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Aldehyde and Ketone Substrate Analogues Inhibit the Collagenase of *Clostridium histolyticum*†

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ABSTRACT: The collagenase from *Clostridium histolyticum* is a mixture of several collagenases, all of which are zinc metalloproteases. This enzyme catalyzes the cleavage of the X–Gly peptide bond in the repeating sequence of collagen: -Gly-Pro-X-Gly-Pro-X-. Thus the S_3, S_2, and S_1 subsites on the enzyme appear to be occupied by the sequence -Gly-Pro-X- and the S_1', S_2', and S_3' subsites also by -Gly-Pro-X-. Short peptides up to and including N^{α}-acyltetrapeptides containing the repeat sequence do not detectably inhibit the enzyme (IC_{50} > 10 mM). However, peptide aldehydes of the form aminoacyl-X-glycinal, presumably occupying the S_1, S_2, ..., S_n subsites, are inhibitors. The most potent of these was Pro_6-Gly-Pro-glycinal, with an IC_{50} of 340 ± 70 μM. The single peptide aldehyde investigated, which could occupy the S_1' and S_2' subsites, 4-oxobutanoyl-L-proline, did not inhibit collagenase (IC_{50} > 20 mM). The peptide ketone 5-benzamido-4-oxo-6-phenylhexanoyl-Pro-Ala (XXV), which could occupy the S_1–S_3' subsites, inhibits collagenase with an IC_{50} of 120 ± 50 μM, over 80-fold more potently than its parent peptide analogue benzoyl-Phe-Gly-Pro-Ala (XXIII). The alcohol analogue of XXV, 5-benzamido-4-hydroxy-6-phenylhexanoyl-Pro-Ala (XXVI), is over 60-fold less potent with an IC_{50} of 8 ± 2 mM. Extending the peptide ketone XXV to occupy the S_2–S_3' subsites gave 5-(N^{α}-carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-Pro-Ala (XXVII). Surprisingly, XXVII had an IC_{50} of only 5.2 ± 2 mM. Neither XXV nor XXVII exhibited the kinetic characteristic of a slow-binding inhibitor. We conclude that 5-benzamido-4-oxo-6-phenylhexanoyl-Pro-Ala (XXV) does not inhibit collagenase by simply forming an enzyme–inhibitor complex similar to the enzyme–substrate Michaelis complex. It must bind to the enzyme by a different mechanism, but not necessarily as an analogue of the transition state in the enzyme-catalyzed hydrolysis of substrate.

The collagenase from *Clostridium histolyticum* (EC 3.4.24.3) is a zinc metalloprotease also known as clostridiopeptidase A and collagenase A (Seifter & Harper, 1971). This collagenase makes a large number of cleavages in native triple-helical collagen, usually at the X–glycine bond in the sequence X-glycyl-L-prolyl-X, where X is frequently alanine or hydroxyproline but can be any amino acid. Synthetic oligopeptides are cleaved with similar specificity. The triple-helical region of native soluble collagen is relatively resistant to nearly every protease except the collagenases (Burleigh, 1977). This enzyme is now known to be a mixture of collagenases (Lwebuga-Mukasa et al., 1976; Sugasawara & Harper, 1984) and has been separated into six individual enzymes (Bond & Van Wart, 1984a,b). These six enzymes fall into two classes, class I (α and β collagenases) having high activity against collagen and lower activity against several small peptide substrates and class II (γ, δ, ε, and ζ collagenases) having the opposite trend of activity (Bond & Van Wart, 1984a,b).

Compounds such as cysteine that are nonspecific inhibitors of all zinc metalloproteases inhibit collagenase (Seifter & Harper, 1971). N^{α}-(Benzyloxycarbonyl)tripeptides, and -tetrapeptides, -pentapeptides containing the sequence glycyl-L-proline have been reported to inhibit the enzyme with K_i's as low as 4 mM (Yagisawa et al., 1965). Similar peptides with a C-terminal chloromethyl ketone group show some

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specificity for inhibition of collagenase compared to other proteases (Balaevskaya et al., 1981). We recently reported a series of phosphoramidate and phosphonamidate inhibitors of the collagenase from *Clostridium histolyticum* (Galardy & Grobelny, 1983). The most potent of these, *P*-isoamylphosphonylglycyl-L-alanine, had an IC_{50} of 17 μM for the enzyme according to the viscometric assay with native soluble collagen as substrate. This type of inhibitor is usually considered to be an example of a transition-state analogue (Wolfenden, 1969), which mimics the transition state (or a reaction intermediate) in the normal substrate hydrolysis reaction. This conclusion is supported by the X-ray crystallographic structure of the complex of phosphoramidon and thermolysin (Weaver et al., 1977).

Aldehydes and ketones have been proposed to be transition state analogues for proteases due to their ability to add either an enzyme-bound nucleophile or a water molecule to form a tetrahedral adduct that mimics a tetrahedral intermediate known to occur in amide hydrolysis [Lewis & Wolfenden (1977a,b) and references cited therein]. Strong inhibition by aldehyde and ketone substrate analogues has been reported for several zinc metalloproteases: angiotensin converting enzyme (Almquist et al., 1980, 1982; Meyer et al., 1981; Gordon et al., 1984; Natarajan et al., 1984), leucine aminopeptidase (Anderson et al., 1982), and carboxypeptidase A [Galardy & Kortylewicz (1984) and references cited therein]. Evidence for formation of a tetrahedral enzyme–inhibitor complex has been acquired by carbon-13 NMR spectroscopy for aldehyde and ketone inhibitors of several proteases that are not metalloenzymes [see review by Mackenzie et al. (1984)].

We show here that several peptide aldehyde and peptide ketone inhibitors of the collagenase from *Clostridium histolyticum* have IC_{50}'s in the micromolar range, using a viscometric assay with native soluble collagen as substrate. The best inhibitor discovered, 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXV), has an IC_{50} of 120 ± 50 μM, over 80-fold more potent than its peptide substrate analogue, *N*-benzoyl-L-phenylalanylglycyl-L-prolyl-L-alanine (XXIII), and over 60-fold more potent than its alcohol analogue 5-benzamido-4-hydroxy-6-phenylhexanoyl-L-prolyl-L-alanine (XXVI). We propose that this inhibitor could either be a transition state analogue for collagenase or interact with the enzyme by some other mechanism that is not analogous to a simple enzyme–substrate Michaelis complex. Figure 1 shows a model for the active site of the collagenase from *Clostridium histolyticum* (Galardy & Grobelny, 1983) and the proposed mode of binding of substrates and carbonyl-containing inhibitors.

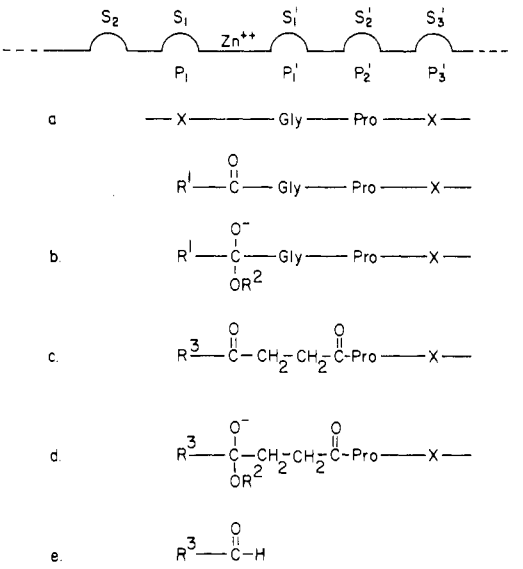

FIGURE 1: A model for the active site of *Clostridium histolyticum* collagenase (Galardy & Grobelny, 1983). S_1, S_2, etc., are the binding subsites for the amino acid residues P_1, P_2, etc., to the left of the scissile amide bond, and S_1', S_2', etc., are the binding substites for the amino acid residues P_1', P_2', etc., to the right according to the nomenclature of Schechter & Berger (1968). (a) Collagen and peptide substrates. R^1 is the continuing peptide chain. (b) Tetrahedral intermediate for the hydrolysis of (a), where R^2 may be H or the side chain of an amino acid residue of collagen. (c) Aldehyde (R^3 = H) or ketone (R^3 = R^1) inhibitors of collagenase. (d) Adduct between an aldehyde or ketone inhibitor and water (R^2 = H or an enzyme-bound nucleophile), which mimics the tetrahedral intermediate shown in (b). (e) Aldehyde inhibitor of collagenase, where R^3 = R^1.

Experimental Procedures

Materials. Protected amino acids, glycyl-L-proline, glycyl-L-prolyl-L-alanine, hippuryl-L-histidyl-L-leucine, acid-soluble calf skin collagen, and collagenase A from *Clostridium histolyticum* (type VII, catalog number C-0773, lot no. 54F-6808, 95% protein) were purchased from Sigma Chemical Co. Acid-soluble rat skin collagen was also obtained as a gift from Dr. Heinz Furthmayr of Yale University. Both the calf skin and rat skin collagens had intrinsic viscosities [η] of between 15 and 18 dL·g^{-1} in 0.05 M Tris-HCl[1]/0.5 M calcium chloride at 20 °C (lit. [η] = 16–18 dL·g^{-1}; Gallop & Seifter, 1963). Angiotensin converting enzyme was partially purified from rabbit lungs as described previously (Galardy, 1982). Gly-Pro, Gly-Pro-Ala, Leu-Gly-Pro-Ala, and Boc-Leu-Gly-Pro-Ala were prepared as previously described (Galardy & Grobelny, 1983). Other chemicals were of analytical grade and were used without further purification. Protected intermediates and inhibitor products were purified by column chromatography on silica gel 60-F254 (EM Reagents). The molecular weight of protected intermediates and inhibitors was determined from mass spectra taken on a Finnigan 3300 GC–MS spectrometer (electron energy = 150 eV, methane chemical ionization). Melting points were taken on a hot stage and are corrected. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390. Chemical shifts are in parts per million (ppm) downfield from tetramethylsilane in organic solvents. Thin-layer chromatography (TLC) was on silica gel 60-F254. Compounds were visualized by the following methods: exposure to hydrogen chloride vapor followed by ninhydrin (0.6 g in 100 mL of acetone) for protected and deprotected peptides and by 2,6-dinitrophenylhydrazine spray (2 g of 2,4-dinitrophenylhydrazine in 100 mL of methanol containing 4 mL of concentrated sulfuric acid) for aldehydes and ketones. The compositions of thin-layer solvent systems were, by volume, as follows: (A) chloroform/methanol, 9:1; (B) chloroform/acetonitrile/hexane, 2:2:1; (C) chloroform/acetonitrile/methanol, 2:2:1; (D) ethyl acetate/methanol, 2:1; (E) 1-butanol/acetic acid/water, 4:1:1; (F) chloroform/methanol/acetic acid, 85:10:5; (G) 2-propanol/concentrated ammonium hydroxide, 2:1; (H) chloroform/acetonitrile/2-propanol/acetic acid, 3:1:0.5:0.2. The TLC of all of the compounds described below gave homogeneous single spots. 4-Oxobutanoyl-L-proline lithium salt (V), (5*RS*)-5-benzamido-4-oxo-6-phenylhexanoyl-L-proline (XXIV), and (5*RS*)-5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine

[1] Abbreviations: DCC, dicyclohexylcarbodiimide; IC_{50}, the concentration of inhibitor giving 50% inhibition of a given enzyme concentration at one given substrate concentration; SD, standard deviation; K_m, the Michaelis constant; K_s, the enzyme–substrate dissociation constant; K_i, the enzyme–inhibitor dissociation constant; Tris, tris(hydroxymethyl)-aminomethane; Tris-HCl, Tris hydrochloride; Boc, *tert*-butyloxycarbonyl; GC, gas chromatography; MS, mass spectroscopy; Cbz, carbobenzoxy.

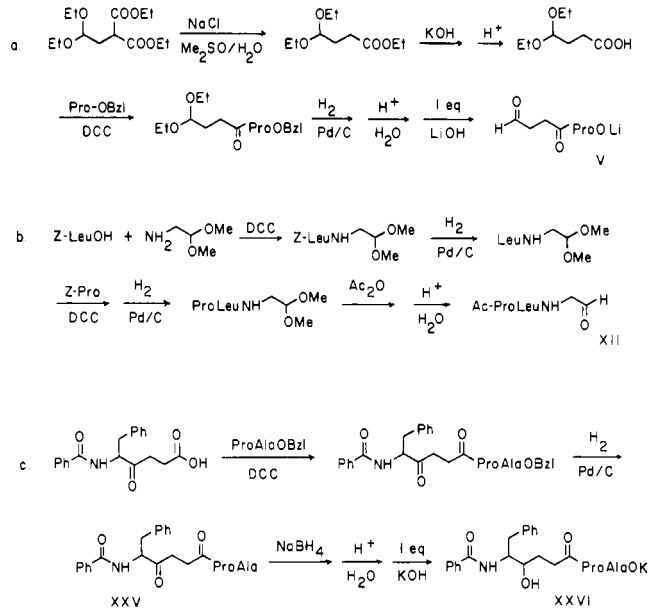

FIGURE 2: Outline of the synthesis of four of the inhibitors. (a) Synthesis of 4-oxobutanoyl-L-proline lithium salt (V) (Grobelny and Galardy, unpublished results) starting from diethyl (2,2-diethoxyethyl)malonate (Galardy & Kortylewicz, 1984). (b) Synthesis of N^{α}-acetyl-L-prolyl-L-leucylaminoacetaldehyde (XII) starting from carbobenzoxy-L-leucine and aminoacetaldehyde dimethyl acetal. (c) Synthesis of 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXV) (Grobelny and Galardy, unpublished results) and 5-benzamido-4-hydroxy-6-phenylhexanoyl-L-prolyl-L-alanine potassium salt (XXVI) starting from 5-benzamido-4-oxo-6-phenylhexanoic acid (Meyer et al., 1981).

(XXV) were prepared as described (D. Grobelny and R. E. Galardy, unpublished results). Figure 2 shows the synthetic pathways used to prepare V and XXV (Grobelny and Galardy, unpublished results) and XII and XXVI, as described below. All asymmetric centers in compounds XXIV, XXV, XXVI, and XXVII that are not α-carbons of L amino acids are assumed to be racemic.

N-Acetylglycinal (VI) was prepared according to Lewis & Wolfenden (1977a) in 95% yield of a mixture of aldehyde (10%) and hydrate (90%): NMR for aldehyde (D_2O) δ 2.00 (s, 3 H, CH_3), 4.05 (s, 2 H, CH_2), and 9.35 (s, 1 H, CHO); NMR for hydrate (H_2O) δ 1.90 (s, 3 H, CH_3), 3.20 (d, 2 H, CH_2CH), and 4.95 [t, 1 H, $CH_2CH(OH)_2$].

N^{α}-Acetyl-L-prolyl-L-leucylglycinal (XII). To a mixture of 6.63 g (25 mmol) of N^{α}-carbobenzoxy-L-leucine (VII) and 2.73 mL (25 mmol) of aminoacetaldehyde dimethyl acetal (VIII) in 15 mL of acetonitrile was added 5.16 g (25 mmol) of dicyclohexylcarbodiimide at −20 °C. The reaction mixture was stirred overnight at 4 °C, dicyclohexylurea was filtered off, and the solvent was evaporated. The residue was crystallized twice from a mixture of chloroform/hexane to give 6.02 g (68% yield) of N^{α}-carbobenzoxy-L-leucylglycinal dimethyl acetal (IX): mp 83–85 °C; NMR ($CDCl_3$) δ 0.95 (m, 6 H, Leu CH_3), 1.65 (m, 3 H, Leu $CH_2CH\gamma$), 3.35 (m, 8 H, Gly CH_2, OCH_3, 5.10 (s, 2 H, $PhCH_2O$), 5.8 (d, 1 H, $NHCH_2$), [m, 2 H, $CH(OCH_3)_2$, Leu CH], 6.75 (m, 1 H, Leu NH), and 7.35 (s, 5 H, Ph). IX (3.98 g, 11.3 mmol) was transformed into L-leucylglycinal dimethyl acetal (X) in 90% yield by hydrogenolysis performed in 15 mL of tetrahydrofuran in the presence of 0.46 g of 5% palladium on carbon under atmospheric pressure at room temperature. The coupling of X (0.736 g, 36 mmol) and *N*-Cbz-L-proline, performed as described for IX, followed by hydrogenolysis and acetylation with acetic anhydride gave 0.95 g (79% yield) of N^{α}-acetyl-L-prolyl-L-leucylglycinal dimethyl acetal (XI): mp 105–107 °C (crystallization from a mixture of ethyl acetate/hexane); R_f(A) = 0.5; NMR ($CDCl_3$) δ 0.90 (m, 6 H, Leu CH_3), 1.35–2.60 (m, 10 H, Leu $CH_2CH\gamma$, Pro $CH_2\beta,\gamma$, $AcCH_3$), 4.20–4.55 [m, 5 H, Leu CHα, Gly CH_2, Pro CHα, $CH(OCH_3)_2$], 6.68 (m, 1 H, Gly NH), and 7.30 (m, 1 H, Leu NH); mass spectrum (chemical ionization) 358 (M + 1), 386 (M + 29), 398 (M + 41). XI (0.174 g, 0.49 mmol) was dissolved in 0.66 g of deuterium oxide, and the pH of the solution was adjusted to 1 with trifluoroacetic acid. The reaction was monitored by NMR [the formation of $-CH(OD)_2$, at δ 4.95, was observed]. After about 20 h at room temperature the reaction mixture was frozen and lyophilized to give 0.150 g (95% yield) of XII: R_f(A) = 0.33; mass spectrum (chemical ionization) 312 (M + 1), 340 (M + 29), 352 (M + 41); NMR ($CDCl_3$) δ 1.60–1.82 (m, 3 H, Leu $CH_2CH\gamma$), 1.80–2.30 (m, 7 H, Pro $CH_2\beta,\gamma$, $AcCH_3$), 3.30 (m, 2 H, Pro $CH_2\delta$), 4.05 (d, 2 H, Gly CH_2), 4.15–4.65 (m, 2 H, Pro CHα, Leu CHα), 7.20 (m, 2 H, NH), 9.6 (s, 1 H, CHO), and 9.90 (m, 6 H, Leu CH_3).

N^{α}-Acetyl-L-prolylglycyl-L-prolylglycinal Dimethyl Acetal (XVII). The coupling of N^{α}-carbobenzoxy-L-proline (5 g, 20 mmol) with VIII (2.18 mL, 20 mmol), performed as described for IX, gave 5.1 g (75% yield) of N^{α}-carbobenzoxy-L-prolylglycinal dimethyl acetal (XIII) after purification by column chromatography (silica gel; chloroform/methanol, 98:2): R_f(B) = 0.55. XIII (4 g, 12 mmol) was transformed into L-prolylglycinal dimethyl acetal (XIV) in 98% yield as described for X. The coupling of XIV (2.66 g, 11.7 mmol) with N^{α}-carbobenzoxyglycine performed as described for IX gave 2.6 g (58% yield) of N^{α}-carbobenzoxyglycyl-L-prolylglycinal dimethyl acetal (XV): R_f(B) = 0.36. Hydrogenolysis of XV (2.6 g, 6.6 mmol), performed as described for X, gave 1.56 g (96% yield) of glycyl-L-prolylglycinal dimethyl acetal (XVI). The coupling of XVI (0.73 g, 4.6 mmol) with *N*-acetyl-L-proline performed as described for IX gave 10.68 g of crude XVII, which was purified by dissolving in a minimal volume of chloroform and precipitating with anhydrous ether to give 0.2 g (65% yield): R_f(D) = 0.3; NMR ($CDCl_3$) δ 2.05 (m, 11 H, Pro $CH_2\beta,\gamma$, $AcCH_3$), 3.10–3.80 [m, 12 H, Pro $CH_2\delta$, $CH_2CH(OCH_3)_2$], 4.05 (d, 2 H, Gly CH_2), 4.50 [m, 3 H, Pro CH, $CH(OCH_3)_2$], 7.05 [m, 1 H, $NHCH_2CH(OCH_3)_2$], and 7.70 (m, 1 H, Gly NH); mass spectrum (chemical ionization) 399 (M + 1).

N^{α}-Acetyl-L-prolylglycyl-L-prolylglycinal (XVIII). XVIII was prepared identically with XII but starting with XVII and *N*-acetyl-L-proline in 98% yield as a hygroscopic foam: R_f(D) = 0.2; NMR (D_2O) δ 1.65–2.40 (m, 11 H, Pro $CH_2\beta,\gamma$, $AcCH_3$), 3.25 [d, 2 H, $CH_2CH(OD)_2$], 3.55 [m, 4 H, Pro $CH_2\delta$], 4.05 (m, 2 H, Gly CH_2), 4.53 (m, 2 H, Pro CH), and 5.00 [t, 1 H, $CH(OD)_2$]; mass spectrum (chemical ionization) 353 (M + 1).

Hexa-L-prolylglycyl-L-prolylglycinal Hydrochloride Salt (XXII). *N^{α}-tert*-Butyloxycarbonylpenta-L-prolyl-L-proline benzyl ester (XIX) was prepared by successive coupling of *N^{α}-tert*-butyloxy-L-proline to the hydrochloride salt of L-proline benzyl ester by using dicyclohexylcarbodiimide in the presence of *N*-ethylmorpholine in CH_2Cl_2 or $HCON(Me)_2$ as solvent. The fully protected intermediates were purified by partition into ethyl acetate or dichloromethane and by washing with dilute acid and base, water, and saturated sodium chloride solution, followed by crystallization. Removal of the *tert*-butyloxy group was accomplished with 2 N hydrogen chloride in acetic acid. Removal of the benzyl group was as described for the carbobenzoxy group of IX. The intermediates were characterized by melting point determinations, thin-layer

chromatography, and NMR spectroscopy. XIX was obtained in 70% yield from Boc-L-proline and the hydrochloride salt of penta-L-proline benzyl ester: mp 231–234 °C; R_f(E) = 0.59; R_f(F) = 0.41; NMR ($CDCl_3$) δ 1.55 [s, 9 H, $(CH_3)_3C$], 2.05 (br s, 26 H, 6 × Pro $CH_2\beta,\gamma$), 4.60 (m, 12 H, 6 × Pro $CH_2\delta$), 5.10 (q, 2 H, $PhCH_2$), and 7.30 (s, 5 H, Ph). Hydrogenolysis of XIX (1.2 g, 1.51 mmol) performed as described for IX gave N^{α}-*tert*-butyloxycarbonylhexa-L-proline (XX) (1 g, 94% yield). A mixture of XX (1 g, 1.42 mmol), XVI (0.4 g, 1.5 mmol), and dicyclohexylcarbodiimide (0.29 g, 0.42 mmol) in 15 mL of chloroform was stirred for 3 days at 4 °C. After evaporation of the solvent, the residue was dissolved in 20 mL of water and the precipitate of dicyclohexylurea was filtered off. The filtrate was frozen and lyophilized to give 1.2 g of crude N^{α}-*tert*-butyloxycarbonylhexa-L-prolylglycyl-L-prolylglycinal dimethyl acetal (XXI). XXI was purified by dissolving in 15 mL of chloroform and precipitating with anhydrous ether (20 mL): yield, 0.84 g (63%); mp 175–185 °C; R_f(C) : 0.16; R_f(G) = 0.8; NMR ($CDCl_3$) δ 1.45 [s, 9 H, $(CH_3)_3$], 1.95 (br s, 28 H, 7 × Pro $CH_2\beta,\gamma$), 2.90–4.15 [m, 24 H, $CH_2CH(OCH_3)$, 7 × Pro $CH_2\delta$, Gly CH_2], 4.20–2.90 [m, 8 H, 7 × Pro CH, $CH(OCH_3)_2$], 6.85 (m, 1 H, $NHCH_2CH$), and 8.15 (m, tH, Gly NH). Anal. Calcd for $C_{46}H_{71}N_9O_{12}$: C, 58.64; H, 7.60; N, 13.38. Found: C, 58.00; H, 7.82; N, 12.98.

Acetal XXI (0.8 g, 0.84 mmol) was dissolved in 10 mL of water, and the pH of the solution was adjusted to 1 with hydrochloric acid. After 48 h at room temperature the mixture was frozen and lyophilized to give 0.71 g (99% yield) of product XXII as a foam: R_f(G) = 0.58; NMR (D_2O, 70 °C) δ 2.35 (m, 28 H, 7 × Pro $CH_2\beta,\delta$), 3.40–4.20 [m, 14 H, 7 × Pro $CH_2\delta$, $CH_2CH(OD)_2$], 4.35 (m, 2 H, Gly CH_2), 4.60–5.15 (m, 7 H, 7 × Pro CH), 5.30 [m, 0.8 H, $CH(OD)_2$], and 9.78 (s, 0.2 H, CHO); NMR (methanol-d_4) δ 2.00 (br s, 28 H, 7 × Pro $CH_2\beta,\gamma$), 3.00–4.10 [m, 16 H, 7 × Pro $CH_2\delta$, $CH_2CH(OD)_2$], and 4.20–5.00 [m, 20 H, 7 × Pro CH, Gly CH_2, $CH(OD_2$, HDO].

N^{α}-Benzoyl-L-phenylalanylglycyl-L-prolyl-L-alanine Potassium Salt (XXIII). N^{α}-Benzoyl-L-phenylalanine (0.39 g, 1.44 mmol, Sigma Chemical Co.) was coupled with the hydrochloride salt of glycyl-L-prolyl-L-alanine benzyl ester (0.53 g, 1.44 mmol; Galardy & Grobelny, 1983) as described for the synthesis of XIX to give 0.79 g (95% yield) of the benzyl ester of XXIII, which was converted to XXIII by hydrogenolysis as described for the removal of the carbobenzoxy group from IX to give the free acid of XXIII. Neutralization of the free acid with 1 N potassium hydroxide followed by lyophilization gave 0.36 g (25% yield) of XXIII: mp 160–165 °C; R_f(G) = 0.65; mass spectrum of free acid (chemical ionization) 495 (M + 1); NMR (D_2O) δ 1.35 (d, 3 H, Ala CH_3), 1.95 (m, 4 H, Pro $CH_2\beta,\gamma$), 2.95–3.70 (m, 4 H, Phe CH_2, Pro $CH_2\delta$), 4.00 (m, 2 H, Gly CH_2), 4.35–5.00 (m, 3 H, Ala CH, Phe CH, Pro CH), 4.67 (HDO), and 7.05–7.70 (m, 10 H, PhCO, Phe Ph).

(5RS,4RS)-5-Benzamido-4-hydroxy-6-phenylhexanoyl-L-prolyl-L-alanine Potassium Salt (XXVI). XXVI was prepared by reduction of XXV with sodium borohydride in a method analogous to the preparation of 5-benzamido-4-hydroxy-6-phenylhexanoyl-L-proline (Grobelny and Galardy, unpublished results) and neutralization of the free acid with 1 N potassium hydroxide in 95% yield: mp 142–145 °C dec, R_f(H) = 0.6; mass spectrum of free acid (chemical ionization) 310 [M − M(Pro-Ala-OH) + 1]; NMR (D_2O) δ 1.30 (d, 3 H, Ala CH_3), 1.90 (m, 4 H, Pro $CH_2\beta,\gamma$), 2.20–3.30 (m, 6 H, hexanoyl CH_2-2, -3, -6), 3.35–3.85 (m, 3 H, Pro $CH_2\delta$, hexanoyl CH-5), 4.00–4.50 (m, 3 H, Pro CH, Ala CH, hexanoyl CH-4), 7.15 (s, 5 H, Phe Ph), and 7.40 (m, 5 H, PhCO).

(5RS)-5-(N^{α}-Carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine Potassium Salt (XXVII). XXVII was prepared similarly to XXIV by starting with (5*RS*)-5-(N^{α}-carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoic acid and the hydrochloride salt of L-prolyl-L-alanine benzyl ester (Grobelny and Galardy, unpublished results). Saponification of the benzyl ester of XXVII in 70% aqueous tetrahydrofuran with 1 N potassium hydroxide (1 equiv) at room temperature gave XXVII in 90% yield: mp 128–135 °C dec; R_f(H) = 0.68; mass spectrum (chemical ionization) 606 [(M − K) + 1], 605 (M − K), 604 [(M − K) − 1]; NMR (D_2O) δ 1.28 (d, 3 H, Ala CH_3), 1.40–3.70 (m, 18 H, Pro $CH_2\beta,\gamma,\delta$, Ala CH_3, hexanoyl CH_2-2, -3, -6), 3.80–4.55 (m, 4 H, Pro CH, Ala CH, hexanoyl CH-5), 4.80 (br s, 2 H, $PhCH_2O$), and 7.10 (m, 10 H, Ph).

Kinetic Studies. Collagenase activity was determined by the viscometric method (Gallop et al., 1957) under the conditions described by McCroskery et al. (1975), in 0.1 M Tris buffer adjusted to pH 7.6 with hydrochloric acid, 0.2 M in sodium chloride but without 0.02% sodium azide, and at 20 °C instead of 35 °C, in 1-mL semimicroviscometers from Canon Instrument Co. As previously described (Galardy & Grobelny, 1983), the inhibitor concentration causing 50% inhibition of collagenase under the above conditions (IC_{50}) was determined from a plot of initial velocity in micromolar collagen hydrolyzed per minute vs. the logarithm of the inhibitor concentration. One inhibitor of this study, 5-benzamido-4-oxo-6-phenylhexanoyl-L-proline (XXIV) (ketoace; Almquist et al., 1980) was assayed by using the spectrophotometric method with the synthetic substrate of Van Wart & Steinbrink (1981) instead of the viscometric assay. The IC_{50} reported for XXIV in Table I is calculated form the IC_{50} found in the spectrophotometric assay by multiplying the spectrophotometric IC_{50} by 8, the ratio of the viscometric IC_{50} to the spectrophotometric IC_{50} for ketone XXV. Although this calculated IC_{50} may be less accurate than the ones measured for the other inhibitors, XXIV is a weak inhibitor and its exact IC_{50} is not crucial to the conclusions of this paper. Reported IC_{50}'s are the averages of at least two IC_{50}'s determined in separate experiments on different days.

The two ketone inhibitors 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXV) and 5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXVII) were also assayed for time-dependent inhibition of collagenase by the spectrophotometric method of Van Wart & Steinbrink (1981) using 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine. This substrate was a gift from Dr. Harold Van Wart of Florida State University. The time dependence of inhibition was determined in order to confirm that the viscometric IC_{50}'s represented steady-state conditions of inhibitor binding to enzyme. The conditions of this spectrophotometric assay were as follows: collagenase, 0.3 $\mu g\ mL^{-1}$; substrate, 0.175 mM; ketone XXV, 200 μM, or ketone XXVII, 6 mM. Inhibitors were assayed against converting enzyme as previously described (Galardy, 1982) in 50 mM Tris-HCl, adjusted at pH 7.5 with sodium hydroxide, and 300 mM sodium chloride, with hippuryl-L-histidyl-L-leucine as substrate.

Results

Figure 3 shows plots of the velocity of the degradation of native collagen by collagenase as a function of the logarithm of the concentrations of three different inhibitors according to the viscometric assay described under Experimental Procedures. The IC_{50} for each inhibitor was taken to be the concentration of inhibitor at half-maximum velocity. The error

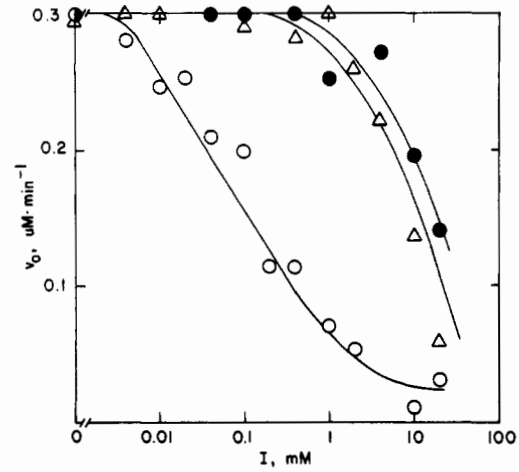

FIGURE 3: Initial velocity vs. inhibitor concentration for the collagenase-catalyzed hydrolysis of native soluble collagen assayed by the viscometric method: ○, 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXVII); ●, benzoyl-L-phenylalanylglycyl-L-prolyl-L-alanine (XXII); △, 5-benzamido-4-hydroxy-6-phenylhexanoyl-L-prolyl-L-alanine (XXVII).

in determining IC_{50}'s by this viscometric assay is large and is apparent in both the scatter in the experimental points in Figure 3 and in the large standard deviations given for the IC_{50}'s in Table I, up to ±40% of the value. IC_{50}'s are given in Table I for several oligopeptides comprising parts of the repeating collagen sequence Gly-Pro-X-Gly-Pro-X for peptide aldehyde inhibitors occupying the S_1'–S_n' subsites on the enzyme as indicated in Figure 1c (R^3 = H), for peptide aldehydes occupying the S_1–S_n subsites as indicated in Figure 1e (R^3 = continuing peptide chain), and for peptide ketones occupying the S_n–S_n' subsites (Figure 1c, R^3 = continuing peptide chain). For the peptide aldehydes occupying the S_1–S_n subsites, IC_{50}'s qualitatively decreased as a function of providing more amino acid residues to occupy more subsites. The most potent aldehyde of this series was Pro_6-Gly-Pro-aminoacetaldehyde (XXII), with an IC_{50} of 340 ± 70 μM. The complete loss of activity observed upon protection of XVIII to give the dimethyl acetal XVII demonstrates the requirement for the aldehyde group to produce inhibition. Only one aldehyde occupying subsites on the S_n' side of the active site zinc atom was tested, 4-oxobutanoyl-L-proline, and it was found to be inactive. Extension of this inhibitor to occupy more subsites was not pursued since we hoped to find a ketone inhibitor spanning the active site zinc and having much greater activity. For the series of ketones, the IC_{50} was minimized at 120 ± 50 μM with 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXV), which is presumed to occupy the S_1–S_3' subsites. This ketone is over 80-fold more potent than its peptide analogue benzoyl-L-phenylalanylglycyl-L-prolyl-L-alanine (XXIII) and over 60-fold more potent than its alcohol analogue 5-benzamido-4-hydroxy-6-phenylhexanoyl-L-prolyl-L-alanine (XXVI). Suprisingly, increasing the length of this peptide ketone by adding a proline in position S_2 to give 5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXVII) increased the IC_{50} by a factor of over 40-fold to 5200 ± 2000 μM.

The two ketones XXV and XXVII were also assayed by the spectrophotometric method of Van Wart & Steinbrink (1981) to ensure that the inhibitor and enzyme had reached a steady-state binding interaction. At the enzyme and substrate conditions given under Experimental Procedures, inhibition by XXV at a concentration of 200 μM gave a constant slope for over 1 h of reaction at a velocity of 15%g of the initial velocity observed in the absence of inhibitor. Inhibitor XXVII

Table I: Inhibition of the Collagenase from *Clostridium histolyticum* by Peptide Aldehydes and Peptide Ketones[d]

	peptide	IC_{50} ± SD (μM)
	Gly-Pro——↓X——Gly—Pro-X	
I	Gly—Pro	> 20,000[a]
II	Gly—Pro-Ala	> 10,000[a]
III	Leu—Gly—Pro-Ala	> 20,000[a]
IV	BOC—Leu—Gly—Pro-Ala	> 20,000[a]
V	$CHOCH_2$-CH_2CO-Pro	> 20,000
VI	Ac-$NHCH_2CHO$	> 20,000
XII	Ac-Pro-Leu-$NHCH_2CHO$	10,300 ± 3,000
XVIII	Ac-Pro-Gly-Pro-$NHCH_2CHO$	9,300 ± 3,000
XVII	Ac-Pro-Gly-Pro-$NHCH_2CH(OMe)_2$	> 20,000
XXII	Pro_6-Gly-Pro-$NHCH_2CHO$	340 ± 70
XXIII	Bz-Phe—Gly—Pro-Ala	10,000
XXIV	Bz-NHCH(Bzl)$COCH_2CH_2CO$—Pro	3,000 ± 1,000[b]
XXV	Bz-NHCH(Bzl)$COCH_2CH_2CO$—Pro-Ala	120 ± 50[c]
XXVI	Bz-NHCH(Bzl)$CHOHCH_2CO$—Pro-Ala	8,000 ± 2,000
XXVII	CBZ-Pro-NHCH(Bzl)$COCH_2CH_2CO$—Pro-Ala	5,200 ± 2,000

[a] Galardy & Grobelny, 1983. [b] K_i of 0.0006 μM for angiotensin converting enzyme (Grobelny & Galardy, 1985). [c] IC_{50} of 16 μM for collagenase according to the spectrophotometric method; K_i of 26 μM for converting enzyme. [d] The arrow shows the proposed site of cleavage in the generalized substrate Gly-Pro-X-Gly-Pro-X.

at a concentration of 6 mM gave a constant slope for nearly 1 h of reaction (at a velocity of 23% of that of the uninhibited reaction). The weak inhibition observed with XXVII compared to XXV was thus confirmed by the spectrophotometric assay and appears to be occurring under steady-state conditions of interaction of enzyme with inhibitor. An IC_{50} of about 16 μM was determined for ketone XXV in the spectrophotometric assay, about 8-fold lower than that found by the viscometric assay. This may be partly due to the fact that the IC_{50} is dependent on the substrate concentration (at least for competitive inhibitors) and to the difference in ionic strength, temperature, and buffer composition between the two assays. In the viscometric assay the collagen substrate concentration was 5 μM, just above the K_m (Galardy & Grobelny, 1983). In the spectrophotometric assay, the substrate concentration was 175 μM, about one-third of its K_m (Van Wart & Steinbrink, 1981).

Discussion

The major conclusions of this study can be summarized as follows. The strength of inhibition of collagenase by the aldehydes examined is qualitatively proportional to the length of the inhibitor, i.e., to the number of subsites on the enzyme that the inhibitor can occupy. This is not true for the ketone inhibitors. The best ketone inhibitor, XXV (IC_{50} = 120 μM), and its longer but weaker homologue XXVII are not slow-binding inhibitors but reach equilibrium with the enzyme in much less than 2 min. The best ketone inhibitor, XXV, probably does not bind in a simple Michaelis-type interaction with the enzyme since its IC_{50} is over 80-fold lower than that of its parent peptide.

In considering the relative IC_{50}'s of the inhibitors of this study, it must be kept in mind that the collagenase preparation employed here is a mixture of six collagenases present in undetermined proportions and that these enzymes fall into two classes with different and not yet fully known substrate spe-

cificities (Bond & Van Wart, 1984a,b; Steinbrink et al., 1985). Thus the IC_{50}'s reported here represent some sort of average IC_{50} against the mixture of enzymes and may vary when tested against individual collagenases.

The Dependence of Inhibitory Potency on Peptide Chain Length. The decrease in IC_{50} with lengthening of the amino acid chain of the aldehyde inhibitors is expected if these subsites actually exist and are involved in binding substrates and substrate analogues. The aldehydes VI, XVII, and XXII thus progressively give lower IC_{50}'s with increasing chain length. The amino acid in the P_1 position in this series is glycine (as glycinal), which is not expected to optimize binding to the enzyme. In our previous study, we found that the side chain of leucine in the P_1 position decreased the IC_{50} by a factor of almost 50 compared to glycine (no side chain) in a series of phosphoric and phosphonic amides designed to occupy the S_1–S_3' subsites (Galardy & Grobelny, 1983). Thus leucinal in position P_1 in the present series of aldehydes may decrease IC_{50} significantly. No attempt made to optimize the sequence of amino acids occupying the other subsites in this series of aldehydes. Minimizing IC_{50} by varying the amino acid residue in each position of the inhibitor would require a massive number of inhibitors to be synthesized and should wait for more detailed structure–activity studies on substrates using purified collagenases as in Steinbrink et al. (1985). Extension of 4-oxobutanoyl-L-proline (V) to occupy more subsites to the right of the zinc atom in Figure 1 was not pursued due to the expectation of finding a much more potent peptide ketone inhibitor occupying subsites on both sides of the zinc atom. The possibility was thought likely because of the strong inhibition of angiotensin converting enzyme by the peptide ketone substrate analogue ketoace (XXIV) (Almquist et al., 1980; K_i = 0.0006 μM; see Table I) and of pepsin by the ketone analogue of pepstatin (K_i = 56 nM; Rich et al., 1982).

The ketones examined as inhibitors of collagenase were based on the sequence Pro-X_1-Gly-Pro-X_2. X_1 is phenylalanine, allowing the synthetic ketone intermediate from the ketoace synthesis to be employed (Almquist et al., 1980). X_2 is alanine as for the phosphorus-containing inhibitors of collagenase previously reported (Galardy & Grobelny, 1983). The first ketone inhibitor tested was ketoace, 5-benzamido-4-oxo-6-phenylhexanoyl-L-proline, XXIV, which had an IC_{50} of 3 mM. Extending ketoace by adding a C-terminal alanine in the P_3' position gave 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXV), with an IC_{50} of 120 μM. However, further extension of XXV by adding a proline in the P_2 position gave 5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (XXVII), a much weaker inhibitor with an IC_{50} of only 5.2 mM. The IC_{50} of 120 μM for XXV, the best ketone inhibitor prepared, is more than 5 orders of magnitude weaker than the K_i of ketoace for converting enzyme (see Table I).

The increase of the IC_{50} of XXV to 5.2 mM by extending XXV to occupy the S_2 subsite was unexpected. However, the same result was found for ketoace and converting enzyme. Extending ketoace (XXIV) by adding one amino acid in the P_2 position gave 5-(carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-L-proline. This longer peptide ketone has a K_i for converting enzyme of 3 μM, compared to 0.0006 μM for ketoace, a 5000-fold increase (Grobelny & Galardy, unpublished results). For these two enzymes then, extension of peptide ketone inhibitors that already occupy the S_1–S_n' subsites to occupy the S_2–S_n' subsites dramatically reduces potency. Clearly, extension of the inhibitor on both sides of the ketone functional group prevents the inhibitor from forming a tightly bound complex with the enzyme and produces an inhibitor that binds in a Michaelis-type complex with a K_i on the order of K_s for substrate (where K_i measured for substrate is assumed to equal K_s). For ketoace it has been shown that the only substituents on the 5-position that give an IC_{50} in the nanomolar range are benzamido and 2-furanamido (Meyer et al., 1981; Almquist et al., 1982). Simply substituting carbobenzoxamido for benzamido in position 5 of ketoace increases the IC_{50} by 2 orders of magnitude (Meyer et al., 1982). Interpreting these and the present results is not possible at this time, but it appears as if an aromatic carboxamido group is required in the 5-position for peptide ketone inhibitors of converting enzyme and may be required for collagenase on the basis of only the results reported here.

The Peptide Ketones Rapidly Reach Equilibrium Binding with Collagenase. Since the inhibition of collagenase by either XXV or XXVII did not increase during a 1-h incubation with the enzyme, neither ketone appears to be a "slow-binding" inhibitor [see discussion of slow-binding inhibitors of angiotensin converting enzyme, Shapiro & Riordan (1984)]. Thus the modest viscometric IC_{50} of 120 μM found for XXV (spectrophotometric IC_{50} = 16 μM), the most potent inhibitor of this study, is a steady-state value indicating equilibrium between enzyme and inhibitor. In spite of its modest IC_{50}, 5-benzamido-4-oxo-6-phenylhexanoyl-L-proline is one of the most potent synthetic inhibitors known for bacterial collagenase (Galardy & Grobelny, 1983).

Peptide Ketone XXV Binds to Collagenase in a Non-Michaelis-Type Complex. The IC_{50} of ketone XXV for collagenase is over 80-fold lower than that of its peptide analogue, benzoyl-L-phenylalanylglycyl-L-alanine (XXIII), and over 40-fold lower than that of its alcohol analogue, 5-benzamido-4-hydroxy-6-phenylhexanoyl-L-prolyl-L-alanine (XXVI). The peptide XXIII is a single optical isomer with all amino acids in the L configuration while the ketone XXV is racemic at the 5-position, and its alcohol analogue, XXVI, is assumed to be racemic at both the 4-position and the 5-position. The IC_{50}'s of a single optical isomer of the ketone XXV and alcohol XXVI could be as much as 2-fold and 4-fold lower than the IC_{50}'s of their respective diastereomeric mixtures. This possibility does not change any of the present conclusions. Assuming that benzoyl-L-phenylalanylglycyl-L-prolyl-L-alanine (XXVI) binds in a Michaelis-type complex, the ketone XXV must have an additional interaction with collagenase. Figure 4 shows several possible modes of binding for the ketone XXV to collagenase. The simple active site model of Figure 4 assumes that there is an active site carboxyl group in collagenase as found in the zinc metalloproteases carboxypeptidase A and thermolysin (Kester & Mathews, 1977; Quiocho & Lipscomb, 1971). The possible modes of binding of carbonyl compounds to collagenase shown in Figure 1 are based on X-ray crystallography of the complex of (−)-2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid (Rees et al., 1980) and 2-benzyl-4-oxobutanoic acid (D. W. Christianson and W. N. Lipscomb, Harvard University, personal communication; Galardy & Kortylewicz, 1984) to carboxypeptidase A.

The binding of the ketone XXV (Figure 4a) to the enzyme could simply displace water from the active site to give the complex shown in Figure 4e. Although structure e appears to be a Michaelis-type complex, it could be argued that the loss of water from the active site leaves an active site without the ability to hydrolyze (add water to) substrate and thus incapable of proceeding to products. This kind of complex between glycyl-L-tyrosine and carboxypeptidase A which does

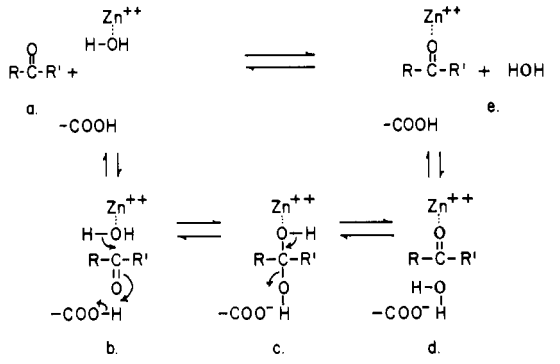

FIGURE 4: Possible modes of binding of ketones to zinc metalloproteases such as collagenase. The ketone could displace water from the enzyme to give structure e directly or could abstract water from the active site proceeding through the tetrahedral intermediate c to give structure e. Structure e is that observed for binding of the ketone (−)-2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid to carboxypeptidase A (Rees et al., 1980). The geminal diol (hydrate) (c) is the structure observed for the binding of 2-benzyl-4-oxobutanoic acid (Galardy & Kortylewicz, 1984) to carboxypeptidase A by X-ray crystallography. In fact, both oxygen atoms of the hydrate are found to be coordinated to the zinc atom (D. W. Christianson and W. N. Lipscomb, Harvard University, personal communication). This schematic representation of binding modes is meant to define neither precise relationships between functional groups nor definite positions of protons in complexes.

not include a water trapped at the active site has been called a nonproductive complex (Kester & Matthews, 1977). Rees et al. (1980) have shown that the ketone (−)-2-benzyl-4-(3-methoxyphenyl)-4-oxobutanoic acid binds to carboxypeptidase A as indicated in Figure 4e, without locating a tightly bound water molecule. Figure 4e therefore could represent this type of nonproductive complex.

An alternative pathway from a to e in Figure 4 goes through the intermediates b, c, and d. The geminal diol structure shown in Figure 4c is observed for the binding of 2-benzyl-4-oxobutanoic acid to carboxypeptidase A by X-ray crystallography (D. W. Christianson and W. N. Lipscomb, Harvard University, personal communication). Therefore, we propose that aldehyde inhibitors of carboxypeptidase such as 2-benzyl-4-oxobutanoic acid and ketone inhibitors such as 2-benzyl-4-oxo-4-(3-methoxyphenyl)butanoic acid could bind via the pathways a, b, c, d, and e in Figure 4. For the highly electrophilic aldehydes (Galardy & Kortylewicz, 1984) and haloketones (Galardy & Kortylewicz, 1985) the tetrahedral state c in Figure 4 is populated. For less electrophilic ketones such as 2-benzyl-4-oxo-4-(3-methoxyphenyl)butanoic acid the tetrahedral structure in Figure 4c is less stable. The less electrophilic ketones therefore could pass through state c to d or e. The demonstration that enzyme-catalyzed exchange of the carbonyl oxygen atom occurs for the ketone inhibitors would prove the existence of the pathway from state a to either d or e.

Extension of this reasoning to bacterial collagenase suggests the two binding modes c and e (or d) in Figure 4 for the peptide ketone inhibitor XXV. We believe that structure c is less likely because of the low electrophilicity of the ketone carbonyl carbon compared to aldehyde carbonyl carbons (Lewis & Wolfenden, 1977a) and *the weak* IC_{50} observed for XXV (120 μM) compared to the strong binding usually associated with transition state analogues assuming the structure shown in Figure 4c. 2-Benzyl-4-oxobutanoic acid, shown to be a transition state analogue by Christianson and Lipscomb (personal communication), has a K_i for carboxypeptidase of 0.48 μM (Galardy & Kortylewicz, 1983). That is, the interaction of the peptide parts of XXV with the enzyme active site is not sufficient to cause the weakly electrophilic ketone group to be stable in its tetrahedrally hybridized form. For the inhibition of angiotensin converting enzyme, we propose that ketoace (XXIV in Table I, K_i for converting enzyme of 0.0006 μM) does interact precisely enough for rehybridization of its weakly electrophilic ketone group to a tetrahedral complex. Therefore, we propose that ketone XXV could bind to collagenase in a complex such as that shown in Figure 4e, which can occur via two different pathways: from state a to state e by direct displacement of water from the active site or from state a to state e via a tetrahedral intermediate as shown in Figure 4c. The decrease in K_2 of XXV compared to its isosteric peptide XXIII could then be due to the expulsion of water from the active site by an addition–elimination mechanism. Alternatively, it could be simply due to the increased electrophilicity of the ketone carbonyl carbon atom compared to the amide carbonyl carbon atom without involving the actual addition or elimination of the water molecule. Hangauer et al. (1984) evaluate different Michaelis complexes, transition states, and zinc coordinating modes for a number of substrates and inhibitors (but not ketone inhibitors) of the zinc metalloprotease thermolysin. The same factors influencing substrate and inhibitor binding modes for thermolysin, i.e., the presence or absence of a basic nitrogen to bind to the active site carboxylate and the identity of the amino acid side chains occupying the S_1 and S_2 sites on the enzyme, could be important in determining the binding interactions and thus the K_i's of the present compounds (XXIII, XXV, XXVI, and XXVII) to collagenase.

In summary, the ketone XXV, 5-benzamido-4-oxo-6-phenylhexanoyl-L-prolyl-L-alanine (viscometric IC_{50} = 120 μM, spectrophotometric IC_{50} = 16 μM), is one of the most potent synthetic inhibitors known for the collagenase of *Clostridium histolyticum*. Although its structure is that of a simple substrate analogue, it must bind to the enzyme in other than a simple Michaelis-type complex since it is bound more than 80-fold more tightly than its peptide analogue benzoyl-L-phenylalanylglycyl-L-prolyl-L-alanine.

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Aggregation Studies on Fluorescein-Coupled Cobra Venom Phospholipase A_2[†]

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ABSTRACT: Phospholipase A_2 from *Naja naja naja* venom (Indian cobra) undergoes a concentration-dependent aggregation, and at an assay concentration of 1 μg mL^{-1}, it exists as a monomer. However, there is some evidence that the enzyme is actually active as a dimer or higher order aggregate. Previous attempts to determine the aggregation state of the enzyme under actual assay conditions were thwarted by experimental difficulties due in part to the low enzyme concentrations required. This aggregation has now been studied by using fluorescence polarization. The extrinsic probe fluorescein isothiocyanate was coupled to the enzyme to serve as the fluorescence marker. Steady-state polarization measurements were made to determine changes in the aggregation state of the fluorescently tagged enzyme. The phospholipases A_2 from *Crotalus adamanteus* (rattlesnake) and porcine pancreas, whose states of aggregation are known, were also labeled with fluorescein isothiocyanate and used as controls. It was found that the divalent metal ions Ca^{2+}, a phospholipase cofactor, and Ba^{2+}, an inhibitor, caused an increase in the cobra venom enzyme polarization, while Mn^{2+}, Mg^{2+}, and Co^{2+} did not. The water-soluble substrate diheptanoylphosphatidylcholine and the lipid analogue dodecylphosphocholine, when present below their respective critical micelle concentrations, also increased the polarization of the phospholipase–fluorescein conjugate. Thus, both cofactor and substrate caused an increase in the polarization, which implies an increase in the aggregation state. It is concluded that under assay conditions the phospholipase A_2 exists in an aggregated form.

Phospholipase A_2 catalyzes the hydrolysis of the *sn*-2 fatty acid of phospholipids with Ca^{2+} required as a cofactor (Dennis, 1983). The enzyme from cobra venom (*Naja naja naja*) appears to have two phospholipid binding sites, an activator site and a catalytic site (Roberts et al., 1979; Adamich et al., 1979; Plückthun & Dennis, 1982). A phosphocholine-containing lipid must be bound to the activator site in order to achieve maximal enzymatic activity. Earlier experiments showed that the enzyme from cobra venom undergoes a concentration-dependent aggregation in the absence of substrate (Deems & Dennis, 1975). However, at an assay concentration of 1 μg mL^{-1}, the enzyme is a monomer. The presence of Ca^{2+} or substrate could cause the enzyme to aggregate. Studies involving gel filtration, cross-linking, and ultracentrifugation suggested that upon contact with substrate the enzyme aggregates (Roberts et al., 1977a,b; Lewis et al., 1977; Dennis

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