Ketone-Substrate Analogues of *Clostridium histolyticum* Collagenases: Tight-Binding Transition-State Analogue Inhibitors†

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ABSTRACT: A series of ketone-substrate analogues has been synthesized for the two classes of collagenases from *Clostridium histolyticum* and shown to be competitive inhibitors. These compounds have sequences that match those of specific peptide substrates for these enzymes. The best inhibitor is the ketone analogue of cinnamoyl-Leu-Gly-Pro-Pro, which has a K_I value of 18 nM for ϵ-collagenase, a class II enzyme. This is the tightest binding inhibitor reported for any collagenase to date. Plots of log K_I for the inhibitors vs log K_M/k_{cat} for the matched substrates for both collagenases are linear with slopes near unity, indicating that the ketones are transition-state analogues. This strongly implies that the ketone carbon atoms of these inhibitors are tetrahedral when bound to the enzymes.

Clostridium histolyticum produces two distinct classes of closely related collagenases[1] (EC 3.4.24.3) that have been purified to homogeneity and characterized extensively (Bond & Van Wart, 1984a,b,c). These enzymes are metalloproteinases that contain a single catalytic zinc ion located at the active site and variable amounts of calcium ions that presumably stabilize the tertiary structure of the enzyme. These two classes of collagenases have different, yet complementary, substrate specificites toward peptides with collagen-like sequences (Steinbrink et al., 1985; Van Wart & Steinbrink, 1985; Mookhtiar et al., 1985) and native, triple-helical type I collagen (French et al., 1987). The active site constituents of these collagenases are very similar to those of other extensively studied zinc proteinases such as thermolysin, carboxypeptidase A, and angiotensin converting enzyme (Bond et al., 1981; Bond & Van Wart, 1985c). Thus, it seems likely that the mechanism of these collagenases is similar to that of these other metalloproteinases and that the same kinds of compounds might act as inhibitors.

There have been few reports of synthetic inhibitors of bacterial collagenases (Yagisawa et al., 1965; Siffert & Pasquet, 1977; Galardy & Grobelny, 1983; Yiotakis et al., 1984; Grobelny & Galardy, 1985; Vencill et al., 1985; Yiotakis & Dive, 1986), and most of these have indeed been patterned after inhibitors of other metalloproteinases. For the clostridial collagenases, for example, Vencill and associates (Vencill et al., 1985) have synthesized peptide inhibitors containing hydroxamic acid, carboxymethyl groups, and thiol groups placed so that they could interact with the active site zinc atom, a strategy known to work for other zinc proteinases. Galardy and Grobelny (1983) have shown that clostridial collagenases are inhibited by several phosphoric and phosphonic amides of peptides. Similar compounds are thought to act as transition-state analogues for carboxypeptidase A (Galardy, 1982; Jacobsen & Bartlett, 1981), thermolysin (Kam et al., 1979; Bartlett & Marlowe, 1983; Weaver et al., 1977), and angiotensin converting enzyme (Thorsett et al., 1982; Goli & Galardy, 1986).

More recently, a series of aldehyde- and ketone-substrate analogues has been shown to inhibit a commercial, unfractionated preparation of these collagenases (Grobelny & Galardy, 1985). Compounds of this type have been shown previously to be good inhibitors of other metalloproteinases (Almquist et al., 1980; Meyer et al., 1981; Galardy & Kortylewicz, 1984; Grobelny & Galardy, 1986). The choice of the particular ketone analogues prepared for clostridial collagenases in the earlier study was based on the limited information available at the time on the specificity of these enzymes, and the best inhibitor had an IC_{50}[2] value of only 16 μM. Since this time, a detailed and comprehensive study of the sequence specificities of both classes of *C. histolyticum* collagenases has been carried out (Steinbrink et al., 1985; Van Wart & Steinbrink, 1985; Mookhtiar et al., 1985). With the aid of this information, a new series of ketone-substrate analogues has been prepared in which the amino acids in specific subsites have been optimized for maximal interaction with the enzymes. It is shown here that these compounds are tight-binding, competitive inhibitors of both classes of collagenases with K_I values as low as 18 nM. Kinetic data for the analogous substrates are also presented, and it is shown that the log K_I values of the ketone analogues vary linearly with the log K_M/k_{cat} values of their matched substrates, sug-

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[1] Unless stated otherwise, collagenase refers to *Clostridium histolyticum* collagenase.

[2] Abbreviations: FALGPA, 2-furanacryloylleucylglycylprolylalanine; IC_{50}, inhibitor concentration resulting in 50% activity at fixed concentrations of enzyme and substrate; K_I, enzyme–inhibitor dissociation constant; CNM, cinnamoyl; HCM, hydrocinnamoyl; Bz, benzoyl; Leu^K-Gly, (5-*RS*)-5-amino-7-methyl-4-oxooctanoic acid; Phe^K-Gly, (5-*RS*)-5-amino-6-phenyl-4-oxohexanoic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; OMe, methyl ester; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; mp, melting point; ppm, parts per million. All amino acids have the L configuration.

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gesting that they act as transition-state analogues.

Materials and Methods

Materials. β- and ϵ-collagenases were purified as described earlier (Bond & Van Wart, 1984a), and all CNM substrates were from a previous study (Steinbrink et al., 1985). Protected and unprotected amino acids were purchased from Sigma Chemical Co. Dicyclohexylcarbodiimide, cinnamoyl chloride, 4-(dimethylamino)pyridine, and other chemicals were obtained from Aldrich Chemical Co. All solvents were of reagent grade or higher in purity and used without further purification or purified by standard procedures.

Synthetic Methods. Melting points were taken on a hot stage and are uncorrected. Proton NMR spectra were recorded on a Varian Model EM-390 spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane. FAB-MS determinations of molecular weight were performed by the Midwest Center for Mass Spectrometry, a National Science Foundation Regional Instrumentation Facility. TLC was performed on silica gel 60-F254, and compounds were visualized by iodine vapor and 2,4-dinitrophenylhydrazine spray (Grobelny & Galardy, 1985). The compositions of TLC solvent systems were as follows (by volume): (A) chloroform/acetonitrile/2-propanol/acetic acid, 3:1:0.5:0.2; (B) 1-butanol/acetic acid/water, 4:1:1; (C) chloroform/methanol/acetic acid, 9:1:0.3; (D) ethyl acetate; (E) 2-propanol/water, 64:36. The TLC of all of the compounds described below gave homogeneous single spots.

Synthesis of Inhibitors. The synthesis and characterization of Bz-Phe^K-Gly-Pro (**1**) and Bz-Phe^K-Gly-Pro-Ala (**2**) have been described earlier (Grobelny & Galardy, 1985, 1986). Bz-Phe^K-Gly-Pro-Pro (**3**) was prepared in a manner identical with that described for **2** by use of the hydrochloride salt of Pro-Pro benzyl ester (Deber et al., 1970): R_f(A) 0.33, R_f(B) 0.65; NMR (D_2O) δ 1.95 (m, 8 H, Pro β,γ-CH_2), 2.2–3.2 (m, 6 H, hexanoyl 2,3,6-CH_2), 3.5 (m, 4 H, Pro δ-CH_2), 4.6 (HDO), 4.0–4.9 (m, 3 H, Pro α-CH, hexanoyl 5-CH), 6.9–7.6 (m, 10 H, Ph). Bz-Leu^K-Gly-Pro-Ala (**4**) was prepared by a similar procedure. The modified procedure of McMurray and Dyckes (1985) was used to prepare CNM-Leu^K-Gly-OMe, which was the precursor for compounds **5–13**. The synthesis and characterization of **5–13** are described in the supplementary material (see paragraph at end of paper regarding supplementary material).

Enzyme Assays and Inhibitor Studies. Enzyme concentrations were determined spectrophotometrically with ϵ_{280} = 1.66×10^5 and 1.37×10^5 M^{-1} cm^{-1} for β- and ϵ-collagenases, respectively (Bond & Van Wart, 1984b). Assays using FALGPA and CNM peptides as substrates were carried out spectrophotometrically as described earlier (Steinbrink et al., 1985). The concentration of the CNM inhibitors was determined spectrophotometrically with ϵ_{279} = 24 600 M^{-1} cm^{-1}, and those of the other inhibitors were determined gravimetrically. Assays for the inhibition studies were carried out at 25 °C in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, with FALGPA as substrate. For the pH-dependence studies, 50 mM Mes and 50 mM sodium borate were substituted for Tricine at pH 6.5 and 8.5, respectively. All inhibitors were dissolved in water immediately prior to use.

To measure the time dependence of inhibition, two procedures were employed. To test for slow onset of inhibition, enzyme was incubated on ice with a concentration of inhibitor that resulted in approximately 60% inhibition at zero time. Aliquots of these incubation mixtures were added to assays carried out at a FALGPA concentration of 0.05 mM ([S] ≪ K_M) containing the same concentration of inhibitor as a function of time over 20 h. Since the assay time is approximately 5 min, an alternative procedure was used to detect shorter time dependences. In this procedure, the assays were carried out at a FALGPA concentration of 1.4 mM (3.6K_M), conditions under which the progress curve for the reaction is linear. Inhibitor was added to the assay after a few minutes, and the time required to establish the new linear rate was used to distinguish between instantaneous and time-dependent inhibition.

Results

Design of Inhibitors. An earlier study demonstrated that ketone-substrate analogues obtained by replacement of the NH group of the scissile peptide bond of several collagenase substrates by a CH_2 group were inhibitors of these enzymes (Grobelny & Galardy, 1985). The best inhibitor prepared was the ketone analogue of Bz-Phe-Gly-Pro-Ala, denoted Bz-Phe^K-Gly-Pro-Ala (**2**). Unfortunately, the presence of the Bz group in subsite P_2 [nomenclature of Schechter and Berger (1967)] makes this pair neither a good substrate nor a potent inhibitor (IC_{50} = 16 μM), respectively. The ketone analogues of more specific substrates for both classes of collagenases, however, should be more potent inhibitors. Detailed studies of the sequence specificities of these collagenases have now been completed (Steinbrink et al., 1985; Van Wart & Steinbrink, 1985; Mookhtiar et al., 1985). Both classes of enzymes prefer substrates with bulky, hydrophobic residues (Leu, Phe, etc.) in subsite P_1, Gly in subsite P'_1, Pro in subsite P'_2, and one of several residues (Ala, Pro, Leu, Arg) in subsite P'_3. The best substrates have subsites P_1–P'_3 occupied with these residues with a blocked N-terminus and unblocked C-terminus. Shorter peptides or tetrapeptides with free NH_2 groups or esterified COOH groups are poor substrates. As far as extension in the P_n direction goes, the best amino acid residues are Pro and Gly in subsites P_2 and P_3, respectively. However, the best substrates have a CNM group in subsite P_2. The class II collagenases selectively prefer CNM-Leu in subsites P_2 and P_1, respectively. Using this knowledge, a series of ketone inhibitors (**1–13**) have been synthesized that are analogues of substrates with CNM, HCM, or Bz in subsite P_2, Phe or Leu in subsite P_1, Gly in subsite P'_1, Pro in subsite P'_2, and Ala, Pro, Leu, or Arg in subsite P'_3.

Time Dependence of Inhibition. Before K_I values for these inhibitors were measured, the rate at which full inhibition was obtained was investigated. For example, ϵ-collagenase was incubated with 10 nM compound **10**, and aliquots of the mixture were assayed in the presence of the same concentration of inhibitor as a function of time (data not shown). Within the time required to perform the assay (<5 min), there was a 60% decrease in activity relative to that of the pure enzyme, and the percentage of inhibition did not change as a function of the time of incubation. To see if the attainment of full inhibition was shorter than 5 min, ϵ-collagenase was added to 1.4 mM FALGPA, and the linear decrease in absorbance was recorded for 3 min (approximately 5% turnover). Compound **10** (1 μL of a 50 μM solution) was then added to the 1-mL assay and the decrease in absorbance recorded again. The new rate was also linear and lowered to 70%, indicating that binding of inhibitor to the enzyme was complete during mixing. Thus, the binding of this inhibitor to ϵ-collagenase reaches equilibrium within 10 s. The same results were obtained with all pairs of inhibitor and collagenase.

Extent and Mode of Inhibition. All of the inhibition studies were carried out with a representative class I (β) and class II (ϵ) collagenase. Values of K_I were measured by three different methods. In the first method, a series of double-reciprocal plots

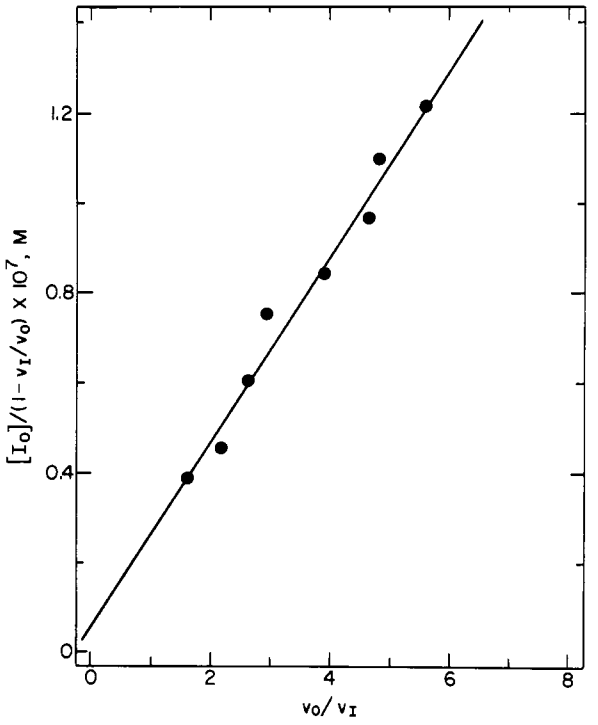

FIGURE 1: Henderson plot for the inhibition of ϵ-collagenase by CNM-Leu^K-Gly-Pro-Pro (**10**). The substrate concentration was 0.05 mM, and the total enzyme concentration was 3.2 nM.

for the hydrolysis of FALGPA by β- and ϵ-collagenases in the presence of various concentrations of inhibitor were obtained. In each of these plots, the lines intersected the $[E_0]/v_i$ axis at the same value, showing that the inhibition was competitive. Replots of the slopes of the lines vs total inhibitor concentration, $[I_0]$, were made and values of K_I estimated from the $[I_0]$ intercepts. These values should be accurate because in all cases both the substrate and inhibitor concentrations were varied both above and below K_M and K_I, respectively. Values of K_I for the inhibition of β-collagenase by compounds **1–3**, **6**, and **8–12** and for ϵ-collagenase by **1–3**, **7**, and **9** were obtained in a similar manner. In the second method, inhibition of β-collagenase by **4**, **5**, **7**, and **13** and ϵ-collagenase by **4**, **5**, and **13** was quantitated by measurement of IC_{50} values which were then converted to K_I values by use of the equation for a competitive inhibitor (Segel, 1975):

$$IC_{50} = K_I(1 + [S]/K_M) \quad (1)$$

In the third method, values of K_I for the tighter binding inhibitors were determined from Henderson plots. Such a plot for the inhibition of ϵ-collagenease by compound **10** is shown in Figure 1. The slope of this plot is related to K_I by (Segel, 1975)

$$\text{slope} = K_I(1 + [S]/K_M) \quad (2)$$

Values of K_I for the binding of compounds **6**, **8**, and **10–12** to ϵ-collagenase were obtained from similar plots.

The K_I values obtained from these experiments for both classes of enzymes are summarized in Table I. Almost all of the compounds inhibit both classes of enzymes. Compounds **1–4** constitute a series of inhibitors in which there is a Bz group in subsite P_2. The tripeptide analogue Bz-Phe^K-Gly-Pro (**1**) is a fair inhibitor (K_I = 160–170 μM) of both collagenases. When an Ala or Pro residue is added to subsite P'_3 to give compounds **2** and **3**, respectively, inhibition of both enzymes is increased substantially (K_I = 4.8–18 μM). A comparison of the K_I values for compounds **2** and **4** shows that Phe is preferred to Leu for both collagenases. These trends are in line with the substrate specificities of these enzymes (Van Wart & Steinbrink, 1985).

Table I: Values of K_I for Inhibition of β- and ϵ-Collagenases by Ketone-Substrate Analogues[a]

	inhibitor (P_2 –P_1 –P'_1–P'_2–P'_3)	K_I (μM) β	K_I (μM) ϵ
1	Bz-Phe^K-Gly-Pro	160	170
2	Bz-Phe^K-Gly-Pro-Ala	10	18
3	Bz-Phe^K-Gly-Pro-Pro	7.0	4.8
4	Bz-Leu^K-Gly-Pro-Ala	52	44
5	CNM-Leu^K-Gly-Pro	1500	26
6	CNM-Leu^K-Gly-Pro-Ala	12	0.11
7	CNM-Leu^K-Gly-Pro-Ala-OMe	60	1.2
8	CNM-Leu^K-Gly-Pro-Leu	2.8	0.030
9	CNM-Leu^K-Gly-Pro-Leu-OMe	37	0.67
10	CNM-Leu^K-Gly-Pro-Pro	2.7	0.018
11	CNM-Leu^K-Gly-Pro-Arg	1.0	0.030
12	CNM-Leu^K-Gly-ProArg-OMe	1.1	0.023
13	HCM-Leu^K-Gly-Pro-Pro	220	2.9

[a] Assays were carried out in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at 25 °C.

A second series of compounds (**5–12**) all have CNM-Leu^K-Gly-Pro in subsites P_2–P'_2 but different residues in subsite P'_3. Compound **5**, in which subsite P'_3 is unoccupied, is a poor inhibitor of β-collagenase, but it inhibits ϵ-collagenase with a K_I value of 26 μM. This difference reflects the strong preference of the class II enzymes for CNM-Leu in subsites P_2 and P_1, respectively, and this differential inhibition is preserved in compounds **6–13**. When subsite P'_3 is occupied by Ala (**6**), Leu (**8**), Pro (**10**), or Arg (**11**), the K_I values decrease dramatically and fall in the 1.0–12 μM and 18–110 nM ranges for β- and ϵ-collagenases, respectively. The K_I value of 18 nM for CNM-Leu^K-Gly-Pro-Pro (**10**) currently makes it the best known synthetic inhibitor of any collagenase. Esterification of compounds **6** and **8** to give **7** and **9**, respectively, gives weaker inhibitors, while esterification of **11** to give **12** has little effect. This matches the trends for the corresponding substrates (Van Wart & Steinbrink, 1985). A comparison of the K_I values of compounds **10** and **13** shows that conversion of the CNM group to the HCM group weakens inhibition over 100-fold for both collagenases.

Correlation of Data for Matched Substrates and Inhibitors. As noted in the previous section, the K_I values for these ketone inhibitors follow qualitatively the substrate specificities (as judged by k_{cat}/K_M values) of these collagenases toward their matched substrates found in an earlier study (Van Wart & Steinbrink, 1985). This earlier investigation, however, did not evaluate the individual kinetic constants k_{cat} and K_M for all of these substrates. Thus, these parameters have been measured here for the available matched substrates of compounds **6–8** and **10–12** to allow comparison with the K_I values of the inhibitors. The k_{cat}/K_M values of the matched substrate for compounds **5** and **9** have also been redetermined (Table II).

A comparison of the K_I values for the ketone inhibitors shown in Table I with the kinetic parameters for the matched substrates shown in Table II provides information on the type of enzyme–substrate interactions that give rise to the inhibition by this series of ketone-substrate analogues. These inhibitors could simply be mimicing the same interactions with the enzyme that are present in the ground state of their matched substrates. Such inhibitors are "ground-state analogues" for which, making the reasonable assumption that $K_M = K_S$, a plot of log K_I vs log K_M for the matched inhibitor/substrate pairs should be linear with a slope of unity (Bartlett & Marlowe, 1983). Since the K_I values of these inhibitors are much lower than the K_M values for their matched substrates, it is clear that these inhibitors have favorable binding interactions with the

Table II: Kinetic Parameters for Hydrolysis of Peptides by β- and ϵ-Collagenases[a]

substrate (P_2 $-P_1$ $-P'_1-P'_2-P'_3$)	β K_M (mM)	β k_{cat} (min^{-1})	β k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})	ϵ K_M (mM)	ϵ k_{cat} (min^{-1})	ϵ k_{cat}/K_m ($\times 10^{-4}$ M^{-1} min^{-1})
CNM-Leu-Gly-Pro			0.24			7.9
CNM-Leu-Gly-Pro-Ala	0.65	350	55	0.33	11000	3300
CNM-Leu-Gly-Pro-Ala-OMe	1.1	0.92	8.4	1.5	5400	360
CNM-Leu-Gly-Pro-Leu	0.10	460	460	0.14	7100	5100
CNM-Leu-Gly-Pro-Leu-OMe			30			460
CNM-Leu-Gly-Pro-Pro	0.13	1100	850	0.33	8800	2700
CNM-Leu-Gly-Pro-Arg	0.13	1100	850	0.080	3500	4300
CNM-Leu-Gly-Pro-Arg-OMe	0.11	1100	1000	0.14	15000	11000

[a] Assays were carried out in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at 25 °C.

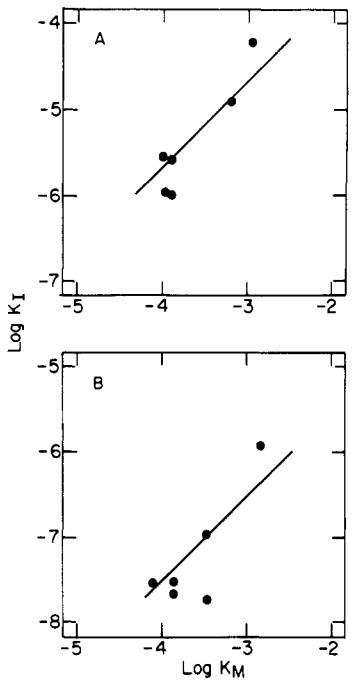

FIGURE 2: Plot of log K_I vs log K_M for the peptides shown in Table II and their matching inhibitors shown in Table I for (A) β- and (B) ϵ-collagenases.

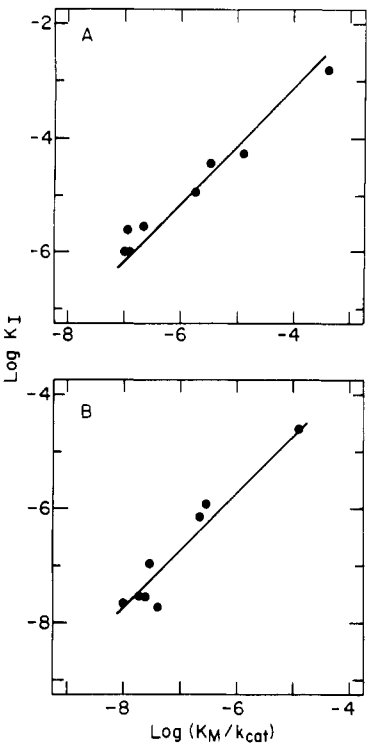

FIGURE 3: Plot of log K_I vs log (K_M/k_{cat}) for the peptides shown in Table II and their matching inhibitors shown in Table I for (A) β- and (B) ϵ-collagenases.

enzymes that *are not* present in the ground state of the substrates. One possible explanation is that the replacement of the NH group of residue P'_1 with a CH_2 group leads to an opportunistic increase in binding to the enzyme. Thus, the ketones could simply be ground-state analogues, each of which has an additional favorable binding interaction with the enzyme. Since the free energy of this new interaction should be constant for each inhibitor, a plot of log K_I vs log K_M should also be linear for a matched series of substrates and inhibitors and have a slope of unity.

Another explanation for the low K_I values is that the ketone inhibitors are acting as transition-state analogues and are taking advantage of favorable binding forces present in the enzyme–substrate transition state. This would lower the K_I values below the K_M values of the matched substrates but would require that the structure of the enzyme-bound inhibitor resemble that of the substrate in the transition state (or a reaction intermediate close in energy and similar in structure to it). Since the carbonyl group of peptide substrates is thought to be tetrahedral in the transition state, this would require that the ketone carbonyl group of these inhibitors become tetrahedral on binding to the enzyme. In fact, Christianson et al. (1987) have shown crystallographically that 5-benzamido-2-benzyl-4-oxopentanoic acid, a ketone-substrate analogue of carboxypeptidase A, binds to the enzyme as the tetrahedral *gem*-diol. Transition-state analogues should have K_I values that correlate inversely with the k_{cat}/K_M values of the corresponding substrates (Thompson, 1973; Westrick & Wolfenden, 1972; Thompson & Bauer 1979), and a plot of log K_I vs log (K_M/k_{cat}) for a series of matched transition-state inhibitor/substrate pairs is predicted to be linear with a slope of unity (Bartlett & Marlowe, 1983).

Plots of log K_I vs log K_M and log K_I vs log (K_M/k_{cat}) for these inhibitors and their matched substrates for both β- and ϵ-collagenases are shown in Figures 2 and 3, respectively. A line with a slope of unity has been drawn in each plot for reference. There is a poor correlation between the log K_I values of the inhibitors and the log K_M values of the matched substrates for either enzyme (Figure 2). Least-squares analysis of the data gives a slope of 1.42 and correlation of 0.94 for β-collagenase and values of 1.30 and 0.84 for ϵ-collagenase, respectively. Thus, these inhibitors cannot be purely ground-state analogues. There is an approximately linear relationship, however, between the log K_I values of the inhibitors and the log (K_M/k_{cat}) values for their matched substrates for both enzymes. Least-squares analysis of the data gives a slope of 0.84 and a correlation of 0.99 for β-collagenase and values of 1.07 and 0.96 for ϵ-collagenase, respectively. The

Table III: pH Dependence of Kinetic Constants for Hydrolysis of FALGPA and K_I Value for Inhibition by Bz-Phe^K-Gly-Pro-Ala of β-Collagenase[a]

pH	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})	K_I (μM)
6.5	0.16	34	21	13
7.5	1.1	666	59	10
8.5	1.7	1100	65	9.0

[a] Assays were carried out in 50 mM buffer, 0.4 M NaCl, and 10 mM $CaCl_2$ at 25 °C. The buffers used were Mes at pH 6.5, Tricine at pH 7.5, and sodium borate at pH 8.5.

values of k_{cat}/K_M for all of the substrates shown in Table II were determined with the identical enzyme samples used in the inhibition studies from double-reciprocal plots. These numbers differ slightly from the k_{cat}/K_M values reported earlier (Steinbrink & Van Wart, 1985) that were measured at a single substrate concentration with different enzyme samples. However, if the earlier values of k_{cat}/K_M are used to construct the plots shown in Figure 3, the results change very little. The slopes and correlations obtained with the old data are 0.98 and 0.91 for β-collagenase and 1.08 and 0.97 for ϵ-collagenase, respectively. Thus, these inhibitors exhibit the behavior expected for transition-state analogues.

pH Dependence of Inhibition. Since the kinetic parameters for the hydrolysis of substrates by these collagenases vary significantly with pH (Kleiman and Van Wart, unpublished data), it is of interest to see whether the changes in K_M or K_M/k_{cat} correlate best with the changes in the K_I values for the ketone-substrate analogues studied here. The kinetic parameters for the hydrolysis of FALGPA by β-collagenase and the K_I values for compound **2** at pH 6.5, 7.5, and 8.5 are listed in Table III. On going from pH 8.5 to pH 6.5, K_M decreases over 10-fold, and k_{cat} decreases over 30-fold with the net result that k_{cat}/K_M decreases 3.1-fold. The value of K_I for inhibitor **2** increases approximately 1.4-fold over this same pH range. Since the large decrease in K_M is accompaned by an *increase* in K_I, there is no correlation between the pH dependence of these parameters. In contrast, the 3.2-fold increase in K_M/k_{cat} correlates reasonably well with the 1.4-fold increase in K_I, especially considering that this is not a perfectly matched substrate–inhibitor pair.

Discussion

A well-established strategy for the design of proteinase inhibitors is to replace the NH or CO groups of the scissile peptide bond of the substrate with other moieties to give a nonhydrolyzable compound that otherwise resembles the substrate. In particular, replacement of the NH group of a scissile peptide bond with a CH_2 group gives a ketone-substrate analogue. Such compounds have been found to be good or excellent, specific inhibitors of zinc metallopeptidases such as carboxypeptidase A (Grobelny et al., 1985) and angiotensin converting enzyme (Meyer et al., 1981; Grobelny & Galardy, 1986; Natarajan et al., 1984). Recently, it has been shown that ketomethylene peptides also inhibit an impure preparation of the collagenases from *C. histolyticum* (Grobelny & Galardy, 1985), but the K_I values were high compared to those for the other zinc proteinases. Since the native substrate for these enzymes is triple-helical collagen, it was not clear whether the moderate inhibition found earlier was attributable to the primary structure of the inhibitors, since the sequence specificity of these enzymes was not well established, or to their lack of a collagen-like conformation.

Recently, the collagenases from *C. histolyticum* have been purified to homogeneity, and it was found that the enzymes could be divided into two distinct classes (Bond & Van Wart, 1984a,b,c). The sequence specificities of these enzymes have been studied in detail (Steinbrink et al., 1985; Van Wart & Steinbrink, 1985; Mookhtiar et al., 1985), and this knowledge has been used here to design ketone-substrate analogues with sequences that match those of the best synthetic substrates. The inhibition measurements presented here show that these compounds are indeed good inhibitors of both classes of collagenases. This dispells the notion that tight-binding inhibitors of collagenases must have a secondary structure that resembles the triple helix of native collagens.

The ketone-substrate analogues studied here inhibit both classes of collagenases competitively with K_I values ranging over 3 orders of magnitude. Full inhibition is achieved within 15 s for both collagenases, and no "slow-binding" phenomena are observed, in agreement with earlier observations (Grobelny & Galardy, 1985). Both enzymes prefer a full collagen-like triplet in subsites P'_1–P'_3, as noted for peptide substrates (Mookhtiar et al., 1985). It is preferable for the residue in subsite P'_3 to have a free carboxyl group, in keeping with the observation that these collagenases prefer to act as tripeptidylcarboxypeptidases toward substrates with a collagen-like triplet in subsites P'_1–P'_3 (Mookhtiar et al., 1985). The lowest K_I values are observed for inhibitors with CN-Leu in subsites P_2–P_1. This is particularly favorable for the class II enzymes such as ϵ-collagenase, for which the inhibition is 30–100 times better than that for β-collagenase. The K_I value of 18 nM for compound **10** is the lowest value reported to date for any collagenase. Since this value is for a mixture of the two stereoisomers of the Leu^K residue, the K_I value for the isomer recognized by the enzyme could potentially be half of this value.

In order to provide some insight into the basis for the inhibition by these ketone-substrate analogues, the kinetic parameters for the hydrolysis of a series of matched substrates by both enzymes have been determined. A comparison of the K_I values of the inhibitors with either the K_M or K_M/k_{cat} values of the matched substrates should indicate whether the ketone analogues are ground-state or transition-state analogues (Bartlett & Marlowe, 1983). The plot of log K_I vs log K_M shown in Figure 2 shows that there is a poor correlation between these two quantities. On the other hand, the plots of log K_I vs log (K_M/k_{cat}) for both collagenases shown in Figure 3 show that there is a good correlation between these parameters for data covering over 3 orders of magnitude. The slopes for both enzymes are close to unity, indicating that structural changes in regions remote from the scissile bond result in the same change in binding energy for both the inhibitors and the transition state of the substrate that they mimic. The pH dependence of the K_I values for **2** also matches more closely the k_{cat}/K_M than the K_M values (Table III), although FALGPA is not a matched substrate for this inhibitor. Thus, it is reasonable to conclude that these compounds are transition-state analogues. Bartlett and Marlowe (1983) have reached a similar conclusion for a series of phosphonamidate inhibitors and matching peptide substrates of thermolysin. However, in their case the inhibitors possessed the tetrahedral transition-state structure *before* interaction with the enzyme.

The conclusion that the ketone-substrate analogues studied here are transition-state analogues implies that their ketone carbon atoms are tetrahedral when bound to the collagenases. It will be interesting to see whether this prediction made on the basis of kinetic data will be confirmed when the structures of these ketone analogue–enzyme complexes are elucidated experimentally. Recently, Christianson et al. (1987) have

reported crystallographic data which indicate that 2-benzamido-2-benzyl-4-oxopentanoic acid, a ketone analogue of the substrate Bz-Gly-Phe, binds to carboxypeptidase A as a tetrahedral covalent hydrate adduct with the two *gem*-diol oxygen atoms close to the zinc and one diol hydrogen bonded to Glu_{270}. Since both the zinc atom and an essential carboxyl residue are known to be in the active site of these collagenases (Bond & Van Wart, 1984c), the ketones studied here could be bound to them in a similar mode. The question then arises as to whether it is the hydrated form of the ketone in solution that first binds to the enzyme or whether water addition across the carbonyl double bond occurs in the enzyme active site. The concentration of the hydrated form in solution is less than 3% of the total ketone concentration because of the low electrophilicity of the ketone carbonyl (Grobelny & Galardy, 1986). Alternatively, it is possible that the inhibitor binds first in the unhydrated form to give a Michaelis-type complex and then undergoes enzyme-assisted water addition to form the hydrate. If all equilibria were rapidly established, no time dependence would be observed. It is not possible to differentiate between the two alternatives at this time.

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Supplementary Material Available

Procedures for the syntheses and characterizations of compounds **5–13** (8 pages). Ordering information is given on any current masthead page.

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