Synthesis of oligopeptide chloromethanes to investigate extended binding regions of proteinases: application to the interaction of fibrinogen with thrombin

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A method was established for the synthesis of oligopeptide chloromethanes which should be useful in the study of serine and cysteine proteinases with extended binding sites. The method involved condensation of an N-terminal peptide fragment obtained by solid-phase synthesis with a C-terminal peptide chloromethane synthesized by solution-phase chemistry. By using this procedure, oligopeptide chloromethanes of up to 16 residues were synthesized. These chloromethanes were based on the sequence of fibrinopeptide A. By using oligopeptide chloromethanes of different length, it was possible to show that the

residues Asp⁷-Phe⁸-Leu⁹ play a crucial role in the recognition of fibrinopeptide A by thrombin. In contrast, the residues Ala¹-Asp²-Ser³-Gly⁴-Glu⁵-Gly⁶ seem to play a minor role. Substitution of valine for Gly¹², which occurs in a dysfibrinogenemia, markedly decreased the rate of inactivation of thrombin by the oligopeptide chloromethane. The results are discussed in terms of the recently published structure of the complex between human thrombin and a chloromethane inhibitor based on fibrinopeptide A.

INTRODUCTION

Peptidyl chloromethanes are effective inactivators of serine and cysteine proteinases. The peptide portion of these reagents directs them to the active site of the target proteinase where they alkylate the catalytic histidine of serine proteinases or the active-site thiol of cysteine proteinases (Kettner and Shaw, 1981; Shaw, 1990). By varying the amino acid residues of the inhibitor, it has been possible to obtain inhibitors that are specific for particular proteinases. In addition, this approach has allowed the binding sites of individual proteinases to be mapped (Kettner and Shaw, 1981). Past studies have been restricted to the use of di- and tripeptidyl chloromethanes because of the length of the peptide moiety that is conveniently synthesized by solution-phase chemistry. However, some proteinases appear to interact with regions of their substrates that extend more than three residues away from the scissile bond. One such proteinase is thrombin. This enzyme plays a central role in blood coagulation. Thrombin cleaves the N-terminal regions of the fibrinogen A α and B β chains to yield fibrinopeptides A and B together with fibrin monomers which polymerize to form the basis of a blood clot. The cleavage of fibrinogen by thrombin is very specific and this specificity appears to be determined by interactions with an acidic region on the C-terminal side of the scissile bond and an extended region on the N-terminal side (Hogg and Blombäck, 1974; Hageman and Scheraga, 1974; Meinwald et al., 1980; Marsh et al., 1982, 1983). Amino acids in the A α chain that are nine residues N-terminal to the scissile bond appear to be important for the recognition of the substrate (Marsh et al., 1983).

In the present study, we report a method by which oligopeptide chloromethanes can be synthesized for the study of the specificity of proteinases that interact with extended regions of substrates.

Oligopeptidyl chloromethanes with peptide portions based on the structure of fibrinopeptide A have been synthesized and tested as inhibitors of human thrombin. Comparison of the rate of inactivation observed with inhibitors of varying length indicates that residues Asp⁷-Phe⁸-Leu⁹ of fibrinopeptide A are important for the specific interaction with thrombin. The results are discussed in terms of the recently determined crystal structures of complexes of thrombin with fibrinopeptide derivatives (Stubbs et al., 1992; Martin et al., 1992).

MATERIALS AND METHODS

Materials

All amino acids, their derivatives and those attached to the Sasrin resin were purchased from Bachem Feinchemikalien AG, 4416 Bubendorf, Switzerland. The chromogenic substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238) was obtained from Kabi-Pharmacia, Mölndal, Sweden. Human α -thrombin was prepared and characterized as described previously (Stone and Hofsteenge, 1986).

Solid-phase synthesis of peptides

This was performed using a Milligen 9050 PepSynthesizer with the pentafluorophenyl esters of Fmoc amino acids and Sasrin resin.

Preparative reversed-phase h.p.l.c.

This was carried out on a Vydac column 218TP1022 (C₁₈, 10 μ m; 2.2 cm $\times$ 25 cm) and was used to purify peptide derivatives with the following solvents: A, 0.1% trifluoroacetic acid (TFA) in water; B, acetonitrile; C, 30% solvent A and 70% solvent B.

Abbreviations used: Fmoc, 9-fluorenylmethyloxycarbonyl; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulphonyl; HOBt, 1-hydroxybenzotriazole; DIC, *N,N'*-di-isopropylcarbodi-imide; Boc, *t*-butoyloxycarbonyl; OSu, *N*-hydroxysuccinimide ester; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; OBzl, benzyl ester; Ac, acetyl.

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Preparation of Ac-Gly-Val-Arg-CH₂Cl

This was carried out by standard methods (Kettner and Shaw, 1981). Briefly, Ac-Gly-OH was coupled with H-Val-OMe by the standard mixed anhydride method. The resulting Ac-Gly-Val-OMe was hydrolysed with 1.5 equivalents of NaOH, and then converted into the OSu ester. Coupling with H-Arg(Mtr)-OH gave the tripeptide Ac-Gly-Val-Arg(Mtr)-OH. Treatment of the mixed anhydride with diazomethane yielded the peptidyl diazomethane which was converted into Ac-Gly-Val-Arg(Mtr)-CH₂Cl with gaseous HCl in ethyl acetate. Ac-Gly-Val-Arg-CH₂Cl was obtained as the TFA salt after deprotection with TFA containing 10% anisole. The identity of the final product was established by n.m.r. { δ (p.p.m.)} (²H₆]dimethyl sulphoxide) 0.85, 0.87 [6H, 2d, J 7 Hz, 2CH₃ (Val)], 1.38–1.85 [4H, m, CHCH₂CH₂CH₂NH (Arg)], 1.85 (3H, s, CH₃CH), 1.95–2.10 [1H, m, CHCH(CH₃)₂ (Val)], 3.02–3.13 [2H, m, CHCH₂CH₂CH₂NH (Arg)], 3.69–3.78 (2H, m, α -CH₂), 4.10 [1H, t, J 7 Hz, α -CH (Val)], 4.30–4.40 (1H, m, α -CH), 4.54 (2H, d, J 3 Hz, CH₂Cl), 6.8–7.7 (5H, broad m, guanidino), 8.08 (1H, d, J 7 Hz, NH), 8.30 [1H, t, J 7 Hz, NH (Gly)], 8.57 (1H, d, J 7 Hz, NH). Field desorption m.s.: ($M + H - H_2O - HCl$)⁺, 351 (C₁₆H₂₉N₆O₄Cl formula 404, M_r 404.899; TFA salt: M_r 518.921).

Preparation of Boc-Gly-Val-Arg(Mtr)-CH₂Cl

Boc-Arg(Mtr)-OH was converted into the diazomethyl ketone as described above. Subsequent treatment with gaseous HCl in ethyl acetate at 0°C for 2 min gave Boc-Arg(Mtr)-CH₂Cl which was purified on silica gel with ethyl acetate. The Boc group was cleaved off with gaseous HCl in ethyl acetate and then coupled with the mixed anhydride of Boc-Gly-Val-OH to yield Boc-Gly-Val-Arg(Mtr)-CH₂Cl (Found: C, 51.36; H, 7.11; N, 11.92; S, 4.44; Cl, 4.57; C₂₉H₄₇N₆O₈SCl requires C, 51.58; H, 7.02; N, 12.45; S, 4.75; Cl, 5.25%), n.m.r. δ (p.p.m.) (²H₆]dimethyl sulphoxide) 0.81, 0.84 [6H, 2d, J 7 Hz, 2CH₃ (Val)], 1.29–1.79 [4H, m, CHCH₂CH₂CH₂NH (Arg)], 1.36 [9H, s, (CH₃)₃C (Boc)], 1.90–2.01 [1H, m, CHCH(CH₃)₂ (Val)], 2.51, 2.58 [6H, 2s, 2CH₃ (Mtr)], 2.98–3.07 (2H, m, CHCH₂CH₂CH₂NH (Arg)], 3.13 [3H, s, CH₃ (Mtr)], 3.56 (2H, d, J 7 Hz, α -CH₂), 3.79 [3H, s, OCH₃ (Mtr)], 4.18 [1H, t, J 7 Hz, α -CH (Val)], 4.27–4.35 (1H, m, α -CH), 4.51 (2H, d, J 3 Hz, CH₂Cl), 6.67 [3H, s, C₆H (Mtr)], 7.01 [1H, t, J 7 Hz, NH (Gly)], 7.68 (1H, d, J 7 Hz, NH), 8.42 (1H, d, J 7 Hz, NH); field desorption m.s.: m/z ($M + H$)⁺ 675. (C₂₉H₄₇N₆O₈SCl formula 674, M_r 675.246).

Preparation of Ac-Asp-Phe-Leu-Ala-Glu-Val-Gly-Gly-Val-Arg-CH₂Cl.TFA

The blocked decapeptide was prepared by a 7 plus 3 procedure. The N-terminal heptapeptide H-Asp(OtBu)-Phe-Leu-Ala-Glu-(OtBu)-Val-Gly-Sasrin (0.260 mmol) from the solid-phase synthesis was acetylated with acetic anhydride (98 μ l, 1.04 mmol) and triethylamine (181 μ l, 1.30 mmol) in tetrahydrofuran (2.6 ml). After 45 min the solid was filtered off and washed with tetrahydrofuran. The acetylated peptide was cleaved off the resin by treating the solid in a glassfrit four times with a mixture of dichloromethane (10 ml) and TFA (100 μ l). The filtrates were evaporated separately. Batches two and three were trituated with ethyl acetate/hexane (1:1, v/v; 10 ml); the solids were combined and trituated again to yield the acetylated peptide as a slightly beige solid (187 mg, 79.6%).

H-Gly-Val-Arg(Mtr)-CH₂Cl (0.248 mmol) was obtained by treating Boc-Gly-Val-Arg(Mtr)-CH₂Cl (168 mg, 0.248 mmol) with TFA (2 ml) at 0°C for 5 min. The solution was concentrated to half its volume in a stream of nitrogen. Dry ether was added

and the precipitate formed was centrifuged and the solvent decanted. The washing with dry ether was repeated twice. The solid was dissolved in dimethylformamide (4 ml) and neutralized with *N*-methylmorpholine (54 μ l, 0.496 mmol).

The decapeptide was obtained by coupling the acetylated heptapeptide (187 mg, 0.207 mmol) to H-Gly-Val-Arg(Mtr)-CH₂Cl (0.248 mmol) with HOBt [32 mg (13% water), 0.207 mmol] and DIC (128 μ l, 0.828 mmol). After 20 h the reaction mixture was evaporated. The solid was twice trituated with water (20 ml), centrifuged and the solvent decanted, twice trituated with dry ether (20 ml), centrifuged and the solvent decanted, three times trituated with methanol (10 ml and twice 6 ml), centrifuged and the solvent decanted to yield a slightly yellowish solid (209 mg, 69.2%).

The solid was stirred for 24 h in a mixture of TFA (1.8 ml) and anisole (0.2 mmol). The reaction mixture was concentrated by half, twice trituated with dry ether, centrifuged and the solvent decanted. Preparative h.p.l.c. (solvent A and B, linear gradient solvent B: 10–50% within 40 min, and then isocratic for the next 20 min; flow rate 8 ml/min) yielded a colourless solid (56 mg, 31.3%); it gave a satisfactory amino acid analysis and a violet spot on t.l.c. with the Sakaguchi reagents; fast atom bombardment m.s.: m/z ($M + H$)⁺ 1136 (C₅₀H₇₈N₁₃O₁₅Cl formula 1135, M_r 1136.703; TFA salt: M_r 1250.725).

Preparation of Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-CH₂Cl.TFA

The blocked decapeptide was prepared by a 7 plus 3 procedure. The N-terminal heptapeptide H-Asp(OtBu)-Phe-Leu-Ala-Glu-(OtBu)-Gly-Gly-Sasrin from the solid-phase synthesis was acetylated with acetic anhydride and triethylamine. The peptide was cleaved off the resin with 1% TFA in dichloromethane. This product was coupled via the mixed anhydride with H-Gly-Val-Arg(Mtr)-CH₂Cl which had been obtained by treating Boc-Gly-Val-Arg(Mtr)-CH₂Cl with TFA at 0°C for 5 min. The resulting decapeptide was deprotected in TFA containing 10% anisole. The product was purified on Sephadex LH-20 by elution with methanol and then further purified by preparative h.p.l.c. to yield a colourless wax (solvents A and C, flow rate 10 ml/min with the following concentrations of solvent C: 0 min, 10%; 5 min, 20%; 35 min, 40%; 45 min, 70% and then isocratic elution with 70% solvent C). Amino acid analysis indicated that the isolated product had the correct composition, and a violet spot was obtained on t.l.c. with the Sakaguchi reagents. Plasma desorption m.s.: ($M + H$)⁺, 1095.1 (C₄₇H₇₂N₁₃O₁₅Cl formula M_r 1094.622; TFA salt: M_r 1208.644).

Preparation of Boc-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(Z₂)-CH₂Cl

This blocked peptide was prepared by a 4 plus 3 procedure using H-Gly-Val-Arg(Z₂)-CH₂Cl as the C-terminal component. The N-terminal fragment, Boc-Ala-Glu(OBzl)-Gly-Gly-OH, was synthesized as follows: reaction of Boc-Ala-OSu and glutamic acid γ -benzyl ester provided Boc-Ala-Glu(OBzl)-OH which was converted into the OSu derivative for reaction with H-Gly-Gly-OH. The tetrapeptide was coupled with H-Gly-Val-Arg(Z₂)-CH₂Cl via the mixed anhydride. The resulting blocked heptapeptide was trituated in methanol. Amino acid analysis confirmed the identity of the heptapeptide.

Preparation of H-Ala-Glu-Gly-Gly-Val-Arg-CH₂Cl.2TFA

The blocked heptapeptide was deprotected in HF containing 5 equivalents of anisole. The product was purified on ion-exchange

Scheme 1

Sephadex C-25 (H⁺ form) by elution with 0.5 M HCl and then further purified by preparative h.p.l.c. to yield the TFA salt (solvents A and C, flow rate 8 ml/min, with the following linear gradients in solvent C: 5–30% over 5 min and 30–60% over the next 30 min). The amino composition of the product confirmed its identity.

Preparation of Ac-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-CH₂Cl.TFA

The blocked hexadecapeptide was prepared by a 9 plus 7 procedure. (The initially selected 13 plus 3 procedure was abandoned because of solubility problems.) The N-terminal peptide Ac-Ala-Asp(OtBu)-Ser-Gly-Glu(OtBu)-Gly-Asp(OtBu)-Phe-Leu-OH was obtained from a solid-phase synthesis as described above. This peptide was activated with HOBt/DIC and coupled with H-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(Z₂)-CH₂Cl that had been obtained by treating Boc-Ala-Glu(OBzl)-Gly-Gly-Gly-Val-Arg(Z₂)-CH₂Cl with TFA at 0 °C for 5 min. The resulting hexadecapeptide was deprotected in HF containing 5 equivalents of anisole. The product was purified by preparative h.p.l.c. to yield the TFA salt as a colourless solid (solvents A and B, linear gradient solvent B 10–50% in 10 min, then 50–100% over the next 20 min; flow rate 8 ml/min). The product gave a satisfactory amino acid analysis and a violet spot on t.l.c. with the Sakaguchi reagents. Plasma desorption m.s.: (M+H)⁺, 1612.4 (C₆₆H₁₀₀N₁₉O₂₆Cl formula M_r 1611.086; TFA salt: M_r 1725.108).

Inactivation of thrombin by peptidyl chloromethanes

The rate constants for the inactivation (k_i) of thrombin by chloromethanes were determined by analysing progress-curve data for the hydrolysis of the substrate D-Phe-pipecolyl-Arg-*p*-nitroanilide in the presence of different concentrations of inhibitor. The assays were performed at 37 °C in 50 mM Tris/HCl buffer, pH 7.8, containing 0.1 M NaCl and 0.2% (w/v) poly(ethylene glycol) M_r 6000 as previously described (Hofsteenge et al., 1986). Each progress-curve experiment consisted of one reaction without chloromethane and five with different concentrations of the inhibitor. The mechanism for the inactivation of thrombin (E) by the chloromethanes (I) in the presence of substrate (S) could be described by Scheme 1.

Data analysis was performed as described by Gray and Duggleby (1989) to yield estimates for the apparent inactivation rate constant (k_i). Eqn. (1) was used to calculate the true value of the k_i from this apparent value:

$$k_i = k_i \{1 + ([S]/K_m)\} \quad (1)$$

The previously determined value of $3.6 \pm 0.1 \mu\text{M}$ for the K_m of D-Phe-pipecolyl-Arg-*p*-nitroanilide was used in these calculations (Hofsteenge et al., 1986).

RESULTS

Synthesis of oligopeptide chloromethanes

A series of oligopeptidyl chloromethanes with sequences based on fibrinopeptide A have been synthesized by a method that involved condensation of an N-terminal peptide fragment obtained by solid-phase synthesis with a C-terminal peptide chloromethane synthesized by solution-phase chemistry. The decapeptidyl inhibitor Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl was obtained by coupling a three-residue C-terminal chloromethane with a heptapeptide N-terminal fragment. Ac-Asp-Phe-Leu-Ala-Glu-Val-Gly-Gly-Val-Arg-CH₂Cl, which is based on the sequence of naturally occurring mutant fibrinogen, was synthesized by a similar procedure. The hexadecapeptide inhibitor Ac-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl, which corresponds to the complete sequence of fibrinopeptide A, was synthesized by coupling a C-terminal heptapeptide chloromethane with an N-terminal nonapeptide fragment. The C-terminal heptapeptide chloromethane was initially synthesized by coupling four- and three-residue fragments made in solution. Thus, by using a strategy of synthesizing fragments by either solution- or solid-phase methods followed by condensation of the fragments, it was possible to synthesize oligopeptide chloromethanes ranging from seven to 16 residues in length. The identity of each of the inhibitors was confirmed by m.s. and amino acid analysis.

Kinetics of inactivation of thrombin by oligopeptide chloromethanes

The rate constants for the inactivation of thrombin by the oligopeptide chloromethanes based on fibrinopeptide A were studied by progress-curve kinetics in the presence of substrate. Data for the inactivation by Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl are shown in Figure 1. Similar data were obtained with all other chloromethanes and the estimates for the

Figure 1 Inactivation of thrombin by Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl

Assays were performed as described in the Materials and methods section with 0.2 nM thrombin and 95 μM D-Phe-pipecolyl-Arg-*p*-nitroaniline. The assays contained 0 (○), 1.63 (●), 3.26 (△), 4.89 (▲), 6.52 (▽) and 8.15 (▼) μM Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl. For the sake of clarity data points at the beginning of the curves (< 1.5–2.0 min), which were used in the regression analysis, are not shown and only every second point thereafter is shown. The data were analysed as described in the Materials and methods section to yield an estimate of k_i ; the lines show the fit of the data to the equation used for analysis (Gray and Duggleby, 1989).

Table 1 Rate constants for the inactivation of thrombin by peptidyl chloromethanes with sequences based on fibrinopeptide A

Assays were performed as described in the Materials and methods section. Progress-curve data such as those shown in Figure 1 were analysed by non-linear regression analysis to yield estimates of the inactivation rate constant (k_i) together with a standard error of the estimate. The values given for k_i represent the weighted means of at least two determinations.

Peptidyl chloromethane	k_i ($M^{-1} \cdot s^{-1}$)
Ac-Gly-Val-Arg-CH ₂ Cl	$(6.31 \pm 0.06) \times 10^2$
Ala-Glu-Gly-Gly-Gly-Val-Arg-CH ₂ Cl	$(1.85 \pm 0.01) \times 10^2$
Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH ₂ Cl	$(7.26 \pm 0.02) \times 10^4$
Ac-Asp-Phe-Leu-Ala-Glu-Val-Gly-Gly-Val-Arg-CH ₂ Cl	$(4.42 \pm 0.01) \times 10^3$
Ac-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Val-Arg-CH ₂ Cl	$(1.09 \pm 0.02) \times 10^5$
D-Phe-Pro-Arg-CH ₂ Cl*	$(2.0 \pm 0.1) \times 10^7$

* From Hofsteenge et al. (1986).

inactivation rate constants (k_i) obtained from the analyses of the data are given in Table 1. The tripeptide corresponding to the C-terminus of fibrinopeptide A (Ac-Gly-Val-Arg-CH₂Cl) inactivated thrombin only relatively slowly with a k_i value of $(6.31 \pm 0.06) \times 10^2 M^{-1} \cdot s^{-1}$. Extension of the peptide to the seven C-terminal residues of fibrinopeptide A (Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl) did not increase the inactivation rate constant (Table 1). A substantial increase in k_i (approx. 100-fold) was obtained, however, when the length of the peptide was increased to 10 residues. Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl exhibited a k_i value of $(7.26 \pm 0.02) \times 10^4 M^{-1} \cdot s^{-1}$. Further increase in the chain length to the 16 amino acids of fibrinopeptide A (Ac-Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl) did not further increase the inactivation rate constant (Table 1).

The substitution of valine for Gly¹² is found in a naturally occurring mutant of fibrinogen. This replacement leads to a bleeding disorder (Henschen et al., 1983; Ménaché, 1983). We have also investigated the effect of this substitution in oligopeptide chloromethanes by synthesizing Ac-Asp-Phe-Leu-Ala-Glu-Val-Gly-Gly-Val-Arg-CH₂Cl. In comparison with the chloromethane of equivalent length based on the wild-type sequence, this chloromethane inactivated thrombin 16-fold more slowly (Table 1).

DISCUSSION

In this manuscript, we have presented a method for the synthesis of oligopeptide chloromethanes and such reagents should be useful in studying the specificity requirements of proteinases (such as thrombin) that interact with extended regions of their substrates. Chloromethanes with a long peptide chain have not previously been synthesized. In order to avoid a long and tedious synthesis in solution, the decapeptide chloromethanes were synthesized by a 7 plus 3 procedure which involved condensation of an N-terminal heptapeptide synthesized by solid-phase chemistry and a C-terminal tripeptide chloromethane made in solution. This method enabled us to make different inhibitors quickly by adding different N-terminal peptides from the solid-phase synthesis to the same C-terminal tripeptide chloromethane. The tripeptide chloromethane was preferred over a single amino acid

chloromethane because coupling of the N-terminal fragment with the tripeptide gave a higher yield in our hands; dipeptide chloromethanes are not suitable because they cyclize. In the case of the hexadecapeptide chloromethane, the 9 plus 7 procedure was preferred over the 13 plus 3 procedure because of solubility problems. The oligopeptide chloromethanes were used to evaluate regions of fibrinopeptide A that make important interactions with thrombin (discussed below). In addition, because of the covalent nature of the bond between the chloromethane and the proteinase, these compounds are also useful in crystallographic studies with peptide fragments that do not bind tightly to the proteinase (Stubbs et al., 1992).

By comparing the observed values of k_i for oligopeptide chloromethanes, it is possible to assess the relative importance of various regions of fibrinopeptide A for interaction with thrombin. The interpretation of the results in structural terms is greatly facilitated by the recently determined structure of the complex between human thrombin and Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl (Stubbs et al., 1992). Figure 2 illustrates this complex. The structure of the complex of the equivalent peptide with bovine thrombin has also recently been reported (Martin et al., 1992). Ac-Gly-Val-Arg-CH₂Cl was a relatively poor inactivator of thrombin; the k_i value for this compound was 3×10^4 times lower than that for the best-known tripeptide chloromethane inhibitor of thrombin (D-Phe-Pro-Arg-CH₂Cl; Table 1). Comparison of the structure of D-Phe-Pro-Arg-CH₂-thrombin (Bode et al., 1989) with that shown in Figure 2 indicates that hydrophobic interactions with the D-phenylalanine in the P₃ position are probably responsible for the increased rate of reaction of D-Phe-Pro-Arg-CH₂Cl. The D-phenylalanine has favourable contacts with the binding site formed by Trp²¹⁵, Leu⁹⁹, Ile¹⁷⁴, Tyr^{60A} and Trp^{60D} (called the aryl-binding site; Bode et al., 1990), whereas the P₃ in the fibrinopeptide analogue (Gly¹⁴) would not interact with this binding site (Figure 2).

Increasing the chain length of oligopeptide chloromethane to seven residues did not increase the value of k_i . This result would suggest that the part of the polypeptide chain comprising Ala¹⁰-Glu¹¹-Gly¹²-Gly¹³ does not make any important interactions with thrombin. However, the crystal structure indicates that Glu¹¹ forms a salt bridge with Arg¹⁷³ of thrombin. For this interaction to occur, the polypeptide chain must assume a specific conformation (Figure 2). If Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl is bound in an extended conformation, this interaction would not be possible. The polypeptide chain of the inhibitor in the Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂-thrombin complex undergoes a chain reversal at Ala¹⁰, and this turn is stabilized by main-chain hydrogen bonds involving Asp⁷-Glu¹¹, Phe⁸-Gly¹³ and Leu⁹-Gly¹². Thus, in the absence of Asp⁷, Phe⁸ and Leu⁹, the polypeptide chain will not be held in the conformation required for the formation of the salt bridge between Glu¹¹ and Arg¹⁷³ of thrombin.

A marked increase in k_i (> 100-fold) was observed when the length of the polypeptide chain was increased to ten residues. This increase indicates an important role for interactions with the residues Asp⁷, Phe⁸ and Leu⁹. The importance of these residues was also suggested from previous studies using peptide substrates (Meinwald et al., 1980; Marsh et al., 1983). As discussed above, intermolecular contacts stabilizing the chain reversal at Ala¹⁰ and the positioning of the side chain of Glu¹¹ are undoubtedly important. In addition, Phe⁸ and Leu⁹ bind to the hydrophobic aryl-binding site (Figure 2). This site was discussed above as the binding site for the D-phenylalanine of D-Phe-Pro-Arg-CH₂Cl; Phe⁸ and Leu⁹, however, enter the pocket from a different angle due to the chain reversal and occupy the site more fully.

Figure 2 Structure of Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg bound to thrombin

Stereo view of the inhibitor (bold) bound to the active site of thrombin (thin lines). Residues in the inhibitor are distinguished from those in thrombin by prefixing the residue number with an 'F'. Dotted lines show intramolecular hydrogen bonds in the inhibitor. The Figure is taken from the structure of Stubbs et al. (1992) with permission.

Further lengthening of the chain to the 16 residues of fibrinopeptide A did not significantly increase the value of k_i which suggests that residues 1 to 6 do not make important intra- or inter-molecular contacts. In the crystal structure presented in Figure 2, the acetylated N-terminus of Asp⁷ points away from thrombin which would allow the first six residues to extend into solution.

The importance of the precise conformation of the polypeptide chain around the turn at Ala¹⁰ is illustrated by the results obtained when valine was substituted for Gly¹². This replacement in fibrinogen causes a bleeding disorder (Henschen et al., 1983; Ménaché, 1983) and, in the oligopeptide chloromethane, it resulted in a 16-fold decrease in k_i . A glycine in position 12 is absolutely conserved in sequences of fibrinopeptide A from different species (Henschen et al., 1983). For the chain to fold back into the active site of thrombin, Gly¹² must assume a left-handed helical conformation (Stubbs et al., 1992). Such a conformation would be energetically unfavourable for a valine residue, which would explain the lower k_i value for the chloromethane with the Gly¹²→Val substitution. The fact that this substitution results in only a 16-fold decrease in k_i suggests, however, that the contacts with one or more of Asp⁸, Leu⁹ and Glu¹¹ may be preserved and that the backbone may assume a slightly different conformation in order to make some of these contacts. This hypothesis is supported by n.m.r. studies which indicate that a peptide with a valine in position 12 binds to thrombin with a different conformation (Ni et al., 1989).

The best inactivators based on fibrinopeptide A sequences reacted more than 100 times more slowly with thrombin than did D-Phe-Pro-Arg-CH₂Cl (Table 1). The reason for the faster reaction of D-Phe-Pro-Arg-CH₂Cl is not immediately apparent from the crystal structures (Bode et al., 1989; Stubbs et al., 1992). A possible reason for the faster reaction of D-Phe-Pro-Arg-CH₂Cl is the formation of a charged hydrogen bond between the α -amino group of the D-phenylalanine and the carbonyl of Gly²¹⁶ of thrombin. A positively charged α -amino group has been

shown to be essential for the optimal rate of reaction of the substrate D-Phe-pipecolyl-Arg-*p*-nitroaniline (Stone et al., 1991). In the case of this substrate, interactions with the positively charged α -amino group increased the value of k_{cat}/K_m by about 40-fold. Such an interaction will not be possible for Ac-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg-CH₂Cl.

In summary, we have developed a method for the synthesis of long peptide chloromethanes that involves the condensation of an N-terminal fragment obtained by solid-phase synthesis to a C-terminal tripeptide (or heptapeptide) chloromethane. This method allows the synthesis of peptide chloromethanes of greater length than has been previously described. Moreover, the sequence of the fragment obtained by solid-phase synthesis can be easily varied and, thus, a series of oligopeptide chloromethanes could be readily synthesized. This approach should be useful for investigating the interactions of substrates and inhibitors with proteinases with extending binding sites and in the present paper, we have examined one such interaction, that of thrombin with the fibrinopeptide A portion of fibrinogen.

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REFERENCES

- Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R. and Hofsteenge, J. (1989) EMBO J. **8**, 3467–3475
- Bode, W., Turk, D. and Stürzebecher, J. (1990) Eur. J. Biochem. **193**, 175–182
- Gray, P. J. and Duggleby, R. G. (1989) Biochem. J. **257**, 419–424
- Hageman, T. C. and Scheraga, H. A. (1974) Arch. Biochem. Biophys. **164**, 707–715
- Henschen, A., Lottspeich, F., Kehl, M. and Southan, C. (1983) Ann. N.Y. Acad. Sci. **408**, 28–43
- Hofsteenge, J., Taguchi, H. and Stone, S. R. (1986) Biochem. J. **237**, 243–251
- Hogg, D. H. and Blombäck, B. (1974) Thromb. Res. **5**, 685–693
- Kettner, C. and Shaw, E. (1981) Methods Enzymol. **80**, 826–842
- Marsh, H. C., Jr., Meinwald, Y. C., Lee, S. and Scheraga, H. A. (1982) Biochemistry **21**, 6167–6171

- Marsh, H. C., Jr., Meinwald, Y. C., Thannhauser, T. W. and Scheraga, H. A. (1983) *Biochemistry* **22**, 4170–4174
- Martin, P. D., Robertson, W., Turk, D., Hober, R., Bode, W. and Edwards, B. F. P. (1992) *J. Biol. Chem.* **267**, 7911–7920
- Meinwald, Y. C., Martinelli, R. A., van Nispen, J. W. and Scheraga, H. A. (1980) *Biochemistry* **19**, 3820–3825
- Ménaché, D. (1983) *Ann. N.Y. Acad. Sci.* **408**, 121–129
- Ni, F., Meinwald, Y. C., Vásquez, M. and Scheraga, H. A. (1989) *Biochemistry* **28**, 3094–3105
- Shaw, E. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol.* **63**, 271–347
- Stone, S. R. and Hofsteenge, J. (1986) *Biochemistry* **25**, 4622–4628
- Stone, S. R., Betz, A. and Hofsteenge, J. (1991) *Biochemistry* **30**, 9841–9848
- Stubbs, M. T., Oschkinat, H., Mayr, I., Huber, R., Angliker, H., Stone, S. R. and Bode, W. (1992) *Eur. J. Biochem* **206**, 187–195
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