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Evidence for Hemiketals as Intermediates in the Inactivation of Serine Proteinases with Halomethyl Ketones†

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ABSTRACT: The mechanism of inactivation of serine proteinases by peptide halomethyl ketone inhibitors was studied through the inhibition of trypsin with a series of model peptide ketones (Lys-Ala-LysCH_2X). In this series, X is a poor leaving group with increasing electron-withdrawing capacity (X = H, $CH_2CO_2CH_3$, $COCH_3$, $OCOCH_3$, and F), and as expected, the peptide ketones are reversible, competitive inhibitors of trypsin. The strength of binding of these inhibitors to trypsin increases with the electron-withdrawing ability of X, indicating that the inhibition constant K_i obtained is a measure of reversible hemiketal formation between the inhibitor ketone carbonyl group and the hydroxyl group of the active site serine. A Hammett plot of $-\log K_i$ vs. σ_I, the inductive substituent constant of X, reveals a linear relationship between the free energy of binding and the electron-withdrawing power of X. The reversible binding constant obtained for the corresponding chloromethyl ketone Lys-Ala-LysCH_2Cl falls on this line, indicating that the reversible binding involves hemiketal formation, which is followed by alkylation of the enzyme.

Halomethyl ketones have found wide use as both in vivo and in vitro probes of serine proteinases. These molecules inhibit the proteinases by N-alkylation of the imidazole ring of His-57 (chymotrypsin numbering system), which forms part of the enzyme catalytic triad (Ser-195, His-57, and Asp-102). Three possible mechanisms of inactivation of the enzyme have been reviewed by Powers (1977): (1) (a) formation of a reversible Michaelis-type enzyme–inhibitor complex, followed by (b) irreversible alkylation of His-57, followed by (c) hemiketal formation between the hydroxyl group of Ser-195 and the ketone carbonyl carbon; (2) (a) formation of a reversible Michaelis-type enzyme–inhibitor complex, followed by (b) hemiketal formation, followed by (c) irreversible alkylation of His-57; (3) (a) formation of a reversible Michaelis-type enzyme–inhibitor complex, followed by (b) hemiketal formation, followed by (c) displacement of the halogen by the original ketone carbonyl oxygen forming an epoxide, followed

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by (d) attack at the original ketomethylene carbon re-forming the hemiketal and irreversibly alkylating His-57.

The following lines of evidence have been cited in favor of mechanism 2 (and possibly 3): Anhydrochymotrypsin is not alkylated by tosylphenylalanine chloromethyl ketone (TPCK).[1] The binding site of anhydrochymotrypsin has been shown to be intact by its ability to bind virtual substrates as well as does the native enzyme. This suggests that the side-chain hydroxyl group of Ser-195 is required for alkylation to take place (Wiener et al., 1966).

Kinetic evidence indicates that during the reaction between TPCK and chymotrypsin the alkylation step is preceded by an adsorption step showing full specificity of the chymotrypsin reaction (Kezdy et al., 1967). On the basis of this evidence and that of Wiener et al. given above, Kezdy et al. first published mechanism 2.

The tetrahedral hemiketal has been observed by X-ray crystallography in subtilisin BPN′ inactivated with chloromethyl ketones (Poulos et al., 1967) and by carbon-13 NMR in trypsin inactivated with tosyllysine chloromethyl ketone (Malthouse et al., 1983, 1985; Mackenzie et al., 1984). The existence of the hemiketal in these alkylated species, however, does not establish the order of events in the deactivation process. Similarly, the fact that hemiacetal formation has been demonstrated for peptide aldehyde inhibitors of serine proteinases (Shah et al., 1984, Brayer et al., 1979) does not indicate that a hemiketal precedes alkylation in halomethylketone inactivation reactions. The fact that the reversible binding of a series of halomethyl ketones to thermitase was stronger than the binding of the corresponding methyl ketones has led Fittkau to suggest (Fittkau et al., 1984) that the bound species in each case was the hemiketal.

In order to further elucidate the mechanism of inactivation of serine proteinases by halomethyl ketones, we have examined the inhibition of trypsin by a series of analogues of a chloromethyl ketone possessing the structure Lys-Ala-LysCH_2X (where X = Cl in the original inhibitor and X = H, $CH_2CO_2CH_3$, $COCH_3$, $OCOCH_3$, and F in the analogues). The substituents represented by X in the analogues were selected to be poor leaving groups with varying degrees of electron-withdrawing capacity. These peptide ketones thus represent a series of potential reversible inhibitors of trypsin. A Hammett plot (Hammett, 1937), $-\log K_i$ vs. σ_I (in which K_i is the dissociation constant of the reversible enzyme–inhibitor complex and σ_I is the inductive substituent constant of X), was used to correlate the strength of binding of each inhibitor with the electron-withdrawing power of X. Our results are consistent with reversible hemiketal formation prior to alkylation of His-57.

Materials and Methods

Materials

Trypsin was obtained from the Sigma Chemical Co., St. Louis, MO, and was purified by ion-exchange chromatography by the method of Walsh (1970) to obtain β-trypsin and to remove chymotryptic contamination. N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide (BAPA), sodium fluoroacetate, 4-(*N*,*N*-dimethylamino)pyridine (DMAP), triethylamine (Et_3N), *N*,*N*-dimethylformamide (DMF), and trifluoroacetic acid (TFA) were obtained from the Aldrich Chemical Co., Milwaukee, WI. DMAP was recrystallized from water, and Et_3N, DMF, and TFA were redistilled prior to use. Dicyclohexylcarbodiimide (DCC) was obtained from Pierce Chemical Co., Rockford, IL, and was vacuum-distilled prior to use. Boc-Lys(Z)-Ala-Lys(Z)-OH was synthesized by classical methods from amino acid intermediates purchased from Aldrich and from Peninsula Laboratories, Belmont, CA. Sephadex G-15 was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. All other reagents and solvents were reagent-grade or better.

Methods

The peptidyl ketones were synthesized from Boc-Lys(Z)-Ala-Lys(Z)-OH and the symmetric anhydride of the appropriately substituted acetic acid by a modification of the Dakin-West reaction, as described earlier (McMurray & Dyckes, 1985). The ketones were deprotected by treatment with cold trifluoroacetic acid followed by hydrogenolysis or by treatment with TFA at 50 °C for 1 h. All final products showed one spot on both thin-layer electrophoresis (TLE) and thin-layer chromatography (TLC) systems. TLE was carried out in pyridine–acetic acid–water (1:10:89) buffer on a Savant Model CVR 300 apparatus. Samples were spotted on precoated cellulose plates (POLYGRAM CEL 300, from Macherey-Nagel, Duren, FRG) and were developed at 200 V for 2 h. The products were visualized with 1% ninhydrin in acetone. TLC was performed on precoated silica plates (5761, EM Reagents, Darmstadt, FRG). The products were visualized by spraying the plates with 1% ninhydrin in acetone and heating at 100 °C for 5 min and/or by exposing the plate to chlorine vapor for 3 s and after 5 min spraying with a 1% starch–1% potassium iodide solution. The synthesis of the fluoromethyl ketone is given as a representative example.

Preparation of Fluoroacetic Acid. Fluoroacetic acid was obtained by distillation under reduced pressure from a solution of 2 g of sodium fluoroacetate in 8 mL of concentrated sulfuric acid (Gryszkiewicz-Trochimowski et al., 1947). The crude liquid was redistilled under reduced pressure to give 1.08 g of clear, colorless crystals: yield 69%; bp (2 mm Hg) 48–51 °C; NMR ($CDCl_3$) δ 11.41 (s, 1 H, COOH), 4.99 (d, J = 47 Hz, 2 H, FCH_2).

Preparation of Symmetric Anhydride of Fluoroacetic Acid. A sample of 0.58 g of DCC (2.8 mmol) was added to 0.44 g of freshly distilled fluoroacetic acid (5.6 mmol) in 20 mL of CH_2Cl_2. After being stirred for 20 min at room temperature, the dicyclohexylurea was removed by filtration, and the solvent was removed in vacuo. The anhydride was used immediately, without further purification.

Preparation of Boc-Lys(Z)-Ala-Lys(Z)CH_2F. Boc-Lys(Z)-Ala-Lys(Z)-OH, 0.50 g (0.7 mmol), was added to the symmetric anhydride of fluoroacetic acid prepared in the previous step, followed by 8 mg of DMAP, 0.1 mL of DMF, and 2.00 mL of Et_3N (1.4 mmol). After being stirred for 30 min at 50 °C, the DMF and Et_3N were removed in vacuo. The gummy residue was dissolved in 50 mL of ethyl acetate and extracted with 2 × 10 mL of 0.1 M acetic acid, 2 × 10 mL of 5% sodium bicarbonate, and 10 mL of brine. The ethyl acetate solution was dried over $MgSO_4$ and filtered and the solvent removed in vacuo to yield a yellow gum. The crude product was purified by silica gel chromatography (2% methanol in chloroform, stepped up to 4 and 6%) to yield 91 mg of a white powder (0.13 mmol, 18%): NMR ($CDCl_3$) δ 7.32 (s, 10 H, 2 C_6H_5), 7.00 (br d, 1 H, Lys^3 α-NH), 6.85 (br d, 1 H, Ala^2 α-NH), 5.13–5.70 (m, 3 H, Lys^1 α-NH, Lys^1 ε-NH, Lys^3 ε-NH), 5.08 (s, 4 H, 2-Z-CH_2), 4.95 (d, J = 47.2

[1] Abbreviations: BAPA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; Et_3N, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; Z, benzyloxycarbonyl.

Table I: Inhibition Constants and Inductive Substituent Constants of the Inhibitors Lys-Ala-LysCH_2S

no.	X	σ_I[a]	K_i (mM)[b]
I	H	0.00	0.29 ± 0.043
II	$CH_2CO_2CH_3$	0.19	0.28 ± 0.035
III	$COCH_3$	0.30	0.090 ± 0.013
IV	$OCOCH_3$	0.38	0.018 ± 0.010
V	Cl	0.47	0.0032[c]
VI	F	0.54	0.0010 ± 0.00024

[a] σ_I values from Charton (1981). [b] Errors are given as ± standard deviation. [c] The value of K_i from Coggins et al. (1974), 0.0065 mM, adjusted to correspond to a 50:50 mixture of diastereomers.

Hz, 2 H, FCH_2), 4.70 (m, 1 H, Lys[3] α-CH), 4.45 (m, 1 H, Ala[2] α-CH), 4.05 (m, 1 H, Lys[1] α-CH), 3.13 (m, 4 H, Lys[1] ϵ-CH_2, Lys[3] ϵ-CH_2), 1.30–1.95 (m, 25 H, BocCH_3, Lys[1] β-CH_2, Lys[1] γ-CH_2, Lys[1] δ-CH_2, Lys[3] β-CH_2, Lys[3] γ-CH_2, Lys[3] δ-CH_2, Ala[2] β-CH_3); TLC R_f 0.58 (CH_3Cl–MeOH, 9:1).

Preparation of Lys-Ala-LysCH_2F·3HCl (VI). A solution of 50 mg of Boc-Lys(Z)-Ala-Lys(Z)CH_2F (0.068 mmol) in 0.5 mL of trifluoroacetic acid was heated at 50 °C for 1 h. The product was precipitated by the addition of 3 mL of 4 N HCl in ether. After this was vortexed and centrifuged, the ether was removed by decanting. The product was washed 3 times with ether. Removal of the final traces of ether with a stream of nitrogen resulted in 32 mg of an off-white, very hygroscopic powder. This was purified by gel filtration on Sephadex G-15 with 1 mM HCl as the eluent. The result was 24 mg of a white powder (0.051 mmol, 75%): TLC R_f 0.45 (1-butanol–pyridine–acetic acid–water, 15:15:12:10); TLE single component, migration relative to arginine = 1.4.

Enzyme Kinetics. The enzymatic activities of solutions of trypsin and trypsin plus inhibitor were determined by a modification of the method of Erlanger et al. (1961), using BAPA as the substrate and monitoring the rate of release of *p*-nitroaniline spectrophotometrically at 405 nm. The following solutions were used: sodium phosphate buffer, 0.05 M, pH 6.5; trypsin, 1 mg/mL, in 1 mM HCl and 20 mM $CaCl_2$; BAPA, solutions of 1, 0.5, $^1/_3$, and 0.25 mg/mL in H_2O. The inhibitors were stored as solutions in 1 mM HCl. Two concentrations of inhibitor were used as well as a blank (1 mM HCl). The assay was carried out by adding sequentially into a cuvette 1.00 mL of buffer, 5 μL of trypsin, and 50 μL of inhibitor followed by 500 μL of BAPA with little or no incubation time. Four assays were performed at each inhibitor–BAPA concentration. The inhibition constants were determined from a Lineweaver–Burk plot of the reciprocal of the mean of the four velocities vs. the reciprocal of the substrate concentration (Lineweaver & Burk, 1934). The slopes and intercepts were calculated with the linear regression program on a Hewlet-Packard 11C calculator.

Results

The Dakin–West reaction results in complete racemization of the carboxy-terminal lysine (McMurray & Dyckes, 1985). All of the K_i values reported here are those of the diastereomeric tripeptides, fully racemic at the C-terminus.

The reversible binding constants determined for each of the inhibitors are shown in Table I. In no case did significant irreversible inactivation of trypsin occur during a run of the inhibition assay, ca. 8 min. In these experiments, the substituted ketones showed reversible, competitive inhibition. (Over a period of hours the fluoromethyl ketone inhibitor does bind irreversibly.) The strength of reversible binding increases with increasing electron-withdrawing power of the substituent, with the strongest binding exhibited by the fluoromethyl ketone.

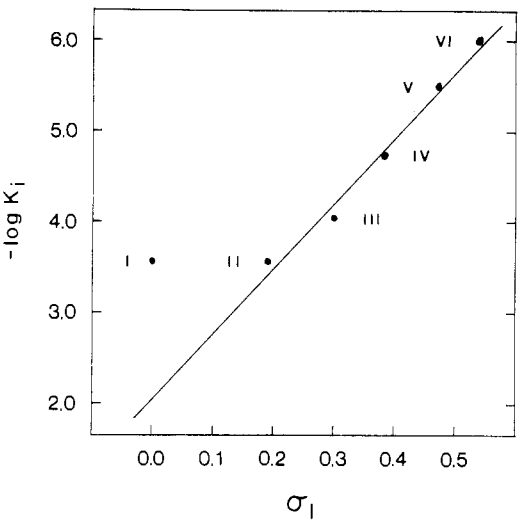

FIGURE 1: Hammett plot of the negative logarithm of the inhibition constant (K_i) vs. the inductive substituent constant (σ_I) of the inhibitors Lys-Ala-LysCH_2X.

A plot of $-\log K_i$ vs. σ_I for the set of inhibitors is shown in Figure 1. With the exception of the methyl ketone (X = H), the relationship between the free energy of binding and the inductive substituent constant is linear and follows the relationship:

$$-\log K_i = 7.2\sigma_I + 2.1 \qquad r = 0.993 \qquad (1)$$

The value of $-\log K_i$ of the corresponding chloromethyl ketone V (Coggins et al., 1974) was not used in the linear regression calculation; however, it falls on this line. (This chloromethyl ketone was not diastereomeric, and the reported value of K_i has been adjusted accordingly, to make it comparable with our data from diastereomers.)

Discussion

The Dakin–West reaction is a useful method for the preparation of a wide variety of peptidyl ketones. Although the reaction produces diastereomers, the product mixtures can still be used for a number of purposes, as illustrated by this study. Preliminary HPLC experiments (to be reported elsewhere) have indicated that in many cases these diastereomers will be readily separated.

Of particular interest is the synthesis of Lys-Ala-LysCH_2F (VI). The synthesis of fluoromethyl ketones of this type has been of interest for some time (Powers, 1977) but has only recently been achieved. Rasnick (1985) has synthesized Z-Phe-AlaCH_2F and Z-Pro-AlaCH_2F by a similar route. Gelb et al. (1985) have recently published the synthesis of the related compounds 2,2-difluorostatine, 2,2-difluorostatone, and pepstatin analogues containing them.

The linear free energy relationship demonstrated in this study shows that the strength of binding to trypsin by the ketone inhibitors Lys-Ala-LysCH_2X is dependent on the electron-withdrawing ability of the substituent X. This is consistant with a hemiketal intermediate (E·I′, Figure 2), and not the Michaelis complex (E·I), as the species whose dissociation is being measured [therefore, $K_I = 1/(K_1K_2)$]. Greater electron density induction by X generates more partial positive charge on the carbonyl carbon, thereby making the carbonyl group more susceptible to nucleophilic attack. On formation of the Michaelis complex, the hydroxyl group of Ser-195 attacks the ketone carbonyl, resulting in the hemiketal (Figure 2), which is itself relatively less destabalized by electron withdrawal than is the carbonyl. Electron-withdrawing groups will thus both increase the rate of hemiketal formation and

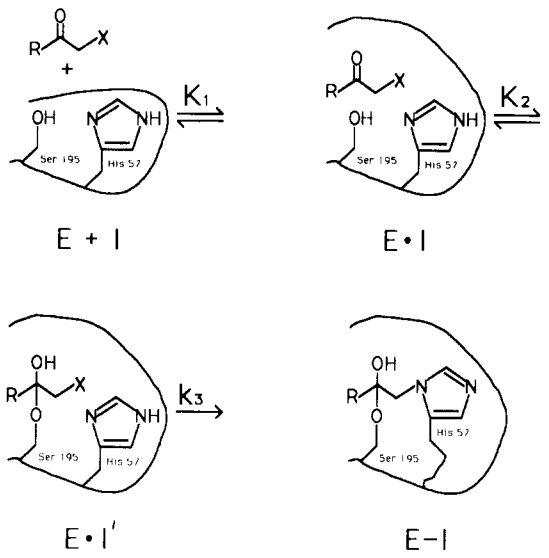

FIGURE 2: Mechanism 2, the proposed mechanism of the inactivation of serine proteinases by halomethyl ketones. Malthouse et al. (1985) have shown in related enzyme–inhibitor conjugates corresponding to E–I, that the pK_a of the hemiketal hydroxyl group is 7.9. Thus, significant amounts of both the protonated and the deprotonated form can be expected in both E·I′ and E–I, and indeed, the deprotonated form may be required for the conversion characterized by k_3.

shift the equilibrium toward the hemiketal intermediate. Only the latter effect has been measured here.

Bell (1966) and Greenzaid et al. (1967) have both found similar free energy relationships for the mechanistically analogous hydration of substituted aldehydes and ketones in aqueous solution. Indeed, the slope of our free energy plot (7.2) is somewhat larger than these. [Greenzaid et al. (1967) found, in a refinement of Bell's (1966) work, a slope of 3.8.] Because the binding equilibrium that we observe is even more sensitive to electron withdrawal than the hydration reaction, the species being observed cannot be noncovalently bound ketone. Furthermore, if the hydrated form of the ketone were the species being bound, the contribution of the electron-withdrawing substituents would be made in the hydration step itself, and the slope of the linear free energy plot for binding could not be greater than that for hydration. (In fact, substituents with greater electron-withdrawing power might well be expected to decrease the binding interaction between the hydrate hydroxyl groups, or the ketone carbonyl oxygen, and the "oxyanion" binding site.) Since the sensitivity of the binding reaction to electron withdrawal is similar to but greater than that for hydration, we conclude the reversibly bound species being observed is the hemiketal. The existance of a hemiketal in the final, alkylated adduct E–I (Figure 2) is well documented (Malthouse et al., 1983, 1985; Mackenzie et al., 1984); a hemiketal as a reversibly bound intermediate is now indicated.

The unsubstituted tripeptidylmethyl ketone [X = H (I)] does not conform to the free energy relationship determined from the other analogues but exhibits a binding stronger than would be predicted from the graph. This may be the result of the fact that the unsubstituted analogue does not lose entropy of rotation upon tight binding, whereas tight binding would certainly be expected to hinder rotation about the carbonyl carbon–α-methylene bond for substituted analogues. On the other hand, the binding exhibited by the unsubstituted analogue may simply be unaffected by interactions at the ketone carbonyl. That is, this binding is very weak and may represent a minimum attraction due primarily to intermolecular interactions at points on the inhibitor away from the ketone carbonyl function.

The value of the dissociation constant for the initial reversible binding of Lys-Ala-LysCH_2Cl (V) to trypsin falls on the line in Figure 1. This close fit to the data from the model inhibitors is strong evidence that the interactions involved in the reversible binding of the chloromethyl ketone are the same as those for the reversible inhibitors: covalent hemiketal formation. Thus, we conclude that mechanism 2, shown in Figure 2, is a better representation of the pathway of halomethyl ketone inhibition reaction than is mechanism 1; the formation of a hemiketal precedes alkylation of the enzyme. Whether the further intermediate step of epoxide formation proposed in mechanism 3 also occurs during the irreversible inhibition process cannot be determined from the evidence presented here.

Registry No. I, 101009-76-1; II, 101009-77-2; III, 101009-78-3; IV, 101009-79-4; V, 52780-80-0; VI, 101009-80-7; VI·3HCl, 101141-38-2; Boc-Lys(Z)-Ala-Lys(Z)-OH, 94904-24-2; Boc-Lys(Z)-Ala-Lys(Z)CH_2F, 101009-81-8; trypsin, 9002-07-7; serine proteinase, 37259-58-8; fluoroacetic acid, 144-49-0; fluoroacetic acid (symmetric anhydride), 407-33-0.

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CORRECTIONS

2-[(4-Azido-2-nitrophenyl)amino]ethyl Triphosphate, a Novel Chromophoric and Photoaffinity Analogue of ATP. Synthesis, Characterization, and Interaction with Myosin Subfragment 1, by Kay L. Nakamaye, James A. Wells, Robert L. Bridenbaugh, Yoh Okamoto, and Ralph G. Yount*, Volume 24, Number 19, September 10, 1985, pages 5226–5235.

Page 5227. In column 2, line 29, ϵ_M = 31 400 M^{-1} cm^{-1} should read ϵ_M = 23 400 M^{-1} cm^{-1}.

Kinetics of Reduction of High Redox Potential Ferredoxins by the Semiquinones of *Clostridium pasteurianum* Flavodoxin and Endogenous Flavin Mononucleotide. Electrostatic and Redox Potential Effects, by C. T. Przysiecki, G. Cheddar, T. E. Meyer, G. Tollin, and M. A. Cusanovich*, Volume 24, Number 20, September 24, 1985, pages 5647–5652.

Page 5649. In Table I, column 1, the letters A through J in parentheses should precede the species names.