Evaluation of inhibitor constants and alkylation rates for a series of thrombin affinity labels

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The kinetics for the inactivation of thrombin (EC 3.4.21.5) by a series of peptides containing C-terminal arginyl chloromethane in the presence of substrate were determined. The inhibitor effectiveness was analysed so as to allow for both the evaluation of the affinity with which the enzyme binds the inhibitor before irreversible modification and also the rate of covalent-bond formation between enzyme and inhibitor. The results obtained show that the observed large range in inhibitor effectiveness can be accounted for almost entirely by marked differences in affinity, with only small variations in rates of formation of covalent complex.

By modelling peptides containing C-terminal arginyl chloromethane on the cleavage sites in the respective protein substrates for a number of plasma proteinases, it has been possible to obtain selective and potent irreversible inhibitors for this class of enzymes. Thus, for example, Kettner & Shaw (1979, 1981b) have demonstrated the selective inactivation of thrombin and Factor Xa by such peptide inhibitors based on their respective cleavage sites in Factors XIII and II. For the design and future development of even more specific and potent inhibitors, it is essential that we be able to assess the individual contributions to overall inhibitor effectiveness made by the binding of the inhibitor to enzyme before covalent-bond formation and by the rate of covalent-bond formation. By defining precisely the changes in affinity and alkylation rate brought about by substitutions in the peptide backbone of the inhibitor one has an exquisite analytical tool, not only for the design of new inhibitors, but also for the exploration of the topography of the secondary binding sites of the enzyme under study.

We have undertaken a kinetic study of the inhibition of human thrombin by a series of peptides containing C-terminal arginyl chloro-

methane in which we have systematically altered the peptide backbone in order to assess the effect of substitution of amino acid residues in the P₂ and P₃ position of the inhibitor (nomenclature of Schechter & Berger, 1968) on the inactivation process.

The kinetic study was achieved by monitoring the progress of inhibition in the presence of the fluorogenic substrate Boc-Val-Pro-Arg-Amc. This approach was adopted because of the difficulties associated with the application of the more commonly used Kitz–Wilson method to the study of fast-acting irreversible inhibitors (Kitz & Wilson, 1962). These difficulties are discussed in detail in the Results and discussion section.

Experimental

Materials

The human thrombin was a freeze-dried preparation obtained from Boehringer–Mannheim, Rotkreuz, Switzerland. The contents of each vial were dissolved in 0.05M-sodium phosphate buffer, pH 8.0, containing 0.15M-NaCl and 0.1% poly(ethylene glycol) and stored on ice before use. The presence of the poly(ethylene glycol) prevents loss of enzyme activity due to adsorption on glass (Lottenberg *et al.*, 1981). The poly(ethylene glycol) (PEG 6000) was purchased from Fluka, Buchs, Switzerland. The thrombin substrate Boc-Val-Pro-Arg-Amc (Morita *et al.*, 1977), 7-amino-4-methylcoumarin and Boc-*p*-nitrophenylalanine were purchased from Bachem Feinchemikalien,

Abbreviations used: Boc, t-butyloxycarbonyl; Z, benzyloxycarbonyl; Bz, benzoyl; Gn, nitroguanidino; Amc, 7-amino-4-methylcoumarin.

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Bubendorf, Switzerland. All other solvents and intermediates were of the best available commercial grade.

Peptides containing C-terminal arginyl chloromethane or phenylalanyl chloromethane

The C-terminal residue was introduced as either Arg(N^G -NO₂)CH₂Cl, HCl (Kettner & Shaw, 1978) or Phe(4-Gn)CH₂Cl, HCl described below. Following elongation with a blocked dipeptide (Kettner & Shaw, 1978, 1979; Kettner *et al.*, 1978), the fully blocked tripeptidyl chloromethane was, in all cases, purified to homogeneity by chromatography on silica-gel columns, typically in methylene chloride/methanol (15:1, v/v) or ethyl acetate/hexane (1:1, v/v). The final product was obtained by HF deblocking and conversion into the hydrochloride salt (Kettner & Shaw, 1978).

Boc-Phe(4-NH₂) (3.7g) obtained by catalytic reduction of the nitro compound (Schwyzer & Caviezel, 1971) was converted into the *p*-nitroguanidino derivative with 3,5-dimethyl-1-nitroguanylpurazole as described for the preparation of *N*^α-Boc-*N*^G-nitrohomoarginine (Kettner *et al.*, 1978). The resultant acid [2.2g; *R*_F 0.88 on t.l.c. in butanol/acetic acid/water (4:1:1, by vol.)] was converted into the mixed anhydride and added to ethereal diazomethane in the standard way (Kettner *et al.*, 1978). The neutral fraction was chromatographed on silica gel in ethyl acetate/hexane (9:1, v/v) containing 0.5% (v/v) methanol and fractions containing only Boc-Phe(4-Gn) diazomethane derivative [*R*_F 0.78 in methylene chloride/methanol (9:1, v/v)] were pooled, yielding 0.91g (Found: C, 49.32; H, 5.35; N, 24.38; C₁₆H₂₁N₇O₅ requires C, 49.10; H, 5.41; N, 25.05). This material was converted into the chloromethane derivative and deprotected with 2M-HCl in ethyl acetate with stirring at room temperature for 1h. After removal of the solvent under reduced pressure, the residue was triturated with anhydrous diethyl ether and used without further purification.

The dipeptides required for the completion of the syntheses were of the general type Xaa-Pro, in which the blocked amino acid was introduced as described previously (Kettner & Shaw, 1979). For D-Lys(Bz)-Pro-ArgCH₂Cl, D-lysine was benzoylated by using the copper salt method of Neuberger & Sanger (1943) followed by introduction of the benzyloxycarbonyl group on the α-amino group with benzyloxycarbonyl chloride under standard conditions. In other cases, Boc-D-Tyr(OBzl)OH and Boc-D-Phe(4-Z-NH)OH (Fahrenholz & Thierach, 1980) were utilized.

Bz-Ala-D-Phe-Pro-ArgCH₂Cl was the only tetrapeptide studied. In this case Z-D-Phe-Pro-Arg(N^G -NO₂)CH₂Cl was treated with HBr in acetic acid to expose the *N*-terminal group, and the

hydrobromide of the partially deblocked peptide was elongated by addition of *N*-benzoylalanine as the mixed anhydride. The blocked tetrapeptide was purified on silica gel in methylene chloride/methanol (15:1, v/v).

Kinetic techniques

(a) *Monitoring of the course of inactivation of the enzyme in the presence of substrate.* To a cuvette maintained at 37°C and housed in the sample compartment of a Perkin-Elmer 650-10S spectrofluorimeter was added a solution (3ml) of the inhibitor under study (0.01–100 μM) and Boc-Val-Pro-Arg-Amc (30–100 μM) in 0.05M-sodium phosphate buffer, pH 8.0, containing 0.15M-NaCl and 0.1% poly(ethylene glycol). A portion (10–20 μl) of a solution (about 5 nM) of thrombin was then added with mixing, and the hydrolysis of the substrate was monitored continuously by measuring the increase in fluorescence at 455nm (excitation at 383nm); the concentration of product was obtained by reference to a 1 μM solution of 7-amino-4-methylcoumarin.

(b) *Evaluation of K_m and k_0 for substrate hydrolysis in the absence of inhibitor.* The K_m and k_0 for the thrombin-catalysed hydrolysis of Boc-Val-Pro-Arg-Amc in 0.05M-sodium phosphate buffer, pH 8.0, containing 0.15M-NaCl and 0.1% poly(ethylene glycol) were determined by monitoring the rate of hydrolysis of a series of concentrations of substrate spanning the range 0.2–5 times K_m and applying the least-squares method directly to the Michaelis-Menten equation (Roberts, 1977).

(c) *Determination of the operational molarity of thrombin solutions.* The concentration of thrombin used in the present study was determined by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate by using the spectrophotometric assay method described by Jameson *et al.* (1973).

(d) *Analysis of kinetic data.* The initial velocities for the hydrolysis of substrate in the presence of various concentrations of inhibitor were evaluated by least-squares linear-regression analysis essentially by the procedure of Hart & O'Brien (1973).

Theory

The kinetic analysis for the irreversible inactivation of an enzyme in the presence of substrate has been worked out by various groups of workers [see, for example, Hart & O'Brien (1973), Tian & Tsou (1982) and Walker & Elmore (1984)]. In the present study the method of Hart & O'Brien (1973) was used to analyse the irreversible inactivation of thrombin by a series of peptide chloromethane derivatives in the presence of a peptide fluorogenic substrate. Assuming that the rate-limiting step for

the hydrolysis of an amide substrate is the acylation of the enzyme, the reaction scheme can be represented as follows:

In this scheme EI is the reversible complex formed between the enzyme and inhibitor before formation of the covalent complex E-I. Hart & O'Brien (1973) have shown that, for such a reaction scheme, the inhibitor dissociation constant K_i can be obtained from the equation:

$$K_i = (K_m \cdot [\text{I}]) / [(K_m + [\text{S}]) \cdot (v_0/v_i - 1)] \quad (1)$$

In this expression v_0 is the initial velocity for the hydrolysis of a substrate S of concentration [S] in the absence of inhibitor, and v_i is the initial velocity for substrate hydrolysis in the presence of inhibitor at concentration [I] at the same substrate concentration [S].

These authors have shown that, by drawing tangents to the progress curves for substrate hydrolysis in the presence of inhibitor and then plotting, in a semi-logarithmic manner, the reaction velocity as a function of time, the initial rate v_i can be obtained by extrapolation back to $t = 0$. Fig. 1 shows progress curves for the thrombin-catalysed hydrolysis of the substrate Boc-Val-Pro-Arg-Amc in the presence of various concentrations of the inhibitor D-Phe-Pro-ArgCH₂Cl. Fig. 2 shows the semi-logarithmic plots of reaction velocity as a function of time that were derived from the curve depicted in Fig. 1. The K_m and v_0 for the thrombin-catalysed hydrolysis of the substrate having been previously evaluated, substitution of the value for the initial rate of substrate hydrolysis in the presence of inhibitor obtained from plots of the type shown in Fig. 2 allows for the evaluation of K_i from eqn. (1).

These authors have additionally shown that the first-order rate constant k_i for the irreversible modification of the enzyme can be evaluated from the slope of the semi-logarithmic plots by using the relationship:

$$k_i = \frac{\Delta(\ln v)}{\Delta t} \cdot \left(\frac{K_i}{[\text{I}] \cdot (1 - \alpha)} + 1 \right) \quad (2)$$

where $\Delta(\ln v)/\Delta t$ is the slope of the semi-logarithmic plots and the term α is a shorthand way of expressing the ratio $[\text{S}]/(K_m + [\text{S}])$. Evaluation of the slope, and substitution of the known values of K_m , [I] and [S] and the determined value of K_i into eqn. (2) yields k_i .

The experimental approach used in this study has a number of advantages over the more commonly used method of Kitz & Wilson (1962). The Kitz-Wilson procedure involves incubating the enzyme with various concentrations of inhibitor, with removal of samples after various time intervals with dilution of the samples by an appropriate substrate to measure residual activity. The arginyl chloromethane inhibitors of thrombin that contain a P₂ proline residue, however, inactivate the enzyme so rapidly that such an approach is of little value, since not enough timed samples can be removed for an accurate assessment of the inhibition process. For example, incubation of human thrombin (0.25 nM) with 0.5 nM-D-Phe-Pro-ArgCH₂Cl results in almost complete inactivation in 1 min (Kettner & Shaw, 1979). In this situation one cannot lower the inhibitor concentration in order to achieve a rate of inactivation that is more amenable to analysis, since the kinetic analysis given by Kitz & Wilson (1962) implicitly assumes that the inhibitor is present in great excess over the enzyme. By incubating enzyme, substrate and inhibitor together we can overcome these limitations. Since the presence of substrate protects the enzyme from inhibition, a greater range of inhibitor concentrations can be used, so that pseudo-first-order conditions always prevail, i.e. $[\text{I}] \gg [\text{E}]$, allowing one to obtain accurate values for K_i and k_i .

Results and discussion

Fig. 1 shows the progress curves for the hydrolysis of Boc-Val-Pro-Arg-Amc (176 μM) by thrombin in the presence of various concentrations of D-Phe-Pro-ArgCH₂Cl (26–65 nM). For the sake of clarity, only the tangents to the curves at an inhibitor concentration of 26 nM have been drawn. Fig. 2 shows the corresponding zero-time semi-logarithmic plots of reaction velocity versus time obtained from the progress curves illustrated in Fig. 1. The initial velocities were obtained by extrapolation to $t = 0$, the data having been subjected to linear-regression analysis. As described above, these plots and eqns. (1) and (2) permitted the evaluation of K_i and k_i .

Table 1 lists the kinetic constants obtained for the inactivation of thrombin by a series of inhibitors. It should be pointed out that the overall second-order rate constant k_i/K_i for the inhibition of thrombin and plasma kallikrein respectively by peptides (1) and (7) have been reported previously (Kettner & Shaw, 1981a,b); however, the individual kinetic constants k_i , K_i and k_i/K_i for the inhibition of thrombin by peptides (2)–(6) were evaluated for the first time in the present study.

Table 1. Inhibitor constants (K_i) and first-order rate constants (k_i) for the inactivation of human thrombin by a series of peptidyl chloromethane derivatives

All of the inactivation studies were carried out at 37°C in 0.05M-phosphate buffer, pH 8.0, containing 0.15M-NaCl and 0.1% poly(ethylene glycol).

	Sequence of inhibitor	K_i (M)	k_i (min ⁻¹)	k_i/K_i (M ⁻¹ ·min ⁻¹)
(1)	D-Phe-Pro-ArgCH ₂ Cl	$2.5 \times 10^{-8} \pm 0.2 \times 10^{-8}$	6.9 ± 0.7	$2.8 \times 10^8 \pm 0.3 \times 10^8$
(2)	D-Tyr-Pro-ArgCH ₂ Cl	$1.9 \times 10^{-7} \pm 0.2 \times 10^{-7}$	44 ± 5	$2.4 \times 10^8 \pm 0.4 \times 10^8$
(3)	4-NH ₂ -D-Phe-Pro-ArgCH ₂ Cl	$4.5 \times 10^{-8} \pm 0.8 \times 10^{-8}$	6.4 ± 0.7	$1.4 \times 10^8 \pm 0.1 \times 10^8$
(4)	ϵ -Bz-D-Lys-Pro-ArgCH ₂ Cl	$7.2 \times 10^{-8} \pm 0.8 \times 10^{-8}$	4.7 ± 0.6	$6.5 \times 10^7 \pm 0.7 \times 10^7$
(5)	Bz-Ala-D-Phe-Pro-ArgCH ₂ Cl	$3.2 \times 10^{-7} \pm 0.4 \times 10^{-7}$	7.6 ± 0.8	$2.4 \times 10^7 \pm 0.3 \times 10^7$
(6)	D-Phe-Pro-Phe(4-Gn)CH ₂ Cl	$5.5 \times 10^{-6} \pm 0.2 \times 10^{-6}$	5.8 ± 0.5	$1.1 \times 10^6 \pm 0.1 \times 10^6$
(7)	D-Phe-Phe-ArgCH ₂ Cl	$2.6 \times 10^{-4} \pm 0.3 \times 10^{-4}$	2.7 ± 0.5	$1.2 \times 10^4 \pm 0.2 \times 10^4$

Fig. 1. Course of hydrolysis of Boc-Val-Pro-Arg-Amc by thrombin in the presence of the affinity label D-Phe-Pro-ArgCH₂Cl

The incubation mixture contained the following: thrombin (0.10 nM), Boc-Val-Pro-Arg-Amc (176 μ M) and D-Phe-Pro-ArgCH₂Cl at 0 mM (curve A), 26 nM (curve B), 36 nM (curve C) or 65 nM (curve D). Tangents are shown drawn for curve B only.

The importance of an amino acid of the D-configuration at the P₃ position of the inhibitor was arrived at empirically from previous studies in which, as pointed out above, only second-order rate constants were determined as a measure of overall effectiveness (Kettner & Shaw, 1979). As a result, the present studies were limited to an exploration of the effect of variations in side-chain structure of amino acids of the D-configuration at the P₃ position on inhibitor effectiveness.

Fig. 2. Semi-logarithmic plot of reaction velocity as a function of time for the inhibition of thrombin by the affinity label D-Phe-Pro-ArgCH₂Cl

The most striking feature that emerges from the present study is the considerable importance of a proline residue at the P₂ position of the inhibitor. Thus comparison of D-Phe-Pro-ArgCH₂Cl (1), which is the most potent inhibitor of thrombin, with D-Phe-Phe-ArgCH₂Cl (7), which is the most potent inhibitor against human urinary kallikrein (Kettner & Shaw, 1981b), shows that the substitution of a phenylalanine residue for proline at P₂ results in a dramatic decrease in k_i/K_i of four orders of magnitude. In particular, this vast difference in potency can be attributed almost exclusively to the very pronounced decrease in affinity with which thrombin binds peptide (7) in comparison with peptide (1) before covalent-bond formation. The rate of formation of the 'Michaelis complex' EI in the case of D-Phe-Pro-ArgCH₂Cl is only 2.5 times faster than that in the case of the analogue with the P₂ phenylalanine. While this paper was in preparation, another study of the inactivation of plasma proteinases by peptide derivatives of arginyl chloromethane appeared (Lijnen *et al.*, 1984),

which included the inactivation of thrombin by D-Phe-Pro-ArgCH₂Cl. Those authors determined the kinetic constants K_i and k_i by the Kitz–Wilson method and obtained the respective values of $1.3 \times 10^{-7} \text{ M}$ and 75 min^{-1} . These values are to be compared with $2.5 \times 10^{-8} \text{ M}$ and 6.9 min^{-1} obtained by us for K_i and k_i respectively. Given the drawbacks of the Kitz–Wilson method detailed above, it seems probable that Lijnen *et al.* (1984) could not achieve the necessary first-order conditions that $[I] \gg [E]$, and that as a result the amount of free inhibitor would be greatly overestimated and this in turn would lead to a calculated value for K_i that would be greater than the true value.

If we compare the inhibitors with common P₁ and P₂ residues but varying in the P₃ position, we observe only a small difference in overall inhibitor effectiveness as measured by the k_i/K_i values. Thus, for the group of peptides (1)–(5), the greatest observed difference between any two members in the group in terms of overall effectiveness is only about one order of magnitude. This was encountered when peptide (1) was elongated by the addition of a benzoylalaninyl group, resulting in a decrease in activity. This difference is again due to differences in affinity as reflected by an approximately 8-fold higher K_i for peptide (5) in comparison with peptide (1). The first-order rate constants for the irreversible modification of thrombin by the two peptides are very similar.

A comparison of the kinetic constants for peptides (1) and (4) reveals that even the presence of a very bulky and unnatural ϵ -benzoyl-D-lysine residue in P₃ is tolerated very well by thrombin. The 4-fold decrease in inhibitor efficacy of peptide (4) in comparison with peptide (1) results from both a decrease in affinity and in rate of covalent modification, although, as before, the variation in the respective K_i values is more pronounced than the variation in first-order rate constants.

The ability to accommodate such an esoteric residue in P₃ implies the existence of a very hydrophobic region on thrombin in the region of the fibrinopeptide-binding sites. Independent work by Sonder & Fenton (1984) has demonstrated the existence of such a region (cleft?) that can bind the very rigid and hydrophobic molecule proflavin. Those authors have shown that human thrombin binds proflavin with a dissociation constant (K_D) of $30 \mu\text{M}$ and that the binding site lies within the so-called 'fibrinopeptide groove' adjacent to the active site. They have also shown that thrombin that has been inactivated with D-Phe-Pro-ArgCH₂Cl binds proflavin with a very marked decrease in affinity (K_D about 1.2 mM), suggesting an overlap in the binding regions for the two reagents. Further investigations designed to explore the topography of this apolar binding region

on thrombin may prove useful in the design of very-tight-binding inhibitors for this enzyme.

The value of analysing the inhibition process in a way that permits the evaluation of k_i and K_i is perhaps best illustrated by comparing the kinetic constants for the inactivation of thrombin by peptides (1) and (2). The specificity constants (k_i/K_i) for these two chloromethane derivatives are almost identical [$2.8 \times 10^8 \pm 0.3 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $2.3 \times 10^8 \pm 0.3 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$ for peptides (1) and (2) respectively], but the contribution of their inhibitory constants K_i and first-order rate constants k_i to the overall effectiveness of each inhibitor is quite different. The replacement of a D-phenylalanine residue by a D-tyrosine results in an 8-fold decrease in affinity but an almost equal and opposing increase in the rate of irreversible enzyme modification. Such a pronounced variation in kinetic constants between the two inhibitors is very difficult to explain, all the more so when one considers that the *p*-amino-D-phenylalaninyl analogue (3) has a k_i essentially identical with that of peptide (1).

The work by Klausner *et al.* (1978) on the inhibition of human thrombin by 4-guanidino- α -N-(*p*-toluenesulphonyl)-L-phenylalaninyl methyl ester [Tos-Phe(4-Gn)-OMe] prompted us to synthesize the *p*-guanidinophenylalanine-containing chloromethane derivative (6). Since the above authors have shown that Tos-Phe(4-Gn)-OMe reversibly inhibits human thrombin with a K_i approximately micromolar, it was hoped that the incorporation of a *p*-guanidinophenylalanine residue into a peptidyl chloromethane derivative would provide an irreversible inhibitor of high potency. However, such a derivative, peptide (6), proved to be much less effective than the parent inhibitor, peptide (1), since the specificity constant for the latter is about 250-fold higher. As in almost all other cases described above, this difference can be attributed to differences in K_i between peptides (1) and (6). Indeed, not only does peptide (6) fail to bind as tightly to thrombin as does peptide (1), but its K_i is only just comparable with that determined for Tos-Phe(4-Gn)-OMe by Klausner *et al.* (1978). This is despite the presence of those residues in P₂ and P₃ that have proven to be particularly efficacious for binding. It is possible that the rigid nature of the aromatic ring at P₁ causes a distortion in enzyme conformation that makes the alignment of the secondary binding sites less favourably disposed towards accommodating the P₂ and P₃ residues of the inhibitor.

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