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THE MECHANISM OF THE CATALYTIC ACTION OF PEPSIN AND RELATED ACID PROTEINASES

By JOSEPH S. FRUTON, *New Haven, Connecticut*

CONTENTS

I. Introduction

Among the enzymes that catalyze the cleavage of peptide bonds, the group of the so-called acid proteinases, of which gastric pepsin A is the best known member, are of special interest. Other enzymes of this group that have been studied extensively are chymosin (rennin), an intracellular enzyme of animal tissues (cathepsin D), and the mold enzymes *Rhizopus*-pepsin (from *Rhizopus chinensis*) and penicillopepsin (from *Penicillium janthinellum*). Considerable effort has been expended during the past 10 years in the study of their catalytic action (for recent reviews, see refs. 1–5). During the course of this work many puzzling features have been encountered and apparently incompatible hypotheses have been advanced. The purpose of the present review is to examine the current status of the problem of the mechanism of the action of acid proteinases as seen from the perspective gained from recent work in our laboratory.

It should be noted at the outset that speculations about the catalytic

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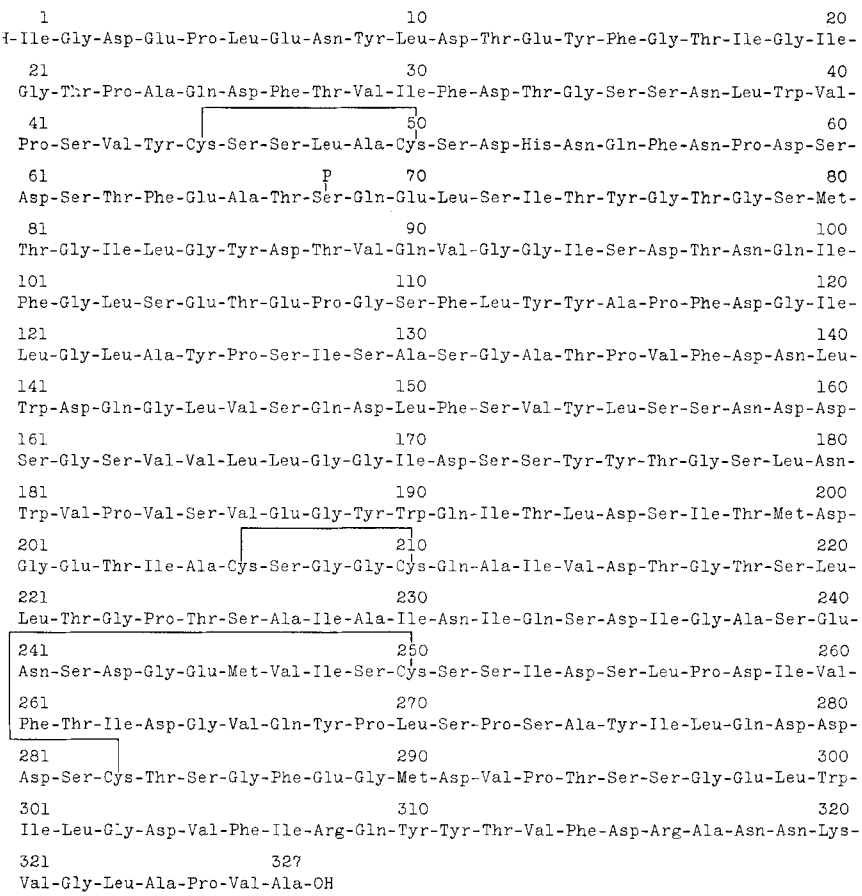


Fig. 1. Amino acid sequence of porcine pepsin (7). According to a recent report (7a) residues 60-61 are Asp-Ser and not Ser-Asp.

mechanism of pepsin have been limited by lack of information about the three-dimensional structure of its active site gained from X-ray crystallographic studies of this enzyme; to my knowledge, the latest published report (6) has given data for the 5.5 Å electron-density map of swine gastric pepsin.* A complete amino acid sequence of gastric swine pepsin

* Dr. N. S. Andreeva has reported data obtained from 2.7 A resolution studies of pepsin at the Fifth Linderstrøm-Lang Conference held at the Vingsted Center, Denmark, in August 1975.

A (Fig. 1) has only recently been deduced in two laboratories (7,8), and attention has been drawn to the many homologies in sequence between pepsin and other acid proteinases, such as chymosin (9) and penicillopepsin (10).

II. Synthetic Substrates for Pepsin

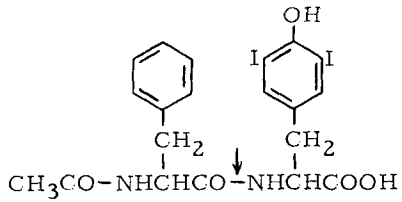
A wide variety of synthetic substrates have been used for the study of the mechanism of pepsin action. For a time most kinetic studies were conducted with acyl dipeptides of the type introduced by Baker (11). The best of these substrates is Ac-Phe-Tyr (I_2)* (I in Fig. 2), which is found to be cleaved at pH 2 and 37°C with $k_{cat} = 0.2$ sec^{-1} and $K_m = 0.08$ mM (12). These data may be compared with $k_{cat} = 0.07$ sec^{-1} and $K_m = 2$ mM for Ac-Phe-Tyr under the same conditions (13); similar kinetic values for the hydrolysis of the Phe-Tyr bond were reported for the methyl or ethyl ester and the amide of this acetyl dipeptide (14–16).

A second large group of synthetic peptide substrates for pepsin are those of the type Z-His-X-Y-OMe (or OEt), where X and Y are L-amino acid residues forming the peptide bond cleaved by the enzyme (17). Systematic variation of the nature of X and Y showed that, of the substrates tested, the most sensitive ones were those in which X = Phe and Y = Trp, Tyr, or Phe (18); for Z-His-Phe-Phe-OMe (II in Fig. 2) $k_{cat} = 0.17$ sec^{-1} and $K_m = 0.33$ mM at pH 4 and 37°C. The results of this study strengthened the conclusion drawn from work with acyl dipeptides that the preferred substrates of pepsin are those in which the sensitive peptide bond is flanked by two aromatic L-amino acid residues.

* The abbreviated designation of amino acid residues denotes the L-form, except where otherwise indicated. Other abbreviations used in this article are: Tyr(I_2), 3,5-diiodo-L-tyrosyl; Tyr(Br_2), 3,5-dibromo-L-tyrosyl; Phe($4NO_2$), *p*-nitro-L-phenylalanyl; PhGly, L-phenylglycyl; Nle, L-norleucyl; Pla, β-phenyl-L-lactyl; Ppa, phenylpyruvoyl; Pol, L-phenylalaninol; Ac, acetyl; Tfa, trifluoroacetyl; Z, benzyloxycarbonyl; Mns, mansyl, 6-(*N*-methylanilino)-2-naphthalenesulfonyl; Dns, dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; OMe, methoxy; OEt, ethoxy; OP4P, 3-(4-pyridinium)propyl-1-oxy. The kinetic parameters mentioned in this article are defined by the equation $v = k_{cat}[E]_t[S]_0/(K_m + [S]_0)$ for the process:

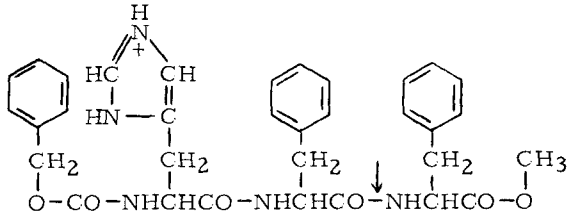
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \ (+ P_1) \xrightarrow{k_3} E + P_2$$

where v = initial velocity, $[E]_t$ = total enzyme concentration, $[S]_0$ = initial substrate concentration, $k_{cat} = k_2k_3/(k_2 + k_3)$, and $K_m = [(k_{-1} + k_2)/k_1][k_3/(k_2 + k_3)]$. Other symbols used are $K_s = K_D = k_{-1}/k_1$.



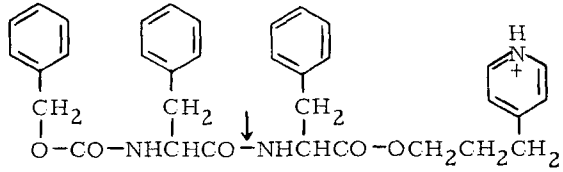
(I)

Ac-Phe-Tyr (I_2)



(II)

Z-His-Phe-Phe-OMe



(III)

Z-Phe-Phe-OP4P

Fig. 2. Synthetic peptide substrates for pepsin.

It should be noted, however, that replacement of either L-phenylalanyl residue in Z-His-Phe-Phe-OMe by a L-phenylglycyl residue rendered the bond resistant to pepsin action (19). On the other hand, the replacement of the L-phenylalanyl residue in the X position by a *p*-nitro-L-phenylalanyl residue did not alter the kinetic parameters significantly and permitted the development of a spectrophotometric method for following

the hydrolysis of the Phe(4NO_2)-Phe bond (20). In contrast to the widely used analytical procedures for estimating the rate of formation of the amine product (e.g., Phe-OMe) by means of its reaction with ninhydrin or fluorescamine, this method measures the rate of formation of the acidic product [e.g., Z-His-Phe(4NO_2)]. In substrates of the type Z-His-X-Y-OMe, the histidyl residue is protonated over the pH range of interest, and these cationic substrates exhibit pH optima for hydrolysis near pH 4 (21) in contrast to the optimal cleavage of the acyl dipeptides in the region pH 2–3.

Cationic substrates of pepsin having a 3-(4-pyridinium)propyloxy group (–OP4P) at the carboxyl-terminus of the peptide have also been studied extensively (22,23). The kinetic parameters of these substrates show relatively little variation over the pH range 2–4.5 (23); for Z-Phe-Phe-OP4P (III in Fig. 2) k_{cat} = 0.7 sec^{-1} and K_m = 0.2 m*M* at pH 3.5 and 37°C.

Perhaps the most important consequence of the introduction of the cationic substrates is the information gained from the modification of the A or B group in substrates of the type A-Phe-Phe-B, where the Phe-Phe [or Phe(4NO_2)-Phe] bond is the only one cleaved under the conditions of the studies (Table I). Thus, for Z-Ala-Ala-Phe-Phe-OP4P, k_{cat} = 282 sec^{-1} and K_m = 0.04 m*M* (pH 3.5, 37°C), and for Z-His-Phe(4NO_2)-Phe-Val-Leu-OMe, k_{cat} = 62 sec^{-1} and K_m = 0.04 m*M* at pH 4 and 37°C (24). In terms of k_{cat}/K_m values, the replacement of the Z group of Z-Phe-Phe-OP4P by a Z-Ala-Ala group leads to a 2000-fold enhancement in the rate of the cleavage of the Phe-Phe bond, while the replacement of the OMe group of Z-His-Phe(4NO_2)-Phe-OMe by a Val-Leu-OMe group leads to a 3000-fold enhancement. Kinetic data are now available for a relatively large number of synthetic cationic substrates of both types in which the A or B group of A-Phe-Phe-B has been altered, and while the values of K_m vary within roughly a single order of magnitude, the values of k_{cat} range over 3–4 orders of magnitude. The availability of such a large collection of cationic substrates of widely different sensitivity has permitted an approach to several problems relating to the mechanism of pepsin action. In this review emphasis is placed on the results obtained in the study of the action of gastric pepsin A on such substrates.

Several acid proteinases other than gastric pepsin have been tested as catalysts of the hydrolysis of the two types of cationic substrates mentioned above, and significant differences have been noted in the specificity of these enzymes with respect to the effect of modification of the A or B

TABLE I

Kinetics of Pepsin Action on Cationic Substrates[a]

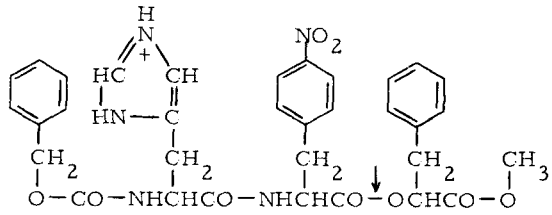
Substrate[b]	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m (m$M^{-1}sec^{-1}$)
Z-Phe-Phe-OP4P(22)	0.7	0.2	3.5
Z-Gly-Phe-Phe-OP4P(22)	3.1	0.4	7.8
Z-$(Gly)_2$-Phe-Phe-OP4P(22)	71.8	0.4	180
Z-$(Gly)_3$-Phe-Phe-OP4P(26)	4.5	0.4	10.1
Z-$(Gly)_4$-Phe-Phe-OP4P(26)	2.1	0.7	3.0
Z-Gly-Ala-Phe-Phe-OP4P(23)	409	0.1	4090
Z-Gly-Pro-Phe-Phe-OP4P(23)	0.06	0.14	0.4
Z-Ala-Gly-Phe-Phe-OP4P(23)	145	0.25	576
Z-Ala-Ala-Phe-Phe-OP4P(22)	282	0.04	7050
Z-His-Phe($4NO_2$)-Phe-OMe(25)	0.26	0.43	0.6
Z-His-Phe($4NO_2$)-Phe-Ala-OMe(25)	3.3	0.40	8.3
Z-His-Phe($4NO_2$)-Phe-Ala-Ala-OMe(25)	28	0.13	215
Phe-Gly-His-Phe($4NO_2$)-Phe-OMe (25)	0.12	0.4	0.3
Phe-Gly-His-Phe($4NO_2$)-Phe-Ala-Ala-OMe (25)	28	0.16	175
Phe-Gly-His-Phe($4NO_2$)-Phe-Ala-Phe-OMe (25)	20	0.04	500
Phe-Gly-His-Phe($4NO_2$)-Phe-Val-Leu-OMe (24)	62	0.04	1540

[a] pH 3.5–4.0, 37°C.

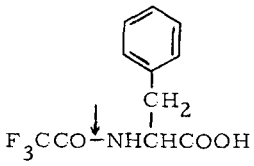
[b] In all cases, cleavage occurred at the Phe-Phe or Phe($4NO_2$)-Phe bond. The numbers in parentheses denote the references from which the data were taken.

group in substrates of the type A-Phe-Phe-B (24–26). In all cases the introduction of hydrophobic amino acid residues into the A or B segment of such substrates leads to a large enhancement of the sensitivity of the Phe-Phe bond; some of the available data for *Rhizopus*-pepsin and cathepsin D are given in Table II. Moreover, with chymosin (rennin), it has been shown that Leu-Ser-Phe($4NO_2$)-Nle-Ala-OMe and Leu-Ser-Phe($4NO_2$)-Nle-Ala-Leu-OMe are cleaved at pH 4.7 and 30°C with k_{cat}/K_m = 0.11 and 11 mM^{-1} sec^{-1}, respectively, with no change in K_m (27). Addition of a Pro-His unit to the latter peptide caused an increase in the k_{cat}/K_m value to 200 mM^{-1} sec^{-1}, largely due to a 12-fold decrease in K_m (28).

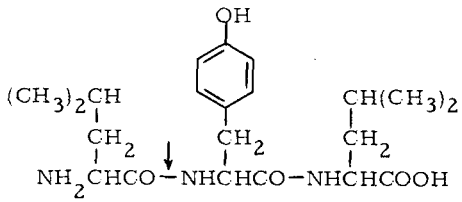
In addition to the three groups of pepsin substrates mentioned above, several others (shown in Fig. 3) have been tested, and the results have suggested hypotheses about the mechanism of pepsin action. Among these other groups are depsipeptide analogs of Z-His-Phe-Phe-OMe, such as Z-His-Phe(4NO_2)-Pla-OMe, for which k_{cat} = 0.8 sec^{-1} and K_m = 0.4 mM at pH 4 and 37°C (20), showing that pepsin can act as an esterase on suitable substrates. Use has also been made of trifluoroacetyl derivatives of aromatic L-amino acids, such as Tfa-Phe, which is cleaved optimally near pH 3.5 with k_{cat} = 0.0006 sec^{-1} and K_m = 16 mM (29). Another type of substrate is exemplified by Leu-Tyr-Leu and Leu-Tyr-



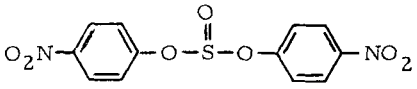
Z-His-Phe(4NO_2)-Pla-OMe



Tfa-Phe



Leu-Tyr-Leu



Bis-p-nitrophenylsulfite

Fig. 3. Other synthetic substrates for pepsin.

TABLE II

Comparative Specificity of Acid Proteinases[a]

Substrate[b]	k_{cat}/K_m ($mM^{-1}sec^{-1}$)	
	Rhizopus-pepsin	Cathepsin D
Z-Phe-Phe-OP4P (26)	0.04	< 0.005
Z-Gly-Phe-Phe-OP4P (26)	1.6	0.07
Z-$(Gly)_2$-Phe-Phe-OP4P (26)	0.5	0.13
Z-$(Gly)_3$-Phe-Phe-OP4P (26)	1.0	0.09
Z-$(Gly)_4$-Phe-Phe-OP4P (26)	0.56	—
Z-Gly-Ala-Phe-Phe-OP4P (26)	5.0	2.0
Z-Ala-Ala-Phe-Phe-OP4P (26)	56	3.6
Z-His-Phe($4NO_2$)-Phe-OMe (25)	0.5	—
Z-His-Phe($4NO_2$)-Phe-Ala-OMe (25)	9.6	—
Z-His-Phe($4NO_2$)-Phe-Ala-Ala-OMe (25)	125	—
Phe-Gly-His-Phe($4NO_2$)-Phe-OMe (24,25)	0.025	< 0.005
Phe-Gly-His-Phe($4NO_2$)-Phe-Ala-Ala-OMe (24)	—	0.1
Phe-Gly-His-Phe($4NO_2$)-Phe-Ala-Phe-OMe (24,25)	10.1	0.4
Phe-Gly-His-Phe($4NO_2$)-Phe-Val-Leu-OME (24)	65	10

[a] pH 3.5–4.0, 37°C.

[b] In all cases cleavage occurred at the Phe-Phe or Phe($4NO_2$)-Phe bond. The numbers in parentheses denote the references from which the data were taken.

NH_2, which are cleaved very slowly (no kinetic parameters are available at present) at the Leu-Tyr bond (30). Finally, considerable attention has been given to the cleavage of sulfite esters by pepsin. This phenomenon, discovered by Reid and Fahrney (31), has been studied extensively; for bis-*p*-nitrophenylsulfite $k_{cat} = 143\ sec^{-1}$ and $K_m = 0.08\ mM$ (32,33).

III. The Mechanism of Pepsin Action

In the cleavage of an amide substrate RCO–NHR′ by a proteinase or peptidase, the mechanism may be considered to involve the following minimum sequence of steps: (*1*) productive binding of the substrate at the active site; (*2*) cleavage of the amide bond; and (*3*) release of the products from the active site.

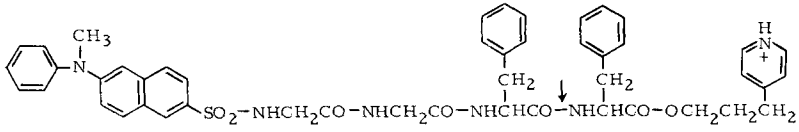
A. BINDING OF THE SUBSTRATE AT THE ACTIVE SITE

The productive reversible interaction of a substrate with the active site of pepsin is characterized by a dissociation constant K_s (or K_D) = k_{-1}/k_1 for the process:

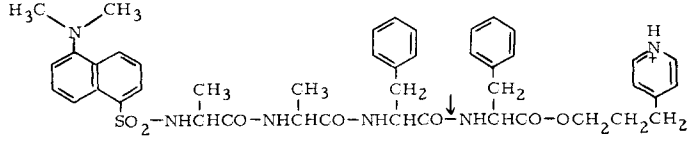
$$E + RCO{-}NHR' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E[RCO{-}NHR']$$

A considerable body of data has been gathered to show that in the action of pepsin on peptide substrates, such as Ac-Phe-Phe (1) and Z-His-Phe-Phe-OMe (2), the value of K_m determined under conditions where $[S]_0 \gg [E]_t$ approximates the value of K_s. This conclusion has recently (34–36) received additional support from results of the study of the interaction of substrates of the type A-Phe-Phe-OP4P, where the A group contains a mansyl or dansyl group (Fig. 4). In aqueous solution, compounds containing either of these groups are only weakly fluorescent, but they become strongly fluorescent when bound to pepsin.

With substances that are completely resistant to pepsin action (e.g., Mns-NH_2, Mns-Gly-Gly-OP4P) or with substrates that are cleaved very slowly (e.g., Mns-Phe-Phe-OP4P), it is possible to perform steady-state fluorescence measurements to determine the fraction of the compound that is bound to pepsin when successively larger amounts of enzyme are added to a constant amount of the compound. If it is assumed that the



Mns-Gly-Gly-Phe-Phe-OP4P



Dns-Ala-Ala-Phe-Phe-OP4P

Fig. 4. Mansyl and dansyl peptide substrates for pepsin.

binding involves a single site that interacts more strongly with the compound under study than do other sites, a Scatchard plot gives an estimate of the value of K_D. In the case of Mns-Phe-Phe-OP4P, K_D was found to be 0.07 mM at pH 2.35 and 25°C; this may be compared with the kinetically determined value of $K_m = 0.095 \pm 0.015$ mM under the same conditions.

The available evidence indicates that the fluorescent probe group of a substrate such as Mns-Phe-Phe-OP4P is drawn into the active site of pepsin by virtue of the interaction of the Phe-Phe unit with complementary active site groups and, in addition, that pepsin has an additional weaker binding locus (or loci) for the mansyl group, distinct from the extended active site of the enzyme. Some of the relevant data are presented in Table III, and it should be noted that the use of pepstatin (Fig. 5) is a key feature of the experiments. From the studies of Umezawa and his associates (37,38) it is known that this inhibitor is bound stoichiometrically at the active site of pepsin to form an enzyme–inhibitor complex whose K_D is approximately 10^{-10} M. The data in Table III indicate that the active site of pepsin has relatively little intrinsic affinity for the mansyl group, as judged by the fact that the increase in fluorescence with Mns-Gly-Gly-OP4P is small and is not altered by the addition of equimolar pepstatin. With Mns-Phe-Phe-OP4P, the large increase in

TABLE III

Interaction of Mansyl Compounds with Pepsin[a]

		Fluorescence[b]			
		+Pepsin		+TPM-pepsin[c]	
Mansyl compound	−Pepsin	−Ptn	+Ptn	−Ptn	+Ptn
Mns-NH_2	0.03(450)	0.20(440)	0.18(440)	—	—
Mns-Gly-Gly-OP4P	0.04(450)	0.22(450)	0.22(450)	0.22(450)	0.22(450)
Mns-Phe-Phe-OP4P	0.05(480)	1.6(435)	0.20(460)	0.60(450)	0.58(450)
Mns-Gly-Phe-Phe-OP4P	0.05(480)	2.9(445)	0.32(465)	—	—

[a] pH 2.35, 25°C. Mansyl compound, pepsin, and pepstatin (Ptn) all at 10 μM.

[b] Expressed in fluorescence units relative to a quinine sulfate standard at emission maximum (in nanometers in parentheses).

[c] Tosyl-L-phenylalanylmethyl-pepsin.

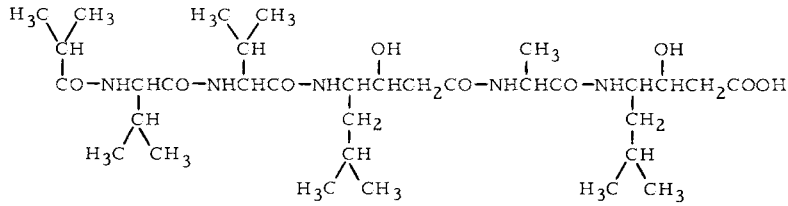


Fig. 5. Structure of pepstatin.

fluorescence was reduced by pepstatin to the value observed with Mns-Gly-Gly-OP4P or mansylamide (35,36).

Further evidence for the conclusion that pepsin has a weak separate binding site for the mansyl group was provided by studies on the change in fluorescence of mansylamide or Mns-Phe-Phe-OP4P in the presence of pepsinogen undergoing activation to pepsin. These experiments showed that, whereas pepsinogen binds the mansyl group of mansylamide more strongly than does pepsin, the reverse is true for Mns-Phe-Phe-OP4P (Fig. 6); this result is concordant with earlier data (39) showing that 6-*p*-toluidino-2-naphthalene sulfonate is bound by pepsin at a locus distinct from the active site. Moreover, with pepsin that had been stoichiometrically inhibited by means of tosyl-L-phenylalanyl diazomethane (40), which combines covalently with a single aspartyl residue (probably Asp-215) at the active site, the fluorescence of both Mns-Gly-Gly-OP4P and Mns-Phe-Phe-OP4P was increased upon the addition of this inactive pepsin derivative (TPM-pepsin), and in neither case was it depressed by pepstatin (Table III). The greater fluorescence of Mns-Phe-Phe-OP4P in the presence of TPM-pepsin as compared with that in the presence of untreated pepsin is not a consequence of tighter binding, since the K_D values (at pH 2.35 and 25°C) for the complexes of the mansyl peptide with TPM-pepsin and with untreated pepsin are 0.7 m*M* and 0.07 m*M*, respectively (35). It would appear, therefore, that in the diazoketone-modified pepsin the protein conformation had been altered so as to decrease the polarity of the separate binding site for the mansyl group to an extent sufficient to overcome the reduced binding affinity. These results show that when the mansyl group of a pepsin substrate is excluded from the active site either by pepstatin or by blockage of the active site with the tosyl-L-phenylalanyl methyl group, it can interact with a binding locus distinct from the active site of pepsin. It may be added that

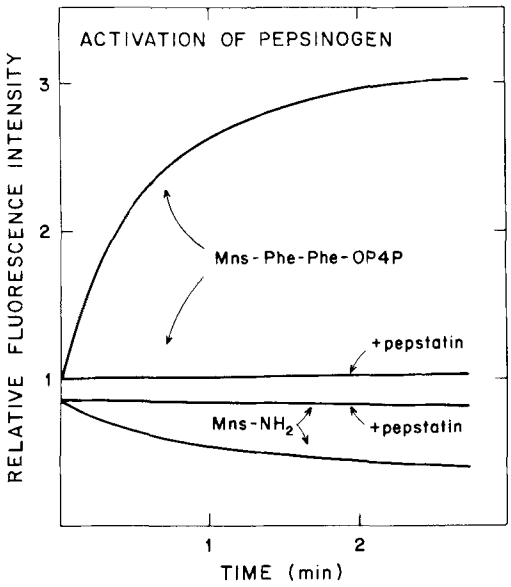


Fig. 6. Fluorescence of Mns-NH_2 and of Mns-Phe-Phe-OP4P in the presence of pepsinogen undergoing activation at pH 2.35 and 25°C. Initial concentrations of pepsinogen, mansyl compound, and pepstatin (when present), 10 μM.

this separate binding site is not as readily detectable with dansylamide, since the dansyl group appears to be a much less sensitive probe.

The fluorescence data thus support the view that a substrate such as Mns-Gly-Phe-Phe-OP4P interacts more strongly with the active site of pepsin than it does with other potential binding sites on the protein. This is in agreement with earlier results of gel-filtration (41,42) and inhibition (43) studies showing that the Phe-Phe unit of cationic pepsin substrates makes the major contribution to their binding at the active site.

As noted above, estimates of K_D from a Scatchard plot for the binding to pepsin of a relatively resistant mansyl peptide substrate gave a value similar to that obtained for K_m under conditions of $[S]_0 \gg [E]_t$. With substrates that are cleaved more rapidly, it is possible to conduct stopped-flow measurements of the rate of decrease of fluorescence under conditions where $[E]_t \gg [S]_0$. When the affinity of the fluorescent cleavage product for the active site is much less than that of the substrate, a first-order decrease in fluorescence intensity is observed (Fig. 7), and the

resulting rate constant (k_{obs}) can be used to estimate both K_s and k_2 in the process:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + \text{products}$$

by means of the equation $k_{obs} = k_2[E]_t/(K_s + [E]_t)$, as shown for several proteolytic enzymes by Kezdy and Bender (44) and others (32,45–47). When such estimations of K_s were performed, the values obtained were in all cases the same (within the precision of the measurements) as the K_m values estimated from kinetic measurements under conditions where $[S]_0 \gg [E]_t$ (48). In one instance, that of Mns-Gly-Phe-Phe-OP4P, the values of K_m, K_D (from a Scatchard plot), and K_s (stopped-flow measurements under conditions of $[E]_t \gg [S]_0$) were the same, namely, 0.03 ± 0.01 mM at pH 2.35 and 25°C. In the special case of Mns-Ala-Ala-Phe-Phe-OP4P, an estimation of K_s and k_2 for the cleavage of this substrate by pepsin was not possible, because the cleavage product Mns-Ala-Ala-Phe is bound at the active site with the same affinity (K_D = 0.065 mM) as the substrate (K_m = 0.065 mM) and no fluorescence change is ob-

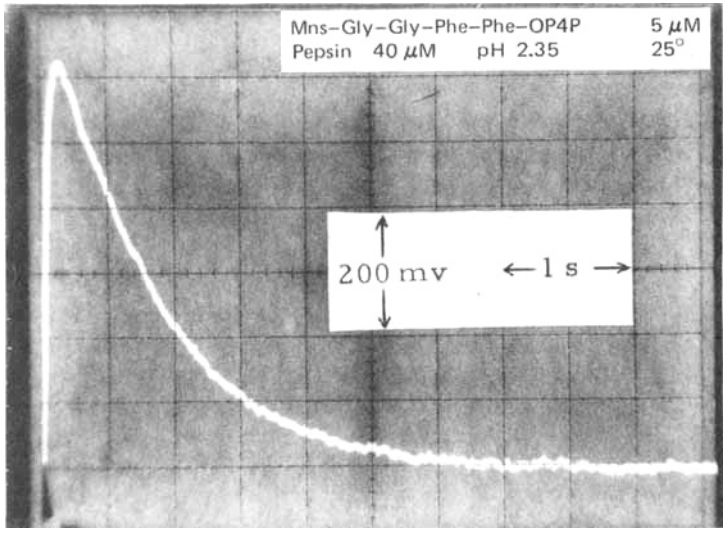


Fig. 7. Time course of the change in fluorescence during the cleavage of Mns-Gly-Gly-Phe-Phe-OP4P by pepsin.

served during the rapid hydrolysis of the Phe-Phe bond (k_{cat} = 112 sec^{-1}). With Dns-Ala-Ala-Phe-Phe-OP4P, however, the cleavage product Dns-Ala-Ala-Phe is bound at the active site much less strongly than the substrate, and values of K_s = 0.06 mM and k_2 = 430 sec^{-1} could be estimated from stopped-flow experiments under conditions where $[E]_t \gg [S]_0$. Some of the available data are collected in Table IV; it is noteworthy that the values of K_s for substrates whose k_2 values range between 0.1 and 430 sec^{-1} are all remarkably similar (0.03–0.11 mM). The small difference in pH in the experiments with mansyl compounds (pH 2.35) and dansyl compounds (pH 3.1) was a consequence of the sparing solubility of the former at pH values above 2.5 and the weak fluorescence of the latter at pH values below 3.

It can be seen in Table IV that, whereas with Dns-Gly-Gly-Phe-Phe-OP4P k_{cat} equals k_2 within the precision of the measurements, k_2 was found to be much greater than k_{cat} with the more sensitive substrates Dns-Gly-Ala-Phe-Phe-OP4P and Dns-Ala-Ala-Phe-Phe-OP4P. Despite efforts to exclude possible sources of error in the determination of k_{cat} at very low enzyme concentrations, such as adsorption on glass (49), this difference was found repeatedly, and the simple mechanism involving only K_s, k_2, and k_3, considered to apply to the action of chymotrypsin

TABLE IV

Kinetics of Pepsin Action on Mansyl and Dansyl Peptides[a]

Substrate	$[S]_0 \gg [E]_t$		$[E]_t \gg [S]_0$	
	k_{cat} (sec^{-1})	K_m (mM)	k_2 (sec^{-1})	K_s (mM)
Mns-Phe-Phe-OP4P	0.002	95	—	—
Mns-Gly-Phe-Phe-OP4P	0.13	37	0.10	34
Mns-Gly-Gly-Phe-Phe-OP4P	16	75	13	94
Dns-Gly-Gly-Phe-Phe-OP4P	4.4	109	4.6	97
Dns-Gly-Ala-Phe-Phe-OP4P	34	60	146	57
Dns-Ala-Ala-Phe-Phe-OP4P	91	54	430	57
Mns-Ala-Ala-Phe-Phe-OP4P	112	65	—	—

[a] pH 2.35 for mansyl compounds, pH 3.1 for dansyl compounds; 25°C. The kinetics under steady-state conditions ($[S]_0 \gg [E]_t$) were performed by following the formation of Phe-OP4P by means of the fluorescamine reaction, and those under conditions of $[E]_t \gg [S]_0$ by means of stopped-flow fluorescence spectroscopy (48).

(44), is not sufficient to explain the action of pepsin on these two substrates, since if k_2 is much greater than k_{cat}, K_s should be much smaller than K_m. Further work is needed to resolve this question, but one possibility is that a step after the one reflected in k_2 becomes kinetically significant when k_2 is relatively large. As is suggested later in this review, such a result may indicate the occurrence of a conformational change at the active site associated with the release of the products of hydrolysis.

The data summarized above indicate, therefore, that with A-Phe-Phe-OP4P peptide substrates of pepsin, the value of K_m for the formation of Phe-OP4P, determined under steady-state conditions ($[S]_0 \gg [E]_t$), closely approximates the dissociation constant K_s of the initial enzyme–substrate complex, as estimated by the determination by fluorescence spectroscopy of the rate of formation of the A-Phe product. This result strengthens the validity of the conclusions that the rate-limiting step in the overall catalytic process under conditions where $[S]_0 \gg [E]_t$ is the decomposition of the first detectable enzyme–substrate complex and that no kinetically significant intermediate accumulates in the process.

The association of pepsin with a mansyl (or dansyl) peptide substrate to produce a fluorescent species is extremely rapid, and stopped-flow measurements could not give reliable estimates of k_1. Under conditions of enzyme excess, the half-time for the association process is near the dead time (about 3 msec) of the Durrum stopped-flow spectrophotometer, and it can only be stated that the calculated second-order rate of association is greater than $10^6\ M^{-1}\ \text{sec}^{-1}$ under the conditions employed (48). In this connection it may be added that the available kinetic data on pepsin substrates of the type A-Phe-Phe-OP4P make it unlikely that synthetic substrates will be found that are much more reactive than compounds such as Z-Ala-Ala-Phe-Phe-OP4P, since the k_{cat}/K_m value for this peptide ($7 \times 10^6\ M^{-1}\ \text{sec}^{-1}$) approaches the limit set by the value of k_1.

From data such as those presented in Tables I, II, and IV, there can be little doubt that pepsin and other acid proteinases possess an extended active site that can accommodate more than the two amino acid residues forming the sensitive bond. In the absence of information about the detailed three-dimensional structure of pepsin, however, estimates of the size of the active site must be considered to be provisional. From a study of the kinetic parameters for the cleavage of a series of substrates having the structure Z-$(\text{Gly})_n$-Phe-Phe-OP4P, where n = 0–4, it was suggested that the active site of pepsin might accommodate a peptide of seven amino acid residues (26); in a fully extended conformation, such a peptide

would be about 25 A long. If, in the enzyme–substrate complex, the substrate is held in some other conformation by virtue of the primary interactions at the catalytic site and the secondary interactions of the substrate with other parts of the extended active site, the length of the active site may not correspond to that of a fully extended heptapeptide.

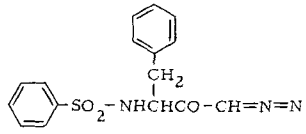
It will be of interest to learn from X-ray diffraction data the nature of the amino acid residues lining the extended active site of pepsin, and presumably involved in the secondary interactions with oligopeptide substrates. The active-site region appears to include Tyr-9 and Tyr-174 in the sequence shown in Figure 1, since these two residues are preferentially iodinated by I_3^- (50), and since such iodination causes the loss of the proteinase, peptidase, and esterase activity of pepsin on suitable substrates (51). It is noteworthy that acetylation of tyrosyl residues by means of acetyl imidazole decreases the proteinase activity but markedly enhances k_{cat} for the cationic substrate Z-His-Phe-Phe-OEt (51). Although the two tryptophan residues of pepsin that are accessible to the attack of 2-hydroxy-5-nitrobenzyl bromide do not appear to be directly involved in the catalytic mechanism, the fact that a significant loss of activity (25–30%) occurs (52) leaves open the possibility that these residues are present in the extended active site of the enzyme.

As noted above, a striking feature of the kinetics of pepsin action on a series of closely related substrates of widely different susceptibility (as measured by k_{cat}) is the relative invariance of K_s, the dissociation constant of the initial enzyme–substrate complex. Clearly, the specificity of pepsin action is expressed in the value of k_{cat}, and not in K_m (in this case equal to K_s). This suggests the possibility that complementary conformational changes in the enzyme and substrate may be associated with a lowering of the energy of activation in the bond-breaking process (21), a view made more plausible by recent theoretical considerations of the contribution of the entropy loss in the formation of the enzyme–substrate complex to the energy required to reach the transition state (53–56). In this connection attention should also be drawn to the possibility that in the association of an oligopeptide substrate with the active site of pepsin there occurs a process of conformational selection in which there is a stepwise binding of the substrate molecule with the active site. In the case of substrates of the type A-Phe-Phe-OP4P, the initial "nucleation" step may be considered to involve the interaction of the Phe-Phe segment with the active site, followed by a cooperative process in which the remaining segments of the oligopeptide are drawn into the site. As has been noted

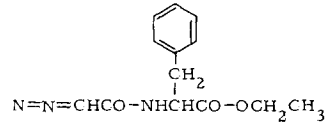
by Burgen et al. (57), if such a process of mutual conformational adjustment of both the substrate and the active site occurs in discrete successive steps, the overall activation energy in the association process may be lower than in the interaction of a substrate with a rigid active site.

B. CLEAVAGE OF THE PEPTIDE BOND

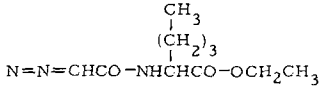
Considerable evidence is available from studies on the pH dependence of pepsin action (14,58–60) and on the chemical modification of pepsin by active-site-directed diazo compounds (40,61–63), epoxides (64,65), and trimethyl oxonium fluoroborate (66) to indicate that at least two carboxyl groups of the enzyme are directly involved in the mechanism of the bond-breaking step. Some of the reagents used are shown in Figure 8. Tang et al. (7) have proposed that the two reactive carboxyl groups are those of Asp-32 (sensitive to epoxides) and Asp-215 (sensitive to diazo compounds) in the sequence they deduced for porcine pepsin. It appears likely that one of these two carboxyl groups is protonated and that the other is in the form of the carboxylate anion. These inferences, based



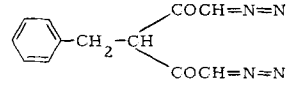
Tosyl-L-phenylalanyl diazomethane



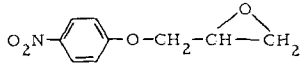
Diazoacetyl-L-phenylalanine ethyl ester



Diazoacetyl-DL-norleucine ethyl ester



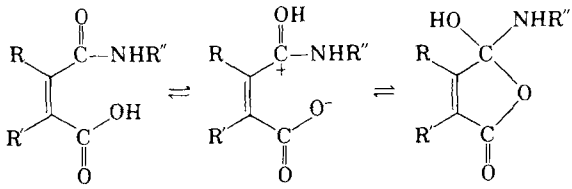
1,1-Bis(diazoacetyl)-2-phenylethane



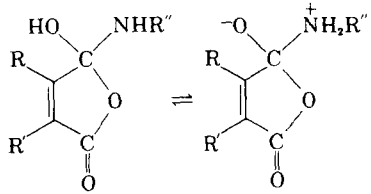
1,2-Epoxy-3(p-nitrophenoxy)propane

Fig. 8. Active-site-directed inhibitors of acid proteinases.

largely on the behavior of model systems, such as the intramolecular hydrolysis of phthalamic acid (70), have led to several suggestions regarding the mechanism of pepsin action (1–4,29,67–69). In particular, Knowles (1) has noted that the similarity of the rates of hydrolysis of the ester bond in Z-Phe(4NO_2)-Pla-OMe and of the corresponding peptide (20) is consistent with a mechanism involving acid catalysis. More recently, important studies by Kirby and his associates (71,72) on the hydrolysis of dialkylmaleamic acids have provided an especially attractive model for pepsin action. According to this model, the attack at the amide bond is initiated by a neighboring carboxyl group that participates as a nucleophilic catalyst with the formation of a tetrahedral intermediate:

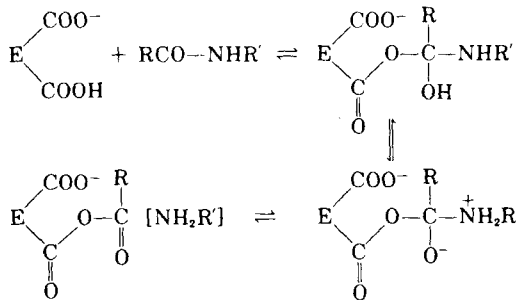


A key feature of the model is the role of an additional carboxyl group (in its carboxylate form) in promoting the interconversion of the neutral and dipolar ionic forms of the tetrahedral intermediate:



According to this model, therefore, the second carboxylate group catalyzes the proton transfer necessary to form the tetrahedral intermediate that undergoes cleavage to the anhydride and the amine product. Presumably the enzymatic group providing this carboxylate ion has a pK_a near 1, as in cyclobutene-1,2-dicarboxylic acid (73). In the model system the anhydride is then hydrolyzed in a process that is subject to specific-acid catalysis by H_3O^+. As applied to the problem of pepsin catalysis, this model suggests, therefore, at least three steps from the

initial enzyme–substrate complex to the formation of a complex composed of an acyl-enzyme and the amine product.



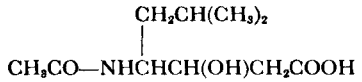
Clearly, any of one of these steps may be rate limiting, and, depending on whether or not proton transfer from water is involved in that step, a D_2O solvent isotope effect will or will not be observed. With pepsin substrates such as Ac-Phe-Tyr-OMe and methyl phenyl sulfite, no D_2O effect has been observed (31,74), whereas with Gly-Gly-Gly-Phe(NO_2)-Phe-OMe and Tfa-Phe, a significant effect has been found [$k_{cat}(H_2O)/k_{cat}(D_2O)$ = 2–3] (29,75). As noted by Knowles (1), the apparent absence of a solvent isotope effect in some cases should not be used for the formulation of a general mechanism of pepsin action, since it does not appear likely that there are no rate-limiting proton transfers in an enzymatic process that resembles acid-catalyzed reactions as much as the reaction catalyzed by pepsin does.

In the proposed mechanism for the carboxylate-assisted cleavage of dialkylmaleamic acids, a decisive role is played by the protonation of the nitrogen in the amine component of the sensitive amide bond. If this mechanism is operative in pepsin catalysis, it may be expected that if all other specificity requirements are met, the pK_a value of the amine product should be related to the rate of bond cleavage. In this connection it may be noted that upon the replacement of the Phe-OMe unit of Z-His-Phe-Phe-OMe by a L-phenylalaninol (Pol) unit, the resulting Z-His-Phe-Pol is resistant to pepsin action and is a competitive inhibitor with a K_i value near the K_m value for Z-His-Phe-Phe-OMe (43). The pK_a values for Phe-OMe and phenylalaninol are approximately 7 and 10, respectively. The possibility has not been excluded, however, that the resistance of Z-His-Phe-Pol is a consequence of the specific requirement,

in pepsin substrates, of a carbonyl group on the amine side of the sensitive peptide bond. It will be of interest to examine the susceptibility of substrates such as Z-His-Phe-Phe-CH_3, where the terminal methoxy group has been replaced by a methyl group.

In this connection it is noteworthy that *O*-acetyl-β-phenyl-L-lactyl-L-phenylalanine appears to be resistant to cleavage by pepsin under conditions where Ac-Phe-Phe is cleaved (76). The depsipeptide analog is bound at the active site, as judged by its ability to inhibit the hydrolysis of Ac-Phe-Tyr competitively with a K_i of 2 mM, a value near the K_m for Ac-Phe-Phe. It seems likely, therefore, that in the formation of the reactive complex leading to bond cleavage, both the *N*-terminal NH group and the *C*-terminal CO group of the sensitive dipeptidyl unit (e.g., Phe-Phe) in the substrate interact with complementary groups at the active site of the enzyme.

In the cleavage of a peptide bond, the transition state may be expected to resemble a tetrahedral intermediate having the structure of an α-amino alcohol. Recent studies have shown that suitable aldehydes are effective inhibitors of several proteinases (37,77,78), and it has been proposed that their hydrates are transition-state analogs (79,80). In the case of pepsin it has been suggested (81) that the potent pepsin inhibitor pepstatin acts by virtue of this principle and that the compound "acetyl statine" (derived from pepstatin)



is an analog of the transition state in pepsin catalysis.

C. IS THERE AN ACYL-ENZYME INTERMEDIATE?

With the early recognition that the rate-limiting step in the overall catalytic process is associated with the decomposition of the first detectable enzyme–substrate complex, efforts have been made to detect intermediates formed after this step by means of trapping reactions. The demonstration (82,83) that pepsin catalyzes the exchange of ^{18}O upon incubation of the enzyme with acylamino acids, such as Ac-Phe with $H_2{}^{18}O$, suggested a mechanism similar to that operative in the case of chymotrypsin. However efforts to trap the presumed acyl-enzyme (in the case of pepsin an acid anhydride) with ^{14}C-labeled methanol have been unsuccessful (84), in contrast to the effectiveness of this method for detect-

ing acyl-enzyme formation in the cases of chymotrypsin and papain (85,86). To explain his negative result, Knowles (1) has proposed that the pepsin-catalyzed ^{18}O exchange of Ac-Phe with $H_2{}^{18}O$ occurs as a consequence of the prior labeling of an enzymatic carboxyl group, since it has been shown (87) that ^{18}O is rapidly incorporated from $H_2{}^{18}O$ into pepsin and that the rate of loss of isotope from previously labeled enzyme is nearly the same as the rate of ^{18}O incorporation into Ac-Phe. This explanation has also been invoked by Silver et al. (88).

The possibility that acyl-enzyme intermediates are involved in pepsin catalysis has been raised anew, however, by the finding that porcine pepsin and penicillopepsin catalyze the transfer of the *N*-terminal leucyl residue of Leu-Tyr-Leu and Leu-Tyr-NH_2 to the substrate to form chromatographically detectable amounts of the Leu-Leu-Tyr-X products, which are then predominantly cleaved to yield Leu-Leu (30,89), as shown in the reactions below. The report that the action of pepsin on

$$\text{Leu-Tyr-Leu} + \text{E} \rightleftharpoons [\text{Leu}]\text{E} + \text{Tyr-Leu}$$

$$[\text{Leu}]\text{E} + \text{Leu-Tyr-Leu} \rightleftharpoons \text{Leu-Leu-Tyr-Leu} + \text{E}$$

$$\text{Leu-Leu-Tyr-Leu} \xrightarrow{\text{E}} \text{Leu-Leu} + \text{Tyr-Leu}$$

Leu-Tyr-Leu leads to the formation of Leu-Leu has been confirmed in experiments by Newmark and Knowles (90), who prepared a sample of the tripeptide labeled with ^{14}C in the amino-terminal residue and with ^{3}H in the carboxyl-terminal residue. The finding that 80–90% of the resulting Leu-Leu is [^{14}C]Leu-[^{14}C]Leu is consistent with the intermediate formation of a [^{14}C]Leu-pepsin that can react with another molecule of the tripeptide to form Leu-Leu-Tyr-Leu, which is cleaved to Leu-Leu and Tyr-Leu. The remainder of the labeled Leu-Leu appears to be [^{3}H]Leu-[^{3}H]Leu, whose formation would be consistent with enzymatic cleavage of the Tyr-Leu bond to form an amino-enzyme.

The principal evidence for the view that a covalent leucyl-enzyme intermediate is formed during the action of pepsin on Leu-Tyr-Leu is the reported absence of incorporation of [^{14}C]leucine into Leu-Leu when the labeled amino acid is added to the incubation mixture (30). The validity of the interpretation of this result rests on the assumption that exchange of the added [^{14}C]leucine with the free leucine generated in the active site is more rapid than the reaction of the latter with Leu-Tyr-Leu.

The hydrolysis of sulfite esters by pepsin appears to involve the same active site as that responsible for the cleavage of peptide substrates, since the hydrolysis of diphenylsulfite is inhibited by Ac-Phe and Ac-Phe-

Tyr(Br_2) (91). It seems likely that a carboxylate group of pepsin attacks the strongly electrophilic sulfur atom of the sulfite to produce an intermediate acyl-enzyme (in this case, an acid anhydride of sulfurous acid and a carboxylic acid). Further studies are needed, however, to determine whether the same active-site groups are responsible for the scission of the sulfite and peptide substrates. Similar questions may be raised in regard to the peptic cleavage of Tfa-Phe (29); the slow hydrolysis of this compound, as compared with the resistance of Ac-Phe, also is consistent with the attack of an enzymatic carboxylate group at the more electrophilic carbonyl carbon of Tfa-Phe.

D. IS THERE AN AMINO-ENZYME INTERMEDIATE?

Much attention has also been given to the proposal (92,93) that pepsin catalyzes the transfer of the amine portion of a substrate to a carboxylic acceptor, with the intermediate formation of an amino-enzyme.

$$\text{RCO—NHR}'' + \text{E} \rightleftharpoons \text{E[RCO—NHR}'] \rightleftharpoons \text{E—NHR}' + \text{RCOOH}$$
$$\text{R}''\text{COOH} + \text{E—NHR}' \rightleftharpoons \text{R}''\text{CO—NHR}' + \text{E}$$

Numerous reports (94–97) have confirmed and extended the finding that acyl dipeptides, such as Z-Phe-Tyr, readily undergo such transpeptidation reactions, with the formation (in this case) of detectable amounts of Tyr-Tyr (arising from the cleavage of the transpeptidation product Z-Phe-Tyr-Tyr). In particular, Antonov et al. (97) have used a spectrophotometric method for following pepsin-catalyzed transpeptidation with Z-Phe($4NO_2$) as the acceptor molecule. This method takes advantage of the decrease in the absorbance at 310–320 nm of the *p*-nitrophenylalanyl residue when the carboxylate group is replaced by a carboxamide (20). An estimate has been made of the relative rates of the hydrolysis of Ac-Phe-Tyr (or Ac-Tyr-Tyr) and of the formation of Z-Phe($4NO_2$)-Tyr from these substrates. It is noteworthy that these rates are extremely slow. For Ac-Phe-Phe $k_{cat} = 0.03$ sec^{-1} and the rate of formation of Z-Phe($4NO_2$)-Tyr is about 0.0005 sec^{-1} at pH 4.6 and 37°C.

If an amino-enzyme is indeed an intermediate, it may be expected that under suitable conditions ([S] $\gg K_m$ of the substrate, [S] $\gg$ [E], and [E] relatively high) a "burst" release of the acyl product would be evident prior to the establishment of the steady-state rate. Efforts to detect such a burst spectrophotometrically have been unsuccessful with Z-His-

Phe($4NO_2$)-Phe-OMe (20) and with *N*-acetyl-3,5-dinitro-L-tyrosyl-L-phenylalanine (84).

With some esters or amides of Ac-Phe-Phe, however, no transpeptidation (with ^3H-labeled Ac-Phe as the acceptor) could be found (96). Antonov et al. (97) have suggested that in this case the initial rate of hydrolysis is so much greater than the rate of transpeptidation that no transfer product is detectable, and it has been reported (97a) that the γ-morpholinopropyl amide of Ac-Phe-Phe undergoes a transpeptidation reaction with Z-Phe($4NO_2$) as the acceptor. An alternative explanation may be that the amine product (e.g., Phe-OEt) can be acylated more readily than the corresponding free amino acid by an enzymatic carboxyl group that is not directly involved in the catalytic mechanism. It has been reported that upon prolonged incubation (1 hr) of pepsin with ^{14}C-labeled Tyr-OEt at pH 5, a covalent bond is formed between the enzyme and Tyr-OEt (98), and that the enzymatic carboxyl group bearing the label is not the one that is alkylated by diazophenylalanine ethyl ester (99).

Aside from the apparent failure of substrates such as Ac-Phe-Phe-OEt to undergo transpeptidation with ^3H-labeled Ac-Phe, additional evidence against the formation of a covalent amino-enzyme during pepsin catalysis has come from studies with a series of substrates of the type A-Phe-Trp, where A = Ac, Z-His, Ac-Gly-Gly, or Z-Ala-His. Silver and Kelleher (99a) have found that the ratio of labeled Ac-Phe-Trp formed at pH 4.5 in the presence of ^{14}C-labeled Ac-Phe to the tryptophan formed by hydrolysis is not constant for this series of substrates, as would be expected from the amino-enzyme hypothesis. Indeed, the substrate that is hydrolyzed most rapidly (A = Z-Ala-His) gave no detectable transpeptidation under the conditions of these experiments.

An analogous result has been obtained from the comparison of the kinetic parameters for the pepsin-catalyzed cleavage of substrates of the type A-Phe($4NO_2$)-Tyr, where A = Z, Z-Gly, and Z-Gly-Gly (100). The role of secondary interactions is evident from the fact that these three substrates are hydrolyzed with a progressively greater k_{cat} (in the order named) but with a similar K_m. Moreover, the increase in the relative rates of cleavage of the substrates of the type A-Phe($4NO_2$)-Tyr are correlated with a decrease in the relative effectiveness of Z-Phe($4NO_2$), Z-Gly-Phe($4NO_2$), and Z-Gly-Gly-Phe($4NO_2$) as "acceptors" in the transpeptidation reaction with Ac-Phe-Tyr as the substrate. These results, taken together with those of Silver and Kelleher, are clearly incompatible

with a mechanism in which a common E-Tyr intermediate is formed. Instead they suggest that the nature of the A-Phe or A-Phe($4NO_2$) product influences the rate of departure of the amine product. At present this suggestion can only be offered as a working hypothesis, and much further work is needed.

As noted above, most of the hypotheses regarding acyl-enzyme or amino-enzyme intermediates in pepsin catalysis have involved the assumption that these intermediates represent compounds in which an acyl group or an amino portion of the substrate is covalently linked to an active-site carboxyl group. In particular, hypotheses have been offered (1,2) suggesting a four-center rearrangement for the conversion of a presumed acyl-enzyme into an amino-enzyme, or the participation of a third carboxyl group to which the amine portion of the substrate is transferred (29). Consideration has also been given (3,90), however, to the possibility that covalent intermediates may not be kinetically significant components in the action of pepsin on peptide substrates, and that it is the sequence of the departure of the two products that may be important.

E. ORDER OF THE RELEASE OF PRODUCTS

It is implicit in the acyl-enzyme mechanism of the enzymatic cleavage of an amide bond that the formation of the acyl-enzyme is accompanied by the rapid release of the amine product. By the same token, in the amino-enzyme mechanism, it is assumed that the carboxylic product leaves the active site, leaving an amino-enzyme that can react with an alternative carboxylic acid to form the transpeptidation product. However, attempts to detect by direct experimental means the sequential release of the products of a pepsin-catalyzed reaction have thus far been largely unsuccessful. For the series of substrates of the type A-Phe-Phe-OP4P, where the A group bears an amino-terminal mansyl or dansyl group, it is possible to conduct stopped-flow fluorescence measurements under conditions of $[E]_t \gg [S]_0$ and to determine the rate of conversion of the initial enzyme–substrate complex to the equilibrium established between the acidic product and its complex with pepsin. For example, with Dns-Gly-Gly-Phe-Phe-OP4P, $K_s \approx 0.1$ mM, and the dissociation constant of the complex of pepsin with Dns-Gly-Gly-Phe is about 0.3 mM (48). Consequently, there is a decrease in the fluorescence of the reaction mixture when the enzyme–substrate complex is converted to enzyme and products. Stopped-flow measurements have shown this decrease to follow

strictly first-order kinetics, with no evidence of any biphasic behavior. A plot of the resulting $1/k_{obs}$ values against $1/[E]_t$ gave values of k_2 that agreed (within the limits of experimental error) with the k_{cat} values obtained under conditions of $[S]_0 \gg [E]_t$ for the release of Phe-OP4P (Table IV). This applied to substrates with $k_2 = k_{cat}$ values ranging from 0.1 to 16 sec^{-1}. It may be inferred, therefore, that under the conditions of these studies, both products leave the active site at the same time. It may be noted that in the pepsin-catalyzed cleavage of bis(*p*-nitrophenyl)sulfite, the two products (*p*-nitrophenol and sulfite) are also liberated concurrently (33).

One of the items of evidence offered in support of the intermediate formation of an amino-enzyme was the observation that the hydrolysis of Ac-Phe-Phe-Gly at pH 2.1 is subject to linear noncompetitive inhibition by Ac-Phe, as well as by Ac-Phe-OEt and Ac-Phe-NH_2 (101). This result was deemed to be consistent with the view that the inhibitor combines with the amino-enzyme, in line with the general formulations of Cleland (102). Recent kinetic studies (103) have cast doubt on this interpretation, as they have shown that the pattern of noncompetitive inhibition of the hydrolysis of a series of substrates A-Phe-Trp (A = Ac, Z-His, Z-Ala-His) is inconsistent with the intermediate formation of a unique amino-enzyme (E-Trp), but that Ac-Phe appears to bind to the initial enzyme–substrate complex or some other intermediate that precedes the rate-limiting step. It should be noted that near pH 4.5, where transpeptidation is observed, acyl products such as Ac-Phe or Z-His-Phe are linear competitive inhibitors of pepsin (43,101), suggesting that the anion of the carboxylic product can combine significantly only with free enzyme. Moreover, the amine products (e.g., Phe-OMe) are linear competitive inhibitors at all pH values over the range pH 1.8–4.5 (16,43), as are the L-enantiomers of Ac-Phe, Ac-Phe-OEt, and Ac-Phe-NH_2 (101). The K_i value for each of these L compounds is equal (within the precision of the measurements) to that of the corresponding D compound at a given pH.

The conclusions drawn by Silver and Stoddard (103) from the pattern of inhibition of the hydrolysis of substrates of the type A-Phe-Trp may help to explain the finding (100) that the relative effectiveness of Z-Phe($4NO_2$), Z-Gly-Phe($4NO_2$), and Z-Gly-Gly-Phe($4NO_2$) as "acceptors" of the tyrosine of Ac-Phe-Tyr decreases in the order indicated, and that the corresponding substrates, Z-Phe($4NO_2$)-Tyr, Z-Gly-Phe($4NO_2$)-Tyr, and Z-Gly-Gly-Phe($4NO_2$)-Tyr, are hydrolyzed at progressively higher rates. It would appear that the nature of the A-Phe($4NO_2$) group

has a marked effect on the tendency of the tyrosine generated in the cleavage reaction to leave the active site. The order of release of the products in a pepsin-catalyzed reaction may depend, therefore, on the structure of both fragments and may in some cases involve the apparent formation of an amino-enzyme or acyl-enzyme intermediate, neither of which involves covalent linkage of the preferentially held fragment to an enzymatic group. In the light of the previous discussion of the possible relation of conformational changes at the active site of pepsin to its catalytic efficiency, it may be suggested that the A-Phe and Phe-B fragments of a substrate of the type A-Phe-Phe-B affect the conformation of a large segment of the extended active site of pepsin in such a manner as to affect the order of release of the products of the hydrolytic process.

An item of evidence consistent with the ordered release of products and the intermediate formation of an amino-enzyme is the report (104) that at pH 4.7 Ac-Phe-Tyr-OEt undergoes pepsin-catalyzed isotopic exchange with ^{14}C-labeled Ac-Phe under conditions of stoichiometric equilibrium (105). This result is incompatible with the apparent failure of Ac-Phe-Tyr-OEt to undergo transpeptidation in the presence of high concentrations of ^{14}C-labeled Ac-Phe, and confirmation of the data reported in reference 104 is needed.

At present, therefore, the question of the ordered release of products, with the intermediate formation of either an amino-enzyme or an acyl-enzyme, appears to be unresolved. The prior release of the $RCOO^-$ product derived from RCO–NHR′ is suggested only by experiments in which substrates such as Ac-Phe-Tyr, Z-Phe($4NO_2$)-Tyr and Ac-Phe-Phe-Gly have been used, and the prior release of the $^+NH_3R'$ has been inferred from experiments with substrates such as Leu-Tyr-Leu and Leu-Tyr-NH_2. All these compounds are cleaved much more slowly than the cationic oligopeptide substrates of the type A-His-Phe-Phe-B or A-Phe-Phe-OP4P, and efforts to discern an ordered release of products from the latter substrates have been unsuccessful thus far.

IV. Some Speculations for Future Research

In the following pages an attempt is made to reformulate the problem of the mechanism of pepsin action in the light of available knowledge and to consider possible experimental approaches to the resolution of the present dilemma. It will be useful to summarize briefly the salient points regarding the specificity of pepsin.

1. In the action of pepsin on small synthetic substrates [e.g., Ac-X-Y, Z-His-X-Y-OMe, Z-$(Ala)_2$-X-Y-OP4P], where X and Y are L-amino acid residues forming the sensitive bond, the nature of both X and Y is important for catalysis, and aromatic protein amino acids are preferred (e.g., X-Y = Phe-Trp). With longer substrates of the type P-X-Y-Q, suitable P and Q groups may make a relatively resistant X-Y bond more susceptible to cleavage. In Z-His-X-Y-OMe or Ac-X-Y, if either X or Y is a D-enantiomer, the X-Y bond is resistant to cleavage and the compound is a competitive inhibitor with K_i equal (within experimental error) to the K_m for the L,L-substrate.

2. Pepsin appears to have an extended active site that may accommodate a heptapeptide. The secondary interaction of structural elements of the P and Q groups of a substrate P-X-Y-Q with a region of the active site relatively distant from the locus of catalytic action may markedly change k_{cat} without comparable change in K_m.

3. In all cases studied thus far, the value of K_m estimated under conditions where $[S]_0 \gg [E]_t$ closely approximates the value of K_s estimated from equilibrium binding studies or from kinetic experiments under conditions where $[E]_t \gg [S]_0$. For a series of closely related substrates (e.g., A-Phe-Phe-OP4P) for which the k_{cat} values are widely different, the K_s values are relatively invariant.

The evidence summarized in the three preceding paragraphs suggests that the rate-limiting step in the cleavage of peptide substrates by pepsin is associated with the transformation of the initial enzyme–substrate complex and that the specificity of binding is directly related to the efficiency of the bond-breaking reaction. Moreover, the possibility must be considered that the estimated K_s (or K_m) values correspond to a fraction of the total binding energy in the productive enzyme–substrate interaction and that the remainder is utilized in the attainment of the transition state for the bond-breaking step (54). Thus, if for the cleavage of a pair of closely related substrates, such as Z-Gly-Ala-Phe-Phe-OP4P and Z-Gly-Pro-Phe-Phe-OP4P, k_{cat} = 410 and 0.06 sec^{-1}, respectively, but K_m = 0.1 m*M* for both compounds, it may be suspected that the total binding energy in the productive interaction of pepsin with the Ala-containing peptide is much greater than with the Pro-containing compound. According to this hypothesis, therefore, the value of K_s (=K_m) estimated from kinetic measurements represents a much larger fraction of the total energy change in the binding of Z-Gly-Pro-Phe-Phe-OP4P than in the binding

of Z-Gly-Ala-Phe-Phe-OP4P. One of the ways in which the energy of enzyme–substrate interaction may contribute to the energy needed to reach the transition state is through changes in the conformation of both the enzyme and the substrate, with possible induction of strain at the CO–NH bond positioned at the catalytic site. If we assume that, in the case of closely related pepsin substrates, the K_s corresponding to the binding energy of a rigid active site with each of the substrates is roughly the same, the differences in catalytic efficiency (as measured by k_{cat}) may then be a consequence of differences in the extent to which particular substrates can induce, at the catalytic site, conformational changes favorable to catalysis. This implies that the enzyme can exist in at least two conformations (E and E*) of different stability and that between the initial enzyme–substrate complex and the transition state, an additional reversible step (or steps) must be considered:

$$\mathrm{E} + \mathrm{RCO{-}NHR'} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E[RCO{-}NHR']} \underset{k^*_{-1}}{\overset{k_1^*}{\rightleftharpoons}} \mathrm{E^*[RCO{-}NHR']} \xrightarrow{k_2}$$

As noted above, such a two-step process in the formation of the labile enzyme–substrate complex could not be detected by stopped-flow fluorescence spectroscopy in the action of pepsin on mansyl or dansyl peptide substrates. If, however, a conformational change does occur during the formation of the Michaelis complex, it is necessary to assume that the release of products is associated with a return of the active site to its original state. Such a conformational change is suggested, but not established, by the kinetic data cited in Table IV for Dns-Gly-Ala-Ala-Phe-Phe-OP4P and Dns-Ala-Ala-Phe-Phe-OP4P. For these two substrates, which are cleaved with k_2 values of 150 sec^{-1} or greater, k_2 was found to be greater than k_{cat}. It seems reasonable to assume, as a working hypothesis, that in these cases the bond-breaking step associated with k_2 is followed by a conformational change in the fluorescent enzyme–product complex and that this change becomes kinetically significant only when k_2 is relatively large. This hypothesis invites further experimental efforts, perhaps temperature-jump measurements, to detect a two-step association mechanism and to seek more direct evidence for conformational changes at the active site of pepsin during the course of its catalytic action.

It should be added that clear evidence for a two-step association mechanism in the case of a proteolytic enzyme has been found in the action of papain on Mns-Gly-Gly-Val-Glu-Leu-Gly, which is cleaved at the Glu-Leu bond (106). In this case a very rapid increase in fluorescence, corresponding to a second-order rate constant greater than

10^6 M^{-1} sec^{-1}, is followed by a slower first-order rate. As expected, the observed rate constant for the slower step shows saturation with increasing enzyme concentration. This finding with papain, as compared with the result with pepsin, suggests the possibility that there may be a continuum of conformational flexibility at the active sites of proteinases. It seems likely that pancreatic trypsin has a rigid active site with a narrowly restricted side-chain specificity, since structural modifications of substrates of the type A-Lys-OMe cause little change in k_{cat} or K_m. The closely related chymotrypsin and elastase may also have rigid active sites, but with less restricted primary specificity, as suggested by the fact that better binding (as measured by K_s) leads to better catalysis; here the value of k_{cat} may be a reflection of the goodness of fit of the substrate at an active site of fixed conformation. If this speculation has validity, pepsin may be considered to be at the other extreme of the continuum, with papain an intermediate case.

As regards the bond-breaking step in the cleavage of peptide substrates by pepsin, it seems necessary to critically reexamine the status of both the amino-enzyme and acyl-enzyme hypotheses and to consider the possibility that no detectable covalent intermediate is involved in pepsin catalysis. In connection with the hypothesis that a covalent acyl-enzyme intermediate is involved in pepsin catalysis, it seems important to institute a more intensive search for pseudosubstrates that are cleaved with a burst release of the amine product. The classical analogy is the cleavage of *p*-nitrophenyl esters of carboxylic acids by chymotrypsin, where a burst release of the *p*-nitrophenolate anion is observed spectrophotometrically under conditions of high $[E]_t$. What appears to be needed are compounds that have appreciable solubility in aqueous buffers in the pH range of 2–5 and that are cleaved at a single bond with relatively low k_{cat} and K_m values. To test for an acyl-enzyme intermediate, a pseudosubstrate AX-YB might therefore require an X-Y such as Gly-Y, Glu-Y, and PhGly-Y, as in Z-His-Gly-Trp-Val-OMe, where the Gly–Trp bond may be expected to be hydrolyzed slowly by pepsin, and the Trp-Val-OMe unit to represent a good leaving group. Alternatively, it may be of interest to examine the behavior of compounds in which the AX component is a phenylacetyl, phenylpyruvoyl, or a β-phenyl-L-lactyl group. In analogy with the action of chymotrypsin on *p*-nitrophenyl acetate, a desirable feature of such pseudosubstrates is that the release of the amine product is accompanied by a significant change in absorbance or fluorescence at wavelengths longer than that of the enzyme.

Moreover, in connection with the search for evidence for a covalent

acyl-enzyme intermediate, substrates in which the Y component is a β-phenyl-L-lactyl compound might be considered, as in Z-His-Gly-Pla-Val-OMe. It is known that pepsin cleaves Z-His-Phe($4NO_2$)-Pla-OMe more rapidly than Z-His-Phe($4NO_2$)-Phe-OMe (107), perhaps because the hydroxylic product is a better leaving group than the amine product. Other explanations for the enhanced reactivity of the Phe($4NO_2$)-Pla bond can be offered, but, regrettably, little work has been done on the kinetics of pepsin action on ester substrates. By the same token, if a covalent amino-enzyme intermediate is involved in pepsin catalysis, the acyl portion of the pseudosubstrate should be a good leaving group, and the amino component should be an "unnatural" one. The kind of substrate that suggests itself is Z-His-Phe($4NO_2$)-Pol, which has hitherto been found to be resistant to pepsin (20) but has not been tested at high enzyme concentrations.

In connection with the amino-enzyme hypothesis, it should be recalled that one of the most puzzling features of pepsin action is the apparent inability of pepsin substrates of the type AX–YOR (e.g., Ac-Phe-Tyr-OEt) to participate in transpeptidation reactions with AX^*O^- (e.g., Ac-[^{14}C]Phe) under conditions where $AX\text{–}YO^-$ yields significant amounts of $AX^*\text{–}YO^-$. Since transpeptidation is observed at pH values (4.5–5.5) above the usual pK_a of carboxylic acids, and not near pH 2, it appears that the reactive species in transpeptidation are the anions of the substrate and the "acceptor" acid. Among the possible explanations for this behavior are the following. If a covalent amino-enzyme intermediate ($E\text{-}YO^-$ or E–YOR) is formed, there may be a large difference in the ratio k_T/k_H in the process (96):

$$\begin{array}{ccccc} E + AX^*\text{—}YO^- & & E\text{—}YO^- & & E + YO^- \\ & \xleftarrow[+AX^*O^-]{k_T} & & \xrightarrow[+H_2O]{k_H} & \\ E + AX^*\text{—}YOR & & E\text{—}YOR & & E + YOR \end{array}$$

Possible reasons for a k_T/k_H ratio more favorable to transpeptidation with $E\text{-}YO^-$ might be that the anion of the amine product is bound at the active site differently from the ester (or the protonated compound) and that the pK_a of the amine may influence the partition between transpeptidation and hydrolysis. An alternative possibility is that in the cleavage of AX–YOR, the process

$$E + AX\text{—}YOR \rightleftharpoons E\text{—}YOR[AXO^-] \rightarrow E + YOR + AXO^-$$

occurs much more rapidly than the exchange of AXO^- by AX^*O^-,

whereas in the cleavage of AX–YO^- such exchange is possible. The systematic study of the influence of changes in the structure of AXO^- acceptors in transpeptidation reactions, as well as of the AX and YOR portions of pseudosubstrates that exhibit a burst release of the AX product, may help to resolve these questions.

A property of the pepsin-catalyzed reactions to which insufficient attention appears to have been given is that the free energy change in the process

$$RCOO^- + R'NH_3^+ \rightleftharpoons RCO{-}NHR' + H_2O$$

at pH values near 4 is approximately zero (108). The condensation of oligopeptides by pepsin has long been known (109), as in the conversion of Tyr-Leu-Gly-Glu-Phe at pH 4 to a polymeric product that is, on the average, a pentadecapeptide. The requirement for a tetrapeptide as a minimum chain length of the monomer clearly points to the importance of the role of secondary interactions in positioning the *C*-terminal Phe of one monomer molecule and the *N*-terminal Tyr of a second monomer unit at the catalytic site of the enzyme. Moreover, the specificity evident in the cleavage of AX-YB substrates, with a preference for a dipeptidyl unit containing two aromatic L-amino acid residues, also applies to these condensation reactions. The kinetics of such condensation reactions require closer study, and more kinetic data are also needed of model systems in which amide bonds are formed by the rapid reaction of suitably positioned ammonium and carboxylate groups. The possibility should be considered, therefore, that no detectable covalent acyl-enzyme or amino-enzyme intermediate can accumulate in pepsin-catalyzed reactions but that either the acid product or the amine product (depending on its affinity for the active site) can stick to the active site longer than its partner. If this should be the case, either apparent acyl transfer or amine transfer would be possible by a direct condensation of the product that is retained preferentially and an acceptor that can readily displace the product that leaves more easily. This may be the situation in the action of pepsin on Leu-Tyr-Leu, where the predominant transpeptidation reaction is apparent acyl transfer, but about 10–20% of the Leu-Leu appears to arise by amino transfer (90).

The objection may be raised that the occurrence of a direct condensation reaction has been excluded in the formation of Leu-Leu from Leu-Tyr-Leu through control experiments in which [^{14}C]leucine was added and no labeled Leu-Leu could be detected. These experiments assumed

that the leucine in the solution was equivalent to the leucine generated at the active site during the enzymatic cleavage of Leu-Tyr-Leu. The possibility must be considered, however, that the active site region of pepsin represents a narrowed cleft that can be widened by specific cooperative interaction with a substrate (or substrate analog). If in the cleavage of Leu-Tyr-Leu, the Tyr-Leu product leaves preferentially, another Leu-Tyr-Leu molecule can enter the active site; however the Leu at the active site may not be exchangeable with [^{14}C]leucine in solution because of the rapid closure of the active site cleft, the expulsion of the leucine generated by hydrolysis, and the inability of the amino acid to enter the narrowed cleft. As noted earlier in this review, the studies with peptide substrates bearing a fluorescent probe group offer indirect evidence in favor of the view that such substrate- (or inhibitor-) induced conformational changes at the active site of pepsin do occur and that the conversion of pepsinogen to pepsin may involve a narrowing of the active-site cleft upon proteolytic removal of the amino-terminal portion of the zymogen (5).

Consequently, if a conformationally mobile active site is present in pepsin, secondary interactions of the acceptor molecule with the extended active site of pepsin may play a significant role in transpeptidation reactions either by apparent amino transfer or by apparent acyl transfer. For the former this possibility is suggested by the report (110) that during the cleavage of Gly-Gly-Gly-Tyr-Tyr at pH 4, the ratio of Gly-Gly-Gly-Tyr to tyrosine is much greater than unity, and that chromatographically detectable Tyr-Tyr is produced rapidly. On the other hand, Gly-Gly-Tyr-Tyr was found to yield approximately equimolar amounts of the expected hydrolytic products, and Tyr-Tyr appears only after prolonged incubation. Although quantitative data are not available for the kinetic parameters, it may be inferred that for these substrates K_m is relatively large (in the range 10–50 mM) and k_{cat} is relatively low (about 0.1 sec^{-1}). In the case of the apparent acyl transfer of a leucyl residue from Leu-Tyr-NH_2 to another molecule of this substrate (30), secondary enzyme–substrate interactions also may be possible (111). In this connection it is significant that the catalytic activity of penicillopepsin toward Leu-Tyr-NH_2 was increased about tenfold by the resistant peptide Leu-Gly-Leu, which was found to cause a change in the circular dichroism spectrum of the enzyme (112). Such a "cosubstrate" effect is reminiscent of the enhancement of the hydrolysis of Gly-Leu by papain upon the addition of Ac-Phe-Gly (113) and was explained by the enzyme-catalyzed condensation of the two peptides to form Ac-Phe-Gly-Gly-Leu, which was then cleaved at the Gly-Leu bond.

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