Proc. Nat. Acad. Sci. USA
Vol. 72, No. 9, pp. 3424–3427, September 1975
Biochemistry

Kinetics of action of pepsin on fluorescent peptide substrates

(stopped-flow kinetics/fluorescence)

GOVERDHAN P. SACHDEV AND JOSEPH S. FRUTON*

Kline Biology Tower, Yale University, New Haven, Connecticut 06520

Contributed by Joseph S. Fruton, June 30, 1975

ABSTRACT **Oligopeptide substrates of porcine pepsin (E) of the type A-Phe-Phe-B (S) that are cleaved solely at the Phe-Phe bond under the conditions of these studies, and bearing an amino-terminal fluorescent probe group (mansyl or dansyl), have been used for stopped-flow measurements of the rate of formation of the A-Phe product. These experiments were conducted under conditions of [E] ≫ [S], and the kinetic data were compared with those obtained under conditions of [S] ≫ [E] for the formation of the Phe-B product (the same in all cases). The results for substrates with A = mansyl-Gly, mansyl-Gly-Gly, and dansyl-Gly-Gly support the conclusion that the rate-limiting step in the over-all catalytic process is associated with the scission of the Phe-Phe bond in the first detectable ES complex. Although the rate of this step varies widely with the nature of the A portion of A-Phe-Phe-B, the magnitude of the dissociation constant of ES is relatively invariant. This supports the view that, in the cleavage of oligopeptide substrates by pepsin, secondary enzyme–substrate interactions may cause conformational changes at the catalytic site, and that a portion of the total binding energy may be used for the attainment of the transition state in the bond-breaking step. With substrates that are hydrolyzed extremely rapidly (A = dansyl-Gly-Ala, dansyl-Ala-Ala), the rate of formation of the A-Phe product appears to be faster than the steady-state rate, suggesting that an additional step has become kinetically significant in the over-all process. This step may be associated with the return of the conformation of the active site to its original state.**

In previous studies from this laboratory (1–6), a series of synthetic oligopeptide substrates of the type A-Phe-Phe-OP4P was described for porcine pepsin and other acid proteinases. The pyridylpropyloxy (OP4P) group was introduced at the carboxyl terminus to render the substrates soluble in aqueous solution at acidic pH values, thus obviating the necessity of adding organic solvents, some of which inhibit pepsin action. These substrates are cleaved solely at the Phe-Phe bond under the conditions of our studies, and the kinetic parameters of the hydrolysis are markedly dependent on the nature of the A group. It was found that the values of k_{cat} obtained under steady-state conditions ($[S]_0 \gg [E]_t$) ranged over several orders of magnitude, whereas the values of K_m varied within much smaller limits. For example, the three substrates with A = Z-Gly-Pro, Z-Gly-Gly, and Z-Gly-Ala were hydrolyzed with k_{cat} values in the relative ratio 1:1200:7000, but the values of K_m were all in the range 0.1–0.4 mM (2). The results of studies on the inhibition of pepsin by substrate analogs (7) and of gel-filtration experiments on the binding of such analogs (8) were consistent with the conclusion that the values of K_m estimated from steady-state kinetic measurements approximate the value of K_s, the dissociation constant of the initial enzyme–substrate complex, and that the rate-limiting step in the over-all catalytic process occurs during the transformation of this complex. In the experiments described below, the validity of this conclusion was tested by means of stopped-flow fluorescence measurements of the kinetics of the cleavage of substrates of the type A-Phe-Phe-OP4P by porcine pepsin and *Rhizopus*-pepsin (9) under conditions of $[E]_t \gg [S]_0$. As shown by Kezdy and Bender (10) for the process

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

the observed rate constant (k_e) for the release of P_1 under these conditions is given by the equation $k_e = k_2[E]_t/(K_s + [E]_t)$; linear plots of $1/k_e$ against $1/[E]_t$ thus provide estimates of the values of K_s ($= k_{-1}/k_1$) and k_2. This equation has been applied to the study of the mechanism of the action of papain (11), chymotrypsin (10, 12), and trypsin (13).

In the present work, advantage was taken of the earlier finding (3, 4, 6) that the fluorescence of the dansyl (Dns) or mansyl (Mns) group of compounds such as Dns- (or Mns-) Gly-Gly-Phe-Phe-OP4P is greatly enhanced when they are bound by pepsin and that cleavage products (e.g., Dns-Gly-Gly-Phe) bearing the fluorescent probe group are bound much more weakly. Consequently, stopped-flow measurements of the rate of decrease of the fluorescence of the enzyme-substrate complex under conditions of $[E]_t \gg [S]_0$ could be expected to give estimates of K_s and k_2, on the assumption that the rate of conversion of the initial ES complex ($-d[ES]/dt$) is given by the rate of decrease in fluorescence intensity, and is equal to the rate of appearance of the first product ($d[P_1]/dt$). In contrast to the case of chymotrypsin, however, the order of the release of the products in pepsin-catalyzed reactions is still uncertain. Although extensive data have been presented in favor of the intermediate formation of an "amino-enzyme", with the carboxylic component of a peptide substrate as the first product (14), some findings (15–17) are inconsistent with this conclusion, and further work is needed. The present studies were performed in the hope of contributing to the solution of this problem, through a comparison of the values of k_2 and K_s estimated from stopped-flow fluorescence measurements with the values of k_{cat} and K_m obtained under steady-state conditions, where the rate of release of the Phe-OP4P product was measured by means of its reaction with fluorescamine (5).

MATERIALS AND METHODS

Porcine pepsin was prepared from crystalline pepsinogen (Worthington Biochemical Corp., lot PG-7KA) in the manner described by Rajagopalan *et al.* (18), and had a specific proteinase activity, with hemoglobin as the substrate (19), of 3400 ± 100 units/mg. *Rhizopus*-pepsin (three times crystallized, Miles Laboratories lot 8U06) was used without further purification; it had a specific proteinase activity of 1800 units/mg. The molecular weights of the two enzymes were assumed to be 34,200 and 35,000, respectively.

Mns-Ala-Ala-Phe-Phe-OP4P was prepared from Z-Ala-

Abbreviations: Z, benzyloxycarbonyl; OP4P, 3-(4-pyridyl)propyl-1-oxy; Mns, mansyl, 6-(*N*-methylanilino)-2-naphthalenesulfonyl; Dns, dansyl, 5-dimethylamino-1-naphthalene sulfonyl. The abbreviated designation of amino-acid residues denotes the L form.

* To whom reprint requests may be sent.

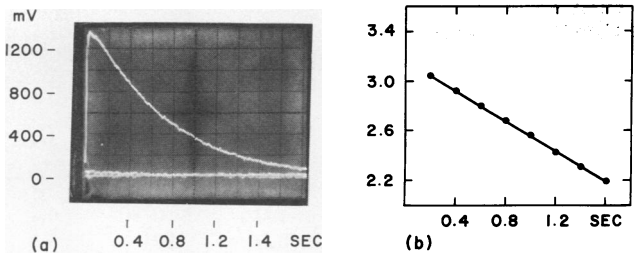

FIG. 1. Time course of the change in fluorescence during the cleavage of Dns-Gly-Gly-Phe-Phe-OP4P (5 μM) by porcine pepsin (50 μM). (a) Oscilloscope trace of the reaction. Time constant, 10 msec. The ordinate denotes the change in fluorescence as photomultiplier voltage (V_t) in millivolts (200 mV per division). The bottom trace denotes the end point (V_i) of the fluorescence change. (b) Evaluation of the same time course as a plot of log ($V_t - V_i$) against time.

Ala-Phe-Phe-OP4P (2) in the manner described for analogous mansyl peptides (4). It melted at 189–191°, and gave a single spot (R_F 0.31) upon chromatography on Eastman silica gel 6061 sheets, with ethyl acetate/methanol (9:1, v/v) as the solvent (ultraviolet light, iodine). Calculated for $C_{49}H_{52}N_6O_7$ (869.1): C, 67.7; H+ 6.0; N, 9.7. Found: C, 67.9; H, 5.9; N, 9.6. The synthesis of the other peptide substrates used in this study has been described (3, 4).

Because of the limited solubility of the mansyl peptides above pH 3, the kinetic studies with these substrates were conducted at pH 2.35 (0.5 M formate buffer), and because of the relatively poor binding of the dansyl peptides at the latter pH value, they were tested at pH 3.1 (0.1 M formate buffer). In all cases, the temperature was 25°.

The kinetic measurements under conditions of $[E]_t \gg [S]_0$ were made with a Durrum Instruments Corp. model D-110 stopped-flow spectrophotometer equipped with a Tectronix R-5103 N storage oscilloscope, and for fluorescence measurements the phototube was mounted at 90° to the xenon or tungsten light source. A Corning cut-off filter 3-72 in front of the phototube excluded light below about 430 nm. The excitation wavelength was 335 nm for the mansyl compounds and 330 nm for the dansyl compounds. The stored oscilloscope traces of the time course of change in fluorescence were photographed on Polaroid 107 film. The increase in fluorescence intensity arising from the formation of the enzyme–substrate complex was within the dead time (2–3 msec) of the instrument, and was followed by a relatively slower decrease that accorded with first-order kinetics up to 90% of the total decrease in fluorescence intensity. All runs were performed at least in quadruplicate, and each value of k_e [estimated by the plotting method of Gutfreund and Sturtevant (20)] was the average of 4 values, with a deviation from the mean of approximately ±10%. All solutions were prepared with doubly-distilled degassed water.

A representative oscilloscope trace and the semilogarithmic plot derived from it are shown in Fig. 1. In Fig. 2 are shown the plots of $1/k_e$ against $1/[E]_t$ for two substrates. Similar linearity was noted for the plots derived from the stopped-flow data for the other substrates tested. The precision (95% confidence limits) of the k_2 values was near ±15% and of the K_s values was in the range ±20–35%.

The determination of k_{cat} and K_m under steady-state conditions ($[S]_0 \gg [E]_t$), with fluorescamine as the reagent for the measurement of the rate of formation of Phe-OP4P, was performed as described previously (6). The results accorded with Michaelis-Menten kinetics. The precision (95% confidence limits) of the k_{cat} values was ±10–25% and of the K_m values, ±20–40%.

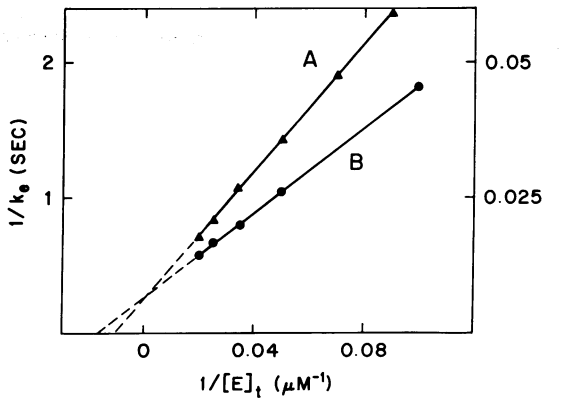

FIG. 2. Representative plots of $1/k_e$ against $1/[E]_t$ for the cleavage of pepsin substrates under conditions of enzyme excess. Substrate concentration, 5 μM. Curve A, Dns-Gly-Gly-Phe-Phe-OP4P (left ordinate); Curve B, Dns-Gly-Ala-Phe-Phe-OP4P (right ordinate).

Separate experiments, using thin-layer chromatography, showed that under the conditions of these studies, the only detectable site of cleavage of the substrates was at the Phe-Phe bond. Upon prolonged incubation of Mns-Ala-Ala-Phe-Phe-OP4P with pepsin under conditions of $[E]_t \gg [S]_0$, it was noted that the rapid cleavage of the Phe-Phe bond was followed by the very slow hydrolysis (k_{cat} = 0.1 sec^{-1}) of the Ala-Phe bond of the acidic cleavage product.

Experiments for determination of the apparent dissociation constant (K_D) of the complex formed between pepsin and the acidic cleavage product were performed with a Perkin-Elmer MPF-3 fluorescence spectrophotometer, in the manner described previously (6).

RESULTS

The kinetic data in Table 1 for the cleavage of Mns-Gly-Phe-Phe-OP4P, Mns-Gly-Gly-Phe-Phe-OP4P, and Dns-Gly-Gly-Phe-Phe-OP4P indicate that for each of these substrates K_m equals K_s and k_{cat} equals k_2, within the precision of the measurements. These results give strong support to the view that the rate-limiting step in the over-all hydrolytic process is associated with the transformation of the first detectable enzyme-substrate complex. Presumably this conclusion also applies to the hydrolysis of Mns-Phe-Phe-OP4P, which is cleaved too slowly for reliable studies under conditions of $[E]_t \gg [S]_0$, and of Dns-Gly-Phe-Phe-OP4P, for which k_{cat} = 0.19 sec^{-1} and K_m = 70 μM (3). Although these five substrates are hydrolyzed with k_{cat} (= k_2) values that differ by as much as 8000-fold, the values of K_m (= K_s) are essentially invariant (37–109 μM). It should be added that the K_D values for the relatively resistant substrates Mns-Phe-Phe-OP4P (70 μM) and Mns-Gly-Phe-Phe-OP4P (30 μM), estimated earlier (6) from Scatchard plots, are in satisfactory agreement with the K_m or K_s values found in the present study.

On the other hand, with two substrates (Dns-Gly-Ala-Phe-Phe-OP4P and Dns-Ala-Ala-Phe-Phe-OP4P) which are hydrolyzed much more rapidly than the ones discussed above, the apparent value of k_2 as estimated from the rate of decrease of fluorescence under conditions of $[E]_t \gg [S]_0$ was found to be significantly greater than the k_{cat} value derived from the determination of the rate of the formation of Phe-OP4P under conditions of $[S]_0 \gg [E]_t$. The possibility must be considered that, under the conditions of $[S]_0 \gg [E]_t$, inhibition by the substrate is more pronounced than under conditions of $[E]_t \gg [S]_0$, but if this were so, the apparent ab-

Table 1. Kinetics of cleavage of mansyl and dansyl peptides by pepsin

Substrate	Steady-state kinetics				Stopped-flow kinetics			
	$[E]_t$ (μM)	$[S]_0$ (μM)	k_{cat} (sec^{-1})	K_m (μM)	$[E]_t$ (μM)	$[S]_0$ (μM)	k_2 (sec^{-1})	K_s (μM)
Mns-Phe-Phe-OP4P	10	30–90	0.002	95	—	—	—	—
Mns-Gly-Phe-Phe-OP4P	0.2	14–109	0.13	37	5–30	1.0	0.10	34
Mns-Gly-Gly-Phe-Phe-OP4P	0.003	15–113	16	75	25–75	5.0	13	94
Dns-Gly-Gly-Phe-Phe-OP4P	0.01	14–134	4.4	109	10–50	5.0	4.6	97
Dns-Gly-Ala-Phe-Phe-OP4P	0.0012	23–218	34	60	10–50	5.0	146	57
Dns-Ala-Ala-Phe-Phe-OP4P	0.0004	18–111	91	54	10–50	5.0	430	57
Mns-Ala-Ala-Phe-Phe-OP4P	0.0004	21–110	112	65	—	—	—	—

sence of substrate inhibition with Dns- (or Mns-) Gly-Gly-Phe-Phe-OP4P would be difficult to understand. Another possible explanation was the adsorption of pepsin on glass, a phenomenon noted for chymotrypsin at enzyme concentrations below 10^{-8} M (21). However, repetition of the pepsin experiments with siliconized glass vessels gave initial rates of hydrolysis similar to those obtained with untreated vessels. Although this explanation has not been excluded by our attempts to reduce the error arising from adsorption on glass, the possibility must also be considered that in the case of these two substrates, a step (or steps) other than that associated with k_2 is kinetically significant in the over-all process. It is noteworthy that the differences between k_{cat} and k_2 do not appear to be reflected in K_m and K_s, which are similar for all the substrates listed in Table 1.

The most sensitive substrate among those tested in this study proved to be Mns-Ala-Ala-Phe-Phe-OP4P, as judged by the value of k_{cat} from steady-state experiments. A determination of k_2 and K_s under conditions of $[E]_t \gg [S]_0$ was not possible in this case, since the rapid decrease in fluorescence intensity seen with corresponding dansyl compound was not evident when 5 μM Mns-Ala-Ala-Phe-Phe-OP4P was mixed in the stopped-flow apparatus with 50 μM pepsin. This behavior was found to be a consequence of the fact that the dissociation constant (K_D) for the complex of pepsin with the cleavage product Mns-Ala-Ala-Phe is approximately 65 μM, the value found for the K_m of the substrate (Table 1) and the extremely rapid conversion of the enzyme–substrate complex to the enzyme–product complex is therefore not accompanied by a detectable change in fluorescence intensity. With the other substrates listed in Table 1 (except for Mns-Phe-Phe-OP4P), measurement of k_2 and K_s was possible because in all cases K_D for the product is significantly greater than K_m or K_s for the substrate. Thus for Mns-Gly-Gly-Phe, K_D = 150 μM, whereas K_D for the fluorescent cleavage products derived from the three dansyl peptides was in the range 235–260 μM under the conditions of our studies.

A more restricted set of experiments was conducted with *Rhizopus*-pepsin; they could not be extended because of curtailment of the commercial supply of this enzyme. With Dns-Ala-Ala-Phe-Phe-OP4P, steady-state experiments ($[E]_t$ = 0.003 μM; $[S]_0$ = 12–93 μM) gave values of k_{cat} = 14 sec^{-1} and K_m = 30 μM, whereas stopped-flow experiments ($[E]_t$ = 5–30 μM; $[S]_0$ = 2.0 μM) gave values of k_2 = 53 sec^{-1} and K_s = 27 μM. It is evident that, as in the cleavage of this substrate by porcine pepsin, k_2 is significantly greater than k_{cat}, and the possibility must be considered that a step other than that associated with k_2 is kinetically significant in the over-all process. The likelihood of error in the determination of k_{cat} because of adsorption on glass is less in the case of *Rhizopus*-pepsin, since higher enzyme concentrations were used in the steady-state experiments.

DISCUSSION

The above results show that in the hydrolytic cleavage by porcine pepsin of substrates that are hydrolyzed under the conditions of our studies with k_{cat} values below about 20 sec^{-1}, the rate-limiting step in the over-all catalytic process is associated with the transformation of the first detectable enzyme-substrate complex. It is reasonable to infer that this step is a component of the bond-breaking reaction, and that the decrease in fluorescence recorded in our experiments represents the formation of a complex of pepsin with the acidic product (e.g., Mns-Gly-Gly-Phe). The lower fluorescence intensity may be a consequence either of poorer binding of the product (a greater K_D) or a change in the environment of the fluorescent probe, or both, but the available data suggest that the poorer binding of the acidic product makes a major contribution to the decrease in fluorescence. Since $k_{cat} = k_2$ for these substrates, it may be concluded that the departure of both the acidic product and the amine product is much more rapid than the bond-breaking step.

The large variation in k_{cat} and the relative invariance of K_m for the cleavage of the Phe-Phe unit of peptides of the type A-Phe-Phe-OP4P emphasizes anew the importance of enzyme-substrate interactions at a distance from the immediate site of catalytic action (22). These data support the view (23, 24) that the specificity of enzyme–substrate interaction is directly related to the efficiency of the bond-breaking reaction, and that the estimated K_m (or K_s) values reflect only a fraction of the total binding energy in the interaction, the remainder being used for the attainment of the transition state in the bond-breaking step. If we assume that, in the case of closely-related pepsin substrates (e.g., A-Phe-Phe-OP4P), the K_s corresponding to the energy of binding of each of the substrates at a rigid active site is roughly the same, the difference in catalytic efficiency may be a consequence of differences in the extent to which particular substrates can induce, through secondary interactions, conformational changes at the catalytic site that are favorable to catalysis.

If there is a conformational change in the initial pepsin-substrate complex (ES) to form a species (ES*) which is closer to the transition state, this change was not detectable by stopped-flow fluorescence spectroscopy. The rate of increase of fluorescence upon mixing enzyme and substrate under conditions of $[E]_t \gg [S]_0$ was too rapid to be estimated with confidence, and corresponded to a second-order rate constant greater than 10^6 M^{-1} sec^{-1}.

The finding that k_2 is greater than k_{cat} for pepsin substrates of the type A-Phe-Phe-OP4P that are cleaved very

rapidly raises questions that invite further experiments. If we assume that the k_{cat} values for Dns-Gly-Ala-Phe-Phe-OP4P and of Dns-Ala-Ala-Phe-Phe-OP4P are not subject to some untraced artifactitious error, it seems that, in the cleavage of these substrates, a step after the one associated with k_2 becomes kinetically significant when k_2 is 150 sec^{-1} or greater. One possibility is that the bond-breaking step associated with k_2 is followed by a conformational change in the fluorescent enzyme–product complex:

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

$$EP^* \xrightarrow{k_2'} EP \xrightarrow{k_3} E + P$$

According to this hypothesis, the initial ES complex is converted to ES* by a conformational change that is too rapid to be detected in our experiments, and that after the bond-breaking step the enzyme must return to its original conformation in a step denoted k_2' which becomes kinetically significant only when k_2 is relatively large. Since there appears to be a proportionality of the k_2/k_{cat} values for the two substrates, the presumed k_2' step cannot be associated with the free enzyme but with a complex that still contains the acidic product.

Clearly, the rate of the departure of the amine product (Phe-OP4P) from the active site should be the same for all substrates of the type A-Phe-Phe-OP4P if it is the second product to leave, so that this process is not likely to be kinetically significant in the cleavage of Dns-Gly-Ala-Phe-Phe-OP4P and Dns-Ala-Ala-Phe-Phe-OP4P. However, if Phe-OP4P leaves either before or together with the acidic product, its rate of departure may be influenced by the nature of the acidic product held at the active site.

These considerations are relevant to the problem of the mechanism of pepsin action, and raise anew the possibility suggested earlier (25) that detectable covalently-bound acyl-enzyme or amino-enzyme compounds may not be intermediates in pepsin catalysis. Instead, they focus attention on the conformational state of the active site after the bond-breaking step has occurred, and suggest that pepsin has an active site cleft that may widen or narrow depending on the presence or absence of substrates (or inhibitors) in it (22), and that the sequence of departure of the two products of hydrolysis may be a reflection of the interaction of each of them with complementary groups in the active site. These interactions may be coupled, so that the nature of one product may influence the rate of departure of the other through the effect it has on the conformational state of the active site, and the two products may therefore leave either in a sequence that suggests apparent acyl transfer or in one that is consistent with apparent amino transfer. In such a situation, the failure to observe rapid exchange of a bound product with isotopically-labeled product in the solution (17) may not be evidence for the formation of a covalent enzyme–product intermediate.

This study was aided by grants from the National Institutes of Health (GM-18172 and AM-15682) and from the National Science Foundation (BMS73-06877). We thank Miss Eleanor Andujar for her assistance in these experiments, and Prof. Jeremy R. Knowles (Harvard University) for his valuable comments about the manuscript of this paper.

1. Sachdev, G. P. & Fruton, J. S. (1969) "Pyridyl esters of peptides as synthetic substrates of pepsin," *Biochemistry* **8**, 4231–4238.
2. Sachdev, G. P. & Fruton, J. S. (1970) "Secondary enzyme-substrate interactions and the specificity of pepsin," *Biochemistry* **9**, 4465–4470.
3. Sachdev, G. P., Johnston, M. A. & Fruton, J. S. (1972) "Fluorescence studies on the interaction of pepsin with its substrates," *Biochemistry* **11**, 1080–1086.
4. Sachdev, G. P., Brownstein, A. D. & Fruton, J. S. (1973) "*N*-Methyl-2-anilinonaphthalene-6-sulfonyl peptides as fluorescent probes for pepsin-substrate interaction," *J. Biol. Chem.* **248**, 6292–6299.
5. Sampath-Kumar, P. S. & Fruton, J. S. (1974) "Studies on the extended active sites of acid proteinases," *Proc. Nat. Acad. Sci. USA* **71**, 1070– 1072.
6. Sachdev, G. P., Brownstein, A. D. & Fruton, J. S. (1975) "Fluorescence studies on the active sites of porcine pepsin and *Rhizopus*-pepsin," *J. Biol. Chem.* **250**, 501–507.
7. Inouye, K. & Fruton, J. S. (1968) "The inhibition of pepsin action," *Biochemistry* **7**, 1611–1615.
8. Raju, E. V., Humphreys, R. E. & Fruton, J. S. (1972) "Gel filtration studies on the binding of peptides to pepsin," *Biochemistry* **11**, 3533–3536.
9. Graham, J. E. A., Sodek, J. and Hofmann, T. (1973) "*Rhizopus* acid proteinases (*Rhizopus*-pepsin): Properties and homology with other acid proteinases,," *Can. J. Biochem.* **51**, 789–796.
10. Kezdy, F. J. & Bender, M. L. (1962) "The kinetics of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate," *Biochemistry* **1**, 1097–1106.
11. Hubbard, C. D. & Kirsch, J. F. (1968) "The presteady-state kinetics of the papain-catalyzed hydrolysis of isomeric nitrophenyl esters of carbobenzoxyglycine," *Biochemistry* **7**, 2569–2573.
12. Hirohara, H., Bender, M. L. & Stark, R. S. (1974) "Acylation of α-chymotrypsin by oxygen and sulfur esters of specific substrates: Kinetic evidence for a tetrahedral intermediate," *Proc. Nat. Acad. Sci. USA* **71**, 1643–1647.
13. Kassera, H. P. and Laidler, K. J. (1970) "Transient-phase studies of a trypsin-catalyzed reaction," *Can. J. Biochem.* **48**, 1793–1802.
14. Clement, G. E. (1973) "Catalytic activity of pepsin," *Progr. Bioorg. Chem.* **2**, 177–238.
15. Silver, M. S. & Stoddard, M. (1972) "Amino-enzyme intermediates in pepsin-catalyzed reactions," *Biochemistry* **11**, 191–200.
16. Silver, M. S. & Stoddard, M. (1975) "Kinetic studies on the mechanism of pepsin action," *Biochemistry* **14**, 614–621.
17. Takahashi, M., Wang, T. T. & Hofmann, T. (1974) "Acyl intermediates in pepsin and penicillopepsin catalyzed reactions," *Biochem. Biophys. Res. Commun.* **57**, 39–46.
18. Rajagopalan, T. G., Moore, S. & Stein, W. H. (1966) "Pepsin from pepsinogen: Preparation and properties," *J. Biol. Chem.* **241**, 4940–4950.
19. Delpierre, G. R. & Fruton, J. S. (1965) "Inactivation of pepsin by diphenyldiazomethane," *Proc. Nat. Acad. Sci. USA* **54**, 1161–1167.
20. Gutfreund, H. & Sturtevant, J. M. (1956) "The mechanism of the reaction of chymotrypsin with *p*-nitrophenyl acetate," *Biochem. J.* **63**, 656–661.
21. Bixler, R. L. & Niemann, C. (1959) "Surface effects in α-chymotrypsin-catalyzed hydrolyses," *J. Am. Chem. Soc.* **81**, 1412–1417.
22. Fruton, J. S. (1974) "The active site of pepsin," *Acc. Chem. Res.* **7**, 241–246.
23. Page, M. I. & Jencks, W. P. (1971) "Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect," *Proc. Nat. Acad. Sci. USA* **68**, 1678–1683.
24. Fersht, A. R. (1974) "Catalysis, binding and enzyme-substrate complementarity," *Proc. Roy. Soc. London Ser. B* **187**, 397–407.
25. Fruton, J. S. (1971) "Pepsin," in *The Enzymes*, ed. Boyer, P. D. (Academic Press, New York), 3rd ed., Vol. III, pp. 119–174.