In summary, with cationic amphiphilic drugs which can bind to substrate liposomes, such as chlorpromazine and propranolol, it has been postulated that the binding of drugs to the phospholipid substrate is responsible for inhibitory activity against liver phospholipase A_1. However, we can see that this is not the case because kinetic parameters shown in Table II indicate that the presence of the ES_bI_b complex itself is negligible when the hydrolytic rate of the ES_bI_b complex is small. Furthermore, in cases where ES_bI_b is significant, its conversion to product is almost as rapid as that of ES_f itself. We conclude that chlorpromazine, propranolol, and chloroquine inhibit liver lysosomal phospholipase A_1 by interacting with the enzyme protein. Binding of drugs to substrate decreases the amount of free drug which results in modulation of velocity–substrate curves.

References

Abraham, R., Hendy, R., & Grasso, P. (1968) *Exp. Mol. Pathol. 9*, 212–229.

Allan, D., & Michell, R. H. (1975) *Biochem. J. 148*, 471–478.

Bickel, M. H., & Steele, J. W. (1974) *Chem.-Biol. Interact. 8*, 151–162.

Hostetler, K. Y., Yazaki, P. J., & van den Bosch, H. (1982) *J. Biol. Chem. 257*, 13367–13373.

Hostetler, K. Y., Reasor, M. J., & Yazaki, P. J. (1985) *J. Biol. Chem. 260*, 215–219.

Kubo, M., Matsuzawa, Y., Yokoyama, S., Tajima, S., Ishikawa, K., Yamamoto, A., & Tarui, S. (1982) *J. Biochem. (Tokyo) 92*, 865–870.

Kunze, H., Hesse, B., & Bohn, E. (1982) *Biochim. Biophys. Acta 713*, 112–117.

Lüllman, H., & Wehling, M. (1979) *Biochem. Pharmacol. 28*, 3409–3415.

Lüllman, H., Lüllman-Rauch, R., & Wassermann, O. (1978) *Biochem. Pharmacol. 27*, 1103–1108.

Lüllman-Rauch, R. (1979) *Lysosomes Appl. Biol. Ther. 6*, 49–130.

Matsuzawa, Y., & Hostetler, K. Y. (1980a) *J. Lipid Res. 21*, 202–214.

Matsuzawa, Y., & Hostetler, K. Y. (1980b) *J. Biol. Chem. 255*, 5190–5194.

Matsuzawa, Y., & Hostetler, K. Y. (1980c) *Biochim. Biophys. Acta 620*, 592–602.

Pappu, A., & Hostetler, K. Y. (1984) *Biochem. Pharmacol. 33*, 1639–1644.

Robinson, M., & Waite, M. (1983) *J. Biol. Chem. 258*, 14371–14378.

Schwendener, R. A., & Weder, H.-G. (1978) *Biochem. Pharmacol. 27*, 2721–2727.

van den Bosch, H., & Aarsman, A. J. (1979) *Agents Actions 9*, 382–389.

Yamamoto, A., Adachi, S., Kitani, T., Shinji, Y., Seki, K., Nasu, T., & Nishikawa, M. (1971) *J. Biochem. (Tokyo) 69*, 613–615.

Complementary Substrate Specificities of Class I and Class II Collagenases from *Clostridium histolyticum*†

Harold E. Van Wart* and D. Randall Steinbrink

Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

Received April 5, 1985

ABSTRACT: The substrate specificities of three class I (β, γ, and η) and three class II (δ, ϵ, and ζ) collagenases from *Clostridium histolyticum* have been investigated by quantitating the k_{cat}/K_M values for the hydrolysis of 53 synthetic peptides with collagen-like sequences covering the P_3 through P_3' subsites of the substrate. For both classes of collagenases, there is a strong preference for Gly in subsites P_1' and P_3. All six enzymes also prefer substrates that contain Pro and Ala in subsites P_2 and P_2' and Hyp, Ala, or Arg in subsite P_3'. This agrees well with the occupancies of these sites by these residues in type I collagen. However, peptides with Glu in subsites P_2 or P_2' are not good substrates, even though Glu occurs frequently in these positions in collagen. Conversely, all six enzymes prefer aromatic amino acids in subsite P_1, even though such residues do not occur in this position in type I collagen. In general, the class II enzymes have a broader specificity than the class I enzymes. However, they are much less active toward sequences containing Hyp in subsites P_1 and P_3'. Thus, the two classes of collagenases have similar but complementary sequence specificities. This accounts for the ability of the two classes of enzymes to synergistically digest collagen.

Collagenases are unique in their ability to efficiently hydrolyze the triple-helical region of collagen under physiological conditions (Seifter & Harper, 1971). The resistance of collagen to digestion by most proteinases is partly due to its triple-helical structure, but is also a consequence of the primary structure of the constituent chains that have a high content of Gly, Pro, and Hyp. In order to detail the mode of attack of collagenases on native, triple-helical collagens, it is first necessary to know the sequence specificities of these enzymes. With this knowledge in hand, the influence of the secondary structure of the substrate in determining the sites and rates of hydrolysis can be addressed.

The bacterium *Clostridium histolyticum* produces two classes (designated I and II) of collagenases[1] (EC 3.4.24.3)

† This work was supported by Research Grant GM27939 and Research Career Development Award AM01066 (to H.E.V.W.) from the National Institutes of Health, U.S. Public Health Service.

* Address correspondence to this author at the Institute of Molecular Biophysics, Florida State University.

0006-2960/85/0424-6520$01.50/0

that hydrolyze collagen at multiple sites to produce a mixture of small, dialyzable peptides (Bond & Van Wart, 1984a,b). The two classes hydrolyze both collagen and synthetic peptides such as FA-Leu-Gly-Pro-Ala at different rates, and it has been suggested that they have different sequence specificities (Bond & Van Wart, 1984a–c). Recently, the rates of hydrolysis of more than 50 tri-, tetra-, penta-, and hexapeptides with collagen-like sequences by β-collagenase, a class I enzyme, have been quantitated in order to elucidate its sequence specificity (Steinbrink et al., 1985). Each substrate contained a single cleavage site with a chromophoric FA or CNM group, or a Nph residue, placed so that hydrolysis produced an absorbance change that could be used to quantitate the k_{cat}/K_M values for the reactions. In the present study, these measurements have been extended to include three class I and three class II enzymes. The resulting sequence specificities provide a basis for understanding the different substrate specificities of the two classes of collagenases. In the following paper (Mookhtiar et al., 1985), the action of these enzymes on longer substrates that contain more than one hydrolyzable bond is reported in order to investigate the endopeptidase vs. exopeptidase activities of these collagenases.

Materials and Methods

Materials. The class I (β and γ) and class II (δ, ϵ, and ζ) collagenases were purified as described previously (Bond & Van Wart, 1984a). The purification of η-collagenase is described in the text. All six enzymes were homogeneous on sodium dodecyl sulfate–polyacrylamide gels and devoid of proteolytic activity toward BAEE and casein. Collagenase activities were measured with a new assay (K. Mookhtiar, S. Mallya, and H. E. Van Wart, unpublished results) based on the hydrolysis of ^{3}H-acetylated type I rat tail tendon collagen. Briefly, a silanized glass tube containing enzyme dissolved in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5, in a total volume of 900 μL was incubated at 30 °C for 10 min and the reaction initiated by addition of 100 μL of a 1 mg/mL solution of collagen. One hundred microliter aliquots were removed as a function of time, pipetted into microfuge tubes containing 100 μL of cold dioxane, vortexed, chilled on ice for 10 min, and centrifuged for 10 min at 4 °C. One hundred microliters of the supernatant was added to 10 mL of scintillation fluid and counted in a scintillation counter. The data were plotted as cpm of ^{3}H released vs. time. Specific activities in units of nanokatals per milligram were calculated by dividing the initial rates by the mass of enzyme present. A molecular weight of 300 000 was assumed for collagen. With this assay, which is more sensitive than those used previously (Van Wart & Bond, 1982; Bond & Van Wart, 1984a), the β-, γ-, η-, δ-, ϵ-, and ζ-collagenases had specific activities of 0.26, 0.22, 0.21, 0.034, 0.034, and 0.043 nkat/mg, respectively. The synthesis and characterization of the synthetic peptides, designated by the numbers 1–52, have been described earlier (Steinbrink et al., 1985). Peptide 53 was prepared by the same procedures. Peptide 49 from our previous study has been renamed peptide 49a for consistency with the numbering scheme used in the following paper (Mookhtiar et al., 1985).

All assays using the synthetic peptides as substrates were carried out at 25 °C in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$ at pH 7.5 (Van Wart & Steinbrink, 1981; Steinbrink et al., 1985). To obtain k_{cat}/K_M values, each reaction was carried out under first-order conditions ($[S] \ll K_M$). For reactions that could be brought to completion within several hours, first-order rate constants were calculated and converted into k_{cat}/K_M values by dividing by the enzyme concentration. For slowly hydrolyzed substrates, k_{cat}/K_M values were calculated from initial rates, v_i, by dividing by both the enzyme and substrate concentrations. The substrate concentrations used in these experiments were 50 μM for all peptides except 34–36, which were studied at 20 μM. Enzyme concentrations ranged from 1 to 100 nM depending on the susceptibility of the peptide and were determined spectrophotometrically by using the extinction coefficients measured earlier (Bond & Van Wart, 1984b). The site of hydrolysis of all peptides was determined by isolating the products by reverse-phase high-pressure liquid chromatography and determining their amino acid compositions (Steinbrink et al., 1985).

The individual kinetic parameters k_{cat} and K_M for peptides 4, 34, 40, and 49a were determined from double-reciprocal plots based on assays carried out at substrate concentrations both above and below K_M. Assays for peptide 40 at substrate concentrations ranging from 0.05 to 0.15 mM were carried out at 324 nm, where the background absorbances range from 0.65 to 1.95, giving absorbance changes on full hydrolysis from 0.11 to 0.33, respectively. Assays at substrate concentrations of 0.2–2 mM were carried out at 345 nm, where the initial absorbances are 0.24 and 2.4, with absorbance changes on full hydrolysis of 0.067 and 0.67, respectively. Assays at a substrate concentration of 3 mM were carried out at 350 nm where the background absorbance is 1.2 and the full absorbance change is 0.49. For peptides 4 and 34, assays at substrate concentrations ranging from 0.02 to 0.15 mM were carried out at 300 nm with background absorbances of 0.28 and 2.1 and absorbance changes on full hydrolysis of 0.019 and 0.14, respectively. Assays at substrate concentrations of 0.2–2 mM were monitored at 315 nm with background absorbances of 0.20–2.0 and full absorbance changes of 0.055–0.55, respectively. Assays for peptide 49a at substrate concentrations of 0.05–0.4 mM were monitored at 308 nm with background absorbances of 0.20–1.6 and full absorbance changes of 0.039–0.31, respectively. A wavelength of 325 nm was used for assays at substrate concentrations in the 0.05–2 mM range. Background absorbances ranged from 0.69 to 2.8, giving total absorbance changes of from 0.185 to 0.74, respectively.

Results

Collagenase Isolation. In our previous purification (Bond & Van Wart, 1984a), three class I (α, β, and γ) and three class II (δ, ϵ, and ζ) collagenases were isolated from the crude starting mixture. This procedure was repeated in order to obtain the enzymes used in the present study. However, the results differed in that the starting material used in this purification did not yield an appreciable amount of α-collagenase, but instead contained a new enzyme, denoted η-collagenase. Its purification and properties are described briefly below.

According to the nomenclature of Bond & Van Wart (1984a) η-collagenase eluted from the Reactive Red-120 dye ligand column in fraction D together with ϵ-collagenase. This fraction was concentrated, dialyzed vs. 5 mM Tris and 5 mM $CaCl_2$, pH 8.0, applied to a DEAE-cellulose column, and eluted with a 5–70 mM Tris gradient followed by a step to 100 mM Tris (Figure 1) to yield three peaks, designated fractions A, B, and C. Fraction A contained impurities, fraction B contained most of the ϵ-collagenase, and fraction

[1] Abbreviations: collagenase, *Clostridium histolyticum* collagenase; FA, 2-furanacryloyl; CNM, cinnamoyl; Nph, 4-nitrophenylalanine; BAEE, benzoyl-L-arginine ethyl ester; Boc, *tert*-butyloxycarbonyl; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)-aminomethane; cpm, counts per minute; nkat, nanokatal; Mes, 2-(*N*-morpholino)ethanesulfonic acid. All amino acids have the L configuration.

Table I: Kinetic Parameters for the Hydrolysis of Synthetic Peptides by β- and ϵ-Collagenases

		β			ϵ		
	P_2—P_1—P_1'–P_2'–P_3'	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})
40	FA-Leu-Gly-Pro-Ala	660	1.1	59	3100	0.43	730
4	CNM-Leu-Gly-Pro-Ala	430	0.46	94	7500	0.41	1800
34	CNM-Phe-Gly-Pro-Ala	2100	0.24	870	12000	0.46	2700
49a	Boc-Gly-Pro-Nph-Gly-Pro-Ala	26000	2.5	1100	21000	1.5	1400

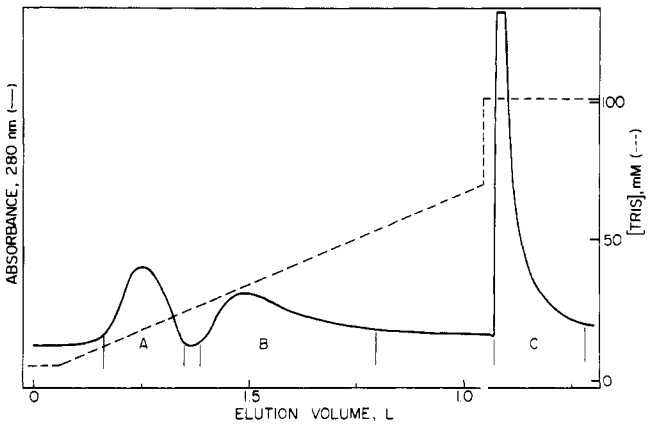

FIGURE 1: Chromatography of fraction D from the Reactive Red-120 agarose column over DEAE-cellulose at 4 °C. The enzyme (110 mg in 11 mL) was applied to the column (1.5 cm × 10 cm) in 5 mM Tris and 5 mM $CaCl_2$, pH 8.0, and eluted with a 1-L, 5–70 mM Tris gradient followed by a step to 100 mM Tris in the same buffer at a flow rate of 25 mL/h.

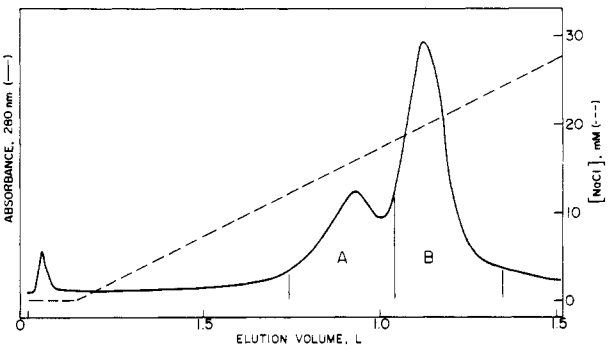

FIGURE 2: Chromatography of DEAE-cellulose fraction C over SP-Sephadex at 4 °C. The enzyme (21 mg in 22 mL) was applied to the column (1 cm × 15 cm) in 1 mM Mes and 1 mM $CaCl_2$, pH 6.0, and eluted with a 1.5-L, 0–30 mM NaCl gradient in the same buffer at a flow rate of 30 mL/h.

C contained all of the η-collagenase. Fraction C was concentrated, dialyzed vs. 1 mM Mes and 1 mM $CaCl_2$, pH 6.0, applied to a SP-Sephadex column, and eluted with a 0–30 mM NaCl gradient. The elution profile (Figure 2) consists of two partially resolved peaks labeled fractions A and B. Fraction B, which contained the η-collagenase, was concentrated, dialyzed, and applied to a second Reactive Red-120 column. The trailing two-thirds of the major peak that eluted with the NaCl gradient (profile not shown) contained pure η-collagenase. The molecular weight determined by gel electrophoresis is 130000, and the molar extinction coefficient determined by the method of Whitaker & Granum (1980) is 1.69×10^5 M^{-1} cm^{-1}. It exhibits high activity toward type I collagen and moderate activity toward FA-Leu-Gly-Pro-Ala and FA-Leu-Gly-Pro-Pro. These activities, along with the results of the substrate specificities to follow, establish that it is a new class I collagenase.

Chromophoric Substrates. The peptides examined in this study span the P_3 through P_3' subsites [nomenclature of Schecter & Berger (1967)] of the substrate. The amino acid content and sequences of the peptides were patterned after those found in type I collagen, which has the Gly-X-Y triplet repeated 338 times (Hofmann et al., 1980). Thus, the P_3 through P_3' subsites of the substrate correspond to two consecutive triplets in the collagen chain, Gly-X-Y-Gly-X'-Y', where X = P_2, Y = P_1, X' = P_2', and Y' = P_3'. The most abundant residues in position X are Pro, Ala, and Glu, while the most frequent Y residues are Hyp, Ala, and Arg.

Each peptide has a chromophoric group placed in such a way that hydrolysis of the P_1–P_1' bond produces an absorbance change that can be used to continuously monitor the progress of the reaction spectrophotometrically. When there is a FA or CNM group in subsite P_2, amino acid substitutions in subsites P_1 through P_3' can be investigated. When the Nph residue is in subsite P_1, amino acid substitutions in subsites P_3 and P_2 can be investigated. The spectral properties of these substrates and the details of the assays have been described previously (Steinbrink et al., 1985).

Results of Assays. Individual kinetic constants have been determined for the hydrolysis of the four representative peptides 4, 34, 40, and 49a by both a class I (β) and a class II (ϵ) collagenase to establish the range of K_M values that would be encountered. Double-reciprocal plots for these reactions (not shown) are linear over the entire range of substrate concentrations studied. Thus, there are no kinetic anomalies, and Michaelis–Menten kinetics are followed. Inspection of the resultant kinetic parameters (Table I) shows that the K_M values range from 0.24 to 2.5 mM, the k_{cat} values from 430 to 26000 min^{-1}, and the k_{cat}/K_M values from 59×10^4 to 2700×10^4 M^{-1} min^{-1}. Thus, the K_M values for all of these reactions are relatively high, and assays carried out at a single substrate concentration in the 20–50 μM range should yield accurate values of k_{cat}/K_M. This is confirmed by the agreement between the k_{cat}/K_M values calculated from such assays (Tables II–IV) and those listed in Table I.

The data in Table I are the first to reveal major differences between the two classes of collagenases. For ϵ-collagenase, K_M is almost constant at 0.43, 0.41, and 0.46 mM for peptides 40, 4 and 34, respectively; however, the k_{cat} values increase from 3100 to 7500 to 12000 min^{-1}. With β-collagenase, the K_M values for peptides 40, 4, and 34 drop from 1.1 to 0.46 to 0.24 mM, while the k_{cat} values vary from 660 to 430 to 2100 min^{-1}, respectively. The comparison of the kinetic constants for the hydrolysis of hexapeptide 49a and the others also exemplifies the differences between the two enzymes. For example, replacement of the CNM-Phe group (peptide 34) with the Boc-Gly-Pro-Nph group (peptide 49a) increases K_M from 0.24 to 2.5 mM and k_{cat} from 2100 to 26000 min^{-1} for β-collagenase. For ϵ-collagenase, the differences are much less pronounced. The K_M values increase only 3-fold and the k_{cat} values less than 2-fold. Thus, these two enzymes clearly interact differently with these substrates.

The k_{cat}/K_m values for the hydrolysis of peptides 1–53 by all six collagenases are listed in Table II–IV. The data for

Table II: P_3' and P_2' Subsite Specificities of Class I and II Collagenases

		k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})					
	$P_2-P_1-P_1'-P_2'-P_3'$	β	γ	η	δ	ε	ζ
1	CNM-Leu-Gly-Pro-OCH_3	<0.30	<0.10	<0.11	<1.6	<0.80	<1.0
2	CNM-Leu-Gly-Pro-NH_2	1.1	0.80	1.1	64	58	120
3	Gly	7.2	2.5	3.1	140	120	270
3a	Gly-OCH_3	2.7	2.2	<1.1	97	130	160
4	Ala	94	64	62	2000	1900	2400
4a	Ala-OCH_3	4.7	4.8	1.4	140	120	190
5	Ser	48	34	30	1500	1200	1900
5a	Ser-OCH_3	3.5	2.5	3.6	85	110	180
6	Leu	160	110	110	1400	1300	1900
6a	Leu-OCH_3	24	16	11	270	290	460
7	Pro	780	460	360	2800	2400	3000
8	Hyp	570	400	310	830	1000	1200
8a	Hyp-OCH_3	4.6	2.7	2.8	25	32	23
9	Arg	500	330	290	4800	4700	5900
9a	Arg-OCH_3	470	390	270	4800	5100	7800
10	FA-Leu-Gly-Pro-Gly	7.3	4.8	5.0	220	170	310
11	Ala	61	44	36	650	640	840
12	Leu	150	110	83	1000	990	1400
13	Pro	460	350	280	1100	950	1300
53	FA-Leu-Gly-Ala-Pro	22	18	15	270	250	440
20	Hyp	24	17	15	200	200	310
14	CNM-Leu-Gly-Ala-Pro	27	17	15	570	560	800
15	Ser	6.0	4.1	4.2	320	260	450
16	Glu	1.8	0.30	<1.1	9.0	9.0	10
17	Leu	3.0	1.2	<1.1	16	7.9	20
18	Hyp	1.1	1.0	1.1	49	31	75
19	CNM-Leu-Gly-Ala-Hyp	32	22	17	580	530	590
22	Glu	2.0	0.63	<1.1	24	17	30
23	Leu	3.4	1.7	1.7	13	10	20
24	CNM-Leu-Gly-Glu-Ala	2.0	0.15	<1.1	25	18	33
25	CNM-Leu-Gly-Ala-Arg	17	8.4	3.9	380	340	380

Table III: P_1 and P_1' Subsite Specificities of Class I and II Collagenases

		k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})					
	$P_2-P_1-P_1'-P_2'-P_3'$	β	γ	η	δ	ε	ζ
4	CNM-Leu-Gly-Pro-Ala	94	64	62	2000	1900	2400
26	Ala	<0.30	<0.10	<1.1	<1.6	<0.80	<1.0
27	Leu	<0.30	<0.10	<1.1	<1.6	<0.80	<1.0
28	CNM-Gly-Gly-Pro-Ala	3.4	2.3	3.1	6.3	4.5	9.8
29	Ala	64	47	39	170	150	240
30	Ser	50	31	23	110	84	130
31	Glu	0.42	0.13	<1.1	<1.6	0.70	2.3
32	Val	2.2	1.1	<1.1	67	57	93
33	Ile	1.2	0.52	<1.1	60	49	94
34	Phe	900	880	780	3000	2800	4100
35	Nph	4400	3100	1800	5100	4900	5100
36	Tyr	1300	1200	930	3500	3200	4500
37	Pro	26	18	19	6.7	6.2	16
38	Hyp	230				14	
39	FA-Gly-Gly-Pro-Ala	1.1	1.3	1.3	0.93	1.6	2.9
40	Leu	61	44	36	640	650	840
41	Phe	690	500	410	1400	1400	1800

β-collagenase have been reported earlier (Steinbrink et al., 1985) but are included here for comparison. For peptides that did not appear to be hydrolyzed, upper bounds for k_{cat}/K_M have been estimated. To illustrate the effect of substitution at each subsite in these tables, the peptides have been separated into groups that differ only by the identity of the residue at a single subsite. In all of these tables, the relative rates of hydrolysis of these peptides by the three class I collagenases are very similar, and those of the three class II collagenases are very similar, but those of the two classes are usually very different.

The relative rates of hydrolysis of peptides 1–13, 20, and 53 listed in Table II provide information on the P_3' subsite specificity of the collagenases. Peptides 1–9a all contain CNM-Leu-Gly-Pro in subsites P_2–P_2'. There is no detectable hydrolysis of the methyl ester (peptide 1) by either class of enzymes. The amide (peptide 2) is hydrolyzed very slowly by class I enzymes, but at an appreciable rate by the class II enzymes. When an amino acid is present in subsite P_3', all rates increase substantially with Pro > Hyp > Arg > Leu > Ala > Ser > Gly for all three class I enzymes and Arg > Pro > Ala > Ser = Leu > Hyp > Gly for all three class II enzymes. Substrates with Gly-Pro-Y triplets in positions P_1'–P_3' are hydrolyzed 5–10 times faster by the class II than by the class I enzymes when the P_3' residue is either Leu, Pro, Hyp, or Arg. When P_3' is a smaller residue, such as Gly, Ala, or Ser, hydrolysis by the class II enzymes is up to 40 times faster. However, when Hyp is in subsite P_3', the rates differ by less than 2-fold. The relative rates of hydrolysis of the FA peptides 10–13, all of which contain FA-Leu-Gly-Pro in positions

Table IV: P_3 and P_2 Subsite Specificities of Class I and II Collagenases

		k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})					
	$P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'$	β	γ	η	δ	ϵ	ζ
42	Nph-Gly-Pro-Ala	<0.30	<0.20	<1.8	<1.6	<0.80	<1.1
42a	Boc	8.0	5.7	4.2	35	23	52
35	CNM	4400	3100	1800	5100	4900	5100
43	Pro	11	9.3	11	100	82	170
44	Boc-Gly-Nph-Gly-Pro-Ala	0.62	0.41	1.2	<1.6	1.0	3.2
45	Leu	26	16	17	7.2	4.4	8.5
43a	Pro	11	8.1	9.3	93	70	140
46	Boc-Gly-Ala-Nph-Gly-Pro-Ala	110	87	79	680	590	870
47	Glu	0.31	<0.20	<1.8	<1.6	1.3	<1.1
48	Leu	220	170	180	68	44	75
49a	Pro	870	660	530	1600	1400	2000
49	Gly-Pro-Nph-Gly-Pro-Ala	820	630	550	1600	1300	1900
50	Boc-Ala-Pro-Nph-Gly-Pro-Ala	43	38	35	580	340	690
51	Leu	4.4	3.0	2.3	23	15	34
52	Hyp	6.1	3.0	2.3	23	15	32

P_2–P_2', by both classes of enzymes are very similar to those of the corresponding CNM peptides 3, 4, 6, and 7, respectively. The lone exception is that peptide 12 with Leu in subsite P_3' is hydrolyzed faster than peptide 11 with Ala at this subsite by the class II enzymes. For both classes of collagenases, esterification of residue P_3' substantially lowers the rate of hydrolysis if the side chain of P_3' is small (Gly, Ala, Ser, or Hyp). When Leu is in this position (peptides 6 and 6a), the reduction is less pronounced, and when Arg is present (peptides 9 and 9a), esterification has almost no effect on the rates.

Table II contains other groups of peptides that differ from one another solely in the identity of the amino acid in the P_3' subsite. All of these groups have either CNM or FA in subsite P_2 and Leu and Gly in subsites P_1 and P_1', respectively. Each group has the same residue in subsite P_2' but a different residue in P_3'. The first group (peptides 16, 22, and 24) contains Glu in subsite P_2' and Pro, Hyp, and Ala, respectively, in subsite P_3'. The class I enzymes hydrolyze these peptides at barely detectable rates, while the class II enzymes hydrolyze all three peptides at appreciable rates. Another group (peptides 14, 19, and 25), which contains Ala in subsite P_2' and Pro, Hyp, and Arg, respectively, in subsite P_3', is hydrolyzed rapidly by class I enzymes and very rapidly by class II enzymes. The same behavior is seen for the analogous FA peptides 20 and 53. Peptides 17 and 23 both contain Leu in subsite P_2' but Pro and Hyp, respectively, in subsite P_3'. Neither is hydrolyzed at an appreciable rate by the class I enzymes. However, the class II enzymes hydrolyze these peptides at a moderate rate.

The P_2' subsite specificity of these collagenases is also shown in Table II. The P_2, P_1, and P_1' subsites of all peptides listed contain CNM or FA, Leu, and Gly, respectively. Peptides 7 and 14–18 all contain Pro in subsite P_3' but Pro, Ala, Ser, Glu, Leu, and Hyp, respectively, in subsite P_2'. Within this group, peptide 7 with Pro in subsite P_2' is hydrolyzed very rapidly, and peptide 14 with Ala in that subsite is hydrolyzed rapidly by the class I enzymes. Peptide 15 with Ser in subsite P_2' is hydrolyzed slowly, whereas peptides 16, 17, and 18 containing Glu, Leu, and Hyp, respectively, are hydrolyzed at barely detectable levels. The class II enzymes, on the other hand, are able to hydrolyze peptides 7, 14, and 15 containing Pro, Ala, and Ser, respectively, in the P_2' subsite at extremely high rates. Peptide 18, containing Hyp, is hydrolyzed reasonably rapidly while peptides 16 and 17 containing Glu and Leu, respectively, are hydrolyzed at slow but appreciable rates.

The same conclusion is reached when the rates of hydrolysis of peptides 8, 19, 22, and 23, all of which contain Hyp in position P_3', are compared. For the class I enzymes, the hydrolysis rates are fast only when Pro or Ala is in subsite P_2' (peptides 8 and 19). Replacement with Glu or Leu (peptides 22 and 23) markedly lowers the rates of hydrolysis. Peptides 8 and 19 containing Pro and Ala, respectively, are hydrolyzed very rapidly by the class II enzymes, but those containing Glu and Leu (peptides 22 and 23) are hydrolyzed at more moderate rates. Peptides 4 and 24 both have Ala in subsite P_3' but Pro and Glu, respectively, in subsite P_2'. Peptide 4 is hydrolyzed rapidly by the class I collagenases and very rapidly by the class II enzymes. Replacement of Pro by Glu lowers the rate nearly 100-fold for the class II enzymes and to barely detectable levels for the class I enzymes. Collectively, these data indicate that the class I enzymes have a very strong preference for Pro or Ala in subsite P_2', while the class II enzymes prefer Pro, Ala, or Ser in P_2', but peptides with other residues at this subsite are still hydrolyzed at appreciable rates.

When the Gly residue in subsite P_1' of peptide 4 is replaced with either Ala (peptide 26) or Leu (peptide 27), the rate of hydrolysis by all six enzymes is reduced below the level of detection (Table III). Apparently, there is a strict requirement for Gly in this position. Replacement of the Leu residue in subsite P_1 of peptide 4 by a wide variety of amino acids (peptides 28–38) markedly alters the hydrolysis rates (Table III). For the class I enzymes, the hydrolysis rate is low when there is Gly (peptide 28), Glu (peptide 31), or a residue with a side chain branched at the β-carbon (Val, peptide 32; Ile, peptide 33) present in subsite P_1. The rates of hydrolysis of peptides with hydrophobic side chains that are not branched at the β-carbon are high with Nph > Tyr > Phe > Leu > Ala. Interestingly, peptide 38 which has Hyp in subsite P_1 is hydrolyzed rapidly, while peptide 37 with Pro in subsite P_1 is hydrolyzed much more slowly. Peptide 30, with Ser in subsite P_1, is hydrolyzed at about the same rate as peptide 29 with Ala in that position. The corresponding FA peptides 39–41 are hydrolyzed in the order Phe > Leu > Gly at rates similar to their CNM counterparts.

The class II enzymes show a similar pattern of hydrolysis rates. Peptides containing P_1 residues that have large hydrophobic side chains unbranched at the β-carbon are hydrolyzed at extremely high rates (Nph > Tyr > Phe > Leu). Peptides containing Ala and Ser are hydrolyzed 10–20-fold more slowly than the larger, more hydrophobic residues, but the rates are still high. Class II enzymes can hydrolyze peptides 32 and 33 that have Val or Ile at subsite P_1 relatively rapidly compared to the class I enzymes, but the rates are at least 25-fold slower than when the unbranched Leu residue is in the P_1 subsite. Peptide 31 containing Glu is not hydrolyzed appreciably, and peptides containing Gly, Pro, and Hyp in subsite P_1 are hydrolyzed relatively slowly. Peptides

37 and 38, which contain Pro and Hyp, respectively, in subsite P_1 are the only peptides not cleaved faster by class II collagenases. The relative rates of hydrolysis of FA peptides 39–41 are very similar to those for the corresponding CNM peptides 28, 4, and 34.

Information on the P_2 subsite specificities of the collagenases is obtained from the relative rates of hydrolysis of the peptides listed in Table IV. All of the peptides listed contain the Nph-Gly-Pro-Ala sequence in subsites P_1 through P_3' but different residues in subsites P_2 and P_3. Peptide 42, which does not have an amino acid in subsite P_2, is not hydrolyzed at a detectable rate by any of the collagenases. When there is a Boc group in subsite P_2 (peptide 42a), all six enzymes hydrolyze the peptide at reasonable rates. A slight increase in the rates for the class I enzymes is observed when Pro (peptide 43) is substituted for the Boc group, while the rates for the class II enzymes triple. Interestingly, when the CNM group is in this subsite (peptide 35), the rate is increased dramatically for both classes of enzymes.

Peptides 44, 45, and 43a are Boc-protected pentapeptides that have Gly, Leu, and Pro, respectively, in subsite P_2. These peptides are hydrolyzed in the order Leu > Pro > Gly for the class I enzymes and Pro > Leu > Gly for the class II enzymes, respectively. Thus, the class II enzymes show a strong preference for Pro in subsite P_2. Peptides 46–48 and 49a are Boc-protected hexapeptides that differ only in the identity of the P_2 residue. When Glu is in subsite P_2 (peptide 47), hydrolysis is very slow or undetectable for both classes of enzymes. The other three peptides are all hydrolyzed rapidly in the order Pro > Leu > Ala by the class I enzymes and Pro > Ala > Leu for the class II enzymes. Again, the class II enzymes show a stronger preference for Pro (Ala substitutes well) in subsite P_2 than the class I enzymes.

Some information on the P_3 subsite specificity of these collagenases can also be obtained from the data in Table IV. Peptide 43a, containing a Boc group in subsite P_3, is hydrolyzed by all enzymes but at a markedly slower rate than peptide 49, which contains Gly in this subsite. The hexapeptides 46 and 50–52 differ only by having Gly, Ala, Leu, and Hyp, respectively, in subsite P_3. The hydrolysis rates for all six collagenases are fastest for peptide 49 which has Gly in subsite P_3, but the rate is also appreciable when Ala is present (peptide 50). The other two peptides are hydrolyzed much more slowly. The class I enzymes have a stronger preference for Gly over Ala in subsite P_3 than the class II collagenases.

Discussion

The k_{cat}/K_M values for the hydrolysis of the 53 synthetic peptides examined in this study provide a basis for understanding the sequence specificities of these six collagenases. It is clear from these data that the six enzymes can be unequivocally assigned to distinct groups on the basis of their specificities. According to the terminology of Bond & Van Wart (1984a–c), β, γ, and η are class I while δ, ϵ, and ζ are class II collagenases. Within each class, the activities of each collagenase toward nearly every peptide are very similar. However, the specificities of the two classes are distinctly different.

By systematic variation in the identity of the residue in subsites P_3 through P_3', these data establish that the residue in each subsite profoundly affects the rate of hydrolysis of these peptides by every collagenase. Thus, these enzymes have extended active sites, a subject discussed in more detail in the following paper (Mookhtiar et al., 1985). With respect to the specificities of the enzymes toward subsites P_1' through P_3', the data shown in Table I are somewhat misleading. In general, all of the rates for the class I enzymes are considerably lower than those for the class II enzymes. This difference was noted earlier for the relative activities of the two classes of enzymes toward FA-Leu-Gly-Pro-Ala and FA-Leu-Gly-Pro-Pro and led to the suggestion that the class II enzymes were generally more active toward synthetic peptides than the class I enzymes (Bond & Van Wart, 1984a–c). However, the basis for this difference is that peptides with Leu in subsite P_1 are much worse substrates for the class I compared to the class II enzymes (see Table III). Thus, the low activities listed for the class I enzymes in Table II are misleading. If all of these peptides contained a P_1 residue that was equally favorable for both classes of enzymes (e.g., Nph, Tyr, or Phe), the rates for the two classes would be closer in magnitude. Assuming that the interactions of the enzymes with the different subsites are additive, the rates for the class I enzymes in Table I would be at least 20-fold higher if all the peptides listed contained Nph in subsite P_1.

With this in mind, several interesting conclusions can be drawn from the data in Table II. Peptides with an amide group in subsite P_3' are hydrolyzed by both classes of collagenases, but the corresponding ester is not, indicating that at least part of the subsite recognition of P_3' lies in the amide bond. The spread in the rates for the class II enzymes is much smaller than that for the class I enzymes, indicating that the former are less specific. Esterification of the P_3' residue (except for Arg) slows the rate of hydrolysis of all peptides by all enzymes considerably. Subsite P_3' corresponds to the Y position in the collagen Gly-X-Y triplet. Interestingly, peptides with P_3' residues that occur most frequently in collagen (Hyp, Ala, and Arg) are hydrolyzed rapidly. However, if we correct for the low rate of the class I enzymes due to the presence of Leu in subsite P_1, the data indicate that the class I enzymes hydrolyze substrates with Hyp in subsite P_3' much faster. For the class I enzymes, Pro can substitute equally well for Hyp at this subsite, even though it occupies the Y position very infrequently in collagen. For the class II enzymes, substitution of Pro for Hyp in subsite P_3' more than doubles the hydrolysis rates.

The class I and II collagenases show marked differences in specificity at the P_2' subsite. The class I enzymes strongly prefer Pro as the P_2' residue. Ala substitutes well, but the rates are 20–30-fold lower than the analogous Pro peptides. Substitution of Ser, Glu, Leu, or Hyp lowers the rates for the class I enzymes to barely detectable limits. The class II enzymes also show a preference for Pro, but peptides with Ala in this subsite are only cleaved 3–4-fold more slowly. Substitution of other amino acids results in lower but still appreciable rates. Thus, the class II enzymes appear to have a broader specificity. The P_2' subsite corresponds to the X position in collagen which is occupied by Pro or Ala over 50% of the time. Glu is the next most abundant amino acid (13%), but peptides with Glu in subsite P_2' are hydrolyzed slowly. This agrees with the data of Bornstein (1967) on the hydrolysis of the α1-CB2 fragment from rat skin and tendon collagen by collagenase. Hyp, which rarely occupies the X position in collagen, cannot substitute well for Pro at subsite P_2' for either class of enzymes. The requirement for Gly in subsite P_1' appears to be strict, since even substitution by Ala lowers the rates to below detectable limits for all enzymes.

The P_1' through P_3' specificities of the class I and II collagenases correspond well with the triplet distribution found in collagen. Seven of the nine most abundant triplets in the α1 chains of type I collagen are, in descending order of occurrences, Gly-Pro-Hyp, Gly-Pro-Ala, Gly-Ala-Hyp, Gly-

Leu-Hyp, Gly-Glu-Hyp, Gly-Pro-Ser, and Gly-Ala-Arg (Hofmann et al., 1980). Collectively, these account for 38% of all triplets in these chains. Inspection of the rates of hydrolysis of the series of peptides with CNM-Leu in subsites P_2 and P_1, and with these triplets in subsites P_1' through P_3' while taking into account the low rates for the class I enzymes due to the presence of Leu in subsite P_1, reveals that all are hydrolyzed by these collagenases. Both classes efficiently hydrolyze peptides with Gly-Pro-Hyp, Gly-Pro-Ala, Gly-Ala-Hyp, Gly-Pro-Ser, and Gly-Ala-Arg in the P_1' through P_3' sites. However, peptides containing Gly-Leu-Hyp and Gly-Glu-Hyp are hydrolyzed more slowly. A comparison of the relative rates also shows that the class II enzymes have a broader specificity on the P′ side of the scissile bond.

Both classes of collagenases exhibit a marked preference at subsite P_1 for large hydrophobic residues, provided they are not branched at the β-carbon. Subsite P_1 corresponds to the Y position in collagen, which contains mostly Hyp, Ala, and polar or charged residues. Peptides with Hyp, Ala, and Ser in this subsite are, in fact, hydrolyzed well, but still 10–20 times more slowly than those containing Nph. β-Collagenase hydrolyzes peptide 38 with Hyp in subsite P_1 16 times faster than ϵ-collagenase. Thus, the class I enzymes are better able to accommodate Hyp in both subsites P_1 and P_3', the position at which it occurs in collagen. The strong preference of these collagenases for hydrophobic residues is odd because they do not occur frequently in the Y position in collagen. The preference for hydrophobic residues in subsite P_1 is very similar to most other bacterial neutral proteinases (Morihara, 1974). This lends support to the suggestion (Bond & Van Wart, 1984) that the collagenases evolved from a neutral proteinase by gene duplication followed by point mutations to yield enzymes that are capable of hydrolyzing collagen.

Peptides with large hydrophobic groups (e.g., FA and CNM) in subsite P_2 are much better substrates for both classes of collagenases than those with amino acids in this subsite. Among the peptides having an amino acid in subsite P_2, the class II enzymes show a fairly strong preference for Pro, while the class I enzymes are less restrictive. Peptides with Glu in subsite P_2 are poor substrates for both classes, even though subsite P_2 corresponds to the X position of collagen which frequently contains Glu. Both classes of enzymes strongly prefer substrates with Gly in subsite P_3. However, Ala can substitute better in this subsite than in P_1'. On extension of the peptides in the N-terminal direction to include Gly in subsite P_3, the hydrolysis rates for both classes increase markedly. Overall, the triplet specificity of the enzymes toward subsites P_3 through P_1 does not correspond nearly as well to the sequences found in collagen as the P_1' through P_3' subsite specificities.

The differences in specificity between the two classes of collagenases noted above are probably significant with respect to their abilities to degrade collagen. For example, the class II collagenases are generally less restrictive with respect to the residues at almost all subsites compared to the class I enzymes, endowing them with broader specificity. Conversely, the class I enzymes are more discriminating, particularly at subsites P_1, P_2', and P_3'. Of particular importance is their ability to more effectively hydrolyze sequences with Hyp in subsites P_1 and P_3' than the class II enzymes. Thus, the two classes of collagenases have sequence specificities that are complementary to one another. This complementarity undoubtedly accounts for the extensive digestion of collagen by mixtures of these enzymes and is the underlying basis for their synergistic action on collagen (Mandl et al., 1964; Kono, 1968; Lwebaga et al., 1976; Van Wart & Bond, 1982). The complementary specificities of the two classes of collagenases also lend support to the suggestion (Bond & Van Wart, 1984c) that one class evolved from the other by gene duplication followed by point mutations that altered the sequence specificities, but not the catalytic machinery, of the enzymes. This is reminiscent of the pancreatic proteinases which have evolved with complementary specificities to maximize the digestion of proteins for nutritional purposes (Neurath et al., 1967; Neurath, 1984). The advantage to this pathogenic bacterium probably lies in the use of these proteinases for invasion, a process that is undoubtedly facilitated by extensive, local collagen degradation.

Acknowledgments

We are indebted to Lyn Kittle for her expert technical assistance.

References

Bond, M. D., & Van Wart, H. E. (1984a) *Biochemistry 23*, 3077–3085.

Bond, M. D., & Van Wart, H. E. (1984b) *Biochemistry 23*, 3085–3091.

Bond, M. D., & Van Wart, H. E. (1984c) *Biochemistry 23*, 3092–3099.

Bornstein, P. (1967) *Biochemistry 6*, 3082–3093.

Hofmann, H., Fietzek, P. P., & Kuhn, K. (1980) *J. Mol. Biol. 141*, 293–314.

Kono, T. (1968) *Biochemistry 7*, 1106–1114.

Lwebuga-Mukasa, J. S., Harper, E., & Taylor, P. (1976) *Biochemistry 15*, 4736–4741.

Mandl, I., Keller, S., & Manahan, J. (1964) *Biochemistry 3*, 1737–1741.

Mookhtiar, K., Steinbrink, D. R., & Van Wart, H. E. (1985) *Biochemistry* (following paper in this issue).

Morihara, K. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 179–243.

Neurath, H. (1984) *Science (Washington, D.C.) 224*, 350–357.

Neurath, H., Walsh, K. A., & Winter, W. P. (1967) *Science (Washington, D.C.) 158*, 1638–1644.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157–162.

Seifter, S., & Harper, E. (1971) *Enzymes, 3rd Ed. 3*, 649–697.

Steinbrink, D. R., Bond, M. D., & Van Wart, H. E. (1985) *J. Biol. Chem. 260*, 2771–2776.

Van Wart, H. E., & Steinbrink, D. R. (1981) *Anal. Biochem. 113*, 356–365.

Van Wart, H. E., & Bond, M. D. (1982) *Anal. Biochem. 120*, 151–158.

Whitaker, J. R., & Granum, P. E. (1980) *Anal. Biochem. 109*, 156–159.