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INHIBITION OF SUBTILISIN BPN' WITH PEPTIDE CHLOROMETHYL KETONES

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Summary

The kinetics of the reaction of a series of peptide chloromethyl ketones with subtilisin BPN' (EC 3.4.21.14) were investigated in order to correlate the rates of reaction in solution with the number of interactions which are observed in the cystallographic model of the inhibited enzyme (Robertus, J.D., Alden, R.A., Birktoft, J.J., Powers, J.C. and Wilcox, P.E. (1972) Biochemistry 11, 2439—2449). The second-order rate constant $k_{obs}/[I]$, measured at pH 7.0 in 9% 1,2-dimethoxyethane at 30°C, varied by a factor of 5900 from the slowest (Z-TrpCH_2Cl) to the fastest inhibitor (Ac-Phe-Gly-Ala-LeuCH_2Cl). Inhibitors with a P_1 leucine or phenylalanine residue are equally effective. The increased reactivity of inhibitors containing alanine as the P_2 residue is the result of a favorable contact between the methyl side chain of the alanyl residue and the S_2 subsite of subtilisin. This result correlates nicely with the previously observed "secondary specificity" of subtilisin for substrates with alanine as the P_2 residue. Tetrapeptide and tripeptide chloromethyl ketone inhibitors have $k_{obs}/[I]$ values of over 100-fold greater than those of most dipeptide and amino acid chloromethyl ketones. This again agrees with the crystallographic model since tripeptide and tetrapeptide inhibitors could form a β-sheet structure involving three or four hydrogen bonds with the enzyme while the others would form fewer hydrogen bonds. The S_4 subsite of subtilisin exhibits a distinct preference for aromatic groups and our four most reactive inhibitors (Ac-Phe-Gly-Aca-LeuCH_2Cl, Z-Gly-Gly-PheCH_2Cl, Z-Gly-Gly-LeuCH_2Cl, and Boc-Ala-Gly-PheCH_2Cl) have either an aromatic (or a large hydrophobic) group as the P_4 residue. The results demonstrate that the solution reactivity of peptide chloromethyl ketones can be explained on the basis of the crystal structures of chloromethyl ketone-inhibited substilisin derivatives and that substrate hydrolysis rates can be used to design effective chloromethyl ketone inhibitors for serine proteases.

Abbreviation: XCH_2Cl, the chloromethyl ketone derivative of peptide X.

Introduction

Studies of the reaction of subtilisin BPN′ (EC 3.4.21.14) with halomethyl ketones have provided revealing insights into the active site region of this serine protease. This enzyme reacts irreversibly with Z-PheCH_2Cl and Z-PheCH_2Br [1], relatives of Tos-PheCH_2Cl, the stereospecific active site-directed inhibitor of chymotrypsin [2]. The active site histidine residue of subtilisin BPN′, presumed to be His-64 on the basis of work with the homologous Carlsberg enzyme and some studies with the BPN′ enzyme, was alkylated by Z-PheCH_2Br [3]. Subsequently, Morihara and Oka [4] showed that the rate of inactivation of subtilisin was over 200 times faster with peptide chloromethyl ketones than with the simple amino acid chloromethyl ketone Z-PheCH_2Cl and Shaw and his coworkers [5] studied the inhibition by lysine containing peptide chloromethyl ketones.

A crystallographic determination of the binding modes of peptide chloromethyl ketones to subtilisin BPN′ has illuminated the nature of the interactions of these inhibitors with this serine protease [6]. The inhibitors are bound to the enzyme via a covalent linkage between the imidazole ring of the active site histidine residue (His-64) and the methylene group of the chloromethyl ketone moiety. The peptide chain of an extended inhibitor and a section of three residues of the peptide backbone of the enzyme form an anti-parallel β-sheet structure. A new study has shown that in addition to the covalent bond between the histidine and the inhibitor moiety, there is a covalent linkage between the γ-oxygen of the catalytic Ser-221 and the carbonyl carbon of the inhibitor [7]. The tetrahedral hemiketal thus formed is stabilized by additional hydrogen bonds.

The rates of inactivation of the structurally homologous serine protease chymotrypsin A_α by peptide chloromethyl ketones in solution are dependent upon the interactions, observed in the crystallographic model [8], between the enzyme and inhibitor both in the region of the primary specificity site and at subsites far removed from the catalytic site [9]. In this paper, we report studies which demonstrate that the rates of reaction of subtilisin with chloromethyl ketones can also be correlated both with the crystallographic model and with the known "secondary substrate specificity" of this enzyme.

Materials and Methods

Peptide chloromethyl ketones. The syntheses of most of the peptide chloromethyl ketones used in the present study were reported earlier [8,10—12]. Four previously unreported chloromethyl ketones synthesized were: Ac-Ala-LeuCH_2Cl, m.p. 95—98° C (recrystallized from ethyl acetate/light petroleum); Z-Gly-Gly-LeuCH_2Cl, an oil that could not be induced to crystallize; Ac-Gly-Gly-Ala-PheCH_2Cl, m.p. 222—224° C (recrystallized from methanol/water); Ac-Phe-Gly-Ala-LeuCH_2Cl, m.p. 167—168° C (recrystallized from ethyl acetate). All amino acids used were of the L-configuration. All chloromethyl ketones were shown to be pure by thin-layer chromatography on silica gel G plates. Elemental microanalyses (performed by Atlantic Microlab, Inc., Atlanta, Ga., U.S.A.), proton magnetic resonance spectra, and mass spectra were consistent

with the assigned structure of each chloromethyl ketone.

The key intermediate in the synthesis of the leucine chloromethyl ketones was L-leucine chloromethyl ketone hydrochloride which was obtained in 71% yield by reaction of gaseous HCl with *t*-butyloxycarbonyl-L-leucine diazoketone. This reaction simplifies the procedure for the preparation of amino acid chloromethyl ketones since the chloromethyl ketone functional group is formed concurrent with the removal of the *t*-butyloxycarbonyl blocking group. In previous syntheses, the chloromethyl ketone moiety and the amino blocking group were formed and removed in separate reactions. Further details concerning the synthesis of the inhibitors may be obtained upon request.

Reaction of subtilisin BPN' with the inhibitors. Subtilisin BPN' was obtained from Nagase and Co., Japan and was used without further purification. The substrate used was Ac-Tyr-OEt which was synthesized by standard methods and recrystallized from ethanol/water to constant melting point (monohydrate, m.p. 79.5—80.5°C). All inhibitor reactions were carried out at 30.0°C and in 0.1 M KH_2PO_4 buffer at pH 7.0. Stock inhibitor solutions were prepared at appropriate concentrations in 1,2-dimethoxyethane which had been redistilled over CaH_2 and stored over 4 Å molecular sieves. In the cases of Ac-Gly-Gly-Ala-PheCH_2Cl and Ac-Ala-Ala-Pro-AlaCH_2Cl, methanol was substituted for 1,2-dimethoxyethane due to the very low solubility of these compounds in 1,2-dimethoxyethane. Stock subtilisin solutions were freshly prepared each day by dissolving 1.9 mg of subtilisin in 1.0 ml of the 0.1 M KH_2PO_4 buffer at pH 7.0. The enzyme concentrations was determined by ultraviolet absorbance ($\epsilon^{1\%}_{1cm}$ = 11.7). The inhibition reaction was started by diluting 0.20 ml of the inhibitor solution to 2.00 ml with 0.1 M phosphate buffer and adding 0.20 ml of the stock subtilisin solutions. The final concentration of enzyme was $5 \cdot 10^{-6}$ M and of 1,2-dimethoxyethane 9% (v/v). Control experiments showed that this amount of 1,2-dimethoxyethane did not cause significant change in the specific activity of subtilisin over the time periods of the inhibition reactions. In all inhibition reactions, the final concentration of inhibitor was at least 10 times greater than the enzyme concentration in order to maintain pseudo first-order kinetics. Inhibitor concentrations were varied in order to obtain measurable reaction rates; concentrations were reduced in the cases of Z-Gly-Leu-PheCH_2Cl and Boc-Gly-Leu-PheCH_2Cl due to the low solubilities of these compounds.

At various time intervals, aliquots (100 or 150 μl) were removed from the reaction vessel and assayed for residual enzyme activity using *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) as substrate. In reactions with Ac-Phe-Gly-Ala-LeuCH_2Cl, the rate of inactivation was so rapid that only one point could be taken per reaction mixture. It was therefore necessary to run several reactions, taking one point at an appropriate time from each reaction. These points were then treated as a single data set as with all of the other reactions. The assay mixture contained 0.011 M substrate in 0.1 M KCl and 5% ethanol by volume (8% tetrahydrofuran was used in some earlier runs). Titration was performed with 0.1 M NaOH in a jacketed reaction vessel thermostated at 30°C. Initial velocities were obtained from the pH stat recorder tracings of base consumption with respect to time using the method of Henderson [13]. The pH stat was composed of a Radiometer titrator type TT11b, pH meter type PHM 26C, Autoburette type ABU 13 and Recorder type SBR 2C.

Under the conditions of the assay, the rate of Ac-Tyr-OEt hydrolysis was directly proportional to the enzyme concentration. For each inhibition reaction, the values of the kinetic parameters k_{obs} and $k_{2nd} = k_{obs}/[I]$ were calculated from the equations

$$v = k_{obsd}\,[E] = k_{2nd}\,[I]\,[E]$$

using a least-squares computer program. For Ac-Ala-PheCH_2Cl, Ac-Ala-Gly-PheCH_2Cl and Ac-Gly-Gly-Ala-PheCH_2Cl, a series of inhibition rates were measured at differing inhibitor concentrations. For these three compounds, the values of the kinetic parameters k_3/K_I, k_3 and K_I were calculated from Eqn. 3 also using a least-squares computer data fit. Correlation coefficients of better than 0.966 were obtained in all cases.

Results

Subtilisin inhibition studies

Subtilisin was irreversibly inhibited by a series of peptide chloromethyl ketones and good psuedo first-order kinetics were observed for all inhibition reactions. The inhibitor solutions were freshly prepared since we had previously observed peptide chloromethyl ketones to undergo a slow hydrolysis reaction upon standing in buffered aqueous solution [9,12]. Inhibitor solutions contained 9% (v/v) 1,2-dimethoxyethane in order to obtain reasonable concentrations of the inhibitors. The tetrapeptide inhibitors Ac-Gly-Gly-Ala-PheCH_2Cl and Ac-Ala-Ala-Pro-AlaCH_2Cl were reacted in a 9% (v/v) methanol solution due to their insolubility in 1,2-dimethoxyethane. A chloromethyl ketone derivative of tryptophan, Z-TrpCH_2Cl, was insoluble in both 9% (v/v) methanol and dimethoxyethane and it was necessary to use 30% (v/v) 1,2-dimethoxyethane in its inhibition reactions.

Table I shows the results for the fixed concentration experiments in which 16 chloromethyl ketones were used to inhibit subtilisin at pH 7.0 and 30°C. The results obtained by Morihara and Oka [4,14] with three chloromethyl ketones at pH 7.0 in 10% dioxane at 40°C are also listed. The best inhibitor is Ac-Phe-Gly-Ala-LeuCH_2Cl which is 5900 times faster than the least reactive inhibitor. Although Z-TrpCH_2Cl was the slowest inhibitor, its reaction rate was the only one measured at the higher 30% 1,2-dimethoxyethane concentration and we choose to use Z-PheCH_2Cl as the base compound for the calculation of relative reaction rates. In general the tri- and tetrapeptide inhibitors are substantially more reactive than chloro ketone derivatives of amino acids or dipeptides. Our results are readily comparable with those obtained by Morihara and Oka [4]. They observed a k_{obs} for the rate of inactivation of subtilisin by Z-PheCH_2Cl in 10% dioxane at 40°C which was 3.3 times faster than the one observed by us in 9% dimethoxyethane at 30°C (different rates are reported in ref. [14]). A rate increase of this magnitude can be explained simply on the basis of the temperature difference between the two studies. In calculating the relative $k_{obs}/[I]$ values from the data of Morihara and Oka [4] which is listed in Table I, we have therefore normalized their $k_{obs}/[I]$ values by a factor of 3.3 in order to compare them with our results at 30°C.

TABLE I

REACTION OF SUBTILISIN BPN' WITH PEPTIDE CHLOROMETHYL KETONES

Subtilisin concentration 5 μM, 9% (v/v) 1,2-dimethoxyethane, pH 7.0, 30°C.

Inhibitor P_5 P_4 P_3 P_2 P_1	[I] (mM)	Nos. of runs	$k_{obs} \times 10^4$ (s^{-1})		$k_{obs} \times 10/[I]$ ($M^{-1} \cdot s^{-1}$)		$k_{obs}/[I]$ relative values	
Z-TrpCH_2Cl [a]	0.1	1	0.009	(0.0006) [b]	0.09	(0.006) [b]	0.3	
Z-PheCH_2Cl	0.1	1	0.03	(0.004)	0.3	(0.04)	1.0	
Ac-Gly-PheCH_2Cl	1.4	4	0.34	(0.02)	0.24	(0.015)	0.8	
Ac-Ala-PheCH_2Cl	1.4	3	5.4	(0.8)	3.83	(0.45)	13	
Ac-Val-PheCH_2Cl	0.7	4	0.31	(0.04)	0.44	(0.06)	1.5	
Ac-Leu-PheCH_2Cl	0.7	2	0.25	(0.004)	0.35	(0.01)		
Ac-Leu-PheCH_2Cl	1.5	2	0.55	(0.03)	0.37	(0.02)	1.2 [c]	
Ac-Ala-LeuCH_2Cl	1.5	4	9.1	(0.94)	6.1	(0.6)	20	
Z-Gly-Gly-PheCH_2Cl	0.125	6	29.7	(2.1)	238	(17)		
Z-Gly-Gly-PheCH_2Cl	0.070	1	17	(2)	240	(20)	800 [c]	
Ac-Ala-Gly-PheCH_2Cl	0.25	2	12	(2)	50	(10)	167	
Boc-Ala-Gly-PheCH_2Cl	0.25	1	28	(3)	110	(10)	370	
Boc-Gly-Leu-PheCH_2Cl	0.25	1	5.8	(0.8)	23	(3)	77	
Z-Gly-Leu-PheCH_2Cl	0.126	1	4.25	(0.07)	34.2	(0.8)	114	
Z-Gly-Gly-LeuCH_2Cl	0.069	1	12	(1)	170	(10)		
Z-Gly-Gly-LeuCH_2Cl	0.073	1	9.9	(1.4)	140	(20)	517 [c]	
Ac-Phe-Gly-Ala-LeuCH_2Cl [d]	0.131	6	74.8	(1.4)	571	(11)		
Ac-Phe-Gly-Ala-LeuCH_2Cl [d]	0.122	6	61.0	(1.9)	500	(16)	1800	
Ac-Gly-Gly-Ala-PheCH_2Cl [e]	0.25	2	25	(4)	100	(17)	333	
Ac-Ala-Ala-Pro-AlaCH_2Cl [e]	0.27	2	15	(1)	57	(12)	191	
Z-PheCH_2Cl [f]	0.1	—	0.1	(0.4) [g]	1	(4) [g]	3.3	(1.0) [h]
Z-Ala-PheCH_2Cl [f]	0.1	—	0.8	(0.9) [g]	8	(9) [g]	27	(8.1) [h]
Z-Ala-Gly-PheCH_2Cl [f]	0.1	—	26	(31) [g]	260	(310) [g]	870	(260) [h]

[a] 30% (v/v) 1,2-dimethoxyethane.

[b] Parenthetical values are standard deviations.

[c] Based on the average value of $k_{obsd}/[I]$.

[d] Data composite of six reactions, one assay measured per reaction due to rapidlity of reaction.

[e] 9% (v/v) methanol.

[f] Results of Morihara and Oka [4], 10% dioxane, pH 7.0, 40°C.

[g] Values in parentheses are based on the results of Morihara et al. [14] using the same conditions as above.

[h] Normalized values so that Z-PheCH_2Cl has the same value in both data sets.

Kinetics of inactivation

The kinetics of inhibition of certain enzymes by irreversible inhibitors reveal the presence of a reversible complex between the enzyme and inhibitor preceding covalent bond formation [15,9]. The irreversible reaction of a site-specific inhibitor with an enzyme may be represented by the overall reaction sequence

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \overset{k_3}{\rightarrow} E - I \tag{1}$$

$$K_I = \frac{[E][I]}{[E \cdot I]} \tag{2}$$

where E · I represents a non-covalently bound complex of the enzyme with the inhibitor and E-I is the final product with the inhibitor irreversibly bound to the enzyme via a covalent linkage. Psuedo first-order kinetics are observed for the inhibition reaction if the initial inhibitor concentration is sufficiently greater than the total enzyme concentration. A pseudo first-order reaction at a fixed value of $[I]$ has the first-order rate constant, k_{obs}, as given by Eqn. 3 [15,9].

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_3[I]} + \frac{1}{k_3} \tag{3}$$

The most appropriate parameter to compare the reactivity of the various inhibitors is the inhibition parameter k_3/K_I which is equal to $k_{obs}/[I]$ if the inhibitor concentrations used are much smaller than K_I. If the inhibitor concentrations used are equal or close to K_I, then $k_{obs}/[I]$ will vary over a range of inhibitor concentrations and the kinetic constants k_3/K_I, k_3 and K_I may be determined using Eqn. 3. In the study of the reaction of peptide chloromethyl ketones with chymotrypsin A_α [9], the concentrations of the inhibitors used were less than K_I and $k_{obs}/[I]$ was invariant in the concentration range studied. In contrast, similar studies with elastase [12] showed that $k_{obs}/[I]$ varied in the range of inhibitor concentrations utilized allowing the evaluation of k_3 and K_I for several inhibitors. Therefore we decided to carry out concentration dependence studies with subtilisin in order to see if these kinetics constants could be measured for several inhibitors.

Concentration dependence studies

Table II shows the results for concentration-dependent experiments in which three chloromethyl ketones were used to inhibit subtilisin over a range of inhibitor concentrations. For each compound a set of inhibition runs was performed at concentrations with the highest inhibitor concentration limited by the solubility of the inhibitor or rapidity of reaction. For Ac-Ala-Gly-PheCH_2Cl and Ac-Gly-Gly-Ala-PheCH_2Cl, the $k_{obs}/[I]$ values increase as $[I]$ decreases over the range of $[I]$, which shows that the $[I]$ values of these inhibitors were large enough to be in the region of K_I. No such trend was observed with Ac-Ala-PheCH_2Cl.

The concentration dependence data listed in Table II was used to evaluate

TABLE II

CONCENTRATION-DEPENDENT INHIBITION OF SUBTILISIN WITH PEPTIDE CHLOROMETHYL KETONES

Subtilisin concentration 5 μM, 9% (v/v) 1,2 dimethoxyethane, pH 7.0, 30°C.

Inhibitor P_4 P_3 P_2 P_1	$[I] \times 10$ (mM)	$k_{obs} \times 10^4$ (s^{-1})	$k_{obs} \times 10/[I]$ ($M^{-1} \cdot s^{-1}$)
Ac-Ala-PheCH$_2$Cl	1.40	6.09	4.35
	1.12	4.61	4.12
	0.90	3.54	3.93
	0.72	2.74	3.81
	0.57	2.10	3.69
	0.46	1.74	3.78
Ac-Ala-Gly-PheCH$_2$Cl	0.25	10.7	42.8
	0.25	14.0	56.0
	0.20	10.1	50.6
	0.16	9.7	60.7
	0.128	6.5	51.0
	0.102	5.7	56.2
	0.082	5.3	64.2
Ac-Gly-Gly-Ala-PheCH$_2$Cl *	0.25	27.9	112
	0.20	26.7	134
	0.16	22.3	139
	0.128	20.9	163
	0.102	18.2	178

* 9% (v/v) methanol.

k_3/K_I, K_I and k_3. The values are listed in Table III. It was not possible to calculate the K_I and k_3 values for Ac-Ala—PheCH$_2$Cl since a plot of $1/k_{obs}$ versus $1/[I]$ for this compound intersected the $1/k_{obs}$-axis near the origin indicating that the inhibitor concentrations used were less than K_I and that Eqn. 3 reduced to $k_{obs}/[I] = k_3/K_I$.

The effectiveness of the three inhibitors can now be compared on the basis of their relative k_3/K_I values, which are superior to $k_{obs}/[I]$ values for the purpose of correlating the effect of structural changes on reactivities. It is apparent that the tripeptide inhibitor is approx. 20-fold better than the dipeptide chloroketone while extending the inhibitor length to a tetrapeptide results only in another 4-fold increase in k_3/K_I. The difference between the k_3/K_I values for Ac-Ala-Gly-PheCH$_2$Cl and Ac-Gly-Gly-Ala-PheCH$_2$Cl is primarily due to differences in K_I values. The limiting rates of inactivation (k_3) for both compounds are very similar, but the tetrapeptide is bound three times more effectively by the enzyme.

In the absence of enough information to determine the inhibition parameter k_3/K_I for all the inhibitors, it is possible to compare the reactivity of a series of inhibitors on the basis of their relative $k_{obs}/[I]$ values (Table I). The differences in the magnitude of the numbers for related inhibitors reflect mostly the effect of structural changes on the binding of the inhibitor to the enzyme (K_I) and on the rate of reaction within the bound complex (k_3). However $k_{obs}/[I]$

TABLE III

CONCENTRATION-DEPENDENT INHIBITION OF SUBTILISIN WITH PEPTIDE CHLOROMETHYL KETONES

Subtilisin concentration 5 μM, 9% (v/v) 1,2-dimethoxyethane, pH 7.0, 30°C. Values in parentheses are standard deviations.

Inhibitor P_4 P_3 P_2 P_1	k_3/K_I – ($M^{-1} \cdot s^{-1}$)	k_3/K_I (relative)	$k_3 \times 10^3$ (s^{-1})	K_I (mM)	[I] range (mM)
Ac-Ala-PheCH$_2$Cl	0.35 (0.008)	1.0	—	—	1.4—0.46
Ac-Ala-Gly-PheCH$_2$Cl	7.0 (0.84)	19	3.7 (1.7)	0.53 (0.26)	0.25—0.082
Ac-Gly-Gly-Ala-PheCH$_2$Cl *	29.4 (2.6)	84	4.56 (0.43)	0.15 (0.019)	0.25—0.102

* 9% (v/v) methanol.

values are subject to distortion from non-linear concentration effects when the inhibitor concentration is close to the inhibitor K_I value, as is probably the case for most of the tri- and tetrapeptide inhibitors in Table I. Comparison of the k_3/K_I (Table III) and the $k_{obs}/[I]$ values for Ac-Ala-Gly-PheCH_2Cl and Ac-Gly-Gly-Ala-PheCH_2Cl shows a small difference (a factor of 2 or 3). Therefore $k_{obs}/[I]$ values are useful in providing a rough guide to the relative reactivities of structurally related inhibitors.

Discussion

X-ray crystallographic studies of subtilisin BPN′ inhibited by several chloromethyl ketones have shown that these inhibitors are bound to the enzyme via covalent linkages between the imidazole ring of His-64 and the methylene group of the chloromethyl ketone moiety, and between the catalytic Ser-221 and the ketone carbonyl group of the inhibitor [6,7,16]. The benzyl group of the phenylalanine chloromethyl ketone residue fits snugly into a hydrophobic crevice. The inhibitor polypeptide chain in each case forms a system of hydrogen bonds of the anti-parallel β-sheet type with an extended segment of backbone chain in the enzyme consisting of residues Ser-125, Leu-126, and Gly-127. In most cases, the β-sheet hydrogen bonding structure was observed in the non-covalently linked complexes of subtilisin with peptide acids [17]. The S_1-P_1 hydrogen bond is not formed, however, and an additional hydrogen bond between the P_5 amino acid residue and the NH of Ser-130 has been proposed on the basis of model building experiments. The present study was undertaken to see if the solution reactivity of a series of peptide chloromethyl ketones could be correlated with the crystal structures of these inhibited subtilisin derivatives.

A schematic drawing of subtilisin inhibited by Ac-Phe-Gly-Ala-LeuCH_2Cl is shown in Fig. 1. The proposed interactions shown are those observed in X-ray studies with other chloromethyl ketones and with peptide acids. The amino acid residues of the inhibitor and the enzyme subsites are designated using the notation of Schechter and Berger [18] for the description of peptide binding subsites of proteolytic enzymes.

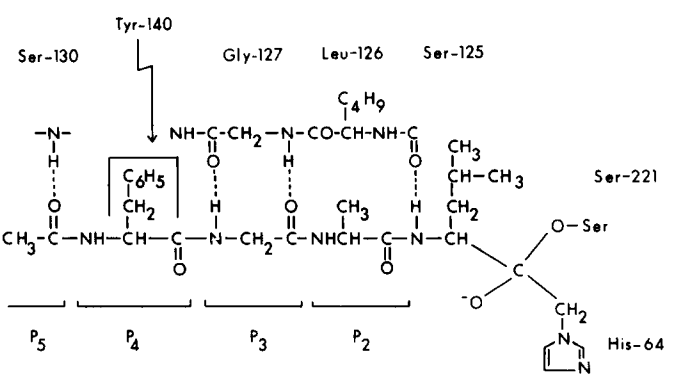

Fig. 1. A schematic representation of the inhibitor moiety Ac-Phe-Gly-Ala-LeuCH_2 bound to the active site of subtilisin BPN′. The probable interactions shown are based on crystallographic studies of subtilisin inhibited with other chloromethyl ketones [6,7,16].

TABLE IV

INTERACTIONS OF PEPTIDE CHLOROMETHYL KETONES WITH SUBTILISIN BPN′ SUBSITES

Inhibitor	$k_{obs}/[I]$	S_5	S_4	S_3		S_2	S_1	
		H bond [a]	Hydrophobic contact [b]	H bond [c]	H bond [d]	Hydrophobic contact [e]	H bond [f]	Hydrophobic contact [g]
Z-TrpCH_2Cl	0.3						+	+
Z-PheCH_2Cl	1						+	+
Ac-Gly-PheCH_2Cl	0.8				+		+	+
Ac-Ala-PheCH_2Cl	13				+	+	+	+
Ac-Val-PheCH_2Cl	1.5				+		+	+
Ac-Leu-PheCH_2Cl	1.2				+		+	+
Ac-Ala-LeuCH_2Cl	20				+	+	+	+
Z-Gly-Gly-PheCH_2Cl	800		+	+	+		+	+
Ac-Ala-Gly-PheCH_2Cl	167			+	+		+	+
Boc-Ala-Gly-PheCH_2Cl	370		+	+	+		+	+
Boc-Gly-Leu-PheCH_2Cl	77		+	+	+		+	+
Z-Gly-Leu-PheCH_2Cl	114		+	+	+		+	+
Z-Gly-Gly-LeuCH_2Cl	517		+	+	+		+	+
Ac-Phe-Gly-Ala-LeuCH_2Cl	1800	+	+	+	+		+	+
Ac-Gly-Gly-Ala-PheCH_2Cl	333	+	+	+	+	+	+	+
Ac-Ala-Ala-Pro-AlaCH_2Cl	191	+		+	+	+	+	
Z-PheCH_2Cl [h]	3.3						+	+
Z-Ala-PheCH_2Cl [h]	27				+		+	+
Z-Ala-Gly-PheCH_2Cl [h]	870		+	+	+		+	+

[a] Possible hydrogen bond with the NH of Ser-130.

[b] Hydrophobic contact with a crevice formed by Cδ1 of Ile-107, Leu-126-Gly-127-Gly-128 peptide bonds, Cδ2 of Leu-135 and the Tyr-104 side chain.

[c] Hydrogen bond with carbonyl group of Gly-127.

[d] Hydrogen bond with the NH of Gly-127.

[e] Hydrophobic contact with Cδ1 of Leu-96 and with the imidazole ring of His-64.

[f] Hydrogen bond with carbonyl group of Ser-125.

[g] Hydrophobic contact with the primary specificity crevice.

[h] Based on the data of Morihara and Oka [4].

The probable interactions between the peptide chloromethyl ketones investigated in this study and by Morihara and Oka [4] are outlined in Table IV along with the relative second-order rate constants $k_{obs}/[I]$, the value of Z-PheCH_2Cl being used as the standard. Examination of the data shows that there is a spread in $k_{obs}/[I]$ of 5900 between the least reactive chloroketone (Z-Trp-CH_2Cl) and the most reactive (Ac-Phe-Gly-Ala-LeuCH_2Cl). In general, the larger the number of interactions an individual inhibitor makes with the enzyme, the larger the second-order rate constant. This rate enhancement due to more favorable subsite interactions is evidenced by the large relative second-order rate constant (1800) for Ac-Phe-Gly-Ala-LeuCH_2Cl which is able to form four hydrogen bonds and participate in at least three favorable hydrophobic interactions with the enzyme subsites.

Extended binding site. The reactivity of the subtilisin inhibitors investigated is strongly influenced by the number of amino acid residues in the peptide chain of the inhibitor. This effect was first observed by Morihara and Oka [4]. The chloromethyl ketone derivatives of phenylalanine and tryptophan (Z-PheCH_2Cl and Z-TrpCH_2Cl) and most of the dipeptide inhibitors are approximately equally reactive. Those inhibitors which possess a P_3 (Ac-Ala-Gly-PheCH_2Cl) or a P_4 residue (Ac-Gly-Gly-Ala-PheCH_2Cl) have $k_{obs}/[I]$ values of over a 100-fold higher. This marked difference in the inhibition rates is evidence for an interaction between the inhibitors and the extended binding site in subtilisin.

Since the overall rate of reaction of an active site-directed irreversible inhibitor depends both on the amount of E · I complex present at equilibrium and the rate-limiting reaction of the bound inhibitor with the enzyme to form the inactivated enzyme (E-I), a change in the inhibitor structure could effect either K_I or k_3 or both. In the one case, (Table III) in which we evaluated the influence of a structural change (tripeptide to tetrapeptide inhibitor) separately on k_3 and K_I, we observed a change in both, although the 4-fold decrease in K_I was primarily the cause for the increased k_3/K_I value of the tetrapeptide inhibitor. However, with the exception of this one case, we are unable at present to discern whether a structural change in the inhibitor (and thus a change in the number of contacts it makes with subtilisin) is affecting the extent of binding to the enzyme, the stereoelectronic relationship between the inhibitor and enzyme in the E · I complex, or both.

P_1 residues. The primary specificity site (S_1) of subtilisin is composed of a crevice and the carbonyl oxygen of Ser-125 which can hydrogen bond with a substrate or inhibitor. The crevice is formed by three short segments of the enzyme's backbone and is lined principally by hydrophobic groups. One side of the crevice is planar and well-defined, while the other is less regular. This accounts for the fact that subtilisin can accommodate substrates which have P_1 amino acid residues either with aromatic rings (Phe, Tyr, Trp) or with branched hydrocarbon side chains (Leu). Chloromethyl ketone inhibitors with leucine as the P_1 residue thus are about as effective as those with a phenylalanine residue. This correlates with the observation of Morihara and Tsuzuki [19] that the k_{cat} and k_{cat}/K_m values for subtilisin-catalyzed hydrolysis of Ac-Leu-OMe and Ac-Phe-OEt vary by less than a factor of two.

A recent crystallographic investigation has shown that the S_1 crevice of sub-

tilisin can accommodate a lysine side chain of the inhibitor Phe-Ala-LysCH_2Cl [7]. The side chain methylenes bend into the crevice while the ϵ-amino group remains outside of the crevice hydrogen bonded to Glu-156 near the outer surface of the enzyme. This is obviously not as favorable an interaction as those observed with inhibitors with P_1 aromatic or alkyl side chains. This manifested in the fact that Z-LysCH_2Cl is not a subtilisin inhibitor [5] in contrast to Z-PheCH_2Cl. Under conditions essentially the same as those used in this study, Ala-Phe-LysCH_2Cl and Phe-Ala-LysCH_2Cl have $k_{obs}/[I]$ of approx. 0.0029 and 0.89 $M^{-1} \cdot s^{-1}$, respectively [5]. The values are similar to dipeptide inhibitors such as Ac-Ala-PheCH_2Cl (0.38 $M^{-1} \cdot s^{-1}$) and Ac-Ala-LeuCH_2Cl (0.61 $M^{-1} \cdot s^{-1}$) but are much lower than any of the tripeptide inhibitors which we investigated.

P_2 residues. Interactions at the S_2 subsite of subtilisin are much less well defined than those at S_1. No hydrogen bonds are formed, but the methyl side chain of inhibitors with alanine as the P_2 residue would make van der Waals contact with Cδ1 of Leu-96 and with the imidazole side chain of His-64. Those inhibitors with alanine as the P_2 residue are among our most effective. In the series of dipeptide chloromethyl ketones Ac-AA-PheCH_2Cl, the order of reactivity is AA = Ala $\gg$ Val $>$ Leu $>$ Gly. This preference has also been observed in amide substrates [20].

The contacts between subtilisin BPN′ and the methyl side chain of a P_2 alanine residue are optimum both for inhibition and substrate hydrolysis. A smaller residue (glycine) allows greater freedom of motion and hence probably retards proper alignment of the substrate or the inhibitor. A larger side chain (leucine or valine) either hinders binding of the inhibitor to the enzyme or proper alignment within the E · I complex. One inhibitor, Ac-Val-PheCH_2Cl, was synthesized in order to examine the possibility that additional branching at the β-carbon of the P_2 side chain might slow or prevent inhibition. This did not occur as Ac-Val-PheCH_2Cl was about as reactive as Ac-Leu-PheCH_2Cl. Probably there is too much flexibility in the E · I complex of dipeptide inhibitors with subtilisin and branched side chains can be accommodated. In longer inhibitors, a leucine residue at P_2 is a distinct disability compared to glycine (compare Z-Gly-PheCH_2Cl (800) with Z-Gly-Leu-PheCH_2Cl (114) and Boc-Ala-Gly-PheCH_2Cl (370) with Boc-Gly-Leu-PheCH_2Cl (77)). These inhibitors form one more hydrogen bond with subtilisin than the dipeptides.

P_3 and P_4 residues. The P_3-S_3 interaction involves two additional hydrogen bonds. Examination of the relative $k_{obs}/[I]$ values in Table IV shows that the extension of the inhibitor from a dipeptide to a tripeptide results in a large increase in the relative rates of inhibition. This large increase can probably be ascribed to tighter binding of the inhibitor to the enzyme because of the two additional hydrogen bonds.

Extension of the inhibitor to the S_4 subsite of subtilisin results in another increase in the relative $k_{obs}/[I]$ values, although not as great as that observed upon extension from a dipeptide to a tripeptide. However, an increase in the inactivation rate is evident if the P_4 residue has an aromatic or large hydrophobic group since the S_4 subsite exhibits a distinct preference for these groups. In subtilisin inhibited by Ac-Ala-Gly-PheCH_2Cl, the acetyl methyl group abuts the Tyr-104 side chain with no discernible motion of the latter. However, in the Z-Ala-Gly-PheCH_2Cl and Z-Gly-Gly-PheCH_2Cl subtilisin derivatives, a rotation of

the C_α-C_β bond of the Tyr-104 side chain has occurred with the formation of a hydrophobic pocket [6]. The phenyl ring of the benzyloxycarbonyl groups of the inhibitors are located in this pocket. Examination of the data in Table IV shows that this interaction is indeed favorable and the solution data can be correlated with the crystallographically observed interactions. Our four more reactive inhibitors (Ac-Phe-Gly-Ala-LeuCH_2Cl, Z-Gly-Gly-PheCH_2Cl, Z-Gly-Gly-LeuCH_2Cl, Boc-Ala-Gly-PheCH_2Cl) and the most effective inhibitor (Z-Ala-Gly-PheCH_2Cl) reported by Morihara and Oka [4], have either an aromatic (Z- or the side chain of phenylalanine) or a large hydrophobic (Boc-) group at the P_4 position. The presence of such a group is worth at least a 2—10-fold increase in inhibition rate. The contribution of the aromatic group at S_4 is readily observed when comparing Ac-Gly-Gly-Ala-PheCH_2Cl (333) with Ac-Phe-Gly-Ala-LeuCH_2Cl (1800). Approximately a 5-fold increase in reaction rate is observed when phenylalanine is substituted for glycine at P_4 (although part of this increase is probably due to the P_1 phenylalanine to leucine change).

P_5 residues. An inhibitor with a P_5 residue could form an additional hydrogen bond with the backbone -NH- of Ser-130 (see Fig. 1) [17]. The contribution of the additional hydrogen bond at S_5 is difficult to estimate. The tetrapeptide inhibitor Ac-Gly-Gly-Ala-PheCH_2Cl (333) has a $k_{obs}/[I]$ value double that of the tripeptide Ac-Ala-Gly-PheCH_2Cl (167). But this difference could be due completely to the favorable interaction of the P_2 alanine in the former with the S_2 binding site of the enzyme. The tetrapeptide inhibitor Ac-Ala-Ala-Pro-AlaCH_2Cl (191), which lacks an aromatic or large hydrophobic residue at P_1 is slightly faster than Ac-Ala-Gly-PheCH_2Cl (167). Again this might be due to a favorable interaction of the P_2 residue, proline in this case, with the S_2 subsite rather than to the addition hydrogen bond at S_5. Substrates with proline at P_2 are hydrolyzed at one-third the rate of those with alanine at P_2 [20]. The fact that Ac-Ala-Ala-Pro-AlaCH_2Cl is a subtilisin inhibitor is not surprising in light of the observation of Morihara and Oka [21] that the k_{cat}/K_m value for the subtilizin BPN′-catalyzed hydrolysis of Ac-Ala-Ala-Ala-OMe is almost as large as that for Ac-Ala-Ala-Phe-OMe.

Comparison with substrates. An excellent correspondence is observed between the rates of inhibition of subtilisin by peptide chloromethyl ketones and the rates of peptide hydrolysis by the enzyme. For example, the relative $k_{obs}/[I]$ values for the series of inhibitors Ac-AA-PheCH_2Cl in which the P_2 residue is varied, are AA = Gly (1) < Leu (1.5) < Ala (14). For the subtilisin-catalyzed hydrolysis of the dipeptide amides Z-AA-Leu-NH_2 [20], the relative rates of hydrolysis (the relative k_{cat}/K_m values are almost the same) are AA = Gly (1) < Leu (2.4) < Ala (14). For the tripeptide amides Z-Gly-AA-Leu-NH_2, the relative hydrolysis rates are AA = Gly (1) < Ala (14) and for the tyrosine amides Ac-AA-Tyr-NH_2 [21], the relative rates are AA = Gly (1) < Ala (11). The correlation between substrates and inhibitors is not only qualitative, but is almost quantitative!

A comparison of the effect of extending the length of the peptide chain in inhibitors or substrates on the rates of inhibition or hydrolysis also shows good correspondence. The substrates Z-Gly-Leu-NH_2 and Z-Ala-Gly-Gly-Leu-NH_2 have hydrolysis rates in the ratio of 1 : 87 [20], while the inhibitors Ac-Ala-PheCH_2Cl and Ac-Gly-Gly-Ala-PheCH_2Cl have $k_{obs}/[I]$ values with a ratio of

1 : 25. The substrates Z-Leu-NH_2 and Z-Gly-Gly-Leu-NH_2 have rates in ratio of 1 : 60, while the chloromethyl ketones Z-PheCH_2Cl and Z-Gly-Gly-PheCH_2Cl have $k_{obs}/[I]$ with the ratio of 1 : 800. The substrates Ac-Phe-OMe, Ac-Ala-Phe-OMe and Ac-Ala-Ala-Phe-OMe have k_{cat}/K_m values with the ratio of 1 : 64 : 260 [21] while the corresponding chloromethyl ketones Z-PheCH_2Cl, Ac-Ala-PheCH_2Cl and Ac-Ala-Gly-PheCH_2Cl have $k_{obs}/[I]$ values with the ratio 1 : 13 : 167. The correspondence is again quite good even though structurally non-identical systems are compared.

The positive correlation between inhibition rates and hydrolysis rates provides convincing evidence for the postulate that the productive binding modes of subtilisin with peptide chloromethyl ketones which leads to alkylation of His-64 is closely related to the productive binding modes of peptide substrates which led to the acylation of Ser-221. Similar correlations have been made with the serine proteases chymotrypsin [9] and elastase [12].

Inhibitor design. It is now well established that the known substrate specificity (both primary and secondary) can be used to design effective chloromethyl ketone inhibitors for serine proteases. The rates of inhibition of both chymotrypsin and subtilisin by this class of inhibitors are correlated both with the crystallographic models and with previous kinetic work with peptide substrates.

A further goal in the design of enzyme inhibitors is specificity. Using Tos-LysCH_2Cl and Tos-PheCH_2Cl, Schoellmann and Shaw [2] first showed that it was possible to selectively inhibit serine proteases the preferred substrates of which varied considerably in stereoelectronic characteristics. This observation was amplified when we showed that the peptide chloromethyl ketone elastase inhibitors were essentially unreactive toward trypsin and chymotrypsin [12]. The question might now be posed whether it is possible to selectively inhibit one enzyme out of two very similar enzymes (subtilisin and chymotrypsin) or an enzyme from one species and not the same enzyme in another species. Obviously this question is of profound importance in drug design. If one lists the subsite preferences for these enzymes (chymotrypsin: S_1, aromatic amino acid residue; S_2, leucine; S_4, non-aromatic group or amino acid residue; subtilisin: S_1, aromatic amino acid residue or leucine; S_2, alanine; S_4, aromatic group or amino acid residue), enough differences are observed to make the design of mutually exclusive inhibitors a practical possibility (it has already been shown that Tos-PheCH_2Cl, an effective chymotrypsin inhibitor, will not inhibit subtilisin [22]). Since the amino acid substitutions that occur when an enzyme from one species is compared to one from another species are likely to occur in regions removed from the primary specificity site, we feel that secondary specificities can be utilized to design more reactive and more selective inhibitors.

As soon as the subsite differences between subtilisin and chymotrypsin became apparent, we designed and synthesized the ideal subtilisin inhibitor Ac-Phe-Gly-Ala-LeuCH_2Cl. This inhibitor contains all the residues which inhibitor studies have shown to be ideal for inhibition of subtilisin. All of these interactions combine to make Ac-Phe-Gly-Ala-LeuCH_2Cl the fastest chloromethyl ketone inhibitor of subtilisin yet reported. (It should be noted that Z-Gly-Ala-Leu-NH_2 with the same positive features as Ac-Phe-Gly-Ala-LeuCH_2Cl is the

best amide substrate known for subtilisin [20]).

The inhibitor Ac-Phe-Gly-Ala-LeuCH_2Cl also inhibits chymotrypsin ($[E]$ = 5 μM, $[I]$ = 125 μM, 0.09 M acetate, pH 5.70, 9% dimethoxyethane, 30°C, $k_{obs} = 3.44 \cdot 10^{-6}\ s^{-1}$, $k_{obs}/[I] = 2.76\ M^{-1} \cdot s^{-1}$). These conditions were selected to be comparable with those used in an earlier study of the reactivity of chloromethyl ketones with chymotrypsin [9]. The rate of inhibition is approximately twice as fast as most dipeptide chloromethyl ketones with a P_1-Phe, but is slower than inhibitors such as Ac-Leu-PheCH_2Cl and all the tripeptide inhibitors. The slow rate of chymotrypsin inhibition by Ac-Phe-Gly-Ala-LeuCH_2Cl is not surprising since the P_4-Phe, P_2-Ala and P_1-Leu are not ideal and may be negative influences on inactivation rate. When the rate of inhibition is measured at pH 7.0 (5 μM chymotrypsin, $[I]$ = 125 μM, $k_{obs} = 2.89 \cdot 10^{-3}\ s^{-1}$, $k_{obs}/[I]$ = $23.09\ M^{-1} \cdot s^{-1}$), the inhibitor reacts 2.5 times more rapidly with subtilisin than chymotrypsin. Thus although Ac-Phe-Gly-Ala-LeuCH_2Cl is a poor inhibitor for chymotrypsin relative to other chloromethyl ketones, its selectivity for subtilisin is only 2.5-fold since subtilisin is less susceptible to inhibition by chloromethyl ketones than is chymotrypsin.

In conclusion, we have shown that crystallographically observed interactions can be used in conjunction with solution kinetic studies to design more effective active site-directed inhibitors of enzymes. In addition, it is possible to obtain inhibitors with kinetic selectivity for two similar enzymes (chymotrypsin and subtilisin) by using the differing subsite preferences.

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