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Rates of Thrombin Acylation and Deacylation Upon Reaction with Low Molecular Weight Acylating Agents, Carbamylating Agents and Carbonylating Agents

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Abstract—Acylated derivatives of thrombin have been made using low molecular weight acylating agents, carbamylating agents and carbonylating agents. The compounds used to acylate the active site serine include isatoic anhydrides, benzoxazinones, benzylisocyanate, N-(benzylcarbonyloxy)succinimide and *p*-(dimethylamino)benzoylimidazolide. The rates of acylation and deacylation were determined. The best overall inhibitors of thrombin are 2-ethoxy-4*H*-3,1-benzoxazin-4-one, isatoic anhydride and *tert*-butyl-2,4-dioxo-2*H*-3,1-benzoxazine-1(4*H*)-acetate, which have k_2/K_i values of 52,700 $M^{-1}s^{-1}$, 48,900 $M^{-1}s^{-1}$ and 5400 $M^{-1}s^{-1}$, respectively. The carbamyl derivative of thrombin formed with benzylisocyanate had the slowest rate of deacylation (2.3×10^{-7} s^{-1}), while the ester derivative formed with 2-(*N*,*N*-dimethylamino)methylimino-4*H*-3,1-benzoxazin-4-one had the fastest rate of deacylation (1.9×10^{-4} s^{-1}).

Introduction

Thrombin is an enzyme that functions in the final step of the blood coagulation cascade to cleave fibrinogen to fibrin which aggregates to form the fibrin clot. It also stimulates platelet secretion and aggregation in blood and activates factors V, VIII, XIII, protein C and protein S.[1,2] Like the other enzymes of the blood coagulation cascade and the complement system, thrombin is a serine protease that exhibits trypsin-like specificity, however, it is more specific and has a preference for Arg–X bonds.[3,4] Since thrombin plays a pivotal role in blood coagulation, it is a well-recognized target for the control of thrombosis. One approach to the control of thrombosis is to prevent propagation of the fibrin clot in various stages of its formation. This could be accomplished with an inactivated acyl thrombin that is resistant to its natural plasma inhibitors such as antithrombin III, and could interact with other factors in the blood coagulation cascade preventing their binding to active thrombin.

The active site serine of serine proteases is acylated by various acyl esters, amides, azapeptides, isocyanates, and heterocyclic compounds.[5,6] Thrombin can be acylated by several esters of guanidino and amidinobenzoic acids.[5,7] The most recognized of these compounds is 4-nitrophenyl-4'-guanidinobenzoate (NPGB), which rapidly acylates thrombin to release stoichiometric amounts of nitrophenol after formation of a stable acyl enzyme, and is thus used as an active site titrant.[8] Other examples of compounds that acylate thrombin include *p*-guanidinobenzoic acid esters of fluorescein[9] and *p*-amidinophenyl esters.[10]

Acylated thrombins can also be generated using heterocyclic acylating compounds. Several classes of such compounds have been reported as serine protease inhibitors, including isatoic anhydrides,[11,12] 3-alkoxy-4-chloroisocoumarins,[4,13] substituted benzoxazin-4-ones,[14] and 5-acyloxyoxazoles.[15] These compounds react with Ser-195 of serine proteases to form acyl enzyme adducts that deacylate at varying rates, depending on the stuctures of the acyl enzymes. A variety of heterocyclic structures incorporating masked reactive functional groups can be utilized, however, it is difficult to obtain specificity. Mechanism-based isocoumarin inhibitors specific for thrombin and other coagulation enzymes have been reported, including some which form stable acyl enzyme derivatives.[4,13] Another heterocyclic acylating inhibitor specifically designed for trypsin-like serine proteases is 7-(aminomethyl)-1-benzylisatoic anhydride.[11] This compound is not a suitable anticoagulant, however, because it is rapidly degraded in serum, and has a half-life of less than one minute. It is possible however, to acylate thrombin with other heterocyclic compounds that are more stable and have longer half-lives.

Acyl enzyme derivatives of other plasma serine proteases have been used therapeutically. Previous investigators have reported the use of acylated derivatives of plasmin and streptokinase–plasmin(ogen) complexes to accomplish fibrinolysis.[16–18] These acylated enzymes are catalytically inert and unreactive toward plasmin inhibitors, but are capable of binding fibrin since the fibrin binding site is separate from the enzyme active site. After *in vivo* deacylation, the enzymes are active and capable of fibrinolysis. The advantages of using acyl-plasmins and other acyl plasminogen activators include (1) evasion of natural inhibitor systems, leading to increased efficacy, (2)

simplified administration and easier clinical control due to sustained-release kinetics, (3) resistance of the enzymes toward autolytic degradation and (4) reduced systemic toxicity.[16] Two acyl-plasmins, *p*-anisoyl human plasmin (BRL 26920), and *p*-anisoyl human plasminogen-streptokinase activator (BRL 26921, APSAC) both cause significant thrombolysis in rabbits[16] and in dogs.[16,19,20] The use of APSAC also results in significant thrombolysis in humans.[18,21] This acylated plasminogen activator does not result in any clinically significant destruction of the haemostatic system.[21] In addition, the side effects resulting from the use of this derivative are few.[21]

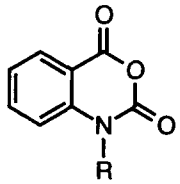
Based on the work done with *p*-anisoyl human plasmin and *p*-anisoyl human plasminogen-streptokinase activator, we believe it is possible that an acylated derivative of thrombin could be used to control thrombosis. A stable acyl thrombin would bind to, and activate, platelets, protein C, protein S, and other factors in the blood coagulation cascade as the enzyme is slowly reactivated. The *in vivo* rate of deacylation of such an acyl thrombin derivative should be dependent upon the reaction with other components in blood, such as thrombomodulin. Few acyl enzyme derivatives of thrombin have been reported,[7,22,23] and such compounds could have possible therapeutic use, as they form stable non-specific acyl enzyme adducts and have varying rates of deacylation. For example, with human thrombin, NPGB has a deacylation rate of 8.8×10^{-4} s^{-1}.[8] With bovine thrombin, benzyl 4'-guanidinothiobenzoate has a deacylation rate of 9.8×10^{-4} s^{-1},[24] and fluorescein mono-4-guanidinobenzoate has a deacylation rate of 1.8×10^{-3} s^{-1}.[9] In this paper we report the rates of acylation and deacylation of additional acyl thrombins which were formed upon reaction of various heterocyclic and non-heterocyclic low molecular weight acylating compounds with thrombin.

Results and Discussion

We have determined the rates of acylation and deacylation of various low molecular weight acylating, carbamylating, and carbonylating compounds (Fig. 1) upon reaction with thrombin. Two of these compounds (**1a** and **1b**) are isatoic anhydrides, and three (**2a–c**) are benzoxazinones. The remaining inhibitors are benzylisocyanate (**3**), *N*-(benzylcarbonyloxy)succinimide (Cbz-OSu, **4**) and *p*-(dimethylamino)benzoylimidazolide (DAB-Im, **5**). Isatoic anhydrides have previously been reported as inactivators of serine proteases,[11,12] as have benzoxazinones,[14,25–28] isocyanates,[29] and substituted *N*-hydroxysuccinimides.[30]

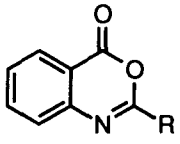
Acylation kinetics

The reaction of inhibitors with serine proteases has been widely investigated and shown to follow the mechanism outlined in Scheme 1. The first step is the formation of the enzyme–inhibitor complex characterized by the dissociation constant K_i. This is followed by an acylation step (k_2), where the active site serine forms a covalent acyl enzyme derivative with the inhibitor upon release of the leaving group P'. The third step is deacylation (k_3), which involves the release of the second product P" (the hydrolyzed or acyl portion

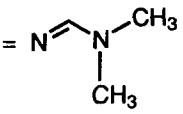


1a, R = H

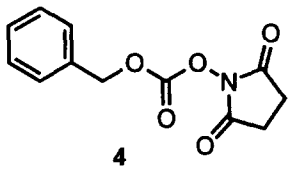
1b, R = CH_2COO^tBu



2a, R = OEt

2b, R = 

2c, R = $NHCH(CH_3)_2$



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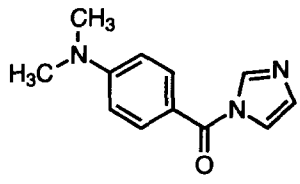

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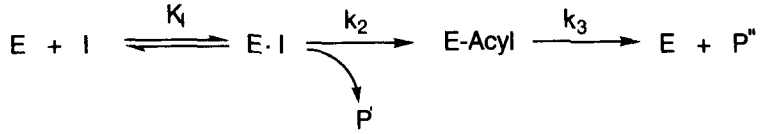
Figure 1. Structures of inhibitors: **1a**, isatoic anhydride; **1b**, *tert*-butyl-2,4-dioxo-2*H*-3,1-benzoxazine-1(4*H*)-acetate; **2a**, 2-ethoxy-4*H*-3,1-benzoxazin-4-one; **2b**, 2-(*N*,*N*-dimethylamino)methylimino-4*H*-3,1-benzoxazin-4-one; **2c**, 2-(*N*-propyl)amino-4*H*-3,1-benzoxazin-4-one; **3**, benzylisocyanate; **4**, *N*-(benzyloxycarbonyloxy)succinimide; **5**, *p*-(dimethylamino)benzoylimidazolide.



Scheme 1.

of the inhibitor) by reaction with H_2O to regenerate the free active enzyme.

The first order rate constants (k_2) for the acylation of thrombin by compounds **1–5** (Fig. 2) are shown in Table 1. Effective inhibition of serine proteases is obtained due to specific recognition of an inhibitor by the enzyme.[29] Thrombin prefers an arginine residue in its P1[31] binding site, a proline residue in its P2 binding site, and a D-phenylalanine in its P3 binding site.[3] The compounds tested in this study have no thrombin recognition features, however, each is intrinsically reactive with thrombin and other serine proteases.

Isatoic anhydrides react with chymotrypsin to form an acyl enzyme derivative containing a carbamate moiety, which decarboxylates to form an anthranoyl–enzyme derivative. Anthranoyl chymotrypsin resists deacylation due to the electron releasing *ortho* amino group.[11] The isatoic anhydrides studied in this investigation react similarly with thrombin. Compound **1b** acylates thrombin almost six times faster than compound **1a** (0.362 s^{-1} and 0.065 s^{-1}, respectively). Compound **1a** however, is a more potent inhibitor of thrombin (k_2/K_i = 48,900 $M^{-1}s^{-1}$) than compound **1b** (k_2/K_i = 5,400 M^{-1} s^{-1}), probably due to its binding to the enzyme.

Benzoxazinones form stable acyl enzyme adducts whose mechanism of acylation and deacylation is shown in Figure 2. The rates of acylation of other benzoxazinones with thrombin have been previously reported.[14] Compounds **2a–c** acylate thrombin at comparable rates, with compound **2b** being slightly faster (0.076 s^{-1}) than compound **2a** (0.059 s^{-1}) or compound **2c** (0.050 s^{-1}). Compound **2a** is the most potent inhibitor of thrombin (k_2/K_i = 52,700 $M^{-1}s^{-1}$), probably because it can fit into the active site more easily than compounds **2b** (k_2/K_i = 23 $M^{-1}s^{-1}$) or **2c** (k_2/K_i = 14 $M^{-1}s^{-1}$), which have large side chains.

Thrombin is acylated by benzylisocyanate (**3**) at a rate of 0.058 s^{-1} to form a stable carbamyl derivative. Other isocyanates (*p*-nitrophenylisocyanate, 1-naphthylisocyanate, 2-carbomethoxyphenylisocyanate, and benzylisothiocyanate) however, do not inhibit this enzyme at a concentration of 1700 μM after 10 minutes incubation. In studies done with alkylisocyanates, Brown and Wold[29] propose that the inactivation of serine proteases requires proper alignment of the two reactive groups (serine of the enzyme and isocyanate of the inhibitor) to permit covalent bond formation. Our results are consistent with this hypothesis. Benzylisocyanate is large enough to fit into the active site of thrombin,

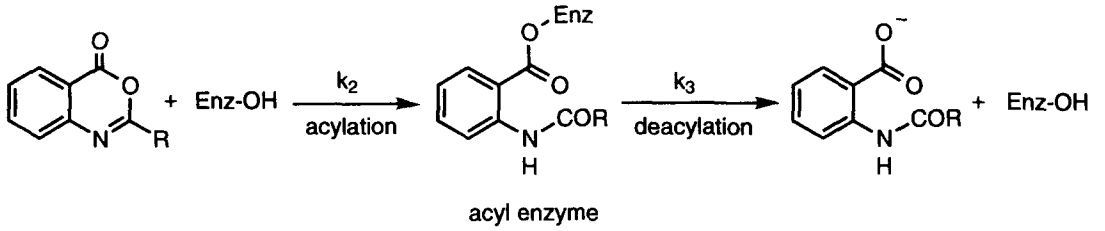


Figure 2. Mechanism of acylation and deacylation of benzoxazinones with serine proteases. The first step involves formation of the covalent acyl enzyme adduct (k_2) between the enzyme and the inhibitor. Deacylation (k_3) results in the release of the hydrolyzed inhibitor and regeneration of the free enzyme.

Table 1. Rates of thrombin acylation* and deacylation

Number	Compound	k_2 (s^{-1})	K_i (M)	k_2/K_i ($M^{-1}s^{-1}$)	k_3 (s^{-1})
1a	isatoic anhydride	0.065	1.3×10^{-6}	48,900	4.4×10^{-6}†
1b		0.362	6.6×10^{-5}	5,400	2.3×10^{-7}†
2a	2-ethoxy-4*H*-3,1-benzoxazin-4-one	0.059	1.1×10^{-6}	52,700	1.3×10^{-5}†
2b		0.076	3.3×10^{-3}	23	1.9×10^{-4}‡
2c		0.050	3.5×10^{-3}	14	1.4×10^{-6}‡
3	benzylisocyanate	0.058	9.5×10^{-4}	61	1.1×10^{-7}‡
4	*N*-(benzyloxycarbonyloxy)succinimide	0.123	3.3×10^{-4}	370	4.3×10^{-5}‡
5	*p*-(dimethylamino)benzoylimidazolide	0.011	4.9×10^{-4}	23	1.1×10^{-6}†

*Conditions were as follows: 0.1 M HEPES and 0.01 M $CaCl_2$, pH 7.5 at 25 °C. Residual enzyme activity was assayed with the same buffer containing 0.23 mM Z-Arg-SBzl, 0.45 mM DTNB and *ca* 10% DMSO.

†Conditions for deacylation were as follows: 0.01 M HEPES, 0.01 M Tris, 0.1 M NaCl, 0.01% PEG_{6000}, pH 7.5 at 25 °C. Residual enzyme activity was assayed with the same buffer containing 0.23 mM Z-Arg-SBzl, 0.45 mM DTNB and *ca* 10% DMSO.

‡Conditions for deacylation were as follows: 0.01 M HEPES, 0.01 M Tris, 0.1 M NaCl, 0.01% PEG_{6000}, pH 7.5 at 25 °C. Residual enzyme activity was assayed with the same buffer containing 2.8 mM H-D-Phe-Pip-Arg-NA and *ca* 10% DMSO.

allowing the isocyanate group and the serine of the enzyme to align for reaction, whereas 1-naphthylisocyanate, and 2-carbomethoxyphenylisocyanate are too large to allow this alignment. The *p*-nitrophenylisocyanate is probably rapidly hydrolyzed in buffer due to its electron-withdrawing nitro group and is destroyed before it can inhibit thrombin.

Derivatives of *N*-hydroxysuccinimide have previously been shown to be efficient inactivators of human leukocyte elastase, chymotrypsin and cathepsin G.[30] Compound **4** (*N*-(benzyloxycarbonyloxy)succinimide) thrombin (0.123 s^{-1}). This compound forms a stable carbonate derivative with thrombin. Succinimide however, is an ineffective inhibitor of thrombin after 10 minutes incubation at 1700 μM.

Compound **5**, *p*-(dimethylamino)benzoylimidazolide (DAB-Im) has been used in Raman difference studies of acyl enzyme intermediates of serine proteases.[32,33] It reacts with chymotrypsin to form a stable acyl enzyme with a deacylation half life of approximately 3 days.[33] This compound acylates thrombin at a very slow rate (0.011 s^{-1}).

Several amide compounds (cyanamide, benzamide, 2-cyanoacetamide, 3-hydroxypicolinamide, hexamethylphosphoramide) and substituted thienothiazine compounds (2*H*-thieno[3,2-*d*][1,3]thiazine-2,4(1*H*)-dione, 2,4-dioxo-2*H*-thieno[3,2-*d*][1,3]thiazine-1(4*H*)-acetic acid, *tert*-butyl-2,4-dioxo-2*H*-thieno[3,2-*d*][1,3]-thiazine-1(4*H*)-acetate) were tested as inhibitors of thrombin at 1700 μM and were found to be ineffective after 10 minutes incubation.

Deacylation kinetics

Each of the compounds tested in this study forms a stable acyl enzyme with thrombin, therefore the rates of deacylation can be determined by following the first-order re-appearance of enzyme activity. The first order deacylation rate constants (k_3) are shown in Table 1.

The deacylation of serine protease acyl enzymes is facilitated by nucleophilic attack of the carbonyl group by water. This reaction is controlled by several factors that affect the stability of acyl enzymes. The first is electronic. If the substituent on the acyl enzyme is electron-donating, the acyl enzyme will be more resistant to deacylation, as the carbonyl carbon will become less electrophilic and nucleophilic attack is less likely. Conversely, if the functional group is electron-withdrawing, the acyl enzyme will deacylate faster because the carbonyl carbon will become more electrophilic, facilitating nucleophilic attack. The second factor that affects the stability of acyl enzymes is geometry. Because nucleophilic attack occurs at the carbonyl group, the highest rate of deacylation should occur when the *p* orbitals of the carbonyl carbon are oriented such that maximum overlap with the orbitals of the attacking water can occur.[34] If the acyl enzyme is twisted such that the orbitals of the carbonyl group and the water molecule cannot overlap properly, then the rate of deacylation would be decreased. Thirdly, the stability of acyl enzymes is affected by steric hindrance. If the functional group on the acyl enzyme is very large, approach of the water molecule to the carbonyl group might be hindered, which would clearly decrease the deacylation rate. The structures of the various acyl enzymes formed with thrombin are shown in Figure 3.

The rate of deacylation of acyl enzyme **6a** (Fig. 3) formed between isatoic anhydride and thrombin is faster (4.4×10^{-6} s^{-1}) than acyl enzyme **6b**, a substituted isatoic anhydride (2.3×10^{-7} s^{-1}). The side chain of acyl enzyme **6b** may be electron donating, which should

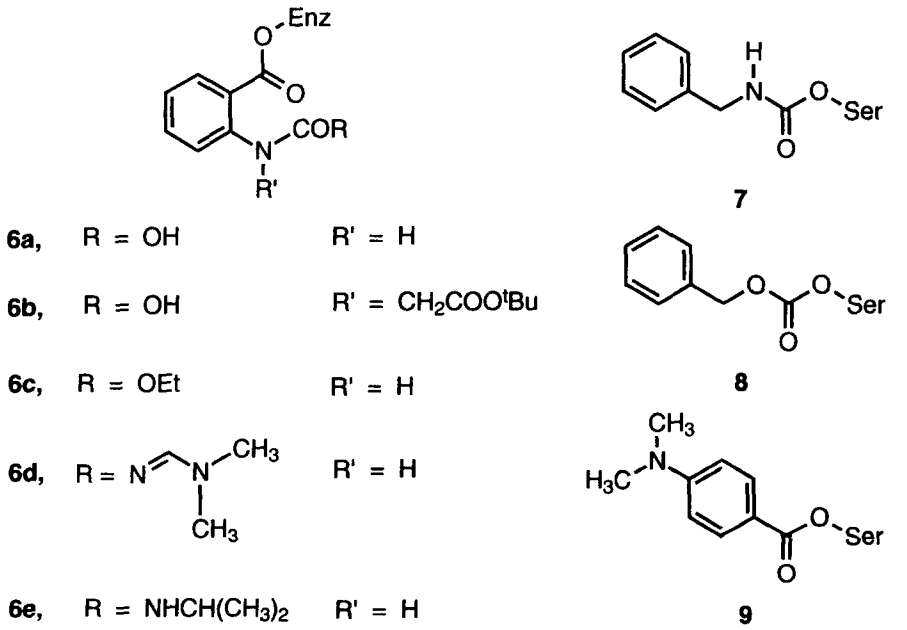


Figure 3. Structures of the acyl enzyme derivatives formed with thrombin.

increase the stability of the acyl enzyme, making it more resistant to deacylation. Alternatively, the *tert*-butyl group may interact with a hydrophobic pocket in the active site of thrombin, twisting the acyl enzyme such that optimum deacylation geometry cannot be obtained and the deacylation rate is decreased.

Krantz *et al.*[26] have done an exhaustive study on the features of benzoxazinones that affect the deacylation of acyl enzyme derivatives of serine protease, particularly human leukocyte elastase, HLE. First, electron-donating groups can enhance the stability of the *o*-aminobenzoyl acyl enzyme (Fig. 3, **6c–e**) as previously discussed. Secondly, small alkyl groups strategically attached to the benzene ring can affect deacylation rates via steric effects on the acyl enzyme adduct.[26] This explains the behavior of acyl enzymes **6c–e** (Fig. 3), which are ester derivatives of thrombin. Acyl enzyme **6c** has a deacylation rate of 1.3×10^{-5} s^{-1}. The alkoxy group at the 2-position in **6c** is more electronegative than the substituents in acyl enzymes **6d** or **6e**, making it more reactive. Acyl enzyme **6e** has a deacylation rate of 1.4×10^{-6} s^{-1}, while **6d** has a faster rate of deacylation (1.0×10^{-4} s^{-1}). The acyl enzyme **6e** in chymotrypsin has a deacylation rate of 8×10^{-4} s^{-1} and that of porcine pancreatic elastase has a deacylation rate of 6×10^{-3} s^{-1}.[27] The acyl enzyme derivative formed with thrombin is clearly more stable.

Benzylisocyanate (**3**) reacts with the active site serine of thrombin to form a carbamate (**7**) with a deacylation rate of 1.1×10^{-7} s^{-1}. Compound **4** (*N*-(benzyloxycarbonyloxy)succinimide) reacts with thrombin to form a carbonate (**8**) which has a deacylation rate of 4.3×10^{-5} s^{-1}. The carbonate derivative deacylates faster because the electronegative oxygen atom increases the electrophilicity of the carbonyl group, facilitating deacylation. The carbamyl derivative is less electrophilic due to the adjacent nitrogen which is less electronegative than the oxygen in the carbonate derivative. This decreases the rate of deacylation.

The benzoyl derivative formed between thrombin and DAB-Im (**9**), DAB-thrombin, is very stable ($k_3 = 1.1 \times 10^{-6}$ s^{-1}). The electron donating *p*-dimethylamino group on the benzene ring stabilizes this acyl enzyme in the same manner as the anthranoyl derivatives previously discussed. The DAB-trypsin has a k_3 value of 6.1×10^{-5} s^{-1} and DAB-chymotrypsin, has a k_3 value of 1.0×10^{-6}.[32] The rates of deacylation of DAB-thrombin and DAB-chymotrypsin are comparable, but the DAB-trypsin deacylates at a slightly faster rate. The reason for the increased stability of the DAB-thrombin and DAB-chymotrypsin compared to DAB-trypsin may be the ability of thrombin and chymotrypsin to bind the aromatic ring in their active sites, producing a geometry unfavorable to deacylation.

Application of acyl thrombins

Thrombin is essential in the blood coagulation cascade, as it cleaves fibrinogen into fibrin, which polymerizes to form a clot. Acyl derivatives of thrombin may be useful in the control of thrombosis. The rationale for this hypothesis is the fact that the acyl plasmin derivatives *p*-anisoyl human plasmin and *p*-anisoyl human plasminogen-streptokinase activator (APSAC) have been shown to be effective alternatives to streptokinase and urokinase for fibrinolysis because they can accomplish successful fibrinolysis without destroying the haemostatic system.[16,21,35] First, acyl thrombins would compete with active thrombin and bind to thrombin binding sites on platelets and thrombomodulin, yet be resistant to antithrombin III. Alternatively, once bound, the acyl thrombin could activate slowly, activating proteins C, protein S, among others, thus giving an anticoagulant effect. Furthermore, acyl thrombins may have additional advantages in the control of thrombosis since other blood components, especially thrombomodulin, may alter their rates of *in vivo* deacylation.

Experimental

Materials

Human prothrombin was activated to thrombin with taipan snake venom[36] and purified as previously described.[37] Tris(hydroxymethyl) aminomethane and $CaCl_2$ were obtained from Fischer Scientific Co., HEPES[38] was purchased from Research Organics, Inc., Cleveland, OH, and Me_2SO was obtained from J. T. Baker Inc., Phillipsburg, NJ. Isatoic anhydride, CDI, DTNB, DAB-OH, and benzylisocyanate were obtained from Aldrich, Milwaukee, WI. Polyethylene $glycol_{6000}$ was obtained from Fluka, Ronkonkoma, NY, and *N*-(benzyloxycarbonyloxy)succinimide was obtained from Bachem California, Torrence, CA. The substrate H-D-Phe-Pip-Arg-NA was obtained from Pharmacia Hepar, Franklin, OH, and Z-Arg-SBzl was synthesized as described previously.[39] Inhibitors **1b**, and **2a–c** were gifts from Dr Michael Gütschow.

Synthesis of DAB-Im

CDI (0.095 g) was added to 0.063 g of DAB-OH dissolved in 5 mL dry THF. The reaction was allowed to stir for 3 h at 25 °C and worked up as previously described.[32] The resulting compound was dissolved in $CHCl_3$ and washed three times with 1 mL aliquots of 8% sodium bicarbonate solution. The $CHCl_3$ phase was dried and a slightly yellow solid was formed: yield, 36% (0.030 g); mp 113–116 °C; ^1H NMR ($CDCl_3$) δ 3.11 (*s*, 6H, CH_3), 6.73 (*d*, 2H, Ph-CH), 7.15 (*s*, 1H, im-CH), 7.54 (*s*, 1H, im-CH), 7.77 (*d*, 2H, Ph-CH), 8.10 (*s*, 1H, im-CH); MS [M^+] = 215.2.

Enzyme inactivation–incubation method

Inactivation reactions were initiated by adding 50 μL of the inhibitor (5–20 mM in Me_2SO) to 0.50 mL of a buffered enzyme solution (0.1 M HEPES, 0.01 M $CaCl_2$, pH 7.5) at 25 °C. The concentration of Me_2SO in the reaction mixture was 8% (v/v).

Aliquots (50 μL) were removed from the reaction mixture at various time intervals and added to a buffered substrate solution (0.1 M HEPES, 0.01 M $CaCl_2$, pH 7.5) at 25 °C. Residual enzyme activity was measured spectrophotometrically using a Beckman Model DU 65 spectrometer with 0.23 mM Z-Arg-SBzl[39] in the presence of 0.45 mM DTNB at 410 nm[40,41] for at least two inactivation half-lives. Duplicate assays were performed and the data averaged to determine the rates.

First order inactivation rate constants, k_{obs}, were obtained from plots of ln v_t/v_o versus time, and the correlation coefficients were greater than 0.96. The rate of acylation (k_2) was calculated from a plot of $1/k_{obs}$ versus 1/[I] at five different inhibitor concentrations from the intercept, while K_i was calculated from the slope as shown in the equation below:[42]

$$\frac{1}{k_{obs}} = \left(\frac{K_i}{k_2}\right)\left(\frac{1}{[I]}\right) + \frac{1}{k_2}$$

Deacylation kinetics

An aliquot of inhibitor (5 mM in Me_2SO) was added to a buffered enzyme solution (10 mM HEPES, 10 mM Tris, 100 mM NaCl, 0.1% PEG_{6000}) and allowed to incubate for 10–30 min. Deacylation rates of inactivated enzymes were measured after the removal of excess inhibitors from the solution by centrifugation at 0 °C for 1 h using Amicon Centricon-10 microconcentrators with no changes in buffer. The residual enzymatic activity was assayed at various time intervals with either 2.8 mM H-D-Phe-Pip-Arg-NA at 405 nm or 0.23 mM Z-Arg-SBzl[39] in the presence of 0.45 mM DTNB at 410 nm[40,41] for at least one deacylation half-life (unless the half-life was longer than 4 days). Duplicate assays were performed and the data averaged to determine the rates. The first-order deacylation rates were obtained from plots of ln $[(v_t - v_f)/(v_o - v_f)]$, where v_o is the enzyme-catalyzed substrate hydrolysis rate of the solution in the absence of inhibitor, v_t is the enzyme-catalyzed substrate hydrolysis rate of the solution in the presence of inhibitor at any time t, and v_f is the maximum inhibition of the enzyme. The correlation coefficients were greater than 0.95.

Conclusion

We have determined the rates of acylation and deacylation of various acylating agents, carbamylating agents and carbonylating agents that acylate the active site serine of thrombin. These compounds have widely varying rates of deacylation, which could allow them to be used therapeutically to control thrombosis. The rate of deacylation of acyl thrombins can easily be manipulated by simply adjusting the length of the side chain or strategically adding substituents to other parts of the molecule.[26] The acyl thrombin derivative **6e**, formed from 2-ethoxy-4*H*-3,1-benzoxazin-4-one, appears to be suitable for further study of the inhibition of thrombosis. This compound is a potent inhibitor of thrombin (k_2/K_i = 52,700 $M^{-1}s^{-1}$), and gives an acyl enzyme that deacylates at a reasonable rate (1.3×10^{-5} s^{-1}) compared to *p*-anisoyl human plasmin, *p*-anisoyl human plasminogen-streptokinase activator, and the 4-aminobenzoyl derivative of *p*-anisoyl human plasminogen-streptokinase activator which have deacylation rates of 1.2×10^{-4} s^{-1}, 2.9×10^{-4} s^{-1}, and 1.1×10^{-5} s^{-1}, respectively.[16,21,35] Acyl thrombin derivatives with varying deacylation rates can be used where it is desirable to regenerate thrombin at various intervals and may be useful in the control of thrombosis.

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38. Abbreviations: Cbz-OSu, *N*-(benzylcarbonyloxy)succinimide; CDI, 1,1'-carbonyldiimidazole; DAB-Im, *p*-(dimethylamino)benzoylimidazolide; DAB-OH, *p*-(dimethylamino)-benzoic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Me_2SO, dimethyl sulfoxide; NA, *p*-nitroanilide; PEG, polyethylene glycol; Pip, piperidyl; SBzl, benzyl thio ester; THF, tetrahydrofuran; Z, benzyloxycarbonyl.

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