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The Inhibition of Pepsin Action*

Ken Inouye† and Joseph S. Fruton‡

ABSTRACT: The spectrophotometric method for following the rate of peptic cleavage of Z-His-Phe(NO_2)-Phe-OMe has been applied to the study of the inhibition of pepsin by amino acid esters, dipeptide esters, derivatives of L-phenylalaninol, and acetylamino acids at pH 4 and 37°. Under these conditions, the cleavage product Phe-OMe and other amino acid esters act as linear competitive inhibitors with K_I values much larger than the K_M of the substrate. Dipeptide esters such as Phe-Phe-OMe are effective linear competitive inhibitors, with K_I near that of K_M for the substrate. The substrate analog Z-His-Phe-Pol is resistant to peptic action, and is a strong competitive inhibitior. Acetyl-L-amino acids inhibit pepsin action weakly at pH 4.

In previous communications from this laboratory (Inouye and Fruton, 1967a,b), a spectrophotometric method for following pepsin kinetics was described. In this method, the substrate Z-His-Phe(NO_2)-Phe-OMe[1] was used, and the change in absorbance at 310 mμ accompanying the specific cleavage of the Phe(NO_2)-Phe bond was measured. In the work described below, this method has been applied to the examination of the inhibitory action of a series of compounds related to synthetic substrates of pepsin.[2]

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† Present address: Shionogi Research Laboratory, Shionogi and Co. Ltd., Fukushima-Ku, Osaka, Japan.

‡ To whom inquiries should be addressed.

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: Phe(NO_2), *p*-nitro-L-phenylalanyl; Pla, β-phenyl-L-lactyl; Pol, L-phenylalaninol; TyrBr_2, 3,5-dibromo-L-tyrosine; Z, benzyloxycarbonyl; OBzl, benzyloxy; Ac, acetyl. The abbreviated designation of amino acid residues denotes the L form, except where otherwise indicated.

[2] The kinetic parameters mentioned in this paper are defined by the equation $v = V_{max}[S]/(K_M + [S])$ for the process

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

where v = initial velocity, $V_{max} = k_{cat} \times$ total enzyme concentration, [S] = initial substrate concentration, $K_M = (k_{cat} + k_{-1})/k_1$, and $K_S = k_{-1}/k_1$. For competitive inhibition, K_I denotes the dissociation constant of EI in the process $E + I \rightleftarrows EI$.

Experimental Section

The synthesis of the substrate Z-His-Phe(NO_2)-Phe-OMe has been described (Inouye and Fruton, 1967b); its concentration was varied between 6.25 and 25 $\times 10^{-2}$ mM. The action of each inhibitor was tested at three to five substrate concentrations [S]. Each substance examined for its inhibitory action was tested at three levels of concentration. The four values of initial velocity (v) obtained for a given substrate concentration (including the value in the absence of inhibitor, always determined in parallel) were used for $1/v$ *vs.* [I] (inhibitor concentration) plots according to the method of Dixon (1953). Also, the slopes derived from $1/v$ *vs.* 1/[S] plots were replotted against [I] (Cleland, 1963).

Before use, all the compounds tested as inhibitors were checked for the absence of impurities detectable by thin-layer chromatography. Except for L-phenylalanine, all the compounds were synthesized in our laboratory. The properties of the amino acid esters and of the acetylamino acids agreed with those recorded in the literature (Greenstein and Winitz, 1961). The preparation of Phe-Phe-OMe, Phe-Tyr-OEt, Z-His-Phe-Phe, Z-His-Phe-Pol, and Pla-OMe has been described previously (Inouye *et al.*, 1966; Inouye and Fruton, 1967b). Phe-Phe was prepared by treatment of Z-Phe-Phe (mp 154–156°) with HBr–acetic acid in the usual manner. His-Phe was prepared from Z-His-Phe (Inouye *et al.*, 1966) in the same manner.

Z-His-Phe-(OAc)Pol. Z-Phe-Pol (0.14 g, 0.25 mmole)

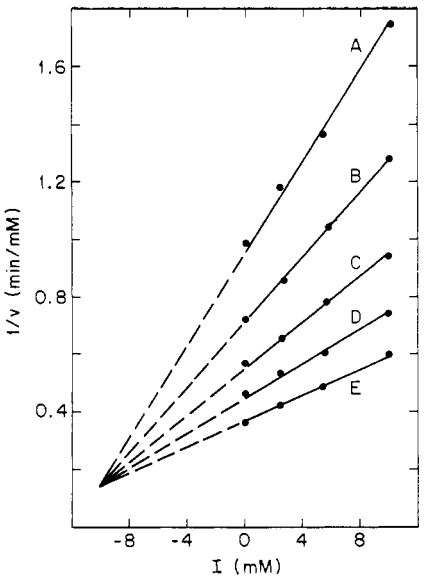

FIGURE 1: Competitive inhibition by Phe-OEt of the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe. Substrate concentration: curve A, 0.0625 mM; curve B, 0.0875 mM; curve C, 0.125 mM; curve D, 0.175 mM; and curve E, 0.25 mM.

(Inouye and Fruton, 1967b) was treated with acetic anhydride (0.1 ml) in anhydrous pyridine (2 ml). After 4 hr at room temperature, the reaction mixture was chilled and water (5 ml) was added to give the crystalline acetyl compound (0.13 g, 87%), mp 168–170°. Thin-layer chromatography (benzene–methanol, 85:15, v/v) gave a single spot of R_F 0.64 (Pauly reagent). *Anal.* Calcd for $C_{34}H_{37}N_5O_6$ (611.7): C, 66.8, H, 6.1; N, 11.4. Found: C, 66.6; H, 6.1; N, 11.3.

Crystalline swine pepsin (Worthington Biochemical Corp., lot PM 708) was used in all experiments. Enzyme solutions were prepared just before the kinetic runs. In all cases, the enzyme concentration was 0.02 mg/ml; based on a molar absorptivity at 278 mμ of 51,000 (Perlmann, 1966) and a molecular weight of 35,000, this corresponds to a pepsin concentration of 5.7×10^{-4} mM. Except where otherwise stated, all experiments were conducted at pH 4.0 ± 0.1 (disodium citrate–HCl buffer, 0.024 M with respect to citrate); pH measurements were made with a Corning expanded-scale pH meter, Model 12.

The rate of enzymic action was followed by means of a Cary 15 recording spectrophotometer equipped with an automatic sample changer, and the absorbance of each sample at 310 mμ was determined at measured intervals of about 1.1 min over a period of 12–20 min. The temperature was controlled at 37.0 ± 0.1° by circulating water from a Formatemp constant-temperature bath (Model 2095-2) around the sample compartment of the spectrophotometer. The solution containing substrate, inhibitor, and buffer was kept in the sample compartment for about 10 min before the addition of pepsin to start the reaction. The reference cuvet contained the substrate and buffer. Except for tryptophan derivatives (at relatively high concentration), none of the inhibitors tested in the present work shows significant absorbance at 310 mμ. In the experiments with Trp-OEt as inhibitor, the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe was followed at 320 mμ.

At pH 4.0, the change in molar absorptivity ($\Delta\epsilon$) at 310 mμ for 100% hydrolysis of Z-His-Phe(NO_2)-Phe-OMe to Z-His-Phe(NO_2) and Phe-OMe is 800 ± 15 (average of five determinations). For an initial substrate concentration of 0.2 mM, this corresponds to a change in absorbance (1-cm cell) of 0.16. At 320 mμ, $\Delta\epsilon$ = 650 ± 15. In all experiments, the 0–0.1 scale of the recorder was used, and satisfactorily linear initial velocities (15–20% hydrolysis) could be determined from the 10–15 points recorded for each sample.

Results

Inhibition by Amino Acid Esters. Under the conditions of these experiments, the amino acid esters tested (see Table I) act as competitive inhibitors of pepsin in the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe. A representative plot of $1/v$ *vs.* [I] is shown in Figure 1 for Phe-OEt. Similar plots were obtained for Phe-OMe, Phe-OBzl, Leu-OEt, Tyr-OEt, and Trp-OEt. Also, when the slopes from $1/v$ *vs.* 1/[S] plots were replotted agsinst [I], straight lines were obtained, as shown in Figure 2 for Phe-OEt. It may be concluded, therefore, that all these amino acid esters, including a product (Phe-OMe) of

TABLE I: Inhibition of Pepsin Action on Z-His-Phe(NO_2)-Phe-OMe at pH 4.0 and 37°.

Inhibitor	Concn Tested (mM)	K_I (mM)
L-Phenylalanine	25, 50, 100	90 ± 5
L-Phenylalaninol	1.25, 2.5, 5	5 ± 1
L-Phe-OMe	5, 10, 20	22 ± 3
L-Phe-OEt	5, 10, 20	10 ± 2
D-Phe-OEt	5, 10, 20	10 ± 2
L-Phe-OBzl	1.25, 2.5, 5	2.0 ± 0.3
L-Leu-OEt	5, 10, 20	19 ± 3
L-Tyr-OEt	2.5, 5, 10	18 ± 3
L-Trp-OEt	2.5, 5, 10	6 ± 1
L-Pla-OMe	5, 10, 20	21 ± 4
His-Phe	5, 10, 20	17 ± 3
Phe-Phe	0.625, 1.25, 2.5	1.1 ± 0.2
Phe-Phe-OMe	0.125, 0.25, 0.5	0.25 ± 0.05
Phe-Tyr-OEt	0.3125, 0.625, 1.25	0.86 ± 0.15
Z-His-Phe-Phe	0.0625, 0.125, 0.25	0.75 ± 0.12
Z-His-Phe-Pol	0.0625, 0.125, 0.25	0.24 ± 0.05
Ac-Phe	12.5, 25, 50	55 ± 4
Ac-Tyr	25, 50, 100	62 ± 5
Ac-Trp	12.5, 25, 50	35 ± 3
Ac-Leu	12.5, 25, 50	70 ± 4

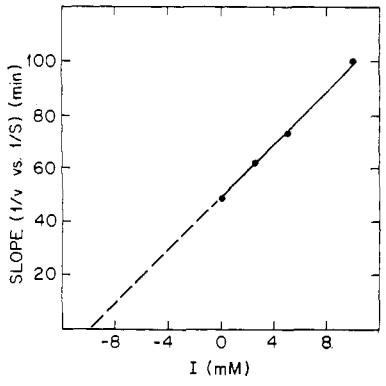

FIGURE 2: Replot of slopes from $1/v$ *vs.* 1/[S] plots for the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe in the presence of Phe-OEt. The experimental data were the same as those used for the Dixon plot in Figure 1.

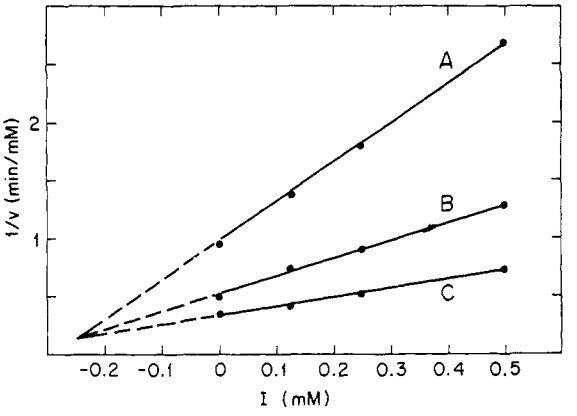

FIGURE 3: Competitive inhibition by Phe-Phe-OMe of the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe. Substrate concentration: curve A, 0.0625 mM; curve B, 0.125 mM; and curve C, 0.25 mM.

the reaction measured, act as linear competitive inhibitors (Cleland, 1963).

It will be noted from Table I that the value of K_I for the esters of L-phenylalanine increases in the order benzyl ester, ethyl ester, and methyl ester. The value of K_I for the reaction product Phe-OMe (22 mM) is approximately 50 times greater than the value of K_M for the substrate (0.46 mM) at pH 4 and 37° (Inouye and Fruton, 1967b).

The identity of the K_I values for the L and D forms of phenylalanine ethyl ester indicates that the binding of these amino acid esters is not stereospecific, and that the amino group (which is completely protonated at pH 4) does not contribute significantly to the binding of the inhibitor at the catalytic site of the enzyme. This conclusion is supported by the identity of the K_I values for Phe-OMe and Pla-OMe; the latter compound also behaves as a linear competitive inhibitor. The difference in the K_I values for the ethyl esters of tryptophan, phenylalanine, leucine, and tyrosine may be attributed therefore to the interaction of the side chains of these amino acids with groups at the catalytic site; in order of increasing K_I, the four esters fall into the series Trp-OEt < Phe-OEt < Leu-OEt, Tyr-OEt.

Inhibition by Dipeptide Derivatives. Of special interest in this study was the finding that Phe-Phe-OMe is a strong competitive inhibitor of pepsin in the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe. As shown in Table I and Figure 3, the K_I for this inhibitor is 0.25 mM, a value near that previously found for the K_M of Z-His-Phe-Phe-OMe (0.37 mM) at pH 4 and 37° (Inouye and Fruton, 1967b). The other dipeptide ester tested, Phe-Tyr-OEt, also acted as a linear competitive inhibitor, with a K_I of 0.86 mM. Comparison of these K_I values with those given above for Phe-OMe and Tyr-OEt shows that the addition of an amino-terminal phenylalanyl residue to these amino acid esters greatly increases the binding of the inhibitor at the catalytic site of pepsin.

The pronounced inhibitory effect of dipeptide units having two phenylalanyl residues is still evident with Phe-Phe, although the replacement of the COOMe group of Phe-Phe-OMe by a carboxyl group (a portion of which is protonated at pH 4) causes a fourfold increase in K_I (Table I). The tighter binding of Phe-Phe, as compared to His-Phe, is also noteworthy. The addition of a Z-His group to Phe-Phe, as in Z-His-Phe-Phe (which is cleaved by pepsin very slowly at pH 4; Inouye *et al.*, 1966), does not appear to change markedly the behavior of the dipeptide unit as a competitive inhibitor of pepsin.

Inhibition by Derivatives of Phenylalaninol. It was reported previously (Inouye and Fruton, 1967b) that Z-His-Phe-Pol is resistant to the action of pepsin. Further work has shown that acetylation of the hydroxyl group of this compound, to form Z-His-Phe-(O-Ac)Pol, does not render the Phe-Pol linkage susceptible to the hydrolytic action of pepsin. The enzymic test was conducted for 18 hr with 0.5 mM peptide and 0.2 mg of pepsin/ml, at pH 4 and 37°; no net liberation of ninhydrin-reactive material was observed. The resistance of the phenylalaninol analogs of Z-His-Phe-Phe-OMe to peptic hydrolysis was further confirmed by the failure to observe a net change in absorbance at 310 mμ when Z-His-Phe(NO_2)-Pol[3] was subjected to the action of pepsin under the conditions given in the Experimental Section for rapid hydrolysis of Z-His-Phe(NO_2)-Phe-OMe.

An examination of the inhibitory effect of Z-His-Phe-Pol showed this compound to be an effective competitive inhibitor of pepsin, with a K_I of 0.24 mM at pH 4 and 37°. It was also of interest to find that L-phenylalaninol itself exhibits a considerable competitive effect, its K_I being 5 mM (Table I). The latter value may be contrasted with that for L-phenylalanine (90 mM) which is present largely as the dipolar ion at pH 4. In view of the previous indication that the protonated amino group of amino acid esters does not contribute significantly to their binding, it would appear that the difference in K_I values for phenylalanine and phenylalaninol is a consequence of the unfavorable effect of the carboxylate group of the amino acid on binding at the catalytic site of pepsin. The somewhat stronger binding of L-phenylalaninol, as compared with Phe-OMe, may be related to

[3] The details of the synthesis of this compound have been reported in connection with studies (Humphreys and Fruton, 1968) on its binding to pepsin.

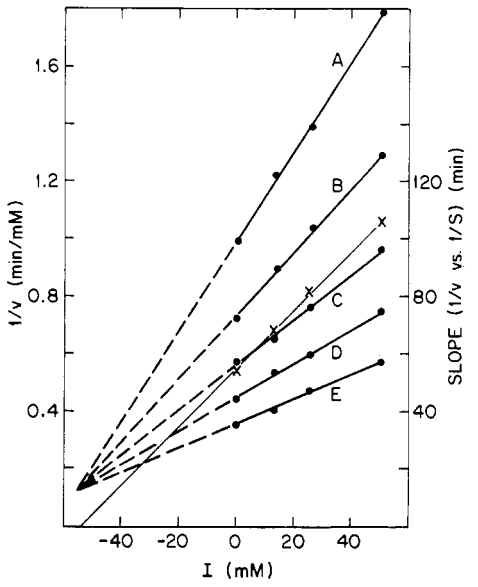

FIGURE 4: Competitive inhibition by acetyl-L-leucine of the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe. Dixon plot, solid points, left ordinate; substrate concentration: curve A, 0.0625 mM; curve B, 0.0875 mM; curve C, 0.125 mM; curve D, 0.175 mM; and curve E, 0.25 mM. The crosses denote slopes of $1/v$ *vs.* 1/[S] plots from the same experimental data (right ordinate).

the difference in their pK_a values, the amino alcohol being a stronger base.

Inhibition by Acetylamino Acids. One of the objectives of this study was to determine the extent and nature of the inhibitory effect of Z-His-Phe(NO_2) on the peptic cleavage of Z-His-Phe(NO_2)-Phe-OMe. In view of the finding that Phe-OMe behaves as a linear competitive inhibitor, it was important to establish the character of the inhibition exerted by the other cleavage product. Such information is often useful in distinguishing between an ordered and random release of products in an enzyme-catalyzed hydrolytic reaction (Hsu *et al.*, 1966). Unfortunately, Z-His-Phe(NO_2) is insufficiently soluble in water at pH 4, and we have eschewed the addition of organic solvents to increase its solubility, as such solvents exert a pronounced inhibitory effect on pepsin action. The question of the nature of the inhibition exerted by the AX split product derived from a pepsin substrate AX–YB is under investigation in more suitable systems, and will be discussed in a future communication.

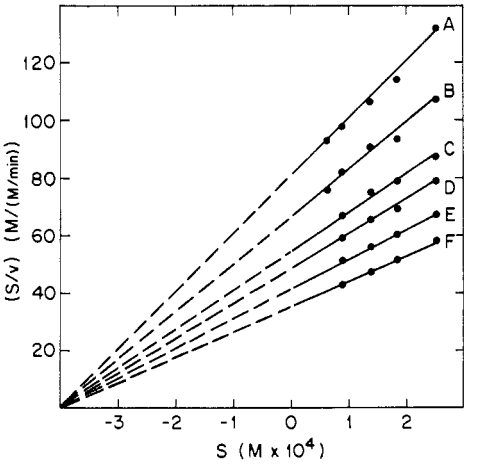

FIGURE 5: Inhibition of peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by pH 4.0 citrate buffer. [S]/v *vs.* [S] plots for buffer concentrations 0.16 M (curve A), 0.08 M (curve B), 0.04 M (curve C), 0.02 M (curve D), 0.01 M (curve E), and 0.005 M (curve F). The buffers were prepared from citric acid and trisodium citrate; the concentrations given refer to the total concentration of all forms of citric acid.

In the present work, the related question of the inhibitory effect of acetylamino acids was examined at pH 4, where they exist extensively in the carboxylate form, their pK_a values being near 3.5. The four compounds tested (Ac-Phe, Ac-Tyr, Ac-Trp, and Ac-Leu) all showed relatively weak inhibition, and the analysis of the kinetic data indicated that these compounds act as linear competitive inhibitors under the conditions of this study. Representative plots are given in Figure 4 for Ac-Leu; the data for the other three compounds gave similar plots. It will be noted in Table I that the apparent K_I values (35–70 mM) of the acetylamino acids are much larger than those found for the amino acid esters and approach the K_I for L-phenylalanine itself.

The weak inhibitory action of the ionized form of the acetylamino acids requires that relatively high concentrations (up to 0.1 M) of the inhibitor be used in the determination of its K_I (see Table I). Consideration must be given therefore to the possible effect of increased ionic strength on the catalytic activity of pepsin. Zeffren and Kaiser (1967) have reported that at pH 2 the rate of peptic hydrolysis of Ac-Phe-TyrBr_2 is the same at $\Gamma/2 = 0.02$ and at $\Gamma/2 = 0.12$, with either KCl or $KClO_4$ as the salt. Unpublished experiments by Dr. T. R. Hollands in this laboratory have given a similar result for the rate of hydrolysis of Z-His-Phe-Phe-OEt at pH 4, with NaCl (up to 0.2 M) as the salt. Although a nonspecific ionic strength effect appears to be absent below $\Gamma/2 = 0.2$, it should be noted that an increase in the concentration of pH 4.0 citrate buffer (citric acid–trisodium citrate) causes a marked decrease in the initial rate of the peptic cleavage of Z-His-Phe(NO_2)-Phe-OMe. An examination of this effect as a function of initial substrate concentration showed that the value of K_M was unchanged (0.4 mM) whereas the value of V_{max} was decreased (Figure 5), indicating a noncompetitive type of inhibition.

Discussion

The procedure used in the present studies for following pepsin kinetics has some advantages over the spectrophotometric method used by previous workers (Silver *et al.*, 1965; Clement and Snyder, 1966). The change in absorbance is measured at 310 mμ, instead of 237 mμ where the base line is much higher; this factor may cause difficulty when relatively high pepsin concentrations (greater than 0.01 mM) are required for the rapid hydrolysis of some synthetic substrates. The difference between the molar absorptivity of substrate and split products for the cleavage of Z-His-Phe(NO_2)-Phe-OMe to Z-His-Phe(NO_2) and Phe-OMe is sufficiently large

(800 at pH 4) for the reproducible measurement of the initial rate of pepsin action on this substrate. A disadvantage of the method is the limited solubility of Z-His-Phe(NO_2)-Phe-OMe in aqueous buffer solutions at pH values (1.0–5.5) of interest in the study of pepsin kinetics. The solubility of this substrate at pH 4 is about 0.4 mM, and may be increased by the addition of organic solvents. In the present studies, we have preferred to omit such solvents because they have been shown to inhibit pepsin action (Tang, 1965; Zeffren and Kaiser, 1967). Studies are in progress in an attempt to develop more soluble pepsin substrates having the Phe(NO_2)-Phe unit as the site of enzymic action.

In the hydrolytic cleavage of a typical pepsin substrate AX–YB (X and Y are the amino acid residues joined by the sensitive bond), the spectrophotometric method measures the rate of formation of AX-OH. It has been found that for the hydrolysis of Z-His-Phe-(NO_2)-Phe-OMe, the rate of formation of H-YB (Phe-OMe), as determined by means of its reaction with ninhydrin, is the same as the rate of release of AX-OH (Inouye and Fruton, 1967b).

Earlier studies had shown that Z-His-Phe-D-Phe-OEt and Z-His-D-Phe-Phe-OEt (both of which are resistant to cleavage by pepsin) are competitive inhibitors of the peptic hydrolysis of Z-His-Phe(NO_2)-Phe-OMe, with K_I values near 0.2 mM at pH 4 and 37° (Inouye and Fruton, 1967b). This K_I value is near that for K_M for the comparable substrate Z-His-Phe-Phe-OEt (Inouye *et al.*, 1966), suggesting that K_M may represent K_S, the dissociation constant of the enzyme–substrate complex. The demonstration in the present work that Phe-Phe-OMe also is an effective competitive inhibitor (K_I = 0.25 mM) indicates that the Z-His portion of the diastereoisomeric forms of Z-His-Phe-Phe-OEt makes a relatively small contribution to the binding at the catalytic site of pepsin. As was indicated by previous work (Baker, 1951; Inouye *et al.*, 1966), the major contribution to binding appears to be provided by the apolar side chains of two adjacent amino acid residues in a substrate or a competitive inhibitor. The interaction of pepsin with its substrates or substrate analogs provides a clear example of the cooperative effect resulting from the presence of adjacent apolar amino acids in the small molecule.

The finding that the K_I values for Phe-OMe and Pla-OMe are the same indicates that the protonated amino group of the amino acid ester does not contribute significantly to binding at the catalytic site. With a larger inhibitor (*e.g.*, Phe-Phe-OMe or Z-His-D-Phe-Phe-OEt), the cooperative effect in the binding of adjacent apolar side chains may bring such a cationic group into the proximity of a carboxylate group of the enzyme to form an ion pair; such additional interaction may contribute somewhat to binding and may also alter the conformation of the protein in the region of the catalytic site.

The weak inhibition exhibited by Ac-Phe, Ac-Tyr, Ac-Trp, and Ac-Leu at pH 4 (K_I = 30–70 mM) may be considered to reflect the unfavorable effect of the carboxylate group of these compounds (pK_a = *ca.* 3.5) on binding at the catalytic site, as Zeffren and Kaiser (1967) have reported a K_I of 2 mM for Ac-Phe at pH 2 and 25° (no organic solvent added). The role of a carboxylate group in decreasing the extent of binding is also suggested by a comparison of the K_I values for the pair phenylalanine and Phe-OMe, as well as the pair Phe-Phe and Phe-Phe-OMe.

The role of carboxyl groups in a pepsin substrate or substrate analog in relation to binding at the catalytic site and to catalysis requires further study. The demonstration that Z-His-Phe-Phe-OEt is hydrolyzed more rapidly than is Z-His-Phe-Phe, and that the pH optimum for the acid is about 3, whereas that for the ester is near 4.5, led to the suggestion (Inouye *et al.*, 1966) that a carboxylate group drawn into the region of the catalytic site is inhibitory to pepsin action. Subsequently, a similar conclusion was offered by Zeffren and Kaiser (1967) in their analysis of the pH dependence of the hydrolysis of Ac-Phe-TyrBr_2. Such inhibitory effects are not evident, however, at relatively low concentrations of carboxylic acid buffers such as acetate, formate, and chloroacetate (Inouye *et al.*, 1966). The finding that citrate buffer inhibits pepsin action, apparently in a noncompetitive manner, raises the possibility that when a sufficiently high local concentration of carboxyl groups is attained in the region of the catalytic site of pepsin, a change occurs in the structure of the catalytic site so as to decrease its efficiency. Further kinetic studies, now in progress, may give an indication of the validity of this hypothesis.

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