Phosphonamidate Inhibitors of Human Neutrophil Collagenase†

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ABSTRACT: A series of phosphonamidates has been synthesized and shown to inhibit human neutrophil collagenase. The compounds all have sequences patterned after the cleavage site in the α1(I) chain of type I collagen, except that the carbonyl group of the Gly residue in subsite P_1 has been replaced by a P(=O)(OH) group (abbreviated Gly^P). As the central Gly^P-Leu unit is lengthened in the N- and C-terminal directions, in accordance with the cleavage sequence found in collagen, inhibition is systematically improved. The best inhibitor is Cbz-Gly^P-Leu-Ala-Gly, which inhibits competitively with a K_I value of 14 μM. These phosphonamidates are thought to be acting as transition-state analogues.

Human neutrophil granules contain a specific collagenase that catalyzes the hydrolysis of native, triple-helical interstitial collagens into characteristic $^3/_4$ and $^1/_4$ fragments (Lazarus et al., 1972; Murphy et al., 1982; Christner et al., 1982). The enzyme is a metalloproteinase that shares many mechanistic features with other well-studied zinc metallopeptidases such as thermolysin, carboxypeptidase A, angiotensin converting enzyme, and the *Clostridium histolyticum* collagenases (Mookhtiar et al., 1986). The development of synthetic inhibitors for these enzymes is an active field of research that has provided insight into the nature of enzyme–substrate interactions during catalysis (Kam et al., 1979; Bartlett & Marlowe, 1983; Yamauchi et al., 1985) and has also led to the use of such inhibitors as pharmaceuticals (Ondetti & Cushman, 1982).

Since the discovery that phosphoramidon, *N*-[(α-L-rhamnopyranosyloxy)phosphinyl]-Leu-Trp, is a potent inhibitor of thermolysin (Komiyama et al., 1975), a number of phosphoramidate and phosphonamidate inhibitors for other bacterial and mammalian zinc metalloproteinases have been developed (Holmquist, 1977; Jacobsen & Bartlett; 1981; Grobelny et al., 1985; Galardy, 1982). There has been one report on the inhibition of *Clostridium histolyticum* collagenases by these compounds (Galardy & Grobelny, 1983). This inhibition is thought to arise partly through the interaction of the phosphoryl oxygen with the active site zinc ion, a result that has been confirmed crystallographically for thermolysin (Weaver et al., 1977) and partly through the interaction of the amino acid side chains of the inhibitor with binding sites on the enzyme. The phosphoryl moiety has a tetrahedral geometry, and these compounds are thought to be transition-state analogues (Bartlett & Marlowe, 1983; Weaver et al., 1977).

The similarity of neutrophil collagenase to other well-studied metalloproteinases suggested that phosphonamidates might inhibit this enzyme as well. Since neutrophil collagenase has been implicated in the pathological connective tissue destruction that accompanies inflammatory conditions such as rheumatoid arthritis (Starkey et al., 1977; Barrett, 1978), inhibitors of this type could provide a means of controlling an overexpression of collagenase activity in vivo.

Materials and Methods

Neutrophil collagenase was purified in latent form from human buffy coats by a new method most similar to that of Christner et al. (1982). After lysis of the white cells and extraction of enzyme from the granules, the crude extract was applied to a Reactive Red-120 agarose (Sigma Chemical Co.) column in 10 mM Tris,[1] 0.5 M NaCl, and 5 mM $CaCl_2$, pH 7.5, containing 0.05% Brij-35 and 50 μM $ZnSO_4$. After the absorbance returned to baseline, collagenase was eluted in the same buffer containing 1 M NaCl. This fraction contains some gelatinase activity and traces of elastase and cathepsin G, which were removed by chromatography over Sephacryl S-200 in the same buffer. Collagenase activity was measured with soluble ^{3}H-acetylated rat tail tendon type I collagen as substrate (Mallya et al., 1986). The synthesis of **2**–**6** has been described earlier (Bartlett & Marlowe, 1983). The synthesis and properties of **1** and **7**–**10** are described in detail in the supplementary materials (see paragraph at the end of paper regarding supplementary material).

All inhibitors were freshly dissolved in 25 mM Tricine, pH 8.5, and kept on ice prior to use. Latent collagenase was activated with 0.1 mM *p*-(chloromercuri)benzoate for 30 min at room temperature and then incubated for 20 min with inhibitor at 30 °C before starting the assay by addition of collagen. K_I values for inhibitors **3** and **10** were determined graphically both by Dixon analysis and from double-reciprocal plots of initial rate vs. substrate concentration at four different inhibitor concentrations. In both cases, the substrate concentration was varied from 0.33 to 3.3 μM. Since K_M for neutrophil collagenase under these conditions is 1.5 μM, the substrate range corresponds to 0.22K_M to 2.2K_M, respectively. All other K_I values were estimated from the IC_{50} values, the inhibitor concentrations resulting in 50% inhibition of the enzyme at a fixed substrate concentration (0.33 μM), by using the equation for competitive inhibitors (Segel, 1975):

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

Most assays were carried out for 4 h. However, assays in the

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[1] Abbreviations: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; Cbz, benzyloxycarbonyl; Gly^P, (aminomethyl)phosphonyl; OLeu, 2-hydroxy-4-methylpentanoyl.

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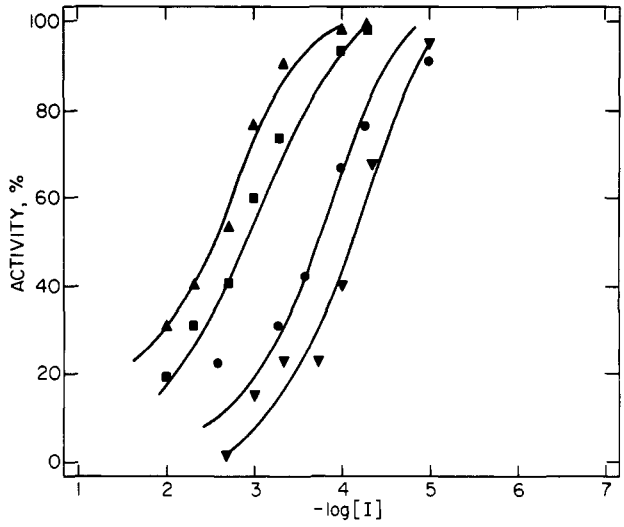

FIGURE 1: Inhibition of human neutrophil collagenase by (▼) Cbz-Phe-Gly^P-Leu-Ala, (●) Cbz-Pro-Ala-Gly^P-Leu-NH_2, (■) Cbz-Gly^P-Leu-NH_2, and (▲) Cbz-Gly^P-Leu. Assays were performed at 30 °C in 50 mM Tricine, 0.2 M NaCl, and 10 mM $CaCl_2$, pH 7.5, at a collagen concentration of 0.33 μM.

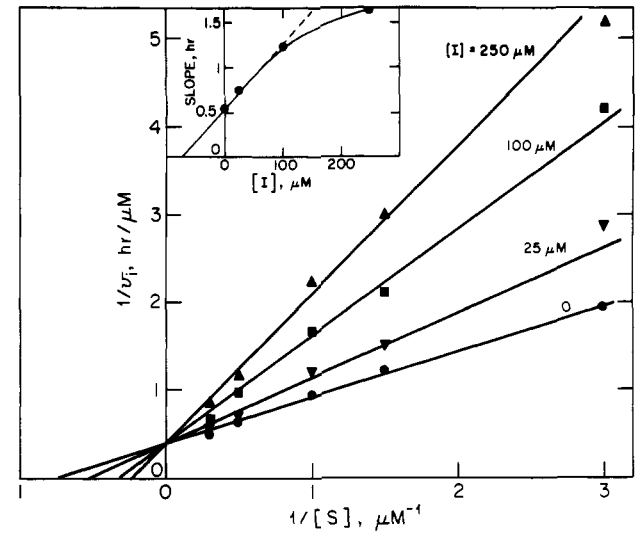

FIGURE 2: Double-reciprocal plots of initial velocity, v_i, vs. substrate concentration for the hydrolysis of collagen by human neutrophil collagenase in the presence of (●) 0, (▼) 25, (■) 100, and (▲) 250 μM Cbz-Gly^P-Leu-Ala. The slopes of the lines obtained are plotted vs. inhibitor concentration in the inset. Assays are described in the text.

presence of **8** were carried out for 1 h because of its reduced stability in aqueous solution.

RESULTS

Tissue collagenases hydrolyze the Gly–Ile bond of the α1(I) chains in type I collagen, which contain the sequence Pro-Gln-Gly-Ile-Ala-Gly (Gross et al., 1974). All of the phosphonamidates synthesized (**1–6**, **8–10**) as potential inhibitors contain related sequences, except that the carbonyl group of the Gly residue in subsite P_1 [nomenclature of Schecter and Berger (1967)] has been replaced by a P(=O)(OH) group. The resulting (aminomethyl)phosphonyl residue is abbreviated Gly^P. In one compound (**7**), the NH group of the Leu residue in subsite P_1' has been replaced by an oxygen atom (abbreviated OLeu) to give a phosphonate ester.

All 10 compounds inhibit neutrophil collagenase to some extent. The degree of inhibition produced by **1**, **2**, **8** and **9** as a function of inhibitor concentration is plotted in Figure 1. These assays were carried out at a fixed collagen concentration of 0.33 μM. Similar curves were obtained for **4–7**, and IC_{50} values were estimated from these plots. The curves shown in Figure 1 have a tendency to "tail off" at high (>0.1 mM) inhibitor concentrations. That is, the activity does not decrease as fast as expected for the poorer inhibitors as the inhibitor concentration is increased. We attribute this to interactions between the phosphonamidates and collagen, both of which are highly charged. Thus, the IC_{50} values obtained from these plots are upper estimates. No time dependence of inhibition was observed in these studies. However, since the collagenase assay is relatively long (1–4 h), the question of whether these compounds are slow-binding inhibitors cannot be addressed.

Double-reciprocal plots for the inhibition of neutrophil collagenase by **3** at inhibitor concentrations of 0, 25, 100, and 250 μM are shown in Figure 2. The intersection of these lines on the y axis indicates that **3** is a competitive inhibitor. The slopes of these lines are plotted vs. inhibitor concentration in the inset, and the x intercept gives a K_I value of 78 μM. The slope for the plot carried out at 250 μM inhibitor falls below the line. It is likely that this is due to its binding with collagen (see above). Dixon analysis of these data gives the same value of K_I. Similar analysis for **10** shows that it is also a competitive inhibitor. A Dixon plot of inhibition data for **10** is shown in

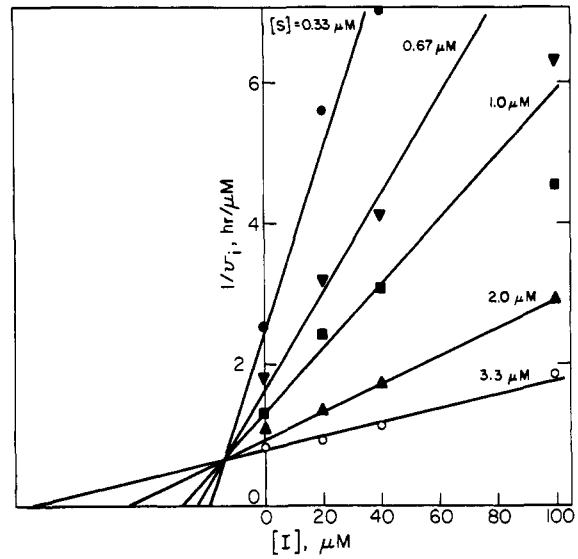

FIGURE 3: Dixon plot for the inhibition of human neutrophil collagenase by Cbz-Gly^P-Leu-Ala-Gly. The substrate concentrations were (●) 0.33, (▼) 0.67, (■) 1.0, (▲) 2.0, and (○) 3.3 μM.

Figure 3. This plot gives a K_I value of 14 μM. The quality of the kinetic data shown in Figures 1–3 has only recently been possible due to the development of a new, accurate assay for hydrolysis of soluble collagen (Mallya et al., 1986). By assuming that **1**, **2**, and **4–9** also inhibit competitively, K_I values have been calculated from eq 1. The K_I values for all of the inhibitors are listed in Table I.

Inhibitor **1**, the phosphonamidate analogue of Cbz-Gly-Leu, is a poor inhibitor of neutrophil collagenase. Amidation of the carboxyl group (**2**) improves the inhibition slightly. It is not clear whether this is due to elimination of the negative charge, the need for an amide group in this position, or both. Inhibitors **3–6** all have amino acids in subsite P_2'. The best inhibitor has Ala in this subsite, which is consistent with the fact that Ala is found in this position in the collagen cleavage site. Substitution of Gly (**5**) or Phe (**6**) for this residue results in at least a 10-fold increase in K_I. Since replacement with D-Ala results in a 30-fold increase in K_I, the recognition of Ala is stereospecific. Inhibitor **10** has a Gly residue in subsite

Table I: Inhibition of Human Neutrophil Collagenase by Phosphonamidates

	Inhibitor[a]	K_I, mM
	P_4 - P_3 - P_2 - P_1 -P_1'-P_2'-P_3'	
1	Cbz-Gly^P-Leu	2.1
2	Cbz-Gly^P-Leu-NH_2	1.3
3	Cbz-Gly^P-Leu-Ala	0.078
4	Cbz-Gly^P-Leu-D-Ala	2.6
5	Cbz-Gly^P-Leu-Gly	6.8
6	Cbz-Gly^P-Leu-Phe	0.98
7	Cbz-Gly^P-OLeu-Ala	2.5
8	Cbz-Pro-Ala-Gly^P-Leu-NH_2	0.15
9	Cbz-Phe-Gly^P-Leu-Ala	0.071
10	Cbz-Gly^P-Leu-Ala-Gly	0.014

[a] Gly^P is (aminomethyl)phosphonyl; OLeu is 2-hydroxy-4-methylpentanoyl.

P_3'. This compound is the best inhibitor in the series with a K_I value of 14 μM.

Inhibitor **8** has subsites P_2 and P_3 occupied by Ala and Pro, respectively. The compound is more unstable in aqueous solution than the others, apparently because of intramolecularly assisted hydrolysis of the phosphonamidate bond. By carrying out 1-h assays, an upper limit for K_I was estimated to be 150 μM. Since this inhibitor is approximately 10-fold better than **2**, the recognition of the inhibitor by the enzyme includes the P_2 and P_3 subsites. Inhibitor **9** has subsites P_2 and P_2' occupied by Phe and Ala, respectively. There is essentially no increase in inhibition by having Phe (**9**) rather than a Cbz group (**3**) in P_2. Phe could be an unfavorable residue for this subsite, which is occupied by Gln in the α1(I) chain of type I collagen, thereby negating any effect of extending the inhibitor in the amino-terminal direction.

Inhibitor **7** is identical with **3** except that the amide nitrogen of the phosphonamidate has been replaced by an oxygen, resulting in a phosphonate ester. This simple change causes a 30-fold increase in the K_I value, indicating the importance of the NH group in the enzyme–inhibitor interaction. This importance of the amide functionality has been noted for the binding of such inhibitors to other zinc metalloproteinases (Thorsett et al., 1982; Gordon et al., 1984; P. A. Bartlett and C. K. Marlowe, unpublished experiments).

Discussion

There have been few reports on the inhibition of tissue collagenases by synthetic compounds. The majority of the known inhibitors such as polyureides (Bernstein, 1980) and antiinflammatory drugs (Wojtecka-Lukasik & Dancewicz, 1974) are not substrate analogues and are nonspecific. Two reports have appeared that have used sulfhydryl-containing peptides (Gray et al., 1981) and carboxyalkyl peptides (Gray et al., 1985) to inhibit tadpole and rat carcinoma collagenases, respectively. The present data show that phosphonamidates, which have proven to be tight binding inhibitors of other zinc metalloproteinases, are also promising inhibitors of collagenases.

It is clear from the data in Table I that inhibition is systematically improved by extending the peptide portion in both the N- and C-terminal directions. Specifically, extending the basic Gly^P-Leu sequence in the C-terminal direction leads to tighter binding when Ala and Gly are in subsites P_2' and P_3', respectively. Extension in the N-terminal direction also leads to better binding when Pro and Ala occupy subsites P_3 and P_2, respectively. This conforms to the known specificity of tissue collagenases toward collagen (Gross et al., 1974) and synthetic peptides (Nagai et al., 1976).

The tetrahedral geometry around the phosphorus atom resembles that of the putative transition state for peptide-bond hydrolysis, suggesting that phosphonamidates are transition-state analogue inhibitors of zinc metalloproteinases (Jacobsen & Bartlett, 1981; Weaver et al., 1977). A distinguishing feature of a series of transition-state analogues is that their K_I values correlate with the K_M/k_{cat} values for a series of matched substrates. In fact, a plot of log K_I vs. log (K_M/k_{cat}) for such a series has a slope near unity, indicating that changes in the sequence of the inhibitor affect binding in the same manner as changes for the matched substrate affect catalysis. Bartlett and Marlowe (1983) have shown this to be the case for phosphonamidate inhibitors of thermolysin. It has also been observed that many transition-state analogues, including phosphoramidate and phosphonamidate inhibitors of zinc metalloproteinases, are slow binding inhibitors (Kam et al., 1979; Holden, Mathews, P. A. Bartlett, and C. K. Marlowe, unpublished data).

Unfortunately, practical limitations prevent these criteria from being applied to the inhibition of neutrophil collagenase by the compounds described here. Since the assay time is minimally 1 h and the time dependence of inhibition is usually of the order of minutes, we would not have been able to detect the slow binding effects observed for other enzymes. Nor was a comparison between the K_I values of the inhibitors studied here and the corresponding substrates feasible because of the relative insensitivity of short peptides toward hydrolysis by this enzyme. For example, no appreciable hydrolysis of the peptide Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln could be detected at a 20-fold higher concentration of the neutrophil collagenase than used in collagenase assays. However, since the K_M value for the hydrolysis of this peptide by neutrophil collagenase is greater than 1 mM (Fields, H. E. Van Wart, and Birkedal-Hansen, unpublished experiments), it is clear from the data in Table I that the substitution of the tetrahedral P(=O)(OH) group for the carbonyl group of the scissile peptide bond greatly enhances binding.

Inhibitor **7** provides evidence for the importance of the NH group of the scissile bond in the binding of the transition state of the substrate to the enzyme. Replacement of the NH group of **3** by an oxygen atom in **7** results in 30-fold weaker binding. The importance of the basic nitrogen has been noted before (Thorsett et al., 1982; Gordon et al., 1984), and it has been postulated to accept a proton in the transition state (Kam et al., 1979; Gordon et al., 1984; Hangauer et al., 1984). The lower basicity of the oxygen could result in the loss of this important interaction and account for the dramatic increase in K_I.

Although the inhibitors studied here do not bind as tightly to collagenase as the natural substrate (K_M = 1.5 μM), they do provide a first step in designing potent inhibitors for this important enzyme. Tissue collagenases are among the most specific proteinases known. Whether this specificity has any basis in the unique tertiary structure of collagen is yet to be determined. We expect that longer inhibitors with residues on both sides of the phosphonamide moiety will prove to be even better inhibitors of collagenases.

Acknowledgments

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SUPPLEMENTARY MATERIAL AVAILABLE

Synthesis and properties of inhibitors **1** and **7–10** (6 pages). Ordering information is given on any current masthead page.

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ADP-ribosyl Transferase and NAD Glycohydrolase Activities in Rat Liver Mitochondria†

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ABSTRACT: ADP-ribosyl transferase and NAD glycohydrolase activities have been estimated in mitochondria in mitoplasts as well as in other submitochondrial fractions. A high activity of these two enzymes was present in mitoplasts as compared to the outer membrane preparation or intermembrane compartment. Inhibitor studies provide strong evidence for the involvement of ADP-ribosyl transferase in the process of ADP-ribosylation of mitochondrial proteins. When NAD glycohydrolase was blocked by nicotinamide or 3-aminobenzamide, the incorporation of ADP-ribose into mitochondrial proteins still occurs. ADP-ribosyl transferase activity could also be detected when NAD glycohydrolase was separated by hydroxylapatite chromatography. The protein-linked ADP-ribose moiety appears to be an oligomer in mitochondria.

ADP-ribosylation is a covalent posttranslational modification of proteins catalyzed by specific enzymes (Mandel et al., 1982; Ueda & Hayaishi, 1985). The ADP-ribose moiety of NAD is transferred to acceptor proteins producing an acceptor-linked monomer or polymer. Nuclear ADP-ribosyl transferase discovered by Chambon et al. (1966) is established to be a chromatin-linked enzyme synthesizing a protein-bound homopolymer. Histones (Okazaki et al., 1980a; Poirier et al., 1982) and various non-histone proteins serve as acceptor proteins for the elongation of poly(ADP-ribose) chains. Apparently, the predominant acceptor is the enzyme itself, i.e., ADP-ribosyl transferase (Okazaki et al., 1980b; Kawaishi et al., 1981).

Enzymatic transfer of ADP-ribose from NAD to acceptor proteins has also been reported in free messenger ribonucleoprotein particles (Thomassin et al., 1985) in mitochondria of rat liver and testis (Kun et al., 1975; Burzio et

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