Employing Mutants to Study Thrombin Residues Responsible for Factor XIII Activation Peptide Recognition: A Kinetic Study†

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ABSTRACT: In the last stages of coagulation, thrombin helps to activate Factor XIII. The resultant transglutaminase introduces covalent cross-links into fibrin thus promoting clot stability. To better understand the roles of individual thrombin residues in recognition and hydrolysis of the Factor XIII activation peptide, mutations within thrombin's aryl and apolar binding site were explored. The thrombin mutants W215A, E217A, W215A/E217A, L99A, and I174A were examined through HPLC kinetics against the substrates FXIII (28−41) V34 AP and FXIII (28−41) V34L AP. Several mutants responded differently to FXIII (28−41) V34 AP vs the cardioprotective V34L AP. W215 provides an important platform for binding and directing FXIII APs for proper hydrolysis. Loss of this platform leads to decreases in kinetics, particularly to the k_{cat} of FXIII V34L AP. E217 also plays a supporting role, but the E217A mutation is not as detrimental as W215A. W215A/E217A is unfavorable for both activation peptides and its coupling effect has been characterized. This mutant can readily bind the peptides but cannot orient them for effective hydrolysis. Kinetic studies with I174A indicate that this thrombin residue is more crucial for interactions with the larger V34L AP segment. The L99A mutation causes deleterious effects to binding and hydrolysis of both APs. The V34L, however, is able to partially compensate for the loss perhaps by increasing contact within the aryl and apolar sites. Understanding how specific FXIII and thrombin residues participate in binding and control hydrolysis may lead to the design of coagulation enzymes whose degree of activation and optimal target site can be controlled.

Blood coagulation involves a series of reactions in which plasma zymogens of serine proteases are activated to their enzyme forms. The activated proteases function sequentially such that the reaction products serve as the next enzyme in a process amplifying the velocity of the overall reaction: formation of an insoluble fibrin clot (*1*). In the last stages of this cascade, thrombin cleaves specific Arg-Gly bonds within the N-terminal portion of the Aα and Bβ chains of fibrinogen $(A\alpha B\beta\gamma)_2$ thereby releasing fibrinopeptides A and B. This cleavage leads to exposure of fibrin polymerization sites promoting noncovalent aggregation of the resultant fibrin molecules (*1*−*3*). Thrombin also helps to activate Factor XIII (FXIII)[1] by hydrolyzing the Arg37-Gly38 bond. Activated FXIII catalyzes the formation of γ-glutamyl-ε-lysyl covalent cross links in the fibrin network resulting in the formation of a proteolytically stable clot. The protease activated receptors (PARs) are also activated by thrombin. PAR1 is cleaved by thrombin and the new N-terminus then becomes a tethered ligand helping to initiate a signaling cascade (*4*).

Thrombin regulates substrate binding by utilizing anion binding exosites as well as by exploiting different residues within the extended active site region. Additionally, thrombin is a sodium activated type-II enzyme (*5*). Sodium ion binding helps to orient the aspartate residue within the S_1[2] site to accept a substrate for effective hydrolysis (*5*, *7*−*13*). Once the P_1[2] arginine is engaged by thrombin D189, residues N-terminal to the cleavage site help direct the binding of substrates to thrombin taking advantage of the apolar and aryl binding sites surrounding the active site. Some substrates also take advantage of the anion binding exosites (ABE) on thrombin to enhance activity of the enzyme. ABE-I and ABE-II lie to the right and left of the active site, respectively. See Figure 1A. Fibrin-I binding at ABE-I increases thrombin hydrolysis of the FXIII activation peptide (FXIII AP) by 80-fold (*14*).

In NMR and X-ray structures, fibrinogen (Fbg) Aα (7−16) has been shown to bind thrombin by participating in a β-turn conformation whereby the phenylalanine at the P_9 position is returned to the hydrophobic pocket of the active site. This additional contact is the key stabilizing interaction for this substrate (*15*−*17*). The X-ray crystal structure (*18*) of FXIII (28−37) V34 AP bound to thrombin depicts the N-terminus of the peptide in a β-turn conformation much

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[1] The abbreviations used are as follows: FXIII (28−41) AP, blood clotting Factor XIII activation peptide; V34 AP, wild type Factor XIII activation peptide; V34L AP, Val to Leu polymorphism of Factor XIII activation peptide; IIa, thrombin; ABE-I, anion-binding exosite-I; ABE-II, anion-binding exosite-II; PAR1, protease activated receptor 1.

[2] The P nomenclature system is used to assign the individual amino acid positions on the substrate peptides (*6*). The scissile bond is designated by P_1-P_1'. The substrate amino acids to the left of the hydrolysis site are labeled P_2, P_3, P_4, etc. whereas those to the right are labeled P_2', P_3', and so on. Likewise, the S nomenclature is used to assign positions on the enzyme. S_1 accepts the P_1 residue from the peptide substrate and so on.

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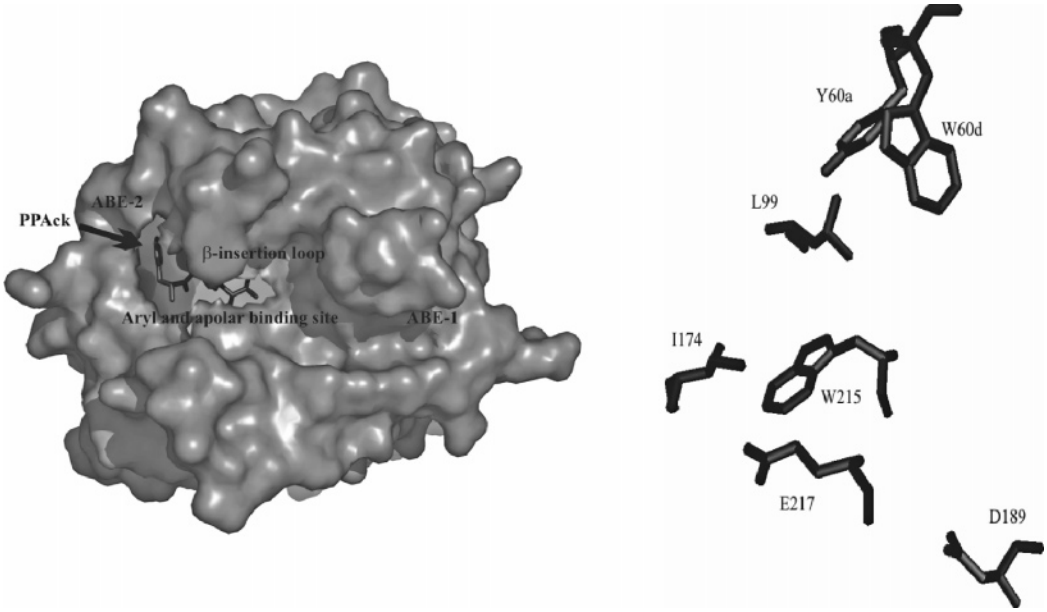

FIGURE 1: Viewing the Thrombin Active Site and the Two Surrounding Exosites. (A) Contour of thrombin with inhibitor D-Phe-Pro-Arg-chloromethyl ketone (PPAck) bound in the active site. This image represents thrombin regions important for binding and hydrolysis of substrates (PDB 1PPB). (B) Individual thrombin residues. Diagram of individual residues within the aryl and apolar binding sites on thrombin. Y60a and W60d are members of the Trp60 β-insertion loop. L99, I174, and W215 are members of the apolar and aryl binding sites. E217 is an integral residue stabilizing D189 in the S_1 pocket. D189 accommodates the positively charged arginine side chain of the hydrolyzable R−X peptide bond (PDB 1PPB). The molecular graphics program PyMOL (*44*) was used to create the structures displayed in Figures 1−3.

like that of Fbg Aα (7−16). In contrast, 2D-transferred NOESY data have suggested that the N-terminus of the bound FXIII AP is flexible and that the P_4−P_1 positions dominate the structure (*19*, *20*). Factor XIII utilizes the P_4−P_1 residues to exploit the apolar and aryl region when binding within the thrombin active site (*19*, *20*).

A polymorphism exists within the activation peptide segment at the P_4 position resulting in substitution of leucine for valine (V34L). FXIII V34L is found in approximately 30% of the population worldwide. This conservative change generates a FXIII which is more easily activated by thrombin and has been associated with protection against myocardial infarction (*21*−*26*). Our laboratory has utilized kinetic and NMR methods to probe FXIII AP residues involved in creating a better substrate for thrombin (*19*, *20*, *27*−*29*). Obtaining a greater understanding of the thrombin residues that regulate interactions with the FXIII APs is also critical.

Figure 1 indicates the residues within the aryl and apolar binding sites examined in this work using human recombinant thrombin. (See Figure 1B for key residues; reference residues are included for orientation of the enzyme.) Functionally important interactions between thrombin residues and various substrates have been extensively documented by site-directed mutagenesis studies as well as by many crystal structures of thrombin and its mutants (*7*−*11*, *17*, *18*, *30*−39). Thrombin residue W215[33] is critical for thrombin hydrolysis of the Fbg Aα chain but less so for PAR1 and Protein C (*34*, *38*). E217A reduces the procoagulant amidolytic activity of thrombin by nearly 50% (*30*, *39*). This residue is part of the Na^+ allosteric network responsible for promoting prothrombotic events (*7*−*9*, *11*, *37*). D189, in the S_1 specificity pocket of thrombin, is linked through a hydrogen-bonding network to this key allosteric system (*10*, *37*). These contacts help to stabilize the 220-loop in the Na^+ binding site of thrombin.

Table 1: Amino Acid Sequences of Thrombin Substrates[a]

	P_9..... P_4....P_1...
Factor XIII AP WT segment	^{28}TVELQGVVPRGVNL^{41}
Factor XIII AP V34L segment	^{28}TVELQGLVPRGVNL^{41}
fibrinogen Aα chain segment	^{7}DFLAEGGGVRGPRV^{20}
thrombin receptor PAR1 segment	^{32}KATNATLDPRSFLL^{45}

[a] These human sequences were taken from the following sources: Factor XIII (*40*), fibrinogen Aα chain (*41*), and thrombin receptor PAR1 (*4*). Key residue positions are underlined.

In the crystal structure of the thrombin WE mutant (W215A/E217A), considerable disorder exists within the 186 and 220-loops which define the Na^+ site. Crucial polar interactions involving E217, T172, and K224 which help to stabilize thrombin are also lost in the WE mutant, leading to a collapse in the primary specificity pocket (*37*). A redirection of D189 in the S_1 pocket hinders interactions with the arginine side chain of the substrate. Furthermore, the S195 of the thrombin catalytic triad is no longer H-bonding with catalytic H57 (*37*).

L99 is part of the S_2 site within the apolar binding pocket on thrombin. I174, also within the apolar site, plays a supporting role in the S_4 site on thrombin. A review of the crystal structures of thrombin bound to the natural substrates Fbg Aα, FXIII AP, and PAR1 (see Table 1 and Figure 2) indicates that the I174 is involved in surrounding the F8 in Fbg Aα, in making van der Waals contacts with the side chain residues on PAR1, and in contributing a minor supporting role for the P_4 and P_5 residues on FXIII AP (*17*, *18*, *42*). In contrast, L99 is less versatile in its interactions with substrates. This residue acts in concert with the

[3] The amino acid residues of the thrombin are designated by the single letter abbreviation for the amino acid residue. W215A is tryptophan residue 215 mutated to alanine. W215A/E217A (WE) is a double mutant with tryptophan 215 and glutamic acid 217 both mutated to alanine. WT corresponds to wild type thrombin.

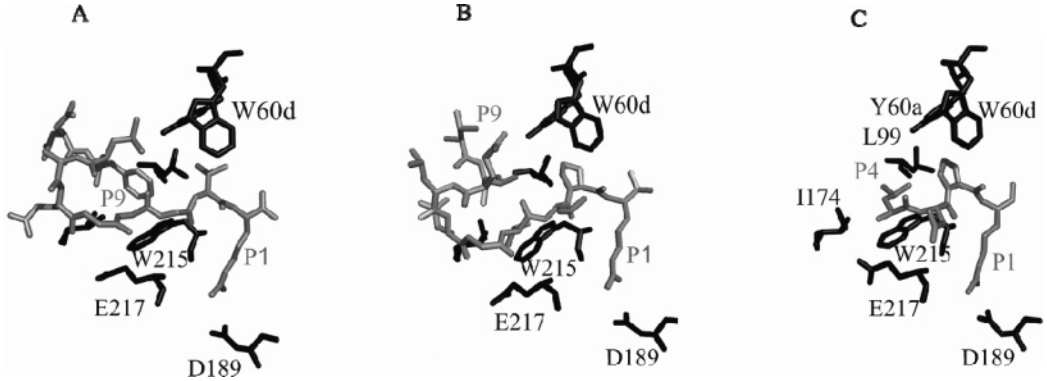

FIGURE 2: Key thrombin residues with various substrates bound. (A) Selected thrombin residues with fibrinogen Aα (7−16) bound in the active site (PDB 1BBR). (B) Selected thrombin residues with Factor XIII (28−37) V34 AP bound in the active site (PDB 1DE7). (C) Selected thrombin residues with PAR1 (38−41) bound in the active site (PDB 1NRS).

β-insertion loop residue Y60a to cage the P_2 residue of the incoming substrate as evidenced in crystal structures of thrombin (*17*, *18*, *33*, *35*, *42*).

The current work elucidates how key residues comprising the apolar and aryl binding site on thrombin interact in a distinct fashion with the activation peptides of FXIII. The cardio-protective FXIII V34L polymorphism is more susceptible to thrombin cleavage than the native V34 sequence. This simple methylene addition within the activation peptide segment is enough to alter not only the rate of the reaction but also the specificity. The different single mutants (W215A, E217A, I174A, and L99A) are each hindered to varying degrees in their ability to hydrolyze FXIII (28−41) V34 AP and FXIII (28−41) V34L AP. Although some improvements in binding can occur with thrombin W215A/E217A, it remains the most dramatically affected mutant with both FXIII polymorphisms. The information obtained from these kinetic studies will contribute to understanding the unique features of FXIII V34L and may aid in the design of new FXIII and thrombin analogs that could be targeted to function under specific physiological conditions. Thrombin's ability to hydrolyze the FXIII AP in vivo is critical for clot structure and stability.

MATERIALS AND METHODS

Synthetic Peptides. Peptides based on residues 28−41 of the human FXIII activation peptide were synthesized by SynPep (Dublin, CA). The amino acid sequences of the peptides are as follows: FXIII (28−41) V34 AP, Ac-TVELQGVVPRGVNL-amide; FXIII (28−41) V34L AP, Ac-TVELQGLVPRGVNL-amide. Some experiments were carried out with the fibrinogen Aα-like sequence Fbg Aα (7−20), Ac-DFLAEGGGVRGPRV-amide. All three peptides were soluble in aqueous environment to at least 10 mM. The purity of all peptides was evaluated by analytical reversed-phase HPLC. Matrix assisted laser desorption time-of-flight (MALDI-TOF) measurements on an Applied Biosystems Voyager DE-Pro mass spectrometer were used to verify the peptide *m*/*z* values. The concentrations of the peptides in solution were determined by quantitative amino acid analysis (AAA Service Lab Inc., Boring, OR).

Thrombin Preparation. All human recombinant thrombin was generously provided by Dr. Enrico Di Cera, Washington University, St. Louis, MO. The expression of these mutants was previously described (*35*). The thrombins used in this project include: wild type (WT), W215A, W215A/E217A (WE), E217A, I174A, and L99A. All mutants listed are single mutations with the exception of the double mutant WE. Thrombin concentrations were determined from absorbance measurements at $E_{280} = 1.83$ mL/(mg cm) and MW= 36 500 (*35*). Active site titration with hirudin indicated that the thrombin was >90% active in all cases. Thrombin was stored at −70 °C until use in kinetic assays.

Kinetics Procedure. The HPLC-based kinetic assay methods were described previously by Trumbo and Maurer (*27*). Briefly, a solution of peptide and assay buffer (50 mM H_3PO_4, 100 mM NaCl, 0.1% PEG, adjusted to pH 7.4 with NaOH) was heated to 25 °C in a heat block. Hydrolysis was begun with the addition of WT or mutant human thrombin. The thrombin concentration for the hydrolysis reactions was 33.6 nM with the exception of the W215A/E217A (WE) mutant which was 1 μM for both FXIII peptides, and the L99A mutant which was 0.7 μM for just the FXIII (28−41) V34 AP. The peptide concentrations for FXIII (28−41) V34 AP, FXIII (28−41) V34L AP, and Fbg Aα (7−20) were within the range of 100−3000 μM. At regular intervals, an aliquot of the reaction mixture was removed and quenched in 12.5% H_3PO_4. Peptide peaks were separated by RP-HPLC using a Brownlee Aquapore octyl RP-300 C8 cartridge column on a Waters HPLC system. Thrombin concentration and kinetic time points were chosen such that less than 15% of the total peptide concentration was hydrolyzed within 30 min.

Initial velocities (in micromolar per second) for the thrombin-catalyzed reactions were determined for each peptide concentration from the slopes of product concentrations vs time plots. The results reported represent averages for at least three independent experiments. Kinetic values were calculated using nonlinear regression analysis fit to the equation: $V = V_{max}/(1 + K_m/[S])$ using the Marquardt−Levenberg algorithm in Sigma Plot (Jandel Scientific). K_m, V_{max}, and k_{cat} were calculated from the coefficients of this equation. The individual kinetic values from such Michaelis−Menten equation fits were compared to the values obtained from linear fits of the Lineweaver−Burk and Eadie−Hofstee plots. Similar results were obtained for both the nonlinear and linear regression analysis approaches.

RESULTS

Kinetic Evaluation of Thrombin Hydrolysis of Factor XIII Activation Peptides. An HPLC assay was employed to

Table 2: Kinetic Constants for Hydrolysis of FXIII (28−41)V34 AP, FXIII (28−41) V34L AP, and Fbg Aα (7−20) by Human Recombinant Thrombins[a]

FXIII (28−41)V34 AP	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
WT IIa	298 ± 42	2.57 ± 0.005	0.0086 ± 0.001
W215A IIa	2374 ± 372	5.53 ± 0.017	0.0023 ± 0.0004
E217A IIa	1242 ± 392	2.84 ± 0.014	0.0023 ± 0.0007
W215A, E217A IIa	745 ± 98	0.117 ± 0.008	0.00015 ± 0.00002
I174A IIa	204 ± 36	5.37 ± 0.009	0.023 ± 0.0005
L99A IIa	2074 ± 414	0.89 ± 0.073	0.00043 ± 0.0001
FXIII (28−41)V34L AP	**K_m (μM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (μM^{-1} s^{-1})**
WT IIa	315 ± 42	22.9 ± 0.003	0.073 ± 0.012
W215A IIa	3619 ± 470	5.31 ± 0.002	0.0015 ± 0.0002
E217A IIa	1025 ± 121	9.93 ± 0.02	0.0097 ± 0.001
W215A, E217A IIa	142 ± 28	0.052 ± 0.003	0.00036 ± 0.0001
I174A IIa	1109 ± 247	10.2 ± 0.04	0.0091 ± 0.002
L99A IIa	1930 ± 354	5.94 ± 0.02	0.0031 ± 0.0002
Fbg Aα (7−20)	**K_m (μM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (μM^{-1} s^{-1})**
WT IIa	569 ± 76	31 ± 0.005	0.0545 ± 0.007
W215A IIa	NA	NA	NA
W215A, E217A IIa	NA	NA	NA

[a] Kinetic constants for the thrombin-catalyzed hydrolysis of peptides based on the FXIII activation peptide segments were determined from an HPLC assay as described in the Experimental Procedures. The results shown here represent the averages of at least three independent experiments. Kinetic values reported were calculated using nonlinear regression analysis methods. The error values correspond to the standard error of the mean (SEM).

monitor rates of hydrolysis of a series of Factor XIII activation peptide segments by thrombin. The product peptides FXIII (28−37) V34 AP, FXIII (28−37) V34L AP, or Fbg Aα (7−16) eluted from the Brownlee Aquapore C8 column as distinct peaks from the parent substrates FXIII (28−41) V34 AP, FXIII (28−41) V34L AP, or Fbg Aα (7−20). The product identities were verified by MALDI-TOF mass spectrometry. Nonlinear regression analysis values K_m, k_{cat}, and k_{cat}/K_m for the three substrate peptides cleaved by wild type and mutant thrombins are shown in Table 2.

The experimentally determined kinetic constants for the FXIII activation peptides indicate that the FXIII (28−41) V34L AP (FXIII V34L AP) is a better substrate for WT thrombin than the V34 sequence as previously reported (*20*, *27*, *39*). This improvement is in agreement with results obtained with plasma Factor XIII isolated from patients containing the L34 vs the V34 forms (*23*). Furthermore, peptide truncation studies (*20*) have revealed that the key amino acids of FXIII AP making contact with the thrombin active site region involve the P_4-P_1 residues ^{34}L/V V P R^{37}. This segment is present within the peptides being examined.

The current studies using the FXIII (28−41) AP substrates employ an HPLC method that readily allows for determination of the individual kinetic parameters K_m and k_{cat} for thrombin catalyzed hydrolysis reactions. While the K_m is nearly identical for both FXIII activation peptides with WT thrombin, the k_{cat} for the V34L peptide is nearly 9-fold improved over V34. These values bring about a 9-fold improvement in specificity (k_{cat}/K_m), making the FXIII V34L AP as attractive a substrate for WT thrombin as the Fbg Aα (7−20) peptide. See Table 2.

The thrombin W215A mutation strongly affects recognition of fibrinogen and also plays an important supporting role with PAR1 (*34*, *35*, *38*). In the current work, cleavage of the peptide Fbg Aα (7−20) was undetectable with the W215A mutant (See Table 2). In contrast, the FXIII (28−41) APs could still be hydrolyzed by this mutant thrombin but far less efficiently than by the wild type enzyme. The K_m values for FXIII V34 AP and FXIII V34L AP are both about 10-fold higher with W215A thrombin than WT thrombin. The k_{cat} value for the V34 AP cleaved by W215A thrombin is increased 2-fold over WT thrombin leading to a 3.7-fold total decrease in k_{cat}/K_m. The k_{cat} for FXIII V34L AP shows a 4-fold decrease vs cleavage by WT thrombin yielding an overall 48-fold decrease in k_{cat}/K_m. The significant increases in K_m and decreases in k_{cat} cause the FXIII V34L AP to be far more affected by the loss of the thrombin W215 than the FXIII V34 AP.

The E217A mutant is proposed to affect hydrolysis of substrates due to interruption of key structural contacts involving the thrombin 220 loop and the S_1 specificity pocket (*36*, *37*). The K_m value for E217A interacting with FXIII V34 AP is increased 4-fold over the WT enzyme, while the k_{cat} is unchanged leading to a 4-fold reduction in specificity. The specificity for FXIII V34L AP with E217A is 7.5-fold, reduced due to a 3.3-fold increase in K_m coupled with a 2.3-fold decrease in k_{cat}. See Table 2. It is interesting to note that the studies with thrombin W215A and thrombin E217A yield comparable k_{cat}/K_m values for hydrolysis of FXIII V34 AP. These values, however, have different contributions from K_m vs k_{cat}. With W215A, the k_{cat} dominates the k_{cat}/K_m value whereas with E217A the K_m dominates. In contrast to the FXIII V34 AP work, the k_{cat}/K_m value for hydrolysis of FXIII V34L AP by thrombin E217A is 6-fold higher than that by thrombin W215A. Better values for both K_m and k_{cat} participate in this effect.

Moving more to the thrombin apolar binding region, FXIII V34 AP experiences a slightly reduced K_m with the I174A mutant as compared to WT thrombin and a 2-fold increase in k_{cat} leading to an overall increase in specificity of 2.7-fold. Thrombin I174A is the only single site mutant of the series that is able to more efficiently hydrolyze FXIII V34 AP than the WT sequence. In contrast, the kinetic constants for the FXIII V34L AP are not improved with this mutant.

The K_m is 3.5-fold increased, the k_{cat} 2.2-fold decreased and the k_{cat}/K_m is 8-fold decreased relative to WT thrombin. The individual kinetic parameters for hydrolysis of FXIII V34L AP by thrombin I174A are quite comparable to those of thrombin E217A.

The L99A mutant experiences a decrease in binding and catalytic turnover with both FXIII V34 AP and V34L AP. See Table 2. Furthermore, the specificity of this mutant for each AP is reduced greater than 20-fold. FXIII V34L AP remains the stronger of the two substrate peptides by 10-fold. Of all the single site thrombin mutants examined in this study, the k_{cat}/K_m value is the weakest for hydrolysis of FXIII V34 AP by thrombin L99A.

The single thrombin mutants respond differently depending upon whether the P_4 residue of FXIII (28−41) AP is a V34 or a L34. How a double mutant involving W215A and E217A would interact with the FXIII APs is also of much interest. Assays employing this WE mutant with FXIII V34 AP and V34L AP reveal that the K_m values return to nearly the levels of WT thrombin. Surprisingly, the K_m value for FXIII V34L AP becomes better than that with WT. In contrast, the k_{cat} values are greatly reduced for both peptides. The k_{cat} value for the FXIII V34 AP cleaved by WE is decreased 22-fold over WT leading to a 57-fold decrease in k_{cat}/K_m. The FXIII V34L AP has a nearly 440-fold decrease in k_{cat} value causing a 200-fold decrease in the k_{cat}/K_m compared to WT. For comparison, there is no detectable hydrolysis of Fbg Aα (7−20) with the WE mutant.

Effects of combining the W215 and E217 mutations into a single thrombin species can be quantified using the coupling free energy (*36*, *43*). The equation employed in these calculations is $\Delta G_c = -RT \ln[(S_{W215A/E217A}S_{WT})/(S_{W215A}S_{E217A})]$, where S corresponds to the k_{cat}/K_m value, R to the gas constant, and T to the absolute temperature (*36*, *43*). When $\Delta G_c > 0$, the single mutations act synergistically in reducing the specificity in the double mutant. Together they reduce the specificity of the double mutant beyond simple additivity ($\Delta G_c = 0$). In contrast, a value of $\Delta G_c < 0$ indicates that the single mutations oppose each other in reducing the specificity in the double mutant. The observed result is that they instead enhance the specificity beyond simple additivity (*36*, *43*). Interactions between FXIII V34 AP and the thrombin WE mutant yielded a ΔG_c value of +0.85 kcal/mol whereas the value for FXIII V34L AP was a ΔG_c of −0.36 kcal/mol.

DISCUSSION

There has been a great deal of effort directed toward parsing the coagulant/anticoagulant properties of thrombin. While functional epitopes have been deciphered, the residues involved in these opposing functions have yet to be fully correlated (*30*). A critical enzyme−substrate system to investigate further includes thrombin interactions with the FXIII activation peptide segment. An important prior study (*39*) focused on a series of single-site thrombin mutants that targeted surface exposed, charged, and polar residues of this serine protease. In the current work, additional crucial thrombin residues within the apolar and aryl binding sites were explored. These residues are located within the extended thrombin active site region and influence both binding and hydrolysis of FXIII (28−41) V34 AP and the cardio-protective V34L AP.

Examining Thrombin Interactions with a Focus on W215 and E217. The crystal structures of Fbg Aα (7−16), FXIII (28−37) V34 AP, and PAR1 (38−41) that are each in complex with thrombin represent different binding modes with the enzyme active site (*17*, *18*, *42*). See Figure 2. In the Fbg Aα (7−16) structure, the F8 (P_9) residue is making contacts with thrombin residues in the apolar binding site including Y60a, L99, I174, and W215. Fbg Aα residue F8 is surrounded intramolecularly, as well, by Fbg Aα residues L9, G13, and V15 (*15*−*17*). The most crucial contact for this peptide is the edge to face pi−pi stacking between Fbg Aα F8 and thrombin W215 brought about by the β-turn conformation adopted by the enzyme-bound peptide (*15*−*17*). Losing that key interaction in the W215A mutant helps to explain the undetectable hydrolysis of Fbg Aα (7−20).

The crystal structure of FXIII (28−37) V34 AP in complex with thrombin displays the V29 at P_9 participating in the same β-turn location as the F8 of the Fbg Aα. This V29, however, is not positioned to interact at the thrombin aryl and apolar binding sites but is instead directed toward R97, E97a, N98, and Y60a (*18*). A focus on the aryl W215 binding site is therefore far less apparent. Nonetheless, the K_m values for both activation peptides have increased 9-fold with the W215A mutant suggesting difficulty binding within the active site. It is important to note that the k_{cat} values for the two FXIII APs with W215A are comparable indicating benefits in k_{cat} occurring for the FXIII V34L AP with WT enzyme are lost with W215A. This k_{cat} benefit has been viewed as one of the hallmark features of the V34L sequence. For the first time, the k_{cat} for FXIII V34L AP is not improved over that of the more common V34. The combined hindrance of both K_m and k_{cat} severely compromises the substrate specificity of thrombin W215A for the FXIII V34L AP. Analogous effects on the k_{cat}/K_m value have been shown with peptides containing the PAR1 (38−41) segment LDPR (34). This sequence is quite similar to the FXIII (33−37) AP segment LVPR. See Figure 2 for the PAR1 bound structure. In contrast to FXIII V34L AP, the V34 AP is not as critically affected in the presence of thrombin W215A. The K_m value for FXIII V34 AP has weakened substantially over that observed with WT, but the k_{cat} has undergone a slight increase.

Unlike Fbg Aα, FXIII AP is not hypothesized to rely heavily on interactions of the N-terminal P_9 residue with the thrombin surface based on both kinetic and solution NMR studies (*19*, *20*, *27*). The current studies indicate that W215 assists in creating an environment for promoting effective binding and hydrolysis of FXIII activation peptides. A review of the crystal structure of FXIII (28−37) V34 AP shows W215 plays more of a role in supporting the main chain backbone of residues V34 and V35 (*18*). The kinetic results reveal that the V34L sequence has a greater dependence on this environment to optimally orient substrate residues to promote thrombin-catalyzed hydrolysis.

E217 is also a key supporting residue for thrombin-ligand contacts. E217A compromises sodium binding by disrupting hydrogen-bonding networks and also seems to destabilize the S_1 site on thrombin (*30*, *36*, *37*, *39*). The single mutant E217A experiences some difficulty binding the FXIII V34 AP. Once bound, however, hydrolysis of FXIII V34 AP by E217A is a bit improved over the WT enzyme. The kinetic

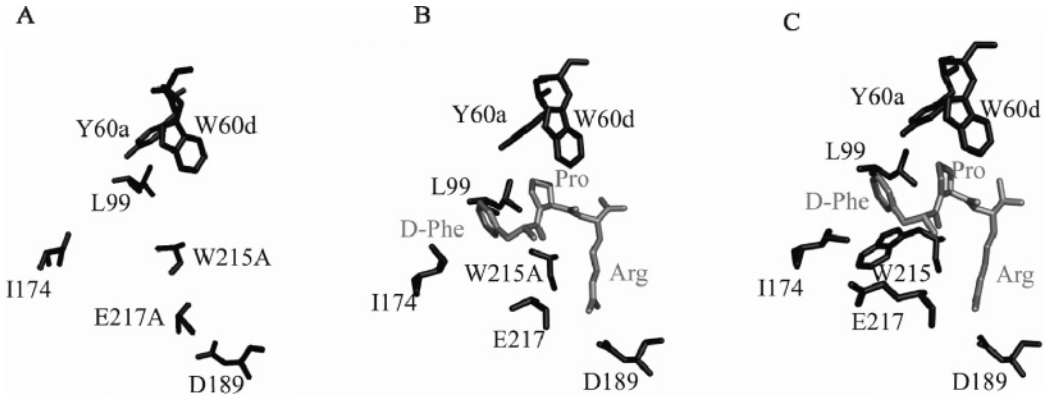

FIGURE 3: Examining the thrombin WE structure in its free and ligand bound states. (A) Free thrombin WE mutant crystal structure representing residues within the aryl and apolar binding site (PDB 1TQ0). (B) Thrombin WE mutant crystal structure with inhibitor PPAck bound (PDB 1TQ7). (C) Crystal structure of wild type thrombin with PPAck bound (PDB 1PPB).

constants for E217A interacting with the FXIII V34L AP indicate binding is slightly less affected than with the V34 AP, but the turnover is decreased, resulting in a significant decrease in specificity. FXIII V34 AP thus becomes a better substrate for the E217A mutant. This overall result is in agreement with earlier work with intact Factor XIII enzymes V34 and V34L (*39*). It may be that there are greater main-chain contacts of P_5 (G) and P_4 (L) with E217 as a result of adjustments within the aryl and apolar sites to accommodate the increased hydrophobicity of the leucine side chain.

Mutating both W215 and E217 to alanine greatly hinders interactions with Fbg Aα (*36*). The current report of no detectable hydrolysis of the peptide model Fbg Aα (7−20) by this WE mutant further confirms this observation. In contrast, the kinetic assays employing the WE mutant with FXIII V34 and V34L APs indicate that the K_m values return impressively to the levels of WT thrombin. The turnover and specificity constants for both APs are, however, greatly reduced. In the crystal structures of WE, there is a collapse in the specificity pocket. See Figure 3. Furthermore, the residue that accommodates the P_1 arginine of the incoming substrate, D189, is rotated nearly 90° in the WE free structure. With this rotation, the double mutant W215A/E217A is still able to locate and bind the APs but interactions crucial for orienting the FXIII APs for cleavage and catalytic turnover are severely hindered (*36*, *37*). The FXIII V34L AP may be able to achieve more effective contacts with the thrombin surface than a V34. A valine at this P_4 position is likely not large or hydrophobic enough to make up for the lack of the W215/E217 platform. In the WE-PPAck structure, the enzyme appears to have corrected for the nonoptimal arrangement of the active site region as a result of binding the inhibitor. Even with the ability to make such corrections, this thrombin mutant is still virtually noncatalytic toward procoagulant substrates (*36*, *37*).

Combining W215 and E217 mutations into a single thrombin species has previously been quantified using the coupling free energy term ΔG_c (*36*, *43*). Thrombin interactions with the two FXIII APs are a new system to explore this coupling effect. With the FXIII V34 AP, the ΔG_c value of +0.85 kcal/mol is clearly greater than zero and indicates that the single thrombin mutations are working toward synergistically reducing specificity in the double mutant. For this peptide substrate, both single site mutants generated the same S (or k_{cat}/K_m) value, the E217A more dominated by K_m and the W215A more dominated by k_{cat}. The double mutant exhibits a k_{cat}/K_m value decreased 15-fold from the individual single mutants. The coupling effect of W215A and E217A in reducing specificity for FXIII V34 AP is similar to the +1.6 kcal/mol value recorded previously for the substrate H−D-Phe-Pro-Arg−*p*-nitroaniline (*36*). In contrast for the FXIII V34L AP, the k_{cat}/K_m value with thrombin W215A is 49-fold less than the wild-type whereas the E217A is 7.5-fold less than the wild-type. The thrombin W215A experiences greater hindrances in both k_{cat} and K_m values than the E217A. For the double mutant W215A/E217A, a ΔG_c value of −0.36 kcal/mol was obtained in the presence of FXIII V34L AP. The two combined thrombin substitutions respond to this V34L substrate with a ΔG_c value that approaches zero indicating the absence of coupling. A mostly additive effect thus occurs with this enzyme−substrate system. This additive property was also observed by Cantwell and Di Cera (*36*) with fibrinogen ($\Delta G_c = -0.3$) and the PAR1 segment ($\Delta G_c = 0.0$). The coupling free energy values provide another example of the distinct features of thrombin interacting with FXIII V34 AP vs the cardioprotective V34L AP.

The Roles Played by Other Thrombin Residues in Differentiating the FXIII APs. Two other thrombin residues to consider include L99A and I174A. L99 is part of the S_2 site on thrombin acting in concert with Y60a in the β-insertion loop to stabilize the substrate P_2 residue during hydrolysis (*11*, *17*, *18*, *30*, *32*). The kinetic results indicate that L99A has great difficulty binding to and hydrolyzing V34 and V34L APs. The resultant specificity constant for FXIII V34 AP in the presence of thrombin L99A is the worst of all the single site mutant systems examined in this work. L99A appears to hinder thrombin's capacity to align FXIII AP substrates in the active site cleft for effective hydrolysis. Structural features that helped to accommodate substrate residues at the P_2 and surrounding positions are likely lost (*11*, *15*−*18*, *30*, *32*).

In the X-ray crystal structure of the FXIII (28−37) V34 AP−thrombin complex, the backbone of the Val residues at the P_3 and P_4 positions of V34 AP are positioned above thrombin W215 (*18*) and the Pro at the P_2 residue interacts with thrombin L99, Y60a, and W60d. Clearly, the L99A mutant is limited in ability to make hydrophobic contacts with P_2 and its neighboring residues which together are

necessary for appropriate binding and catalysis of thrombin substrates. In a published study involving L99G (*33*), the now larger S_2 pocket was proposed to impair stabilization of the transition state for selected thrombin substrates. Similar effects may be occurring with the slightly larger L99A and the FXIII APs.

Additional clues about thrombin's ability to accommodate the FXIII APs are revealed from exploring the thrombin I174 position. I174A interactions with the FXIII V34 AP are the least affected of all the mutants with kinetic constants quite similar to those with the WT enzyme. See Table 2. The FXIII V34L AP is more sensitive to the substitution as reflected in its K_m and k_{cat} values. Residue I174 within the apolar binding site of thrombin aids in supporting F8 within the Fbg Aα chain. This residue plays less of a role in binding PAR1 peptide and appears to act as minor support for the P_4 and P_5 residues in the FXIII AP structure (18, 42). See Figure 2.

X-ray crystal results indicate that the thrombin I174 side-chain is shifted about 1 Å in the presence of FXIII V34 AP compared to thrombin complexed with Fbg Aα (7−16). The thrombin W215 is also shifted in the presence of FXIII V34 AP. These changes indicate that thrombin residues within the apolar and aryl sites may move to accommodate the bulkier hydrophobic P_3 and P_4 residues of FXIII APs (VVPR or LVPR) over those of Fbg Aα (GGVR) (*18*). If this is true for a valine at the P_4 position, it must be even more so for the bulkier leucine at the same position. We have proposed, based on 2D transferred NOESY data that, in fact L34 is able to occupy a different space in the enzyme binding site than V34, interacting more with L99 near the β-insertion loop (*19*, *20*).

There remains no published X-ray structure for the common polymorphism FXIII AP (28−37) V34L in complex with thrombin. In earlier work, we showed that removal of the N-terminal P_{10}−P_6 residues of the activation peptides, V34 and V34L, does not hinder interactions with thrombin (*20*). The key contacts of thrombin and FXIII AP are within the P_4−P_1 residues. NMR studies suggested that the overall solution conformations for these residues in the two FXIII peptides would be similar in the presence of thrombin (*20*). The L34 would have the added feature of being long enough to be positioned toward P36 generating a stabilizing P_4 to P_2 interaction. This type of stabilizing interaction is also observed for the PAR1 segment ^{38}LDPR^{41}. We have gone back to the NOEs from FXIII (33−37) V34L AP in the presence of thrombin (*20*) and have used molecular modeling to generate a solution structure. A representative structure shown in Figure 4 confirms that this FXIII AP segment has a similar conformation to that of the thrombin bound PAR1 peptide segment, LDPR. Furthermore, the L43 of FXIII AP appears to be interacting with thrombin L99 through the side chain and thrombin W215 through the main chain. There were not enough NOEs to fully model the solution structure of FXIII (33−37) V34 AP in the presence of thrombin. However, a review of the X-ray crystal structure of thrombin-bound FXIII AP (28−37) V34 reveals that the overall conformation of the 33−37 residue segment is comparable to that of FXIII (33−37) L34 and PAR1.

Conclusions. Each of the thrombin mutants studied has provided valuable information on FXIII AP - thrombin interactions. The FXIII V34L AP is a better substrate for

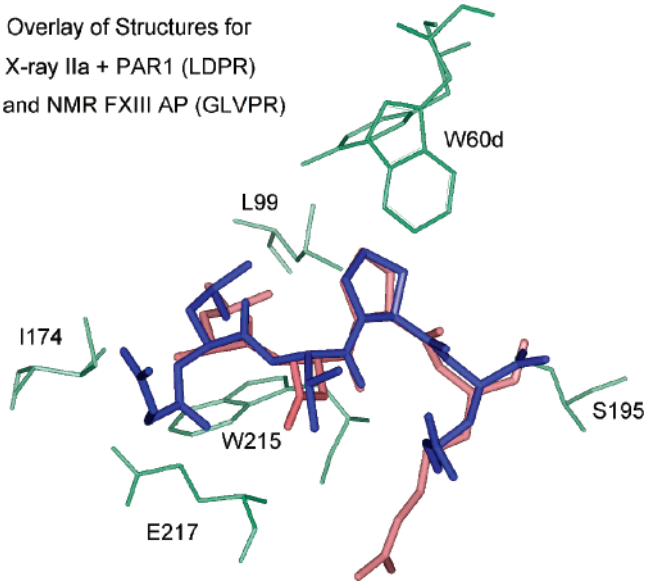

FIGURE 4: Representative solution structure for thrombin-bound FXIII (33−37) V34L AP that satisfies the NOE distance constraints. FXIII (33−37) V34L AP in blue binds to thrombin in a conformation very similar to that of PAR1. The PAR1 segment (38−41) (PDB 1NRS) is shown in red whereas key thrombin residues are in green. Solution structures for the FXIII peptide were calculated using InsightII/Discover (Accelrys). The CVFF force field was applied. NOE-derived distances, medium (1.5−2.5 Å) and weak (2.5−3.5 Å) were used as input constraints (*20*). These constraints were derived from transferred NOESY spectra collected at 250ms. An energy minimization protocol of steepest decents was begun using 100 cycles. The temperature was raised to 1000 K and reduced by 100 K every 100 steps of the minimization until a final temperature of 300 K was reached. Energy minimization was continued with 15 000 iterations using a conjugate gradient until the maximum derivative was less than 0.0001 kcal/Å.

WT thrombin than V34 AP. This improvement is in agreement with studies carried out using plasma FXIII isolated from patients. The beneficial effects observed with the FXIII APs are abrogated with the W215A mutant. W215 provides an important platform for binding these peptides and helping to direct them for proper hydrolysis. E217 also plays a supporting role in binding substrates but its E217A mutation is not as detrimental as the W215A. FXIII V34L AP is less affected than V34. The double thrombin mutant W215A/E217A (WE) is quite unfavorable for both activation peptides. The FXIII V34L AP is better able to adapt to binding to the WE mutant apolar binding site; however, its ability to be hydrolyzed is severely compromised. The WE double mutant appears to impart a synergistic effect in the presence of FXIII V34 AP whereas its effect is mostly additive on V34L AP. Kinetic results with I174A indicate that this thrombin residue is more crucial for interactions with the larger V34L AP segment. Finally, a reduction in the side chain of L99A causes deleterious effects to binding and hydrolysis for both APs. The V34L is able to partially make up for the hydrophobic loss in L99A perhaps due to increased contacts within the aryl and apolar site. Further knowledge on how thrombin discriminates between substrates by utilizing unique residues for recognition and hydrolysis will enable the design of molecules which can inhibit or enhance features necessary for coagulation. For the thrombin and FXIII AP system, there

is interest in ability to control sites of interaction and degree of activation.

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