The dye procedure is well suited for the rapid determination of enzyme activity in multiple samples. Thus, differences observed between the production of *Bam*HI and *Pal*I (Figures 3 and 4) indicate that this parameter should be routinely evaluated for other enzymes and/or growth conditions.

In summary, since the Cibacron chromatography gave similar results with four crude and four purified restriction nucleases, we believe that it will also be applicable to the purification of other type II enzymes.

Acknowledgments

We thank Drs. R. W. Hartley, P. Leder, and R. T. Simpson for reading the manuscript.

References

Bickle, T. A., Pirotta, V., and Imber, R. (1977), *Nucleic Acid Res. 8,* 2561.
De Filippes, F. M. (1974), *Biochem. Biophys. Res. Commun. 58,* 586.
Dingman, C. W., and Peacock, A. C. (1971), *Proced. Nucleic Acid Res. 2,* 623.
Drocourt, J. L., Thang, D. C., and Thang, M. N. (1978), *Eur. J. Biochem. 82,* 355.
Eshaghpour, H., and Crothers, D. M. (1978), *Nucleic Acids Res. 5,* 13.
Gingeras, T. R., Myers, P. A., Olson, J. A., Hanberg, F. A., and Roberts, R. J. (1978), *J. Mol. Biol. 118,* 113.
Gromkova, R., and Goodgal, S. H. (1972), *J. Bacteriol. 109,* 987.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Roberts, R. J. (1976), *CRC Crit. Rev. Biochem. 7,* 123.
Roberts, R. J., Myers, P. A., Morrison, A., and Murray, K. (1976), *J. Mol. Biol. 102,* 157.
Rushizky, G. W., Mozejko, J. H., Woodford, P. C., and Sober, H. A. (1972), *Anal. Biochem. 46,* 443.
Sharp, P. A., Sugden, B., and Sambrook, J. (1973) *Biochemistry 12,* 3055.
Smith, H. O. (1974), *Methods Mol. Biol. 7,* 71.
Smith, H. O., and Wilcox, K. W. (1970), *J. Mol. Biol. 51,* 379.
Sugden, B., de Troy, B., Roberts, R. J., and Sambrook, J. (1975), *Anal. Biochem. 68,* 36.
Thompson, S. T., Cass, R., and Stellwagen, E. (1975), *Proc. Natl. Acad. Sci. 72,* 669.
Weiss, B., Jaquemin-Sablon, A., Live, T. R., Fareed, G. C., and Richardson, C. C. (1968), *J. Biol. Chem. 243,* 4543.
Wilson, J. E (1976), *Biochem. Biophys. Res. Commun. 72,* 816.

Postproline Cleaving Enzyme: Kinetic Studies of Size and Stereospecificity of Its Active Site†

Roderich Walter* and Tadashi Yoshimoto

ABSTRACT: Postproline cleaving enzyme [EC 3.4.21.–] has recently been purified from lamb kidney and tentatively identified as a serine endopeptidase with a high specificity for proline-containing peptides. The interaction of postproline cleaving enzyme with peptide substrates and competitive inhibitors has been studied in an effort to explore the size and stereospecificity of the active site of the protease. The substrates and inhibitors included proline-containing peptide amides, *p*-nitrophenyl esters, and free acids with increasing numbers of amino acid residues and residues of L and D configuration. Oligopeptides of alanine, which can also be recognized by the protease, were also tested as substrates. This series included Ala_3, Ala-D-Ala-Ala, Ala-Ala-D-Ala, Z-$(Ala)_3$, Ala_4 through Ala_6. The contribution of each of the three amino acid residues flanking the primary specificity site (S_1) of postproline enzyme to such kinetic parameters as K_m, k_{cat}, and k_{cat}/K_m in the case of substrates and K_i with inhibitors was determined. The results suggest that postproline cleaving enzyme has an extended substrate binding region in addition to the primary specificity site, S_1. It seems to be comprised of three sites located at the amino-terminal site (S_1, S_2, and S_3) and two sites at the carboxyl site from the catalytic point (S_1', S_2'). High stereospecificity was observed for subsites S_1, S_2, and S_1'.

Postproline cleaving enzyme [EC 3.4.21.–] was discovered by Walter et al. (1971) in human uterus. The enzyme has recently been purified from lamb kidney (Walter, 1976; Koida & Walter, 1976) and has tentatively been characterized as a serine protease using active site-directed, irreversible inhibitors (Yoshimoto et al., 1977).

It has been shown that the specificity of proteases is determined not only by the two amino acid residues flanking the scissile bond of a peptide substrate, but also by amino acid residues more distant from the point of hydrolysis (Schechter & Berger, 1967; Fruton, 1970; Shaw, 1970; Bauer et al., 1973a,b; Powers, 1977). Thus, it became apparent that the active site of a protease has a more or less extended substrate binding region with subsites for secondary interactions in addition to the primary specificity site, and each of these sites of the enzyme was postulated to interact with one amino acid residue of the peptide substrate (Schechter & Berger, 1967). These concepts have been confirmed by extensive kinetic studies using substrates with systematic structural modifications of the amino acid residues in locations close to and distant from the scissile bond (e.g., Morihara, 1974). Furthermore, X-ray crystallographic investigations of proteases, irreversibly

† From the Department of Physiology and Biophysics, University of Illinois at the Medical Center, Chicago, Illinois 60612. *Received March 1, 1978.* Supported by National Institutes of Health Grant AM-18399.

0006-2960/78/0417-4139$01.00/0

inhibited by active site-directed inhibitors, support and descend to particulars of the structures proposed for enzyme–peptide complexes (Robertus et al., 1972; Tulinsky et al., 1973; Drenth et al., 1976; Powers, 1977).

For postproline cleaving enzyme, a high primary substrate specificity for the -Pro-X- bond of proline-containing peptide substrates has been found (Walter, 1976; Koida & Walter, 1976), although the endopeptidase can also cleave the -Ala-X- bond at a rate of 1/100 to 1/1000 that of the -Pro-X- bond (Yoshimoto et al., 1977). In the present kinetic study, the size and stereospecificity of the active site of postproline cleaving enzyme are investigated with the aid of proline- and alanine-containing peptide substrates and inhibitors.

Materials and Methods

Postproline cleaving enzyme was purified from lamb kidney by ion-exchange and affinity chromatography according to the method of Koida & Walter (1976). Known peptides used were either purchased or resynthesized in this laboratory. For newly synthesized peptides, the information on experimental detail of preparation, physicochemical properties, and analytical data is presented in supplementary material (see paragraph concerning supplementary material at the end of this paper).

Analysis of Peptide Digests by High Voltage Paper Electrophoresis. The formation of products upon digestion of NH_2-terminal protected and free peptides by postproline cleaving enzyme was determined by high voltage paper electrophoresis of the digest mixture on Whatman 3MM paper in a pyridine–acetate buffer, pH 3.5 (5% acetic acid containing 0.5% pyridine), using 46 V/cm for 1 h (Savant Instruments). Authentic standards (peptide and/or amino acid) were subjected to coelectrophoresis. A cadmium–ninhydrin reagent (Fisher & Dörfel, 1953) was used to develop the electrophoretogram. For quantitative determinations, the cadmium–ninhydrin color developed was measured by absorption through a scanning densitometer (Biomedical Instruments). For example, in the case of the digestion of Ala_6, the enzymatic digestion and quantitative product analysis was performed as follows: Ala_6 (1.2 mM) dissolved in 500 μL of 0.05 M sodium phosphate buffer, pH 7.0, was incubated with 100 μL of protease (2.56 units/mL) (Koida & Walter, 1976) at 37 °C for 0.10, 20, 30, 60, 90, 120 min and 4 h. Sixty microliter aliquots of solution were applied on filter paper and subjected to high voltage electrophoresis. Amounts of products were calculated from calibration curves of mono-, di-, tri-, tetra-, penta-, and hexaalanine standards obtained in parallel experiments.

Initial Velocity Measurements for Peptidase and Esterase Activities of Postproline Cleaving Enzyme. Initial rate measurements using substrates in the presence and absence of inhibitors were made by one or more of the following methods.

Peptidase Activities. 1. The rates of hydrolysis of NH_2-terminal protected or free peptides were measured at 37 °C and pH 7.7 by a procedure already described (Walter, 1976). In brief, 50 μL of enzyme was mixed with 200 μL of substrate dissolved in 50 mM sodium phosphate buffer, pH 7.7, containing 1 mM EDTA and dithiothreitol. After incubation at 37 °C for 2 to 15 min, the enzyme reaction was stopped by the addition of 0.5 mL of 2% ninhydrin solution and kept in boiling water for 15 min. After cooling to room temperature, 1.5 mL of 50% methanol was added and the absorbance at 570 nm was determined using a Zeiss spectrophotometer, PQ-3. When an NH_2-terminal protected peptide substrate was tested, the appropriate amino acid or free peptide formed as a result of the action of the protease was quantitatively determined by comparison with absorption plots obtained in parallel experiments using authentic standard. The same approach was taken with NH_2-terminal free substrates. The assumption was made that the NH_2-terminal portion of the product formed exhibits the same degree of ninhydrin color as the starting substrate: since this assumption may not always hold, results obtained with NH_2-terminal free peptide substrates are less reliable by this method than those with NH_2-protected substrates. In order to substantiate the results, the data were therefore also analyzed by the pH-stat procedure (see below) and by quantitative analysis of the absorption in the cadmium–ninhydrin test following high voltage paper electrophoresis (see preceding paragraph).

2. Rates of hydrolyses of peptide amide or NH_2-terminally free peptide substrates were determined by titration according to the method of Thompson & Blout (1973) using a Radiometer titrator TTT-60 and autoburet SUB-11 (Radiometer Copenhagen). In the case of peptide amides enzyme reactions were carried out at 25 °C, and the pH was maintained at 9.0 by the addition of 2×10^{-3} N NaOH under nitrogen.

Esterase Activities. 3. The initial rate of hydrolysis of a peptide *p*-nitrophenyl ester was determined at 25 °C at pH 7.0 using a double-beam Beckman spectrophotometer (Model Acta MVI) following the method of Yoshimoto et al. (1977). Enzyme solution (50 μL) was added to 1 mL of 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT and EDTA. Z-Gly-Pro-ONp dissolved in 50 μL of 1,4-dioxane was added and incubated for various periods of time at 25 °C. The reference contained 50 μL of sodium phosphate buffer in place of enzyme solution.

Steady-State Parameters. K_m and k_{cat} (V/E) were determined from initial velocity measurements determined at various substrate concentrations of peptides and peptide *p*-nitrophenyl esters. Lineweaver–Burk plots were obtained by a least-squares computer program using a PPDP/8L computer, Digital Equipment Corp. Enzyme concentrations were calculated on the basis of a specific activity of 45 units/mg for postproline cleaving enzyme (Koida & Walter, 1976). In the calculations of k_{cat}, the molecular weight of postproline cleaving enzyme monomer was taken as 58 000 (Koida & Walter, 1976). Inhibition constants (K_i values) of peptides were calculated from Dixon plots (1953) using Z-Gly-Pro-Leu-Gly or Z-Gly-Pro-ONp as substrate, respectively.

Results

Effects of Various NH_2-Terminal Protecting Groups of a Given Proline-Containing Peptide on Hydrolysis by or Inhibition of Postproline Cleaving Enzyme. The results in Table I show that different NH_2-terminal protecting groups significantly affect K_m, k_{cat}, and k_{cat}/K_m of a substrate with a given peptide structure.

When the NH_2-protecting groups of the competitive inhibitor Z-Gly-Pro (Yoshimoto et al., 1978) were varied in the same fashion, it was found in the present study that also in this series the Z group[1] was most effective and endowed the dipeptide with the highest inhibitory property among the series tested. The K_i of Z-Gly-Pro was 5.32×10^{-4} M and that of Boc-Gly-Pro was 5.47×10^{-3} M, while all other X-Gly-Pro peptides (X equaled H, Tos, Pht, and For) failed to compete with the substrate Z-Gly-Pro-Leu-Gly (and Z-Gly-Pro-ONp in the case of X = H).

Kinetic Effects of Residues Located NH_2-Terminally from the Scissile Bond of Proline-Containing Peptide p-Nitrophenyl Esters and Amides. Increasing the peptide chain length in going from the *p*-nitrophenyl ester of Z-Pro to Z-Gly-Pro or Z-Ala-Pro, decreased K_m and increased k_{cat}/K_m (Table II). However, Z-Gly-Gly-Pro or Z-Ala-Gly-Pro showed an in-

TABLE I: Effects of NH_2-Terminal Protecting Groups on the Rate of Hydrolysis of a Proline-Containing Peptide by Postproline Cleaving Enzyme.[a]

P_3 P_2 $P_1 \downarrow P_1'$ P_2'	pH	[S] (mM)	[E_0] ($\times 10^{-8}$ M)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
H-Gly-Pro-Leu-Gly[b]	9.0	2.00	2.85	negligible rate of hydrolysis		
Z-Gly-Pro-Leu-Gly	7.8	0.05–1.60	1.25	0.06	52.0	850
Tos-Gly-Pro-Leu-Gly	7.8	0.05–1.60	2.85	0.07	25.0	350
Boc-Gly-Pro-Leu-Gly	7.8	0.10–1.60	1.25	0.38	46.0	120
Pht-Gly-Pro-Leu-Gly	7.8	0.10–1.60	1.25	0.13	21.0	160
For-Gly-Pro-Leu-Gly	7.8	0.10–1.60	2.85	negligible rate of hydrolysis		
Ac-Gly-Pro-Leu-Gly	7.8	0.10–1.60	2.85	negligible rate of hydrolysis		

[a] Enzymatic digest products are determined colorimetrically. [b] Estimated by titration using a pH-stat unit after separation of digest products by high voltage paper electrophoresis; for detail see text.

TABLE II: Kinetic Parameters for Postproline Cleaving Enzyme-Catalyzed Hydrolysis of Peptide Esters and Amides Extended NH_2-Terminally from the Scissile Bond.[a]

P_4 P_3 P_2 $P_1 \downarrow P_1'$	pH	[S] (mM)	[E] ($\times 10^{-8}$ M)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Z—Pro—ONp	7.0	0.065–0.25	0.433	0.14	8.9	62.2
Z-DPro—ONp	7.0	0.065–0.25	0.433	no hydrolysis		
Z—Pro—NH_2[b]	9.0	0.1–2.0	50.0	negligible rate of hydrolysis		
Gly—Pro—NH_2[b]	9.0	0.1–2.0	50.0	negligible rate of hydrolysis		
Z—Gly—Pro—ONp	7.0	0.013–0.105	0.433	0.07	32.7	488
Z—Ala—Pro—ONp	7.0	0.013–0.05	0.17	0.07	61.0	884
Z-DAla—Pro—ONp	7.0	0.013–0.05	1.20	negligible rate of hydrolysis		
Z—Gly—Pro—NH_2[b]	9.0	0.078–0.625	50.0	0.39	1.25	3.24
Ala—Gly—Pro—NH_2[b]	9.0	0.125–0.5	50.0	0.96	3.68	3.82
DAla—Gly—Pro—NH_2[b]	9.0	0.125–0.5	50.0	0.99	0.34	0.34
Z—Gly—Gly—Pro—ONp	7.0	0.063–0.5	0.433	0.27	27.0	98.2
Z—Ala—Gly—Pro—ONp	7.0	0.063–0.5	1.00	0.38	30.0	78.9
Z-DAla—Gly—Pro—ONp	7.0	0.063–0.5	1.00	1.02	38.2	37.5
Z—Ala—Gly—Pro—NH_2[b]	9.0	0.625–5.0	50.0	3.55	1.65	0.46
Z-DAla—Gly—Pro—NH_2[b]	9.0	1.25–7.25	105.0	4.44	0.40	0.09

[a,b] See legends of Table I.

crease of K_m and a decrease of k_{cat}/K_m as compared with the dipeptides. The same phenomenon was observed with the NH_2-terminally protected peptide amide substrates.

When P_1 or P_2 residues were replaced by D amino acids, hydrolysis occurred only at a negligible rate or not at all. However, the replacement of an L residue by the corresponding D isomer in position P_3 of the substrate has only minor effects on the kinetic parameters, as evaluated by a comparison of D,L-Ala-Gly-Pro-NH_2, Z-D,L-Ala-Gly-Pro-ONp, and Z-D,L-Ala-Gly-Pro-NH_2 (Table II). The decrease in k_{cat}/K_m observed with both NH_2-terminally protected and free peptide amides is due to the significant decrease of the k_{cat} value rather than to a change in the K_m value.

Kinetic Effects of Residues Located at the COOH-Terminal Site of the Scissile Bond of Proline-Containing Peptides. The chain length of the substrate was increased at the carboxyl terminal site while keeping the Z-Gly-Pro (P_3, P_2, P_1) sequence constant (Table III). When Ala or Leu was added (P_1') essentially the same K_m and k_{cat}/K_m were found. Further elongation of Z-Gly-Pro-Leu by Gly or Ala (P_2') resulted in a decrease of K_m and substantial increase of k_{cat}/K_m. However, further elongation of Z-Gly-Pro-Leu-Gly with Gly, Ala, or Pro (P_3') resulted in a decrease of k_{cat}/K_m.

Kinetic Effects of Chain Length and Configuration of Peptide Inhibitors. As shown in Table IV, K_i decreased in going from Z-Pro to Z-Gly-Pro or Z-Ala-Pro. Additional peptide chain elongation in the NH_2-terminal direction of P_1 in comparing Z-Gly-Pro to Z-Gly-Gly-Pro or Z-Ala-Gly-Pro resulted in increased values of K_i. Replacement of Ala in location P_2 by its D isomer resulted in a 100-fold increase in K_i, while the same substitution in P_3 was without effect.

When Z-Pro was extended at the carboxyl end to Z-Pro-Leu in order to include P_1', there was no significant decrease in K_i; further chain elongation, however, to include P_2' resulted in a decrease of K_i as the study of Z-Pro-Leu-Gly revealed. The protected tetrapeptide, Z-Pro-Leu-Gly-Gly, was practically void of inhibitory properties.

Rate of Hydrolysis of Oligoalanine Peptides by Postproline Cleaving Enzyme. Table V summarizes apparent rates of hydrolysis of oligoalanines by postproline cleaving enzyme. Figure 1 shows the time course of hydrolysis of Ala_6 by postproline cleaving enzyme. In early time periods of incubation

[1] Abbreviations used follow the tentative Rules and Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature for amino acids and peptides ((1972) *J. Biol. Chem. 247*, 977). All optically active amino acids are of L configuration unless otherwise stated. Additional abbreviations used are: Z, benzyloxycarbonyl; Ac, acetyl; Pht, phthalyl; For, formyl; ONp, nitrophenyl ester. In this paper, the nomenclature introduced by Schechter & Berger (1967) is used to facilitate discussion of the interactions between postproline cleaving enzyme and peptide substrates and inhibitors. Amino acid residues (and NH_2-protecting groups of these peptides) are designated P_1, P_2, P_3, etc., numbering from the primary specificity site (in the case of substrates the amino acid that supplies the carbonyl group of the cleaved bond) and continuing to number in the direction of the amino terminal end of the peptide; the locations of amino acid residues in the COOH-terminal direction from the scissile bond of the peptides are identified by P_1', P_2', P_3', etc. The complementary subsites of the enzyme's active site are referred to as S_1, S_2, S_3 and S_1', S_2', S_3', etc.

TABLE III: Kinetic Parameters for Postproline Cleaving Enzyme-Catalyzed Hydrolysis of Peptides Extended COOH-Terminally from the Scissile Bond.[a]

P_3 P_2 $P_1 \downarrow P_1'$ P_2' P_3'	pH	[S] (mM)	[E] ($\times 10^{-8}$ M)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Z—Gly—Pro—NH_2[b]	9.0	0.078–0.63	50.0	0.39	1.25	3.24
Z—Gly—Pro—Ala	7.8	0.05–0.80	1.25	0.08	15.6	188
Z—Gly—Pro-DAla	7.8	0.05–0.80	1.25	0.25	1.52	6.1
Z—Gly—Pro—Phe	7.8	0.025–0.80	1.7	0.16	3.2	19.8
Z—Gly—Pro—Leu	7.8	0.10–1.60	1.25	0.17	26.0	150
Z—Gly—Pro-DLeu	7.8	0.10–0.40	6.95	0.32	0.67	2.1
Z—Gly—Pro—Leu—Gly	7.8	0.05–1.60	1.25	0.06	52.0	852
Z—Gly—Pro—Leu—Ala	7.8	0.10–0.8	1.25	0.12	38.4	334
Z—Gly—Pro—Leu-DAla	7.8	0.10–1.60	1.25	0.37	46.0	124
Z—Gly—Pro—Leu—Gly—Gly	7.8	0.05–1.60	1.25	0.13	28.0	214
Z—Gly—Pro—Leu—Gly—Ala	7.8	0.10–1.60	1.25	0.18	34.0	187
Z—Gly—Pro—Leu—Gly-DAla	7.8	0.10–1.60	1.25	0.14	25.0	181
Z—Gly—Pro—Leu—Gly—Pro	7.8	0.025–0.40	1.5	0.68	53.3	78.4

[a,b] See legends in Table II.

TABLE IV: Kinetic Parameters for the Inhibition of Postproline Cleaving Enzyme by Peptides[a]

inhibitor P_4 P_3 P_2 P_1 P_1' P_2' P_3'	[I] (mM)	K_i (M)
Z—Pro	0.62–5.0	3.8×10^{-3}
Z—Gly—Pro[b]	0.12–2.5	5.3×10^{-4}
Z—Ala—Pro	0.008–0.06	5.1×10^{-6}
Z-DAla—Pro	0.46–2.1	2.2×10^{-4}
Z—Gly—Gly—Pro	0.25–5.0	1.2×10^{-3}
Z—Ala—Gly—Pro	0.63–5.0	1.6×10^{-2}
Z-DAla—Gly—Pro	0.63–10.0	1.5×10^{-2}
Z—Pro—Leu[b]	0.12–0.22	1.2×10^{-3}
Z—Pro—Leu—Gly	0.12–0.22	4.7×10^{-4}
Z—Pro—Leu—Gly—Gly	0.10–5.0	$>10^{-2}$

[a] [S] = Z-Gly-Pro-Leu-Gly, 0.5, 1, and 2 mM, pH 7.0; [E] = 1.2×10^{-6} M. [b] Reported by Yoshimoto et al. (1978).

TABLE V: Apparent Rate of Hydrolysis of Oligoalanine Peptides by Postproline Cleaving Enzyme.

peptide	rate of hydrolysis (μmol/(min·mg))
Ala—Ala—Ala[a]	0.01
Ala—Ala-DAla	negligible rate
Ala-DAla—Ala	no hydrolysis
Z—Ala—Ala—Ala[a]	0.33
Ala—Ala—Ala—Ala[a]	0.39
Ala—Ala—Ala—Ala—Ala[a]	1.19
Ala—Ala—Ala—Ala—Ala—Ala	1.03

[a] Site of cleavage was reported by Yoshimoto et al. (1978).

(0 to 45 min), Ala_2, Ala_3, and Ala_4 were formed from Ala_6 in ratios of about 1:2:1. After 1 h, Ala_4 levels decreased rapidly and Ala_3 levels slowly. As a result, Ala_2 and Ala concentrations increased.

Discussion

The present study concerns the identification of enzyme–substrate interactions remote from the point of hydrolysis of the substrate by postproline cleaving enzyme. Since postproline cleaving enzyme appears to be a serine protease (Yoshimoto et al., 1977), the three-step mechanism of acid amide and ester hydrolyses, well established first for the serine protease α-chymotrypsin (Bender & Kézdy, 1964; Brot & Bender, 1969),

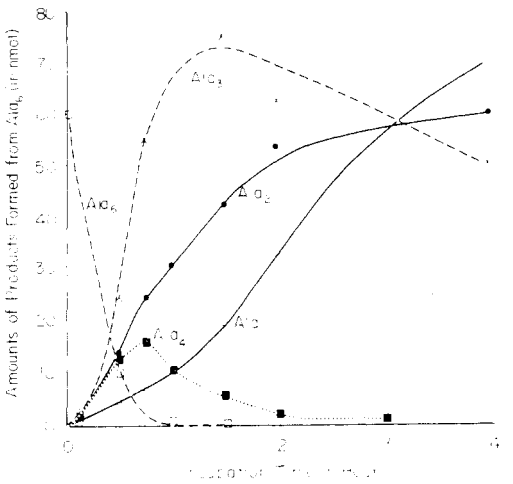


FIGURE 1: Formation of hydrolysis products from hexaalanine catalyzed by postproline cleaving enzyme as a function of time. Ala_6, 1.2 mM, in 50 μL of 50 mM sodium phosphate buffer, pH 7.8, and 100 μL of protease E, 56.8 μg, was incubated at 37 °C for 0–4 h. Sixty-microliter aliquots of digest mixture were withdrawn at the time periods indicated and spotted on filter paper, Whatman 3MM. Following high voltage paper electrophoresis at pH 3.5 in a pyridine–acetate buffer using 46 V/cm for 4 h, the chromatogram was developed with cadmium–ninhydrin spray and the color spots were quantitatively measured by a scanning densitometer as detailed in the text.

is assumed to also apply to postproline cleaving enzyme according to the formula (Bender & Kézdy, 1965)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{P_1}{\overset{k_2}{\rightarrow}} EA \xrightarrow{k_3} E + P_2 \qquad (1)$$

K_m and k_{cat} relate the individual rate constants of eq 1 as follows:

$$K_m = K_s \frac{k_3}{k_2 + k_3} \qquad (2)$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \qquad (3)$$

From eq 2 and 3 follows $k_{cat}/K_m = k_2/K_s$, where the parameter k_{cat}/K_m represents the overall reactivity, and it has been proposed that this kinetic parameter most accurately reflects the specificity of substrates to hydrolases. In the case of acid amide hydrolysis, acylation is the rate-limiting step (Bender et al., 1964); i.e., $k_2 \ll k_3$, which reduces eq 2 and 3 to $k_{cat} = k_2 = K_m = K_s$, respectively.

The effectiveness of postproline cleaving enzyme to catalyze the hydrolysis of X-Gly-Pro-Leu-Gly was studied, where X represents various NH_2-protecting groups (Z, Tos, Boc, Pht, For, and Ac). Z-Gly-Pro-Leu-Gly exhibited the lowest K_m and highest velocity value (k_{cat}) and proteolytic coefficient (k_{cat}/K_m) (Table I). Also, in a similar series of competitive dipeptide inhibitors, protection of the primary amino group with the Z group yielded Z-Gly-Pro with the lowest K_i. Kinetic data listed in Table I suggest that the Z-protecting group acts in an amino acid residue-like manner in position P_3 of the tetrapeptide and interacts with the S_3 subsite of postproline cleaving enzyme (a comparison of Z-Gly-Pro-NH_2 and Ala-Gly-Pro-NH_2 as well as Z-Ala_3 and Ala_4 leads to similar conclusions). Contributions of NH_2-protecting groups on kinetic parameters of protease substrates and inhibitors have been studied extensively by Morihara (1974).

Effects resulting from an increase of the number of residues of the substrate in the NH_2-terminal direction away from the scissile bond were investigated next using peptide nitrophenyl esters and carboxamides (Table II). Comparison of the data for ester or amide of Z-Pro with those of Z-Gly-Pro or Z-Ala-Pro suggests a strong interaction in the substrate–enzyme complex at the level of P_3–S_3 in addition to P_1–S_1 and P_2–S_2. Further elongation of the substrate ester and amide in order to test for possible interactions between P_4–S_4 results in a significant increase of K_m and a decrease of k_{cat}/K_m. The similarity of the values for k_{cat}/K_m of Z-Gly-Pro-NH_2 and Ala-Gly-Pro-NH_2 (Table II) lends further evidence to the above contention that the Z group of the protected dipeptide acts like the NH_2-terminal Ala residue of the tripeptide.

When the stereospecificities of subsites S_1, S_2, and S_3 of postproline cleaving enzyme are studied using substrates with amino acid substitutions of L and D configuration, it is found that the P_1–S_1 and P_2–S_2 interactions exhibit a high degree of stereospecificity, while rather low stereospecific requirements seem to exist for the P_3–S_3 interaction of the substrate–enzyme complex (Table II). Since the kinetic parameters of Z-Ala-Pro-ONp were unaffected in the presence of increasing amounts of Z-D-Ala-Pro-ONp, it seems that the substitution of the L-Ala by the D isomer results in a substrate with a low affinity for the protease. Studies with proline-containing competitive inhibitors of postproline cleaving enzyme, which are summarized in Table IV, lead to similar conclusions in terms of the relative importance of the various subsites for the formation of the inhibitor–enzyme complex. In conclusion, the kinetic analysis of substrates and competitive inhibitors with increasing chain length in the NH_2-terminal direction from residue P_1 reveals that binding of the peptides to postproline cleaving enzymes involves interactions between P_1–S_1, P_2–S_2, and P_3–S_3; from a stereochemical viewpoint interactions at the level of P_1–S_1 and P_2–S_2 appear most important.

It is now well established, however, that the specificity of a protease is determined by the nature of amino acid residues at both sides of the peptide bond subject to hydrolysis. The effects on K_m, k_{cat}, and k_{cat}/K_m of proline-containing substrates with structural variations of amino acid residues located at the carboxyl-terminal site of the scissile bond (at positions P_1', P_2', and P_3'), but with a constant sequence of Z-Gly-Pro (at positions P_1, P_2, and P_3), were evaluated (Table III). Hydrolysis of Z-Gly-Pro-Ala and Z-Gly-Pro-Leu was efficient, and almost identical kinetic values for k_{cat}/K_m were obtained, while the hydrolysis of Z-Gly-Pro-NH_2 or Z-Gly-Pro-Phe was poor. Elongation of the peptide chain to include P_2', i.e., Z-Gly-Pro-Leu-Gly and Z-Gly-Pro-Leu-Ala, further decreased K_m and increased k_{cat}/K_m values. However, addition of residues in location P_3' caused a decrease in the k_{cat}/K_m value.

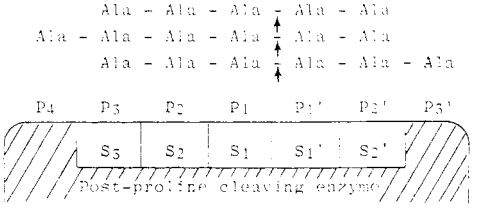
The effect of the configuration of residues located in P_1', P_2', and P_3' was also tested (Table III). The replacement of an L residue by the D isomer in P_1' had a dramatic effect on both K_m and k_{cat}. While such a replacement in location P_2' also had a significant effect on both parameters, there was practically no change in the apparent k_{cat}/K_m upon the replacement of Ala in P_3' by its optical antipode. These conclusions, reached from the study of postproline cleaving enzyme substrates, are also supported by preliminary results obtained with competitive inhibitors which possess peptide chains stepwise elongated at the carboxyl terminus of Z-Pro (Table IV). In summary, the important interactions in the substrate- and inhibitor–enzyme complexes at the carboxyl site of the substrate cleavage point seem to involve P_1'–S_1' and P_2'–S_2' contacts.

It has been demonstrated previously that postproline cleaving enzyme fails to catalyze the hydrolysis of oligoproline peptides (Koida & Walter, 1976). While the protease degrades oligopeptides of alanine, the rate of hydrolysis is too slow to permit, in general, the determination of K_m, k_{cat}, and k_{cat}/K_m (Yoshimoto et al., 1978). However, it is feasible to measure the relative rates of hydrolysis of oligoalanines, which have been used in this investigation to give further insight into the mechanism of interaction between substrate and postproline cleaving enzyme (Table V). The hydrolysis of Ala_3 as compared with the negligible or lack of hydrolysis of Ala-Ala-D-Ala or Ala-D-Ala-Ala seems to be a further indication of the high stereospecificity of the interactions of the P_1–S_1 and P_1'–S_1' residues. As expected from results described above, the conversion of Ala_3 to Z-$(Ala)_3$ results in a 30-fold increase in its initial rate of hydrolysis, which is practically identical with that of Ala_4 (Table V). An additional and significant increase in the initial rate of hydrolysis is observed with Ala_5 as compared with Ala_4, while there is no further enhancement of the relative rate of hydrolysis in going from Ala_5 to Ala_6 (Table V). When the postproline cleaving enzyme-catalyzed hydrolysis of Ala_6 was followed over a 4-h period (Figure 1), it was found that the initial hydrolysis products Ala_2, Ala_3, and Ala_4 were formed at an approximate molar ratio of 1:2:1. These data are interpreted to indicate that the Ala^3-Ala^4 and Ala^4-Ala^5 peptide bonds of Ala_6 are hydrolyzed at similar initial velocities.

In summary, the results presented in this study using proline- and alanine-containing substrates, and proline-containing competitive inhibitors as well as results from an earlier study using labeled pentaalanine ([^{14}C]Ala-Ala_4; Yoshimoto et al., 1978), suggest that postproline cleaving enzyme has five subsites. Among the alanine oligopeptides studied, Ala_5 is the substrate with the highest initial rate of velocity of hydrolysis and for all practical purposes only one peptide bond is cleaved in Ala_5 (i.e., the peptide bond between residues Ala^3 and Ala^4). The fact that there is no further increase in the relative rate of hydrolysis in comparing results with Ala_5 to those of Ala_6 and that Ala_6 is cleaved at similar rates at two sites can be explained by assuming that Ala_6 forms the two types of substrate–enzyme complexes depicted in Diagram I.

This interpretation is also supported by the finding that extension of the peptide chain of substrates and inhibitors to

DIAGRAM I



include P_4–S_4 and P_3'–S_3' interactions failed to enhance, and in some instances actually decreased, favorable peptide–enzyme interaction. Moreover, the results obtained with postproline cleaving enzyme are reasonable in light of the active sites found for several other peptidases. Seven subsites (S_4, S_3, S_2, S_1, S_1', S_2', S_3') were reported for papain by Schechter & Berger (1967), which were confirmed by X-ray crystallography by Drenth et al. (1976). Six subsites (S_4, S_3, S_2, S_1, S_1', S_2') were demonstrated for elastase (EC 3.4.4.7) by Atlas et al. (1970) and Thompson & Blout (1970) and for subtilisin BPN' by Morihara et al. (1970); these were found in the crystalline state (Robertus et al., 1972). Six subsites, but involving a different pattern (S_3, S_2, S_1, S_1', S_2', S_3'), were found for pepsin by Tang (1963), Oka & Morihara (1970), Medzihradszky et al. (1970), and Sachdev & Fruton (1970), five subsites (S_2, S_1, S_1', S_2', S_3') for *Bacillus subtilis* neutral protease by Morihara & Oka (1968)) and five subsites (S_4, S_3, S_2, S_1, S_1') for carboxypeptidase by Abramowitz et al. (1967). Postproline cleaving enzyme also appears to have five subsites for substrate and inhibitor interactions, and these seem to be comprised of S_3, S_2, S_1, S_1', and S_2'.

Acknowledgments

The authors are grateful to Drs. S. Hase Nakagawa and R. C. Orlowski for the preparation of the peptides used in this study, to Ms. M. Fischl for enthusiastic assistance in the kinetic studies, to Mr. C. Botos for reliably performing the amino acid analyses, and to Mr. W. Simmons for his valuable critique.

Supplementary Material Available

Information for newly synthesized peptides on experimental details of preparation, physicochemical properties, and analytical data (7 pages). Ordering information is available on any current masthead page.

References

Abramowitz, N., Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 29*, 862.

Anderson, G. W., Zimmerman, J. E., & Callahan, F. M. (1964) *J. Am. Chem. Soc. 86*, 1839.

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

Atlas, D., Levit, S., Schechter, I., & Berger, A. (1970) *FEBS Lett. 11*, 281.

Bauer, C.-A., Thompson, R. C., & Blout, E. R. (1973a) *Biochemistry 15*, 1291.

Bauer, C.-A., Thompson, R. C., & Blout, E. R. (1973b) *Biochemistry 15*, 1296.

Beecham, A. F. (1957) *J. Am. Chem. Soc. 79*, 3257.

Bender, M. L., & Kézdy, F. J. (1964) *J. Am. Chem. Soc. 86*, 3704.

Bender, M. L., & Kézdy, F. J. (1965) *Annu. Rev. Biochem. 34*, 49.

Bender, M. L., Clement, G. E., Gunter, C. R., & Kézdy, F. J. (1964) *J. Am. Chem. Soc. 86*, 3697.

Bodanszky, M., Sheehan, J. T., Ondetti, M. A., & Lande, S. (1963) *J. Am. Chem. Soc. 85*, 991.

Brot, F. E., & Bender, M. L. (1969) *J. Am. Chem. Soc. 91*, 7187.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Drenth, J., Kalk, K. H., & Swen, H. M. (1976) *Biochemistry 15*, 3731.

Fisher, F. G., & Dörfel, H. (1953) *Biochem. Z. 324*, 544.

Fruton, J. S. (1970) *Adv. Enzymol. 33*, 401.

Goodman, M., & Steuben, K. C. (1959) *J. Am. Chem. Soc. 81*, 3980.

Koida, M., & Walter, R. (1976) *J. Biol. Chem. 251*, 7593.

Kolac, J., Zaoral, M., & Sorm, F. (1967) *Collect. Czech. Chem. Commun. 32*, 2667.

Medzihradszky, K., Voyniek, I. M., Medzihradszky-Schweiger, I. M., & Fruton, J. S. (1970) *Biochemistry 9*, 1154.

Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819.

Morihara, K. (1974) *Adv. Enzymol. 41*, 179.

Morihara, K., & Oka, T. (1968) *Biochem. Biophys. Res. Commun. 30*, 625.

Morihara, K., Oka, T., & Tsuzuki, H. (1970) *Arch. Biochem. Biophys. 138*, 515.

Oka, T., & Morihara, K. (1970) *FEBS Lett. 10*, 222.

Powers, J. C. (1977) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins* (Weinstein, B., Ed.) pp 65–178, Marcel Dekker, New York, N.Y.

Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, S., Powers, J. C., & Wilcox, P. E. (1972) *Biochemistry 11*, 2439.

Sachdev, G. P., & Fruton, J. A. (1970) *Biochemistry 9*, 4465.

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

Shaw, E. (1970) *Physiol. Rev. 50*, 244.

Sheehan, J. T. (1965) U.S. Patent 3,196,144 (Olin Matheson Chem. Corp.), July 20.

Tang, J. (1963) *Nature (London) 199*, 1094.

Thompson, R. C., & Blout, E. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1734.

Thompson, R. C., & Blout, E. R. (1973) *Biochemistry 12*, 51.

Tulinsky, A., Vandlen, R. L., Morimoto, C. N., Mani, N. V., & Wright, L. H. (1973) *Biochemistry 12*, 4185.

Walter, R. (1976) *Biochim. Biophys. Acta 422*, 138.

Walter, R., Shlank, H., Glass, J. D., Schwartz, I. L., & Kerenyi, T. D. (1971) *Science 173*, 827.

Weygand, F., & Kaelicke, J. (1962) *Chem. Ber. 95*, 1031.

Wieland, T., Lapatsanis, L., Faisal, J., & Kanz, W. (1971) *Justus Liebigs Ann. Chem. 747*, 194.

Wünsch, E., & Deimer, K. H. (1972) *Z. Physiol. Chem. 353*, 1246.

Wünsch, E., Jaeger, E., & Schonsteiner-Altmann, G. (1971) *Hoppe-Seyle's Z. Physiol. Chem. 352*, 1568.

Yoshimoto, T., & Walter, R. (1977) *Biochim. Biophys. Acta 485*, 391.

Yoshimoto, T., Orlowski, R. C., & Walter, R. (1977) *Biochemistry 16*, 2942.

Yoshimoto, T., Fischl, M., Orlowski, R. C., & Walter, R. (1978) *J. Biol. Chem. 253*, 3708.

Zahn, H., & Rexroth, E. (1955) *Z. Anal. Chem. 148*, 181.