A chain in the back of the molecule (*Right*), are missing in the electron density map, as opposed to 36 residues in meizothrombin desF1. The site of cleavage between the A and B chains at R320 is fully exposed to solvent. In the front view (*Left*), the perturbed conformation of the B chain of prethrombin-1 is readily apparent. The side chains of Y60a, W60d, L99, W148, and W215 (orange) coalesce and occlude access to the active site.

atom of S195 precluding access of substrate to what remains of the oxyanion hole.

The changes imposed by D194 on the 141–144 peptide segment have additional and important consequences on the structure of prethrombin-1 and seed a helix turn in the autolysis loop never before documented in a thrombin structure (Fig. 5). The helix enables W148 to move >8 Å from the position assumed in meizothrombin desF1 and prethrombin-2 and to join W60d and W215 in a tightly packed cluster of aromatic residues capped by Y60a and L99 (Fig. 5). The cluster obliterates access to the active site, which is a feature not observed in other zymogen forms of trypsin (18, 19), chymotrypsin (20), chymase (21), factor VIIa (22), and thrombin itself, as documented by prethrombin-2 (8, 9). Although these zymogen forms have a disrupted oxyanion hole, they all share an open conformation of the active site and an intact architecture of the catalytic triad. The collapsed conformation of prethrombin-1, along with the open conformation observed in all other zymogen structures, vouch for the presence of alternative forms with the active site closed by the hydrophobic cluster (Fig. 5) or open as in prethrombin-2 (8, 9). An intriguing connection is established with the E*-E equilibrium of thrombin (31), meizothrombin (11), factor Xa (32), and activated protein C (32), where E* is an inactive form of the protease with the active site closed that interconverts on the millisecond timescale with the active form E where the active site is accessible to substrate.

Indeed, the side chain of W215 in the hydrophobic cluster of prethrombin-1 occupies a position that is very similar to that observed in the E* form of thrombin (12–15), where it relocates >10 Å from the position assumed in the active E form (33).

In the case of thrombin and meizothrombin desF1, Na⁺ binds between the 186 and 220 loops (11, 33, 34) and pulls the E*-E equilibrium in favor of E that converts to the more active form E:Na⁺ (11, 31). It is unclear if Na⁺ binds to thrombin precursors. Available structures of prethrombin-2 do not document a bound cation (8, 9). The structure of prethrombin-1 was solved in the absence of Na⁺ and shows a highly perturbed environment around the 186 and 220 loops. The 186 loop assumes a twisted conformation similar to that of prethrombin-2 and the 220 loop collapses into the space occupied by Na⁺ in the mature enzyme bringing the backbone O atom of K224, one of the ligands in the Na⁺ coordination shell, within H-bonding distance of the backbone N atom of D189 in the primary specificity pocket. The two atoms are >8 Å apart in the mature enzyme. Notably, the backbone O atom of K224 occupies a position analogous to that of the bound Na⁺ in the E:Na⁺ form of thrombin and meizothrombin desF1 (11, 33). Another notable change affects the side chain of D189 that moves >6 Å from the position assumed in meizothrombin desF1 and H bonds to the backbone O atom of R187 and the backbone N atom of Y184. Na⁺ binding to prethrombin-1 would require rearrangement of the 186 loop and up-

Fig. 3. Contacts between the B chain with the A chain and fragment 2 of prothrombin-1. Fragment 2 (gold cartoon and sticks) assumes the expected fold for a kringle domain but makes few contacts (sticks) with the B chain rendered as a surface in wheat (atoms <4 Å away are in cyan). Details of these contacts are given in the text. Three disulfide bonds involving the Cys pairs 170–248 (A), 219–243 (B), and 191–231 (C) are fully resolved in fragment 2, and so are W194 and W230 that are stacked. The A chain (green cartoon and sticks) decorates the back of the prothrombin-1 molecule (atoms <4 Å away are in orange) without making contacts with fragment 2. The site of mutation at A284 is clearly visible in the $2F_o - F_c$ density map (contoured at 1σ , green mesh) and so is the trident formed by F280, F281, and F286 that penetrates a deep crevice of the B chain. Shown are selected residues (sticks) that make contacts with the B chain, along with the terminal residues Q169 and E254 for fragment 2 and T274 for the A chain. Residues of fragment 2 and the A chain are labeled in black and relevant residues on the surface of the B chain are labeled in white.

ward shift of the 220 loop. That would likely pull the 215–217 segment along, reposition the side chain of W215 as seen in the active E:Na⁺ form, and disassemble the hydrophobic cluster formed by Y60a, W60d, L99, W148, and W215 restoring access to the active site. In this scenario, Na⁺ binding would be linked to a large change in fluorescence because of the change in environment experienced by major fluorophores like W215 and W148 (31). Contrary to this expectation, no significant fluorescence change is observed upon Na⁺ titration of prothrombin-1 up to [Na⁺] = 800 mM, which suggests that Na⁺ binding requires the zymogen → protease conversion to take place.

Discussion

We have recently proposed that E* interconverts with the active form E and that the E*-E equilibrium is a basic feature of the trypsin fold affording a simple mechanism of allosteric regulation of protease activity after the irreversible zymogen → protease conversion has taken place (5). Collapse of the 215–217 segment into the active site and disruption of the oxyanion hole due to a flip of the 192–193 peptide bond are distinctive features of the E* form of thrombin and occur with the I16–D194 ion pair intact (12–15). A collapsed conformation of the 215–217 segment is also seen in the structures of α 1-trypsin (35), the high-temperature-requirement-like protease (36), complement factor D (37), granzyme K (38), hepatocyte growth factor activator (39), prostate kallikrein (40), and prostatic (41, 42). The physiological relevance of the E*-E equilibrium is illustrated by numerous examples. Stabilization of E* is particularly important in maintaining the resting state of complement factor D that does not have a

Fig. 4. Activation domain and zymogen architecture of the oxyanion hole in prothrombin-1. (A) The segment around the cleavage site at R320 (R15 in the chymotrypsin numbering) defines the activation domain and shows an intact R15–I16 peptide bond and a conformation similar to that of prothrombin-2 (8). The electron density $2F_o - F_c$ map (green mesh) is contoured at 1σ . (B) Lack of the I16–D194 ion pair forces the side chain of D194 to seek alternative H-bonding partners in the backbone N atoms of W141, G142 and N143. The latter backbone N atom forms a key H bond with the backbone O atom of E192 in the active protease (15). Disruption of this H bond causes the E192–G193 peptide bond to flip (arrow) with resulting disruption of the oxyanion hole formed by the backbone N atoms of G193 and S195. Added perturbation to the architecture of the oxyanion hole comes from the side chain of N143 that H bonds to the backbone N atom of the catalytic S195. The electron density $2F_o - F_c$ map (green mesh) is contoured at 2σ .

zymogen form or known natural inhibitors (37). In this case, binding of substrate induces the transition to the active E form. In the case of complement factor B (26), hepatocyte growth factor activator (39), or clotting factor VIIa (22, 30), stabilization of E* is achieved after the zymogen → protease conversion has taken place and transition to the active E form relies on the binding of substrate and/or cofactors. The observation that prothrombin-1 can assume a collapsed conformation similar to E* and that an open conformation similar to E is documented in other zymogens (8, 9, 18–21) suggests that the E*-E equilibrium may also exist in the zymogen form of the protease.

Evidence of conformational plasticity in prothrombin-1, which differs from prothrombin only for the lack of fragment 1 and is therefore a better model of this zymogen form than prothrombin-2 (Fig. 1), has significant implications for the mechanism of action of prothrombinase. The conformational plasticity of prothrombin supported by the structural results reported here and the existence of the E*-E-E:Na⁺ equilibria recently uncovered for factor

Fig. 5. Hydrophobic cluster of prethrombin-1. A cluster of hydrophobic/aromatic residues completely occludes access to the active site of prethrombin-1 (gold cartoon and sticks). The cluster is formed by the collapse of W215 and W148 into the active site, with the indole rings moving 8–10 Å away from their positions in prethrombin-2 [cyan, Protein Data Bank (PDB) ID 1HAG] (8) or meizothrombin desF1 (11). Y60a, W60d, L99, W148, and W215 (gold) are in van der Waals interaction (dotted lines with distances). The collapse of W215 into the active site is similar to that observed in the E* form of thrombin (white, PDB ID 3BEI) (12–15). Crystal contacts with symmetry related molecules in the lattice are >4.6 Å away in the region surrounding the hydrophobic cluster. The electron density $2F_o - F_c$ map (green mesh) is contoured at 2σ .

Xa (32) call for a mechanism of prothrombin activation where multiple conformations are accessible to both enzyme and substrate. Conformational plasticity may play an important role when the protein functions as a substrate before the irreversible zymogen → protease conversion has taken place. In this scenario, the repertoire of biological activities and regulatory interactions accessible to an enzyme cascade is greatly expanded. Distinct forms of the protease can target distinct conformations of the

zymogen form of another protease acting as substrate. In turn, different forms of the protease can be stabilized upon interaction with biological cofactors. The coagulation and complement cascades are two relevant examples where conformational plasticity may dictate the fate of each enzymatic step. The cascades share a common evolutionary origin (4) and feature several Na^+ -activated enzymes (43). Structural detection of multiple conformations for the zymogen and protease forms of such enzymes remains challenging because it requires crystallization of the free form. However, the growing appreciation of this key aspect of protease biology (5, 22) will undoubtedly broaden our understanding of how key biological processes unfold at the molecular level.

Materials and Methods

The prethrombin-1 construct carrying the double substitution R271A/R284A, used in our recent structural characterization of meizothrombin desF1 (11), proved suitable for crystallization of prethrombin-1 that was achieved at 22 °C by the vapor diffusion technique using an Art Robbins Instruments Phoenix™ liquid handling robot and mixing equal volumes (0.2 μL) of protein (9 mg/mL) and reservoir solution. Optimization of crystal growth was achieved by hanging drop vapor diffusion method mixing 3 μL of protein (11 mg/mL) with equal volumes of reservoir solution (Table 1). Diffraction quality crystals grew in about 2 wk and were cryoprotected in a solution similar to the reservoir solution but containing 15% glycerol prior to flash freezing. X-ray diffraction data were collected on the Area Detector Systems Corporation Quantum-315 CCD detectors at the Northeastern Collaborative Access Team beamline 24-ID-E at the Advanced Photon Source, Argonne National Laboratory. The data were indexed, integrated, and scaled with the HKL2000 package (44). The structure was solved by molecular replacement using MOLREP from the CCP4 suite (45) and Protein Data Bank ID code 3E6P for meizothrombin desF1 (11) as a search model. Refinement and electron density generation were performed with REFMAC5 from the CCP4 suite and 5% of the reflections were randomly selected as a test set for cross-validation. Model building and analysis of the structures were carried out using COOT (46). In the final stages of refinement for both structures, translation, libration, screw tensors modeling rigid-body temperature factors were calculated and applied to the model. Ramachandran plots were calculated using PROCHECK (47). Statistics for data collection and refinement are summarized in Table 1.

ACKNOWLEDGMENTS. We are grateful to Dr. Narayanasami Sukumar for his assistance with synchrotron data collection and to Ms. Tracey Baird for her help with illustrations. This work was supported in part by the National Institutes of Health Research Grants HL49413, HL58141 HL73813, and HL95315 (to E.D.C.).

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