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Kinetics of the Reaction of Chymotrypsin A_α with Peptide Chloromethyl Ketones in Relation to its Subsite Specificity†

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ABSTRACT: The kinetics of the reaction of a series of peptide chloromethyl ketones with chymotrypsin A_α were investigated in order to relate rates of reaction in solution with the number of interactions which are observed in the crystallographic model of the inhibited enzyme (Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971), *Biochemistry 10*, 3728). The second-order rate constant (k_{obsd}/[I]) obtained at pH 5.02 and 5.80 in either 9 or 30% 1,2-dimethoxyethane varied by a factor of 27 from the slowest (CHO-PheCH_2Cl) to the fastest inhibitors (Z-Gly-Leu-PheCH_2Cl and Boc-Gly-Leu-PheCH_2Cl). The variation in the rates of inhibition with changing inhibitor structure could be a function of strength of binding of the inhibitor to the enzyme, stereoelectronic effects on the bond-forming step between enzyme and enzyme-bound inhibitor, or both. The increased reactivity of inhibitors containing a leucyl residue as the P_2 residue (Ac-Leu-PheCH_2Cl/Ac-Ala-PheCH_2Cl = 2.9, Boc-Gly-Leu-PheCH_2Cl/Boc-Ala-Gly-PheCH_2Cl = 3.5–3.9) is the result of a hydrophobic interaction between the Leu residue of the inhibitor and Ile-99 of the enzyme. This result correlated nicely with the previous observed "secondary specificity" of chymotrypsin for substrates with bulky aliphatic side chains as the P_2 residue. Inhibitors containing three amino acid residues reacted faster than Ac-PheCH_2Cl or dipeptide chloromethyl ketones. This again agrees with the crystallographic model since tripeptide inhibitors could form a β-sheet structure involving three hydrogen bonds with the enzyme while the others would form fewer hydrogen bonds. These results strengthen the view that the solution reactivity of peptide chloromethyl ketones and substrates can be explained on the basis of the crystal structures of chloromethyl ketone inhibited chymotrypsin derivatives.

The crystallographic determinations of the binding modes of peptide chloromethyl ketones to chymotrypsin A_γ (Segal *et al.*, 1971a,b) and to subtilisin BPN′ (Kraut *et al.*, 1971; Robertus *et al.*, 1972) have provided revealing insights into the interactions of inhibitors with these serine proteases. The inhibitors used in these studies are related to Tos-PheCH_2Cl, the stereospecific, active site-directed inhibitor of chymotrypsin which was designed by Schoellmann and Shaw (1963) and which is known to react irreversibly with His-57 in the enzyme (Ong *et al.*, 1964, 1965). For the crystallographic studies, the tosyl group of Tos-PheCH_2Cl was replaced by peptide chains of varying length so that the inhibitors would more closely resemble natural peptide substrates and would interact with subsites of the enzyme on the N-terminal side of the scissile bond of a peptide substrate. It was found that both enzymes presented an extended binding site composed of at least three subsites in the case of chymotrypsin and four in the case of subtilisin. Furthermore, the extended binding sites in the two proteases were found to be very similar in three-dimensional structure (Kraut *et al.*, 1971).

Although the crystallographic results are very convincing, great care must be exercised in extrapolating the structural features of crystalline enzyme–inhibitor complexes to models of the intermediates which take part in the dynamic processes of enzyme catalysis in solution. Relevant data from various types of physical chemical experiments in solution are required before conclusions drawn from the crystalline state can be satisfactorily evaluated.

One direct approach is to examine the crystallographic model for possible interactions between subsites and various amino acid residues substituted into the inhibitor chain, predict how these interactions would affect the binding of analogous substrates, and test the prediction by kinetic measurements of the hydrolysis rates of the corresponding synthetic peptide substrates. The results of some studies of this kind have been reported (Segal *et al.*, 1971b; Segal, 1972) and they

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‡ Dr. Philip E. Wilcox died on Nov 2, 1971. This is a great loss to those who have known him personally, or followed his work in protein chemistry.

TABLE I: Reaction of α-Chymotrypsin with Peptide Chloromethyl Ketones.[a]

Inhibitor	[I] × 10^3 (M)	k_{obsd} × 10^4 (sec^{-1})	k_{obsd}/[I] (M^{-1} sec^{-1})	Av k_{obsd}/[I] (Rel Values)
CHO-PheCH$_2$Cl	0.81	0.41 (0.02)[b]	0.05	
	0.608	0.21 (0.08)	0.04	0.05 (.33)
	0.405	0.20 (0.90)	0.05	
	0.203	0.11 (0.07)	0.05	
Ac-PheCH$_2$Cl	0.81	1.17[c] (0.07)	0.14	
	0.608	0.93[c] (0.08)	0.15	0.15 (1.0)
	0.405	0.60[c] (0.07)	0.15	
	0.203	0.30[c] (0.05)	0.15	
Tos-PheCH$_2$Cl	0.27	0.30 (0.03)	0.11	0.12 (.8)
	0.135	0.18 (0.02)	0.13	
Ac-Gly-PheCH$_2$Cl	0.81	1.03[c] (0.12)	0.13	
	0.608	0.80[c] (0.06)	0.13	0.13 (.87)
	0.405	0.55[c] (0.09)	0.14	
	0.203	0.28[c] (0.06)	0.14	
Ac-Ala-PheCH$_2$Cl	0.81	0.86[c] (0.04)	0.10	
	0.608	0.66[c] (0.09)	0.11	0.11 (.73)
	0.405	0.44[c] (0.06)	0.11	
	0.203	0.20[c] (0.03)	0.10	
Ac-Leu-PheCH$_2$Cl	0.81	2.68[c] (0.19)	0.33	
	0.608	1.86[c] (0.18)	0.31	0.32 (2.1)
	0.405	1.29[c] (0.11)	0.32	
	0.203	0.65[c] (0.07)	0.32	
Z-Gly-Gly-PheCH$_2$Cl	0.81	2.64 (0.32)	0.33	
	0.608	1.97 (0.17)	0.32	0.32 (2.1)
	0.405	1.33 (0.29)	0.33	
	0.203	0.65 (0.04)	0.32	
Boc-Ala-Gly-PheCH$_2$Cl	0.81	2.56[c] (0.29)	0.32	
	0.608	1.87[c] (0.25)	0.33	0.33 (2.2)
	0.405	1.32[c] (0.21)	0.31	
	0.203	0.69[c] (0.17)	0.34	
Ac-Ala-Gly-PheCH$_2$Cl	0.81	7.56[c] (0.77)	0.93	
	0.608	4.88[c] (0.58)	0.80	0.88 (5.9)
	0.405	3.30[c] (0.34)	0.82	
	0.203	1.95[c] (0.21)	0.96	
Boc-Gly-Leu-PheCH$_2$Cl[d]	0.18	2.30 (0.17)	1.28	
	0.135	1.65 (0.23)	1.22	1.29 (8.6)
	0.09	1.19 (0.16)	1.32	
	0.045	0.59 (0.18)	1.32	

[a] Chymotrypsin concentration 40 μM, pH 5.02 acetate buffer, 9% (v/v) 1,2-dimethoxyethane, 30.0°. [b] Values in parentheses are standard deviations. [c] Averages of at least five runs. [d] 8 μM enzyme solution was employed with this inhibitor.

provide support for proposals that certain enzyme–substrate intermediates have structures very similar to the structures of the enzyme–inhibitor complexes in the crystal.

The approach of the work presented in this paper is along a different line in that it is directed toward the following questions. (1) Are the rates of reaction of peptide chloromethyl ketones with chymotrypsin related to the number of interactions which are observed in the crystallographic model of the inhibited enzyme? (2) Are these rates correlated with the known "secondary specificities" of this enzyme (Neil *et al.*, 1966; Yamamoto and Izumiya, 1966; Yamashita, 1960)? Positive correlations would add convincing evidence to the postulate that the productive binding mode for chloromethyl ketones which leads to alkylation of His-57 is closely related to the productive binding modes of peptide substrates which lead to the acylation of Ser-195.

Materials and Methods

Chymotrypsin A_α was obtained from Worthington Biochemical Corp. (lot CDIOBK) and was used without further purification. The substrate, Ac-Tyr-OEt, was synthesized in the laboratory by standard methods and recrystallized from ethanol–water to constant melting point (79.5–81°). *1,2-Dimethoxyethane* was purchased from J. T. Baker Chemical Co. and was analytical grade. All other common chemicals were also analytical grade.

The synthesis and characterization of the inhibitors, all derivatives of phenylalanine chloromethyl ketone, have been described in a previous publication (Segal *et al.*, 1971a,b) with the exception of the two described below.

N-Formyl-L-phenylalanyl Chloromethyl Ketone (CHO-PheCH$_2$Cl) was prepared by a method similar to that reported by

TABLE II: Reaction of α-Chymotrypsin with Peptide Chloromethyl Ketones.[a]

Inhibitor	[I] × 10^3 (M)	k_{obsd} × 10^4 (sec^{-1})	k_{obsd}/[I] (M^{-1} sec^{-1})	Av k_{obsd}/[I] (Rel Values)
CHO-PheCH$_2$Cl	0.81	4.48 (0.55)[b]	0.55	0.49 (0.48)
	0.608	3.42 (0.46)	0.56	
	0.405	2.00 (0.23)	0.50	
	0.203	0.71 (0.10)	0.35	
Ac-PheCH$_2$Cl	0.81	8.16 (0.52)	1.01	1.03 (1.0)
	0.608	6.70 (0.17)	1.10	
	0.405	3.98 (0.15)	0.99	
	0.203	2.04 (0.08)	1.01	
Ac-Gly-PheCH$_2$Cl	0.81	16.10 (3.01)	1.76	1.78 (1.7)
	0.608	12.38 (0.22)	1.80	
	0.405	7.76 (1.10)	1.69	
	0.203	4.10 (1.03)	1.79	
Ac-Ala-PheCH$_2$Cl	0.81	11.40 (0.49)	1.40	1.33 (1.3)
	0.608	8.02 (0.54)	1.32	
	0.405	5.79 (0.27)	1.29	
	0.203	2.62 (0.41)	1.29	
Boc-Ala-Gly-PheCH$_2$Cl	0.81	32.50 (3.00)	3.41	3.37 (3.3)
	0.608	22.00 (2.50)	3.30	
	0.405	13.50 (0.88)	3.34	
	0.203	6.91 (0.64)	3.41	

[a] Chymotrypsin concentration 40 μM, pH 5.80 acetate buffer, 9% (v/v) 1,2-dimethoxyethane, 30.0°. [b] Values in parentheses are standard deviations.

Shaw and Ruscica (1971) and had mp 148.5–149.5 (ethanol–water).

Anal. Calcd for $C_{11}H_{12}ClNO$: C, 58.53; H, 5.36; N, 6.18. Found: C, 58.36; H, 5.43; N, 6.29.

*N-Benzyloxycarbonylglycyl-*L*-leucyl-*L*-phenylalanyl Chloromethyl Ketone* (Z-Gly-Leu-PheCH$_2$Cl). The mixed anhydride formed from 3.2 g (10 mmol) of benzyloxycarbonylglycyl-L-leucine, 1.1 ml (10 mmol) of *N*-methylmorpholine and 1.34 ml (10 mmol) of isobutyl chloroformate in 60 ml of H_4furan was reacted with L-phenylalanine chloromethyl ketone hydrobromide and 1.3 ml of *N*-methylmorpholine for 1.25 hr. Work-up of the reaction mixture involved evaporation of the H_4furan, extraction of the residue into ethyl acetate, washing with citric acid and sodium bicarbonate solutions, drying over magnesium sulfate, and evaporation. The product was recrystallized from ethyl acetate–cyclohexane to yield 3.41 g (68%) of product with mp 140.5–143°. *Anal.* Calcd for $C_{26}H_{32}ClN_3O_5$: C, 62.20; H, 6.43; N, 8.39. Found: C, 62.00; H, 6.14; N, 8.16.

Reaction of Chymotrypsin with Inhibitors. All inhibition experiments were carried out at 30.0° and at pH 5.02 or 5.80. A solution of inhibitor was prepared by dissolving a weighed amount of inhibitor in 1 or 3 ml of dimethoxyethane and then diluting to 10.0 ml with an 0.09 M acetate buffer. The reaction was started by mixing 0.90 ml of the inhibitor solution with 0.10 ml of 400 μM chymotrypsin A_α. The final concentration of enzyme was 40 μM and of dimethoxyethane 9 or 30%. Control experiments showed that this amount of dimethoxyethane did not cause any change in the specific activity of native chymotrypsin over the time periods of the experiments. The concentration of inhibitors was varied from 0.203 to 0.8 mM except that all concentrations were reduced fourfold in the case of Z-Gly-Leu-PheCH$_2$Cl and Boc-Gly-Leu-Phe-CH$_2$Cl because of the low solubilities of these compounds.

At various time intervals, an aliquot (10 μl) was removed from the inhibition mixture and spectrophotometrically assayed for residual enzyme activity using acetyltyrosine ethyl ester as a substrate. The initial rate of liberation of *N*-acetyl-L-tyrosine was measured at 237 mμ in a 0.1 M potassium phosphate–5% ethanol (v/v) (pH 7.0) buffer using a Cary 15 recording spectrophotometer with the cell compartment thermostated at 25.0°.

Values of k_{obsd} for each experiment were calculated from the equation $\log v/v_0 = -k_{obsd}t$ using $\log v/v_0$ and t as input into an Olivetti Underwood Programma 101 computer operating on a least-squares program. The initial rate of hydrolysis of Ac-Tyr-OEt for an aliquot taken at zero time is v_0 and v is the initial rate of hydrolysis for an aliquot taken at time t.

Results

Inhibitions of chymotrypsin A_α with a series of peptide chloromethyl ketones were followed as a function of time at 30.0°, pH 5.02 or 5.80. Good pseudo-first-order kinetics were observed in all cases. In Table I, the results at 30.0°, pH 5.02, and 9.0% 1,2-dimethoxyethane are shown. This concentration of dimethoxyethane was employed to be as low as possible but high enough to completely dissolve every inhibitor at this temperature. Dimethoxyethane was chosen as the solvent because peptide chloroketones have greater solubility than in tetrahydrofuran or dioxane. Although ethanol is a good solvent, higher concentrations result in denaturation of chymotrypsin. All inhibitor solutions were freshly prepared since it was observed that Ac-Gly-PheCH$_2$Cl was hydrolyzed to the extent of 30% after 20-hr standing in a buffer solution as measured by a lower k_{obsd} of inhibition. In Table II, the results of inhibitions at pH 5.80 and 9% dimethoxyethane are shown.

TABLE III: Reaction of α-Chymotrypsin with Peptide Chloromethyl Ketones.[a]

Inhibitor	[I] × 10^3 (M)	k_{obsd} × 10^4 (sec^{-1})	k_{obsd}/[I] (M^{-1} sec^{-1})	Av k_{obsd}/[I] (Rel Values)
Ac-PheCH$_2$Cl	0.706	1.85 (0.26)[b]	0.26	
	0.530	1.46 (0.10)	0.28	0.27 (1.0)
	0.353	0.99 (0.29)	0.28	
	0.177	0.48 (0.14)	0.27	
Ac-Leu-PheCH$_2$Cl	0.81	10.86 (0.67)	1.34	
	0.608	8.39 (0.92)	1.38	1.34 (5.0)
	0.405	4.76 (0.47)	1.17	
	0.203	2.96 (0.63)	1.46	
Boc-Ala-Gly-PheCH$_2$Cl	0.75	6.10 (0.33)	0.81	
	0.564	5.27 (0.74)	0.93	0.83 (3.1)
	0.375	2.94 (0.28)	0.78	
	0.187	1.52 (0.03)	0.81	
Boc-Gly-Leu-PheCH$_2$Cl	0.81	23.80 (1.85)	2.94	
	0.608	17.60 (3.16)	2.90	2.88 (10.7)
	0.405	11.33 (1.34)	2.80	
	0.203	5.86 (0.51)	2.89	
Z-Gly-Leu-PheCH$_2$Cl[c]	0.196	6.32 (0.38)	3.23	
	0.147	4.23 (1.08)	2.87	2.99 (11.1)
	0.098	2.85 (0.95)	2.91	
	0.049	1.45 (0.39)	2.95	

[a] Chymotrypsin concentration 40 μM, pH 5.80 acetate buffer, 30% (v/v) 1,2-dimethoxyethane, 30.0°. [b] Values in parentheses are standard deviations. [c] 9 μM enzyme was employed for the inhibition with this inhibitor.

This series of inhibitions was performed to study the effects of pH on k_{obsd} values. Generally, k_{obsd} values are about 10-fold greater at pH 5.80 than at pH 5.02. The pH optimum for the inhibition of chymotrypsin with Tos-PheCH$_2$Cl is 7.8 (Kézdy *et al.*, 1967) and even at pH 6, the inhibition was very sensitive to small pH changes. Since the X-ray crystallographic studies were performed on chymotrypsin A_γ crystals grown and inhibited with peptide chloromethyl ketones at *ca.* pH 5.6 (Powers and Wilcox, 1970; Segal *et al.*, 1971a), the pH's for the solution kinetic studies were chosen to be close to that value. In Table III, the results of inhibitions at pH 5.80 and in 30% dimethoxyethane are shown. This high concentration of dimethoxyethane was employed for this series of inhibitors since Z-Gly-Leu-PheCH$_2$Cl and Boc-Gly-Leu-PheCH$_2$Cl have low solubility in 9% dimethoxyethane. This concentration of dimethoxyethane did not cause any change in the specific activity of native chymotrypsin over the time periods of the experiment.

Kinetics of Inactivation. The kinetics of inhibition of certain enzymes by irreversible inhibitors reveal the presence of a reversible complex between enzyme and inhibitor preceding covalent bond formation. Examples of this behavior are the inhibition of cholinesterase by certain methanesulfonic acid esters (Kitz and Wilson, 1962), trypsin by Tos-LysCH$_2$Cl (Shaw and Glover, 1970), carboxypeptidase A by *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Petra, 1971) and chymotrypsin by MeSO$_2$-DL-PheCH$_2$Cl (Kumar and Hein, 1970). The irreversible reaction of a site-specific inhibitor with an enzyme may be represented by the overall reaction sequence

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot I \xrightarrow{k_3} E\text{–}I \qquad (1)$$

$$K_I = \frac{[E][I]}{[E\cdot I]} \qquad (2)$$

where E·I represents a noncovalently 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. If the inhibitor concentration is sufficiently greater than the total enzyme concentration, it can be shown (above references) that the decrease in E + E·I concentration in the inhibition mixture follows pseudo-first-order kinetics at any fixed value of I and the observed first-order rate constant is represented by eq 3 or its reciprocal form, eq 4. Therefore, in

$$k_{obsd} = \frac{k_3[I]}{K_I + [I]} \qquad (3)$$

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_3[I]} + \frac{1}{k_3} \qquad (4)$$

general k_{obsd} is not constant with changing values of [I]. If data are available over a sufficient range of inhibitor concentrations, both K_I (dissociation constant of the E·I complex) and k_3 (the limiting rate of inactivation) may be evaluated by the use of a double-reciprocal plot and eq 4. Examination of the data in Tables I, II, and III shows, contrary to the prediction of eq 3, the ratio of k_{obsd}/[I] for most chloromethyl ketones is constant within the limits of experimental error for a range of inhibitor concentrations up to 0.81 mM. This situation occurs when the K_I value is much greater than the chosen inhibitor concentrations and eq 3 reduces to

$$\frac{k_{obsd}}{[I]} = \frac{k_3}{K_I} \qquad (5)$$

which predicts that a reciprocal plot of $1/k_{obsd}$ *vs.* 1/[I] will pass through the origin and K_I cannot be evaluated.

An attempt was made to evaluate K_I for all the chloroketones listed in Tables I, II, and III using an Olivetti Underwood Programma computer operating on a least-squares program and using $1/k_{obsd}$ and $1/[I]$ values as input. In the majority of the cases the intercept was within one or two standard deviations of the origin indicating that K_I was much larger than the inhibitor concentrations employed. In a few cases we were able to evaluate K_I, but due to a lack of any significant change in $k_{obsd}/[I]$ values with changing inhibitor concentrations, we have no confidence in the calculated values.

The question now arises of whether a lower limit can be placed on K_I. Petra (1971) was able to evaluate K_I using a range of inhibitor concentrations with the highest being 13% of K_I. With an inhibitor concentration of 10% of K_I, a 9% change in $k_{obsd}/[I]$ would be expected. Since no changes in $k_{obsd}/[I]$ of this magnitude are observed with most of the chloroketones studied, we can place a lower limit on K_I of *ca.* 8 mM for these inhibitors. Kumar and Hein (1970) have determined a K_I of 14.9 mM for the inhibition of chymotrypsin A_α by $MeSO_2$-DL-$PheCH_2Cl$ at pH 6.15 and if only the L isomer is reacting a value of *ca.* 7.5 mM is obtained. Therefore a lower limit of *ca.* 8 mM for the K_I of inhibition of chymotrypsin A_α by most peptide chloromethyl ketones seems reasonable.

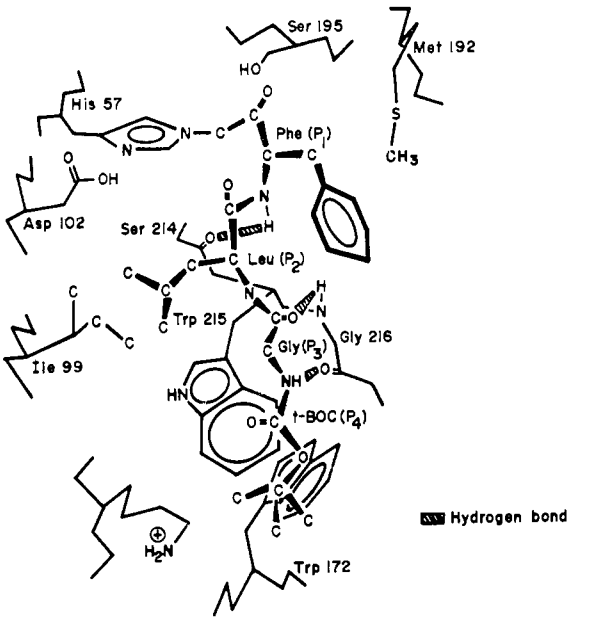

FIGURE 1: A schematic drawing of the inhibitor moiety, Boc-Gly-Leu-$PheCH_2Cl$, bound to chymotrypsin A_α. Only the portion of the enzyme which can interact with the inhibitor is shown.

Discussion

An X-ray crystallographic study of chymotrypsin A_γ inhibited by three peptide chloromethyl ketones (Ac-$PheCH_2Cl$, Ac-Ala-$PheCH_2Cl$, and Ac-Ala-Gly-$PheCH_2Cl$) has shown that these inhibitors are bound to the enzyme *via* a covalent linkage between the imidazole ring of His-57 and the methylene group of the chloromethyl ketone moiety (Segal *et al.*, 1971a,b). The benzyl group of the phenylalanyl residue occupies the so-called "tosyl pocket" (Matthews *et al.*, 1967; Birktoft *et al.*, 1970; Cohen *et al.*, 1969; Steitz *et al.*, 1969) and the longest inhibitor forms three hydrogen bonds with the enzyme, producing a β-sheet structure with main chain residues Ser-214, Trp-215, and Gly-216. A similar binding scheme has been observed with the bacterial protease, subtilisin BPN′ (Kraut *et al.*, 1971; Robertus *et al.*, 1972). The present study was undertaken to determine whether the solution reactivity of a series of peptide chloromethyl ketones could be correlated with the crystal structures of these inhibited chymotrypsin A_γ derivatives. Since no significant differences have been observed between chymotrypsin A_α and chymotrypsin A_γ upon direct comparison of their 5.5-Å resolution electron density maps (Cohen *et al.*, 1970) or upon examination of models of their active-site regions, we have carried out our solution kinetic work on the more readily available chymotrypsin A_α.

A proposed binding scheme for one of the peptide chloromethyl ketones used in this study is shown in Figure 1. This illustration is based on the crystallographically determined model of chymotrypsin inhibited with Ac-Ala-Gly-$PheCH_2Cl$ and shows one of our best inhibitors, Boc-Gly-Leu-$PheCH_2Cl$, linked to His-57 by a covalent linkage between the methylene group of the inhibitor and the imidazole nitrogen of the enzyme.

In the following discussion of the reactivity of various inhibitors in terms of this model, we have adopted the notation originally proposed by Schechter and Berger (1967) for describing peptide binding subsites for proteolytic enzymes. The individual amino acid residues of a substrate are designated P_1, P_2, etc., numbering from the amino acid which supplies the carbonyl of the peptide bond which is cleaved by the enzyme and numbering in the direction of the amino-terminal end of the substrate. The corresponding subsites of the enzyme which interact with substrate are designated S_1, S_2, etc. All inhibitors described in this paper have a phenylalanyl residue at P_1, the side chain of which would fit into the tosyl pocket while the NH would form a hydrogen bond with the carbonyl group of Ser-214. Those portions of the enzyme would be referred to as the S_1 binding subsite. Clearly this notation refers only to amino acid residues and their corresponding binding subsites and not to "abnormal" groups such as the *tert*-butyloxycarbonyl (Boc). However in order to avoid making the notation more complex by introducing further terms we will simply refer to such groups as if they were an additional amino acid residue. Thus Boc is the P_4 residue of the inhibitor Boc-Gly-Leu-$PheCH_2Cl$.

The possible interactions between all peptide chloromethyl ketones investigated and chymotrypsin are outlined in Table IV along with relative second-order rate constants $k_{obsd}/[I]$, the value of Ac-$PheCH_2Cl$ being set equal to 1.0 for various inhibition pH's and conditions. Examination of the data shows that there is a spread of a factor of 27 between the least reactive chloroketone (CHO-$PheCH_2Cl$) and the most (Z-Gly-Leu-$PheCH_2Cl$). And in general, the larger the number of interactions an individual inhibitor makes with the enzyme, the larger the second-order rate constant.

The overall rate of reaction of an active-site-directed irreversible inhibitor should depend 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 E–I, the inactivated enzyme. Covalent bond formation would certainly be influenced by the steric arrangement of inhibitor with enzyme in the E·I complex and by electronic effects. On the other hand, the dissociation constant K_I of the E·I complex is determined primarily by the increase in entropy upon binding the inhibitor to the enzyme. This increase is due to the destruction of some water structure around the water-separated inhibitor and enzyme when the E·I complex forms. It is

TABLE IV: Interactions of Peptide Chloromethyl Ketones with Chymotrypsin A_α Subsites.

Inhibitor	Rel k_{obsd}/[I] pH 5.02[a]	pH 5.80[a]	5.80[b]	S_4 Hydrophobic Contact[c]	S_3 H Bond[d]	S_3 H Bond[e]	S_2 Hydrophobic Contact[f]	S_1 H Bond[g]	S_1 Hydrophobic Contact[h]
CHO-PheCH$_2$Cl	0.33	0.48						+	+
Ac-PheCH$_2$Cl	1.0	1.0	1.0					+	+
Tos-PheCH$_2$Cl	0.80							+	+
Ac-Gly-PheCH$_2$Cl	0.87	1.7				+		+	+
Ac-Ala-PheCH$_2$Cl	0.73	1.3				+		+	+
Ac-Leu-PheCH$_2$Cl	2.1		5.0			+	+	+	+
Z-Gly-Gly-PheCH$_2$Cl	2.1			+	+	+		+	+
Boc-Ala-Gly-PheCH$_2$Cl	2.2	3.3	3.1	+	+	+		+	+
Ac-Ala-Gly-PheCH$_2$Cl	5.9				+	+		+	+
Z-Gly-Leu-PheCH$_2$Cl			11.1	+	+	+	+	+	+
Boc-Gly-Leu-PheCH$_2$Cl	8.6		10.7	+	+	+	+	+	+

[a] 9% 1,2-dimethoxyethane. [b] 30% 1,2-dimethoxyethane. [c] Hydrophobic contact with the indole ring of Trp-172 and Trp-215. [d] Hydrogen bond with the carbonyl group of Gly-216. [e] Hydrogen bond with the NH of Gly-216. [f] Hydrophobic contact with the side chain of Ile-99. [g] Hydrogen bond with the carbonyl group of Ser-214. [h] Hydrophobic contact with the tosyl pocket.

obvious that a structural change in the inhibitor could affect the extent of binding to the enzyme (measured by K_I), the stereoelectronic relationship between inhibitor and enzyme in the E·I complex (measured by k_3) or both. It has already been shown for inhibition of trypsin with Tos-LysCH$_2$Cl (K_I = 0.21 mM, k_3 = 0.16 min^{-1}) and LysCH$_2$Cl (K_I = 1.3 mM, k_3 = 0.50 min^{-1}) that the affinity of the inhibitor for the enzyme is not necessarily related to the rapidity of inactivation (Shaw and Glover, 1970). In the present study, due to the low inhibitor concentrations utilized, we were unable to evaluate these two effects. Thus the second order rate constant k_{obsd}/[I] reflects both the influence of K_I and k_3 (eq 5).

The slowest inhibitor is CHO-PheCH$_2$Cl which can only interact with the S_1 subsite of chymotrypsin. A two- or threefold increase in rate is observed upon changing the formyl to an acetyl group. The greater k_{obsd}/[I] value of Ac-PheCH$_2$Cl is probably caused by an additional hydrophobic interaction between the methyl group of the inhibitor and C_α–C_β of Trp-215. Ac-Gly-PheCH$_2$Cl and Ac-Ala-PheCH$_2$Cl are slightly slower than Ac-PheCH$_2$Cl at pH 5.02 or slightly faster at pH 5.80. It appears that the one additional hydrogen bond that these compounds could form with S_3 of the enzyme (between the C=O of the inhibitor and the NH of Gly-216) does not substantially alter the reactivity. The C=O and NH groups of a P_2 residue point away from the enzyme and would not interact. The tosyl group of Tos-PheCH$_2$Cl seems to have little effect when compared to Ac-PheCH$_2$Cl. This was a surprising result since we felt that the tosyl group should make better contact with the S_2 subsite of the enzyme, though it could be binding quite well and have a low k_3.

One of the strongest reasons for our belief that the solution reactivity of peptide chloromethyl ketones and of substrates can be correlated with the crystallographically based model (Figure 1) is the effect on reactivity that a leucyl residue has in P_2 of a substrate or inhibitor. The k_{obsd}/[I] value of Ac-Leu-PheCH$_2$Cl is greater than Ac-Gly-PheCH$_2$Cl and Ac-Ala-PheCH$_2$Cl by a factor of 2.5–3.0, although they have the same structure except for the P_2 residue. A factor of 3.5–3.9 is also observed upon comparison of Boc-Ala-Gly-PheCH$_2$Cl with Boc-Gly-Leu-PheCH$_2$Cl. These differences can be explained by van der Waals contact between the side chain of a leucyl residue in P_2 and Ile-99 of the enzyme. The side chain of Leu in P_2 extends about 4 Å away from C_α and can make contact with Ile-99 whereas Gly and Ala have side chains too small (1–2 Å from C_α) to interact with Ile-99. These results agree beautifully with results obtained by other research groups. Izumiya, Yamashita, and coworkers have shown in a series of papers (Yamamoto and Izumiya, 1966; Yamashita, 1960; and earlier papers) studying the hydrolysis of tyrosine containing peptide amides by chymotrypsin that the enzyme shows a marked preference for a P_2 residue (Val, Leu, Ile, etc.) containing a bulky aliphatic side chain. Both lower K_M values and higher k_3 rates are observed when these substrates are compared with similar substrates having Gly or Ala as the P_2 residue. In addition, Neil *et al.* (1966) has shown in a study of sequences around chymotryptic splits in proteins of known sequence that Val and Ile promote hydrolysis when located at P_2. Thus the well-documented "secondary specificity" of chymotrypsin for certain residues at P_2 is explicable in terms of the model illustrated in Figure 1.

Tripeptide chloromethyl ketone inhibitors are 2–11 times more reactive than Ac-PheCH$_2$Cl. Ac-Ala-Gly-PheCH$_2$Cl, the longest inhibitor for which crystallographic data were determined, forms three hydrogen bonds with the enzyme and is six times more reactive than Ac-PheCH$_2$Cl. The possibility of forming an antiparallel β structure beyond S_3 is prevented by a sharp bend in the backbone of the enzyme between Gly-216 and Ser-217 and the side chain of a P_3 residue points away from the enzyme and should make no contact. Thus groups in the P_4 position of inhibitors would interact mainly with the side chain of Trp-172. It was somewhat surprising to us that Z-Gly-Gly-PheCH$_2$Cl and Boc-Ala-Gly-PheCH$_2$Cl were poorer inhibitors than Ac-Ala-Gly-PheCH$_2$Cl. A good hydrophobic interaction should be possible between the P_4 Z or Boc group of the inhibitors and the side chain of Trp-172. If the model (Figure 1) is correct, this must not be a favorable interaction. It is possible that due to the size of these groups and the orientation of the two indole rings in the "S_4" site that this

hydrophobic interaction results in disruption of the hydrogen bonds between the P_3 residue and S_3 of the enzyme.

The best inhibitors we have thus far observed are Z-Gly-Leu-PheCH_2Cl and Boc-Gly-Leu-PheCH_2Cl which are 9–11 times more reactive than Ac-PheCH_2Cl. Both are tripeptides and can form three hydrogen bonds with the enzyme (Table IV) and in addition have a leucyl residue at P_2. Boc-Gly-Leu-PheCH_2Cl is 11 times more reactive than Tos-PheCH_2Cl, the standard chymotrypsin active-site-directed irreversible inhibitor.

It should be noted that the relative k_{obsd}/[I] values listed in Table IV are pH dependent. For example the k_{obsd}/[I] of Ac-Leu-PheCH_2Cl is 2.1 times that of Ac-PheCH_2Cl at pH 5.02, but 5.0 times larger at pH 5.80. At pH 7.0 Shaw and Ruscica (1971) have shown that Z-PheCH_2Cl is 51 times more reactive than CHO-PheCH_2Cl, while our fastest inhibitor is only 27 times faster than our slowest. After this observation was pointed out to us by a referee, the rate of inactivation of chymotrypsin by Z-PheCH_2Cl was measured at pH 5.80 and 30% 1,2-dimethoxyethane (J. C. Powers and P. Andrews, unpublished results) and was found to be two to three times slower than Ac-Leu-PheCH_2Cl (Table III) which would indicate that it was at most four to five times more reactive than CHO-PheCH_2Cl at pH 5.80. This was in line with our predictions since we expected the benzyloxy group of Z-PheCH_2Cl to interact favorably with the S_2 subsite of the enzyme. The fact that the relative rates of inactivation vary with pH indicates that the extent of protonation of histidine-57 is not the sole controlling factor. We feel that the explanation for these observations lies in the fact that some or all of the subsite interactions between the enzyme and various inhibitors are pH dependent. It is not unexpected that these interactions might vary more with pH at some subsites than at others, and variable relative reactivities at different pH's would thus be the result.

In conclusion, the kinetic results presented in this paper are consistent with the model illustrated in Figure 1 and show although the range is small that in general the greater the number of interactions between the enzyme and the inhibitor, the larger the second-order rate constant k_{obsd}/[I]. The rates are also correlated with "secondary substrate specificity" of chymotrypsin and support the hypothesis that the productive binding mode of this enzyme with peptide chloromethyl ketones which leads to alkylation of His-57 is similar to the productive substrate binding mode which leads to acylation of Ser-195. Since a similar binding scheme is observed with subtilisin BPN′ (Kraut *et al.*, 1971; Robertus *et al.*, 1972), it appears that there is a common binding scheme among most serine proteases, although another binding mode has been proposed for elastase (Shotton *et al.*, 1971).

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