Mapping of the Thrombin des-ETW Conformation by Using Site-Directed Mutants of Hirudin. Evidence for the Induction of Nonlocal Modifications by Mutagenesis†

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ABSTRACT: Deletion of Glu^{146}, Thr^{147}, and Trp^{148} from thrombin dramatically alters its interactions with substrates, ligands, and inhibitors, and the changes appear to result from nonlocal modification of thrombin's structure [Le Bonniec, B. F., Guinto, E. R., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 19341–19348]. In an attempt to localize conformational change in this thrombin mutant (des-ETW), its affinity for 43 site-directed mutants of hirudin have been compared with that of native thrombin. The inhibition constants (K_I) of recombinant and natural hirudin with des-ETW were found to be ≈2300-fold higher than those with thrombin. The reduced affinity was predominantly the result of an increase in the dissociation rate constant rather than a decrease in the association rate constant. For most of the hirudin mutants tested, the difference in binding energy between thrombin and des-ETW [$\Delta\Delta G_b^\circ$(IIa-ETW)] was about 19.9 kJ mol^{-1} and comparable to that of hirudin; in particular, all mutations in the C-terminal region of the inhibitor did not affect $\Delta\Delta G_b^\circ$(IIa-ETW). Thus, the conformation of thrombin's anion-binding exosite seems unaltered by the des-ETW mutation. In contrast, hirudin substitutions involving Val$^{1'}$, Val$^{2'}$, Thr$^{4'}$, Asp$^{5'}$, and Ser$^{19'}$, as well as the addition of an N-terminal glycine, resulted in values of $\Delta\Delta G_b^\circ$(IIa-ETW) which were 2 to 10 kJ mol^{-1} lower than that for hirudin. Furthermore, a Leu$^{15'}$→Ala hirudin mutant behaved, with des-ETW, as a partial competitive inhibitor rather than a tight-binding inhibitor, in contrast to all other hirudin variants tested. Results are interpreted in terms of the thrombin–hirudin tertiary structure, and it is concluded that the des-ETW mutation at least affects the conformations of (1) the catalytic serine, (2) the primary specificity pocket, and (3) the S_2 binding subsite. These nonlocal modifications of thrombin's structure subsequent to the des-ETW mutation may be caused by loss of the internal salt bridge between Glu^{146} and Arg^{221A} that seems to play a key role in thrombin's functions.

Hirudin is a 65 amino acid inhibitor that was originally isolated from the medicinal leech *Hirudo medicinalis* (Markwardt, 1970; Bagdy et al., 1976; Dodt et al., 1988). It exhibits an absolute specificity for thrombin (Walsmann & Markwardt, 1981), the serine protease directly responsible for blood clot formation (Furie & Furie, 1988; Davie et al., 1991). This inhibition is achieved by a unique interaction that involves the catalytic site but not the primary specificity pocket of the proteinase (Grütter et al., 1990; Rydel et al., 1990; Bode & Huber, 1992). The dissociation constant of the thrombin–hirudin complex is in the femtomolar range (Stone & Hofsteenge, 1986), and the tightness of the complex is due to multiple interactions that occur over a large area of contact between the two molecules, involving over 40% of the hirudin residues (Grütter et al., 1990; Rydel et al., 1990, 1991;, Vitali et al., 1992; Stone et al., 1989). Extensive mutagenesis studies with recombinant hirudin (rhir)[1] have provided an understanding of the contribution of most interactions to the stability of the complex (Dodt et al., 1988; Braun et al., 1988; Stone et al., 1989; Degryse et al., 1989; Lazar et al., 1991; Winant et al., 1991; Wallace et al., 1989; Betz et al., 1991a,b, 1992a). Thus, the rhir–thrombin complex is one of the best detailed proteinase–inhibitor interactions.

The thrombin mutant des-ETW (Le Bonniec et al., 1992b) results from the deletion of Glu^{146}, Thr^{147}, and Trp^{148} within the autolysis loop of thrombin[2] (Bode et al., 1989). This deletion impairs most of thrombin's functions, including fibrinogen cleavage and protein C activation, although thrombomodulin binding is only slightly affected. The mutation reduces the reactivity of the charge-stabilizing system, and a comparison of the kinetic parameters between des-ETW and thrombin with a series of *p*-nitroanilide substrates suggested a major modification of thrombin's P_2 specificity. In addition, while the des-ETW mutation dramatically lowers the rate of association (k_{on}) with antithrombin

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[1] Abbreviations: des-ETW, mutated thrombin where amino acids Glu^{146}, Thr^{147}, and Trp^{148} have been deleted; rhir, recombinant hirudin variant 1 (hirudin refers to natural hirudin, as isolated from leeches, with Tyr$^{63'}$ sulfated); H-D-Phe-Pip-Arg-pNA, H-D-Phe-pipecolyl-Arg-*p*-nitroanilide.

[2] The amino acid sequence numbering of thrombin, as suggested by Bode et al. (1989), is based on its three-dimensional topological identity with chymotrypsin, and insertion residues are marked by capital letters in alphabetical order. Primed numbers are used for residues in rhir to distinguish them from thrombin residues.

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III, it markedly increases the susceptibility to inhibition by soybean trypsin inhibitor (Le Bonniec et al., 1992b). The genetic variant thrombin *Salakta* (Glu^{146}→Ala mutation) has similar, although attenuated, dysfunctional properties to those of des-ETW (Miyata et al., 1992). In particular, clotting activity, protein C activation, and peptidyl substrate hydrolysis are all impaired by the mutation, and the k_{on} values for antithrombin III, tosyl-$LysCH_2Cl$, and diisopropyl phosphofluoridate are all reduced in comparison with thrombin (Bezeaud et al., 1984, 1988). The effects on activity observed with the des-ETW and Glu^{146}→Ala mutations probably are not due to a perturbation of adjacent residues (Ala^{149A} to Lys^{149E}) in the autolysis loop. Except for a possible role in thrombomodulin binding (Suzuki et al., 1990), no specific function has yet been assigned to the autolysis loop of thrombin. Indeed, functional studies with the ϵ-thrombin derivative, prepared by pancreatic or neutrophil elastase digestion of thrombin between Ala^{149A} and Asn^{149B} (Kawabata et al., 1985; Brower et al., 1987), or with ζ-thrombin obtained by neutrophil cathepsin G or chymotrypsin proteolysis between Trp^{148} and Thr^{149} (Brezniak et al., 1990) tend to exclude the autolysis loop of thrombin as a critical motif for clotting and amidolytic activities, protein C activation, or inhibition by antithrombin III and hirudin (Stone et al., 1987; Hofsteenge et al., 1988; Brezniak et al., 1990; Rogers et al., 1992).

In an attempt to identify nonlocal conformational changes introduced by the des-ETW mutation in thrombin, we have compared the inhibition constants (K_I) of 43 rhir mutants for des-ETW and thrombin. Consideration of the mutational effects in relation to the crystal structure of the rhir–thrombin complex suggests that the structural differences between thrombin and des-ETW include, in addition to the S_1 primary specificity pocket with segment Ser^{214}–Lys^{224A}, the S_2 binding subsite with residues Tyr^{60A}, Pro^{60C}, Trp^{60D}, Leu^{99}, and Glu^{192}.

MATERIALS AND METHODS

Proteins and Reagents. Plasma-derived thrombin (Stone & Hofsteenge, 1986), wild-type recombinant thrombin (Le Bonniec et al., 1991), and the des-ETW mutant (Le Bonniec et al., 1992b) were prepared as previously reported. Hirudin (sulfated), isolated from leeches, was a gift from Plantorgan AG (Bad Zwischenahn, Germany). Wild-type rhir variant 1 was a gift from Ciba-Geigy (Basel, Switzerland). The preparation of the rhir mutants used in this study has been reported previously (Braun et al., 1988; Wallace et al., 1989; Betz et al., 1991a,b, 1992b), and their concentrations were determined by titration with 2 nM thrombin as described (Wallace et al., 1989). The substrate H-D-Phe-pipecolyl-Arg-*p*-nitroanilide (H-D-Phe-Pip-Arg-pNA, S-2238) was purchased from Chromogenix (Molndal, Sweden). Concentrations of intact substrate and of pNA released after cleavage were determined spectrophotometrically by using absorption coefficients of 8270 M^{-1} cm^{-1} at 342 nm and 9230 M^{-1} cm^{-1} at 400–410 nm, respectively (Lottenberg & Jackson, 1983; Stone et al., 1991).

Kinetic Data Analysis. Assays were performed at 37 °C in 0.05 M Tris-HCl, pH 7.8, containing 0.1 M NaCl and 0.2% (w/v) poly(ethylene glycol) (M_r 6000). In this buffer system, and at 37 °C, the K_M for H-D-Phe-Pip-Arg-pNA was 202 μM with des-ETW and 3.6 μM with thrombin. To minimize the effect of substrate depletion with des-ETW, but to lessen competition with the secondary, low-affinity binding site of thrombin (K_d = 0.6–0.9 mM; Stone & Hofsteenge, 1986; Stone et al., 1987), kinetics was performed in the presence of 200 μM substrate. Inhibition kinetics was started by the addition of 20–50 pM thrombin (plasma-derived and recombinant) or 50–100 pM des-ETW.

When the inhibition reactions proceeded with a discernible lag phase, the apparent K_I (K_I') and the apparent rate of association (k_{on}') were estimated by global nonlinear regression analysis using the equation for a progress curve for slow, tight-binding inhibition (Cha, 1975, 1976; Williams & Morrison, 1979) with data obtained with six different concentrations of inhibitor (I_t) in the presence of the same amount of enzyme (E_t), as described (Stone & Hofstengee, 1986):

$$P = v_s t + (v_o - v_s)(1 - d)/(dk') \times \ln\{[(1 - d)\exp(-k't)]/(1 - d)\} \quad (1)$$

In this equation, P is the amount of product formed at time t, v_o is the initial velocity in the absence of inhibitor, and v_s, given by eq 2, is the steady-state velocity in the presence of inhibitor. Parameters d and k' are themselves functions of the two parameters F_1 and F_2, such that $d = (F_1 - F_2)/(F_1 + F_2)$ and $k' = k_{on}'F_2$, where $F_1 = K_I' + I_t + E_t$ and $F_2 = (F_1^2 - 4E_tI_t)^{1/2}$.

A discernible lag phase was, however, barely detectable with des-ETW for any of the rhir variants, and reliable estimations of k_{on}' and K_I' using eq 1 were not feasible with the mutated thrombin. Therefore, K_I' values were systematically estimated by nonlinear regression analysis of the dependence of v_s on I_t using the equation for tight-binding inhibition (Cha, 1975; Williams & Morisson, 1979; Stone & Hofsteenge, 1986):

$$v_s = (v_o/2E_t)\{[(K_I' + I_t - E_t)^2 + 4K_I'E_t]^{1/2} - (K_I' + I_t - E_t)\} \quad (2)$$

In this approach, v_s was determined, once the steady-state equilibrium was reached, by linear regression analysis of the changes in absorbance at 400–410 nm using only data obtained at less than 10% hydrolysis of the substrate. For each rhir variant, 12–18 concentrations were used with the degree of inhibition varying from 10 to 90%. The true K_I value was calculated by correcting for the concentration of the substrate (S) and its Michaelis constant (K_M) for the proteinase (Braun et al., 1988):

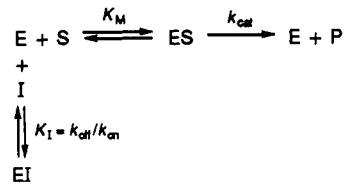
$$K_I = K_I'/(1 + S/K_M) \quad (3)$$

The K_I values obtained from the analysis of steady-state velocity data according to eq 2 were, on average, 25% higher than those obtained through analysis of progress curve data using eq 1 (when feasible). This variation was probably due to a slight, systematic overestimation of the true v_s. Since the purpose of this study was to compare the K_I values between des-ETW and thrombin, and since these values could not be accurately estimated by progress curve kinetics for des-ETW, all of the values reported were determined using eq 2.

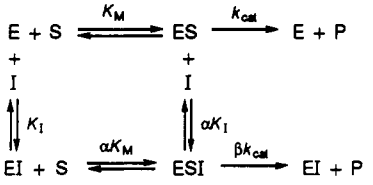
In all but one case, almost complete inactivation of both thrombin and des-ETW could be achieved with saturating amounts of the rhir variant; the observed interaction of the inhibitor with the proteinase in the presence of a substrate can be adequately described by Scheme 1. Almost complete inhibition of thrombin was achieved with saturating concentrations of the rhir mutant L15A,[3] but only partial inhibition

[3] Standard nomenclature is used to denote mutants of rhir. The residue mutated is given first in the single letter code followed by its sequence number and then the substituted residue; G^0 represents the mutant rhir with an N-terminal glycine extension.

Scheme 1



Scheme 2



was observed with des-ETW. Thus, Scheme 1 could no longer describe the interaction of des-ETW with L15A, which was best described by Scheme 2, where α and β represent the factors by which K_M and k_{cat} respectively change upon inhibitor binding. For the L15A–des-ETW interaction, K_I, α, and β were estimated by nonlinear regression analysis of the dependence of v_s on S and I_t, according to the equation for partial mixed-type inhibition (Segel, 1975; Schmitz et al., 1991):

$$v_s = k_{cat}E_tS/\{\alpha K_M(I_t + K_I)/(\beta I_t + \alpha K_I) + S(I_t + \alpha K_I)/(\beta I_t + \alpha K_I)\} \quad (4)$$

For this analysis, v_s was measured at five concentrations of S, varying from 25 to 400 μM, and six concentrations of L15A, with at least one of them approaching saturation.

The Gibbs standard free energy (ΔG_b°) values for the formation of the complexes were calculated from the estimated K_I using eq 5, where R is the gas constant and T the absolute temperature:

$$\Delta G_b^\circ = RT \ln(K_I) \quad (5)$$

It is possible to define two changes in binding energy for each of the rhir variants. The first is the change in binding energy for an rhir variant relative to wild-type rhir with the same proteinase [$\Delta\Delta G_b^\circ$(ETW) or $\Delta\Delta G_b^\circ$(IIa)], and the second is the change in binding energy between the two proteinases for the same rhir variant [$\Delta\Delta G_b^\circ$(IIa-ETW)].

RESULTS

Inhibition Kinetics of des-ETW by Hirudin and rhir. In previous studies, we reported that the K_I of crude hirudin for des-ETW is much higher than that for thrombin (Le Bonniec et al., 1992b). Using detailed kinetics at 37 °C, the affinity of des-ETW for pure recombinant hirudin (rhir) appeared to be even more affected than we initially estimated. Compared with thrombin, the K_I value of des-ETW with rhir was 2271 times higher (Table 1). When 40 pM thrombin is mixed with 50 pM rhir, equilibrium formation takes ≈8 min, thus allowing for an accurate estimation of k_{on} (1.4×10^8 M^{-1} s^{-1}; Braun et al., 1988) using eq 1 for the progress curve for slow, tight-binding inhibition (Figure 1). In contrast, at concentrations of rhir (≥2 nM) necessary to observe inhibition with des-ETW, the steady-state rates of hydrolysis were attained in less than 10 s whether the reaction was started by (1) addition of 100 pM des-ETW to a mixture of rhir and substrate (Figure 1), (2) addition of rhir to a mixture of des-ETW and substrate in steady-state hydrolysis, or (3) addition of the substrate to a mixture of des-ETW and rhir preincubated for 4 h.

Table 1: Parameters for the Interaction of Mutants in the C-Terminal Region of rhir with Thrombin and des-ETW[a]

mutant	K_I (ETW) (nM)	K_I (IIa) (pM)	$\Delta\Delta G_b^\circ$(ETW) (kJ mol^{-1})	$\Delta\Delta G_b^\circ$(IIa) (kJ mol^{-1})	$\Delta\Delta G_b^\circ$(IIa-ETW) (kJ mol^{-1})
rhir	0.73	0.32			19.9
hirudin	0.09	0.04	−5.4	−5.4	19.9
D55N	2.88	1.30	3.5	3.6	19.9
F56Y	1.04	0.43	0.9	0.7	20.1
F56W	2.04	0.69	2.6	1.9	20.6
F56I	27.7	10.3	9.4	8.9	20.4
F56V	60.5	27.3	11.4	11.4	19.9
F56S	69.0	28.60	11.7	11.5	20.1
F56T	68.8	25.80	11.7	11.3	20.3
F57Q	4.19	2.26	4.5	5.0	19.4
F58Q	2.43	1.19	3.0	3.3	19.6
P60K	4.19	2.10	4.5	4.8	19.6
P60G	14.7	6.42	7.7	7.7	19.9
P60A	9.41	4.60	6.6	6.8	19.7
E62Q	1.96	0.80	2.5	2.3	20.1
Y63F	0.46	0.21	−1.2	−1.1	19.8
Y63L	1.43	0.73	1.7	2.1	19.5
Y63A	0.69	0.30	−0.2	−0.2	19.9
Y63V	5.02	2.30	5.0	5.0	19.8

[a] K_I values were estimated by nonlinear curve fitting to eq 2 and corrected for the substrate concentration according to eq 3. Standard errors were ±19% or less. Values of ΔG_b° were calcualted using the relationship given in eq 5. The values of $\Delta\Delta G_b^\circ$(ETW) and $\Delta\Delta G_b^\circ$(IIa) represent the decrease in binding energy caused by a particular mutation relative to rhir. The value of $\Delta\Delta G_b^\circ$(IIa-ETW) reflects the decrease in binding energy between thrombin and des-ETW for a particular rhir mutant.

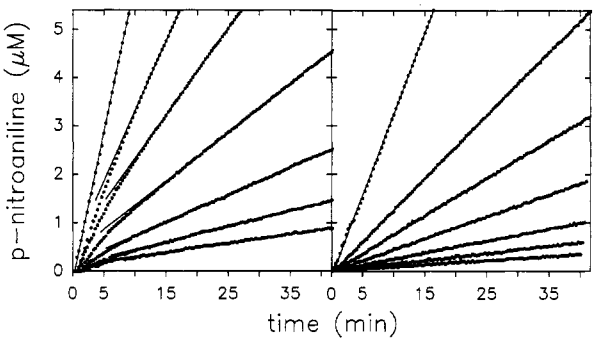


FIGURE 1: Time courses of thrombin and des-ETW inhibition by rhir. Reactions were started by the addition of 40 pM thrombin (left panel) or 100 pM des-ETW (right panel) to a mixure of 200 μM H-D-Phe-Pip-Arg-pNA and (from top to bottom) 0, 0.03, 0.06, 0.12, 0.25, 0.5, and 1 nM rhir (left panel) or 0, 3.1, 6.25, 12.5, 25, 50, and 100 nM rhir (right panel). Steady-state velocities (solid lines) were estimated by linear regression of the absorbance values at equilibrium versus time.

An attempt was made to estimate k_{on} by using progress curve kinetics on a short time scale. Simulations indicated that the half-life of the reaction would be longest when the concentrations of enzyme and inhibitor are equal. Under such conditions, rapid transition to the steady-state velocity could be detected with nanomolar concentrations of both des-ETW and rhir (Figure 2). Analysis of data similar to those shown in Figure 2 according to eq 1 reproducibly yielded estimates for k_{on} in the range of $(0.8–1.1) \times 10^8$ M^{-1} s^{-1} and estimates for the K_I values that were comparable to those obtained from analyses of the steady-state velocities using eq 2. Given the low signal in the progress curve experiments, this estimate for k_{on} must be treated with some caution. However, simulations indicated that the value for k_{on} could not be 10-fold lower (Figure 2). Thus, the k_{on} value for rhir with des-ETW appears to be on the same order of magnitude as that with thrombin. This observation would imply that the ≈2300-fold decrease

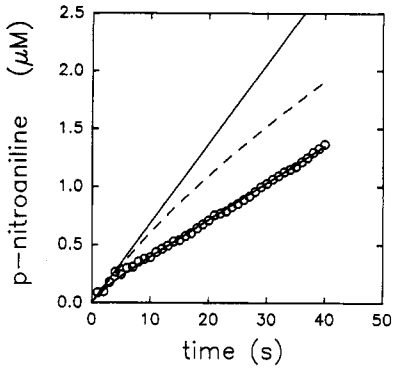


FIGURE 2: Estimation of the k_{on} value of rhir for des-ETW. The progress curve of 2 nM des-ETW inhibition by 2 nM rhir in the presence of 200 μM H-D-Phe-Pip-Arg-pNA (O) is shown. The solid line through the open circles represents the result of the analysis of these data by nonlinear curve fitting to eq 1 with v_o fixed at the velocity observed in the absence of rhir (upper solid line). The dashed line is a simulation of a curve where k_{on} is 10-fold lower.

in the affinity of rhir for des-ETW primarily results from an increase in the dissociation rate constant ($k_{off} = K_I k_{on}$).

While a reliable determination of k_{on} was difficult to obtain with des-ETW, the steady-state velocities allowed the estimation of K_I, using eq 2 and, thus, of the binding energy (ΔG_b°), using eq 5. The ΔG_b° for rhir with des-ETW represented about 75% of that observed with thrombin (−54.2 versus −74.1 kJ mol^{-1}, Table 1). In hirudin (isolated from leeches) Tyr$^{63'}$ is sulfated, and this posttranslational modification is not present in rhir (Riehl-Bellon et al., 1989). The ΔG_b° for hirudin with des-ETW also represented ≈75% of that observed with thrombin (−59.6 versus −79.5 kJ mol^{-1}). Thus, the change in binding energy between thrombin and des-ETW ($\Delta\Delta G_b^\circ$(IIa-ETW)) was the same for rhir and hirudin (19.9 kJ mol^{-1}), suggesting that sulfation of Tyr$^{63'}$ improves the binding to des-ETW and thrombin in a similar fashion.

Interactions within Anion-Binding Exosite 1. In addition to five negatively charged residues that make direct or indirect electrostatic interactions with anion-binding exosite 1 of thrombin, the C-terminal tail of rhir contains several hydrophobic residues (Phe$^{56'}$, Ile$^{59'}$, Pro$^{60'}$, and Tyr$^{63'}$) that also bind to this region of the proteinase. Mutants of rhir in which each of the acidic residues between Asp$^{55'}$ and Glu$^{62'}$ was replaced by a neutral residue were tested together with substitutions of Phe$^{56'}$ (Tyr, Phe, Trp, Ile, Val, Ser, or Thr), Pro$^{60'}$ (Lys, Ala or Gly) and Tyr$^{63'}$ (Phe, Leu, Val, or Ala). For all of these rhir C-terminal variants, the difference in affinity between des-ETW and thrombin was similar to that observed with rhir and hirudin, i.e., the $\Delta\Delta G_b^\circ$(IIa-ETW) values were essentially equal (mean = 19.9 ± 0.3 kJ mol^{-1}, Table 1). The observation that all of the 17 mutations within the carboxyl tail of rhir affect the interactions with thrombin and des-ETW to a similar extent suggests that the conformation of thrombin's anion-binding exosite 1 is preserved in des-ETW. This conclusion is consistent with a k_{on} value that is not markedly different in des-ETW since, under the conditions of the progress curve assay, the rate-determining step in complex formation is binding of the rhir C-terminal tail to anion-binding exosite 1 (Stone & Hofsteenge, 1986; Stone et al., 1989; Jackman et al., 1992). Moreover, the affinity of des-ETW for thrombomodulin, which also binds to anion-binding exosite 1 (Hofsteenge et al., 1986, 1988; Hofsteenge & Stone, 1987; Tsiang et al., 1990; Wu et al., 1991; Ye et al., 1991, 1992), is only moderately increased compared with thrombin (Le Bonniec et al., 1992b). The binding to thrombin of rhir residues 52–65 involves Lys^{149E}, Gln^{151}, and segments

Table 2: Parameters for the Interaction of Mutants in the N-Terminal Region of rhir with Thrombin and des-ETW[a]

mutant	K_I (ETW) (nM)	K_I (IIa) (pM)	$\Delta\Delta G_b^\circ$(ETW) (kJ mol^{-1})	$\Delta\Delta G_b^\circ$(IIa) (kJ mol^{-1})	$\Delta\Delta G_b^\circ$(IIa-ETW) (kJ mol^{-1})
rhir	0.73	0.32			19.9
G0	9.69	17.82	6.7	10.0	16.6
V1R	0.96	0.45	0.7	0.8	19.8
V1S	14.0	13.20	7.6	3.8	16.5
V2S	2.59	1.44	3.2	3.8	19.3
V2K	2.11	1.60	2.7	4.1	18.5
V2R	0.13	0.14	−4.5	−2.2	17.6
V2T	0.30	0.35	−2.3	0.2	17.4
Y3A	73.4	39.10	11.9	12.3	19.4
T4A	2.37	6.53	3.0	7.4	15.5
T4S	0.68	2.70	−0.2	5.5	14.2
T4W	0.66	11.20	−0.3	9.1	10.5
D5A	7.14	1.67	5.9	4.2	21.5
D5E	1.56	1.57	1.9	4.1	17.8
E8Q	0.88	0.31	0.5	−0.1	20.5
L15A[b]	67.2	84.8	12.0	14.4	17.8
S19A	1.32	0.57	1.5	1.4	20.0
S19D	0.47	0.68	−1.2	1.9	16.8
N20A	11.0	4.55	7.0	6.8	20.1
N20D	0.71	0.52	−0.1	1.2	18.6
V21A	1.95	0.89	2.5	2.6	19.8
D33N	1.41	0.48	1.7	1.0	20.6
E43Q	0.76	0.27	0.1	−0.5	20.5
P46A	1.88	1.46	2.4	3.9	18.5
P48A	1.79	1.05	2.3	3.0	19.2
Q49E	0.62	0.25	−0.4	−0.7	20.1
H51R	0.40	0.30	−1.6	−0.2	18.5

[a] K_I, ΔG_b°, and $\Delta\Delta G_b^\circ$ values were determined as in Table 1. Standard errors with K_I values were ±18% or less. [b] rhir mutant L15A is a tight-binding inhibitor of thrombin, but a partial competitive inhibitor of des-ETW with an α value of 4.22.

Phe^{34}–Leu^{41} and Lys^{70}–Lys^{81} of the proteinase (Grütter et al., 1990; Rydel et al., 1991; Tulinsky & Qiu, 1993).

rhir Mutant L15A Is a Partial (Hyperbolic) Competitive Inhibitor of des-ETW. The Leu$^{15'}$→Ala mutation decreased the affinity of rhir for thrombin by more than any other variant evaluated; ΔG_b° represented 81% of that observed with rhir (−59.8 versus −74.1 kJ mol^{-1}, Table 2). As with the other rhir mutants tested, greater than 90% thrombin inhibition was achieved with L15A concentrations 10-fold in excess of K_I'. With des-ETW, however, only 67% inhibition was achieved with saturating amounts of L15A. In contrast to all other rhir variants evaluated, the remaining amidolytic activity plateaued at 33%. Thus, simple competitive inhibition (Scheme 1) could no longer describe the overall inhibition kinetics of des-ETW by L15A. Stopped-flow fluorescence studies revealed that the initial binding of rhir to anion-binding exosite 1 of thrombin is followed by two intramolecular steps that result in binding of the inhibitor N-terminal core to the proteinase active site (Jackman et al., 1992). If the combination of mutations L15A in rhir and des-ETW in thrombin impedes one or both of these intramolecular steps, it may be possible for the inhibitor (L15A) and the *p*-nitroanilide substrate to bind simultaneously to the enzyme. Such a situation would result in the L15A rhir variant being a partial inhibitor of des-ETW. This partial inhibition will be competitive if, in addition, the substrate has a greater affinity for the free enzyme than for the L15A–des-ETW complex, whereas the bi- and trimolecular complexes yield the product with equal facility (eq 4 for partial mixed-type inhibition with $\alpha > 1$ and $\beta = 1$). To test whether L15A is a partial competitive inhibitor of des-ETW, the steady-state velocities of hydrolysis of increasing concentrations of H-D-Phe-Pip-Arg-pNA were determined in the presence of various concentrations of L15A (Figure 3). Analysis of these data according to eq 4 yielded a β value constantly close to unity, an α value of 4.22 ± 0.37,

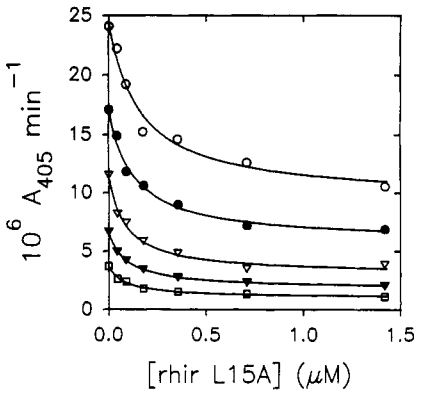


FIGURE 3: Partial inhibition of des-ETW by rhir L15A. Plot of the steady-state velocities of H-D-Phe-Pip-Arg-pNA hydrolysis (400, 200, 100, 50, and 25 μM from top to bottom) by des-ETW (56 pM) as a function of L15A concentration. Solid lines were obtained by nonlinear curve fitting to the equation for partial competitive inhibition (eq 4 with $\beta = 1$ and the values of k_{cat} and K_M fixed at those values determined in the absence of inhibitor). In the presence of 200 μM substrate and saturating amounts of L15A, the remaining activity was 33% of that in the absence of inhibitor with des-ETW, whereas it represented less than 3% that of the free enzyme with thrombin.

and a K_I value of 67.2 ± 7.2 nM. Taken together, these observations are consistent with L15A being a partial competitive inhibitor of des-ETW, which increases the K_M of the free enzyme for H-D-Phe-Pip-Arg-pNA 4.22 times, whereas k_{cat} remains unchanged.

Interactions within the Catalytic Groove and the B-Insertion. The three N-terminal amino acids of rhir form a parallel β-sheet with the Ser^{214}-Trp^{215}-Gly^{216} motif of thrombin, and numerous interactions with the proteinase are made by the first five residues of the inhibitor (Figure 4). In contrast to the C-terminal rhir variants, many of the N-terminal mutations were less detrimental for binding to des-ETW than to thrombin, and the variants exhibited a smaller $\Delta\Delta G_b^\circ$(IIa-ETW) than that observed with rhir (Table 2). When an extra glycine was added to the N-terminus of rhir ($Gly^{0'}$ extension), $\Delta\Delta G_b^\circ$(IIa-ETW) was only 16.6 kJ mol^{-1}, compared with 19.9 kJ mol^{-1} for rhir. A similar effect was observed for the $Val^{1'}$→Ser and $Val^{2'}$→Thr mutations; the $\Delta\Delta G_b^\circ$(IIa-ETW) values were 3.4 and 2.5 kJ mol^{-1} less than with rhir, respectively. The $Val^{2'}$→Arg mutation not only improved the inhibitory activity of rhir toward des-ETW, as it does with thrombin, but also decreased the value of $\Delta\Delta G_b^\circ$(IIa-ETW) by 2.3 kJ mol^{-1} relative to that for rhir. In contrast, the $Val^{1'}$→Arg, $Val^{2'}$→Ser, and $Tyr^{3'}$→Ala variants exhibited $\Delta\Delta G_b^\circ$(IIa-ETW) values comparable to that for rhir.

In the X-ray structure, $Thr^{4'}$ of rhir contacts Glu^{146} of thrombin's ETW motif (Figure 4). The greatest difference in the value of $\Delta\Delta G_b^\circ$(IIa-ETW) was observed with the rhir variant in which $Thr^{4'}$ was replaced by tryptophan (9.4 kJ mol^{-1}). Compared with rhir, the K_I value for the T4W mutant increased 35-fold with thrombin, but only 9-fold with des-ETW. The other replacements of $Thr^{4'}$ (Ser or Ala) also resulted in $\Delta\Delta G_b^\circ$(IIa-ETW) values that were 4–5 kJ mol^{-1} less than that for rhir. When $Asp^{5'}$ was substituted with Glu, the mutation was also less detrimental for the association to des-ETW than that to thrombin; the $\Delta\Delta G_b^\circ$(IIa-ETW) value was 2 kJ mol^{-1} lower than that for rhir. However, the $Asp^{5'}$→Ala mutation resulted in a more harmful effect on binding to des-ETW than to thrombin; the $\Delta\Delta G_b^\circ$(IIa-ETW) value was 1.6 kJ mol^{-1} higher than that for rhir. $Asp^{5'}$ of rhir makes an ionic interaction with thrombin Arg^{221A}, itself ion-paired in the wild-type proteinase to Glu^{146} of the ETW motif (Figure 4). Within the core of rhir, $Ser^{19'}$ also interacts with Arg^{221A} of thrombin. Interestingly, if $Ser^{19'}$ is substituted with Asp, the mutation is less detrimental for binding to des-ETW than to thrombin, whereas if it is substituted with Ala, $\Delta\Delta G_b^\circ$(IIa-ETW) is similar to that for rhir.

It is possible to examine graphically which residues in rhir interact differently with des-ETW than with thrombin by plotting the change in binding energy relative to rhir for a particular mutant with des-ETW [$\Delta\Delta G_b^\circ$(ETW)] against the change observed for the same mutant with thrombin [$\Delta\Delta G_b^\circ$(IIa)]. If the residue makes the same interactions with des-ETW and thrombin, $\Delta\Delta G_b^\circ$(ETW) should equal $\Delta\Delta G_b^\circ$(IIa). Thus, a straight line with a slope of 1 should be obtained if the residues interact equivalently with both proteinases (Figure 5). Deviations from this line will be observed for residues that do not make the same contacts. Considering that a change in $\Delta\Delta G_b^\circ$value higher than 1.8 kJ

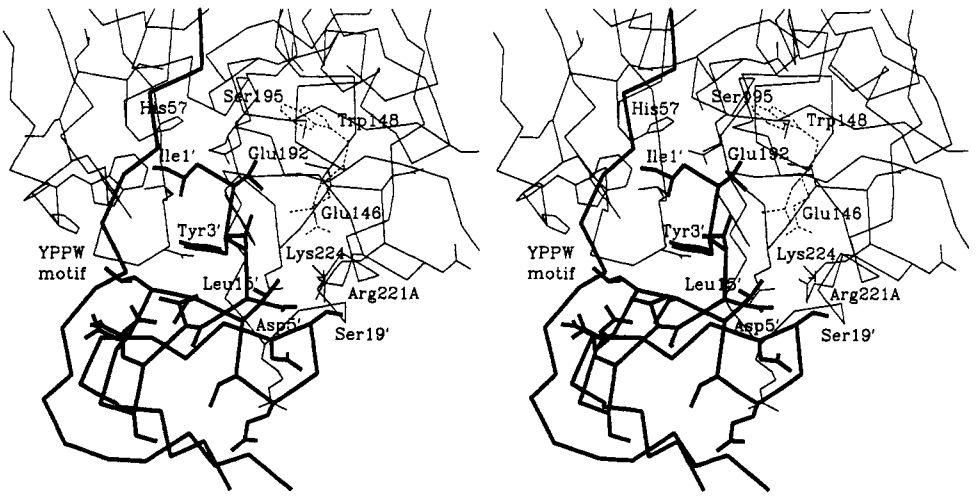


FIGURE 4: Interaction of the rhir N-terminus with the catalytic groove of thrombin. Coordinates are from Rydel et al. (1990). The N-terminal region of rhir variant 2 is shown in thick lines, while thrombin residues are drawn in thinner lines. The ETW motif with Glu^{146} and Trp^{148} (deleted in des-ETW) is drawn in dotted lines. Only the amino acids mentioned in this study are fully drawn; the others are represented by their α-carbon chain. Arg^{221A} of thrombin interacts with Glu^{146} of the ETW motif, as well as with $Asp^{5'}$ of rhir. Substitution of rhir $Leu^{15'}$ (underneath $Asp^{5'}$) by Ala induces the greatest drop in affinity for thrombin among the rhir variants tested. This rhir variant (L15A) is a partial competitive inhibitor of des-ETW rather than a tight-binding inhibitor, contrary to all other mutants tested.

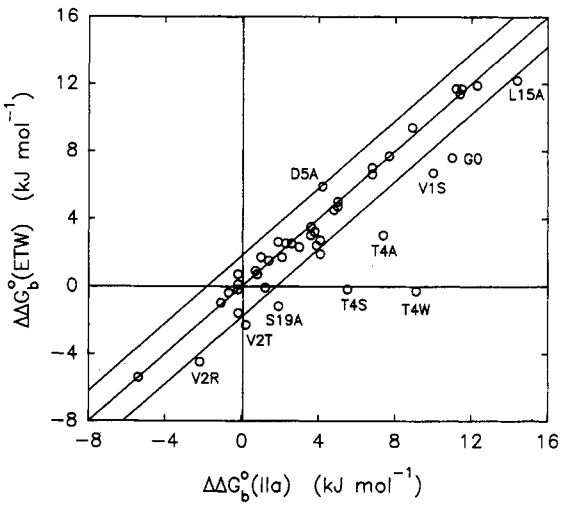


FIGURE 5: Plot of $\Delta\Delta G_b^\circ$(ETW) against $\Delta\Delta G_b^\circ$(IIa). If an rhir mutated residue makes the same interactions with des-ETW and thrombin, $\Delta\Delta G_b^\circ$(ETW) should equal $\Delta\Delta G_b^\circ$(IIa), and the point corresponding to this mutation should fall on the solid line with a slope of 1. For most rhir variants, the ratio of the K_I values with thrombin and des-ETW changed by less than 2-fold with respect to the ratio with rhir. This difference corresponds to a change in the absolute value of $\Delta\Delta G_b^\circ$(IIa) − $\Delta\Delta G_b^\circ$(ETW) of less than 1.8 kJ mol^{-1}. This limit is bound by the dotted lines on the graph. Mutants G0, V1S, V2T, V2R, V2K, T4A, T4S, T4W, D5E, S19A, and L15A fall outside this limit and were considered to exhibit an altered interaction with des-ETW.

mol^{-1} is significant suggests that the following residues interact differently with des-ETW and thrombin: Gly$^{0'}$, Val$^{1'}$, Val$^{2'}$, Thr$^{4'}$, Asp$^{5'}$, Leu$^{15'}$, and Ser$^{19'}$. In the structures of thrombin complexed with rhir variants 1 (Grütter et al., 1990) and 2 (Rydel et al., 1990), these rhir amino acids neighbor thrombin residues His^{57}, Tyr^{60A}, Trp^{60D}, Glu^{97A}, Leu^{99}, Glu^{146}, Arg^{173}, Cys^{191}, Glu^{192}, Ser^{195}, Lys^{224}, and every amino acid of segment Ser^{214}–Arg^{221A}. These observations do not imply that all of these thrombin residues adopt a different conformation in des-ETW and thrombin. In addition, other areas of thrombin, which do not interact with hirudin, might also be modified. However, these results are consistent with our previous functional studies suggesting that the charge-stabilizing system, the primary binding pocket, and the B-insertion are affected by the des-ETW mutation (Le Bonniec et al., 1992b).

DISCUSSION

By using mutants of rhir, it has been possible to show that the des-ETW mutation introduces a relatively large conformational change in thrombin. This alteration, possibly initiated from Arg^{221A} at the bottom of the primary specificity pocket, extends up to subsite S_2 of the proteinase, but does not appear to affect the conformation of anion-binding exosite 1. This study also reveals the critical role of rhir Leu$^{15'}$ in the effective inactivation of thrombin catalysis.

That mapping of des-ETW is feasible by using site-directed mutants of rhir relies on the assumption that each point mutation only introduces a local modification in hirudin. The inhibitor comprises an N-terminal core firmly maintained by three disulfide bridges and a C-terminal tail that is essentially disordered in solution (Clore et al., 1987; Folkers et al., 1989; Haruyama & Wüthrich, 1989; Szyperski et al., 1992a,b). Modifications in the C-terminal tail appear unlikely to affect the conformation of the core (Szyperski et al., 1992a). In addition, no cooperativity was detected when comparing the effects of single and multiple mutations in the C-terminal tail, suggesting that the modifications investigated induced only local changes in the rhir structure (Braun et al., 1988; Betz et al., 1991a,b, 1992a,b). In fact, the K_I for thrombin of most rhir mutants can reasonably be rationalized by studying the crystal structure of the complex. Among the three rhir variants tested involving residues not employed in the interaction with thrombin, the Glu$^{8'}$→Gln and Glu$^{43'}$→Gln alterations have little or no effect on the affinity of rhir for thrombin or des-ETW. The Pro$^{48'}$→Ala mutation, however, diminishes the binding energy with des-ETW and thrombin by 2–3 kJ mol^{-1} and, thus, may somehow introduce a nonlocal modification in rhir's structure. Furthermore, both thrombin and rhir undergo structural modifications upon complex formation. When the rhir structure in solution is compared with that of the rhir–thrombin complex, residues Glu$^{17'}$ to Val$^{21'}$ adopt a somewhat different conformation and the three N-terminal amino acids rotate slightly (Szyperski et al., 1992a,b). In thrombin, the autolysis loop, together with the ETW motif, adopts various conformations among the X-ray structures available (Bode et al., 1989; Banner & Hadváry, 1991; Grütter et al., 1990; Rydel et al., 1990, 1991; Skrzypczak-Jankun et al., 1991). Although these subtle structural rearrangements potentially limit the conclusions that can be drawn from mapping of des-ETW by rhir variants, it is reasonable to assume that most, if not all, effective interactions in the rhir-thrombin and rhir–des-ETW complexes are similar. Thus, a variation in $\Delta\Delta G_b^\circ$(IIa-ETW) observed with a particular mutation in rhir must reflect, in part, the loss of contacts normally formed between that particular rhir residue and thrombin.

All mutations in the C-terminal region of rhir caused a similar decrease in binding energy with des-ETW and thrombin; $\Delta\Delta G_b^\circ$(ETW) was comparable to $\Delta\Delta G_b^\circ$(IIa) in all cases. These results indicate that rhir makes the same interactions with anion-binding exosite 1 of des-ETW and thrombin, and it can be concluded that the des-ETW mutation has not affected the exosite's structure. In contrast, for a number of mutations in the N-terminal domain of rhir, $\Delta\Delta G_b^\circ$(ETW) was not equal to $\Delta\Delta G_b^\circ$(IIa). Several variations of $\Delta\Delta G_b^\circ$(IIa-ETW) can be interpreted in terms of steric hindrance and/or direct interaction losses resulting from the des-ETW mutation, but others implicate modifications in thrombin that are remote from the ETW motif. These nonlocal alterations apparently include Glu^{192} and the S_2 binding subsite, in addition to Ser^{195} and the primary binding pocket; all seem to radiate from the destroyed Arg^{221A}–Glu^{146} ion pair.

The largest effects observed involved substitutions of Thr$^{4'}$ which, in the crystal structure of rhir with thrombin, makes contacts of less than 4 Å with Glu^{146} and Glu^{192}. This latter residue is only 4 Å away from Trp^{148} of the ETW motif in the H-D-Phe-Pro-ArgCH$_2$-thrombin structure (Bode et al., 1989). Substitution of Thr$^{4'}$ for a tryptophan is much more detrimental for binding to thrombin than to des-ETW. The lack of an unfavorable interaction with Glu^{146} (deleted in the mutated thrombin) might explain the marked reduction in the effect of the Thr$^{4'}$→Trp mutation with des-ETW. However, a serine or alanine in position 4 of rhir would not be expected to make any unfavorable contacts with Glu^{146}, yet they both are more detrimental for binding to thrombin than to des-ETW. If one or both of the contacts between the β-methyl group of Thr$^{4'}$ and the carboxyl oxygens of either Glu^{146} and Glu^{192} are not formed in the rhir–des-ETW complex, the mutation of Thr$^{4'}$ to alanine would have a smaller effect, because the substitution would not be removing a contact that contributes to the stability

of the complex. While the des-ETW mutation obviously will remove contacts with Glu^{146}, it seems possible that the deletion has also affected the conformation of Glu^{192}.

A role of Glu^{192} in the P_2 specificity of thrombin was uncovered by the ability of the Glu^{192}→Gln thrombin mutant to activate bovine, but not human, coagulation factor X (Le Bonniec et al., 1992a). The P_2 specificity of des-ETW for *p*-nitroanilide substrates differs considerably from that of thrombin (Le Bonniec et al., 1992b). The first residue of rhir roughly occupies the binding site for the P_2 residues of thrombin substrates (Grütter et al., 1990; Rydel et al., 1990, 1991; Vitali et al., 1992; Bode et al., 1989, 1992; Stubbs et al., 1992; Martin et al., 1992). Thus, the effect observed with V1S would be consistent with the des-ETW mutation, causing an alteration in the S_2 subsite that could be due in part to a change in the conformation of Glu^{192}. It seems unlikely, however, that the change in the S_2 subsite is solely due to a modification in the conformation of Glu^{192}. An alteration in the structure of other residues forming this site (Tyr^{60A}, Trp^{60D}, Leu^{99}, and Ser^{214}–Gly^{216}) seems probable; each of these residues also contacts the first amino acid of rhir.

In the rhir–thrombin crystal structures, the α-amino group of the first residue in rhir forms hydrogen bonds with Ser^{195}, Ser^{214}, and possibly His^{57} (Rydel et al., 1990, 1991; Grütter et al., 1990; Vitali et al., 1992), and these bonds have been shown to make a considerable contribution to the stability of the complex (Wallace et al., 1989; Betz et al., 1992a,b). The rhir with a glycine extension ($G^{0'}$) would not be able to make the same hydrogen bonds. The fact that the $G^{0'}$ mutation is less detrimental for binding to des-ETW suggests that these bonds are less optimal in the rhir–des-ETW complex and, thus, that the conformation of Ser^{195} might be altered by the des-ETW mutation. This hypothesis is consistent with the observation that des-ETW is more slowly inactivated by diisopropyl phosphofluoridate than thrombin (Le Bonniec et al., 1992b). Alternatively, a change in the conformation of segment Ser^{214}–Gly^{216} would also lead to a less optimal hydrogen bond between the α-amino group of rhir and the carbonyl of Ser^{214}.

The second residue of rhir binds to the entrance of thrombin's primary specificity pocket (Rydel et al., 1990, 1991; Grütter et al., 1990; Vitali et al., 1992), and it has been proposed that an arginine in position 2 of rhir could interact favorably with the primary specificity pocket, although an interaction with Glu^{192} is also possible (Betz et al., 1992a). Thus, the effects of the $Val^{2'}$→Arg mutation, which improves the tightness of the complex even more with des-ETW than with thrombin, could be explained in terms of an altered interaction with either Glu^{192} or the primary specificity pocket. A modification in the structure of the primary specificity pocket would be consistent with the higher K_I for benzamidine of des-ETW compared to thrombin (Le Bonniec et al., 1992b). Disruption of the S_1 binding pocket caused by the deletion of Glu^{146} is conceivable due to the numerous intramolecular contacts that this residue makes with Arg^{221A} and Lys^{224}. The primary specificity pocket of thrombin is built by the junction of loop Cys^{182}–Ser^{195} and segment Val^{213}–Tyr^{228} (which includes Arg^{221A} and Lys^{224}). Any perturbation in this area of a serine protease results in dramatic modification of the P_1 specificity (Craik et al., 1985; Miyata et al., 1987; Spitzer et al., 1988; Ware et al., 1988; Geddes et al., 1989; Henriksen & Mann, 1989; Bajaj et al., 1990; Hedstrom et al., 1992). Thus, the absence of the Arg^{221A}–Glu^{146} ion pair in des-ETW could modify the conformation of residues within segment 214–224. An alteration in the structure of a number of these residues can, however, be excluded by the results obtained with the $Tyr^{3'}$→Ala and $Val^{21'}$→Ala substitutions, which had similar effects on the interactions with thrombin and des-ETW. In the rhir–thrombin structure, $Tyr^{3'}$ interacts with Glu^{97A} and every residue of segment Trp^{215}–Glu^{219}, while $Val^{21'}$ makes six contacts of less than 4 Å with Gly^{217} (Rydel et al., 1991). Thus, large alterations in the structure of residues within segment 215–219 can be excluded.

The effects of the mutations involving $Asp^{5'}$, $Ser^{19'}$, and $Leu^{15'}$ are probably also due to an alteration in the structure of Arg^{221A}, which they all neighbor in the rhir–thrombin complex. The $Asp^{5'}$→Glu substitution is less detrimental for the rhir–des-ETW interaction, whereas the replacement of $Asp^{5'}$ by Ala is more damaging. The result with D5E suggests that the $Asp^{5'}$–Arg^{221A} interaction is less optimal with des-ETW than with thrombin, yet it is conceivable that this interaction (and the $Glu^{5'}$–Arg^{221A} interaction in the D5E–des-ETW complex) partially substitutes for the Arg^{221A}–Glu^{146} ion pair missing in des-ETW. If this were the case, when $Asp^{5'}$ is replaced by Ala, the conformation of Arg^{221A} would be maintained neither by Glu^{146} (deleted in des-ETW) nor by $Ala^{5'}$ of the mutated rhir. Thus, an alanine in position 5 of rhir would leave the Arg^{221A} conformation un-repaired, and the effect of the rhir mutation could be more detrimental with des-ETW than that observed with thrombin.

$Ser^{19'}$ of rhir makes five contacts of less than 4 Å with Arg^{221A}. Again, the $Ser^{19'}$→Asp mutation may create an $Asp^{19'}$–Arg^{221A} link and, thus, partially replace the missing Glu^{146}–Arg^{221A} ion pair, whereas the $Ser^{19'}$→Ala substitution would remove any interaction with Arg^{221A}. Finally, in addition to Glu^{217} and Gly^{219}, $Leu^{15'}$ of rhir also interacts with Arg^{221A} of thrombin. The L15A rhir variant caused only partial competitive inhibition of des-ETW, indicating that the N-terminal core of the mutated rhir does not fully occlude the active site of the thrombin mutant. Thus, the result with L15A suggests that interaction between rhir $Leu^{15'}$ and Arg^{221A} of thrombin may be critical for active-site occlusion. The dramatic impact of the L15A mutation may, however, result from disruption of the numerous intramolecular contacts made by $Leu^{15'}$, in particular with residues 3–6 and 19–21. These two regions of the inhibitor contain precisely the residues that, when mutated, lead to major differences in the value of $\Delta\Delta G^{\circ}_{b}$(IIa-ETW). Therefore, the $Leu^{15'}$→Ala substitution could induce nonlocal conformational changes in rhir that are repaired in part upon complex formation with thrombin, but not with des-ETW, resulting in rhir L15A being a partial competitive inhibitor of the mutated thrombin.

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