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Mechanism of Inactivation of Human Leukocyte Elastase by a Chloromethyl Ketone: Kinetic and Solvent Isotope Effect Studies

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ABSTRACT: The mechanism of inactivation of human leukocyte elastase (HLE) by the chloromethyl ketone MeOSuc-Ala-Ala-Pro-Val-CH_2Cl was investigated. The dependence of the first-order rate constant for inactivation on concentration of chloromethyl ketone is hyperbolic and suggests formation of a reversible "Michaelis complex" prior to covalent interaction between the enzyme and inhibitor. However, the observed K_i value is 10 μM, at least 10-fold lower than dissociation constants for complexes formed from interaction of HLE with structurally related substrates or reversible inhibitors, and suggests that K_i is a complex kinetic constant, reflecting the formation and accumulation of both the Michaelis complex and a second complex. It is proposed that this second complex is a hemiketal formed from attack of the active site serine on the carbonyl carbon of the inhibitor. The accumulation of this intermediate may be a general feature of reactions of serine proteases and chloromethyl ketones derived from specific peptides and accounts for the very low K_i values observed for these reactions. The solvent deuterium isotope effect (SIE) on the inactivation step (k_i) is 1.58 ± 0.07 and is consistent with rate-limiting, general-catalyzed attack of the active site His on the methylene carbon of the inhibitor with displacement of chloride anion. The general catalyst is thought to be the active site Asp. In contrast, the SIE on the second-order rate constant for HLE inactivation, k_i/K_i, is inverse and equals 0.64 ± 0.05. The proton inventory (rate measurements in mixtures of H_2O and D_2O) for this reaction is "bowed down" from a straight line connecting the points in pure H_2O and D_2O and indicates multi-proton reorganization. These results are consistent with a mechanism for k_i/K_i in which (i) initial formation of the Michaelis complex is accompanied by solvent reorganization and (ii) the ketone moiety of the inhibitor exists as a fully formed hemiketal in the rate-limiting transition state.

Peptide-derived chloromethyl ketones are irreversible inhibitors of serine proteases (Powers, 1977). The stable complexes formed from interaction of CMKs[1] with several proteases have been examined by X-ray crystallographic methods and reveal two important structural features (James et al., 1980; Poulos et al., 1976): (i) a covalent bond between the methylene carbon of the ketone and $N^{\epsilon 2}$ of the active-site histidine imidazole and (ii) a covalent bond between the ketone carbonyl carbon and O^{γ} of the active site serine. These results support earlier studies demonstrating the presence of alkylated histidine residues in acid hydrolysates of CMK-inactivated proteases (Schoellmann & Shaw, 1963; Shaw & Ruscica, 1971) and the inability of anhydrochymotrypsin to interact with *N*-tosyl-PheCH_2Cl (Weiner et al., 1966).

Kinetic studies of the interaction of serine proteases with CMKs indicate the reversible formation of an enzyme–inhibitor complex prior to alkylation and thus adherence of these reactions to the minimal mechanism of Scheme I (Collen et al., 1980; Kurachi et al., 1973; Powers, 1977; Walker et al., 1985).

Scheme I

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_i} E\text{–}I$$

Kinetic investigations also reveal a correlation between inhibitor structure and inactivation rates. In general, peptide structural features known to enhance catalytic efficiency during substrate hydrolysis also tend to increase rates of inactivation by CMKs (Collen et al., 1980; Kurachi et al., 1973; Powers, 1977).

Despite this level of understanding, the mechanistic picture for the inactivation of serine proteases by CMKs is still incomplete. Important unanswered questions include the following: What is the structure of E·I? Is E·I the initial Michaelis complex or the hemiketal formed from interaction of the active site serine with the carbonyl carbon of the inhibitor? What elementary reaction steps rate limit the processes governed by k_i and k_i/K_i? What are the structures of the rate-limiting transition states for these steps? Are these transition states stabilized by protolytic catalysis? Finally, are these mechanistic features sensitive to the structure of the inhibitor? In this paper we will try to answer these questions for the inhibition of human leukocyte elastase by the chloromethyl ketone MeOSuc-Ala-Ala-Pro-Val-CH_2Cl.

[1] Abbreviations: CMK, chloromethyl ketone; Tos, *N*-tosyl; MeOSuc, *N*-(methoxysuccinyl); pNA, *p*-nitroanilide; HLE, human leukocyte elastase; CT, bovine chymotrypsin.

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Materials and Methods

MeOSuc-Ala-Ala-Pro-Val-pNA was available from previous studies (Stein, 1983). HLE was prepared as previously described (Stein, 1985b; Viscarello et al., 1983). Buffer salts and Me_2SO were analytical grade from several sources. Buffer solutions in H_2O and D_2O (99% from Sigma Chemical Co.) were prepared as outlined previously (Stein, 1983). Synthetic methods for the preparation of MeOSuc-Ala-Ala-Pro-Val-CH_2Cl and MeOSuc-Ala-Ala-Pro-D-Val-pNA appear in the supplementary material (see paragraph at end of paper regarding supplementary material).

Determination of Inactivation Kinetics for Reaction of HLE and MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. The kinetics of inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl was determined by both a continuous method and a discontinuous method.

The continuous, spectrophotometric method is similar to that recently described by Tian and Tsou (1982). In a typical experiment, 50 μL each of substrate and inhibitor solution in Me_2SO was added to a cuvette containing 2.88 mL of buffer (0.10 M phosphate, 0.50 M NaCl, 7.6). The cuvette was placed in a jacketed holder in the cell compartment of a Cary 210 spectrophotometer, and the reaction solution was allowed to reach thermal equilibrium (10–20 min). The temperature was maintained at 25 °C by water circulated from a Lauda K-2/RD bath. Injection of 20 μL of enzyme solution initiated the reaction. The absorbance at 410 nm, due to the release of *p*-nitroaniline, was continuously measured, digitized, averaged, and stored in an Apple II microcomputer.

As predicted from theory (Tian & Tsou, 1982), the temporal dependence of the absorbance change was exponential and could be fit to a first-order rate law. First-order rate constants were determined by iterative fit of the data to the linearized exponential function:

$$\ln (A_\infty - A_t) = -k_{obsd}t + \ln (A_\infty - A_0) \quad (1)$$

where A_∞ is the absorbance at infinite time, A_t is the absorbance at time t, A_0 is the initial absorbance, and k_{obsd} is the observed first-order rate constant. The parameters optimized were A_∞, $A_\infty - A_0$, and k_{obsd}. Data were collected for no less than three half-times and frequently for more than five half-times to demonstrate the complete inactivation of HLE.

The discontinuous method is quite general and has been described by several authors (Powers, 1977). Typically, a buffered solution of CMK was brought to thermal equilibrium and an aliquot of HLE added. Periodically, small aliquots of this reaction solution were withdrawn, and the residual enzymatic activity was determined by a standard HLE assay. Values of k_{obsd} were then calculated by the method of half-lives from semilog plots of residual activity vs. time.

Results

Kinetics of Inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. Values of k_{obsd} were determined at several inhibitor concentrations by the continuous method described above. The dependence of k_{obsd} on [I] was found to be hyperbolic and thus suggested adherence of this reaction to the minimal mechanism of Scheme I. Equations 2 and 3 describe

$$k_{obsd} = k_i[I]/\{(K_i)_{app} + [I]\} \quad (2)$$

$$(K_i)_{app} = K_i(1 + [S]/K_m) \quad (3)$$

the dependence of k_{obsd} on [I] for an inactivation process that proceeds according to Scheme I and predict that a double-reciprocal plot of $1/k_{obsd}$ vs. $1/[I]$ will be linear with x-axis intercept equal to $-1/(K_i)_{app}$, y-axis intercept equal to $1/k_i$, and slope equal to $(K_i)_{app}/k_i$. In Figure 1 is shown the double-reciprocal plot for a typical inactivation experiment. The data from this experiment were fit to eq 2 by nonlinear least squares, and the best fit values were found to be $k_i = (3.71 \pm 0.03) \times 10^{-2}$ s^{-1} and $(K_i)_{app} = 188 \pm 20$ μM. These experiments were conducted at a substrate concentration of 864 μM, which is equal to $16K_m$ ($K_m = 54$ μM). Dividing $(K_i)_{app}$ by the appropriate value of $1 + [S]/K_m$ (see eq 3) yields a value of K_i equal to 11 μM.

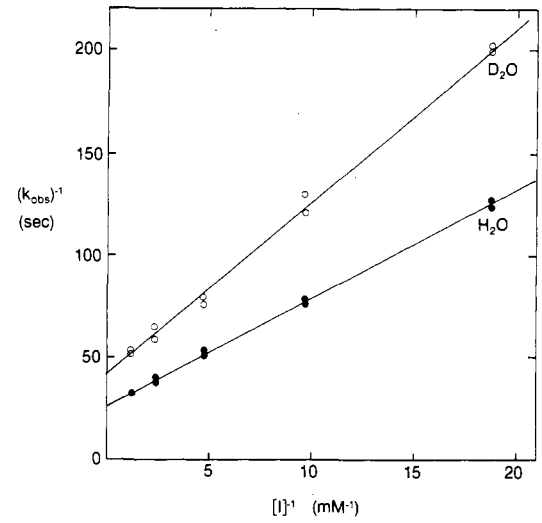

FIGURE 1: Double-reciprocal plots for dependence of first-order rate constant for inactivation of HLE in H_2O and D_2O on concentration of MeOSuc-Ala-Ala-Pro-Val-CH_2Cl.

Results from three inactivation experiments yield average values: $k_i = (3.6 \pm 0.1) \times 10^{-2}$ s^{-1}, $K_i = 10.2 \pm 0.6$ μM, and $k_i/K_i = 3500 \pm 150$ M^{-1} s^{-1}. A previous kinetic study of the reaction between MeOSuc-Ala-Ala-Pro-Val-CH_2Cl and HLE was performed at a single concentration of CMK equal to 25 μM and at a temperature of 30 °C and yielded a value of $k_{obsd}/[I]$ equal to 1600 M^{-1} s^{-1} (Powers et al., 1977). Using the kinetic parameters determined in this investigation, we calculate a $k_{obsd}/[I]$ of 1000 M^{-1} s^{-1} at 25 °C. Given the difference in temperature, the agreement between the results from the two laboratories is satisfactory.

Solvent Deuterium Isotope Effects for Inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. The continuous method was used to obtain values of k_{obsd} at several CMK concentrations in both light and heavy water. These data are shown as double-reciprocal plots in Figure 1. By nonlinear regression methods, the following values were obtained: H_2O, $k_i = (3.71 \pm 0.03) \times 10^{-2}$ s^{-1} and $(k_i/K_i)_{app} = 190 \pm 4$ M^{-1} s^{-1}; D_2O, $k_i = (2.35 \pm 0.10) \times 10^{-2}$ s^{-1} and $(k_i/K_i)_{app} = 125 \pm 6$ M^{-1} s^{-1}.

The solvent isotope effect on k_i (expressed as ^Dk_i) can be calculated directly as the ratio of rate constants in the two solvents and is equal to 1.58 ± 0.07. To calculate a value for $^D(k_i/K_i)$, however, the expression of eq 4 must be used. If

$$^D(k_i/K_i)_{app} = {}^D(k_i/K_i)/{}^DK_m \quad (4)$$

$^D(k_i/K_i)_{app}$ and ^DK_m are taken as 1.52 ± 0.08 and 2.34 ± 0.11 (Stein, 1983), respectively, then $^D(k_i/K_i)$ can be calculated to equal 0.65 ± 0.05.

The inverse solvent isotope effect of 0.65 is unusual for serine protease catalyzed reactions but was confirmed by two additional methods. The first of these methods was the discontinuous assay described above. Solutions of HLE and CMK were prepared in light and heavy water with [I] = 1.0 μM $\ll K_i$. Under these conditions, eq 2 simplifies to

$$k_{obsd} = (k_i/K_i)[I] \quad (5)$$

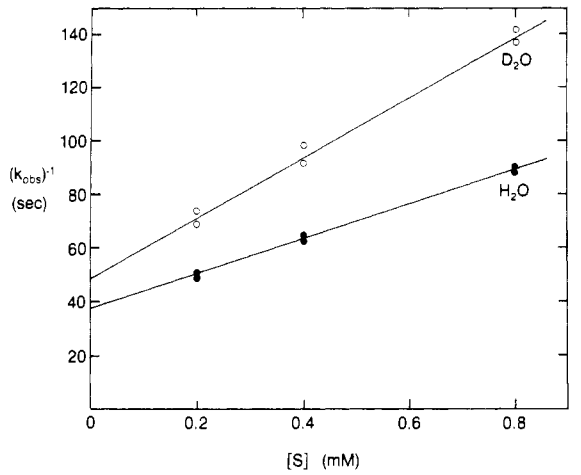

FIGURE 2: Dependence of reciprocal inactivation rate constant on concentration of substrate. k_{obsd} values are for the inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl and were determined in H_2O and D_2O.

Triplicate experiments yielded the following: H_2O, k_i/K_i = 2730 ± 190 M^{-1} s^{-1}; D_2O, k_i/K_i = 3990 ± 170 M^{-1} s^{-1}; $^D(k_i/K_i)$ = 0.68 ± 0.06.

The second method took advantage of continuous spectrophotometry and involved the determination of k_{obsd} values at a single concentration of CMK but over a range of substrate concentrations. Under these conditions, the following holds:

$$1/k_{obsd} = \{(K_i/k_i)/([I]K_m)\}[S] + (1/k_i)(1 + K_i/[I]) \quad (6)$$

According to this relationship, a plot of $1/k_{obsd}$ vs. [S] should be linear with a y-axis intercept of $(1/k_i)(1 + K_i/[I])$. In Figure 2 are plots of $1/k_{obsd}$ vs. [S] for the inactivation of HLE by the CMK at [I] = 30 μM. The y intercepts were found to be 37.8 ± 1.3 and 49.0 ± 2.9 s for the reactions in H_2O and D_2O, respectively. With these values and the previously determined values for k_i, K_i was calculated to be 12.1 ± 0.4 μM and 4.5 ± 0.3 μM in H_2O and D_2O, respectively, and ^DK_i = 2.7 ± 0.2. Finally, we can calculate $^D(k_i/K_i)$ = 0.59 ± 0.05.

From these three methods the solvent isotope effect on k_i/K_i for the inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl can be confidently estimated as 0.64 ± 0.05.

Proton Inventory of k_i/K_i for Inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. Discontinuous assay provided values of k_i/K_i in three binary mixtures of H_2O and D_2O. For mole fraction of solvent D_2O (n_{D_2O}) equal to 0, 0.49, and 0.98, values of k_i/K_i are 2730 ± 190, 3170 ± 130, and 3990 ± 170 M^{-1} s^{-1}, respectively. These data are plotted in Figure 3 as n_{D_2O} vs. k_i/K_i and constitute a proton inventory (Venkatasubban & Schowen, 1985) of k_i/K_i. The proton inventory of k_i/K_i has an overall inverse isotope effect of 0.68 ± 0.06 and is bowed down from a straight line drawn between the points for pure H_2O and D_2O. The general shape of proton inventory indicates multi-proton reorganization in the rate-limiting transition state (Venkatasubban & Schowen, 1985).

Solvent Isotope Effect for Inhibition of HLE by MeO-Suc-Ala-Ala-Pro-D-Val-pNA. Dissociation constants were calculated for the reversible inhibition of HLE by MeOSuc-Ala-Ala-Pro-D-Val-pNA in light and heavy water from a nonlinear least-squares fit of the dependence of k_{obsd} on inhibitor concentration to the expression

$$k_{obsd} = k\{K_i/([I] + K_i)\} \quad (7)$$

where k_{obsd} and k are pseudo-first-order rate constants determined at $[S]_0 \ll K_m$ in the presence and absence of inhibitor. The constants determined are as follows: H_2O, K_i = 218 ± 25 μM; D_2O, K_i = 169 ± 15; ^DK_i = 1.29 ± 0.19.

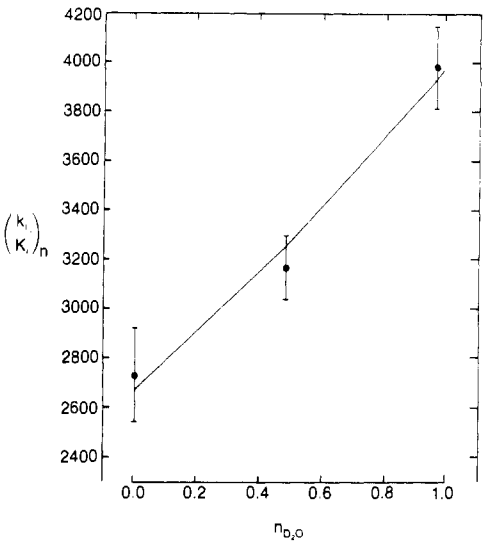

FIGURE 3: Proton inventory of k_i/K_i for inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. The solid line was drawn according to eq 18 with k_i/K_i = 2,674 M^{-1} s^{-1}, Z = 1.20, and ϕ_T = 1.25.

Discussion

A principle finding of this study is the K_i value of 10 μM for the inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. If this were a simple dissociation constant for an initially formed encounter complex of enzyme and inhibitor, we would expect it to be similar in magnitude to K_s or K_i values for structurally related substrates or reversible inhibitors. As shown in this work and elsewhere, however, dissociation constants for complexes of HLE with substrates or inhibitors are greater than 100 μM (Stein, 1985c; Stein et al., 1986) and thus suggest that the K_i value determined in this work is a complex kinetic constant reflecting the formation and accumulation of at least two intermediates on the reaction pathway. Kinetic Scheme II incorporates this view. According to this mechanism, leukocyte elastase, E, and chloromethyl ketone, I, reversibly form an encounter complex, $(E{\cdot}I)_1$, which forms yet another, more stable intermediate, $(E{\cdot}I)_2$. Alkylation of the active site histidine occurs from $(E{\cdot}I)_2$ to produce the inactivated species, E–I.

Scheme II

$$E + I \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} (E{\cdot}I)_1 \underset{k_{-h}}{\overset{k_h}{\rightleftharpoons}} (E{\cdot}I)_2 \xrightarrow{k_i'} E\text{–}I$$

The most likely candidate for $(E{\cdot}I)_2$ is the hemiketal formed from interaction of the active site serine with the ketone moiety of the inhibitor. This is consistent with X-ray crystallographic results (James et al., 1980; Poulos et al., 1976) and experiments with anhydrochymotrypsin (Weiner et al., 1966). Thus, k_h and k_{-h} are the first order rate constants for the formation and decomposition of the hemiketal intermediate and k_i' is the rate constant for histidine alkylation.[2]

[2] Two mechanisms for histidine alkylation (k_i' of Scheme II) have been proposed (Powers, 1977). One involves attack of histidine on the methylene carbon of the hemiketal intermediate [$(E{\cdot}I)_2$ of Scheme II], while the other involves attack of the oxyanion of the hemiketal on the methylene carbon to form an epoxide intermediate, which subsequently undergoes addition by the histidine. The results presented in this paper are consistent with both mechanisms and thus do not allow us to distinguish them. However, in accordance with Occam's razor (Carpenter, 1984), we are obliged to give preference to the simpler mechanism. Therefore, the results of this study will be interpreted in the context of the mechanism involving the direct attack of histidine on the methylene carbon of the hemiketal.

The kinetic formulation of this mechanism is

$$k_{obsd} = k_i[I]/(K_i + [I]) \quad (8)$$

where

$$k_i = \frac{k_h k_i'}{(k_h + k_{-h} + k_i')} \quad (9)$$

and assuming that dissociation of $(E{\cdot}I)_1$ is very fast relative to subsequent steps

$$K_i = \frac{k_{-a}}{k_a}\frac{k_{-h} + k_i'}{k_h + k_{-h} + k_i'} \quad (10)$$

The second-order rate constant for reaction of free enzyme and inhibitor is expressed as

$$k_i/K_i = \frac{k_a}{k_{-a}}\frac{k_h k_i'}{k_{-h} + k_i'} \quad (11)$$

The rate constants k_i and k_i/K_i are of principle interest to us here since they reflect properties of inactivation transition states and therefore provide insights into the catalytic mechanisms that are operative during the processes governed by these rate constants. k_i and k_i/K_i are both complex; however, the latter reflects a virtual transition state, and the former reflects both a virtual transition state and virtual reactant state (Schowen, 1978; Stein, 1981). As we will see below, simplification of these equations is possible, yielding more easily interpretable expressions.

According to the mechanism of Scheme II, $(E{\cdot}I)_2$ must accumulate if K_i is to be lower in magnitude than the simple dissociation constant, k_{-a}/k_a. If $(E{\cdot}I)_2$ is to accumulate, certain kinetic requirements must be met. These are derived as follows: First, k_{-a}/k_a is set to 150 μM, a value similar to K_s values for peptide-based substrates, such as MeOSuc-Ala-Ala-Pro-Val-pNA [K_s = 120 (Stein, 1985c); K_s = 240 μM (Stein et al., 1986)], and K_i values for reversible, peptide-based inhibitors, such as MeOSuc-Ala-Ala-Pro-D-Val-pNA (K_i = 220 μM). Substitution of this value and the observed K_i of 10 μM for the reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-CH_2Cl into eq 10 and rearrangement yields

$$\frac{k_h + k_{-h} + k_i'}{k_{-h} + k_i'} = \frac{k_{-a}/k_a}{K_i} \simeq 15 \quad (12)$$

or

$$\frac{k_h}{k_{-h} + k_i'} + 1 \simeq 15 \quad (13)$$

We see then that for $(E{\cdot}I)_2$ to accumulate

$$k_h \gg k_{-h} + k_i' \quad (14)$$

Incorporating this into eq 9 results in the simplified expression for k_i:

$$k_i = k_i' \quad (15)$$

We see then that under conditions where $(E\text{-}I)_2$ accumulates k_i reflects the single step of histidine alkylation from this intermediate.

Unlike the expression for k_i, k_i/K_i still remains complex (see eq 11). However, there are two limiting cases that we should consider:

$$k_{-h} \gg k_i' \qquad k_i/K_i = k_i'/K_a K_h \quad (16)$$

$$k_{-h} \ll k_i' \qquad k_i/K_i = k_h/K_a \quad (17)$$

In these expressions, $K_a = k_{-a}/k_a$ and $K_h = k_{-h}/k_h$. The effective reactant state for both forms of k_i/K_i is the same, as it must be: enzyme and inhibitor free in solution. The two expressions for k_i/K_i differ only in the transition state they reflect: if k_{-h} is greater than k_i', the transition state corresponds to histidine alkylation, while if k_{-h} is less than k_i' the transition state is for hemiketal formation. Solvent isotope effects allow us to distinguish the two alternatives.

Table I: Solvent Deuterium Isotope Effects for Inhibition of Human Leukocyte Elastase by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl and MeOSuc-Ala-Ala-Pro-D-Val-pNA

reaction	parameter	solvent isotope effect	method
inactivation by CMK	k_i	1.58 ± 0.07	*a*
inactivation by CMK	k_i/K_i	0.65 ± 0.05	*a*
		0.68 ± 0.06	*b*
		0.59 ± 0.05	*c*
		0.65 ± 0.05[e]	
reversible inhibition by peptide anilide	K_i	1.29 ± 0.19	*d*

[a] Dependence of k_{obsd} on [I] according to eq 2 and 4. k_{obsd} determined in the presence of substrate by a continuous spectrophotometric method. [b] k_i/K_i determined directly by discontinuous method. [c] Dependence of k_{obsd} on [S] according to eq 6. [d] Dependence of k_{obsd} on [I] according to eq 7. [e] Average.

The solvent isotope effects on k_i is normal and equal to about 1.6 (Table I). We suggest that it arises from general-base-catalyzed attack of the histidine imidazole on the methylene of the chloromethyl ketone and that the general catalyst in this reaction is the carboxylate of the active site aspartic acid residue.

The solvent isotope effect on k_i/K_i, on the other hand, is inverse and equals 0.65 (Table I). As indicated by the proton inventory (Figure 3), this effect arises from multiple proton reorganization in the rate-limiting transition state for k_i/K_i (Venkatasubban & Schowen, 1985). Previous studies with similar systems have shown that the general expression of eq 18 should account for the proton inventory of k_i/K_i[3] (Stein,

$$(k_i/K_i)_n = (k_i/K_i)_0 Z^n(1 - n + n\phi_T) \quad (18)$$

1985c,d; Stein & Matta, 1985). In this equation, Z is the product of many small fractionation factors originating from the solvent reorganization that accompanies binding of the CMK to HLE, and ϕ_T is a transition-state fractionation factor for a single hydrogenic site. According to eq 16 and 17, if $k_{-h} \gg k_i'$, ϕ_T is the fractionation factor for the proton transferred from His to Asp in the transition state for His alkylation, while if $k_{-h} \ll k_i'$ ϕ_T corresponds to some yet unidentified hydrogenic site in the transition state for hemiketal formation.

We feel it unlikely that $k_{-h} \gg k_i'$. If this were the case, the transition state for k_i/K_i would be the same transition state as occurs in k_i', which we know has a ϕ_T of 0.63 ($\phi_T = 1/^D k_i$). If ϕ_T were 0.63, Z would have to equal 2.45 to produce an isotope effect on k_i/K_i of 0.65 (eq 18; n = 1). Typically, Z is similar in magnitude to solvent isotope effects on dissociation constants and ranges from 1.2 to 1.6 (Stein, 1985c). This is illustrated in Table I, where the solvent isotope effect on K_i for the inhibition of HLE by MeOSuc-Ala-Ala-Pro-D-Val-pNA is found to be 1.3.

We conclude then that hemiketal formation rate limits k_i/K_i. At this stage of the analysis, we would like to be able to fit the proton inventory data of Figure 1 to eq 18, solve for Z and

[3] The model represented by eq 18 assumes that all reactant-state fractionation factors are unity. In the present case, it is conceivable that if the CMK is hydrated, this reactant state would generate a nonunity fractionation factor (Mata-Segreda et al., 1974). However, it has been demonstrated (Lewis & Wolfenden, 1977) that CMKs of the sort used in this study are not hydrated in solution. Therefore, our assumption of a reactant-state fractionation factor of 1 remains valid.

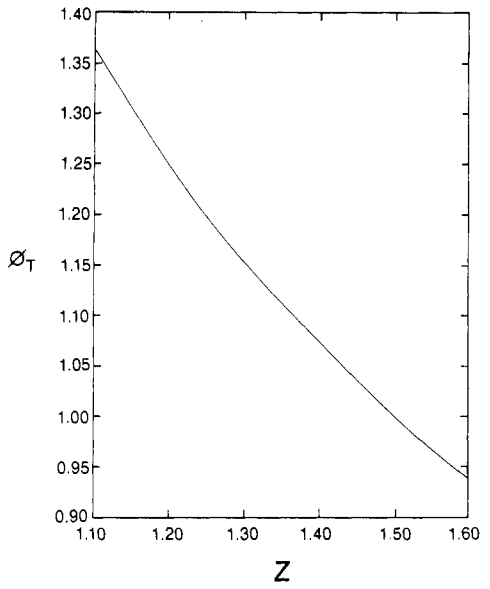

FIGURE 4: Theoretical dependence of Z on ϕ_T according to eq 18 and assuming $(k_i/K_i)_1/(k_i/K_i)_0$ equal to 1.5.

ϕ_T, and through a mechanistic interpretation of ϕ_T gain some feeling for the structure of the rate-determining transition state. Unfortunately, these parameters cannot be simultaneously extracted from the proton inventory data because they are dependent parameters (Stein, 1985c). However, the dependence of ϕ_T on Z according to eq 18 can be simulated and is shown in Figure 4. In this simulation, n is set equal to 1, $(k_i/K_i)_1/(k_i/K_i)_0$ is set to its observed value of 1.54 [i.e., the inverse of $^D(k_i/K_i)$], and Z is varied over a range of 1.1–1.6.

Over the range of allowed Z values, ϕ_T is seen to vary from unity to 1.4. These values of ϕ_T indicate that there is tighter binding at this hydrogenic site in the rate-limiting transition state than in the reactant state. On the basis of this, we can rule out certain conceivable rate-limiting steps, including (i) general-base catalysis by His of the attack of Ser on the carbonyl carbon of the ketone and (ii) deprotonation of the resultant histidinium cation. Such processes, with a proton "in flight" in the rate-limiting transition state, generate large normal isotope effects ($\phi_T \sim 0.25$–0.5) (Stein, 1983; Stein et al., 1986; Venkatasubban & Schowen, 1985) and therefore cannot be reconciled with the present proton inventory data.

A number of transition states are allowed by the data, however. At values of Z between 1.1 and 1.3, ϕ_T is roughly equal to 1.25. This value is in the range of hemiacetal fractionation factors as estimated by Schowen and co-workers (Mata-Segreda et al., 1974) and is consistent with the hemiketal intermediate being fully formed and protonated by the time the rate-limiting transition state for k_i/K_i is reached. At Z values closer to 1.68 ϕ_T approaches 1. This values is similar to more recent estimates of hemiacetal fractionation factors (Bone & Wolfenden, 1985) and is again consistent with a fully formed and protonated hemiketal in the rate-determining transition state. Other rate-limiting steps consistent with ϕ_T equal to 1 include conformational changes of the initially formed E·I complex (Stein, 1985b,c) and processes in which the hemiketal is formed but unprotonated.

Except for the case of a rate-limiting conformational change of E·I, we have proposed a transition state for k_i/K_i in which the hemiketal exists fully formed. It still is not clear, however, what reaction step is actually rate limiting, i.e., what reaction step this transition state represents. Since we know that the overall deuterium fractionation factor for this transition state must be close to 1, likely candidates for this step include (i) positioning of His for attack on the methylene carbon of the inhibitor and (ii) protonation of the hemiketal anion, if in fact this intermediate's most stable state is protonated. The unit fractionation factor for the protonic site in the latter transition state presumably results from a cancellation of two effects: a weakening of force constants that is characteristic of transition-state hydrogen bridges in "solvation catalysis" (Swain et al., 1965; Schowen, 1972; Eliason & Kreevoy, 1978) and a strengthening of force constants that may be characteristic of hemiacetals (Mata-Segreda et al., 1974).

Elucidation of structural features of rate-limiting transition states for inactivation of other serine proteases by chloromethyl ketones has received little attention. A solvent isotope effect was determined for the inactivation of chymotrypsin by Tos-Phe-CH_2Cl ($k_i/K_i = 10\ M^{-1}\ s^{-1}$; Kezdy et al., 1967). The isotope effect was on k_i/K_i and found to equal 1.2. This value is very different from the isotope effect on k_i/K_i of 0.65 determined in this study and, at first glance, calls into question the validity of the mechanism presented here. However, the two results can be easily reconciled when we understand what step rate limits k_i/K_i for the reaction of CT with Tos-Phe-CH_2Cl.

The K_i for the reversible interaction of CT with Tos-Phe-CH_2Cl is large and greater than 1 mM (Kezdy et al., 1957) and is similar to K_s values for structurally similar substrates. This suggests that K_i for this reaction reflects the simple dissociation of the initially formed Michaelis complex, $(E\cdot I)_1$ of Scheme II, and that the hemiacetal, $(E\cdot I)_2$, does not accumulate. Kinetically, this requires that k_{-h} be much greater than both k_i' and k_h. According to eq 16, k_i/K_i now equals $k_i'/(K_aK_h)$. Thus, the rate-limiting step for the inactivation of CT by Tos-Phe-CH_2Cl is k_i' and not k_h as it is for the inactivation of HLE by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl.

The solvent isotope effect for k_i/K_i now becomes

$$^D(k_i/K_i) = 1/(Z\phi_h\phi_{Ti}) \qquad (19)$$

where ϕ_h is the fractionation factor for the hemiketal intermediate, $(E\cdot I)_2$, and ϕ_{Ti} is the fractionation factor for the transition state of k_i'. It we assume that ϕ_{Ti} is equal to the fractionation factor of 0.6 observed for k_i' during reaction of HLE with MeOSuc-Ala-Ala-Pro-Val-CH_2Cl, then $^D(k_i/K_i)$ values similar to 1.2 can be readily calculated with values of Z and ϕ_h ranging from 1.1 to 1.4 and from 1.0 to 1.25, respectively. As we saw previously, exact assignments for Z and ϕ_h are impossible.

CONCLUSIONS

A key intermediate that forms during the inactivation of serine proteases by chloromethyl ketones is the hemiketal. For inhibitors derived from specific peptides, the hemiketal is stable relative to the Michaelis complex and dissociates slower than it alkylates the active site His (Figure 5A). The stability enjoyed by this intermediate results from utilization of the free energy released as the peptide portion of the inhibitor interacts with the protease at remote subsites (Fersht, 1974; Jencks, 1975). For less specific CMKs, there will be fewer opportunities for favorable interactions at remote subsites, and the hemiketal intermediate will be less stable (Figure 5B).

Kinetically, this translates into rate-limiting hemiketal formation in k_i/K_i for specific CMKs (eq 17, Figure 5A) and rate-limiting alkylation in k_i/K_i for nonspecific CMKs (eq 16, Figure 5B). For both specific and nonspecific inhibitors, k_i appears to be rate-limited by general-base-catalyzed attack of His on the methylene carbon of the inhibitor.

The accumulation of the hemiketal intermediate that we postulate to occur during the inactivation of serine proteases

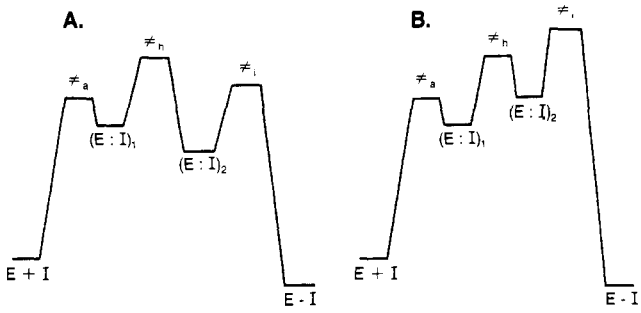

FIGURE 5: Free energy–reaction progress diagrams for inactivation of serine proteases by chloromethyl ketones under conditions of [I] $\ll K_i$. (A) Reaction of a CMK derived from a specific peptide. The stability of the hemiketal intermediate, $(E{\cdot}I)_2$, relative to the Michaelis complex, $(E{\cdot}I)_1$, and the slow rate of dissociation of $(E{\cdot}I)_2$ relative to alkylation (i.e., $k_{-h} \ll k_i'$) result in hemiketal formation being the rate-limiting step in the process governed by k_i/K_i. (B) Reaction of a CMK derived from a nonspecific peptide. The instability of $(E{\cdot}I)_2$ results in histidine alkylation being the rate-limiting step in k_i/K_i.

by specific chloromethyl ketones is suggested by the low K_i values observed for these reactions (Coggens et al., 1974; Collen et al., 1980; Cottenberg et al., 1983; Kettner & Shaw, 1977; Lijnen et al., 1984; Walker et al., 1985) and higher K_i values similar in magnitude to simple dissociation constants for substrates and reversible inhibitors (Powers, 1977) observed for reactions of less specific CMKs. This interpretation of K_i contrasts with previously proposed interpretations that consider only the stability of the initially formed Michaelis complex. There earlier views are incomplete and require provisions for the possible accumulation of the hemiketal intermediate.

Supplementary Material Available

Synthetic methods for the preparation of MeOSuc-Ala-Ala-Pro-Val-CH_2Cl and MeOSuc-Ala-Ala-Pro-D-Val-pNA (5 pages). Ordering information is given on any current masthead page.

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