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Temperature dependence of the thrombin-catalyzed proteolysis of prothrombin

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Abstract

Measurement of the temperature-dependence of thrombin-catalyzed cleavage of the $Arg_{155}-Ser_{156}$ and $Arg_{284}-Thr_{285}$ peptide bonds in prothrombin and prothrombin-derived substrates has yielded Arrhenius parameters that are far too large for classical mechanistic interpretation in terms of a simple hydrolytic reaction. Such a difference from the kinetic behavior exhibited in trypsin- and chymotrypsin-catalyzed proteolysis of peptide bonds is attributed to contributions by enzyme exosite interactions as well as enzyme conformational equilibria to the magnitudes of the experimentally determined Arrhenius parameters. Although the pre-exponential factor and the energy of activation deduced from the temperature-dependence of rate constants for proteolysis by thrombin cannot be accorded the usual mechanistic significance, their evaluation serves a valuable role by highlighting the existence of contributions other than those emanating from simple peptide hydrolysis to the kinetics of proteolysis by thrombin and presumably other enzymes of the blood coagulation system.

Keywords: Blood coagulation; Proteolysis; Thrombin; Prothrombin; Meizothrombin; Prethrombin 1; Temperature dependence; Conformation changes

1. Introduction

Under in vitro conditions thrombin catalyzes the cleavage of the $Arg_{155}-Ser_{156}$ peptide bond of prothrombin, but the extent of such scission in vivo is minimal [1]. Thrombin also catalyzes the in vitro proteolysis of $Arg_{284}-Thr_{285}$ peptide bond of prothrombin in prethrombin 1 [2,3], but accessibility of the same bond in prothrombin [4] to thrombin is not clearly established, although it is accessible to other proteinases. One purpose of the present investigation is to ascertain the susceptibility of the prothrombin peptide bond at Arg_{284} to thrombin-catalyzed cleavage in vitro by determining whether the cleavage of $Arg_{284}-Thr_{285}$ in prothrombin occurs by a sequential mechanism requiring prior proteolysis at Arg_{155} to generate prethrombin 1, or by a parallel mechanism

Abbreviations: F1, prothrombin fragment 1; $F2A_{1-13}$, prothrombin fragment 2 formed by thrombin action on prothrombin; gla, γ-carboxyglutamic acid.

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involving its scission in both prothrombin and prethrombin 1.

Another aim is to investigate the temperature dependence of thrombin-catalyzed cleavage of the Arg_{155}–Ser_{156} and Arg_{284}–Thr_{285} peptide bonds of human prothrombin. Variation of temperature clearly has the potential to alter bond accessibility. This is likely because temperature dependence of thrombin activity modulation results from prothrombin's activatory and inhibitory interactions with its exosites [5–10]. It is therefore, of interest to ascertain the manner in which the existence of these additional interactions impinge upon Arrhenius analysis of thrombin's kinetic behavior. This study of the proteolysis of three related protein substrates, prethrombin 1, prothrombin and meizothrombin, follows the example of the classical studies of the chymotrypsin-catalyzed hydrolysis of small peptides [11–13].

2. Experimental

2.1. Materials

Human prothrombin and α-thrombin were from batches used previously [9,14]. Enzyme concentrations were determined by active-site titration [15], whereas prothrombin concentrations were estimated spectrophotometrically at 280 nm [3]. Previous procedures [16,17] were used to prepare prethrombin 1, the concentration of which was based on the absorption coefficient reported therein. Ecarin-catalyzed cleavage of Arg_{320}–Ile_{321} in prothrombin [18] was again used to generate meizothrombin, to which the molar absorptivity of prothrombin was considered to apply.

2.2. Kinetic studies of prothrombin and prethrombin 1 hydrolysis

The kinetics of thrombin-catalyzed proteolysis of prothrombin and prethrombin 1 were followed by sampling reaction mixtures at appropriate times during incubations at 15–37 °C of enzyme–substrate mixtures in Hepes–chloride buffer (0.05 M Hepes/HCl—0.125 M NaCl). pH 7.4, I 0.15, supplemented with PEG6000 (1 mg/ml) to minimize protein losses by adsorption. The reaction was terminated by the addition of D-Phe–Pro–Arg–chloromethylketone. Proteolytic products were separated by HPLC on a MonoQ (HR 5/5) column (Pharmacia) at a flow rate of 1.0 ml/min [9]. Experimental data were fit by non-linear regression analysis to the appropriate equation by means of the program Scientist (Micromath Corp., Salt Lake City, UT).

2.3. Distinction between sequential and random pathways of thrombin-catalyzed cleavage of prothrombin

Proteolysis of prothrombin is usually considered to reflect the sequential mechanism in which Arg_{155}–Ser_{156} is cleaved to yield F1 and prethrombin 1 (i.e.

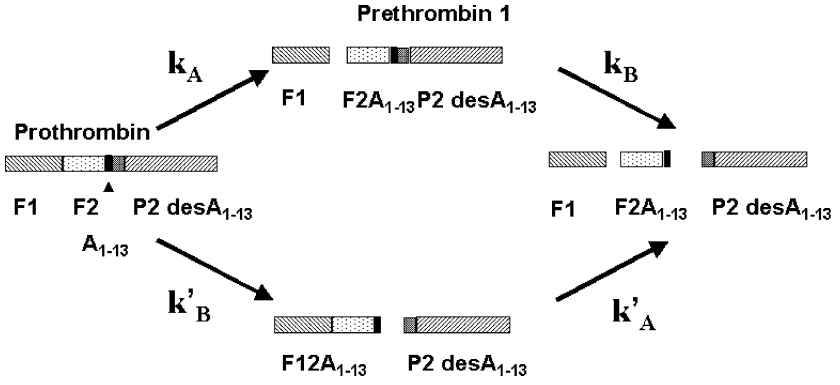

Fig. 1. Schematic representation of the possible mechanisms for cleavage of the two thrombin-susceptible peptide binds in prothrombin. The sequential mechanism (upper pathway) presumes initial cleavage at Arg_{155}–Ser_{156}, whereas the random pathway also allows for the possibility of initial cleavage at Arg_{284}–Thr_{285}. The designation of prothrombin fragments is also indicated.

$F2A_{1-13}P2$), after which $Arg_{284}-Thr_{285}$ in the latter product is hydrolysed to produce $F2A_{1-13}$ and P2 (upper pathway in Fig. 1). However, in view of the observation that $F1F2A_{1-13}$ is a product of prothrombin activation in plasma [4], the possibility of concurrent operation of the alternative pathway involving initial scission of $Arg_{284}-Thr_{285}$ (lower pathway in Fig. 1) must be considered. By analogy with the expressions used previously for the Factor Xa-catalyzed cleavage of prothrombin [18], the integrated rate equations for hydrolysis of prothrombin (initial concentration $[P]_o$) via the sequential pathway are

$$[P] = [P_o]\exp(-k_A t) \tag{1a}$$

$$[\mathrm{F1}] = [P_o]\{1-\exp(-k_A t)\} \tag{1b}$$

$$[\mathrm{P1}] = \{[P_o]k_A/(k_B-k_A)\}\{\exp(-k_A t) - \exp(-k_B t)\} \tag{1c}$$

$$\begin{aligned}[\mathrm{P2desA_{1-13}}] &= [\mathrm{F2A_{1-13}}] \\ &= [P_o]\{(1+k_A-k_B)/(k_A-k_B)\} \\ &\quad \times \{k_B\exp(-k_A t)-k_A\exp(-k_B t)\}\end{aligned} \tag{1d}$$

For random scission four rate constants are required to describe the proteolysis (Fig. 1). However, inability to detect $F1F2A_{1-13}$ experimentally signifies that the rate of hydrolysis of this putative product greatly exceeds its rate of formation ($k'_A k'_B$), whereupon the alternative pathway can only be described in terms of the reaction-limiting rate constant k_B'. The integrated rate equations describing the random mechanism then become

$$[P] = [P_o]\exp\{-(k_A + k_B')t\} \tag{2a}$$

$$[\mathrm{F1}] = [P_o]\exp\{1-\exp[-(k_A + k_B')t]\} \tag{2b}$$

$$\begin{aligned}[\mathrm{P1}] = \{[P_o]k_A/(k_B-k_A-k_B')\}\{&\exp[-(k_A + k_B')t] \\ &-\exp(-k_B t)\}\end{aligned} \tag{2c}$$

$$\begin{aligned}[\mathrm{P2desA_{1-13}}] &= [\mathrm{F2A_{1-13}}] \\ &= [P_o]\{[(1+k_A-\mathrm{k_B})/(k_A-k_B)] \\ &\quad \times [k_B\exp(-k_A t)-k_A\exp(-k_B t)] \\ &\quad + [k_B'/(k_A+k_B')] \\ &\quad \times [1-\exp\{-(k_A+k_B')t\}]\}\end{aligned} \tag{2d}$$

3. Results

3.1. Accessibility of the $Arg_{284}-Thr_{285}$ bond of prothrombin

Progress curves for the proteolysis of prothrombin (3 μM) by α-thrombin (0.2 μM) at 15 °C (pH 7.4, I 0.15) in the presence of 10 mM Ca^{2+} are presented in the two panels in the upper section of Fig. 2. The left-hand panel shows best-fit descriptions of time-courses for substrate depletion and product formation for the random model involving scission of $Arg_{284}-Thr_{285}$ in prothrombin as well as in prethrombin 1 [Eqs. (2a), (2b), (2c) and (2d)]. The right-hand panel illustrates the corresponding best-fit description in terms of the sequential mechanism, [Eqs. (1a), (1b), (1c) and (1d)], in which cleavage of $Arg_{284}-Thr_{285}$ is restricted to thrombin-catalyzed proteolysis of prethrombin 1. Several points are noted. (i) The decline in prothrombin concentration (●) as the result of $Arg_{155}-Ser_{156}$ scission is accompanied by increases in the concentrations of the products, F1 (○) and prethrombin 1 (□). The concentration of prothrombin 1 then declines due to cleavage of the $Arg_{284}-Thr_{285}$ bond to form equal amounts of two additional fragments ($F2A_{1-13}$ and $P2desA_{1-13}$). (ii) Discrimination between the random and sequential models needs to be based on the relative merits of the two predicted time-courses for the generation of these two products (▲, △)—a decision that can only be made during the early stage when prothrombin is still present. (iii) Comparison of the theoretical plots reveals that the random model provides the better description of the results for $F2A_{1-13}$ and $P2desA_{1-13}$ formation. (iv) This conclusion also applies to results for hydrolysis of prothrombin by thrombin at 37 °C (lower panels of Fig. 2). We therefore conclude that the $Arg_{284}-Thr_{285}$ peptide bond is susceptible

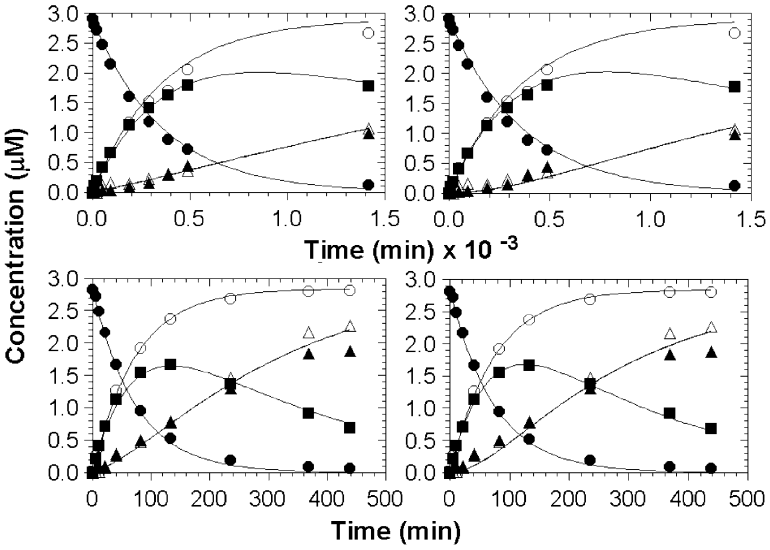

Fig. 2. Progress curves for reactant and product concentrations in the thrombin-catalyzed hydrolysis of prothrombin (3 μM) in Hepes–chloride buffer (pH 7.4, I 0.15) at 15 and 37 °C (upper and lower panels respectively): , prothrombin (P); ○, Fragment 1 (F1); □, prethrombin 1; ▲, $F2A_{1-13}$; △, $P2desA_{1-13}$. Left-hand panels contain the best-fit descriptions in terms of random hydrolysis of the two susceptible peptide bonds [Eq. (2a) Eqs. (2b), (2c) and (2d)], whereas the right-hand panels refer to the corresponding descriptions in terms of sequential cleavage [Eqs. (1a), (1b), (1c) and (1d)].

to thrombin proteolysis in prothrombin as well as in prethrombin 1. Although an addition bond can be cleaved in F1, no consistent evidence was observed to indicate that this was occurring during these experiments.

The results shown in Fig. 2 have substantiated interpretation of the hydrolysis of prothrombin by thrombin according to the random model, and hence analysis in terms of three rate constants, k_A, k_B, and k'_B. However, the amount of information available for evaluating the magnitude of the rate constant for cleavage of $Arg_{284}–Thr_{285}$ in prothrombin (k'_B) is very limited because the proportion of $F2A_{1-13}$ and $P2desA_{1-13}$ produced from prothrombin (rather than prethrombin 1) clearly diminishes with the rapidly decreasing concentration of the substrate (prothrombin). Because global fitting in terms of Eqs. (2a), (2b), (2c) and (2d) may therefore fail to provide a sufficiently accurate value of k'_B, and consequently of k_B, the values reported in Table 1 have been obtained by constraining k_B to the value obtained for prethrombin at the corresponding temperature. (See Section 3.3 for the data that validate this constraint).

3.2. Temperature-dependence of $Arg_{284}–Th_{285}$ hydrolysis in prethrombin 1

The temperature-dependence of $k_B=k_c/K_m$ for the thrombin-catalyzed hydrolysis of the $Arg_{284}–Thr_{285}$ bond in prethrombin 1 is summarized in Table 1. It is evident that the increase in this pseudo-first-order rate constant over a 10° temperature range exceeds the two-fold increase expected with reactions for simple kinetic systems. The results (Fig. 3) are, however, consistent with a simple kinetic reaction in that they may be described by the Arrhenius relationship,

$$k = \mathrm{A}\exp(-E_a/RT) \quad (3)$$

in the presence (■) or absence (□) of Ca^{2+}. Magnitudes of the two Arrhenius parameters obtained by non-linear curve-fitting of the untransformed data to Eq. (3) are summarized in the top line of Table 2.

It should be noted that the estimates of A are several orders of magnitude greater than the maximal value of 4.5×10^{12} M^{-1} s^{-1} (kT/h, where k and h are the Boltzmann and Planck constants, respectively) predicted at 25 °C on the basis of reaction rate theory

Table 1
Temperature dependence of pseudo-first-order rate constants (k_c/K_m) for thrombin-catalyzed peptide bond hydrolysis in prothrombin-derived protein substrates (pH 7.4, I 0.15)

Substrate	k_c/K_m (M^{-1} s^{-1}) 10 mM Ca^{2+}			k_c/K_m ($M^{-1}s^{-1}$) 1 mM EDTA		
	15 °C	25 °C	37 °C	15 °C	25 °C	
Cleavage of Arg284–Thr285						
Prethrombin	28 (±1)[a,b]	88 (±3)	320 (± 93)	20 (±1)	81 (±3)	385 (±17)
Prothrombin (k_B)	46 (±11)	120 (±10)	352 (± 20)	11 (±8)	52 (±5)	285 (±9)
Prothrombin (k_B)[c]				24	73	355
Prothrombin (k'_B)	9.1 (±3.6)	28 (±11)	98 (±16)	–	–	–
Meizothrombin (k_B)	75 (±2)	214 (±8)	686 (± 34)	35 (±1)	132 (±5)	579 (±40)
Meizothrombin (k_B)[c]				81	186	721
Cleavage of Arg_{155}–Ser_{156}						
Prothrombin	286	550 (±15)	1140 (±23)	11 600 (±500)	34 900 (±1 300)	119 000 (±4 000)
Prothrombin[c]				24 700	49 400	148 000
Meizothrombin	10 500 (±500)	16 100 (±40)	25 800 (±900)	33 500 (±28 000)	164 000 (±37 000)	964 000 (±186 000)
Meizothrombin[c]				70 900	232 000	1 200 000

[a] Values obtained on the basis of the best-fit relationship obtained by non-linear regression analysis of results in terms of Eq. 3.
[b] Numbers in parentheses denote the standard deviation of the estimate.
[c] Values corrected for non-competitive inhibition by the Fragment 1 domain.

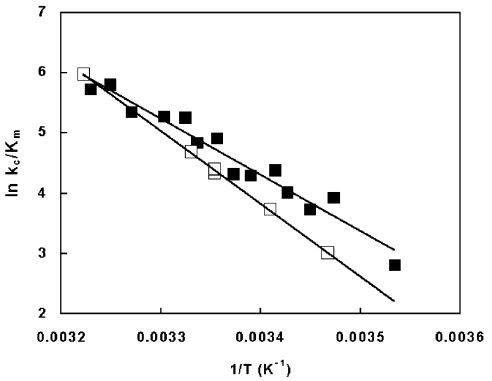

Fig. 3. Arrhenius plots of the temperature-dependence of the pseudo-first-order rate constant (k_{cat}/K_m) for scission of the Arg_{284}–Thr_{285} bond in prethrombin 1 by thrombin in the presence (■) and absence (□) of Ca^{2+} ion. Solid lines correspond to the best-fit descriptions obtained by non-linear regression analysis of the individual data sets in terms of Eq. (3), whereas the broken line reflects the corresponding analysis of the combined data set.

for the kinetics of a simple first-order reaction. Although values of A greater than kT/h can, in principle, be rationalized on the basis of the contribution from a positive entropy of activation [19], a more realistic estimate of the pre-exponential parameter is in the range 7×10^9 M^{-1} s^{-1} for uncharged molecules to 10^{11} M^{-1} s^{-1} in the event of favorable electrostatic interaction [20]. Potential sources of the large deviation from reaction rate predictions clearly need to be examined.

Although the present reactions are pseudo-first order in that k_c/K_m varies linearly with substrate concentration, this ratio of kinetic parameters is described by the expression

$$k_c/K_m = k_1[(k_2/k_{-1})/\{1 + (k_2/k_{-1})\}] \quad (4)$$

where the subscripts 1 and 2 designate respective rate constants for reversible formation of the enzyme–substrate complex and the subsequent acyl–enzyme intermediate [21–23]. The physically unrealistic magnitudes of the pre-exponential terms in Arrhenius descriptions of the present data may thus reflect, at least in part, the necessity to regard k_c/K_m as a composite of rate constants (each with its unique temperature-dependence) rather than as the single rate constant to which the Arrhenius equation applies.

An additional source of deviation from classical Arrhenius behavior stems for the temperature-dependence of k_c/K_m from the protein nature of the two reactants. Any temperature-dependence of pre-existing [24–27] or substrate-induced [5–10]] equilibria affecting the enzyme and/or substrate must also be manifested in the magnitudes of parameters derived from the Arrhenius relationship. In this case, substrate-induced changes occur via thrombin exosite interactions. Fig. 4 illustrates the effect of temperature on the non-competitive inhibition by F1 of the thrombin-catalyzed hydrolysis of the Arg_{284}–Thr_{285} bond of prethrombin 1 in Hepes-chloride buffer (pH 7.4, I

Table 2
Apparent Arrhenius parameters for thrombin-catalyzed peptide bond hydrolysis in prothrombin-derived protein substrates (pH 7.4. I 0.15)

Substrate	Parameters in 10 mM $CaCl_2$		Parameters in 1 mM EDTA	
	A (M^{-1} s^{-1})[a]	E_a (kcal/mol)[b]	A (M^{-1} s^{-1})[a]	E_a (kcal/mol)[b]
Cleavage of Arg_{284}–Thr_{285}				
Prethrombin 1	2×10^{16}	20 (±1)	3×10^{19}	24 (±1)
Prothrombin (k_B)	1×10^{14}	16 (±1)	7×10^{20}	26 (±1)
Prothrombin (k_B)[c]			2×10^{19}	24 (±1)
Prothrombin (k'_B)	3×10^{15}	19 (±4)	–	–
Meizothrombin (k_B)	2×10^{15}	18 (±1)	5×10^{18}	23 (±2)
Meizothrombin (k_B)[c]			6×10^{16}	20 (±2)
Cleavage of Arg_{155}–Ser_{156}				
Prothrombin	8×10^{10}	11 (±1)	2×10^{18}	19 (±1)
Prothrombin[c]			2×10^{16}	16 (±1)
Meizothrombin	3×10^{9}	7 (±1)	1×10^{25}	27 (±1)
Meizothrombin[c]			3×10^{23}	25 (±1)

[a] Only the order of magnitude is given.
[b] Numbers in parentheses denote the standard deviation.
[c] Values corrected for non-competitive inhibition by the Fragment 1 domain.

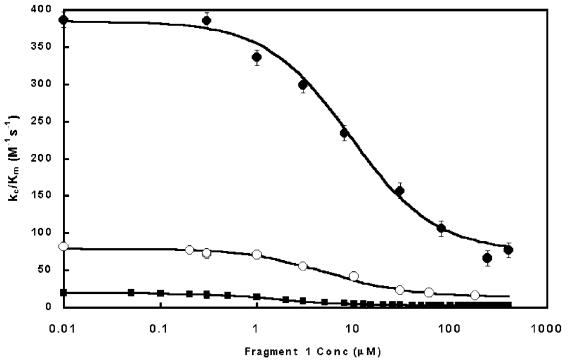

Fig. 4. Effect of temperature on the non-competitive inhibition by F1 of the thrombin-catalyzed hydrolysis of prethrombin 1 (Arg_{284}–Thr_{285}) in Hepes–chloride buffer (pH 7.4, I 0.15) supplemented with 1 mM EDTA: ●, 37 °C; ○, 25 °C; ■, 15 °C. The lines, which correspond to best-fit descriptions of the individual data sets in terms of Eq. (2) of [9], signify inhibition constants (K_I) of 9.2, 5.4 and 1.8 μM in order of decreasing temperature.

0.5) supplemented with 1 mM EDTA. These results, analyzed in terms of Eq. (2) of [9], produce inhibition constants (K_I) of 9.2, 5.4 and 1.8 μM at 37, 25 and 15 °C. Inasmuch as F1 is part of the substrate (prothrombin), this temperature dependence of K_I would be incorporated into that of k_c/K_m if it were not for the absence of inhibition in the presence of Ca^{2+} [9]. Nevertheless, Fig. 4 serves to illustrate the potential for additional contributions to the temperature-dependence of k_c/K_m from exosite interactions.

The consequences of temperature-dependent transitions within the enzyme [22–29] should be common to Arrhenius plots for the protein substrates investigated here, because they compare the same peptide sequences (those surrounding either Arg $_{284}$ or Arg_{155}) in all substrates. Differences between the apparent A and E_a values for different substrates under the same conditions should stem from temperature effects on enzyme–substrate interactions additional to those involving the active site. Such substrate–exosite equilibria include interactions of the $F2A_{1-13}$ region of substrates with (i) the putative heparin-binding site and (ii) the fibrinopeptide-binding site of thrombin [9].

Substrate exosite equilibria must also be considered, although an intramolecular interaction between the $F2A_{1-13}$ domains present in prethrombin 1 and thrombin is seems unlikely for two reasons. First, interaction between $F2A_{1-13}$ and thrombin appears to involve the same residues as those involved in the interaction between $F2A_{1-13}$ and prethrombin 2 [30]. Secondly, the effect of isolated $F2A_{1-13}$ on the thrombin-catalyzed hydrolysis of prethrombin 1 is independent of Ca^{2+} ion (Fig. 4 of [9]). Fig. 3 indicates a real, but relative small effect of Ca^{2+} on the temperature dependence of rate constants for proteolysis of Arg $_{284}$–Thr_{285} in prethrombin 1 (Table 1). Such a small difference is not surprising in view of the weakness of interaction between Ca^{2+} and either thrombin or prethrombin 1 [31].

3.3. Temperature dependence of prothrombin proteolysis by thrombin

A switch to prothrombin as a substrate introduces the need to consider temperature effects on the proteolysis of Arg $_{155}$–Ser $_{156}$ as well as on Arg_{284}–Thr_{285}.

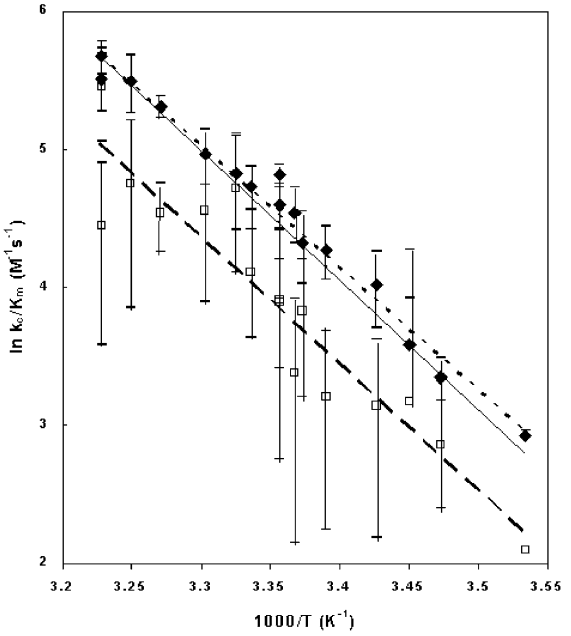

Fig. 5. Arrhenius plot of results for thrombin-catalyzed cleavage of the Arg_{284}–Thr_{285} bond of prothrombin in Hepes–chloride buffer (pH 7.8, I 0.15) supplemented with 10 mM $CaCl_2$: ◆, values of k_B for hydrolysis of prethrombin 1 generated by the serial pathway; □, values of k_B', the rate constant for hydrolysis of this bond in intact prothrombin. ——, Temperature-dependence of k_B deduced for prethrombin 1 from Fig. 3: ····, best-fit description of the present data for k_B: – – –, best-fit description of the temperature-dependence of k_B' in terms of Eq. (3).

The previously demonstrated effects of the F1 and F2A(1–13) domains must also be taken into account. In the presence of Ca^{2+} the rate constants for the scission of Arg_{284}–Thr_{285} in the generated prethrombin (♦) are indistinguishable from those observed in prethrombin 1 alone (Fig. 5) inasmuch as the experimental values of k_B are well described by the dotted line, which is taken from Fig. 3 for the temperature dependence of scission of this bond in isolated prethrombin 1: indeed, this dependence is indistinguishable from the best-fit description of the present data (——). Understandably, the results obtained for k'_B, the rate constant for scission of Arg_{284}–Thr_{285} in prothrombin, by global fitting of all three rate constants exhibit greater scatter than their k_B counterparts. The precision of their estimation was therefore improved by refitting all data with k_B fixed at the corresponding value for prethrombin 1 (□). From Table 1 the rate of Arg_{284}–Thr_{285} cleavage in prothrombin (k'_B) seems to be approximately three-fold less than in prethrombin 1 (k_B), but the activation energy deduced from the temperature dependence of k'_B is essentially identical (Table 2).

Substitution of EDTA for Ca^{2+} in reaction mixtures had a dramatic effect on the temperature-dependence of k_B (Fig. 6a). No information on the magnitude of k'_B could be deduced from experiments in the absence of Ca^{2+} because the rapidity with which the prothrombin concentration decreased to zero meant that the reaction essentially followed the serial pathway. Although a significant effect of Ca^{2+} was certainly observed for thrombin-catalyzed scission of Arg_{284}–Thr_{285} in prethrombin 1 (Fig. 3), the consequences of Ca^{2+}-binding are much greater for prothrombin. This difference presumably reflects to some extent the elimination of non-competitive inhibition at the fibrinopeptide exosite by the F1 present either as a domain of prothrombin or as the released product (Fig. 4). Only in the absence of Ca^{2+} is F1 an inhibitor of the thrombin-catalyzed cleavage of Arg_{284}–Thr_{285}

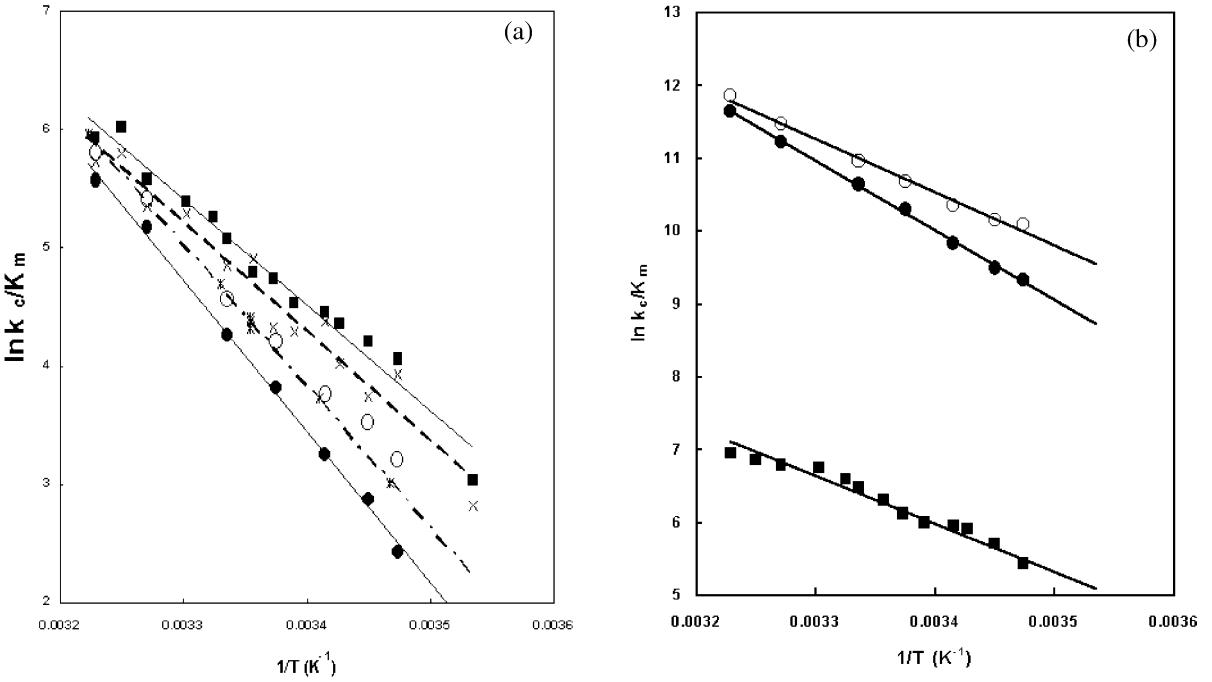

Fig. 6. Effects of Ca^{2+} on the temperature dependence of pseudo-first-order rate constants for prothrombin proteolysis by thrombin. (a) Arrhenius plots of rate constants (k_B) for the scission of Arg_{284}–Thr_{285} in the presence (■) and absence (●) of the metal ion, the former data being taken from Fig. 5 (b) Corresponding comparison of the effect of Ca^{2+} on the rate constant for scission of Arg_{155}–Ser156 (k_A). Open symbols refer to values of k_B and k_A that have been corrected for effects of F1 inhibition by means of Eq. (5). Broken lines and crosses in (a) are the corresponding results for prethrombin 1 (taken from Fig. 3).

in prethrombin 1 [9]. However, an effect of the F1 domain cannot be completely excluded. For the substrates used in the present investigation, this consideration only applies to prothrombin and meizothrombin proteolysis by thrombin.

In order to gain some insight into the extent to which allowance for the effects of such inhibition might affect the form of the Arrhenius plot, the value of (k_c/K_m) obtained in the absence of Ca^{2+} (plus EDTA) at each temperature has been corrected for effects of non-competitive inhibition via the expression [9]

$$k_c/K_m = [\{(k_c/K_m)_E + (k_c/K_m)_{EI}[I]/K_I\}[E_t]] /(1 + [I]/K_I) \quad (5)$$

where $(k_c/K_m)_E$ and $(k_c/K_m)_{EI}$ are the respective rate constants for native and inhibited enzyme, and where $[I]$ and $[E_t]$ denote the concentrations of inhibitor and total enzyme, respectively. Values of K_I at the various temperatures were presented in the caption to Fig. 4. Although the inherent assumption that Fragment 1 is a quantitative mimic of this portion of prothrombin from the viewpoint of thrombin inhibition in the absence of $Ca^{2+}$is open to question, it is noted (open symbols in Fig. 6a) that this allowance for effects of F1 inhibition nearly eliminates the difference observed in the rate constants for the Ca^{2+} effect on hydrolysis of prothrombin at Arg_{284}. Indeed, there is now correspondence between the temperature dependences of Arg_{284}–Thr_{285} scission in prothrombin and prethrombin 1 in the presence and absence of Ca^{2+}—a point illustrated by inclusion of the data (×) and best-fit descriptions (– – –; –·–·–) for prethrombin 1 cleavage presented in Fig. 3.

Attention is now directed to proteolysis of the other thrombin-susceptible peptide bond, Arg_{155}–Ser_{156}, in prothrombin. Fig. 6b presents the Arrhenius plots for the thrombin-catalyzed scission of Arg_{155}–Ser_{156}, in the presence (■) and absence (●) of Ca^{2+} ion: open symbols denote the latter results after allowance for non-competitive inhibition by the F1 domain of prothrombin (as in Fig. 6a). For this cleavage the inclusion of the metal ion also gives rise to a decreased value for the apparent energy of activation (Table 2). Of particular interest, however, is that the results obtained in the presence of Ca^{2+} provide the first unequivocal evidence of a systematic departure from description in terms of a single exponential in reciprocal time. The pronounced curvilinearity of this Arrhenius plot for scission of the Arg_{155}–Ser_{156} bond in the presence of metal ion (■) strengthens the earlier inference that the physically unrealistic magnitudes returned for E_a and A may signify the concomitant operation of separate temperature-dependences for the different steps that occur during enzyme–substrate interactions, e.g. binding, acylation and deacylation [Eq. (4)]. Indeed, such curvilinearity has been interpreted quantitatively in terms of the energy-of-activation equivalent of a thermodynamic heat capacity effect (the analog of ΔC_p) for the thrombin-catalyzed hydrolysis of fibrinogen—a phenomenon attributed to temperature-dependence of thrombin interconversion between slow and fast isomeric forms [27]. Comparable studies of the energetics of the thrombin–hirudin [26] and thrombin–thrombomodulin [29] interactions have also been reported. An additional observation of relevance is the recent demonstration of a substrate-induced isomerization of thrombin to an expanded [or more asymmetric] transition state prior to the irreversible steps that lead to substrate hydrolysis [32].

In the above experiments the deviation from classical Arrhenius behavior can reflect the consequences of conformational changes within the substrate as well as within the enzyme. We therefore attempt to identify the extent of contributions from the latter source by re-examining published data for the thrombin-catalyzed hydrolysis of chromogenic substrates [22,23,33].

3.4. Temperature-dependence of the thrombin-catalyzed hydrolysis of chromogenic substrates

There are two published studies of the temperature-dependence of chromogenic substrate hydrolysis by thrombin that allow evaluation of the Arrhenius parameters [22,33]. For H-D-Phe–L-Pip–L-Arg–*p*NA (S2238) hydrolysis at pH 7.8 the temperature dependence of k_c reported by Lottenberg et al. [33] yields respective values of 2×10^{10} M^{-1} s^{-1} and 11 kcal/mol for A and E_a, an essentially identical value of the latter parameter having been reported by Di Cera

and coworkers [22]. On the other hand, the corresponding considerations of k_c/K_m values [33] yield a pre-exponential factor of 3×10^9 M^{-1} s^{-1} and an apparent activation energy of 2 kcal/mol. From an alternative set of published results for k_c/K_m [22] the respective values were 2×10^{11} M^{-1} s^{-1} and 5 kcal/mol. Although the parameter estimates vary considerably, the temperature-dependence of kinetic parameters is clearly described by much smaller values of A and E_a than those reported in Table 2 for prothrombin-derived substrates. Furthermore, the pre-exponential terms are now smaller than kT/h but still larger than the k_c/K_m values of 10^8 M^{-1} s^{-1} that are obtained for the best p-nitroanilide substrates [33]. These values for E_a and A are more in keeping with reaction rate theory [20].

Results for Tos–Gly–Pro–Arg–*p*NA (Chromozym TH) yield higher values of the pre-exponential factor, 2×10^{12} M^{-1} s^{-1} [30] and 6×10^{11} M^{-1} s^{-1} [33], which signify the probable existence of additional substrate-induced contributions to the energetics. Indeed, the results reported by Stone et al. [22] exhibit not only curvature but also a maximum in the Arrhenius plot—an unequivocal indication of energetic contributions additional to those involved in simple bond hydrolysis. The desirability of extending the experimental temperature range beyond 37 °C is emphasized by studies of the thrombin–fibrinogen interaction, the Arrhenius plot for which also exhibited a maximum at approximately 30 °C [27]. Such behavior is now expected to be the norm rather than the exception—particularly with thrombin as the enzyme. A major contributing factor to such behavior of Arrhenius plots for thrombin-catalyzed hydrolysis of chromogenic substrates is undoubtedly the sodium- and temperature-dependent isomerization equilibrium between the slow and fast conformer states of thrombin [23–29]; the isomerization to an expanded transition state [32] is also a potential contributor.

3.5. Comparison of the thrombin-catalyzed proteolysis of meizothrombin and prothrombin

Separation of the effects of temperature, F1, and Ca^{2+} on the enzyme, as described above, from their effects on the protein substrates has been facilitated by monitoring cleavage of the two peptide bonds, Arg_{284} and Arg_{155}, in three prothrombin-derived substrates, prethrombin 1, prothrombin and meizothrombin. Meizothrombin, the sole proteolytic product arising from hydrolysis of Arg_{320}–Ile_{321} in prothrombin, is much less structured than the parent molecule. Whereas the two halves of meizothrombin are held together by a single disulfide bond, the structure of the intact prothrombin molecule is constrained not only by that disulfide bond but also by the restrictions imposed by the presence of the Arg_{320}–Ile_{321} peptide bond as part of the polypeptide chain. The fact that these two thrombin substrates have the same structural domains (F1 and F2) and same molecular mass affords an opportunity to assess the effect of greater structural flexibility on the temperature dependence of proteolysis by thrombin. Because of its enzymatic nature, the active site of meizothrombin has been inactivated by reaction with D-Phe–Pro–Arg–chloromethylketone to prevent substrate autolysis.

In the presence of Ca^{2+}, the thrombin-catalyzed hydrolysis of Arg_{284}–Thr_{285} in meizothrombin is governed by a rate constant (k_B) which is approximately two-fold greater than that for the hydrolysis of the same bond in either prothrombin or prethrombin 1 (Table 1). Consequently, the values of apparent activation energies and the pre-exponential factors obtained by classical Arrhenius analysis are very similar (Table 2). On the other hand, the greater flexibility of meizothrombin is reflected in rate constants (k_A) for Arg_{155}–Ser_{156} hydrolysis that are twenty to forty times larger than those pertaining to prothrombin (Table 1). This is manifested in a decrease in E_a, (11 kcal/mol to 7 kcal/mol), and a decrease in the pre-exponential factor from 8×10^{10} M^{-1} s^{-1} for Arg_{155}–Ser_{156} cleavage in prothrombin to 3×10^9 M^{-1} s^{-1}—a value comparable with those calculated for chromogenic substrates.

The removal of Ca^{2+} leads to enormous changes in the energetics parameters obtained by classical Arrhenius analysis (Table 2). As noted in Section 3.3, rate constants for bond scission in the absence of Ca^{2+} seemingly reflect non-competitive inhibition arising from enzyme exosite interaction with the F1 portion of prothrombin (Figs. 4 and 6). Application of the same allowance for this inhibition of meizothrombin brings the energetics of Arg_{284}–Thr_{285} cleavage into line with description that is common to scission of this bond in prothrombin and prethrombin 1. It would therefore seem that inhibition by the F1 portions of

prothrombin and meizothrombin is largely responsible for the observed differences in energetics between thrombin-catalyzed scission of the $Arg_{284}-Thr_{285}$ bond in prothrombin-derived substrates. In other words, the surface of the portion of prothrombin containing the $Arg_{284}-Thr_{285}$ bond is relatively unaltered by the consequences of the proteolytic events that give rise to meizothrombin and prethrombin 1.

Similar considerations of the results for cleavage of the $Arg_{155}-Ser_{156}$ bond suggest that inhibition by F1 also affects the magnitudes of activation energies and pre-exponential factors, but does not alter the conclusion that the structuring of the Gla domain by Ca^{2+} has markedly different effects on the apparent Arrhenius parameters for this scissile bond in meizothrombin and prothrombin, the consequences being even more profound when the substrate is the more loosely structured meizothrombin.

The dramatic effects of Ca^{2+} are most simply understood in terms of probable differences in the accessibility of peptide bonds as the result of expansion and contraction of the F1 portion of the substrate. The larger rate constants (k_A and k_B) for meizothrombin presumably reflect greater access to either scissile bond in this looser assembly of constituent segments compared with the situation in prothrombin. The effect is greater for $Arg_{155}-Ser_{156}$ bond scission because of its closer proximity to the F1 region; and more noticeable in the absence of Ca^{2+} because of the expanded, disordered and highly charged state of the Gla domain [34,35].

4. Discussion

The reaction scheme for proteolysis by the classical serum proteinases (trypsin and chymotrypsin) is also the basis of thrombin catalysis, which is, however, under the control of additional regulatory mechanisms—interactions involving exosites that are distinct from the catalytic site [5–10,23–29,36,37].

In spite of obvious limitations of the theory for such complex systems, the utility of investigating the temperature-dependence of enzyme-catalyzed processes has been shown by studies of the effects of the equilibrium coexistence of two thrombin conformers on the enzyme's interactions with fibrinogen and thrombomodulin [24–29]. Results of the present investigation of thrombin-catalyzed proteolysis of two different peptide bonds in prothrombin and protein substrates derived from them have confirmed the value of such studies; and have extended their scope to include modulation by domains within the protein substrate. The first clear indicator of interaction additional to the classical enzyme–substrate interaction is an elevated magnitude of the Arrhenius pre-exponential factor, A. Values of the apparent activation energy also tend to be high; and for some systems there is demonstrable deviation from the linear dependence of $\ln k$ upon $1/T$ that is predicted by the Arrhenius equation.

It is evident from the data in Table 1 that the environments of the scissile bond and the gross conformation of the substrate must differ dramatically in the presence and absence of Ca^{2+}, and also with the relaxation of constraints that occurs when $Arg_{320}-Ile_{321}$ is cleaved in prothrombin to generate meizothrombin. Whereas the ratio of rate constants for $Arg_{284}-Thr_{285}$ cleavage increases two- to three-fold in the presence or Ca^{2+}, the corresponding increase for the scission of $Arg_{155}-Ser_{156}$ is up to 40-fold. Variations in exosite interactions are clearly having a pronounced effect on the kinetics of hydrolysis at $Arg_{155}-Ser_{156}$. On the other hand, the similarity of Arrhenius parameters for scission of the $Arg_{284}-Thr_{285}$ bond, for which the similarity of Arrhenius parameters in the presence and absence of Ca^{2+} implies relative insensitivity of the structural topography in this region of prothrombin either to the presence of metal ion or to the peptide scissions leading to the generation of prethrombin 1 and meizothrombin.

In summary, this investigation has established that the mechanism for thrombin-catalyzed hydrolysis of prothrombin and prothrombin-derived substrates is more complicated than that for proteolysis by trypsin and chymotrypsin. Whereas Arrhenius parameters for the latter serine proteinases are seemingly amenable to classical interpretation, the magnitudes of the parameters emanating from temperature–dependence studies of thrombin-catalyzed hydrolysis of peptide bonds are in general far too high for consideration as pre-exponential factors and energies of activation for simple chemical reactions. By pointing out this inability to ascribe the usual mechanistic significance to the magnitudes deduced for A and E_a it is not our

intent to deter temperature-dependence studies of the kinetics of hydrolysis catalyzed by blood coagulation proteinases. To the contrary, the present investigation has highlighted the value of such studies as a potential means of identifying the existence of contributions from enzyme exosite interactions as well as from enzyme conformational rearrangements to the kinetics of proteolysis by thrombin and other enzymes of the blood coagulation system.

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