Canfield, R. E. (1963) *J. Biol. Chem. 238*, 2691-2697.
Carraway, K. L., & Koshland, D. E., Jr. (1968) *Biochim. Biophys. Acta 160*, 272-274.
Combarnous, Y., & Henner, G. (1974) *FEBS Lett. 44*, 224-228.
Flory, P. J. (1956) *J. Am. Chem. Soc. 78*, 5222-5235.
Gerken, T. A., Jentoft, J. E., Jentoft, N., & Dearborn, D. G. (1982) *J. Biol. Chem. 257*, 2894-2900.
Hampe, O. G. (1972) *Eur. J. Biochem. 31*, 32-37.
Hoare, D. G., & Koshland, D. E., Jr. (1966) *J. Am. Chem. Soc. 88*, 2057-2058.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., & Rupley, J. A. (1972) *Enzymes, 3rd Ed. 7*, 665-868.
Imoto, T., Fukuda, K., & Yagishita, K. (1976) *J. Biochem. (Tokyo) 80*, 1313-1318.
Johnson, R. E., Adams, P., & Rupley, J. A. (1978) *Biochemistry 17*, 1479-1484.
Kramer, K. J., & Rupley, J. A. (1973) *Arch. Biochem. Biophys. 156*, 414-425.
Lin, T.-Y. (1970) *Biochemistry 9*, 984-995.
Lin, T.-Y., & Koshland, D. E., Jr. (1969) *J. Biol. Chem. 244*, 505-508.
Linnell, R. H. (1960) *J. Org. Chem. 25*, 290.
Marfey, P. S., Gill, T. J., III, & Kunz, H. W. (1965) *Biopolymers 3*, 27-41.
Nakanishi, M., Tsuboi, M., & Ikegami, A. (1973) *J. Mol. Biol. 75*, 673-682.
Okazaki, K., Imoto, T., Yamada, H., Kuroki, R., & Fujita, K. (1982) *J. Biol. Chem. 257*, 12559-12562.
Pace, N. C., & McGrath, T. (1980) *J. Biol. Chem. 255*, 3862-3865.
Paiva, A. C. M., Juliano, L., & Boschcov, P. (1976) *J. Am. Chem. Soc. 98*, 7645-7648.
Poland, D. C., & Scheraga, H. A. (1965) *Biopolymers 3*, 379-399.
Sheehan, J. C., & Hlavka, J. J. (1957) *J. Am. Chem. Soc. 79*, 4528-4529.
Sophianopoulos, A. J. (1969) *J. Biol. Chem. 244*, 3188-3193.
Timkovich, R. (1977) *Biochem. Biophys. Res. Commun. 74*, 1463-1468.
Weare, J. A., & Reichert, L. E., Jr. (1979) *J. Biol. Chem. 254*, 6964-6971.
Yamada, H., Imoto, T., Fujita, K., Okazaki, K., & Motomura, M. (1981) *Biochemistry 20*, 4836-4842.

Inhibition of Collagenase from *Clostridium histolyticum* by Phosphoric and Phosphonic Amides†

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ABSTRACT: Di- and tripeptides with sequences present in collagen that are known to occupy the S_1' through S_3' subsites at the active site of the collagenase from *Clostridium histolyticum* do not themselves inhibit this zinc protease. Thus glycylproline, glycylprolylalanine, and their C-terminal amides are not inhibitors. N^{α}-Phosphorylglycylproline, N^{α}-phosphorylglycyl-L-prolyl-L-alanine, and their C-terminal amides are weak inhibitors with IC_{50}'s (concentration causing half-maximal inhibition) of 4.6, 0.8, 3, and 1.5 mM, respectively. Extension of glycyl-L-prolyl-L-alanine to L-leucyl-glycyl-L-prolyl-L-alanine gives a tetrapeptide known to occupy the S_1, S_1', S_2', and S_3' subsites of collagenase when present in collagen but that still does not itself inhibit the enzyme. (Isoamylphosphonyl)glycyl-L-prolyl-L-alanine, a peptide containing a tetrahedral phosphorus atom at the position of the amide carbonyl carbon of the L-leucylglycyl amide bond of the parent tetrapeptide, inhibits collagenase with an IC_{50} of 16 μM, at least 1000-fold more potent than the parent peptide. Substitution of the two-carbon ethyl chain of alanine for the five-carbon isoamyl chain of leucine increases the IC_{50} to 46 μM. Substitution of the *n*-decyl chain for the isoamyl chain does not change the IC_{50}. (Isoamylphosphonyl)glycyl-glycyl-L-proline contains a tripeptide that does not occupy the S_1' through S_3' subsites of collagenase when this peptide is present in collagen and thus has an IC_{50} of 4.4 mM. (Isoamylphosphonyl)glycyl-L-prolyl-L-alanine may be an analogue of the tetrahedral transition state for the hydrolysis of the natural collagen substrate. However, the IC_{50} of this inhibitor is 3-4 orders of magnitude higher than those of the best phosphorus-containing transition-state analogues of other zinc proteases. In addition, this inhibitor lacks specificity for its target, having a K_i for angiotensin converting enzyme of 11 μM, about equal to its IC_{50} for collagenase.

The collagenase from *Clostridium histolyticum* (EC 3.4.24.3) is a zinc metalloprotease also known as clostridiopeptidase A and collagenase A (Seifter & Harper, 1971). This collagenase makes a large number of cleavages in native triple-helical collagen, usually at the X-glycine bond in the sequence X-glycine-proline-Y where X is any amino acid and Y is frequently alanine or hydroxyproline. Synthetic oligopeptides are cleaved with similar specificity. Exceptions to this specificity that have been observed could be due to heterogeneity of the enzyme preparations (four homogeneous collagenases from *C. histolyticum* have been described; Lwebuga-Mukasa et al., 1976) or to the natural activity of pure collagenase (Seifter & Harper, 1971). The triple-helical region of native soluble collagen is highly resistant to nearly every protease except the collagenases (Burleigh, 1977). Compounds that are nonspecific inhibitors of all zinc metalloproteases such as cysteine inhibit collagenase (Seifter &

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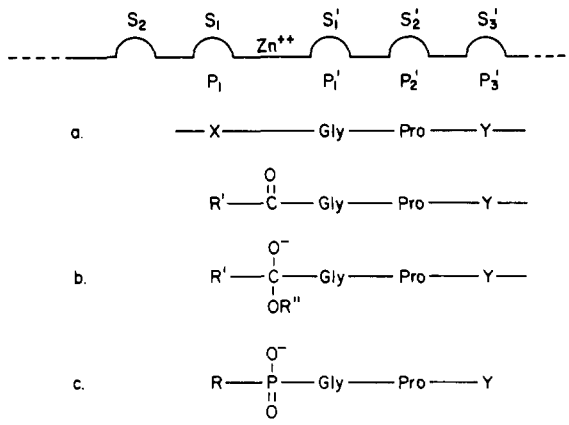

FIGURE 1: A model for active site of *C. histolyticum* collagenase adapted from the model proposed by Cushman et al. (1977) for angiotensin converting enzyme. S_1, S_2, etc. are the binding sites for the amino acid residues P_1, P_2, etc. to the left of the scissile amide bond, and S_1', S_2', etc. are binding sites for the amino acid residues P_1', P_2', etc. to the right, according to the nomenclature of Schechter & Berger (1968) for papain. (a) Collagen and peptide substrates. R′ is the continuing peptide chain. (b) Tetrahedral intermediate for the hydrolysis of (a) where R″ may be H or the side chain of an amino acid residue of collagenase. (c) Inhibitors containing a tetrahedral phosphorus atom where R is OH, ethyl, isoamyl, or *n*-decyl.

Harper, 1971). N^{α}-(Benzyloxycarbonyl) tri-, tetra-, and pentapeptides containing the sequence glycine-proline inhibit the enzyme with K_i's as low as 4 mM (Yagisawa et al., 1965). Similar peptides with a C-terminal chloromethyl ketone group show some specificity for inhibition of collagenase compared to other proteases (Balaevskaya et al., 1981), but specific, potent (K_i in the micromolar or nanomolar range), low molecular mass inhibitors of this collagenase have not been reported.

Phosphoric and phosphonic amides of peptides known to bind to the active sites of zinc metalloproteases are potent inhibitors of this class of enzymes. Phosphoric and phosphonic amide inhibitors with K_i's in the micromolar or nanomolar range have been reported for angiotensin converting enzyme (Galardy et al., 1983; Galardy, 1982; Thorsett et al., 1982; Holmquist & Vallee, 1979), enkephalinase (Alstein et al., 1982), and thermolysin and carboxypeptidase A (Jacobsen & Bartlett, 1981; Kam et al., 1979; Holmquist & Vallee, 1979). These inhibitors containing a tetrahedral phosphorus atom are presumed to act by mimicking the tetrahedral carbon intermediate occurring in the transition state for amide hydrolysis and thus approximating a species having very high affinity for the enzyme's active site (Wolfenden, 1969). An X-ray crystallographic study of the enzyme–inhibitor complex shows that phosphoramidon, a potent inhibitor of thermolysin, has its tetrahedral phosphorus atom in the position of the tetrahedral carbon in the presumed catalytic transition state (Weaver et al., 1977). On the basis of results with the above proteases, N^{α}-phosphoryl and N^{α}-phosphonyl peptides of the sequence glycylprolylalanine should be potent inhibitors of the collagenase from *C. histolyticum*. Figure 1 shows a model for the active site of this zinc protease, adapted from the model proposed for angiotensin converting enzyme by Cushman et al. (1977). This model contains binding subsites for substrate amino acid residues both to the left (S_1, S_2, etc.) and to the right (S_1', S_2', etc.) of the scissile X–glycyl bond.

Experimental Procedures

Protected amino acids, glycyl-L-proline, glycyl-L-prolyl-L-alanine, hippuryl-L-histidyl-L-leucine, acid-soluble calf skin collagen, and collagenase A from *Clostridium histolyticum* were purchased from Sigma Chemical Co. Acid-soluble rat skin collagen was a gift of Dr. Heinz Furthmayr of Yale University. Both the calf skin and rat skin collagens had intrinsic viscosities, $[\eta]$, of between 15 and 18 $dL \cdot g^{-1}$ in 0.05 M Tris[1]-HCl/0.5 M calcium chloride at 20 °C (lit. $[\eta]$ 16–18 $dL \cdot g^{-1}$, Gallop & Seifter, 1963). Angiotensin converting enzyme was partially purified from frozen rabbit lungs as described previously (Galardy, 1982).

Dicyclohexylcarbodiimide and dibenzyl phosphite were obtained from Aldrich Chemical Co. Other chemicals were of analytical grade and were used without further purification. Protected intermediates were purified by column chromatography on silica gel 60-F254 (EM Reagents). The molecular weight of protected intermediates was determinated by mass spectra taken on a Finnigan 3300 GC–MS spectrometer (EMV = 2100, electron energy = 150 eV, methane chemical ionization). Melting points were taken on a hot stage and are corrected. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM-390. Chemical shifts are in ppm downfield from tetramethylsilane in organic solvents. Paper electrophoresis was at approximately 60 $V \cdot cm^{-1}$ for 1 h on Whatman No. 3 MM paper in 1.7% *N*-ethylmorpholine adjusted to pH 7.9 with acetic acid. Relative migration is given as R_f(elec), the distance of migration relative to the distance between the origin and the end of the paper nearest the anode (20 cm). Thin-layer chromatography was on silica gel 60-F254. Compounds were visualized by the following methods: exposure to hydrogen chloride vapor followed by ninhydrin (0.6 g in 100 mL of acetone) for protected and deprotected peptides, phosphomolybdate spray for phosphoryl and phosphonyl peptides (Bandurski & Axelrod, 1951), ultraviolet light, and iodine vapor. The compositions of thin-layer solvent systems were, by volume, as follows: (A) chloroform/hexane/acetonitrile, 1.9:1.1:1; (B) 1-propanol/concentrated ammonium hydroxide, 8.6:3.7; (C) 2-propanol/water/concentrated ammonium hydroxide, 10:2:8; (D) chloroform/acetonitrile/2-propanol, 2:2:1; (E) ethyl acetate/hexane, 7:3.

N^{α}-Phosphoryl-L-alanine Tripotassium Salt (II). L-Alanine benzyl ester hydrochloride (Sigma) was phosphorylated by dibenzyl chlorophosphite prepared from dibenzyl phosphite by the method of Atherton et al. (1948). To a solution of dibenzyl chlorophosphite (4.5 mmol) and 0.863 g of L-alanine benzyl ester hydrochloride (4 mmol) in 15 mL of chloroform was added 1.12 mL (8 mmol) of triethylamine at 0 °C. After 20 h at 0 °C, the reaction mixture was diluted to 150 mL with chloroform and washed successively with 0.1 N hydrochloric acid (2 × 25 mL), water (2 × 25 mL), 0.1 N sodium hydroxide (2 × 25 mL), water (2 × 25 mL), and saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated, yielding 1.4 g (82% yield) of N^{α}-(dibenzylphosphoryl)-L-alanine benzyl ester (I): mp 44–45 °C; R_f(A) 0.47; NMR ($CDCl_3$) δ 1.28 (d, 3 H, Ala CH_3), 4.1 (m, 2 H, Ala NHCH), 4.9–5.2 (m, 6 H, $PhCH_2$), 7.25 (m, 15 H, Ph). Hydrogen gas was bubbled for 1.5 h through a mixture of 0.516 g (1.2 mmol) of I and 3.6 mL of 1.0 N potassium hydroxide (3.6 mmol) in 10 mL of methanol in the presence of 0.22 g of 10% palladium on carbon. After removal of the catalyst by filtration, the filtrate was partially evaporated, frozen, and lyophilized to give 0.35 g (100%) of crude III. This material was precipitated from the minimum amount of methanol with anhydrous tetrahydrofuran to give II as a

[1] Abbreviations: IC_{50}, concentration of inhibitor producing 50% inhibition of collagenase at a given enzyme and substrate concentration; Tris, tris(hydroxymethyl)aminomethane; Boc, *tert*-butyloxycarbonyl.

colorless solid: R_f(B) 0.01 [R_f(B) Ala 0.34]; NMR (D_2O) δ 1.3 (d, 3 H, Ala CH_3), 3.6 (m, 1 H, Ala CH).

N^{α}-Phosphoryl-L-leucyl-L-phenylalanine Tripotassium Salt (VI). To 1.5 g (5.1 mmol) of L-phenylalanine benzyl ester hydrochloride and 1.277 g (5.1 mmol) of *N*-(*tert*-butyloxycarbonyl)-L-leucine/H_2O in 30 mL of methylene chloride at 0 °C were added 0.66 mL (5.1 mmol) of *N*-ethylmorpholine and 1.057 g (5.1 mmol) of dicyclohexylcarbodiimide. After an overnight standing at 4 °C, the dicyclohexylurea was removed by filtration, and the filtrate was diluted to 150 mL with methylene chloride, extracted as described for I, and dried under high vacuum to yield 2.49 g of crude N^{α}-(*tert*-butyloxycarbonyl)-L-leucyl-L-phenylalanine benzyl ester. Recrystallization from hexane/ethyl acetate yielded 1.9 g (76.3%) of pure III: mp 95–97 °C [lit. mp 98–100 °C; Kam et al. (1979)]; R_f(A) 0.7. A 1.9-g (3.9-mmol) aliquot of III was deprotected by stirring in 3 mL of trifluoroacetic acid at room temperature for 1 h. After evaporation of excess acid under reduced pressure, 150 mL of anhydrous ether was added to the residue. The precipitate was collected, washed several times with ether, and dried to give 1.5 g (79.8% yield) of IV, the trifluoroacetate salt of L-leucyl-L-phenylalanine benzyl ester: mp 183–185 °C. IV was phosphorylated as described for the synthesis of I to give V in 78% yield: mp 78–79 °C [lit. mp 100–101 °C; Kam et al. (1979)], R_f(A) 0.6; NMR ($CDCl_3$) δ 0.83 (d, 6 H, Leu CH_3), 1.5 (m, 3 H, Leu CH_2 β, CH γ), 3.05 (d, 2 H, Phe CH_2), 3.7 (m, 2 H, PNH, Leu CH α), 4.65–5.1 (m, 7 H, $PhCH_2$, Phe CH α), 6.9–7.35 (m, 16 H, benzyl Ph, Phe Ph, Phe NH); mass spectrum, *m/e* (relative intensity) 629 (M + 1), 657 (M + 29), 669 (M + 41), 521 (M − OCH_2Ph), 369 [M − $P(O)(OCH_2Ph)_2$], 261 (M − Leu-Phe-OBzl).

N^{α}-Phosphorylglycyl-L-proline Tripotassium Salt (VII). VII was prepared identically with VI, starting with N^{α}-(*tert*-butyloxycarbonyl)glycine and L-proline benzyl ester hydrochloride. Hydrogenolysis of the tribenzyl ester of VII gave VII in 95% yield: R_f(C) 0.36, R_f(elec) 0.54 [R_f(elec) Gly-Pro 0.05]; NMR (D_2O) δ 1.9 (m, 4 H, Pro CH_2 β,γ), 3.3 (m, 4 H, Gly CH_2, Pro CH_2 δ), 4.3 (m, 1 H, Pro CH α).

N^{α}-Phosphorylglycyl-L-proline Amide Dipotassium Salt (VIII). VIII was prepared identically with VI, starting with N^{α}-(*tert*-butyloxycarbonyl)glycine and L-proline amide (Sigma). Hydrogenolysis of the dibenzyl ester of VIII gave VIII in 91% yield: R_f(C) 0.32, R_f(elec) 0.35; NMR (D_2O) δ 2.1 (m, 4 H, Pro CH_2 β,γ), 3.7 (m, 4 H, Gly CH_2, Pro CH_2 δ), 4.3 (m, 1 H, Pro CH α).

N^{α}-Phosphorylglycyl-L-prolyl-L-alanine Tripotassium Salt (IX). IX was prepared identically with VI, starting with N^{α}-(*tert*-butyloxycarbonyl)glycyl-L-proline (Anderson et al., 1967) and L-alanine benzyl ester hydrochloride. Hydrogenolysis of the tribenzyl ester of IX gave IX in 90% yield: R_f(B) 0.06 [R_f(B) Gly-Pro-Ala 0.41], R_f(elec) 0.48; NMR (D_2O) δ 1.4 (d, 3 H, Ala CH_3), 2.1 (m, 4 H, Pro CH_2 β,γ), 3.7 (m, 4 H, Gly CH_2), 4.2 (m, 1 H, Ala CH α), 4.45 (m, 1 H, Pro CH α).

N^{α}-Phosphorylglycyl-L-prolyl-L-alanine Amide Dipotassium Salt (XI). XI was prepared identically with VI, starting with N^{α}-(*tert*-butyloxycarbonyl)glycyl-L-proline (Anderson et al., 1967) and L-alanine amide hydrobromide (Sigma). The intermediate glycyl-L-prolyl-L-alanine amide trifluoracetate salt (X) was isolated in 81% yield: mp 100–125 °C dec; R_f(B) 0.52 [R_f(B) Boc-Gly-Pro-Ala-NH_2 0.77]. Hydrogenolysis of the dibenzyl ester of XI gave XI in 76% yield: R_f(C) 0.4, R_f(B) 0.04 [R_f(B) Gly-Pro-Ala-NH_2 0.52], R_f(elec) 0.31; NMR (D_2O) δ 1.4 (d, 3 H, Ala CH_3), 2.0 (m, 4 H, Pro CH_2 β,γ), 3.68 (m, 4 H, Gly CH_2, Pro CH_2 δ), 4.38 (m, 2 H, Ala CH α, Pro CH α).

N^{α}-(Alkylphosphonyl)glycyl-L-prolyl-L-alanine Dipotassium Salts (XII–XIV). Dibenzyl esters of alkylphosphonic acids were prepared with yields of 75–78% according to previously described methods (Thorsett et al., 1982), starting with dibenzyl phosphite and alkyl bromides. For dibenzyl ethylphosphonate, R_f(E) 0.28 and NMR ($CDCl_3$) δ 0.08–1.9 (m, 5 H, Et), 4.9 (d, 4 H, Ph CH_2), 7.2 (5, 10 H, Ph). For dibenzyl isoamylphosphonate, R_f(E) 0.40 and NMR ($CDCl_3$) δ 0.8 (d, 6 H, isoamyl CH_3 δ), 1.1–1.9 (m, 5 H, isoamyl CH_2 α,β, CH γ), 5.0 (d, 4 H, Ph CH_2), 7.3 (s, 10 H, Ph). For dibenzyl *n*-decylphosphonate, R_f(E) 0.47 and NMR ($CDCl_3$) δ 0.7–1.9 (m, 21 H, *n*-decyl), 4.9 (d, 4 H, Ph CH_2), 7.2 (s, 10 H, Ph). The dibenzyl esters were converted to the monobenzyl phosphochloridates with phosphorus pentachloride by the method of Petrov et al. (1959) and coupled with glycyl-L-prolyl-L-alanine benzyl ester trifluoroacetate salt (mp 165–167 °C) identically as for I in 35–67% yields. Hydrogenolysis of the resulting dibenzyl ester gave the three following products.

N^{α}-(Ethylphosphonyl)glycyl-L-prolyl-L-alanine Dipotassium Salt (XII): 100% yield; R_f(B) 0.30 [R_f(B) Gly-Pro-Ala 0.41]; NMR (D_2O) δ 0.8–1.75 (m, 8 H, PCH_2CH_3, Ala CH_3), 2.05 (m, 4 H, Pro CH_2 β,γ), 3.5 (m, 2 H, Pro CH_2 δ), 3.65 (d, 2 H, Gly CH_2), 4.15 (m, 1 H, Ala CH α).

N^{α}-(Isoamylphosphonyl)glycyl-L-prolyl-L-alanine Dipotassium Salt (XIII): 100% yield; R_f(B) 0.37; NMR (D_2O) δ 0.8 (d, 6 H, isoamyl CH_3), 1.20–1.45 [m, 8 H, isoamyl CH_2 α,β, CH γ, Ala CH_3 (1.25)], 1.95 (m, 4 H, Pro CH_2 β,γ), 3.45 (m, 2 H, Pro CH_2 δ), 3.6 (d, 2 H, Gly CH_2), 4.0 (m, 1 H, Ala CH α), 4.3 (m, 1 H, Pro CH α).

N^{α}-(n-Decylphosphonyl)glycyl-L-prolyl-L-alanine Dipotassium Salt (XIV): 89% yield; R_f(B) 0.4; NMR (D_2O) δ 0.75–1.65 (m, 24 H, *n*-decyl, Ala CH_3), 1.95 (m, 4 H, Pro CH_2 β,γ), 3.45 (m, 2 H, Pro CH_2 δ), 3.6 (d, 2 H, Gly CH_2), 4.0 (m, 1 H, Ala CH α), 4.25 (m, 1 H, Pro CH α).

N^{α}-(Isoamylphosphonyl)glycylglycylproline Dipotassium Salt (XV). XV was prepared identically with XII, starting with glycylglycyl-L-proline benzyl ester trifluoroacetate salt and benzyl isoamylphosphochloridate. Hydrogenolysis of the dibenzyl ester of XV gave XV in 80% yield: R_f(B) 0.32; NMR (D_2O) δ 0.75 (d, 6 H, isoamyl CH_3), 1.2–1.6 (m, 5 H, isoamyl CH_2 α,β, CH γ), 1.95 (m, 4 H, Pro CH_2 β,γ), 3.3–3.68 (m, 4 H, Pro CH_2 δ, Gly CH_2), 3.72–4.4 (m, 3 H, Gly CH_2, Pro CH α).

N^{α}-(tert-Butyloxycarbonyl)-L-leucylglycyl-L-alanine (XVI). XVI was synthesized with 96% yield by hydrogenolysis of the benzyl ester of XV, obtained identically with IV, starting with N^{α}-(*tert*-butyloxycarbonyl)-L-leucine and glycyl-L-prolyl-L-alanine benzyl ester trifluoroacetate salt: mp 103–106 °C; R_f(B) 0.65.

L-Leucylglycyl-L-prolyl-L-alanine Trifluoroacetate Salt (XVII). XVII was prepared identically with V, starting with XVI and trifluoroacetic acid, in 85% yield: mp 120–126 °C; R_f(B) 0.55.

Kinetic Studies. Collagenase activity was determined by the viscometric method (Gallop et al., 1957) under the conditions described by McCroskery et al. (1975), 0.1 M Tris buffer adjusted to pH 7.6 with hydrochloric acid, 0.2 M in sodium chloride and 5 mM in calcium chloride but without 0.02% sodium azide, and at 20 instead of 35 °C, in 1-mL semimicroviscometers from Canon Instrument Co. with a flow

time for distilled water of around 75 s. Collagen solutions were prepared in buffer at 4 °C, and insoluble material was removed by centrifugation according to Gallop et al. (1957). The first viscosity measurement was taken 3 min after addition of the collagenase. Collagenase (63 units in 10 μL of the above buffer) was added to 1 mL of 5 μM collagen (1.76 mg·mL^{-1}) in the viscometers after the specific viscosity of the collagen alone had been determined (~5.4 × 10^{-2} P). The actual time elapsed at this point was calculated as 3 min plus half the time required for the collagen solution to flow between the marks on the viscometer. This flow time varied from about 7.5 (5.4 × 10^{-2} P with no collagenase or 100% inhibition) to 1.5 min (0.20 × 10^{-2} P at nearly complete digestion). Under these conditions, 100 μg of trypsin caused a total specific viscosity change in 15 min of 7% of that caused by complete digestion with collagenase. After 15-min exposure to trypsin, no further significant change in viscosity occurred. This result indicates tryptic cleavage only in the nonhelical end regions of native collagen and implies that the collagen is in its native triple-helical form under the assay conditions (Hodge et al., 1959). Treatment of the collagenase with 10 mM hydrogen peroxide (Mitchell & Harrington, 1968) caused no significant change in the velocity of the reaction, indicating that the collagenase was free of the thiol protease contaminant clostripain.

The inhibitor concentration causing 50% inhibition of collagenase under the above conditions (IC_{50}) was determined from a plot of initial velocity vs. the logarithm of the inhibitor concentration. The initial velocity at complete inhibition (or with no collagenase added) was small but not zero due to very slow denaturation of the collagen in the viscometer. Where 50% inhibition could not be reached even at very high inhibitor concentrations, the IC_{50} is given as being greater than the highest inhibitor concentration used. All IC_{50}'s reported are the averages of two or more separate determinations. K_i was determined from a Dixon plot at several substrate concentrations for one inhibitor (XIII) by converting specific viscosity to collagen concentration with a standard curve and calculating initial velocity in nanomoles of collagen hydrolyzed per minute. K_m was determined similarly from a Lineweaver–Burk plot. Inhibitors were assayed against converting enzyme as previously described (Galardy, 1982) in 50 mM Tris-HCl adjusted to pH 7.5 with sodium hydroxide, 300 mM in sodium chloride, and with hippuryl-L-histidyl-L-leucine as substrate.

Results

The K_m of *Clostridium histolyticum* collagenase for native soluble collagen in 0.1 M Tris buffer adjusted to pH 7.6 with hydrochloric acid, 0.2 M sodium chloride, and 5 mM calcium chloride was found to be 4 ± 2 μM at 20 °C. Although our enzyme was shown not to contain significant clostripain activity, this K_m may represent that of a mixture of collagenases (Lwebuga-Mikasa et al., 1976). *Clostridium* collagenase is known to attack denatured collagen at a much faster rate than native collagen (von Hippel & Harrington, 1959). The presence of small amounts of denatured collagen in our native collagen cannot be ruled out. Therefore, the initial reaction velocities determined for the enzyme could contain a component resulting from the more rapid digestion of denatured collagen. K_m's (and IC_{50}'s) determined from initial velocities (at 3 min of digestion) did not differ significantly from those determined from velocities at later times (10–30 min), where complete digestion did not occur before 200 min.

Figure 2 shows a plot of initial velocity vs. the logarithim of the inhibitor concentration for (isoamylphosphonyl)-glycyl-L-prolyl-L-alanine at a collagen concentration of 5 μM and a Dixon plot for this and two additional collagen concentrations. The K_i from the Dixon plot is 20 μM. This is the only inhibitor for which a K_i was determined. Table I gives IC_{50}'s for collagenase and K_i's for angiotensin converting enzyme for peptides containing amino acid sequences known to bind to collagenase when these sequences are present in collagen and for N^{α}-phosphorylated and N^{α}-phosphonylated peptides. Cysteine is included as a reference compound. Di-, tri-, and tetrapeptides related to collagen do not inhibit collagenase with IC_{50}'s less than 10 mM. However, the dipeptide glycyl-L-proline inhibits angiotensin converting enzyme with an IC_{50} of 450 μM. Phosphonylation of the di- and tripeptides decreases both the IC_{50} for collagenase and the K_i for converting enzyme in every case. Every N^{α}-phosphonylated compound is a better inhibitor of converting enzyme than collagenase. N^{α}-Phosphoryl-L-alanine and N^{α}-phosphoryl-L-

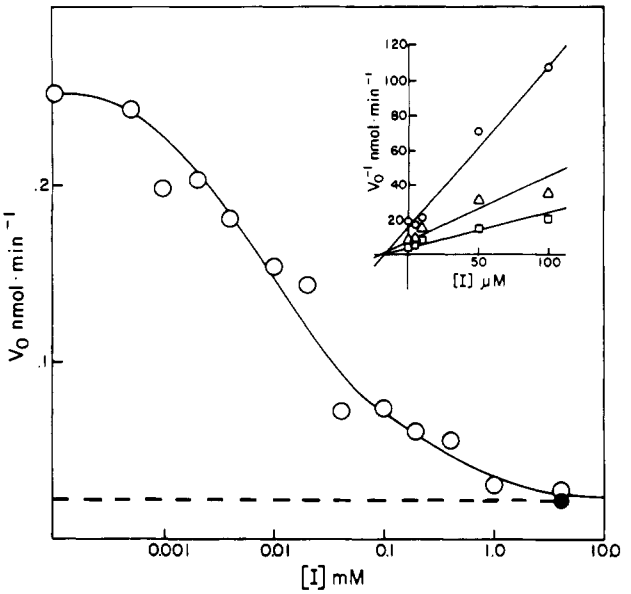

FIGURE 2: Initial velocity vs. logarithm of inhibitor concentration for inhibition of collagenase-catalyzed hydrolysis of 5 μM native collagen by (isoamylphosphonyl)glycyl-L-prolyl-L-alanine. The filled circle is the apparent velocity in the absence of enzyme at the indicated inhibitor concentration. The apparent velocity in the absence of enzyme was not significantly dependent on inhibitor concentration, as shown by the dashed line. The inset shows a Dixon plot of inhibition at 5 (□), 3 (△), and 2 μM (○) collagen concentration.

Table I: Inhibition of Collagenase (IC_{50} ± Standard Deviation) and Angiotensin Converting Enzyme (K_i ± Standard Deviation) by Collagen Substrate Analogues

inhibitor	IC_{50} (μM)	K_i (μM)
Gly-Pro	>20 000	450[a]
Gly-Pro-NH_2	>20 000	
Gly-Pro-Ala	>10 000	>2 000
Gly-Pro-Ala-NH_2	>20 000	
Leu-Gly-Pro-Ala (XVII)	>20 000	3 400 ± 1 700
Boc-Leu-Gly-Pro-Ala (XVI)	>20 000	
PO_3-Ala (II)	>10 000	16 ± 2
PO_3-Leu-Phe (VI)	>20 000	0.07 ± 0.01
PO_3-Gly-Pro (VII)	4 600 ± 1 000	0.05 ± 0.01
PO_3-Gly-Pro-NH_2 (VIII)	3 000 ± 1 000	3.4 ± 0.8
PO_3-Gly-Pro-Ala (IX)	780 ± 170	4.2 ± 0.8
PO_3-Gly-Pro-Ala-NH_2 (XI)	1 500 ± 400	45 ± 9
Et-PO_2-Gly-Pro-Ala (XII)	46 ± 9	4 ± 1
isoamyl-PO_2-Gly-Pro-Ala (XIII)	16 ± 1	11 ± 1
n-decyl-PO_2-Gly-Pro-Ala (XIV)	14 ± 6	0.8 ± 0.4
isoamyl-PO_2-Gly-Gly-Pro (XV)	4 400 ± 500	130 ± 24
Cys	7 200 ± 280	~100[b]

[a] IC_{50}, Cheung et al. (1980). [b] Assayed by the spectrophotometric method of Cushman & Cheung (1971) but in pH 7.5 50 mM Tris-HCl/300 mM sodium chloride. Lineweaver-Burk plots were linear, but Dixon plots were not.

leucyl-L-phenylalanine do not inhibit collagenase as expected from their lack of resemblance to the natural substrate. Substitution of an alkyl chain for one of the phosphorus oxygen atoms in the most potent phosphoramidate, N^{α}-phosphoryl-glycyl-L-prolyl-L-alanine, decreases the IC_{50} by from 1 to 2 orders of magnitude, depending on the alkyl chain. The same substitution increases the K_i for converting enzyme, with the exception of the *n*-decyl substituent that decreases the K_i by about 5-fold. No inhibitor was selective for collagenase compared to converting enzyme. N^{α}-Phosphoryl-L-leucyl-L-phenylalanine was selective for converting enzyme by more than 300 000-fold.

(Isoamylphosphonyl)glycylglycyl-L-proline, in which the tetrahedral phosphorus atom cannot simultaneously occupy the position of the scissile amide carbonyl carbon with the glycine and proline occupying positions S_1' and S_2', is a weak inhibitor, as expected from this misalignment.

Discussion

The K_m found for native soluble collagen and *Clostridium histolyticum* collagenase, 4 μM, is in the same range as the K_m's reported for two vertebrate tissue collagenases for the same substrate [human skin fibroblast collagenase, K_m = 1–2 μM (Welgus et al., 1981); rheumatoid synovial collagenase, K_m = 2.6 μM (Harris & Vater, 1980)] even though the bond cleaving specificities of bacterial and vertebrate collagenases are totally different (Seifter & Harper, 1971). The IC_{50}'s of the collagenase inhibitors in Table I are consistent with the active site model shown in Figure 1. Increasing the number of subsites occupied on the enzyme decreases the IC_{50}. This is illustrated in the series phosphorylglycyl-L-proline (VII), phosphorylglycyl-L-prolyl-L-alanine (IX), and (isoamylphosphonyl)glycyl-L-prolyl-L-alanine (XIII), in which an increasing number of subsites on the enzyme are occupied. The isoamyl chain in XIII presumably mimics the carbon chain of leucine to occupy the S_1 subsite on the enzyme. Alternatively, the aliphatic side chains in XII, XIII, and XIV could be occupying a nonspecific hydrophobic site on the enzyme or merely helping to exclude water from the inhibitor–active site complex since the enzyme does not prefer the isoamyl side chain found naturally in collagen compared to the unnatural *n*-decyl chain. Amidation of the C-terminal of the phosphorylated peptides VII and IX to give VIII and XI does not significantly affect their IC_{50}'s for the endopeptidase collagenase but dramatically increases their K_i's for the exopeptidase angiotensin converting enzyme. The di-, tri-, tetra-, and N^{α}-protected tetrapeptides themselves do not inhibit collagenase. However, the protected tetrapeptide N^{α}-(benzyloxycarbonyl)-L-prolylglycylglycyl-L-proline inhibits the enzyme with a K_i of 3.9 mM (Yagisawa et al., 1965).

A tetrahedral phosphorus atom correctly aligned in the position of the scissile carbonyl carbon is required for measureable inhibition of collagenolysis. The requirement for correct alignment is illustrated in (isoamylphosphonyl)-glycylglycylproline (XV), which incorrectly positions the tetrahedral phosphorus atom if its glycyl-L-proline dipeptide binds to the S_1' and S_2' sites. This misaligned inhibitor is nearly 300-fold less active than correctly aligned XIII. In spite of the specificity demonstrated for correctly aligned collagen-derived sequences, no specificity for collagenase compared to converting enzyme is found for any inhibitor in Table I. The best collagenase inhibitor found (XIII) has a K_i (20 μM) that is higher than the K_m (4 μM) found for the natural substrate. The K_i's of phosphorus-containing converting enzyme inhibitors (in the range of 1 nM; Galardy, 1982) are orders of magnitude lower than the K_m's of the natural substrates (an apparent K_m of 30 μM for angiotensin I and 0.85 μM for bradykinin; Cheung et al., 1980). These results illustrate important differences between collagenase and converting enzyme. Small peptides and their N^{α}-phosphonylated derivatives bind very tightly to converting enzyme, which has small flexible peptide as its natural substrates (angiotensin I and bradykinin), while small peptides and their phosphorylated derivatives do not bind as tightly to collagenase, which has a large ordered protein (collagen) as its specific natural substrate. Collagenase may, therefore, require occupation of many more amino acid residue subsites in order to properly position the tetrahedral phosphorus atom for maximum interaction with its site. Alternatively, collagenase may recognize the local secondary structure of its substrates in addition to a few specific amino acid residues in a small number of binding subsites. Inhibitors possessing the correct secondary structure, presumably the polyproline helix found in the triple helical region of soluble collagen, may bind much more tightly.

Similar considerations seem to apply to vertebrate collagenase, which has a K_m for native collagen of around 1 μM (vide supra). Small polypeptides containing a sulfhydryl function, known to strongly complex with zinc in converting enzyme (Cushman et al., 1977), have IC_{50}'s for vertebrate collagenase of 10 μM or higher (Gray et al., 1981). However, specific naturally occurring vertebrate collagenase inhibitors that are proteins (M_r ~25 000) are known. An inhibitor from human tendon has an IC_{50} of between 20 and 40 nM (Vater et al., 1979). The small peptide inhibitors thus far reported for vertebrate collagenase and *Clostridium* collagenase lack strong interactions with their binding subsites on the enzyme or fail to maximize the interaction between their specific zinc ligands (a thiol or a tetrahedral phosphorus atom, respectively) and the active site zinc atom.

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Registry No. I, 86563-67-9; II, 86594-84-5; VI, 70691-50-8; VII, 86563-68-0; VIII, 86563-69-1; IX, 86563-70-4; X, 86563-71-5; XI, 86563-72-6; XII, 86563-73-7; XIII, 86563-74-8; XIV, 86563-75-9; XV, 86563-76-0; XVI, 86563-77-1; XVII, 86563-79-3; Ala-OCH_2Ph·HCl, 5557-83-5; Phe-OCH_2Ph·HCl, 2462-32-0; Boc-Leu, 13139-15-6; Boc-Leu-Phe-OCH_2Ph, 70637-26-2; Leu-Phe-OCH_2Ph·F_3CCO_2H, 70637-28-4; Boc-Gly, 4530-20-5; Pro-OCH_2Ph·HCl, 16652-71-4; Pro-NH_2, 7531-52-4; Boc-Gly-Pro, 14296-92-5; Ala-NH_2, 7324-05-2; Gly-Gly-Pro-OCH_2Ph·F_3CCO_2H, 86563-81-7; benzyl isoamylphosphochloridate, 86563-82-8; Gly-Pro-Ala-OCH_2Ph·F_3CCO_2H, 86563-84-0; Gly-Pro, 704-15-4; Gly-Pro-Ala, 837-83-2; Cys, 52-90-4; collagenase, 9001-12-1; angiotensin converting enzyme, 9015-82-1.

References

Alstein, M., Blumberg, S., & Vogel, Z. (1982) *Eur. J. Pharmacol. 76*, 299–300.

Anderson, J. C., Barton, M. A., Hardy, P. M., Kenner, G. W., Preston, J., & Sheppard, R. C. (1967) *J. Chem. Soc.*, 108–113.

Atherton, F. R., Howard, H. T., & Todd, A. R. (1948) *J. Chem. Soc.*, 1106–1110.

Balaevskaya, T., Solovieva, N. J., & Orekhovich, V. N. (1981) *Bioorg. Khim. 7*, 1553–1559.

Bandurski, R. S., & Axelrod, B. (1951) *J. Biol. Chem. 193*, 405–410.

Burleigh, M. C. (1977) *Proteinases in Mammalian Cells and Tissues* (Barret, A. J., Ed.) pp 285–209, Elsevier/North-Holland Biomedical Press, New York.

Cheung, H. S., Wang, F. L., Ondetti, M. A., Sabo, E. F., & Cushman, D. W. (1980) *J. Biol. Chem. 255*, 401–407.
Cushman, D. W., & Cheung, H. S. (1971) *Biochem. Pharmacol. 20*, 1637–1648.
Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5484–5491.
Galardy, R. E. (1982) *Biochemistry 21*, 5777–5781.
Galardy, R. E., Kontoyiannidou-Ostrem, V., & Kortylewicz, Z. P. (1983) *Biochemistry 22*, 1990–1995.
Gallop, P. M., & Seifter, S. (1963) *Methods Enzymol. 6*, 635–641.
Gallop, P. M., Seifter, S., & Meilman, E. (1957) *J. Biol. Chem. 227*, 891–906.
Gray, R. D., Sanei, H. H., & Spatola, A. F. (1981) *Biochem. Biophys. Res. Commun. 101*, 1251–1258.
Harris, E. D., & Vater, C. A. (1980) in *Collagenase in Normal and Pathological Connective Tissues* (Wooley, D. E., & Evanson, J. M., Eds.) pp 37–63, Wiley, New York.
Hodge, A. J., Highberger, J. H., Deffner, G. J., & Schmitt, F. O. (1959) *Proc. Natl. Acad. Sci. U.S.A. 46*, 197–206.
Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216–6220.
Jacobsen, N. E., & Bartlett, P. A. (1981) *J. Am. Chem. Soc. 103*, 654–657.
Kam, C. M., Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 3032–3038.
Lwebuga-Mukasa, J. S., Harper, E., & Taylor, P. (1976) *Biochemistry 15*, 4736–4741.
McCroskery, P. A., Richards, J. F., & Harris, E. D. (1975) *Biochem. J. 152*, 131–142.
Mitchell, W. M., & Harrington, W. F. (1968) *J. Biol. Chem. 243*, 4683–4692.
Petrov, K. A., Neimysheva, A. A., & Smirnov, E. V. (1959) *J. Gen. Chem. USSR (Engl. Transl.) 29*, 1465–1467.
Schechter, I., & Berger, A. (1968) *Biochem. Biophys. Res. Commun. 32*, 898–902.
Seifter, S., & Harper, F. (1971) *Enzymes, 3rd Ed. 3*, 649–697.
Thorsett, E. D., Harris, E. E., Peterson, E. R., Greenlee, W. J., Patchett, A. A., Ulm, E. H., & Vassil, T. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2176–2180.
Vater, C. A., Mainardi, C. L., & Harris, E. D. (1979) *J. Biol. Chem. 254*, 3045–3053.
von Hippel, P. H., & Harrington, W. F. (1959) *Biochim. Biophys. Acta 36*, 429–447.
Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114*, 119–132.
Welgus, H. G., Jeffrey, J. J., & Eisen, A. Z. (1981) *J. Biol. Chem. 256*, 9511–9515.
Wolfenden, R. (1969) *Nature (London) 223*, 704–705.
Yagisawa, S., Morita, F., Nagai, Y., Noda, H., & Ogura, Y. (1965) *J. Biochem. (Tokyo) 58*, 407–416.

An Insulin Analogue Possessing Higher in Vitro Biological Activity than Receptor Binding Affinity. [21-Proline-B]insulin†

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ABSTRACT: To study the effect of an increase in the potential for β-turn formation of the B^{20}–B^{23} segment of the B-chain moiety on the biological behavior of insulin, the [21-proline-B]insulin ([Pro^{21}-B]insulin) was synthesized. The in vitro biological activity and the receptor binding affinity of this analogue were compared with that of insulin. In stimulating labeled glucose incorporation into lipids in rat fat cells, the analogue displayed 33.2% potency relative to insulin; receptor binding affinity for the analogue was 15.9% in rat liver membranes and 17.8% in isolated fat cells. [Pro^{21}-B]insulin is thus the first example of a modified insulin for which the biological activity exceeds the receptor binding potency. The secondary structure of this analogue was investigated by circular dichroism studies. Although no significant differences in the conformation of monomeric insulin and analogue could be discerned, their difference in behavior with respect to dimerization and biological properties indicates that these forms are not equivalent. We suggest that the intrinsic activity of receptor-bound [Pro^{21}-B]insulin is greater than that of insulin, although the receptor displays greater affinity for insulin than for the analogue. We consider a model for the interaction between insulin and its receptor that accommodates our findings.

X-ray analysis of the insulin molecule indicates that the B chain segment -Gly-Glu-Arg-Gly-, occupying positions B^{20}–B^{23}, forms a turn within the molecule so that residues B^{24}–B^{30} lie antiparallel and against the B^{9}–B^{19} α-helical segment (Blundell et al., 1972). This arrangement generates interactions which have been postulated to be important in the maintenance of the structure and hence the activity of insulin (Blundell et al., 1972).

By the Chou–Fasman method for prediction of secondary structure (Chou & Fasman, 1978), the sequence B^{20}–B^{23} has a relatively low potential for β-turn formation with $(P_t) = 1.2$ and $p_t = 9.2 \times 10^{-5}$, slightly exeeding the minimum values of these parameters for β-turn prediction [$(P_t) > 1$ and $p_t > 7.5 \times 10^{-5}$]. It was therefore of interest to investigate what effect an increase in the potential for β-turn formation of the B^{20}–B^{23} segment would have on the biological activity of insulin. Replacement of Glu found in the second position of the above-mentioned tetrapeptide sequence by Pro would considerably increase its potential for β-turn formation with $(P_t) = 1.4$ and $p_t = 4.6 \times 10^{-4}$. The present paper describes the synthesis and biological evaluation of [Pro^{21}-B]insulin in which the glutamic acid residue found at the B^{21} position in the

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