Studies on the Catalytic Mechanism of Pepsin Using a New Synthetic Substrate†

Michael W. Hunkapiller and John H. Richards*

ABSTRACT: The proteolytic enzyme, porcine pepsin, has been found to catalyze the hydrolysis of several *N*-trifluoroacetyl aromatic L-amino acids. This catalytic activity is lost when pepsin is chemically modified so as to impair its protease activity. Highly purified preparations of two closely related enzymes, human pepsin and human gastricsin, also manifest catalytic activity toward *N*-trifluoroacetyl-L-amino acids. Nuclear magnetic resonance techniques were used to follow the catalytic activity of porcine pepsin in the pH range 1.7–5.4. The pH dependence revealed that nonproductive binding strongly influences the observed catalytic activity and that productive enzyme–substrate binding requires an anionic substrate (pK_a = 2.8) and an undissociated group on the free enzyme of apparent pK_a = 3.7. Binding is also strongly influenced by ionization of a group on the free enzyme with an apparent pK_a near 4.8. L-Tryptophan ethyl ester competes with productive binding of *N*-trifluoroacetyl-L-phenylalanine. A kinetic isotope effect (k_{H_2O}/k_{D_2O} = 3) has been observed for the hydrolysis of *N*-trifluoroacetyl-L-phenylalanine which, being the largest reported for pepsin-catalyzed reactions, suggests that proton transfer may be involved in the rate-limiting step of these reactions. A new mechanism—one involving three carboxylic acid groups on the enzyme and an intermediate in which the amino moiety is noncovalently held by the enzyme after release of the acyl moiety—is proposed to explain these and previous observations on catalysis by pepsin.

Porcine pepsin is an endopeptidase with maximal activity at acidic pH. It can also catalyze the hydrolysis of a wide range of small synthetic peptides displaying a preference for substrates with hydrophobic substituents on the amino acid residues on both sides of the sensitive bond (Tang, 1963; Fruton, 1970). Pepsin does not hydrolyze simple amides or esters of amino acids, nor does it normally cleave *N*-acetyl or *N*-carbobenzoxy groups from amino acids (Knowles, 1970). Its only reported carboxyl esterase activity is toward the depsipeptide methyl *N*-carbobenzoxy-L-histidyl-L-*p*-nitrophenylalanyl-L-β-phenyllactate, in which the ester bond between the *p*-nitrophenylalanyl and β-phenyllactate residues is cleaved (Inouye and Fruton, 1967). Hydrolysis of sulfite esters can also be catalyzed by pepsin (Reid and Fahrney, 1967). Also, pepsin can catalyze transpeptidation reactions in which the amino moiety is transferred from the original acyl moiety to another carboxylic acid group (Neumann *et al.*, 1959; Fruton *et al.*, 1961) and exchange of ^{18}O between $H_2{}^{18}O$ and the carboxyl group of *N*-acetylamino acids (Kozlov *et al.*, 1962). All these catalytic activities (hydrolysis, transpeptidation, and ^{18}O exchange) show similar specificity and probably occur at a single catalytic locus on the enzyme (Knowles, 1970).

Knowles (1970) and Fruton (1970) have proposed a mechanism for pepsin catalysis which involves two enzymic carboxyl groups. They suggest that one, with an apparent pK_a in the free enzyme of 4.7, protonates the carbonyl oxygen of the sensitive peptide bond while the other, with an apparent pK_a of 1.1, as the anion, nucleophilically attacks the carbonyl carbon of the protonated amide. The resulting tetrahedral intermediate undergoes a reversible four-center exchange reaction to liberate the free acid product and generate an amino enzyme intermediate. Subsequent reaction with water liberates the free amine product and regenerates active enzyme.

Though attractively simple, this mechanism suffers from several disadvantages (i) the assumption of a four-center exchange reaction in the breakdown of the initial tetrahedral intermediate, (ii) the so far unsuccessful isolation of the proposed covalent amino enzyme, (iii) the absence of kinetic isotope effect upon hydrolysis of dipeptide substrates such as *N*-Ac-L-Phe-L-TyrOMe (Clement and Snyder, 1966) (Knowles (1970) postulates proton transfer in the rate-limiting steps), and (iv) indications that more than two carboxyl groups may play important roles at pepsin's active site (Hollands and Fruton, 1968; Lundblad and Stein, 1969).

The purpose of the present paper is to report our results on the activity of pepsin in catalyzing the hydrolysis of some *N*-CF_3CO-L-amino acids (an activity serendipitously observed when attempting to study binding of these substances to pepsin by magnetic resonance techniques (Gammon *et al.*, 1971) and our conclusions, based on this catalytic activity, about the mechanism of pepsin action.

Experimental Section

Materials. Crystalline porcine pepsin (1:60,000; lot 70C-9280) and pepsinogen (grade 1; lot 117B-1130) were obtained from Sigma and used without further purification. Standard hemoglobin assay for pepsin (described below) showed an activity of 4100 ± 200 units/mg of pepsin. Human gastric juice was supplied by the University of Southern California Medical Center. Human pepsin and gastricsin were prepared by chromatography on Amberlite CG-50 ion-exchange resin as described by Richmond *et al.* (1958). *N*-CF_3CO-amino acids were synthesized according to the procedure of Fones (1952). *N*-CF_3CO-L-Phe had a melting point of 119.5–120.5°. Fones (1952) reported mp 119.4–120.6°. N_2AcGlyOEt, prepared by diazotizing Gly-GlyOEt hydrochloride according to the procedure of Kozlov *et al.* (1967), had a melting point of 107–108° (lit. mp 107–108°). L-Tryptophan ethyl ester hydrochloride, prepared by treating L-tryptophan with HCl in

† Contribution No. 4390 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91109. *Received December 14, 1971.* Supported by a grant from the U. S. Public Health Service NIGMS-10218.

ethanol, had a melting point of 224–225° (lit. mp 225–226°; Greenstein and Winitz, 1961). 2,4′-Dibromoacetophenone was obtained from Sigma. Deuterium oxide (99.7%) was obtained from Columbia Organic Chemicals.

Determination of Rate of Hydrolysis of N-CF_3CO-amino Acids by Pepsin. A weighed sample of the *N*-CF_3CO-amino acid was dissolved in 2.50 ml of 0.040 M sodium citrate buffer (pH 4.5), 0.50 ml of a freshly prepared 5.0 mM pepsin solution was added, and the pH was adjusted to the desired value by addition of 0.20 M citric acid, 0.20 M sodium citrate, or 0.20 M citric acid–HCl buffer (pH 1.0). The solution was then made up to 5.0 ml by addition of 0.20 M sodium citrate buffer of the desired pH and placed in a water bath maintained at 34 ± 1°. In order to measure the per cent hydrolysis, the solution was transferred to a 12-mm nuclear magnetic resonance (nmr) tube and inserted in a Varian XL-100-15 nmr spectrometer with probe temperature at 34 ± 1°. The hydrogen resonance of the solvent (water or deuterium oxide) was used for field-frequency stabilization, and the fluorine spectrum of trifluoroacetate anion and the *N*-CF_3CO-amino acid was time averaged (4 scans) using a Fabritek Model C-1062 time-averaging computer. Digital integration of the fluorine spectrum was also performed with the computer. The per cent hydrolysis for each time point was calculated from the relative areas of the trifluoroacetyl and trifluoroacetate fluorine peaks. The hydrolysis of any given sample was followed until 10–15% hydrolysis had been obtained, and v_0 was then calculated by averaging the values for six to eight times points taken around the 10–15% hydrolysis level. Alternatively, v_0 was calculated by fitting the data for time points taken at intervals during the 2–15% hydrolysis range to a straight line with the aid of a least-squares computer program.

All pH measurements were made on a Radiometer Model 26 pH meter zeroed on pH 4.00 and 7.00 standard buffers. Values of pD were calculated by adding 0.4 to the observed pH meter reading for D_2O solutions (Bell, 1959). Measurements at the start and end of hydrolysis runs showed essentially no change in pH during the reaction (±0.02 pH unit).

Proteolytic Activity. The procedure of Anson and Mirsky (1932) was used. The hydrolysis of acid-denatured bovine hemoglobin at pH 1.8, 37°, was followed by measuring the 280-mμ absorbance of trichloroacetic acid soluble hydrolysis products in a Beckman DU spectrophotometer. Activity is defined as (1000 × ΔOD/10 min) units/mg of enzyme. Hemoglobin assays with pepsin incubated at 34° for 24 hr in 0.10 M sodium citrate buffer (pH 2.0 or 5.4) showed 3900 ± 300 units/mg. This rules out significant loss of activity (<5%) during the kinetic experiments.

Preparation of Pepsin Inactivated by N_2AcGlyOEt. The procedure of Lundblad and Stein (1969) was used. Five milliliters of a 0.1 M ethanolic solution of cupric acetate was mixed with 5 ml of a 0.06 M ethanolic solution of N_2AcGlyOEt and allowed to stand for 10 min at room temperature. This solution was then added with gentle stirring to a solution of 500 mg of pepsin in 90 ml of 0.1 M sodium acetate buffer (pH 5.4). The solution was allowed to stand at room temperature for 15 min, dialyzed against 3 l. of distilled water for 2 hr at 4°, and lyophilized. The dried material was dissolved in a minimum amount of 0.1 M sodium acetate buffer (pH 5.4), and the inactivated enzyme was freed from excess reagents by passage through a column of Sephadex G-25 (3 × 60 cm) equilibrated with the same buffer. The eluted protein fraction was dialyzed against distilled water at 4° and lyophilized. Hemoglobin assay showed no pepsin activity.

Preparation of Pepsin Inactivated by 2,4′-Dibromoacetophenone. The procedure of Erlanger *et al.* (1967) was used. Pepsin (500 mg) was dissolved in 90 ml of 0.1 M sodium citrate buffer (pH 3.0) and 10 ml of a 0.03 M ethanolic solution of 2,4′-dibromoacetophenone was added with gentle stirring. The solution was allowed to stand at room temperature for 24 hr, dialyzed against 3 l. of distilled water for 2 hr at 4°, and lyophilized. The inactivated enzyme was then freed from excess reagents on Sephadex G-25 as above. Hemoglobin assay showed that only 22% of the initial pepsin activity remained.

Determination of pK_a of N-CF_3CO-L-Phe. A 0.01 M solution of *N*-CF_3CO-L-Phe was made up in 0.2 M citric acid–HCl buffer (pH 1.15) and trifluoroacetamide (to 0.01 M) was added as an internal reference. The fluorine nmr spectrum of the solution at this and higher pH's was observed, the pH being changed by addition of 0.2 M sodium citrate buffer (pH 7.0) containing 0.01 M *N*-CF_3CO-L-Phe and 0.01 M trifluoroacetamide to the original sample. The separation, δ, in Hertz between the fluorine peaks of the two compounds was measured as a function of pH over the range of pH 1.15–5.00. δ decreased from 48.6 to 43.2 Hz over this range and showed a smooth titration curve with a pK_a of 2.85.

Determination of K_M and k_{cat}. The initial velocity, v_0, of the pepsin-catalyzed hydrolysis of *N*-CF_3CO-L-Phe at each pH and pD studied was measured at substrate concentrations, $[S_0]$, ranging from 7.5 to 40.0 mM. The Michaelis constant, K_M, and the catalytic constant, k_{cat}, were calculated from a plot of $[S_0]/v_0$ *vs.* $[S_0]$. The intercept on the abscissa is equal to $-K_M$ and the slope is equal to $1/(k_{cat}[E_0])$, where $[E_0]$ is the molar concentration of enzyme.

Kinetic Analysis. Examination of the pH dependence of the observed kinetic parameters for hydrolysis of *N*-CF_3CO-L-Phe suggested the occurrence of significant nonproductive binding which would greatly complicate a complete kinetic analysis. Indeed, such nonproductive binding might be expected in light of studies showing that pepsin has two binding sites with high affinity for the side chains of hydrophobic amino acids (Fruton, 1970). Thus, good pepsin substrates contain two hydrophobic amino acid residues, both with L configurations, which can fill both binding pockets simultaneously. *N*-CF_3CO-amino acids, however, having only one hydrophobic side chain might be able to bind in either pocket, although binding in only one of these would lead to catalytic activity.

Hein and Niemann (1962) have developed equations describing the effect of nonproductive binding on the observed kinetic parameters, K_M and k_{cat}, which they show are related to the true values for the productive reaction by eq 1 and 2

$$\frac{1}{K_M} = \frac{1}{K_{ES}^*} + \frac{1}{K_{N1}} + \frac{1}{K_{N2}} + \ldots + \frac{1}{K_{Nj}} \qquad (1)$$

$$k_{cat} = \frac{k_{cat}^*}{K_{ES}^*(1/K_{ES}^* + 1/K_{N1} + 1/K_{N2} + \ldots + 1/K_{Nj})} \qquad (2)$$

where K_{ES}^* is the dissociation constant for the active complex, K_{Nj} is the dissociation constant for a nonproductive complex, and k_{cat}^* is the actual value for the catalytic constant. If K_{ES}^* is greater than K_{Nj}, the observed K_M will reflect the value of K_{Nj} more than that of K_{ES}^*, and the observed k_{cat} will be significantly less than k_{cat}^*. Moreover, since each dissociation constant can have a different pH dependence, an analysis of the ionizations affecting either k_{cat} or K_M can easily become impossible if several binding modes contribute

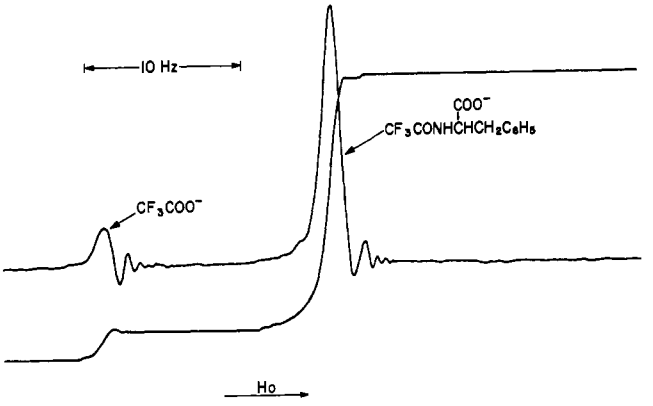


FIGURE 1: Fluorine nmr spectrum of *N*-CF_3CO-L-Phe hydrolysis experiment. $[E_0]$, 5.0×10^{-4} M; $[S_0]$, 4.0×10^{-2} M; 0.10 M sodium citrate buffer, pH 4.05; temperature, 34°, incubation time, 550 min. The peak at right is *N*-CF_3CO-L-Phe fluorine. The smaller peak is trifluoroacetate fluorine. Per cent hydrolysis is 9.6%.

significantly to observed binding. Equation 3, however, shows

$$\frac{k_{cat}}{K_M} = \left[\frac{k_{cat}^*}{K_{ES}^*\left(\frac{1}{K_{ES}^*} + \frac{1}{K_{N1}} + \frac{1}{K_{N2}} + \ldots + \frac{1}{K_{Nj}}\right)}\right] \times \left(\frac{1}{K_{ES}^*} + \frac{1}{K_{N1}} + \frac{1}{K_{N2}} + \ldots + \frac{1}{K_{Nj}}\right) = \frac{k_{cat}^*}{K_{ES}^*} \quad (3)$$

that k_{cat}/K_M does not depend on nonproductive binding but only upon those ionizations that affect the probuctive reaction.

Dixon and Webb (1960) have developed equations for the dependence of k_{cat}^* on pH in terms of $\tilde{k}_{cat}^*$ (the pH-independent velocity constant for breakdown of the active complex) and the appropriate pH function (f) which determines the concentration of this complex at any pH (eq 4). Similarly,

$$k_{cat}^* = \tilde{k}_{cat}^*/f_{ES*} \quad (4)$$

K_{ES*}, is represented by equation 5. Equations 4 and 5 show

$$K_{ES*} = \tilde{K}_{ES*}\left(\frac{f_{E*}f_{S*}}{f_{ES*}}\right) \quad (5)$$

that k_{cat}/K_M is only dependent upon ionizations on the free enzyme and substrate that affect the productive reaction (eq 6). For a system involving an enzyme active only when a

$$\frac{k_{cat}}{K_M} = \frac{k_{cat}^*}{K_{ES*}} = \frac{\tilde{k}_{cat}^*}{\tilde{K}_{ES*}}\left(\frac{1}{f_{E*}f_{S*}}\right) \quad (6)$$

critical ionizable group is undissociated and a substrate active only when it is ionized, k_{cat}/K_M may be represented by eq 7,

$$\frac{k_{cat}}{K_M} = \frac{\tilde{k}_{cat}^*}{K_{ES}}\left[\frac{1}{(1 + K_E/[H^+])(1 + [H^+]/K_S)}\right] \quad (7)$$

where K_E and K_S are defined by

$$EH \overset{K_E}{\rightleftharpoons} E + H$$

$$SH \overset{K_S}{\rightleftharpoons} S + H$$

TABLE I: Hydrolysis of Several *N*-CF_3CO-amino Acids by Pepsin.[a]

	Time (hr)	% Hydrolysis
N-CF_3CO-L-Phe	12	58
	48	98
N-CF_3CO-D-Phe	48	0
N-CF_3CO-D,L-Phe	12	23
	48	49
N-CF_3CO-L-*p*-F-Phe	12	59
N-CF_3CO-D,L-*p*-F-Phe	12	30
	48	50
N-CF_3CO-D,L-*o*-F-Phe	12	19
N-CF_3CO-L-Trp	12	37
N-CF_3CO-D-Trp	48	0
N-CF_3CO-D,L-Trp	12	17
N-CF_3CO-D,L-Tyr	12	16
N-CF_3CO-L-phenylglycine	12	0
N-CF_3CO-Gly	12	0

[a] $[E_0]$, 5.0×10^{-4} M, based on molecular weight of 34,200 (Rajagopalan *et al.*, 1966); $[S_0]$, 5.0×10^{-3} M; temperature, 34°; buffer, 0.10 M sodium citrate, pH 3.0.

In order to determine K_E and K_S, a computer program based upon eq 7 was written the outputs of which were a tabulation of values for k_{cat}/K_M as a function of pH for a set of assumed values of K_E and K_S. The input consisted of trial values for each quantity needed to define k_{cat}/K_M and was varied until the best fit was achieved as determined by visual comparison of the curves generated from these trial values of $\tilde{k}_{cat}^*/\tilde{K}_{ES*}$, K_E and K_S and the experimental pH profile.

Results

A typical fluorine nmr spectrum of a hydrolysis experiment is shown in Figure 1. Digital integration by a Fabritek computer showed an accuracy of better than 2% in determining concentrations of trifluoroacetate and *N*-CF_3CO-amino acids. This was demonstrated by taking spectra of several mixtures of known concentrations of trifluoroacetic acid and *N*-CF_3CO-L-Phe.

Table I lists data for the per cent hydrolysis of several *N*-CF_3CO-amino acids by porcine pepsin, and Table II lists data for the per cent hydrolysis of *N*-CF_3CO-L-Phe by several enzyme preparations. Under similar conditions, these substrates are stable to nonenzymic hydrolysis.

A plot of per cent hydrolysis *vs.* time for a typical hydrolysis experiment is shown in Figure 2; v_0 was calculated by a least-squares fit. Hydrolysis was, however, essentially linear even after 35–40% reaction. In practice, therefore, most determinations of v_0 were made from averages of several spectra taken in the range of 10–15% hydrolysis.

The range of substrate concentration used to evaluate the kinetic constants was limited somewhat by the maximum solubility of the substrate and the need to keep $[E_0] \ll [S_0]$. These factors restricted the kinetic studies to a 4- to 6-fold range of substrate concentration (7.5×10^{-3} to 4.0×10^{-2} M). However, as the K_M values fell within this range, they could be accurately determined from the $[S_0]/v_0$ *vs.* $[S_0]$ plots.

TABLE II: Hydrolysis of *N*-CF_3CO-L-Phe by Several Enzymes.[a]

Enzyme	pH	Time (hr)	% Hydrolysis
Pepsin (commercial, twice crystallized)	3.5	12	55
Pepsin (from pepsinogen activated according to Rajagopalan *et al.*, 1966)	3.5	12	50
Pepsin (N_2AcGlyOEt treated)	3.5	12	0
Pepsin (2,4′-dibromoacetophenone treated)	3.5	12	10
Human pepsin	3.5	12	53
Human gastricsin	3.5	12	13
None	1.0	24	0
None	5.4	24	0

[a] $[E_0]$, 5.0×10^{-4} M; $[S_0]$, 0.010 M, temperature, 34°; buffer, 0.10 M sodium citrate (pH 3.5 and 5.4), 0.10 M citric acid–HCl (pH 1.0).

Kinetic data for hydrolysis of *N*-CF_3CO-L-Phe are shown for the pH range 1.7–5.4 in Table III and for the pD range 3.4–4.3 in Table IV. The errors quoted are probable errors from the plots of $[S_0]/v_0$ *vs.* $[S_0]$ and are not accuracy estimates. However, duplicate experiments performed at pH 4.05 sug-

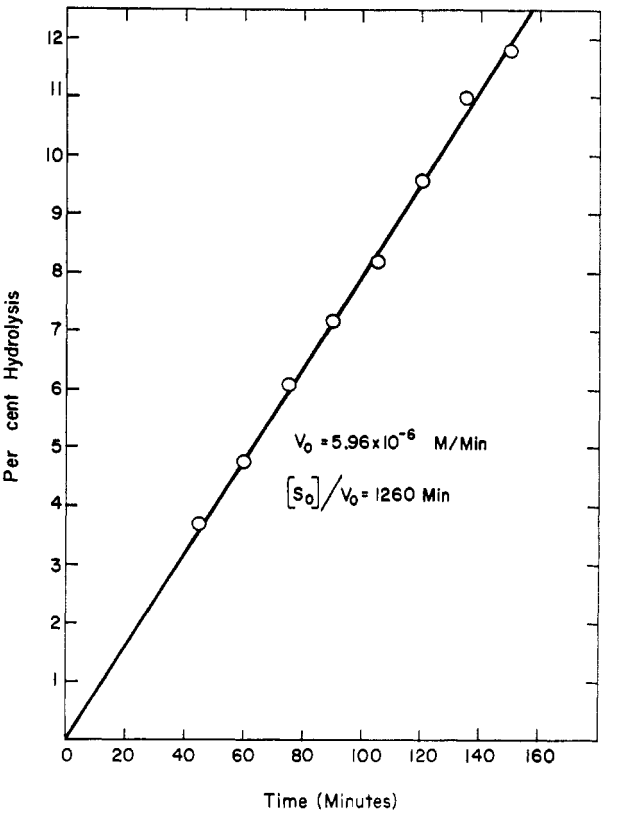


FIGURE 2: Hydrolysis of *N*-TFA-L-Phe by pepsin. $[E_0]$, 5.0×10^{-4} M; $[S_0]$, 7.5×10^{-3} M; 0.10 M sodium citrate buffer, pH 3.60; temperature, 34°. v_0 was calculated from a least-squares fit.

TABLE III: Kinetic Parameters for the Hydrolysis of *N*-CF_3CO-L-Phe by Pepsin.[a]

pH	$[S_0]$ (mM)	k_{cat} ($min^{-1} \times 10^3$)	K_M (mM)	k_{cat}/K_M (min^{-1} M^{-1})
1.70[b]	10.0–40.0	2.4 ± 0.2	6.6 ± 0.8	0.36 ± 0.02
2.25[b]	10.0–40.0	6.0 ± 0.6	5.9 ± 1.2	1.01 ± 0.10
2.80[b]	10.0–40.0	14.3 ± 0.8	8.0 ± 0.8	1.79 ± 0.10
3.05[c]	10.0–40.0	26.8 ± 1.0	14.0 ± 0.8	1.91 ± 0.06
3.35[c]	7.5–30.0	34.1 ± 3.5	16.6 ± 3.4	2.06 ± 0.21
3.60[c]	7.5–30.0	31.7 ± 1.0	15.5 ± 0.8	2.05 ± 0.06
4.05[c]	10.0–40.0	21.7 ± 0.4	21.6 ± 0.8	1.01 ± 0.02
4.50[c]	7.5–30.0	15.8 ± 1.0	28.6 ± 4.0	0.55 ± 0.04
4.65[c]	7.5–30.0	13.1 ± 0.6	29.0 ± 3.2	0.45 ± 0.02
5.40[c]	10.0–40.0	5.5 ± 1.0	81.8 ± 15.0	0.07 ± 0.02

[a] $[E_0]$, 5.0×10^{-4} M; temperature, 34 ± 1°. [b] 0.10 M citric acid–HCl buffer. [c] 0.10 M sodium citrate buffer.

TABLE IV: Kinetic Parameters for the Hydrolysis of *N*-CF_3CO-L-Phe by Pepsin in D_2O.[a]

pD	$[S_0]$ (mM)	k_{cat} ($min^{-1} \times 10^3$)	K_M (mM)	k_{cat}/K_M (min^{-1} M^{-1})
3.45	10.0–40.0	8.1 ± 1.0	14.1 ± 1.6	0.57 ± 0.06
3.65	10.0–40.0	10.7 ± 1.8	14.1 ± 2.4	0.76 ± 0.12
3.85	10.0–40.0	10.8 ± 0.6	14.3 ± 0.8	0.76 ± 0.04
4.05	10.0–40.0	12.5 ± 1.2	18.7 ± 1.8	0.67 ± 0.12
4.35	10.0–40.0	6.7 ± 0.4	21.0 ± 1.0	0.32 ± 0.02

[a] $[E_0]$, 5.0×10^{-4} M; temperature, 34 ± 1°; buffer, 0.10 M sodium citrate in D_2O.

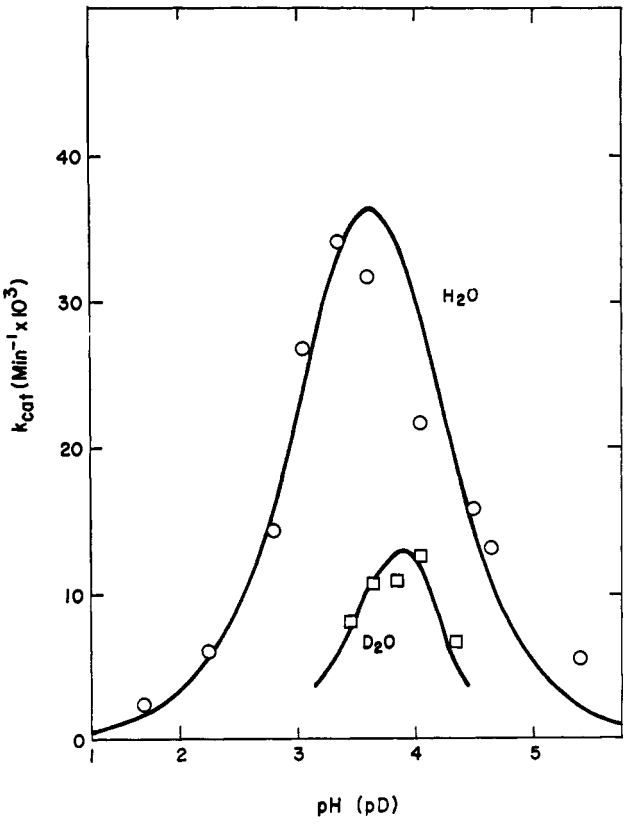


FIGURE 3: Plot of k_{cat} *vs.* pH for the hydrolysis of *N*-CF_3CO-L-Phe by pepsin at 34°. The circles are data for experiments in H_2O; the squares are data for experiments in D_2O.

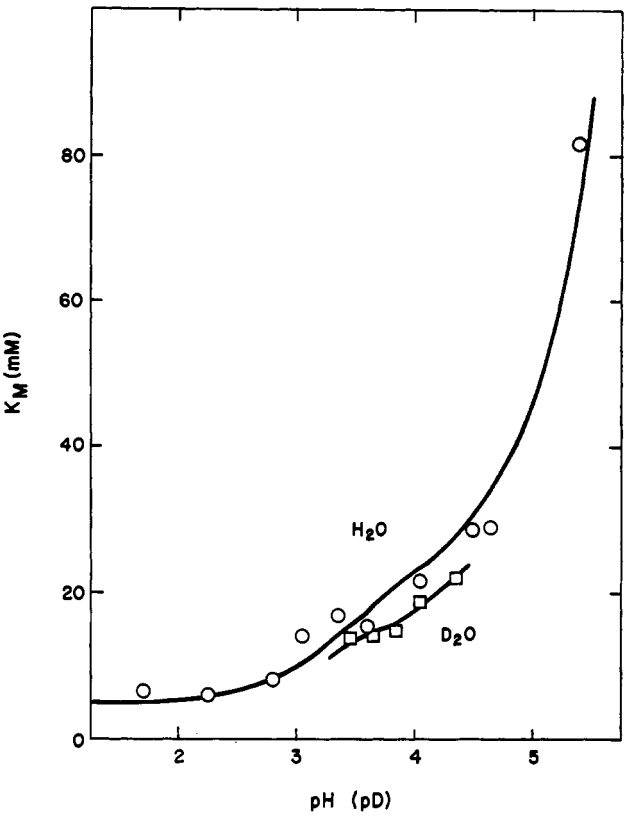


FIGURE 4: Plot of K_M *vs.* pH for the hydrolysis of *N*-CF_3CO-L-Phe by pepsin at 34°. The circles are data for experiments in H_2O; the squares are data for experiments in D_2O.

gested that data have a precision of ±4–8%. Plots of the kinetic parameters from Tables III and IV *vs.* pH and pD are shown in Figure 3 (for k_{cat}), Figure 4 (for K_M), and Figure 5 (for k_{cat}/K_M).

The computer fit of the data for k_{cat}/K_M to eq 7 gave a value for pK_E of 3.7 and a value for pK_S of 2.8. Figure 5 shows the theoretical curve obtained using these values for K_E and K_S and compares it to the experimental dependence of k_{cat}/K_M on pH.

Calculation of the deuterium isotope effect on k_{cat}/K_M was made from the formula $K_{H_2O}/k_{D_2O} = k_{max\ H_2O}/k_{max\ D_2O}$ where k_{max} was the apparent maximum value of the curves for k_{cat}/K_M *vs.* pH and pD. (Such a comparison depends on the independence of K_M on H_2O or D_2O as solvent; within experimental error, D_2O caused a decrease in k_{cat} without affecting K_M.) The value of the isotope effect (k_{H_2O}/k_{D_2O}) so determined was 2.8 ± 0.3.

Discussion

Analytical Method. The nmr technique described above has been found to be a convenient and accurate method for studying the kinetics of the pepsin-catalyzed hydrolysis of *N*-CF_3CO-amino acids. Commonly used assay procedures for studying pepsin kinetics involve spectrophotometric measurements of ninhydrin-reactive products released during hydrolysis of peptides (Inouye *et al.*, 1966) or differences in the ultraviolet (uv) absorption of substrates and products (Inouye and Fruton, 1967). The former often suffers from the need to correct for "enzyme blank" caused by ninhydrin-positive material in pepsin preparations, and the latter suffers

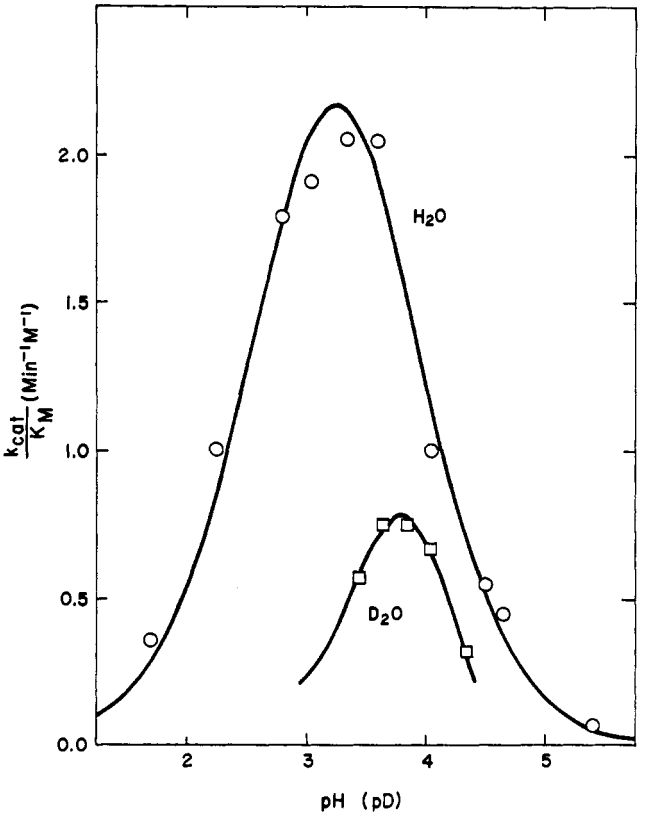


FIGURE 5: Plot of k_{cat}/K_M *vs.* pH for the hydrolysis of *N*-CF_3CO-L-Phe by pepsin at 34°. The circles are data for experiments in H_2O; the squares are data for experiments in D_2O. The solid line for the H_2O data is the theoretical line calculated from eq 7 for pK_S = 2.8 and pK_E = 3.7.

from the small change in absorbance observed upon hydrolysis of most substrates.

In our procedure, the only fluorine nmr signals came from the substrate and one of the hydrolysis products (trifluoroacetate) and the signals were far enough apart (10–18 Hz depending upon pH) that they could be integrated without overlap.

Pepsin kinetic studies have also suffered from the low solubility of pepsin substrates in aqueous solution, and failure to obtain substrate concentrations greater than K_M without adding organic solvents to the reaction solution has often prevented accurate separate determinations of k_{cat} and K_M. *N*-CF_3CO-amino acids are soluble to concentrations well above K_M, and such determinations were easily possible in our experiments. The chief limitation of the nmr technique involved the relative slowness of the hydrolysis of *N*-CF_3CO-amino acids (even at the pH optimum for the reaction) which necessitated use of fairly high enzyme concentrations (5×10^{-4} M).

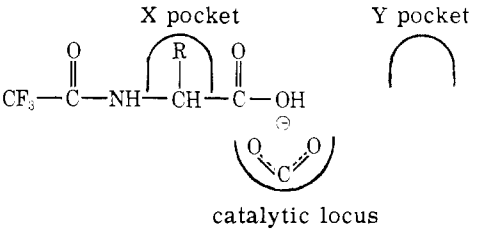
Specificity. The specificity of pepsin in hydrolysis of *N*-CF_3CO-amino acids parallels its specificity in hydrolysis of peptides. Only *N*-CF_3CO-amino acids with the L configuration are cleaved, and aromatic amino acids are preferred. Pepsin's failure to hydrolyze *N*-CF_3CO-Gly and *N*-CF_3CO-L-phenylglycine is consistent with its known specificity (Fruton, 1970). He found that, although a hydrophobic side chain is normally required of a pepsin substrate, branching at the β carbon of an amino acid involved in a peptide bond prevents hydrolysis of that bond (*i.e.*, a β-methylene group is essential for catalytic activity).

Human and porcine pepsin, known to possess almost identical catalytic specificity and activity (Mills and Tang, 1967), hydrolyzed *N*-CF_3CO-L-Phe at approximately the same rate. Human gastricsin, which possesses a very similar, but not identical, catalytic specificity (Huang and Tang, 1969), also hydrolyzes this substrate, but at a markedly slower rate. A highly purified porcine pepsin sample obtained by activation of commercial pepsinogen according to the method of Rajagopalan *et al.* (1966) showed the same activity toward *N*-CF_3CO-L-Phe as commercial twice-crystallized pepsin.

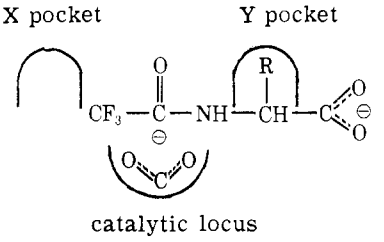
The only products detected during hydrolysis of *N*-CF_3CO-L-Phe by pepsin were trifluoroacetic acid and phenylalanine. No transpeptidation producing *N*-CF_3CO-L-Phe-L-Phe was observed. There was no appearance of a fluorine resonance for *N*-CF_3CO-L-Phe-L-Phe (which comes 15–20 Hz upfield of the fluorine resonance for *N*-CF_3CO-L-Phe), and the combined area of the peaks for *N*-CF_3CO-L-Phe and trifluoroacetic acid was constant.

Porcine pepsin previously treated with N_2AcGlyOEt and thereby inactivated toward hemoglobin substrate was also inactive toward *N*-CF_3CO-L-Phe. Porcine pepsin previously treated with 2,4′-dibromoacetophenone and possessing only 22% of its initial activity in hemoglobin assay retained only 18% of activity toward *N*-CF_3CO-L-Phe. Accordingly, hydrolysis of *N*-CF_3CO-amino acids seems to be a true pepsin-catalyzed reaction involving the same catalytic site as that used in pepsin's other catalytic activities.

pH Dependence and Mode of Substrate Binding. The pH dependence of the kinetic parameters for the hydrolysis of *N*-CF_3CO-amino acids suggests that, although the undissociated substrate binds more strongly to the enzyme than does the ionized substrate, only ionized substrate is hydrolyzed appreciably. Neutral substrate probably binds in an unproductive mode with its side chain "R" filling the hydrophobic pocket normally occupied by the "X" side chain of a dipeptide substrate such as CH_3–CO–NH–CHX–CO–NH–CHY–COOH. For anionic substrates, binding will be expected to



decrease due to electrostatic repulsion between the negatively charged substrate and the enzymic carboxylate anion. This would be particularly important if the side chain were to bind in the "X" pocket as this would position the carboxylate group of the substrate near the carboxylate anion on the enzyme catalytic locus. Accordingly, the substrate, when bound, will be more likely to have its side chain in the "Y" pocket which will minimize the electrostatic repulsion of the two carboxylate anions and position the sensitive amide bond near the catalytic locus.



In fact, this type of binding accounts for the observed pH dependence of hydrolysis at low pH. The substrate has a pK_a of 2.85 and the kinetic measurements show that the hydrolysis is dependent upon an ionization of $pK_a = 2.8$ for the free substrate or enzyme (presumably the substrate).

We find that the basic side of the curve of k_{cat}/K_M *vs.* pH depends on ionization of a group on the free enzyme with an apparent pK_a of 3.7 which does not correspond to that of either of the catalytic carboxyl groups (pK_a's = 1.1 and 4.7) implicated in pepsin-catalyzed hydrolysis of peptide substrates (Cornish-Bowden and Knowles, 1969). However, esterification of the β-carboxyl group of an aspartyl residue of pepsin by 2,4′-dibromoacetophenone is known to partially inactivate pepsin by interfering with substrate binding without affecting k_{cat} (Erlanger *et al.*, 1967). Further, Hollands and Fruton (1968) have shown that a noncatalytic carboxyl group with an apparent pK_a of 3.8 strongly influences both binding and hydrolysis of a series of cationic substrates. Thus, this acidic group (of $pK \sim 3.7$) is apparently located sufficiently near the site occupied by the free carboxyl group of a bound substrate (as, for example, *N*-Ac-L-Phe-L-Phe) to interfere significantly with binding when both it and the substrate are negatively charged (Cornish-Bowden and Knowles, 1969). Accordingly, ionization of this group on the enzyme (pK_a = 3.7) decreases binding of *N*-CF_3CO-amino acids (whose carboxyl groups are already ionized, having a pK_a of 2.8) in the "Y" pocket which is the productive mode. This decrease in productive binding as the enzyme group of $pK_a = 3.7$ is ionized will lead, in turn, to the observed decrease in hydrolysis in this pH region.

Finally, the dependence of K_M on pH (Figure 5) suggests that ionization of a group on the free enzyme with an apparent

pK_a around 4.8 decreases binding drastically. This ionization corresponds closely to that of one of the catalytic carboxyl groups, and might be expected to prevent binding since it would create significant negative charge in both the preferred "X" site and the productive "Y" site. Ionizations of the catalytic carboxyl groups with pK_a's 1.1 and 4.7 are not manifest in the plot of k_{cat}/K_M *vs.* pH as their states of ionization are effectively unchanged over the pH range where activity against *N*-CF_3CO-amino acids is observed.

Why are *N*-CF_3CO-amino acid derivatives hydrolyzed by pepsin when *N*-acetyl derivatives are not? Failure to observe hydrolysis of *N*-acetylamino acids probably results from the small fraction that would be bound in a productive mode. With pK_a's generally around 3.5–3.7, they ionize at essentially the same pH as the enzymic carboxyl group with pK_a = 3.7. In contrast, for the *N*-CF_3CO derivatives of amino acids which have a significantly lower pK_a than the corresponding acetylamino acids, there should exist an appreciable pH range over which the *N*-CF_3CO-amino acid is ionized while the enzymic carboxyl group of pK = 3.7 is still neutral. Over this pH range, the *N*-CF_3CO-amino acid will bind to pepsin in a productive mode, with the aromatic residue in the "Y" pocket and the trifluoroacetamide bond in the catalytic locus. Even so, the favorable pH range will be small, and the catalytic rate, further decreased by the presence of appreciable nonproductive binding, might be considerably slower than that for most good pepsin substrates. The observed rates are, in fact, slower by a factor of 100–1000.

These effects of pH on binding and catalysis of normal acid substrates (*N*-Ac-L-Phe-L-Phe) and *N*-CF_3CO-amino acids (*N*-CF_3CO-L-Phe) are shown graphically in Figure 6. Whereas a normal acid substrate binds productively in the low pH range, an *N*-CF_3CO-amino acid derivative does not begin to bind in a productive manner until its carboxyl group is ionized (pK_a = 2.8). Above this pH, its activity as a substrate increases until titration of the carboxylic acid group on the enzyme with pK_a of 3.7 leads to decreased binding both for normal acid substrates and *N*-CF_3CO-amino acids. Thus, in the higher pH region both curves fall off with ionization of this enzymic carboxyl group (pK_a = 3.7).

Essentially the scheme just discussed proposes that *N*-CF_3CO-L-Phe usually binds to the enzyme in the "X" pocket in a nonproductive mode; only when bound in the "Y" pocket does hydrolysis occur. We sought further substantiation of this proposal by studying the effect on the hydrolysis of *N*-CF_3CO-L-Phe of L-tryptophan ethyl ester which has been shown (Inouye and Fruton, 1968) to behave as a linear competitive inhibitor (K_I = 6 mM at pH 4.0) for the pepsin-catalyzed hydrolysis of *N*-Cbz-L-His-L-Phe(NO_2)-Phe-OMe and to bind preferentially in the "Y" pocket (Fruton, 1970). Thus L-tryptophan ethyl ester should, according to our scheme, compete only with the productive binding of *N*-CF_3CO-L-Phe.

The kinetic manifestations of such competition between L-tryptophan ethyl ester and hydrolysis of *N*-CF_3CO-L-Phe can be described by the following equations

$$E + S \overset{K_{ES}^*}{\rightleftharpoons} ES \xrightarrow{k_{cat}^*} E + P$$

$$E + S \overset{K_N}{\rightleftharpoons} ES_N$$

$$E + I \overset{K_I}{\rightleftharpoons} EI$$

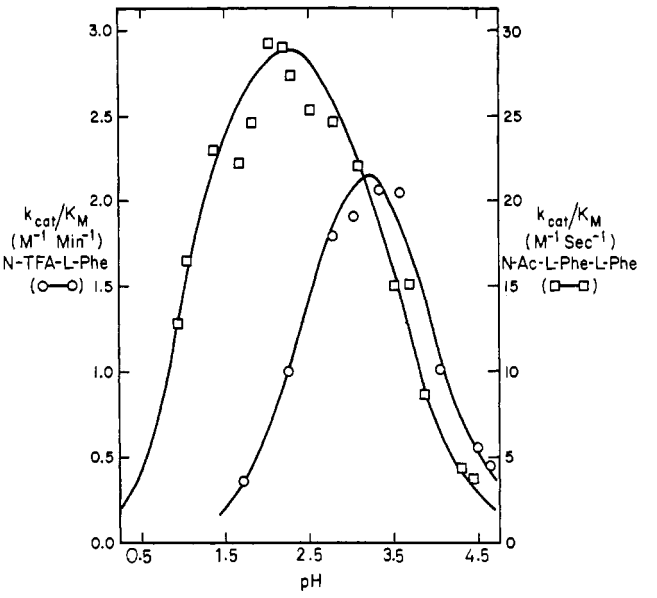


FIGURE 6: Comparison of pH dependence of k_{cat}/K_M for pepsin-catalyzed hydrolysis of *N*-CF_3CO-L-Phe and *N*-Ac-L-Phe-L-Phe. Data for *N*-CF_3CO-L-Phe from Table I. Data for *N*-Ac-L-Phe-L-Phe from Cornish-Bowden and Knowles (1969).

$$EI + S \overset{K_N}{\rightleftharpoons} ES_NI; \quad ES + I \overset{K_N}{\rightleftharpoons} ES_NI$$

Solution of these equations leads to the following relations between the molecular and observed parameters (k_{cat} and K_M)

$$k_{cat} = \frac{k_{cat}^*}{\left(1 + \frac{K_{ES}^*}{K_N} + \frac{K_{ES}^*}{K_N}\frac{[I]}{K_I}\right)}$$

$$K_M = \frac{K_{ES}^*\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{K_{ES}^*}{K_N} + \frac{K_{ES}^*}{K_N}\frac{[I]}{K_I}\right)}$$

Under conditions when $K_{ES}^* > K_N$ (*i.e.*, most binding occurs in a nonproductive fashion as in the present case) and $[I]/K_I > 1$ (a condition which can be—and in the present case was—imposed on the system by keeping $[I] > K_I$) these equations simplify to

$$k_{cat} \sim \frac{k_{cat}^*}{\frac{K_{ES}^*}{K_N}\left(1 + \frac{[I]}{K_I}\right)}$$

$$K_M \sim K_N$$

Thus, such an inhibitor under these conditions, though competing with the productive binding of substrate will cause no observable change in K_M, but will cause a decrease in the observed k_{cat} which will be a function of [I]; the inhibitor will manifest the symptoms of noncompetitive inhibition (decrease in k_{cat}, no change in K_M).

A study of the pepsin-catalyzed hydrolysis of *N*-CF_3CO-L-Phe at pH 3.60 in the presence of 12.0 mM L-TrpOEt showed that k_{cat} was reduced to 11.3 × 10^{-3} min^{-1} (compared to 31.7 × 10^{-3} min^{-1} in the absence of inhibitor) and that K_m

was 15.1 mM in the presence of inhibitor, essentially unchanged from its value of 15.5 mM without inhibitor. Moreover, the value of K_I of 6.6 mM based on these results and calculated from the relationship

$$\frac{k_{cat}}{K_M} = \frac{k_{cat}^*}{K_{ES}^*\left(1 + \frac{[I]}{K_I}\right)} \text{ (note } K_M \sim K_N\text{)}$$

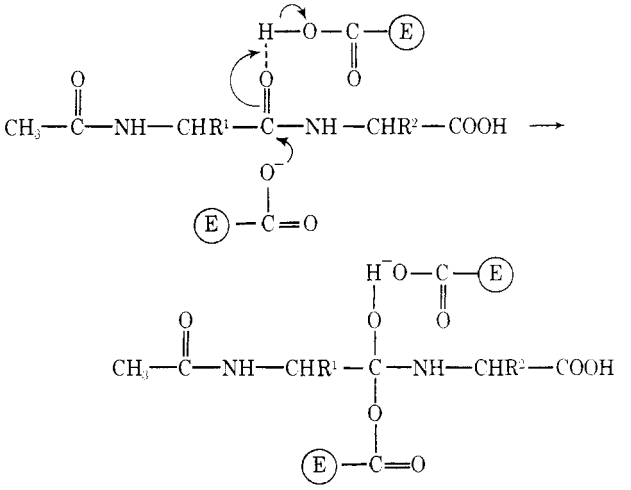
agrees well with the value of 6 mM reported by Inouye and Fruton (1968). The central point of this particular experiment is, however, the support it lends to the general scheme proposed in this paper for the interaction of *N*-CF_3CO-L-Phe with pepsin.

Isotope Effect. Replacement of water by deuterium oxide reduces the rate of pepsin-catalyzed hydrolysis of *N*-CF_3CO-L-phenylalanine by a factor of three. The effect is apparently entirely on k_{cat}; K_M within experimental error remains unchanged. An isotope effect of this type manifested in k_{cat} suggests that proton transfer occurs in the rate-determining catalytic step. The magnitude of this effect and its relation to the nature of the trifluoroacetyl group has significance in regard to the mechanism of pepsin-catalyzed reactions and will be discussed after some preliminary comments on the probable nature of the catalytic groups and mechanism.

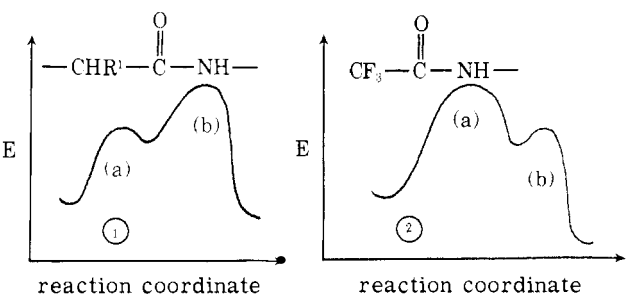
Mechanism. An important question concerns the identity and nature of the carboxylic acid groups (or carboxylate anions) involved in pepsin-catalyzed hydrolysis. Kinetic studies of the hydrolysis of substrates such as *N*-Ac-L-Phe-L-$PheNH_2$ which do not ionize in the range of pepsin activity have shown that at least two carboxyl groups at pepsin's active-center function in catalysis (Cornish-Bowden and Knowles, 1969). One of these, with an apparent pK_a of 1.1, must be present in the ionized form. The other, with an apparent pK_a of 4.7, must be present in the undissociated form. Modification studies involving active-site-directed irreversible epoxide inhibitors have shown that esterification of a single carboxyl group with a pK_a below 2 (presumably the same one implicated in kinetic studies) destroys pepsin's catalytic activity (Tang, 1971). Modification studies involving active-site-directed irreversible diazocarbonyl inhibitors have shown that esterification of a different carboxyl group, one with a much higher pK_a, also destroys catalytic activity (Bayliss *et al.*, 1969). Lundblad and Stein (1969) have argued that the Cu(II)-catalyzed reaction of diazocarbonyl compounds with carboxylic acids invariably involves reaction with an undissociated carboxyl group. Thus, the generally accepted notion that the catalytic carboxyl group with $pK_a = 4.7$ is the group blocked by diazocarbonyl inhibitors seems unlikely as the pH dependence of this inactivation indicates that the group with $pK_a = 4.7$ must be ionized for inactivation to occur. In fact, Lundblad and Stein (1969) proposed a reaction in which the positively charged, copper-complexed inhibitor is attracted to the negatively charged active site in which the carboxyl group with $pK_a = 4.7$ is ionized. In this way, the inhibitor is positioned for reaction with a third, still undissociated, catalytic carboxyl group with a pK_a around 6. This conclusion, that several carboxyl groups may function in catalysis, is also supported by the work of Fruton (1970) who found that the pH optimum for pepsin hydrolysis may vary from 2.0 to 4.5 depending upon the substrate.

Though the exact function of these various carboxyl groups is presently unknown, an electrophilic and a nucleophilic component seem to play a role in pepsin catalysis. The presence of an electrophilic component in the catalytic mechanism is suggested, for example, by the relative rates of the hydrolysis of depsipeptide (ester) and peptide (amide) substrates ($k_{depsipeptide}/k_{peptide} = 2$) (Inouye and Fruton, 1969). Also, the observed isotope effect ($k_{H_2O}/k_{D_2O} = 2$) for hydrolysis of Gly-Gly-Gly-L-Phe(NO_2)-L-PheOMe at the Phe(NO_2)-Phe bond reveals that the appropriate amino acid residues in the peptide substrates can cause the electrophilic component to dominate (Hollands and Fruton, 1969).

On the other hand, the absence of a singificant deuterium isotope effect with peptides such as *N*-Ac-L-Phe-L-TyrOMe ($k_{H_2O}/k_{D_2O} = 1.05 \pm 0.30$) or sulfite esters such as methyl phenyl sulfite ($k_{H_2O}/k_{D_2O} = 1.1 \pm 0.1$) suggests that the electrophilic component is not the rate-limiting step in hydrolysis of these pepsin substrates (Clement and Snyder, 1966; Reid and Fahrney, 1967), and implies, in turn, that a nucleophilic component occurs in pepsin's mechanism and can become the rate-limiting step. Accordingly, the initial hydrolytic step may be a concerted attack by two enzymic carboxyl groups, one in the ionized form acting as a nucleophile and the other in the undissociated form acting as a proton or hydrogen-bond donor (Knowles, 1970). The influence on the deuterium



isotope effect observed in this work by replacing the $CH_3CONHCHR^1$ group with a CF_3 group supports such a mechanism. The electron-withdrawing nature of the trifluoromethyl group will decrease electron density at the carbonyl group at which reaction occurs. This will decrease the ability of the oxygen atom of this carbonyl group to function as a hydrogen bond or proton acceptor, but at the same time it will increase the susceptibility of the carbon atom to nucleophilic attack. This effect may be represented by the following energy profiles in which the first transition state (a) represents proton transfer (the electrophilic component of the mechanism) and the second (b) represents nucleophilic attack.



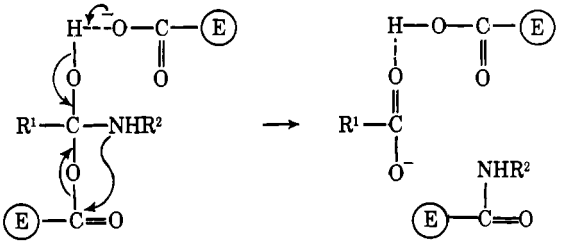
The isotope effect of 3 associated with hydrolysis of *N*-CF_3CO-amino acids by pepsin suggests that the energy profile for this reaction may resemble diagram 2 in which proton transfer is rate limiting for the overall reaction (which will result from the freezing out of an oxygen–hydrogen stretching vibration in the transition state (Jencks, 1969)). The absence of an isotope effect in the hydrolysis of *N*-Ac-L-Phe-L-TyrOMe (Clement and Snyder, 1966) suggests that this reaction may have an energy profile like diagram 1. The zero-point energy of the O–H and O–D bonds in the ground state of the reactant is lost in the initial state involving proton transfer, but it is regained in new O–H and O–D bonds on the substrate before the rate-determining step (nucleophilic attack) occurs. This variation in the deuterium isotope effect of pepsin-catalyzed reactions with the nature of the substrate supports a mechanism of pepsin action involving concerted electrophilic–nucleophilic attack. (Incidentally, the order along the reaction coordinate of electrophilic or nucleophilic attack is not essential to this argument.)

The electrophilic–nucleophilic cooperativity of a carboxylic acid and carboxylate anion of pepsin in the attack on the carbonyl group of the substrate leads to a tetrahedral intermediate whose formation probably represents the slow step in the overall reaction and whose breakdown leads to eventual release of products. Attempts to isolate any stable intermediate have been inconclusive (Fruton, 1970) which one might expect if formation of the tetrahedral intermediate is very slow compared to subsequent steps. In this connection, Knowles (1970) has shown that the rate-limiting step is that which immediately follows formation of the Michaelis complex (i) by failure to observe any burst release of either of the two products of hydrolysis, (ii) by the nonequivalence of k_{cat} for a series of substrates in which either the acyl or amino moiety is the same, and (iii) by the apparent equivalence of K_M and the enzyme–substrate dissociation constant (*i.e.*, $k_2 \ll k_{-1}$ in the normal Michaelis–Menten kinetic scheme).

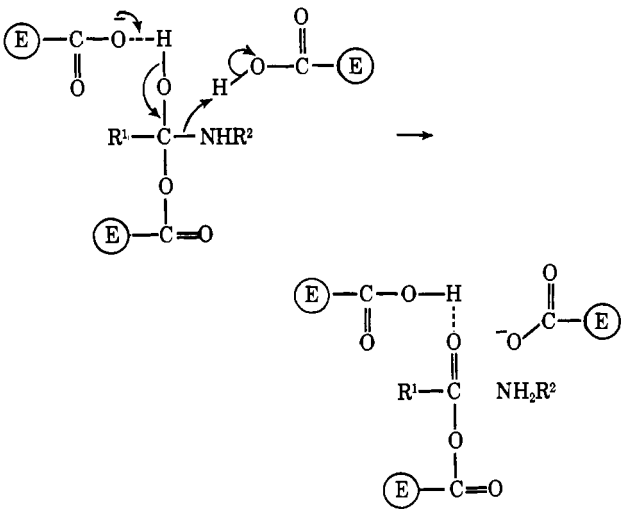
The nature of pepsin-catalyzed transpeptidation indicates, however, that the release of products is not random and that the amino moiety is retained for a finite time. With pepsin, transpeptidation occurs by amino transfer (*i.e.*, transfer of enzyme bound amino moiety to a new acceptor acyl moiety) but does not occur by analogous acyl transfer (Neumann *et al.*, 1959; Fruton *et al.*, 1961). Accordingly, the amino moiety must remain bound long enough for the acyl product to leave and be replaced by a new amino acceptor.

Kitson and Knowles (1971a,b) have shown by the nature of the inhibition of hydrolysis of *N*-Ac-L-Phe-L-PheOMe by *N*-Ac-L-Phe and L-PheOMe that there is indeed an ordered release of products with acyl moiety leaving first. L-PheOMe acts as a linear competitive inhibitor and apparently binds only to free enzyme. At Ph 2.1, when it is largely undissociated, *N*-Ac-L-Phe behaves as a linear noncompetitive inhibitor and binds both to the free enzyme and to the enzyme–amino moiety complex in a nonproductive manner such that transpeptidation is not possible. At pH 4.3, however, when it is largely ionized, *N*-Ac-L-Phe approximates a linear competitive inhibitor, binding both to the free enzyme and to the complex in a productive manner so that transpeptidation is possible. The change in the nature of the inhibition indicates that the acyl product leaves as the anion during hydrolysis and enters as the anion during transpeptidation. This explains the failure of *N*-Ac-L-PheOMe and *N*-Ac-L-PheSMe to serve as transpeptidation acceptors whereas *N*-Ac-L-Phe can (Kitson and Knowles, 1971b). *N*-Ac-L-Phe must be ionized for this reaction, the reverse of hydrolysis, but *N*-Ac-L-PheOMe and *N*-Ac-L-PheSMe cannot form the ionized species necessary to attack the enzyme–amino moiety complex.

Whether this "amino–enzyme" is a covalent intermediate in the catalytic scheme is unknown. The amino moiety could simply be bound more tightly to the enzyme than the acyl moiety as is suggested by the finding that K_I for *N*-Ac-Gly-L-Phe is one-fifth that of K_I for *N*-Ac-L-Phe-Gly (Knowles *et al.*, 1969). Knowles (1970) has argued, however, for a mechanism in which the tetrahedral intermediate undergoes a 1 → 3 nitrogen shift to yield an amino–enzyme with a new amide bond between the amino moiety of the original peptide bond and a carboxylate group on the enzyme.

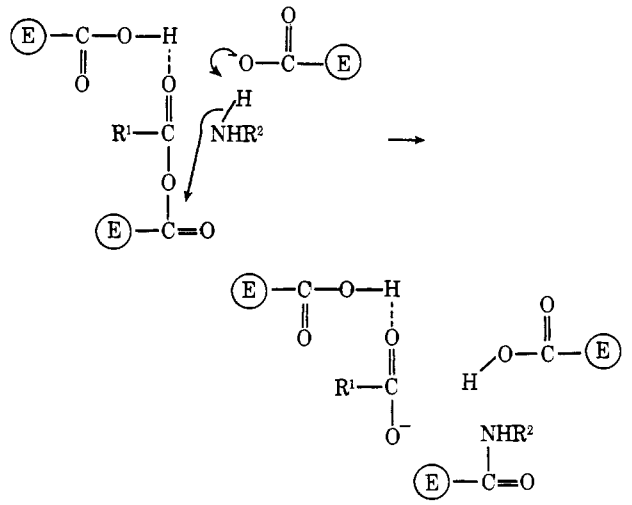


While this mechanism would account for the ordered release of products, the distortions in bond angles required for such a shift to occur directly make this an unlikely mechanism for efficient catalysis. The difficulty can, however, easily be circumvented by utilization of a third carboxyl group (one of the two with high pK_a's) implicated by kinetic and inactivation studies. Subsequent release of the acyl moiety

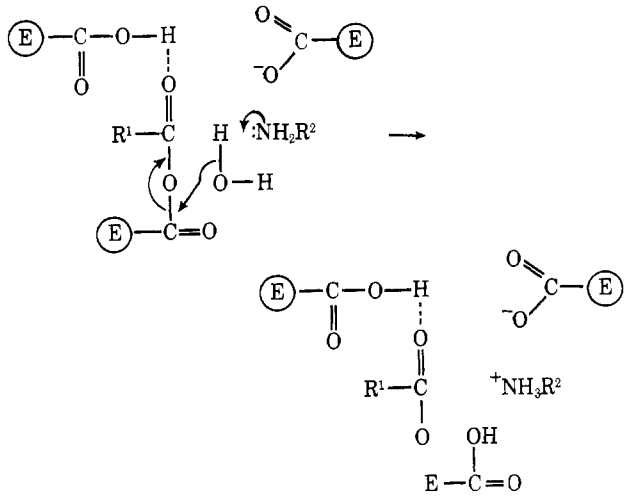


of the substrate and production of a covalent amino–enzyme could then be effected by an attack from the highly nucleophilic amine nitrogen on the anhydride link at the enzymic carboxyl carbon. This intermediate could then be hydrolyzed by water to liberate the free amine in a reaction catalyzed by one of the free carboxyl groups.

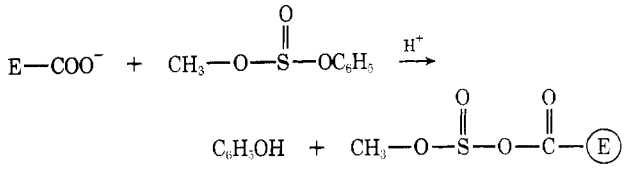
Alternatively, water might enter the scheme in the attack on the acyl (anhydride) intermediate. In this reaction, no covalent amino enzyme is formed, and the ordered release of products and transpeptidation would be accounted for by stronger binding of the amino moiety to the enzyme, an alternative we favor for the following reasons. First, it accounts for the inconclusiveness of efforts to isolate a specific, covalent amino–enzyme (Fruton, 1970). Second, it eliminates the seeming inefficiency of breaking one peptide bond and form-



ing another. Third, it accounts for results of ^{18}O-tracer experiments on the source of the oxygen added to the acyl moiety during hydrolysis. Stein and Fahrney (1968) have shown that an oxygen atom is transferred from an enzymic carboxyl



group to the sulfite group of the substrate during hydrolysis of methyl phenyl sulfite. They suggested that nucleophilic attack by an enzymic carboxylate anion on the sulfite ester would yield a mixed anhydride. This mixed anhydride would



be expected to undergo further nucleophilic attack primarily at the enzymic carbon, resulting in transfer of an oxygen atom originally in the enzymic carboxylate group to the substrate. In the case of the anhydride formed during peptide hydrolysis, however, the subsequent attack might just as likely occur at the substrate carbon. This is in accord with unpublished results quoted by Stein and Fahrney (1968) that in hydrolysis of *N*-Cbz-L-Phe-L-Tyr by pepsin in $H_2{}^{18}O$ the initial *N*-Cbz-L-Phe released by the enzyme contains excess ^{18}O (that is, the product formed before any ^{18}O could have been incorporated into the enzymic carboxylate anion). The mechanism involving the covalent amino enzyme does not allow for this latter result, and further ^{18}O-tracer studies are needed to confirm its occurrence and distinguish between the two mechanisms. Lastly, electrostatic effects should lead to facile ejection of the negatively charged carboxylate anion (acyl) moiety and some retention of the positively charged amino moiety of the substrate since the active site of pepsin must carry an overall negative charge.

Conclusion

The main point from the work of the present paper is that pepsin catalysis of the hydrolysis of *N*-CF_3CO-L-phenylalanine leads to insight into the dramatic effect of pH on binding into the two aromatic pockets of pepsin. The observed kinetic isotope effect supports a mechanism for formation of a tetrahedral intermediate involving two carboxyl functions (one as a neutral carboxylic acid, the other as a carboxylate anion) which mount a concerted electrophilic–nucleophilic attack on the substrate leading to a tetrahedral intermediate, whose subsequent breakdown probably requires the intervention of a third carboxylic acid group. Moreover, the stronger binding of the amino moiety than of the carboxylate moiety of the substrate may be caused by secondary forces and may not require covalent attachment of the amino fragment to the enzyme.

Acknowledgment

We are grateful to R. B. Moon and K. L. Gammon for help in synthesis of some of the *N*-CF_3CO-amino acids used in this study and to S. H. Smallcombe for helpful suggestions about experimental procedures.

References

Anson, M. L., and Mirsky, A. E. (1932), *J. Gen. Physiol. 16*, 59.

Bayliss, R. S., Knowles, J. R., and Wybrandt, G. B. (1969), *Biochem. J. 113*, 377.

Bell, R. P. (1959), The Proton in Chemistry, Ithaca, N. Y., Cornell University Press, p 188.

Clement, G. E., and Snyder, S. L. (1966), *J. Amer. Chem. Soc. 88*, 5338.

Cornish-Bowden, A. J., and Knowles, J. R. (1969), *Biochem. J. 113*, 353.

Dixon, M., and Webb, E. C. (1960), Enzymes, New York, N. Y., Academic Press, p 129.

Erlanger, B. F., Vratsanos, S. M., Wassermann, N., and Cooper, A. G. (1967), *Biochem. Biophys. Res. Commun. 28*, 203.

Fones, W. S. (1952), *J. Org. Chem. 17*, 1661.

Fruton, J. S. (1970), *Advan. Enzymol. 33*, 401.

Fruton, J. S., Fujii, S., and Knappenberger, M. H. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 759.

Gammon, K. L., Smallcombe, S. H., and Richards, J. H. (1972), *J. Amer. Chem. Soc.* (in press).

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, N. Y., Wiley, p 932.

Hein, G. E., and Niemann, C. (1962), *J. Amer. Chem. Soc. 84*, 4495.

Hollands, T. R., and Fruton, J. S. (1968), *Biochemistry 7*, 2045.

Hollands, T. R., and Fruton, J. S. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1116.

Huang, W. Y., and Tang, J. (1969), *J. Biol. Chem. 244*, 1085.
Inouye, K., and Fruton, J. S. (1967), *Biochemistry 6*, 1765.
Inouye, K., Voynick, I., Delpierre, G. R., and Fruton, J. S. (1966), *Biochemistry 5*, 2473.
Kitson, T. M., and Knowles, J. R. (1971a), *Biochem. J. 122*, 241.
Kitson, T. M., and Knowles, J. R. (1971b), *Biochem. J. 122*, 249.
Knowles, J. R. (1970), *Phil.. Trans. Roy. Soc. London, Ser. B 257*, 135.
Knowles, J. R., Sharp, H., and Greenwell, P. (1969), *Biochem. J. 113*, 343.
Kozlov, L. V., Ginodman, L. M., and Orekhovich, V. N. (1967), *Biokhimiya 32*, 1011.
Kozlov, L. V., Ginodman, L. M., Zolotarev, B. M., and Orekhovich, V. N. (1962), *Dokl. Akad. Nauk SSSR 146*, 943.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 246.
Lundblad, R. L., and Stein, W. H. (1969), *J. Biol. Chem. 244*, 154.
Mills, J. N., and Tang, J. (1967), *J. Biol. Chem. 242*, 3093.
Neumann, H., Levin, Y., Berger, A., and Katchalski, E. (1959), *Biochem. J. 73*, 33.
Rajagopalan, T. G., Moore, S., and Stein, W. H. (1966), *J. Biol. Chem. 241*, 4940.
Reid, T. W., and Fahrney, D. (1967), *J. Amer. Chem. Soc. 89*, 3941.
Richmond, V., Tang, J., Wolf, S., Trucco, R., and Caputto, R. (1958), *Biochim. Biophys. Acta 29*, 453.
Stein, T. P., and Fahrney, D. (1968), *Chem. Commun.*, 555.
Tang, J. (1963), *Nature (London) 199*, 1094.
Tang, J. (1971), *J. Biol. Chem. 246*, 4510.

Specific Synthesis of 1-(5-Glutamyl)-2-methylhydrazine by Glutamine Synthetase†

Melvin L. Rueppel, Susan Lord Lundt, Jerald D. Gass, and Alton Meister*

ABSTRACT: Incubation of methylhydrazine in place of ammonia in the glutamine synthetase system leads to the formation of a specific isomer of 5-glutamylmethylhydrazine, namely, 1-(5-glutamyl)-2-methylhydrazine (I). This was established by the unambiguous synthesis of I and its isomer 1-(5-glutamyl)-1-methylhydrazine (II); these were obtained by reaction of 1-carbobenzoxy-1-methylhydrazine or 1-carbobenzoxy-2-methylhydrazine, respectively, with the mixed anhydride obtained by treatment of α-*tert*-butyl *N*-carbobenzoxy-L-glutamate with ethyl chloroformate, followed by removal of the blocking groups. In the presence of relatively high concentrations of methylhydrazine the product of the enzyme-catalyzed reaction (I) is converted to II; this conversion is nonenzymatic. The findings indicate that methylhydrazine attaches to the ammonia binding site of glutamine synthetase in a specific manner which makes only the nonalkylated nitrogen atom of methylhydrazine available for reaction with enzyme-bound γ-glutamyl phosphate.

In the reaction catalyzed by glutamine synthetase (reaction 1), ammonia may be replaced by hydroxylamine, hydrazine, methylamine, glycine ethyl ester (Speck, 1949; Elliott, 1951; Levintow and Meister, 1954), methylhydrazine (Willis, 1966), and several other amines; under these conditions, the corresponding 5-glutamyl compounds are formed. Methylhydrazine is of particular interest since it possesses two nucleophilic centers. As a consequence, depending upon the nature of the amine-binding site on the enzyme, one might expect either 1-(5-glutamyl)-1-methylhydrazine (II, Figure 1) or 1-(5-glutamyl)-2-methylhydrazine (I) to be the enzymatic product; indeed there is no reason, *a priori*, to exclude formation of both I and II. Study of the available literature on the nonenzymatic reactions of methylhydrazine is consistent with the possibility that both I and II might be formed by glutamine synthetase. Thus, it has been shown that reaction of methylhydrazine with an anhydride or with an ester gives both possible hydrazine derivatives (Hinman and Fulton, 1958; Pedersen, 1964). Willis (1966) reported that incubation of methylhydrazine in the glutamine synthetase system gave two enzymatic products which he characterized as I and II; these were not distinguished.

$$\text{glutamate} + NH_3 + ATP \overset{M^{2+}}{\rightleftharpoons} \text{glutamine} + ADP + P_i \quad (1)$$

While we have in the present work confirmed the experimental observations of Willis (1966), a further investigation of the interaction of methylhydrazine in the glutamine synthetase system has shown that there is only one enzymatic product and that the second product arises by a nonenzymatic reaction between the enzymatically formed product and methylhydrazine. In the work reported here, we have established the identity of both the enzymatic product (I) and the nonenzymatic product (II).

Experimental Approach

In the studies reported by Willis (1966), sheep brain glutamine synthetase was incubated with [^{14}C]methylhydrazine, glutamate, ATP, imidazole-HCl buffer, 2-mercaptoethanol,

† From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. *Received March 30, 1972*. Supported in part by the National Institutes of Health, U. S. Public Health Service, and the National Science Foundation.