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Role of the A chain in thrombin function

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Abstract

The A chain of thrombin is covalently linked to the catalytic B chain but is separate from any known epitope for substrate recognition. In this study we present the results of the Ala replacement of 12 charged residues controlling the stability of the A chain and its interaction with the B chain. Residues Arg4 and Glu8 play a significant role in substrate recognition, even though they are located >20 Å away from residues of the catalytic triad, the primary specificity pocket and the Na⁺ site. The R4A mutation causes significant perturbation of Na⁺ binding, fibrinogen clotting and PAR1 cleavage, but modest reduction of protein C activation in the presence of thrombomodulin. These findings challenge our current paradigm of thrombin structure-function relations focused exclusively on the properties of the catalytic B chain, and explain why certain naturally occurring mutations of the A chain cause serious bleeding.

Keywords

Thrombin; allostery; fibrinogen; protein C; PAR1

Introduction

Thrombin is a Na⁺-activated, allosteric serine protease involved in blood clotting [1] and is composed of two polypeptide chains of 36 (A chain) and 259 (B chain) residues covalently linked through the Cys1--Cys122 (chymotrypsinogen numbering) disulfide bond. Because catalytic function and all known regulatory interactions involve the B chain, the shorter A chain has received little attention in functional and mutagenesis studies and is considered an appendage from the activation process of prothrombin. The original structure of thrombin solved by Bode [2] has cemented this view in a widely used orientation where the B chain is displayed prominently with its catalytic site in the middle and eclipsing the A chain in its back. The role of the A chain of thrombin has not been studied in terms of Ala-scanning mutagenesis [3], so most of our knowledge is currently based on a previous study on bovine thrombin where the A and B chains were separated after reduction of all disulfide bonds including Cys1-Cys122, denaturation, removal of the A chain and refolding of the B chain [4]. The study concluded that bovine thrombin devoid of the A chain in this manner retains most of its catalytic activity, a conclusion at odds with the moderately to severe bleeding phenotype associated with numerous naturally occurring mutations affecting residues of the A chain [5–8]. A possible solution to this discrepancy may be that the A chain is important for the activation process of prothrombin by the prothrombinase complex, but once the mature enzyme is generated, the A and B chains can be separated without significant loss of catalytic activity. A direct test of the role of the A chain would require detailed characterization of the properties of selected mutants

to establish if the constructs can be activated efficiently from the zymogen form and retain the functional properties of wild type. To date, only a single mutant of the A chain of thrombin has been characterized functionally. Deletion of Lys9 in the A chain is associated with a bleeding phenotype [8]. The thrombin mutant $\Delta K9$ shows defects in the activation of its zymogen form [9], but the mature enzyme features impaired Na^+ binding and significantly reduced activity toward a chromogenic substrate, fibrinogen, PAR1 and protein C [9] due to long-range perturbation of the active site moiety and catalytic triad [9,10]. These findings underscore the importance of the A chain in thrombin function and warrant a thorough analysis in terms of mutagenesis.

Materials and methods

Site-directed mutagenesis of human thrombin was performed as described [11–13] using the Quik-Change site-directed mutagenesis kit from Stratagene (La Jolla, CA) in an HPC4-modified pNUT expression vector containing the human prothrombin-1 gene. Thrombin mutants were expressed in baby hamster kidney cells. Mutants E1cA, D1aA, K9A, K10A, R14dA, E14eA, E14hA and D14IA were activated completely with the prothrombinase complex for 40 min at 37 °C. On the other hand, <10% activation was obtained for mutants R4A, E8A, D14A and E14cA even after 2 days of incubation with the prothrombinase complex at 37 °C. For these four mutants, prothrombinase cleaved efficiently at Arg15 to generate meizothrombin-desF1, but cleaved very slowly at Arg271 (prothrombin numbering) to separate fragment 2 from the A chain to generate thrombin. Enzymes used in the activation were from Enzyme Research (South Bend, IN). All mutants were purified to homogeneity by FPLC using Resource Q and S columns with a linear gradient from 0.05 to 0.5 M choline chloride, 5 mM MES, pH 6.0 at room temperature. Active site concentrations were determined by titration with hirudin and found to be >95% for all mutants.

Values of $s = k_{\text{cat}}/K_m$ for the hydrolysis of H-D-Phe-Pro-Arg-p-nitroanilide (FPR) were determined as reported [12] under experimental conditions of 5 mM Tris, 0.1 PEG8000, pH 8.0 at 25 °C in the presence of 200 mM NaCl or choline chloride to study the properties of the E: Na^+ and E forms, respectively [1]. The interaction with fibrinogen leading to release of fibrinopeptide A (FpA) and B (FpB), cleavage of the protease activated receptor 1 (PAR1) and activation of protein C with and without thrombomodulin were studied as reported [11,14, 15] under experimental conditions of 5 mM Tris, 0.1% PEG8000, 145 mM NaCl, pH 7.4 at 37 °C.

Stopped-flow fluorescence measurements were carried out with an Applied Photophysics SX20 spectrometer, with excitation at 280 nm and a cut-off filter at 305 nm [16]. Samples of thrombin at a final concentration of 100 nM in 5 mM Tris, 0.1% PEG8000, pH 8.0 at 15 °C were mixed 1:1 with 60 μl of the same buffer containing variable amounts of NaCl (up to 400 mM) kept at constant ionic strength of 400 mM with choline chloride. The baseline was measured with 400 mM choline chloride in the mixing syringe. Each trace was determined in quadruplicate.

Results and discussion

When the structure of thrombin was first solved [2], it was noted that the A chain is rich in charged residues engaged in intra- and inter-molecular interactions. Specific interactions that stabilize the fold are the Asp1a-Lys9 and Arg14d-Glu13 ion pairs and the Arg4-Glu8-Asp14-Glu14c ion quartet (Fig. 1). In addition, the A chain contributes to stabilization of the B chain through numerous ionic interactions. There is a conspicuous polarity in the interface, with the A chain contributing several acidic residues that neutralize basic residues from the B chain. Surface interactions Glu1a-Arg206 and Lys14a-Glu23 may be energetically dispensable. On

the other hand, interactions of the ion quartet with Arg137 and Lys202 are less exposed to solvent and may be quite relevant. Few hydrophobic interactions involve Tyr14j with Pro204 and residue Arg4 with the side chains of Trp29 and Trp207.

Figure 2 summarizes the catalytic activity of the Ala mutants of 12 charged residues of the A chain toward a chromogenic and several natural substrates. Eight mutations (E1cA, D1aA, K9A, K10A, R14dA, E14eA, E14hA and D14lA) show no appreciable effect on substrate recognition. Of the remaining four mutants, E8A, D14A and E14cA show up to tenfold decrease in the k_{cat}/K_m for the hydrolysis of FPR, FpA, FpB, protein C in the absence of thrombomodulin and PAR1. The mutant R4A is the most compromised. The value of k_{cat}/K_m for the hydrolysis of FPR drops 20- and 65-fold in the Na^+ -bound (E: Na^+) and Na^+ -free (E) forms of thrombin, respectively. The increased difference between the catalytic activities of the E and E: Na^+ forms of thrombin is reminiscent of the effect observed with the S214A mutation within the active site [12,17]. Comparable effects are observed for the cleavage of fibrinogen, PAR1 and protein C in the absence of thrombomodulin, confirming a molecular origin of the perturbation within the active site of the enzyme. Binding of thrombomodulin partially relieves the perturbation, as documented for other mutants of the active site region [18]. However, the gain in relative anticoagulant potency, measured as the ratio between protein C activation in the presence of thrombomodulin relative to fibrinogen cleavage, is modest compared to wild type and makes the R4A mutant a poor competitor of other well-established protein C activators [19,20].

The R4A mutant was characterized further in view of its intriguing functional properties toward chromogenic and natural substrates. We specifically sought to explain the molecular nature of the perturbation affecting a residue located $>20 \text{ \AA}$ away from residues of the catalytic triad, the S1 pocket or the Na^+ site. Binding of Na^+ to thrombin elicits a 10–15% increase in intrinsic fluorescence [13,16,21,22] that is contributed by changes in the environment of all nine Trp residues of the enzyme distributed over the entire surface up to $35 \text{ \AA}$ away from the bound cation [16]. The fluorescence increase can be decomposed into a fast phase due to Na^+ binding to the E form to generate E: Na^+ and a slow phase due to the interconversion of the Na^+ -free forms E^* and E [16,23]. The biphasic response was observed for all mutants reported in this study except R4A, for which no slow phase was detected but only a fast transition to decreasing levels of fluorescence without appreciable saturation (Fig. 3). This kinetic response to Na^+ binding is unprecedented for a thrombin mutant and vouch for a drastic perturbation of the structure of the enzyme. Mutation of some Trp residues to Phe abrogates the fast phase, but not the slow phase [16]. In no case has Na^+ binding been associated with a decrease rather than an increase in intrinsic fluorescence. We conclude that Na^+ binds very weakly to the R4A mutant and the mutation drastically perturbs the environment of fluorophores reporting the E^* -E interconversion. Alternatively, the R4A mutation could abrogate the E^* form of thrombin and stabilize the E form. In this case, the E form would show very weak affinity for Na^+ because of the lack of saturation of the fluorescence change measured up to 200 mM NaCl (Fig. 3). The side chain of Arg4 is stabilized by strong ionic contacts with Glu8 and Glu14c in the ion quartet (Fig. 1), but is also in van der Waals interaction with Trp29 ($3.8 \text{ \AA}$) and Trp207 ($3.6 \text{ \AA}$) in the B chain. Both the E^* -E and E-E: Na^+ interconversions affect the environment of all nine Trp residues of thrombin, including Trp29 and Trp207 that behave as a paired fluorophore due to their proximity [16]. Reciprocity of linkage demands perturbation of the A chain around Arg4 to propagate long-range to the Na^+ site in the B chain *via* Trp29 and Trp207. We believe that the energetic perturbation of Na^+ binding resulting from this long-range effect (Fig. 3) may account for the compromised catalytic activity of the mutant toward synthetic and natural substrates (Fig. 2). Recent successes in mapping the pathway of long-range communication in an allosteric enzyme like thrombin [24] make crystal studies of the R4A mutant a high priority.

The results reported in this study demonstrate that residues of the ion quartet Arg4-Glu8-Asp14-Glu14c, and especially Arg4, play an unanticipated important role in thrombin function by affecting long-range the Na⁺ site and the active site. This conclusion provides a possible explanation for the severe bleeding phenotypes associated with naturally occurring mutations of residues of the A chain [5–8]. The functional defects in prothrombins Denver (E8K and E14cK) [5], Segovia (G14mR) [6] and San Antonio (R15H) [7] have been attributed to perturbation of the zymogen → enzyme conversion and processing by factor Xa, resulting in severe bleeding. Such an explanation is obvious for the G14mR and R15H mutations that affect the P1 (Arg15) and P2 (Gly14m) sites of recognition by factor Xa. Whether a similar explanation holds for residues Glu14c and Glu8 located at the P12 and P21 positions of substrate is highly unlikely. One cannot discount the possibility that mutations of the A chain alter the structure and function of thrombin itself, following activation from prothrombin. Indeed, our study demonstrates that the E8A and E14cA mutations cause significant perturbation of fibrinogen cleavage and PAR1 activation (Fig. 2). Charge reversal substitutions of Glu8 and Glu14c as seen in prothrombins Denver [5] should be even more disruptive, thereby explaining the observed phenotype. Naturally occurring deletions of Lys9 or Lys10 in the A chain are also associated with bleeding [8].

Functional investigation of the ΔK9 mutant has revealed significant impairment of FpA release (64-fold), PAR1 activation (116-fold), protein C activation in the presence of thrombomodulin (34-fold) and hydrolysis of chromogenic substrate in both the E (6-fold) and E:Na⁺ (18-fold) forms [9]. The results have been interpreted in terms of molecular dynamics calculations as a gross perturbation of the active site moiety propagating long-range from the site of deletion in the A chain [9]. Notably, the perturbed structure produced by modeling contains features documented in the X-ray crystal structure of the inactive form of thrombin E* [25]. Recent measurements of the pKa of the catalytic His57 further support a direct effect on active site residues [10]. Our study shows that the K9A and K10A mutants have functional properties comparable to those of wild type. The drastic perturbation seen in the ΔK9 mutant is therefore due to non-local effects beyond shaving of the Lys side chain. Some properties of the ΔK9 mutant echo those of the R4A mutant reported here. It is likely that deletion of residue Lys9 or Lys10 shifts the register of the A chain downstream and causes a disruption of the ion quartet Arg4--Glu8-Asp14-Glu14c thereby producing functional effects that are similar to those produced by the R4A and E8A substitutions. This could also explain the similarity of phenotypes observed between deletions of Lys9 or Lys10 [8] with those of prothrombins Denver (E8K and E14cK) [5] that affect the ion quartet directly.

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References

1. Di Cera E. Thrombin. *Mol. Aspects Med* 2008;29:203–254. [PubMed: 18329094]
2. Bode W, Turk D, Karshikov A. The refined 1.9-Å X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human alpha-thrombin: Structure analysis, overall structure, electrostatic properties, detailed active-site geometry, and structure-function relationships. *Protein Sci* 1992;1:426–471. [PubMed: 1304349]
3. Kolodzeiskaya MV, Sokolovskaya LI, Volkov GL. Role of A-chain in functioning of the active site of human alpha-thrombin. *Biochemistry (Mosc)* 2008;73:237–244. [PubMed: 18393757]
4. Hageman TC, Endres GF, Scheraga HA. Mechanism of action of thrombin on fibrinogen. On the role of the A chain of bovine thrombin in specificity and in differentiating between thrombin and trypsin. *Arch. Biochem. Biophys* 1975;171:327–336. [PubMed: 1103742]

5. Lefkowitz JB, Haver T, Clarke S, Jacobson L, Weller A, Nuss R, Manco-Johnson M, Hathaway WE. The prothrombin Denver patient has two different prothrombin point mutations resulting in Glu-300→Lys and Glu-309→Lys substitutions. *Br. J. Haematol* 2000;108:182–187. [PubMed: 10651742]
6. Akhavan S, Rocha E, Zeinali S, Mannucci PM. Gly319→Arg substitution in the dysfunctional prothrombin Segovia. *Br. J. Haematol* 1999;105:667–669. [PubMed: 10354128]
7. Sun WY, Burkart MC, Holahan JR, Degen SJ. Prothrombin San Antonio: A single amino acid substitution at a factor Xa activation site (Arg320 to His) results in dysprothrombinemia. *Blood* 2000;95:711–714. [PubMed: 10627484]
8. Akhavan S, Mannucci PM, Lak M, Mancuso G, Mazzucconi MG, Rocino A, Jenkins PV, Perkins SJ. Identification and three-dimensional structural analysis of nine novel mutations in patients with prothrombin deficiency. *Thromb. Haemost* 2000;84:989–997. [PubMed: 11154146]
9. De Cristofaro R, Akhavan S, Altomare C, Carotti A, Peyvandi F, Mannucci PM. A natural prothrombin mutant reveals an unexpected influence of A-chain structure on the activity of human {alpha}-thrombin. *J. Biol. Chem* 2004;279:13035–13043. [PubMed: 14722067]
10. De Cristofaro R, Carotti A, Akhavan S, Palla R, Peyvandi F, Altomare C, Mannucci PM. The natural mutation by deletion of Lys9 in the thrombin A-chain affects the pKa value of catalytic residues, the overall enzyme's stability and conformational transitions linked to Na⁺ binding. *FEBS J* 2006;273:159–169. [PubMed: 16367756]
11. Dang QD, Guinto ER, Di Cera E. Rational engineering of activity and specificity in a serine protease. *Nat. Biotechnol* 1997;15:146–149. [PubMed: 9035139]
12. Pineda AO, Carrell CJ, Bush LA, Prasad S, Caccia S, Chen ZW, Mathews FS, Di Cera E. Molecular dissection of Na⁺ binding to thrombin. *J. Biol. Chem* 2004;279:31842–31853. [PubMed: 15152000]
13. Prasad S, Wright KJ, Roy DB, Bush LA, Cantwell AM, Di Cera E. Redesigning the monovalent cation specificity of an enzyme. *Proc. Natl. Acad. Sci. USA* 2003;100:13785–13790. [PubMed: 14612565]
14. Ayala YM, Cantwell AM, Rose T, Bush LA, Arosio D, Di Cera E. Molecular mapping of thrombin-receptor interactions. *Proteins* 2001;45:107–116. [PubMed: 11562940]
15. Vindigni A, Di Cera E. Release of fibrinopeptides by the slow and fast forms of thrombin. *Biochemistry* 1996;35:4417–4426. [PubMed: 8605191]
16. Bah A, Garvey LC, Ge J, Di Cera E. Rapid kinetics of Na⁺ binding to thrombin. *J. Biol. Chem* 2006;281:40049–40056. [PubMed: 17074754]
17. Krem MM, Prasad S, Di Cera E. Ser(214) is crucial for substrate binding to serine proteases. *J. Biol. Chem* 2002;277:40260–40264. [PubMed: 12181318]
18. Xu H, Bush LA, Pineda AO, Caccia S, Di Cera E. Thrombomodulin changes the molecular surface of interaction and the rate of complex formation between thrombin and protein C. *J. Biol. Chem* 2005;280:7956–7961. [PubMed: 15582990]
19. Cantwell AM, Di Cera E. Rational design of a potent anticoagulant thrombin. *J. Biol. Chem* 2000;275:39827–39830. [PubMed: 11060281]
20. Tsiang M, Paborsky LR, Li WX, Jain AK, Mao CT, Dunn KE, Lee DW, Matsumura SY, Matteucci MD, Coutre SE, Leung LL, Gibbs CS. Protein engineering thrombin for optimal specificity and potency of anticoagulant activity *in vivo*. *Biochemistry* 1996;35:16449–16457. [PubMed: 8987977]
21. De Filippis V, De Dea E, Lucatello F, Frasson R. Effect of Na⁺ binding on the conformation, stability and molecular recognition properties of thrombin. *Biochem. J* 2005;390:485–492. [PubMed: 15971999]
22. Wells CM, Di Cera E. Thrombin is a Na(+)-activated enzyme. *Biochemistry* 1992;31:11721–11730. [PubMed: 1445907]
23. Gianni S, Ivarsson Y, Bah A, Bush-Pelc LA, Di Cera E. Mechanism of Na⁺ binding to thrombin resolved by ultra-rapid kinetics. *Biophys. Chem* 2007;131:111–114. [PubMed: 17935858]
24. Gandhi PS, Chen Z, Mathews FS, Di Cera E. Structural identification of the pathway of long-range communication in an allosteric enzyme. *Proc. Natl. Acad. Sci. USA* 2008;105:1832–1837. [PubMed: 18250335]
25. Pineda AO, Chen ZW, Bah A, Garvey LC, Mathews FS, Di Cera E. Crystal structure of thrombin in a self-inhibited conformation. *J. Biol. Chem* 2006;281:32922–32928. [PubMed: 16954215]

Figure 1.

Interactions between the A chain (stick model, residues labeled in black) and B chain (wheat surface, residues labeled in white) of thrombin based on the structure 1PPB [2]. The A chain is stabilized by the Asp1a-Lys9 and Arg14d-Glu13 ion pairs and the ion quartet Arg4-Glu8-Asp14-Glu14c (H bonds are in green). The interaction between the A and B chain depends on the Cys1--Cys122 bond (hidden behind Arg206), the ionic interactions Asp1a-Arg206, Glu8-Glu14c-Lys202, Asp14-Arg173, Lys14a--Glu23 and Glu14e-Lys186d-Tyr184a, and the hydrophobic stacking Tyr14j-Pro204. H bonds for these interactions are omitted for clarity. The Na⁺ site is located below the surface of Lys186d and Tyr184a. Trp29 and Trp207 in van der Waals contact with Arg4 are also noted.

Figure 2.

Functional properties of the 12 mutants of the A chain. Shown are the values of $s = k_{\text{cat}}/K_{\text{m}}$ for the hydrolysis of the chromogenic substrate FPR in the E:Na⁺ (black bar) and E (white bar) forms, the cleavage of fibrinopeptides (Fps) A (black bar) and B (white bar) from fibrinogen, the activation of protein C (PC) with (black bar) or without (white bar) 100 nM thrombomodulin, and the activation of PAR1. Values of s_{mut} for each mutant are expressed in units of s_{wt} determined for wild type as follows: $88 \pm 3 \mu\text{M}^{-1} \text{s}^{-1}$ (FPR, E:Na⁺ form); $5.9 \pm 0.4 \mu\text{M}^{-1} \text{s}^{-1}$ (FPR, E form); $17 \pm 1 \mu\text{M}^{-1} \text{s}^{-1}$ (FpA); $8.1 \pm 0.5 \mu\text{M}^{-1} \text{s}^{-1}$ (FpB); $0.22 \pm 0.01 \mu\text{M}^{-1} \text{s}^{-1}$ (protein C in the presence of 100 nM thrombomodulin and 5 mM Ca²⁺); $0.15 \pm 0.01 \text{mM}^{-1} \text{s}^{-1}$ (protein C in the absence of thrombomodulin but in the presence of 5 mM Ca²⁺); $30 \pm 1 \mu\text{M}^{-1} \text{s}^{-1}$ (PAR1).

Figure 3.

(A, B) Kinetic traces of Na^+ binding to the thrombin mutants D141A (A) and R4A (B) in the 0–250 ms time scale. Shown are the traces obtained at 0 mM (black circles), 6.25 mM (red circles), 12.5 mM (green circles), 25 mM (yellow circles), 50 mM (magenta circles), 100 mM (blue circles) and 200 mM (brown circles) Na^+ . The binding of Na^+ obeys a two-step mechanism in (A), with a fast phase completed within the dead time (<0.5 ms) of the spectrometer, followed by a single-exponential slow phase. The k_{obs} for the slow phase decreases with increasing $[\text{Na}^+]$, as seen for wild type [16]. On the other hand, binding of Na^+ to R4A in (B) shows a decrease in intrinsic fluorescence and lacks the well-defined slow phase seen for the wild-type [16] or the mutant D141A in (A). Continuous lines in (A) were drawn using the expression $a \exp(-k_{\text{obs}} t) + b$ with best-fit parameter values: (black circles) $a = 0 \pm 0$ V, $k_{\text{obs}} = 0 \pm 0$ s^{-1} , $b = 8.08 \pm 0.01$ V; (red circles) $a = -0.08 \pm 0.02$ V, $k_{\text{obs}} = 210 \pm 20$ s^{-1} , $b = 8.64 \pm 0.02$ V; (green circles) $a = -0.17 \pm 0.02$ V, $k_{\text{obs}} = 150 \pm 10$ s^{-1} , $b = 8.89 \pm 0.02$ V; (yellow circles) $a = -0.25 \pm 0.02$ V, $k_{\text{obs}} = 130 \pm 10$ s^{-1} , $b = 9.04 \pm 0.02$ V; (magenta circles) $a = -0.28 \pm 0.02$ V, $k_{\text{obs}} = 130 \pm 10$ s^{-1} , $b = 9.22 \pm 0.02$ V; (blue circles) $a = -0.30 \pm 0.02$ V, $k_{\text{obs}} = 120 \pm 10$ s^{-1} , $b = 9.36 \pm 0.02$ V; (brown circles) $a = -0.36 \pm 0.02$ V, $k_{\text{obs}} = 120 \pm 10$ s^{-1} , $b = 9.38 \pm 0.02$ V. Continuous lines in (B) refer to flat lines ($a = 0$ V) with best-fit values: (black circles) $b = 7.94 \pm 0.02$ V; (red circles) $b = 7.94 \pm 0.02$ V; (green circles) $b = 7.83 \pm 0.03$ V; (yellow circles) $b = 7.77 \pm 0.03$ V; (magenta circles) $b = 7.68 \pm 0.02$ V; (blue circles) $b = 7.49 \pm 0.02$ V; (brown circles) $b = 7.37 \pm 0.02$ V.