

Critical role of W60d in thrombin allostery

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Received 27 June 1996; revised 26 July 1996; accepted 31 July 1996

Abstract

Mutation of residue W60d of thrombin, located 17 Å away from the Na⁺ binding site, suppresses Na⁺ binding and the functional differences between the slow and fast forms. The molecular basis for the long-range effect of this mutation is provided by a conspicuous network of water molecules which connects the Na⁺ binding environment to the specificity sites S1 and S2 of the enzyme. The mutation appears to stabilize thrombin in a hybrid conformation that is overall similar to the slow form, but with the fibrinogen recognition site functioning as in the fast form. It also affects the switch in specificity from fibrinogen to protein C linked to the release of Na⁺ and the fast → slow conversion. Under physiological conditions of pH, temperature and NaCl concentration, the W60dS mutant behaves as an anticoagulant. It has a reduced activity toward fibrinogen by 22-fold, while the reduction of protein C activation in the presence of saturating concentrations of thrombomodulin is less than 2-fold. Even more remarkable is the cleavage of fibrin I monomer leading to release of fibrinopeptide B, which is reduced by more than 130-fold. This property is reminiscent of the snake venom ancrod, which only releases fibrinopeptide A, and adds substantially to the anticoagulant potency of the W60dS mutant. In fact, the clotting time in the presence of this mutant is prolonged more than 40-fold compared to the wild-type.

Keywords: Thrombin; Allosteric enzymes; Na⁺; Site-directed mutagenesis

1. Introduction

The serine protease thrombin plays its crucial procoagulant and anticoagulant roles in hemostasis [1] by virtue of an allosteric equilibrium involving

two forms, slow and fast, that interconvert rapidly upon binding and dissociation of Na⁺ [2,3]. This cation binds in the loop connecting the last two β-strands of the B chain [4]. The discovery of the allosteric nature of thrombin and the differential role of its two forms suggest new ways of developing a control of the enzyme function in vivo. Essentially two strategies can be pursued. One is based on the design of allosteric effectors capable of switching the enzyme to the slow or fast form. The other is based on the design of thrombin mutants that are devoid of allosteric regulation and behave as either the anti-coagulant slow form or the procoagulant fast form. Important progress has been made in both directions.

Abbreviations: Ch, choline; DRR, H-D-Asp-Arg-Arg-*p*-nitroanilide; FGR, H-D-Phe-Gly-Arg-*p*-nitroanilide; FpA, fibrinopeptide A; FpB, fibrinopeptide B; FPR, H-D-Phe-Pro-Arg-*p*-nitroanilide; Hir^{1–49}, hirudin N-terminal fragment 1–49; Hir^{55–65}, hirudin C-terminal fragment 55–65; HPLC, high-performance liquid chromatography; PEG, poly(ethylene glycol); S2238, H-D-Phe-pipecolyl-Arg-*p*-nitroanilide; TM, thrombomodulin; Tris, tris(hydroxymethyl)aminomethane

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Mutations in and around the Na^+ binding site reduce the Na^+ affinity and stabilize the anticoagulant slow form [5,6]. A core of residues controlling the allosteric equilibrium has been identified [7] and provides the target for synthetic allosteric effectors.

A surprising finding was reported while studying a number of thrombin mutations in the region of the allosteric core. Mutation of W60d, a residue defining part of the apolar S2 site of the enzyme [8] and located 17 Å away from the Na^+ binding site (see Fig. 1), was found to reduce the differences between

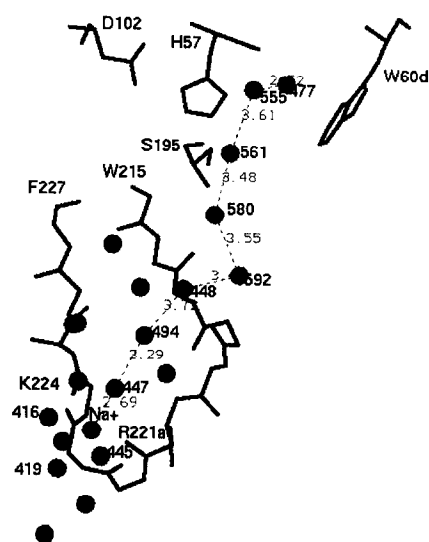


Fig. 1. Molecular environment of the Na^+ binding site of thrombin (1HAH). The bound Na^+ (black circle) is accommodated in the loop connecting the last two β -strands of the B chain (sequence 215–227 in the chymotrypsin numbering) and is coordinated octahedrally by the carbonyl O atoms of R221a and K224 and four water molecules (gray circles, numbered 416, 419, 445 and 447). W60d sits on the short insertion loop that caps the entrance to the catalytic pocket from above and defines the S2 specificity site (8). This residue is 17 Å away from the bound Na^+ and its mutation to Ser abolishes Na^+ binding and the allosteric properties of the enzyme. The long-range communication between the Na^+ binding site and the S2 specificity site is mediated by a thread of water molecules that also embed the Na^+ binding site and the specificity site S1 around D189 (22, 23). The hydrogen-bonding network involving these water molecules (gray circles, numbered 447, 494, 448, 692, 580, 561, 555 and 477) is shown by dotted lines along with the relevant interatomic distances in Å. Water molecules of particular interest are water 477, 3.0 Å from the carbonyl oxygen atom of the catalytic H57, and water 555, 4.0 Å from the hydroxyl oxygen atom of Y60a in the S2 site.

the slow and fast forms [7]. In order to characterize better the behavior of the W60dS mutant, we have studied the interaction with a number of molecules that probe distinct regions of the enzyme in an attempt to map structurally the origin of the perturbed energetics. The theoretical basis of this approach has been described in our previous work on thrombin–hirudin interaction [9,10] and exploits the properties of thermodynamic cycles involving the binding of a ligand and the conformational slow \rightarrow fast equilibrium. We have also tested the possibility that this mutant may be stabilized in the anticoagulant slow form, which would reduce its ability to cleave fibrinogen but enhance activation of protein C. Such a possibility is clearly of interest in view of its potential therapeutic applications.

2. Materials and methods

The thrombin mutant W60dS was constructed, purified, and tested for activity as described [7]. The hirudin N-terminal fragment 1–49 was obtained by proteolytic digestion of the intact hirudin as reported elsewhere [11]. The hirudin C-terminal fragment 55–65 was synthesized by solid phase, purified by HPLC and analyzed for purity by mass spectrometry. Human protein C, activated protein C and human antithrombin III were purchased from Enzyme Research or Hematologic Technologies. Heparin (Ca^{2+} -salt) was from Sigma. Recombinant human thrombomodulin with the chondroitin sulfate moiety was obtained from Dr. Brian Grinnell (Eli-Lilly). The chromogenic substrates FPR, FGR and DRR were synthesized by solid phase by Dr. John Tomich (Kansas State University, Manhattan, KS). The chromogenic substrate S2238 was purchased from Chromogenix.

The monovalent cation specificity of the W60dS mutant was tested under conditions of 5 mM Tris, 0.1% PEG, pH 8.0, 25°C and 0.2 M LiCl, NaCl, KCl or ChCl [3,5]. The Na^+ binding affinity was measured from the effect of $[\text{Na}^+]$ on the specificity constant k_{cat}/K_m [12] using the theory of allosteric effects in serine proteases [13]. The hydrolyses of FPR and FGR were studied under conditions of 100 mM ChCl (slow form) or NaCl (fast form), pH 8.0 at 25°C. These measurements were also carried out in the presence of saturating amounts of hir^{55–65} or

thrombomodulin. The binding of hir^{1–49} was quantified from the competitive effect on the k_{cat}/K_m for the hydrolysis of a chromogenic substrate [11]. Binding of hir^{55–65} and thrombomodulin was quantified from the competitive inhibition of the release of fibrinopeptide A [14]. The release of fibrinopeptides A and B was quantified by HPLC [15]. The hydrolysis of protein C in the presence of saturating amounts of thrombomodulin was quantified from a progress curve of the hydrolysis of DRR, a chromogenic substrate highly specific for activated protein C, using the method of Dang and coworkers [3,6]. The interaction with antithrombin III in the presence of heparin was studied from analysis of progress curves by exploiting the competitive inhibition of substrate hydrolysis [4,16]. The interactions of W60dS with antithrombin III, protein C and fibrinogen were studied under conditions of physiological interest, i.e., 145 mM NaCl, pH 7.4 at 37°C. Similar interactions for the wild-type were studied under identical solution conditions and also as a function of [NaCl] to obtain the properties of the slow and fast forms. The properties of the slow form were derived from experiments carried out at 145 mM ChCl. Those of the fast form were derived from measurements as a function of [NaCl], keeping the ionic strength constant at 145 mM with ChCl, and extrapolating at $[\text{Na}^+] \rightarrow \infty$ [9,10]. Clotting curves were measured from the increase in turbidity at 350 nm and the clotting time was derived from the inflection point of the progress curve [15].

3. Results

The values of the specificity constant for the hydrolysis of the chromogenic substrate S2238 in the presence of different monovalent cations are shown in Fig. 2 for the wild-type thrombin and the W60dS mutant. Mutation of W60d abolishes the monovalent cation sensitivity of the enzyme. A similar result has been reported recently for mutation of residues in the Na⁺-binding loop [4–6] and is due to the lack of monovalent cation binding. Even at 800 mM ionic strength, no appreciable binding of Na⁺ can be seen for this mutant, suggesting that mutation of W60d may stabilize the anticoagulant slow form of the enzyme.

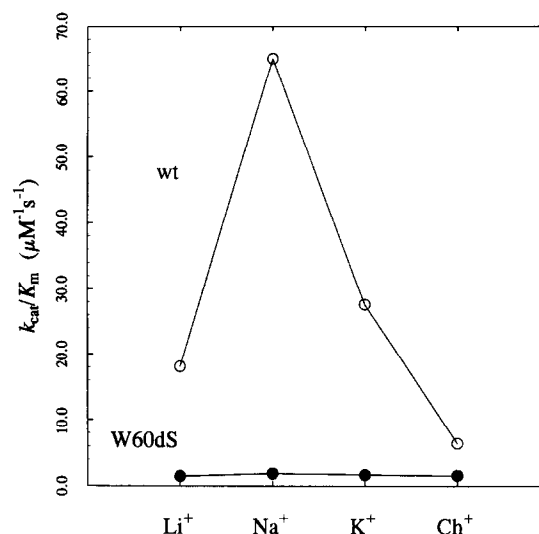


Fig. 2. Value of the specificity constant for the hydrolysis of S2238 by wild-type thrombin and the W60dS mutant, as indicated. Experimental conditions are: 5 mM Tris, 0.1% PEG, pH 8.0, 25°C. The salt concentration was kept constant at 200 mM with the chloride salts indicated on the abscissa. The large difference seen between Na⁺ (fast form) and Ch⁺ (slow form) for the wild-type is not observed in the W60dS mutant, which behaves functionally like the slow form of the wild-type.

This hypothesis was tested by studying the interaction of W60dS with hirudin and its fragments hir^{1–49} and hir^{55–65}, which are sensitive probes of the energetics of the enzyme [11]. Hir^{1–49} probes the region surrounding the access to the active site and the adjacent Na⁺-binding loop, while hir^{55–65} probes the fibrinogen recognition site, or exosite 1 [17]. The results of these measurements are summarized in Table 1. Consistent with the results obtained with S2238 (see Fig. 2), the slow and fast forms of the mutant behave identically with a negligible value of the coupling free energy for allosteric switching, ΔG_c [9,10]. Interestingly, hir^{1–49} binds with an affinity comparable to that of the slow form of the wild-type, whereas binding of hir^{55–65} takes place with an affinity similar to that of the fast form of the wild-type. This splitting of the energetics is confirmed by the binding of thrombomodulin, which mostly explores the region around exosite 1 [18] and also exosite 2 [14] and binds with an affinity similar to that of the fast form of the wild-type. When both exosite 1 and the region surrounding the catalytic

Table 1

Functional properties of the W60dS mutant. Values in bold refer to wild-type

	Slow	Fast	ΔG_c
K_d values:			
Hirudin	31.8 ± 0.4 pM	27.3 ± 0.3 pM	-0.1 ± 0.1
	4.4 ± 0.4	0.18 ± 0.02	-1.9 ± 0.1
Hir ^{55–65}	1.30 ± 0.1 μ M	1.6 ± 0.1 μ M	-0.1 ± 0.1
	4.9 ± 0.3	0.9 ± 0.1	-1.0 ± 0.1
Hir ^{1–49}	4.9 ± 0.2 nM	4.9 ± 0.2 nM	$+0.0 \pm 0.1$
	7.0 ± 2.0	0.23 ± 0.05	-2.0 ± 0.1
Thrombomodulin	0.52 ± 0.04 nM	0.58 ± 0.04 nM	-0.1 ± 0.1
	2.0 ± 0.1	0.20 ± 0.01	-1.4 ± 0.1
k_{cat}/K_m values:			
FPR	3.0 ± 0.2 μ M ⁻¹ s ⁻¹	3.3 ± 0.3 μ M ⁻¹ s ⁻¹	-0.0 ± 0.1
	1.8 ± 0.1	27.0 ± 1.0	-1.6 ± 0.1
FPR + hir ^{55–65}	6.1 ± 0.4 μ M ⁻¹ s ⁻¹	7.1 ± 0.6 μ M ⁻¹ s ⁻¹	-0.1 ± 0.1
	4.0 ± 0.2	38.0 ± 2.0	-1.3 ± 0.1
FPR + TM	9.3 ± 0.7 μ M ⁻¹ s ⁻¹	8.7 ± 0.7 μ M ⁻¹ s ⁻¹	$+0.0 \pm 0.1$
	6.3 ± 0.4	27.0 ± 1.0	-0.9 ± 0.1
FGR	0.16 ± 0.02 μ M ⁻¹ s ⁻¹	0.18 ± 0.03 μ M ⁻¹ s ⁻¹	-0.1 ± 0.1
	0.9 ± 0.1	2.0 ± 0.2	-0.5 ± 0.1
FGR + hir ^{55–65}	0.31 ± 0.4 μ M ⁻¹ s ⁻¹	0.33 ± 0.06 μ M ⁻¹ s ⁻¹	-0.1 ± 0.1
	6.8 ± 0.7	2.0 ± 0.2	$+0.7 \pm 0.1$
FGR + TM	0.61 ± 0.07 μ M ⁻¹ s ⁻¹	0.71 ± 0.07 μ M ⁻¹ s ⁻¹	-0.1 ± 0.1
	7.6 ± 0.9	2.8 ± 0.3	$+0.6 \pm 0.1$

ΔG_c measures the specificity of the fast form relative to the slow form in kcal mol⁻¹ [9,10].

pocket are probed, as in the case of intact hirudin, the mutant behaves like the slow form of the wild-type.

Further information on the properties of W60dS comes from the energetic mapping of the specificity sites using site-specific thermodynamics [10]. The wild-type discriminates quite well between FPR and FGR in the fast form, but not in the slow form (see Table 1). The slow \rightarrow fast transition makes the apolar site S2 more specific for a Pro at P2. The W60dS mutant again shows a hybrid behavior. It recognizes these substrates with a specificity similar to that of the slow form of the wild-type, but it discriminates between Pro and Gly at P2 much like the fast form. We speculate that W60d occupies different positions in the slow and fast forms of the wild-type, which enables discrimination between Pro and Gly at P2 in the fast form. The side chain of W60d may hinder more the binding to the apolar site in the slow form, reducing the rotation around the P2–P3 bond of the bound substrate and creating an environment where Pro or Gly at P2 in the transition state become sterically equivalent. Mutation to Ser reduces the

specificity, due to the introduction of the polar hydroxyl in the apolar site, but eliminates the steric constraints and re-instates the differences between Pro and Gly at P2. These differences persist upon binding of hir^{55–65} or thrombomodulin to exosite 1, which, however, induces no differences between the slow and fast forms of the W60dS mutant. In the wild-type, these ligands enhance the specificity of the slow form, with minimal changes in the fast form. The intriguing result is that the slow form has higher specificity for FGR than the fast form upon binding to exosite 1, with the coupling free energy in the transition state reversing its sign (see Table 1). This peculiar effect is not seen in the W60dS mutant which, on binding to exosite 1, elicits a small enhancement of specificity which is the same for FPR and FGR. Mutation of W60d changes, both qualitatively and quantitatively, the allosteric communication between the active site and the fibrinogen recognition site.

The properties of the W60dS mutant were also studied under experimental conditions of physiological interest and compared to those of the wild-

Table 2

Properties of the W60dS mutant under physiological conditions compared to those of the slow and fast forms of the wild-type.

	FpA	FpB	Protein C ^a	Antithrombin III ^b
Slow form	1.51 ± 0.05	0.73 ± 0.04	0.32 ± 0.01	4.0 ± 0.2
Fast form	35.0 ± 8.0	17.0 ± 3.0	0.21 ± 0.01	20.0 ± 2.0
Wild-type	17.5 ± 0.6	9.4 ± 0.5	0.22 ± 0.01	13.0 ± 1.0
W60dS	0.79 ± 0.05	0.07 ± 0.01	0.15 ± 0.02	4.3 ± 0.1

Experimental conditions: 5 mM Tris, 145 mM NaCl, 0.1% PEG, pH 7.4, 37°C. The values of the slow and fast forms of the wild-type were obtained as described in Section 2. All values are in $\mu\text{M}^{-1}\text{s}^{-1}$. ^a In the presence of 100 nM human thrombomodulin and 5 mM Ca^{2+} . ^b In the presence of 0.5 USP units/ml of heparin. All values, except those for the W60dS mutant, are from Ref. [6].

type. This was done in view of the fact that even a partial stabilization of the slow form upon W60dS substitution could convert the enzyme into an anticoagulant. Compared to wild-type, the W60dS mutant shows a 22-fold reduction in the rate of fibrinogen cleavage leading to release of fibrinopeptide A, but a less than 2-fold reduction in the rate of cleavage of the anticoagulant protein C (see Table 2). The rate of inhibition by antithrombin III in the presence of heparin is reduced by 3-fold. The cleavage of fibrin I monomer leading to release of fibrinopeptide B is even more affected by the mutation, with a reduction of more than 130-fold. This property of the

mutant W60dS is of particular interest since it mimics that of the snake venom anacrod, which only releases fibrinopeptide A and produces a clot defective in lateral aggregation and growth [19]. Indeed, the clotting curve is profoundly altered in the presence of the W60dS mutant (see Fig. 3) and the clotting time is prolonged by more than 40-fold compared to wild-type. When these properties are compared to those of the slow and fast forms of the wild-type, it is clear that mutation of W60dS not only affects the allosteric slow \leftrightarrow fast equilibrium in favor of the slow form, but also produces a direct interference with the binding of fibrinogen, fibrin I monomer, protein C and antithrombin III.

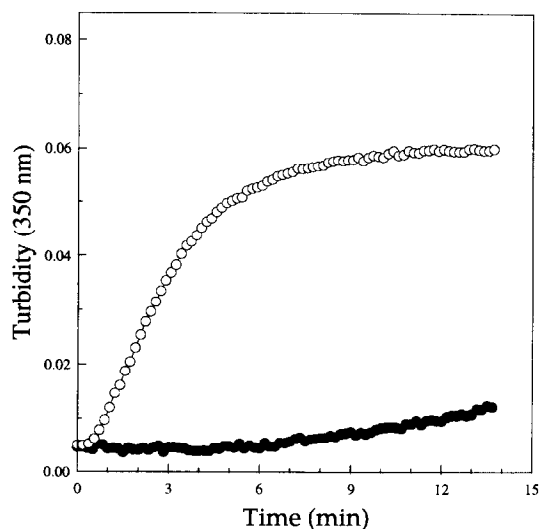


Fig. 3. Clotting curves measured as changes in turbidity at 350 nm as a function of time. Experimental conditions are: 2 nM thrombin, 0.25 M fibrinogen, 5 μM Tris, 145 mM NaCl, 0.1% PEG, pH 7.4 at 37°C. Data refer to wild-type (○) and the W60dS mutant (●). The clotting time is 0.5 min (wild-type) and > 20 min (W60dS).

4. Discussion

The hallmark of allosteric proteins is that effects can be propagated at a distance through perturbation of the equilibrium between states with different functional properties [20]. The equilibrium can be perturbed by ligands that target one state with higher affinity, or by temperature and pressure. These phenomena have been studied in great detail in classical allosteric systems. Similar allosteric effects can be produced by structural modifications in the form of site-directed mutations, as illustrated convincingly by hemoglobin [21] and our recent work on thrombin [5–7].

The effect of mutation of W60d of thrombin reported here should be understood within the framework of the allosteric theory. Although this residue is 17 Å away from the Na^+ binding site, its mutation suppresses Na^+ binding and stabilizes a conformation that is similar overall to the anticoagulant slow

form. The molecular basis for this long-range effect is to be found in the conspicuous network of water molecules [22] that connects the Na^+ binding environment to the specificity site S1 around D189 and the apolar site S2 (see Fig. 1). Changes in the Na^+ site can propagate to S1 and S2 by altering the hydrogen bonding network in the region, so that Na^+ binding and dissociation can be felt at other regions of the enzyme [23]. The importance of this network has been emphasized recently in connection with the large heat capacity change linked to Na^+ binding [12]. W60d is a critical residue in thrombin allostereism and may control the transduction of signals from the Na^+ binding site to the active site of the enzyme, the specificity site S2 and exosite 1. Interestingly, while the W60dS mutant has lost the allosteric regulation of the active site and exosite 1 by the Na^+ binding site, it retains part of the allosteric regulation of the active site by exosite 1. This demonstrates the existence of multiple allosteric pathways in the thrombin molecule that can be decoupled by ad hoc mutations. Regulation of the active site by exosite 1 [9] may involve an allosteric transition that is structurally distinct from the slow \rightarrow fast transition, although energetically similar.

If the W60dS mutation stabilizes the slow form, why are the properties of the W60dS mutant different from this allosteric state? In an allosteric system like thrombin, a mutation can produce three kinds of effects. A type-1 effect is observed when the mutation affects exclusively the slow \leftrightarrow fast equilibrium by reducing or enhancing Na^+ binding. In this case the effects on recognition of any substrate or ligand can be predicted from the properties of the slow and fast forms of the wild-type. A mutant devoid of Na^+ binding capability and stabilized in the slow form should cleave fibrinogen and protein C at the rates found for the slow form of the wild-type (see Tables 1 and 2). A type-2 effect is observed when the mutation affects the slow \leftrightarrow fast equilibrium and, in addition, the direct recognition of a given substrate or ligand. In this case the effects may be predicted only partially from the properties of the slow and fast forms of the wild-type. An example of type-2 effect is given by the double mutation D221A/D222K [4]. Fibrinogen and synthetic substrates are cleaved by this mutant at a rate consistent with perturbation of the slow \leftrightarrow fast equilibrium of

the wild-type. Protein C is cleaved at a rate that cannot be predicted from the slow \leftrightarrow fast equilibrium of the wild-type due to direct interference with recognition of this substrate. A type-3 effect is observed when the mutation does not affect the slow \leftrightarrow fast equilibrium but interferes with direct recognition of a given substrate. In this case the effects cannot be predicted from the properties of the slow and fast forms of the wild-type. The effect seen for the W60dS mutant is type-2. The cleavage of synthetic substrates is similar to that of the slow form of the wild-type and so is the release of fibrinopeptide A. However, the release of fibrinopeptide B, the cleavage of protein C and the inhibition by antithrombin III occur at rates that cannot be predicted from simple perturbation of the slow \leftrightarrow fast equilibrium. This suggests that W60d is involved in recognition of fibrin I monomer, protein C and antithrombin III, consistent with recent findings [24].

The W60dS mutant has intriguing properties and distinguishes itself by the significantly reduced activity toward fibrinogen linked to an insignificant reduction of cleavage of protein C. A number of mutations have been reported to show similar effects [25–27]. Particularly interesting is a mutation of K60f, a residue close to W60d, that affects fibrinogen but not thrombomodulin binding [26]. This observation is consistent with the results presented here on the W60dS mutant and suggests a similar underlying mechanism. We have shown that the side chain of K60f must be oriented differently in the slow form [11], much like that of W60d. It would be of interest to explore whether the K60fE mutant has altered Na^+ binding properties like the W60dS mutant. This would support the idea that the entire W60d insertion loop may be quite flexible, contrary to the suggestion of Bode et al. [8], and that it may occupy a different position in the slow form or upon binding of fibrinogen, thrombomodulin and other molecules. More recently, mutation of E217 has been shown to convert thrombin into an anti-coagulant by reducing binding of fibrinogen by 40-fold and binding of protein C by only 2-fold [27]. The E217A substitution disrupts the salt-bridge between E217 and K224, a residue directly involved in Na^+ coordination [4], impairs Na^+ binding, and stabilizes the slow form. The mutation K224A produces effects similar to E217A [6]. The reduction of

protein C cleavage in the E217A and K224A mutants indicates the presence of a type-2 effect. Integrity of the K224–E217 salt-bridge is clearly important for direct recognition of protein C.

The W60dS mutant is a potential anticoagulant, at least as effective as the mutants K60fE [26] and E217A [27]. All these mutants, however, show type-2 effects that partially compromise their reduced procoagulant activity and make them less potent than the Y225P mutant [5,6] which perturbs the slow \leftrightarrow fast equilibrium. The properties of the W60dS mutant and the knowledge gained from recent mutational studies [4–6,26,27] reinforce the dominant role played by Na⁺ binding in nearly every aspect of thrombin function and points to promising new strategies for the development of effective anticoagulants.

Acknowledgements

This work was supported in part by NIH Research Grant HL49413 and by a grant from the American Heart Association. E.D.C. is an Established Investigator of the American Heart Association and Genentech.

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