Inhibition of Thermolysin by Dipeptides

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ABSTRACT: The inhibition of the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 by dipeptides was studied. Competitive inhibition was observed with dipeptides containing a hydrophobic amino acid such as leucine or phenylalanine and with Leu-NH_2, L-Phe, and D-Phe. The position in the dipeptide of the leucine or phenylalanine was not critical for inhibition unlike the substrate specificity requirement. A free amino or carboxyl group diminished the inhibition and it was suggested that the Arg-203 in the active site was responsible for the charge effects observed. The temperature dependence of K_I was determined for a few inhibitors. Linear Arrhenius plots were obtained through a temperature range of over 50° indicative of a stable conformation. Comparison of the ΔS and ΔH values for binding Z-Phe-Gly-NH_2, Z-Leu-Gly-NH_2, and Z-Phe-Gly yielded progressively less negative entropies and smaller enthalpies as the K_I values decreased. Displacement of solvent molecules from the active site by inhibitor binding was suggested as an explanation of these results. The pH dependence of inhibition by Z-Phe-Gly-NH_2 indicated a dependence for binding upon a group or groups with pK in the region of 7–8.

Thermolysin is the thermostable metallo endopeptidase produced by *Bacillus thermoproteolyticus*. Characteristic of the class of microbial neutral proteases, thermolysin exhibits a specificity toward peptide bonds in which the amino group was contributed by amino acids having hydrophobic side chains such as phenylalanine and leucine (Matsubara *et al.*, 1965; Matsubara, 1966a,b; Morihara, 1967; Morihara and Oka, 1968; Morihara *et al.*, 1968; Feder and Schuck, 1970). Morihara and Tsuzuki (1970) also have demonstrated that five additional amino acid subsites surrounding this position influence the specificity. Although specificity information is generally deduced from substrate activity studies, examination of inhibition by model peptides offers complementary information on the nature of the binding site of the enzyme.

Experimental Section

Materials. Crystalline thermolysin (*B. thermoproteolyticus* Rokko) was obtained from Daiwa Kasei Co., Ltd., Osaka, and used without further purification. All peptides were available from commercial sources. Gly-Phe, Gly-D-Leu, D-Leu-Gly, Leu-Gly, Phe-Gly-NH_2, Z-Phe-Gly-NH_2,[1] Z-Leu-Gly-NH_2, Phe-Gly, Z-Tyr-Gly-NH_2, Bz-Gly-Phe, Z-Gly-Leu, Z-Gly-Phe, Phe-Phe, and Gly-Pro were purchased from Mann Research Laboratories.

Gly-D-Phe, Z-Gly-Arg, Gly-Tyr-NH_2·Leu-Gly-Gly-Gly-Ala, Gly-Val, Z-Leu-Gly, Leu-Gly-NH_2, Z-Phe-Gly-Leu-NH_2, L-Phe, D-Phe, Z-Gly, Z-Ala-Gly, Gly-NH_2, Ac-Gly, Gly-Gly, Bz-Gly-Lys, Leu-Leu, Phe-Phe, Z-Gly-Trp, and Z-Try-Gly were purchased from Cyclo Research Corp.

Furylacryloyl-Gly-Leu-NH_2 was synthesized as described (Feder and Schuck, 1970).

Only reagent grade salts and deionized water were used throughout these studies.

Methods. The enzyme-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 was monitored spectrophotometrically at 345 mμ on a Cary 14 PM recording spectrophotometer equipped with a thermostated cell compartment maintained at ±0.1° of the designated temperature (Feder, 1968; Feder and Schuck, 1970).

The enzyme molarity was determined from the pseudo-first-order rate constant for the catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 ($k = k_{cat}(E)/K_m$) divided by k_{cat}/K_m. Values of 9.46×10^3 and 19.11×10^3 M^{-1} sec^{-1} for k_{cat}/K_m were used for reactions in pH 7.2 Tris buffer (0.1 M) and pH 7.2 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (0.1 M), respectively.

The furylacryloyl-Gly-Leu-NH_2 was prepared in pH 7.2 Hepes buffer (0.1 M) at a concentration of 9.60×10^{-4} M for all the inhibition studies. At this concentration, the condition of $S \ll K_m$ is established and good pseudo-first-order kinetics were obtained. All inhibition studies were carried out at 25.0 ± 0.1° unless otherwise indicated.

Results

The inhibition of the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 by simple dipeptides was examined under pseudo-first-order conditions. When $S \ll K_m$ competitive inhibition is described by the rate equation

$$dp/dt = k_{cat}(E)(S)/K_m(1 + (I)/K)$$

Under these conditions the complete reactions yield a first-order rate constant $k = k_{cat}(E)/K_m(1 + (I)/K_I)$. The ratio of the first-order rate constants in the presence and absence of inhibitor, π, is equal to $(1 + (I)/K_I)$ from which K_I can be

$$\pi = k_0/k_1 = 1 + (I)/K_I$$

determined. A plot of π *vs.* the inhibitor concentration would be linear and the reciprocal of the slope would give the K_I.

Figure 1 shows the inhibition of the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 by Z-Tyr-Gly, Z-Gly-Trp, Bz-Gly-Phe, and Z-Gly-Tyr. This offers a simple accurate method for the determination of K_I.

Under pseudo-first-order conditions, one cannot distinguish competitive from noncompetitive inhibitors because both yield the same rate expression. However, only the competitive

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[1] Abbreviations used are: Z, benzyloxycarbonyl; Bz, benzoyl.

TABLE I: Effect of a Carboxyl or Amino Group on the Inhibition of Thermolysin by Dipeptides.[a]

Inhibitor	Inhibitor × 10^3 (M)	$K_I \times 10^3$ (M)
Z-Phe-Gly-NH_2	0.32–3.18	0.35
Z-Phe-Gly	2.72–27.2	4.50
Phe-Gly-NH_2	11.2–56.1	10.90
Phe-Gly	2.01–20.1	10.30
Z-Leu-Gly-NH_2	0.95–9.55	3.07
Z-Leu-Gly	2.25–45.2	4.03
Leu-Gly-NH_2	8.7–43.4	8.3[c]
Leu-Gly	3.65–21.90	23.5[c]
Leu-Gly-Gly	3.36–33.60	27.4
D-Leu-Gly	20.2–67.5	108.0[c]
Z-Gly-Phe-NH_2		K_m = 14.7 × 10^{-3} M[b]
Z-Gly-Phe	2.45–24.50	14.7
Bz-Gly-Phe	2.11–21.12	38.5
Gly-Phe-NH_2	3.45–17.20	53.0[c]
Gly-Phe	13.2–87.4	141.0
Gly-D-Phe	13.2–65.4	16.0
Z-Gly-Leu-NH_2		K_m = 20.6 × 10^{-3} M[b]
Z-Gly-Leu	1.65–16.48	5.65
Gly-Leu-NH_2	4.71–47.07	11.60
Gly-Leu	9.51–95.10	47.5
Gly-D-Leu	77.4	No inhibition
Z-Tyr-Gly-NH_2	0.16–0.95	0.22 (K_m = 20.8 × 10^{-3} M)[b]
Z-Tyr-Gly	2.60–12.90	6.90
Z-Gly-Tyr-NH_2		K_m = 14.7 × 10^{-3} M[b]
Z-Gly-Tyr	5.20–26.1	55.0
Gly-Tyr-NH_2	6.02–30.1	78.9

[a] All reactions carried out in pH 7.2 Hepes buffer (0.1 M), 25.0 ± 0.1°. (E_0) = 1.16 × 10^{-6} M; (S_0) = 9.76 × 10^{-4} M; additional details in text. [b] From Morihara and Tsuzuki (1970). [c] Inhibition plots deviate from theoretical linearity.

component of the noncompetitive inhibition is observed and, therefore, competitive inhibition constants are obtained.

Table I shows that the inhibition of thermolysin by dipeptides is greatest when both the carboxyl and amino groups are blocked and least when both are free. The K_I for Phe-Gly was 29-fold greater than that for Z-Phe-Gly-NH_2 while the K_I for Z-Phe-Gly was about 13-fold greater than that for the fully blocked dipeptide. Likewise, the K_I values for Leu-Gly, Leu-Gly-NH_2, and Z-Leu-Gly were about eight-, three-, and 1.3-fold greater, respectively, than Z-Leu-Gly-NH_2.

The substrate specificity for dipeptides requires that the phenylalanine or leucine be at the carboxyl end of the molecule. This restriction was tested with respect to inhibition Table II shows the effect on the K_I of the position of the hydrophobic amino acid in the dipeptide. In all cases better binding of the dipeptide was observed when the phenylalanine, leucine, tyrosine, and tryptophan were at the amino end of the molecule. This is just the reverse of what is required for catalysis. Thus ratios of K_I values for Z-Gly-X/Z-X-Gly of 3.3, 8, 3.9, 13.7, 2.0, and 5.0 were obtained for Z-Phe-Phe/Z-Phe-Gly, Z-Gly-Tyr/Z-Tyr-Gly, Z-Gly-Trp/Z-Trp-Gly, Gly-Phe/Phe-Gly, Gly-Leu/Leu-Gly, and Z-Gly-Ala/Z-Ala-Gly, respectively.

The presence of two hydrophobic amino acids such as Leu-Leu (K_I = 8.4 × 10^{-4} M) and Phe-Phe (K_I = 2.13 × 10^{-3} M) further enhanced the inhibition (Table III). No inhibition was

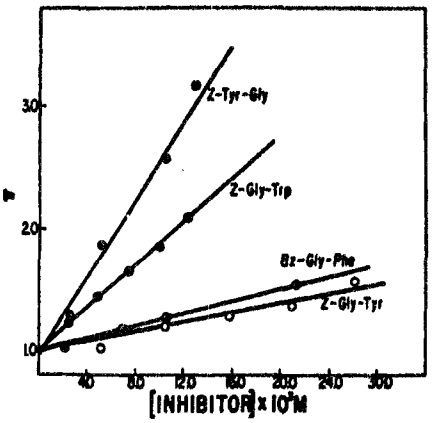

FIGURE 1: The inhibition of thermolysin by dipeptides, π plots: pH 7.2 Hepes buffer (0.1 M), 25.0 ± 0.1°, (S_0) = 9.76 × 10^{-4} M, (E_0) = 1.1 × 10^{-6} M. Additional details in text.

observed with lysine or arginine containing dipeptides while Leu-NH_2 and phenylalanine were inhibitory (Table III). D-Phenylalanine was found to be a better inhibitor than the L-phenylalanine both singly and in a dipeptide. Although Gly-Gly, Ala-Gly, and Gly-NH_2 showed negligible inhibition, the Z-Gly yielded a K_I of 11.7 × 10^{-3} M (Table III). This might be a function of the carbobenzoxyl group. Further work is needed to determine its contribution to the binding of Z-dipeptides.

The thermal stability of thermolysin permitted an examination of the temperature dependence of K_I over a range of over 50°. Figure 2 shows the Arrhenius plots for the temperature dependence of the K_I's for Z-Phe-Gly-NH_2, Z-Leu-Gly-NH_2, and Z-Phe-Gly. The temperature dependence of the K_I for Z-Phe-Gly-NH_2 also was determined in the presence of added 0.1 M $CaCl_2$ and essentially identical values were obtained. It should, however, be mentioned that the enzyme used

TABLE II: Effect of the Position of the Hydrophobic Amino Acid in Dipeptide on the Inhibition of Thermolysin.[a]

	Inhibitor × 10^3 (M)	$K_I \times 10^3$ (M)
Z-Phe-Gly	2.72–27.2	4.50
Z-Gly-Phe	2.45–24.50	14.70
Z-Tyr-Gly	2.60–12.90	6.9
Z-Gly-Tyr	5.20–26.1	55.0
Z-Leu-Gly	2.25–45.2	4.0
Z-Gly-Leu	1.65–16.48	5.65
Z-Trp-Gly	2.45–12.20	4.8[b]
Z-Gly-Trp	2.44–24.40	18.9
Phe-Gly	2.01–20.1	10.3
Gly-Phe	13.2–87.4	141.0
Phe-Gly-NH_2	11.2–56.1	10.90
Gly-Phe-NH_2	3.45–17.2	53.00
Gly-Leu	9.51–95.1	47.5
Leu-Gly	3.65–21.90	23.5
Gly-Leu-NH_2	4.71–47.07	11.60
Leu-Gly-NH_2	8.70–43.40	8.30[b]
Z-Gly-Ala	6.94–34.6	38.5
Z-Ala-Gly	6.90–34.7	7.67

[a] All reactions carried out in pH 7.2 Hepes buffer (0.1 M), 25.0 ± 0.1°. (E_0) = 1.16 × 10^{-6} M; (S_0) = 9.76 × 10^{-4} M; additional details in text. [b] Inhibition plots deviate from linearity.

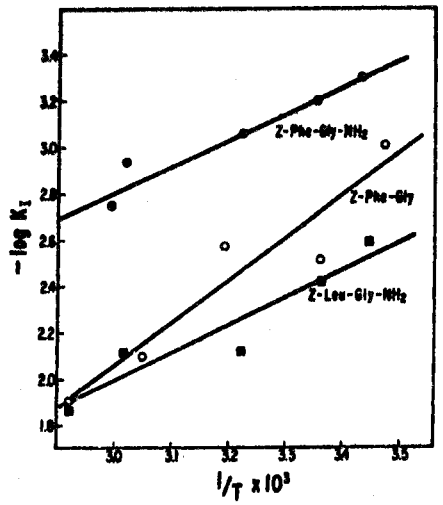

FIGURE 2: The effect of temperature on the K_I values for Z-Phe-Gly-NH_2, Z-Leu-Gly-NH_2, and Z-Phe-Gly: pH 7.2 Hepes buffer (0.1 M), $(S_0) = 9.76 \times 10^{-4}$ M, $(E_0) = 1.1 \times 10^{-6}$ M.

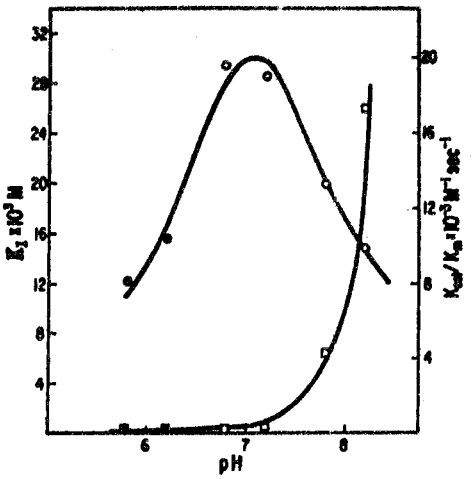

FIGURE 3: Comparison of the pH dependence of the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu-NH_2 (●○) and the pH dependence of the K_I for Z-Phe-Gly-NH_2 (■□): (●■) cacodylate buffer, 0.1 M; (○□) Hepes buffer, 0.1 M; $(E_0) = 6.02 \times 10^{-7}$ M.

was crystallized from $CaCl_2$ and, therefore, significant levels of calcium were present even without the addition of $CaCl_2$. No determinations of the calcium levels were made. Good linearity was obtained over the entire temperature scale indicative of maintenance of conformational integrity over this entire range of temperature. Table IV gives the enthalpies and entropies of binding for the inhibitor to enzyme. Z-Phe-Gly-NH_2, which had the best binding constant ($1/K_I$ = 3000 M^{-1}), yielded the most positive ΔS (−0.38 cal/(mol deg)) and the smallest ΔH (−4465 cal/mol); while Z-Leu-Gly-NH_2 and Z-Phe-Gly with progressively lower binding constant (300 and 250 M^{-1}, respectively) yielded progressively more negative ΔS values (−4.76 and −14.96 cal/(mol deg), respectively) and larger ΔH values (−5557 and −8507 cal/mol, respectively). An explanation for this contribution of binding by entropy will be suggested in the discussion.

Figure 3 shows the pH dependence of the K_I for Z-Phe-Gly-NH_2. The sharp increase in K_I in the region of pH 7–8 suggested the possible role in binding of a group(s) with pK in this region.

TABLE III: Inhibition of Thermolysin by Simple Dipeptides and Amino Acids.[a]

Inhibitor	Concn × 10^3 (M)	$K_I \times 10^3$ (M)
Leu-Leu	0.51–20.2	0.84
Phe-Phe	0.57–5.69	2.13
Asp-Phe-OMe	2.82–28.15	18.7
Gly-Pro	29.7–44.5	No inhibition
Gly-Gly	160.0	No inhibition
Bz-Gly-Lys	30.0	No inhibition
Z-Gly-Arg	41.0	No inhibition
Z-Gly	3.86–15.4	11.7
Ac-Gly	8.28–82.8	208.0
Gly-NH_2	14.6–87.6	252.0
Leu-NH_2	4.86–29.2	11.8
L-Phe	5.86–29.40	15.60
D-Phe	1.96–19.60	4.98

[a] All reactions carried out in pH 7.2 Hepes buffer (0.1 M), 25.0 ± 0.1°. $(E_0) = 1.16 \times 10^{-6}$ M; $(S_0) = 9.76 \times 10^{-4}$ M; additional details in text.

Discussion

A study of competitive inhibition of an enzyme can yield important information complementary to substrate specificity about the active site and about structure requirements for binding to the active site. The results reported here reemphasize the requirement for the presence of an amino acid with a hydrophobic side chain such as leucine and phenylalanine. Although the substrate specificity designates that this amino acid be at the imino end of the peptide bond cleaved, it is evident that for binding this is not necessary. Even free leucine, phenylalanine, and D-phenylalanine were competitive inhibitors with this substrate. Thus Z-Phe-Gly-NH_2 and Z-Leu-Gly-NH_2 yielded K_I values of 0.35×10^{-3} and 3.07×10^{-3} M, respectively, which compare to K_m values of 14.7×10^{-3} and 20.6×10^{-3} M for Z-Gly-Phe-NH_2 and Z-Gly-Leu-NH_2, respectively, as reported by Morihara and Tsuzuki (1970). This also is indicated by the data presented in Table II where in every case the dipeptides with the hydrophobic amino acid at the amino end of the molecule were more inhibitory than those with the reverse sequence. Recently the primary structure and conformation of thermolysin have been reported (Titani *et al.*, 1972; Matthews *et al.*, 1972a,b; Colman *et al.*, 1972). Colman *et al.* (1972) found that the molecule was folded into two lobes with a cleft containing the essential zinc atom. A hydrophobic pocket was found in the cleft about 8 Å from the zinc. One might suggest that this is the binding site for the hydrophobic side chain of leucine or phenylalanine. Any molecule which would bind at this site would act as a competitive inhibitor. However, this binding alone is not sufficient for the right orientation for catalysis unless the phenylalanine or leucine would be at the imino end of

TABLE IV: Some Thermodynamic Parameters of Inhibition of Thermolysin.[a]

Inhibitor	$K_I \times 10^3$ (M)[b]	ΔH (cal/mol)	ΔS (cal/(mol deg))
Z-Phe-Gly-NH_2	0.35	−4465	−0.38
Z-Leu-Gly-NH_2	3.07	−5557	−4.76
Z-Phe-Gly	4.50	−8507	−14.96

[a] Experimental details in text. [b] 25.0 ± 0.1°.

the peptide bond. The exceptions to this were the peptides Z-Phe-Gly-Ala and Z-Tyr-Gly-NH_2 which Morihara and Tsuzuki reported to be substrates (Morihara and Tsuzuki, 1970). Perhaps additional factors contributing to productive orientation such as the tripeptide with a free carboxyl or binding of the carbobenzoxy group contributed to this catalysis.

The effect of a free carboxyl or amino group on the enzyme-catalyzed hydrolysis of dipeptide substrates has been reported earlier. Matsubara (1966b) found that Gly-Phe and Gly-Phe-NH_2 were not cleaved by thermolysin while the Z-Gly-Phe and Z-Gly-Leu were slowly hydrolyzed. Likewise, Morihara and coworkers (Morihara *et al.*, 1968) reported that Gly-Leu-NH_2 was not hydrolyzed while both Z-Gly-Leu-NH_2 and Ac-Gly-Leu-NH_2 were cleaved. This indicates that the presence of a free amino group prevents enzymatic hydrolysis. We have observed that the k_{cat}/K_m for the thermolysin-catalyzed hydrolysis of furylacryloyl-Gly-Leu was about 1/1000 that for furylacryloyl-Gly-Leu-NH_2. Similar effects were observed for the dipeptide inhibitors. However, it depended on the position of the charged group relative to the leucine or phenylalanine. A smaller K_I was observed for dipeptides in which the hydrophobic amino acid was at the amino end of the molecule than those dipeptides in which the leucine or phenylalanine was at the carboxyl end. Thus the ratios for the K_I values of Z-Gly-Phe/Z-Phe-Gly, Z-Gly-Tyr/Z-Tyr-Gly, Z-Gly-Trp/Z-Trp-Gly, Gly-Phe/Phe-Gly, and Z-Gly-Ala/Z-Ala-Gly were 3.3, 8.0, 3.9, 13.7, and 5.0, respectively. Inversely, greater inhibition was observed when the free amino group was on the phenylalanine then removed by an amino acid as evidenced by a K_I of 10.9×10^{-3} M for Phe-Gly-NH_2 and a K_I of 53.0×10^{-3} M for Gly-Phe-NH_2. Colman *et al.* (1972) pointed out that thermolysin had an arginine-203 in the active site which appeared to be an analog to arginine-145 in carboxypeptidase. In carboxypeptidase Arg-145 is thought to bind the carboxyl group of the substrate. No role has been assigned to Arg-203 in thermolysin, but it might be involved in the interaction of thermolysin with charged substrates and inhibitors. If a distance of about an amino acid length separates the binding site for the hydrophobic side chain from Arg-203, a molecule containing a sequence such as $-Phe-XCOO^-$ could be bound simultaneously by an ionic bond and a hydrophobic interaction. However, if the hydrophobic amino acid carried the free carboxyl group, the multiple binding could not occur and ionic binding could result in a nonproductive displacement for catalysis or competitive inhibition. This could explain why the peptides with general structure of Z-X-Phe-Ala reported by Morihara and Tsuzuki (1970) functioned as good substrates even with a free carboxyl group. Similarly the greater inhibition by dipeptides of general structure Z-X-Gly than Z-Gly-X could be explained. The guanidinium group in the vicinity of the substrate binding site would also effect the interaction of thermolysin with substrates having a free amino group. One would expect less interference if the amino group were removed from the vicinity of the arginine. This would be consistent with the difference in inhibition observed for Phe-Gly-NH_2 and Gly-Phe-NH_2 and the absence of catalytic activity toward Gly-Phe and Gly-Phe-NH_2 (Matsubara, 1966b).

The study of the temperature dependence of the inhibition constants yielded some interesting results. The linearity of the Arrhenius plots was indicative of a fairly stable conformation of the enzyme molecule over the entire temperature range, 15–70°. Comparison of the enthalpies and entropies of binding ($1/K_I$) for Z-Phe-Gly-NH_2, Z-Leu-Gly-NH_2, and Z-Phe-Gly revealed that, as the value of $1/K_I$ became smaller, the ΔS became more negative. The binding thus appeared to be a function of the change in entropy. One might suggest that the progressively less negative entropy change observed as the binding increased reflects a displacement of bound solvent molecules from the active site similar to the positive entropies reported by Klotz for the binding of organic ions to albumin (Klotz and Urquhart, 1949).

Although the pH dependence of K_I was studied for only one inhibitor, Z-Phe-Gly-NH_2, the results invite some speculation. Colman *et al.* (1972) reported 15 salt bridges between amino acids. The majority (12) of these involve lysine and arginine but three of these involve histidines. These ionic bonds would have a pH dependence reflecting the pK of the imidazole. Disruption of these salt bridges could result in conformational changes which would affect both binding and catalysis. This particularly is suggested in light of the salt bridges His-231–Asp-226 and His-142–Asp-170 which are an integral part of the active site (Colman *et al.*, 1972). The bell-shaped pH dependencies for the thermolysin and *B. subtilis* neutral protease catalyzed hydrolyses of furylacryloyl-Gly-Leu-NH_2 and furylacryloyl-Gly-Phe-NH_2 suggested a dependence upon two ionizable groups with pK values close to neutrality (Feder and Schuck, 1970). This is reminiscent of the alkaline pH dependence of chymotrypsin and the role of Ile-16 in maintaining a catalytically active conformation (Oppenheimer *et al.*, 1966; Himoe *et al.*, 1967).

It is important to point out that all of these studies were carried out with small dipeptide inhibitors and a dipeptide substrate and some of the effects observed might be modified with larger substrates. In consideration of the additional subsites proposed by Morihara and Tsuzuki (1970), larger polypeptide substrates might be less effected by small conformational changes since a number of residues would be contributing to the orientation of the molecule in the active site.

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