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

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

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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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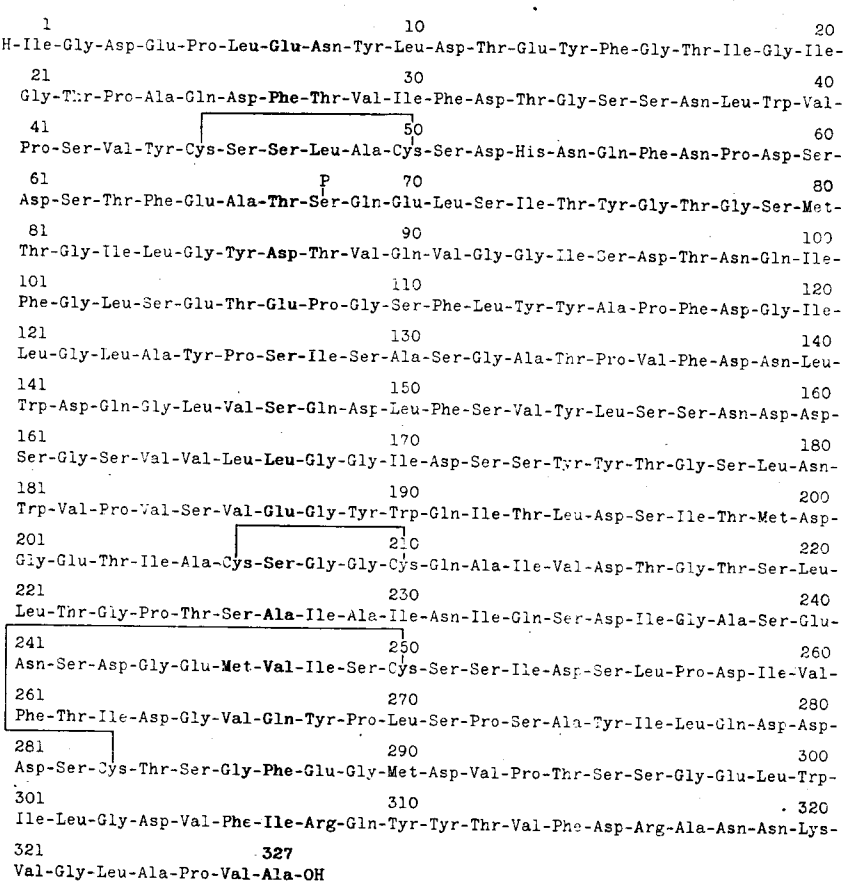


Fig. 1. Amino acid sequence of porcine pepsin (7). According to a recent report (7a) residues 60-61 are Asp-Ser and not Ser-Asp.

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

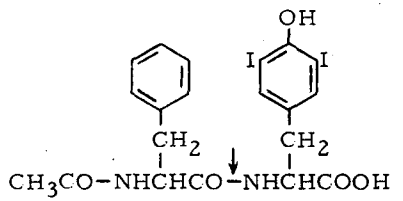
A wide variety of synthetic substrates have been used for the study of the mechanism of pepsin action. For a time most kinetic studies were conducted with acyl dipeptides of the type introduced by Baker (11). The best of these substrates is Ac-Phe-Tyr (I_2)* (I in Fig. 2), which is found to be cleaved 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:

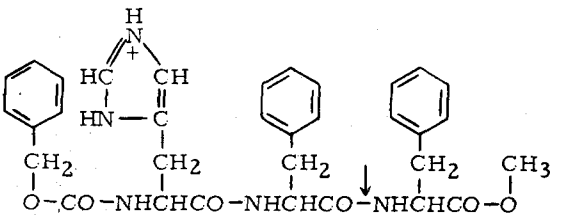
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \ (+ P_1) \xrightarrow{k_3} E + P_2$$

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 $K_s = K_D = k_{-1}/k_1$.



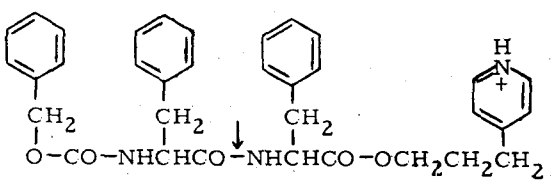
(I)

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 $K_m = 0.2$ mM 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 $K_m = 0.04$ mM (pH 3.5, 37°C), and for Z-His-Phe(4NO_2)-Phe-Val-Leu-OMe, $k_{cat} = 62$ sec^{-1} and $K_m = 0.04$ mM 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]

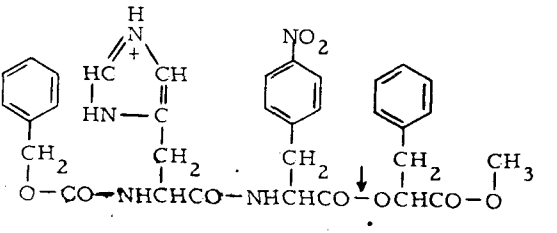
Substrate[b]	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}sec^{-1}$)
Z-Phe-Phe-OP4P(22)	0.7	0.2	3.5
Z-Gly-Phe-Phe-OP4P(22)	3.1	0.4	7.8
Z-$(Gly)_2$-Phe-Phe-OP4P(22)	71.8	0.4	180
Z-$(Gly)_3$-Phe-Phe-OP4P(26)	4.5	0.4	10.1
Z-$(Gly)_4$-Phe-Phe-OP4P(26)	2.1	0.7	3.0
Z-Gly-Ala-Phe-Phe-OP4P(23)	409	0.1	4090
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.

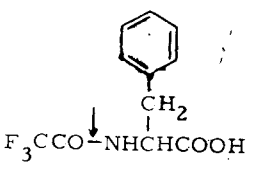
[b] In all cases, cleavage occurred at the Phe-Phe or Phe($4NO_2$)-Phe bond. The numbers in parentheses denote the references from which the data were taken.

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).

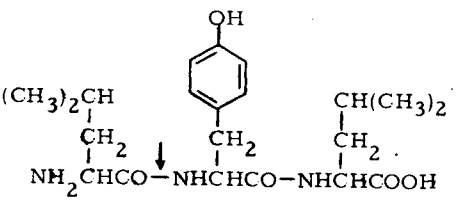
In addition to the three groups of pepsin substrates mentioned above, several others (shown in Fig. 3) have been tested, and the results have suggested hypotheses about the mechanism of pepsin action. Among the other groups are depsipeptide analogs of Z-His-Phe-Phe-OMe, such as Z-His-Phe($4NO_2$)-Pla-OMe, 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-



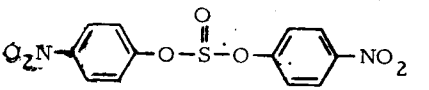
Z-His-Phe(4 NO_2)-Pla-OMe



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 m*M* (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.

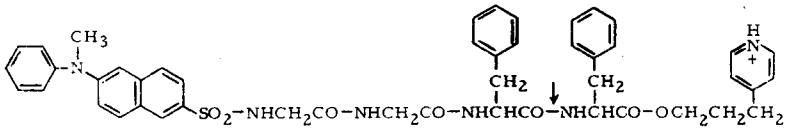
A. BINDING OF THE SUBSTRATE AT THE ACTIVE SITE

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:

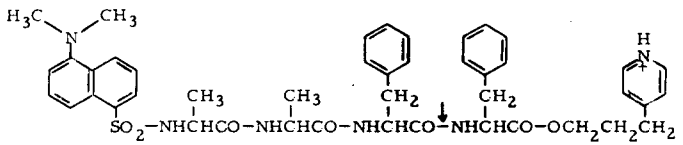
$$E + RCO{-}NHR' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E[RCO{-}NHR']$$

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.

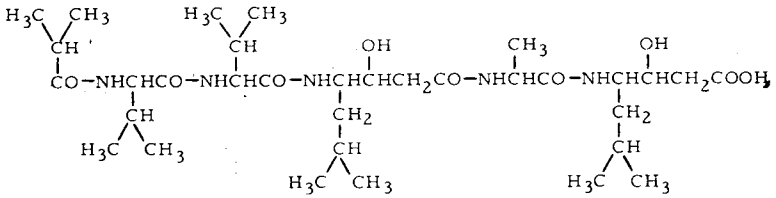


Fig. 5. Structure of pepstatin.

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

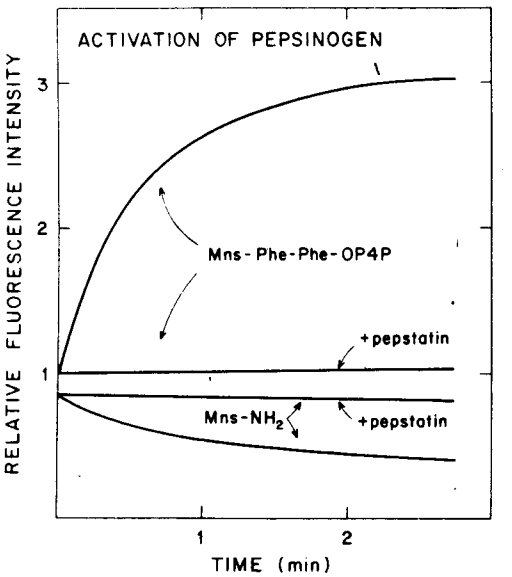


Fig. 6. Fluorescence of Mns-NH_2 and of Mns-Phe-Phe-OP4P in the presence of pepsinogen undergoing activation at pH 2.35 and 25°C. Initial concentrations of pepsinogen, mansyl compound, and pepstatin (when present), 10 μM.

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-

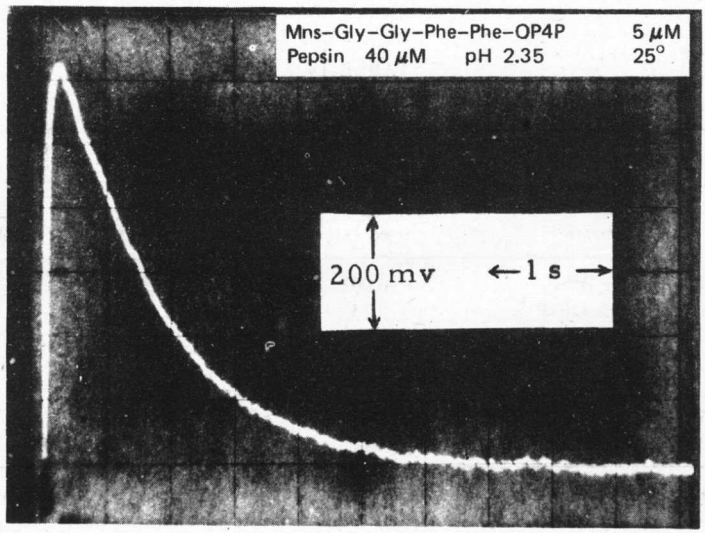


Fig. 7. Time course of the change in fluorescence during the cleavage of Mns-Gly-Gly-Phe-Phe-OP4P by pepsin.