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Letter to the Editor

Do the crystallographic forms of prethrombin-2 revert to a single form in solution?

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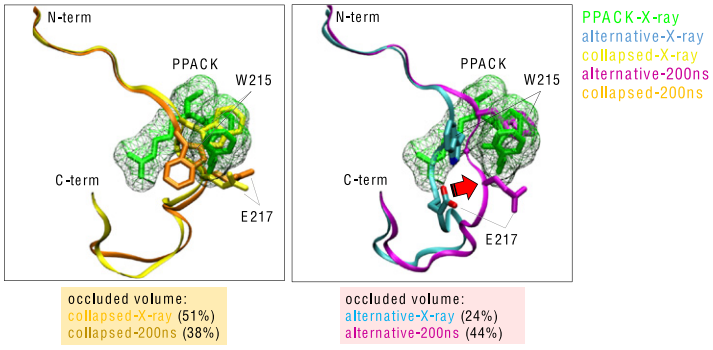
- The two X-ray crystal forms of prethrombin-2 appear to relax into two different forms in solution via MD simulations.
- The two solution forms are characterized by the position of the W215–E217 segment relative to the active site.
- The torsion angle for the disulfide C191–C220 relaxes to unique values for each of the two solution forms.
- The 180s-loop is greatly stabilized in the open solution form derived from the X-ray crystal alternative form.



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ABSTRACT

It has been earlier established (Pozzi et al. Biochemistry 50 (2011) 10195–10202) that prethrombin-2 crystallizes into two similar but distinct forms: a collapsed form and an alternative form. We employed long molecular dynamics (MD) simulations for these two forms to obtain solvent-equilibrated forms. We find that, at 200 ns, the simulated solution collapsed form is quite similar to the X-ray crystal collapsed form, while the simulated solution alternative form deviates from the X-ray crystal alternative form as well as from the solution collapsed form. A detailed structural analysis suggests that the fluctuation of the 140s-loop, in cross-talk with the 220s-loop, may alter the conformation of the W215–E217 segment near the nascent thrombin active site. A rationale is provided for the manner in which interactions of prethrombin-2 with FVa may affect the equilibrium between the two forms of prethrombin-2.

Dear Editor,

Prethrombin-2 is one of two intermediates in the processing of prothrombin to thrombin. It results from cleavage of prothrombin at R271 by factor Xa (FXa) in the presence of cofactor factor Va (FVa). It was found recently that uninhibited prethrombin-2 crystallizes into two distinct forms: an alternative form that appears to have more space for substrate binding at the nascent thrombin active site and a collapsed form which has this active site somewhat blocked [1]. This distinction was mainly caused by the orientation of the W215–E217 segment (Fig. 1A). The structures found for the two crystal forms are quite similar, otherwise, for the structured part of the sequences (RMSD

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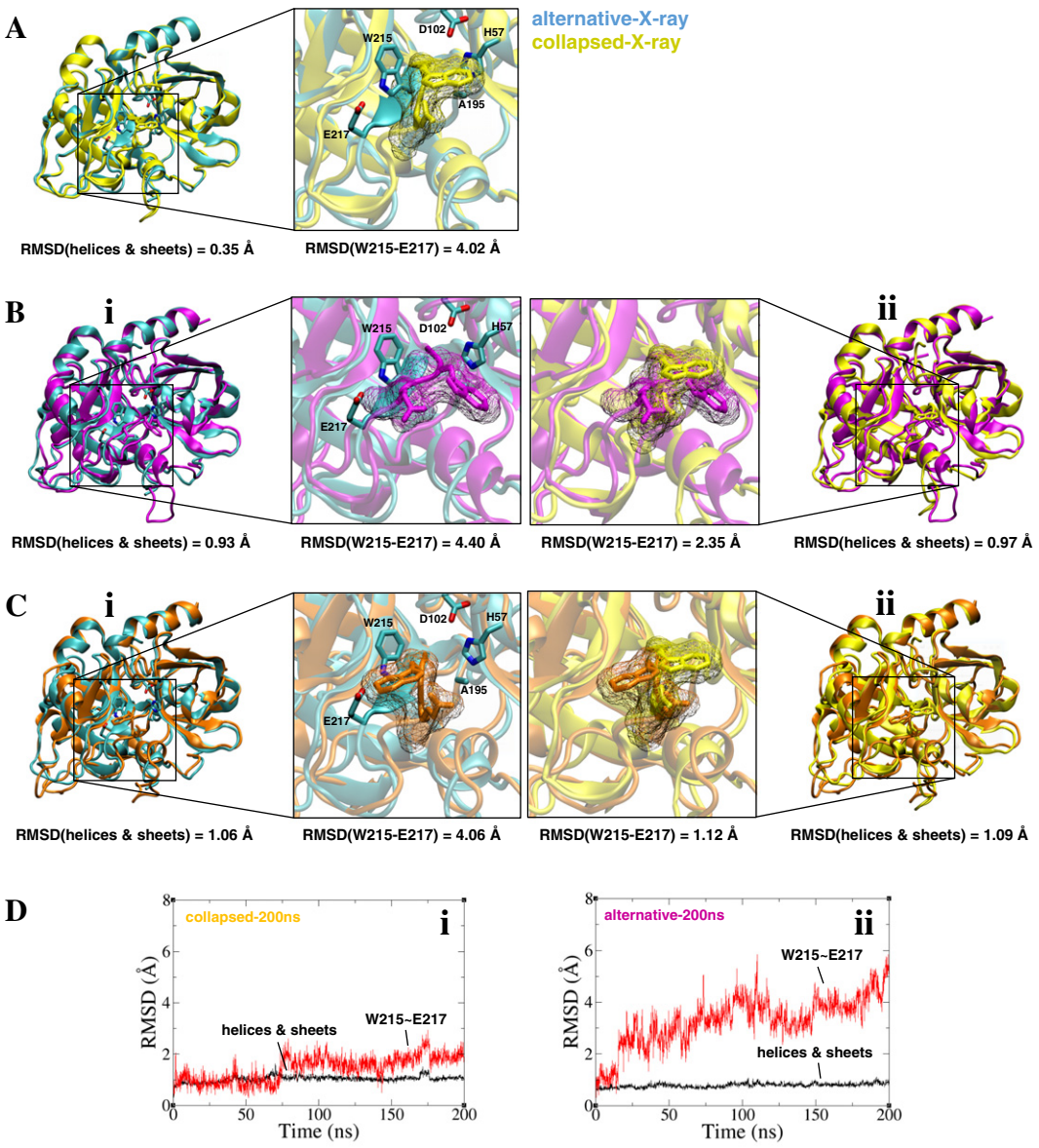

Fig. 1. A. Overlay of the X-ray crystal collapsed form (pdb code 3SQE) [1] (in yellow) to the X-ray crystal alternative form (pdb code 3SQH) [1] (in cyan). B. Overlay of the final solution alternative form (in magenta) to the X-ray crystal alternative form (in cyan) (i) and to the X-ray crystal collapsed form (in yellow) (ii). C. Overlay of the final solution collapsed form (in orange) to the X-ray crystal alternative form (in cyan) (i) and to the X-ray crystal collapsed form (in yellow) (ii). Residues W215–E217 and the catalytic triad of the X-ray crystal alternative form (colored by atom type) are represented by sticks, while residues W215–E217 of the X-ray crystal collapsed form and the two solution forms are represented by sticks and meshes. RMSD(helices & sheets) and RMSD(W215–E217) represent RMSD values (in Å) of the backbone C_α atoms of secondary structure and the C_α atoms of the W215–E217 segment, respectively, of the two solution forms compared to the two X-ray crystal forms. RMSD values were calculated after RMS fitting, with respect to the backbone C_α atoms of the secondary structure. All heavy atoms of residues W215–E217 were used to calculate the RMSD(W215–E217) values in parenthesis. D. (i) The RMSD plots for RMSD(helices & sheets) (in black) and RMSD(W215–E217) (in red) of the solution collapsed form for 200 ns simulation, calculated by using the X-ray crystal collapsed form as the reference structure. (ii) The RMSD plots for RMSD(helices & sheets) (in black) and RMSD(W215–E217) (in red) of the solution alternative form for 200 ns simulation, calculated using the X-ray crystal alternative form as the reference structure.

0.35 Å for backbone Cα atoms of the structured part). An overlay of the two structures is shown in Fig. 1A. We were fascinated by the obvious question: what happens in solution? Do these two forms appear to converge to a single form or do the two forms maintain separate identities? To attempt to answer these questions, we conducted relatively long unrestrained molecular dynamics (MD) simulations of each of the crystal forms.

Methods

The detailed methodology is given in the Supplementary information (SI). Some processing of the X-ray crystal files was necessary: 1) a missing loop between residues N149B–Q151 in the alternate form was modeled with *Modeller ver. 9.7* [2], 2) a missing residue (E247) at the C terminus of the collapsed form was added and 3) the alanine at the putative thrombin active site (i.e. residue 195) was changed to the active serine in both forms. Both forms were equilibrated and then allowed to evolve freely for 200 ns in neutral solution with the program *NAMD ver. 2.8* [3]. Long range electrostatic interactions were accommodated. For details of the simulation protocol, see Supplementary Table S1. The resulting structures at the end of 200 ns simulations of each of the solution forms of prethrombin-2 are freely available at the journal homepage or from the authors.

To calculate the RMSD values, the structures were fitted with respect to the Cα atoms of structured regions (α-helices and β-sheets) common to X-ray crystal structures and both solution forms. RMSD(helices &

sheets) and RMSD(W215–E217) indicate the RMSD values for the secondary structure and for the W215–E217 segment, respectively.

We adapted the method that Gohara and Di Cera [4] used to estimate the differences in substrate binding volume occluded in the active site of the two solvent-equilibrated forms. Assuming that the volume occupied by the active site inhibitor PPACK in thrombin (pdb code 1SHH) [5] represented the available volume for substrate binding (i.e. 100%), we examined the substrate binding volume reduced by the W215–E217 segment volume in the various forms of prethrombin-2. First, the solvent-equilibrated structures of prethrombin-2 were superposed to the structure of thrombin bound to the active site inhibitor PPACK [5] using the Swiss-PdbViewer program [6]. The volumes of the W215–E217 segment and PPACK alone, and the volumes occupied by the W215–E217 segment and PPACK combined were calculated using the program *Mol_Volume* (http://www.ks.uiuc.edu/Development/MDTools/molvolume/) that allowed the measurement of the number of vertices of a dense regular grid within the probe radius of the molecule's atoms. We used a grid radius of 1 Å. The volume of overlap between the W215–E217 segment and PPACK was estimated by subtracting the combined volume from the individual volumes of the W215–E217 segment and PPACK. The substrate binding volume (%) occluded by the W215–E217 segment was then calculated by dividing the volume of overlap between the W215–E217 segment and PPACK by the volume by PPACK.

Results and discussion

RMSDs of the solution forms in comparison with the X-ray crystal forms

The resulting structures at the end of 200 ns simulations of each of the solution forms of prethrombin-2 are displayed in Fig. 1B and C. We wished to find whether the two forms, after solvent-equilibration with molecular dynamics, merged to one in solution, or two separate forms were present. Since the crystal packing in prethrombin-2 (Supplementary Tables S2 and S3) is lost in the solution process, one would expect that the X-ray crystal forms of prethrombin-2 would relax. As shown in Fig. 1D of the RMSD plots, the RMSD(helices & sheets) values of both solution forms compared to either of the X-ray crystal forms of prethrombin-2 remain low (~1 Å) throughout the simulation, suggesting that the overall fold of prethrombin-2, as determined in the X-ray crystal structures [1], is quite stable and little influenced by the physical state. These results also suggested that the overall fold in the X-ray crystal forms of prethrombin-2 was little affected by crystal packing. Comparison of the RMSD(W215–E217) plot for both solution forms (Fig. 1D) shows that the solution collapsed form converged quickly (~2 Å within 75 ns), while the solution alternative form continued to evolve (~6 Å at 200 ns), indicating that the W215–E217 segment of the solution collapsed form is similar to that in the X-ray crystal collapsed form, while the W215–E217 segment of the solution alternative form is deviating from that of the X-ray crystal alternative form.

Pattern of a cluster of four hydrophobic residues W60d, W148, W215, and F227

Since W215, a member of a hydrophobic cluster also involving W60d, W148, and F227, has been found to play a crucial role in distinguishing the two X-ray crystal forms of prethrombin-2[1], we initially examined the pattern of the side chains of these four hydrophobic residues in the solution forms in comparison with the X-ray crystal forms. We noted that for the solution collapsed form the side chain of W215 fluctuated from the beginning to the end of the 200 ns simulation, while for the solution alternative form the side chain of W215 inserted somewhat into the catalytic site (at 70 ns) and retained this position for the remainder of the simulation (Fig. 1B and C). Thus, the examination of the pattern of these four residues near the catalytic site appeared to be less informative for determining whether the two solution forms converge to a single form or maintain separate identities in solution.

Subsequently, we analyzed the segment W215–E217 of our simulated solution structures based upon the following considerations: 1) The conformation of the W215–E217 segment backbone was quite unique in the X-ray crystal structures of prethrombin-2 [1]: the W215–E217 segment backbone moves away from the catalytic site in the alternative form and somewhat blocks the catalytic site in the collapsed form. This segment, which blocks the volume available for substrate binding 24% in the X-ray crystal alternative form and 48% in the X-ray crystal collapsed form [1], appears to be crucial for determining the accessibility of a substrate to the catalytic site. 2) This segment, attached to the 220s-loop (also known as the Na^+-binding loop) that is exposed to solvent, is expected to be sensitive to the state of prethrombin-2, whether it exists as a free form or a bound form and whether it is in crystal or in solution.

Backbone movement of the W215–E217 segment

We compared the backbone movement of the W215–E217 segment in our simulated prethrombin-2 structures with that in the X-ray crystal structures [1]. A detailed analysis of the W215–E217 segment in terms of the Cα distance change of W215 and E217 in the solution forms compared with these residues in the X-ray crystal forms reveals that for the solution collapsed form the W215–E217 segment remains as in the X-ray crystal collapsed form, while for the solution alternative form the W215–E217 segment deviates from that in the X-ray crystal alternative form (Fig. 2i and ii). Thus, in the solution collapsed form only E217 showed some displacements while in the solution alternative form the entire segment showed displacements from the corresponding X-ray

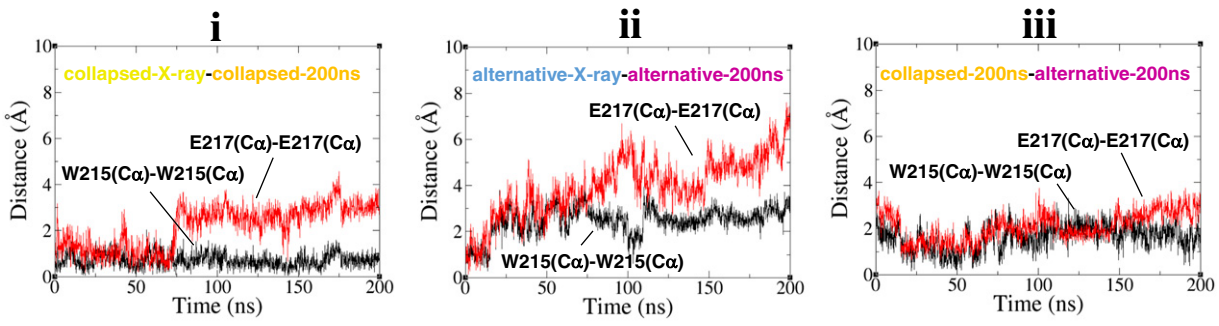

Fig. 2. (i) The distance (in Å) between the Cα atoms of W215 (in black) and between the Cα atoms of E217 (in red) of the solution collapsed form and the X-ray crystal collapsed form of prethrombin-2. (ii) The distance (in Å) between the Cα atoms of W215 (in black) and between the Cα atoms of E217 (in red) of the solution alternative form and the X-ray crystal alternative form of prethrombin-2. (iii) The distance (in Å) between the Cα atoms of W215 (in black) and between the Cα atoms of E217 (in red) of the solution collapsed and alternative forms of prethrombin-2. The distance values were calculated after RMS fitting of the solution form to the X-ray crystal form, with respect to the backbone C_α atoms of secondary structure.

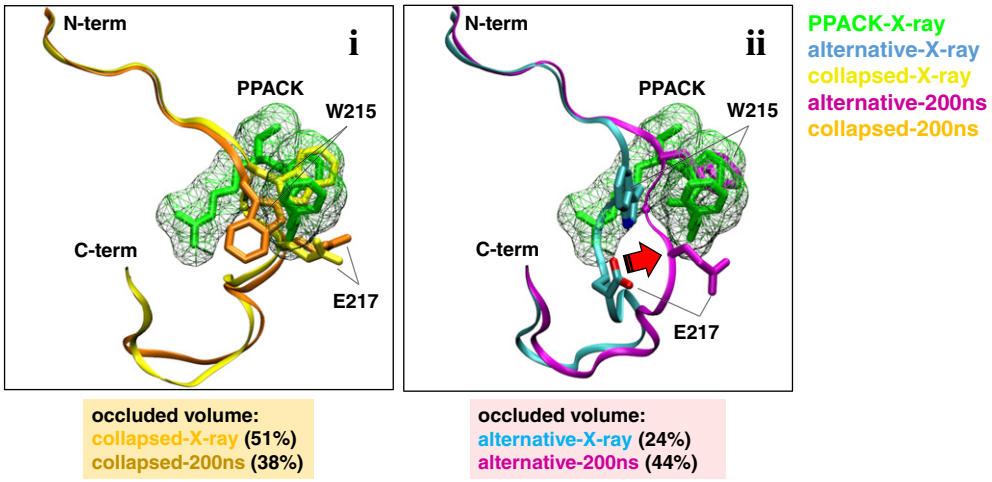

Fig. 3. Substrate volume occluded by the W215–E217 segment in (i) the solution collapsed form (in orange) compared with the X-ray crystal collapsed form (in yellow) and (ii) the solution alternative form (in magenta) compared with the X-ray crystal alternative form (in cyan). The X-ray crystal structure of thrombin bound to PPACK (in green) [5] was used to superimpose all of the structures using the Swiss-PdbViewer program [6]. Only the loop region (M210–Y225) containing the W215–E217 segment is shown. In ii, the backbone movement of the W215–E217 segment from the X-ray crystal alternative form to the solution alternative form is indicated by the red arrow.

forms. As shown in Fig. 2iii, relative distances of W215 and E217 between the two solution forms show that W215 is somewhat converging while E217 is somewhat diverging.

Active site blockage by the W215–E217 segment

In Fig. 3, substrate volumes occluded by the W215–E217 segment in our solution forms are represented. Our estimated volume occluded by the W215–E217 segment in the solution alternative form (44%) is significantly different from that of the X-ray crystal alternative form (24%). Although the corresponding volume in the blocked form (38%) appears to be somewhat different from that in the X-ray crystal collapsed form (51%), we believe that it remained similar, for the decrease in volume in the solution collapsed form was caused by the side chain of W215 that is flipped away from the catalytic site in the solution form (Fig. 3i). Thus, our analysis of the estimated volumes blocked by the W215–E217 segment suggests that the solution collapsed form of this segment remains largely as the X-ray crystal collapsed form, while the solution alternative form diverges from the X-ray crystal alternative form. This result is also suggested in both the RMSD(W215–E217)

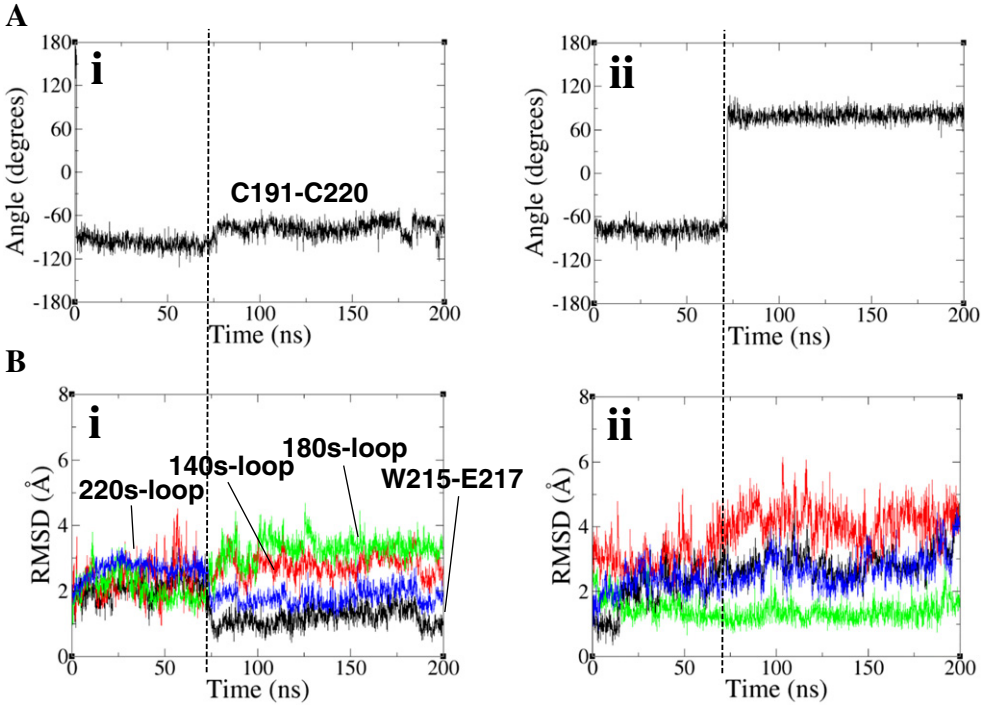

Fig. 4. A. The torsional angle around the disulfide bond between C191 and C220 (i.e. C191(Cβ)–C191(Sγ)–C220(Sγ)–C220(Cβ)) in the solution collapsed form (i) and in the solution alternative form (ii). The dotted lines indicate the time of the change in the torsional angle around the disulfide bond between C191 and C220. B. Possible correlations between the W215–E217 segment (in black) and the loops in the activation domain, including the 140s-loop (in red), 180s-loop (in green), and 220s-loop (in blue) in the solution collapsed form (i) and in the solution alternative form (ii). [loops: 140s = 141–153; 180s = 184–188; 220s = 215–225] RMSD plots were created after RMS fitting with respect to the backbone Cα atoms of the secondary structure.

plots (Fig. 1D) and in the distance analysis of the W215–E217 segment (Fig. 2) of the two solution forms. The finding that the volume blocked by the W215–E217 segment in the solution alternative form is similar to that in the X-ray crystal collapsed form, however, does not guarantee that the solution alternative form is merging to the X-ray crystal collapsed form.

Structural elements that affect the relative position of the W215–E217 segment

Firstly, there are three loops (the 140s-, the 180s- and the 220s-loops) that are (roughly) located in a quadrant for which the W215–E217 segment defines an origin. These loops are dynamical and thus can interact at times. Secondly, the 180s- and 220s-loops are linked with a disulfide bond, C191–C220. When we analyzed the time dependence of the C191–C220 torsion angle over the 200 ns trajectories, we found that at 75 ns, the torsion angle for this disulfide bond underwent changes—a small change for the solution collapsed form and a much larger change for the solution alternative form. Once the changes occurred, the torsion angles remained at the new values (Fig. 4A). We then systematically determined the time dependence of the RMSD (in the context of maximum overlay of structured elements) for each of the loops and for the W215–E217segment for both solution structures. The organizing element for the analysis turns out to be the torsion angle of the disulfide. In Fig. 4Bi, for instance, we see a sharp reduction (stabilization) in the RMSD at 75 ns for the W215–E217 segment of the collapsed form. In Fig. 4Bii, we see an increase in the RMSD of this segment for the solution alternative form after 75 ns. The vertical lines in Fig. 4 emphasize the time of the disulfide changes. The 140s-loop RMSDs increase at this time for both solution forms of prethrombin-2, the greatest increase occurring for the alternative form. It is perhaps not so surprising that for both solution forms, there is strong correlation between the RMSDs of the 220s-loops and the W215–E217 segments (direct connection). More surprising, however, is the behavior of the 180s-loop: it is most displaced for the solution collapsed form and most stabilized for the solution alternative form. In summary, it is clear that the loops that might affect the position of the W215–E217 segment relative to the thrombin active site-to-be behave with different dynamics for each of the two solution forms of prethrombin-2.

Relationship between the fluctuation of the 140s-loop, in cross-talk with the 220s-loop, and the conformation of the W215–E217 segment in the activation pocket

Previous studies on thrombin had led to the suggestion that the 140s-loop [7] and the torsion C191–C220 angle [10] were deeply involved in thrombin allostery. Likewise, in the X-ray crystal structure of a mutant prethrombin-2 (Ala replaces E14e, D141, E18 and S195) [8], the W215–E217 segment is displaced away from the active site-to-be, as is also the case for the solution (here) and for the X-ray crystal alternative forms of wild type prethrombin-2 [1]. In all three cases, the 140s-loop is disordered. Thus the link between the 140s-loop disorder, the C191–C220 torsion angle change and the W215–E217 displacement away from the active site-to-be may facilitate the activation of prothrombin.

Since the 220s-loop appears to play an important role in the W215–E217 displacement, as indicated by our correlation analysis (Fig. 4B) and also as reported in a recent study by Pozzi et al. [1], and perhaps play a biological function for prethrombin-2, we examined prethrombin-2 within the context of the processing of prethrombin-2 by prothrombinase. Upon examination for possible contacts of prothrombinase with prothrombin in our recent ternary complex model of prothrombinase–prothrombin [9], we find that R313 and R316 of the FVa A2 domain form tight salt bridges with E186b and D186a, respectively, of the 180s-loop of prothrombin. If we assume that prethrombin-2 and prothrombin are positioned similarly within prothrombinase, as shown in the overlay of prethrombin-2 to prothrombin within prothrombinase of the ternary model (Supplementary Fig. S1), we would then expect stabilization of the 180s-loop of prethrombin-2 by FVa, as seen in the solution alternative form (Fig. 4Bii). Such stabilization appears to influence (stabilize) the W215–E217 segment to be positioned *away* from the catalytic site, as seen in the X-ray crystal alternative form of human prethrombin-2 [1]. Thus, interactions between the 180s-loop of prethrombin-2 and FVa may be of biological significance by inducing the inactive prethrombin-2 (the collapsed form) into the active thrombin form (the open form).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bpc.2015.05.005.

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