Kinetics of the Pepsin-catalyzed Hydrolysis of *N*-Acetyl-L-phenylalanyl-L-diiodotyrosine*

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ABSTRACT: Kinetics of the hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine by pepsin was studied at *p*H 2 and 4.5, alone and in the presence of inhibitors. In addition, the influence of ionic strength on the *p*H-activity profile for the hydrolysis was also investigated. It is concluded from studies of K_M and K_I that the substrate is bound to the active center on both sides of the peptide bond that is split.

Binding by the active center appears to involve protonated groups with a net apparent *pK* value of approximately 3.2. Values for k_3 of the substrate at *p*H 2 and 4.5 indicate that catalysis of its peptide bond involves a carboxyl group in the enzyme's active center with an apparent *pK* of 4.25.

The enzymic action of pepsin is interesting from several points of view: (a) Its action is expressed only in acidic media. (b) It hydrolyzes a great variety of peptide linkages in both peptides and proteins, albeit at different rates (Bell, 1954; Green and Neurath, 1954; Hirs, 1960; Li, 1956; Konigsberg and Hill, 1962). (c) Its *p*H-activity profile may show different *p*H optima for synthetic substrates (Fruton *et al.*, 1939) and broad *p*H ranges of activity for proteins (Schlamowitz and Peterson, 1959). (d) It can mediate transpeptidation reactions of the "amino" type between peptides (Neumann *et al.*, 1958, 1959; Fruton *et al.*, 1961).

The present investigation was undertaken to enlarge on the information about the active center of pepsin. Such information could contribute to an understanding of the above-described characteristics and their relation to each other. Specifically, it deals with the pepsin-catalyzed hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine. Its main objective is the evaluation of those kinetic parameters that reflect the binding and catalytic functions of the active center,[1] e.g., Michaelis constant, K_M; the dissociation constant of the enzyme-substrate complex, K_S; and the molecular activity coefficient, k_3.

For an enzyme system following Michaelis-Menten kinetics, the basic reactions may be designated as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P$$

where k_1, k_2, and k_3 are the steady-state rate constants. For such a system, the following relations hold:

$$v = \frac{k_3 eS}{K_M + S}$$

where v is the initial velocity, e the enzyme concentration, S the initial substrate concentration, and

$$K_M = \frac{k_2 + k_3}{k_1}$$

Further, in the case where k_3 is negligible compared with k_2, the Michaelis constant reduces to k_2/k_1, which is the dissociation constant of the enzyme-substrate complex.

N-Acetyl-L-phenylalanyl-L-diiodotyrosine was chosen because it is most rapidly hydrolyzed of all low molecular weight peptides reported (Bovey and Yanari, 1960; Herriott, 1962), and because its stereoisomer, *N*-acetyl-D-phenylalanyl-L-diiodotyrosine, was reported to be a pepsin inhibitor (Herriott, 1962). The latter opened the possibility for evaluating the enzyme-substrate binding constant (see Results). Other structurally related compounds studied as substrates or inhibitors were *N*-acetyl-L-phenylalanine and *N*-acetyl-L-diiodotyrosine.

The data indicate that the substrate binds to the active center through both of its amino acid moieties, that catalytic function of the center involves a group with the characteristics of a carboxyl group in its protonated state, and that *p*H influences hydrolysis of this substrate more through its effect on binding than on catalysis.

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[1] The term "active center" will be used in the sense defined by Vallee (1964). It will refer to all features of the primary, secondary, and tertiary structures of pepsin required for its binding and hydrolysis of substrate.

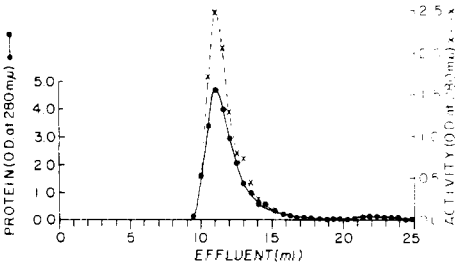

FIGURE 1: Chromatography of pepsin on Sephadex G-50. One mg of pepsin in 1 ml was chromatographed on a 9- × 36-cm column with 0.1 M glycine buffer, *p*H 2, at a rate of 9.5 ml/hr. Protein (absorbance at 280 mμ) and activity (hemoglobin assay) were determined on aliquots of the effluent (see methods).

Materials

Substrate. A preparation of the substrate, *N*-acetyl-L-phenylalanyl-L-diiodotyrosine, was obtained from Cyclo Chemical Corp. (lot S 1022) who reported mp 227–230° (lit mp 230°; Baker, 1951). Cyclo also reported that the material was homogeneous in two systems on paper chromatography and had a nitrogen content of 4.49% (calculated value = 4.51%). Another sample, received from Dr. Roger M. Herriott, had mp 223–224° uncor. Both preparations were hydrolyzed completely and at the same rate by pepsin.

Inhibitors. The *N*-acetyl-D-phenylalanyl-L-diiodotyrosine was obtained from Cyclo (lot S 1052). They reported mp 203–206° (lit 215°; Baker, 1951), nitrogen analysis = 4.49% (calculated value = 4.51%), and that paper chromatography in two solvent systems gave a single spot. *N*-Acetyl-L-diiodotyrosine as purchased from Sigma Chemical Co. (lot 83B-0880) contains 3.7% H_2O, the correct amount for the monohydrate. It chromatographed as a single spot, and had mp 200–202° with decomposition (lit mp 198–200°; Myers, 1932). *N*-Acetyl-L-phenylalanine obtained from Cyclo (lot M 1113) had mp 174–175° (lit mp 172°, Du Vigneaud and Irish, 1938), was homogenous in two solvents on paper chromatography, and was ninhydrin negative.

Anal. Calcd for $C_{11}H_{13}O_3N$: C, 63.8; H, 6.28; N, 6.76. Found: C, 63.7; H, 6.4; N, 6.6.

Enzyme. The pepsin used in these investigations was a three-times-crystallized preparation (Pentex Co., Inc.; lot D-3709) obtained by fractionation with ethanol according to Northrop (1946). In one experiment, described under Results, a similar preparation (Worthington Biochemical Corp., lot M628) was also used.

Chromatography on Sephadex G-50 (Figure 1) showed that all enzymatic activity (hemoglobin assay; Schlamowitz *et al.*, 1964) was in the major fraction (absorbance at 280 mμ). The specific activity of the fraction containing material from the top of the protein peak was 20% greater than the unchromatographed sample. The commercial preparation is thus assumed to contain 1/1.2 or 83% active enzyme with a molecular weight of 34,500 (Herriott, 1954). Where concentrations of pepsin are cited in the text, they have been calculated using these assumptions.

Methods

Determination of K_M and k_3. The initial velocity, v, of the pepsin-catalyzed hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine was measured at different substrate concentrations. The Michaelis constant and the molecular activity coefficient were calculated from a plot of S/v versus S. The intercept on the abscissa is equal to $-K_M$ and the slope is equal to $1/k_3e$, where e is the molar concentration of active pepsin.

Determination of K_I. The inhibition constant was determined from plots of $1/v$ versus I for two substrate concentrations. The value on the abscissa that corresponds to the point where these two lines intersect is equal to $-K_I$.

Operational Methods. Since the procedures in each experiment were similar, they will be illustrated by those used in the determination of K_M and k_3 for the substrate at *p*H 2. A series of tubes was set up, each tube containing 3.2 ml of different concentrations of substrate. Peptic hydrolysis was initiated by adding 0.2 ml of enzyme solution containing 62.2 μg commercial pepsin in 0.001 M acetic acid. All reactions were run at 37°. They were arrested after 5 minutes by adjusting mixtures to *p*H 8–11.5 with the addition of 0.1 ml of an alkaline solution.

The extent of hydrolysis was determined by measuring the ninhydrin color given by the newly formed L-diiodotyrosine, using a modification of Rosen's method (1957). The modifications permitted the use of larger sample volumes. This was necessary since the amounts of diiodotyrosine formed were very small. To each tube 0.5 ml of cyanide-acetate buffer solution and 0.5 ml ninhydrin solution were added. The tubes were heated in boiling water for 15 minutes after which 2.5 ml 2-propanol was added. The absorbance of the solutions was measured in a Klett-Summerson colorimeter with a No. 56 filter. The amount of substrate hydrolyzed was computed from a standard absorbance curve made with L-diiodotyrosine. Assays were carried out in duplicate or triplicate. Readings were corrected for ninhydrin-reacting material present at the start of hydrolysis by subtracting the absorbance of a solution in which enzyme was added after the addition of alkali.

During the 5-minute incubation period, 10–20% of the dipeptide substrate was hydrolyzed. Preliminary experiments with several substrate levels showed that the production of L-diiodotyrosine was a linear function of time until about 30% was utilized. Therefore, it was permissible to calculate the initial velocity (mM/min) from the amount of hydrolysis that occurred in 5 minutes.

The substrate was extremely difficult to dissolve directly at *p*H 2. It was found that the concentrations used could be obtained by first dissolving the compound in 0.01 N NaOH (an amount equal to 2 equivalents of substrate) and then adding this slowly to a large volume

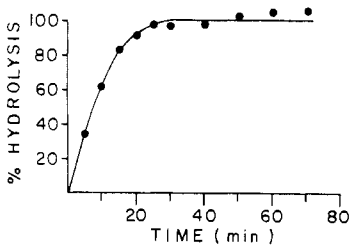

FIGURE 2: Hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine (16×10^{-5} M) by pepsin (4.16×10^{-6} M) at *p*H 2 and 37°. The extent of hydrolysis was determined by the ninhydrin method.

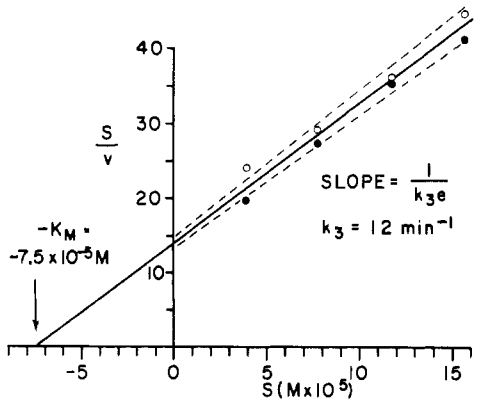

FIGURE 3: Determination of Michaelis constant, K_M, and molecular activity coefficient, k_3, from a plot of S/v versus S for the hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine by pepsin at *p*H 2. Open and solid circles indicate data from separate experiments. The dashed lines indicate the ±5% range of error for these experiments. The concentration of pepsin was 4.44×10^{-7} M.

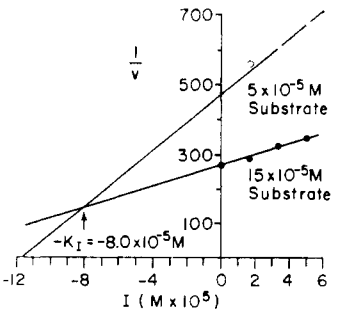

FIGURE 4: Determination of the inhibition constant, K_I, of *N*-acetyl-D-phenylalanyl-L-diiodotyrosine at *p*H 2 from plots of $1/v$ versus I. The concentration of pepsin was 4.44×10^{-7} M.

of water previously adjusted with HCl to *p*H 2. These solutions were used within 30–60 minutes after preparation since in some cases material precipitated on prolonged standing. Essentially the same procedure was employed in making solutions of *N*-acetyl-D-phenylalanyl-L-diiodotyrosine, *N*-acetyl-L-phenylalanine, and *N*-acetyl-L-diiodotyrosine, although the latter two compounds were more soluble.

The factors governing the range of substrate concentration used to evaluate the kinetic constants were the sensitivity of the ninhydrin method and the limited solubility of the substrate at *p*H 2.0. These factors restricted the rate studies to a 4-fold range of substrate concentration (3.9×10^{-5}–15.5×10^{-5} M). However, as will be seen (Results), the K_M value fell within this range, so that it could be accurately determined. Similarly the value of K_I for *N*-acetyl-D-phenylalanyl-L-diiodotyrosine was not much greater than the highest concentration of inhibitor used, so that it too could be accurately determined.

Results

Hydrolysis of N-Acetyl-L-phenylalanyl-L-diiodotyrosine by Pepsin. The course of hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine is not recorded, nor have data from other studies with synthetic substrates ruled out that transpeptidation reactions might complicate the kinetics of hydrolysis. This latter possibility has been pointed out by Bovey and Yanari (1960).

Our investigation of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine at *p*H 2 showed that hydrolysis progressed to completion (Figure 2). Similar results were obtained in another experiment run for 6 hours. An accumulation of "aminotranspeptidation" products (Neumann *et al.*, 1959) would have resulted in failure to attain 100% hydrolysis.

Determination of K_M and k_3 of N-Acetyl-L-phenylalanyl-L-diiodotyrosine at pH 2. Figure 3 shows a plot of S/v versus S from which K_M and k_3 values of 7.5×10^{-5} M and 12 min^{-1}, respectively, were found. The maximum error estimated for K_M is $\pm 0.8 \times 10^{-5}$ M.

As discussed in the next section the K_M value of the substrate is numerically equal to K_S, the dissociation constant for the enzyme-substrate complex. On this assumption the value for the standard free energy of combination of enzyme and substrate, calculated from the K_M at 37°, is 5.85 kcal/mole (Table I).

Determination of K_I of N-Acetyl-D-phenylalanyl-L-diiodotyrosine at pH 2. In Figure 4 is shown a graph of $1/v$ versus I from which a K_I value of 8.0×10^{-5} M was calculated. This K_I value is the same within experimental error as the K_M of the substrate. The value for the free energy of combination of the inhibitor with pepsin is calculated to be 5.81 kcal/mole (Table I).

The data indicated that the inhibition was competitive, since the two straight lines intersect at a point above the abscissa. Had the inhibition been noncompetitive the lines would have intersected at the abscissa (Dixon and Webb, 1959). A $1/v$ versus I plot of the data was used to clearly differentiate competitive from noncompetitive inhibition, since it would do this not only by location of the intersect but also by the relative slopes of

TABLE I: Reaction Constants for Pepsin with Synthetic Substrates and Inhibitors.

Compound	pH	K_M (M × 10^5)	K_I (M × 10^5)	k_3 (min^{-1})	$-\Delta F°$[a] (kcal/mole)
N-Acetyl-L-phenylalanyl-L-diiodotyrosine	2.0	7.5		12	5.85
N-Acetyl-D-phenylalanyl-L-diiodotyrosine	2.0		8.0		5.81
N-Acetyl-L-diiodotyrosine	2.0		88		4.33
N-Acetyl-L-phenylalanyl-L-diiodotyrosine	4.5	84		4.3	4.36

[a] Standard free energies of association are calculated using the expression $\Delta F° = -RT \ln K$ for 37°.

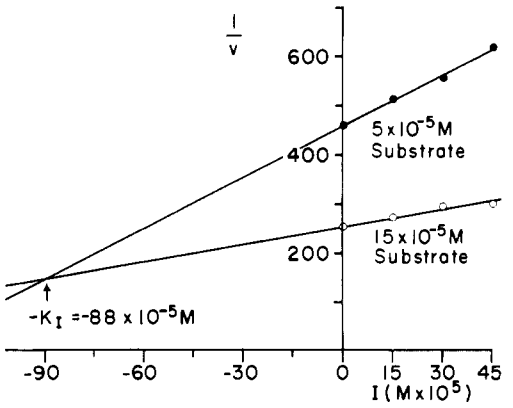

FIGURE 5: Determination of the inhibition constant, K_I, of *N*-acetyl-L-diiodotyrosine at *p*H 2 from plots of $1/v$ versus I. The concentration of pepsin was 4.44 × 10^{-7} M.

the lines. If competitive inhibition had occurred the slope of each line would be equal to K_M/Sk_3eK_I, whereas if noncompetitive inhibition had occurred the slope of each line would be equal to $K_M/Sk_3eK_I + 1/k_3eK_I$. When the ratio of substrate concentrations used is 3 and K_M is 7.5 × 10^{-5} M, as was the case in this experiment, the ratio of the slopes would be 3 if competitive inhibition had occurred and 1.7 if noncompetitive inhibition had occurred. The observed ratio of 2.7 is further indication that the inhibition was competitive.

The data could also represent completely mixed inhibition (Webb, 1963) where α, the factor that relates the dissociation constant of EIS with that of EI, is approximately 10. Limitations imposed by the analytical methods employed do not permit differentiation between completely mixed inhibition with an α greater than 5 and completely competitive inhibition where $\alpha = \infty$. Nevertheless, whether inhibition is competitive or mixed, the experimentally determined K_I, the dissociation constant of EI, was found to be numerically equal to K_M, the Michaelis constant of the substrate.

The above-mentioned finding, coupled with the fact that the inhibitor is a stereoisomer of the substrate, favors the view that $k_2 \gg k_3$ and, therefore, K_M is numerically equal to K_S. Although direct measurement of k_2 has not been made, an approximate value can be calculated from the equation $k_2 = k_1K_M - k_3$. As stated previously, K_M was found to be 7.5 × 10^{-5} M and k_3 to be 12 min^{-1}. Values for k_1 for different enzyme-substrate systems have been reported to vary from 2.1 × 10^7 to >6 × 10^{10} M^{-1} min^{-1} (Eigen and Hammes, 1963; Bloomfield *et al.*, 1962). If it is assumed that k_1 in this pepsin-substrate system lies within this range, then k_2 should be between 16 × 10^2 and 45 × 10^5 min^{-1}. The observed value of k_3 (12 min^{-1}) is less than 1% of the calculated k_2. Of course, direct verification that K_M and K_S are equal must await the measurement of k_1 and k_2.

To establish that inhibition of hydrolysis of the substrate by *N*-acetyl-D-phenylalanyl-L-diiodotyrosine was not peculiar to a single sample of pepsin, the velocity of hydrolysis of 5 × 10^{-3} M substrate in the absence and presence of 5 × 10^{-5} M inhibitor was carried out with another sample of pepsin (Worthington Biochemical Corp., lot M628). The data agreed within experimental error with those found for the pepsin sample from the Pentex Co.

Determination of K_I of N-Acetyl-L-diiodotyrosine and of N-Acetyl-L-phenylalanine at pH 2. The conclusion that the two *dipeptides* are bound to pepsin to a similar degree led us to investigate the importance of their constituent moieties, diiodotyrosine and phenylalanine, on the binding. If only one amino acid moiety is involved, then either *N*-acetyl-L-diiodotyrosine or *N*-acetyl-L-phenylalanine would be a competitive inhibitor[2] with K_I equal to the K_I for *N*-acetyl-D-phenylalanyl-L-diiodotyrosine. If both residues are important to the binding, their separate K_I values would be greater than that for the dipeptide. As will be seen, the latter appears to be the case.

[2] It was first established that neither of these *N*-acetylamino acids was hydrolyzed. This was relevant since each contains a —CONH— linkage, making it a potential substrate.

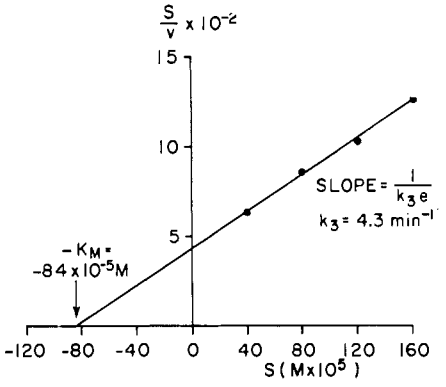

FIGURE 6: Determination of Michaelis constant, K_M, and molecular activity coefficient, k_3, from plot of S/v versus S for the hydrolysis of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine by pepsin at *p*H 4.5. The concentration of pepsin was 4.44×10^{-7} M.

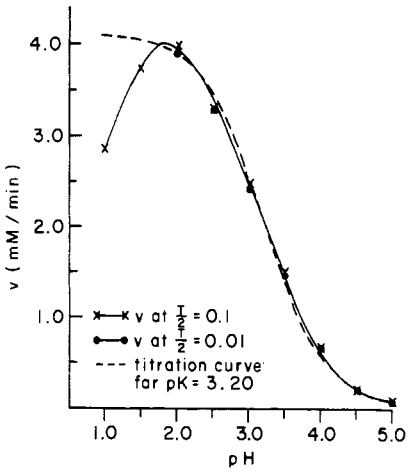

FIGURE 7: The *p*H-activity curve for the action of pepsin (4.44×10^{-7} M) on *N*-acetyl-L-phenylalanyl-L-diiodotyrosine (15.5×10^{-5} M) at ionic strength 0.01 and 0.10 M. The dashed curve is a theoretical titration curve for an acid with apparent *pK* value of 3.2.

In Figure 5 are shown the data from inhibition studies with *N*-acetyl-L-diiodotyrosine. From the $1/v$ versus I plots it was found to have a K_I value of 88×10^{-5} M, about ten times greater than the K_I of the dipeptide inhibitor. Its binding energy was calculated to be 4.33 kcal/mole, i.e., about 75% of the binding energy of the dipeptide (Table I).

Attempts to determine experimentally a K_I value for *N*-acetyl-L-phenylalanine have not been successful. No inhibition was observed when concentrations as high as 300×10^{-5} M were used. If *N*-acetyl-L-phenylalanine is a potential inhibitor of the hydrolysis of the dipeptide substrate, its K_I value must be several times the highest concentration of inhibitor used, that is, greater than 1000×10^{-5} M.[3] This would give it a maximum binding energy of 2.84 kcal/mole. These are conservative values for K_I and $\Delta F°$ of the *N*-acetyl-L-phenylalanine; the real ones may indicate even less binding.

From the fact that the K_I and $\Delta F°$ values for neither amino acid moiety alone accounts for the binding of the substrate, it is concluded that both amino acid residues of the dipeptide are bound to pepsin.[4]

Determination of K_M and k_3 of N-Acetyl-L-phenylalanyl-L-diiodotyrosine at pH 4.5. The kinetic constants of the dipeptide substrate were determined at *p*H 4.5 in the same manner as at *p*H 2 and at the same ionic strength. The two sets of values were compared to find out whether binding, catalysis, or both were responsible for the decline in the rate of hydrolysis with increase in *p*H (see Figure 7). The S/v versus S plot is shown in Figure 6. Values of K_M and k_3 were found to be 84×10^{-5} M and 4.3 min^{-1}, respectively. The molecular activity coefficient at *p*H 4.5 was 0.36 times the value at *p*H 2. This decrease is moderate compared with the more than 10-fold increase in the value of K_M in going from *p*H 2 to *p*H 4.5. Thus it appears that the decline of activity with increasing *p*H is primarily the result of poorer binding of the substrate to pepsin.

The pH-Activity Profile of Pepsin. Figure 7 shows a plot of the initial velocity, v, versus *p*H for the hydrolysis of 15.5×10^{-5} M *N*-acetyl-L-phenylalanyl-L-diiodotyrosine at two ionic strengths, 0.01 and 0.10 M. The velocity at any one *p*H was the same for both ionic strengths. The figure further shows that a titration curve for a functional group with a *pK* of 3.2 can be approximately superimposed on the portion of the *p*H-activity profile between *p*H 2 and 5.

Discussion

Bovey and Yanari (1960) proposed a working hypothesis for studying the mechanism of peptic hydrolysis. They suggested that a substrate, such as an acyl dipeptide, first forms a complex with pepsin in which the side groups of the substrate are bound to the enzyme (Figure 8).[5] A functional group, designated in the figure by Z, which they speculated to be a protonated carboxyl group, then reacts with the peptide

[3] Baker (1954, 1956) reported inhibition of *N*-acetyl-L-phenylalanyl-L-tyrosine by *N*-acetyl-L-phenylalanine. Although her data do not permit a direct calculation of K_I, it appears that she did obtain significant inhibition using *N*-acetyl-L-phenylalanine in the same range of concentration as used in our experiment. Further study of this compound as a pepsin inhibitor in the presence of different substrates should be a very worthwhile investigation.

[4] Since each of the acylamino acids has in common a carboxyl group and an *N*-acetyl group, the difference in their binding strengths can be attributed to the difference in the nature of their aromatic side chains.

[5] The figure schematically depicts some features of the active center, *in the presence of substrate*. It therefore requires no suppositions as to whether the center exists in the free enzyme or is induced by the substrate (Koshland, 1963).

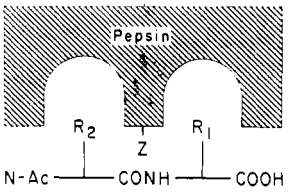

FIGURE 8: Schematic representation of the active center of pepsin combined with an *N*-acyl dipeptide substrate.

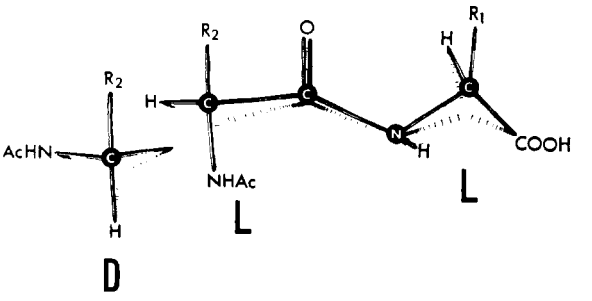

FIGURE 9: Molecular models of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine and its stereoisomer, *N*-acetyl-D-phenylalanyl-L-diiodotyrosine, showing the ability of the isomers to assume the same spatial orientation except for the acetamido group and hydrogen atom on the N-terminal amino acid. Drawings were made from Dreiding stereomodels.

linkage of the substrate. In the process there is release of the N-terminal amino acid and transpeptidation of the amino group of the C-terminal amino acid to the enzyme's postulated carboxyl group, Z. Hydrolysis is completed by cleavage of this enzyme–amino acid intermediate with a molecule of water.

The data of the present study are in agreement with this hypothesis in several respects. According to the scheme, binding of the substrate takes place by means of the amino acid side chains on either side of the —CONH— bond split. There are two experimental facts that are consistent with this view. First, the two acylamino acids studied were poorer inhibitors than the dipeptide inhibitor. Second, the fact that the K_M of the dipeptide substrate, which appears to be numerically equivalent to K_S, is the same as the K_I of the dipeptide inhibitor indicates that the binding properties of the two dipeptides are equivalent.

Diastereoisomers are not always bound to an enzyme to the same degree (Manning and Niemann, 1958). However, molecular models of the dipeptide substrate and the dipeptide inhibitor (Figure 9) show that these isomers can assume the same spatial orientation with respect to their peptide bonds and R_1 and R_2 amino acid side chains. This could account for their equivalent binding properties.

It may be supposed that adequate binding by the two side chains to the active center is one prerequisite for hydrolysis, its purpose being to place the peptide bond in the proper position for hydrolysis. The fact that *N*-acetyl-L-diiodotyrosine, though strongly bound through its aromatic group and containing a —CONH— bond, is not a substrate suggests that its methyl group is unable to fulfil this purpose. Adequate binding of the side chains is obviously not the only requisite for hydrolysis, as may be seen by reference to the two stereoisomeric dipeptides, one a substrate, the other an inhibitor.

The only structural difference seen in the models of these compounds is in the orientation of the acetamido group and the hydrogen atom connected to the α-carbon of the N-terminal amino acid residue. That such transposition should render the D-L compound impotent as a substrate invites speculation that the position of this acetamido group is crucial for catalysis. Insofar as the active center represented in Figure 8 does not take this into account, it depicts at best only a part of the essential features of the center.[6]

With reference to the Z group, the decrease in value of k_3 from 12 min^{-1} at *p*H 2 to 4.3 min^{-1} at *p*H 4.5 seems likely to result from the titration of a group on the enzyme that must be protonated for the enzyme to be active. If we assume that this group is fully protonated at *p*H 2 where the enzyme shows maximum activity, then its *pK* can be calculated from the fact that 36%, i.e., k_3 = 4.3 min^{-1}, remains protonated at *p*H 4.5. This *pK* value is 4.25, which coincidentally is the same as the value for the *pK* of the ω-carboxyl group of glutamic acid (Steinhardt and Beychok, 1964). Several dipeptides containing glutamic or aspartic acid also have *pK* values for their ω-carboxyl groups in this general range (Steinhardt and Beychok, 1964; Cohn and Edsall, 1948). The above provides the experimental support for the carboxyl group postulated by Bovey and Yanari (1960).

Studies of the *p*H-activity profile at different ionic strengths furnished information about the binding of *N*-acetyl-L-phenylalanyl-L-diiodotyrosine by pepsin that has not been previously discussed. Figure 7 shows that activity is maximal at *p*H 2, declines with increasing *p*H, and is the same for both ionic strengths. The decline has been attributed principally to decreased binding of the substrate to pepsin (see Results). The fact that ionic strength does not affect the activity indicates that ion pairs are not involved to any great extent in the binding. Therefore the effect of *p*H seems to result from the requirement for uncharged groups on the enzyme and/or the substrate for maximum binding. These groups may participate in binding of enzyme to substrate in a direct sense, e.g., hydrogen bonds, or indirectly, perhaps as protonated groups necessary for maintaining the enzyme's active center in a conformation which permits binding to take place.

[6] It must also be pointed out that this representation of the active center still does not account for the ability of pepsin to catalyze the exchange of oxygen atoms between water and carboxylic acids described by Sharon *et al.* (1962).

The fact that a titration curve for an acid with apparent *pK* value of 3.2 could be superimposed on the *p*H-activity profile (Figure 7) may be relevant to the nature of the groups discussed. Of the possible explanations for such an apparent *pK* value, the one which at present seems most likely is a simultaneous requirement for more than one protonated carboxyl group. This would have the effect of lowering the apparent *pK* value.

It is of interest to note the contrast between the effects of ionic strength (0.01–0.10 M) on the hydrolysis of small and large substrates. With *N*-acetyl-L-phenylalanyl-L-diiodotyrosine, no effect was noticed between *p*H 2 and 5; with bovine serum albumin at *p*H 3.5 a marked increase in peptic activity was noted (Schlamowitz and Peterson, 1961). This reinforces the view, previously expressed, that the influence of ionic strength in the bovine serum albumin system was on the substrate rather than on the enzyme.

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References

Baker, L. E. (1951), *J. Biol. Chem. 193*, 809.
Baker, L. E. (1954), *J. Biol. Chem. 211*, 701.
Baker, L. E. (1956), *Nature 178*, 145.
Bell, P. H. (1954), *J. Am. Chem. Soc. 76*, 5565.
Bloomfield, V., Peller, L., and Alberty, R. A. (1962), *J. Am. Chem. Soc. 84*, 4375.
Bovey, F. A., and Yanari, S. S. (1960), *Enzymes 4*, 63.
Cohn, E. J., and Edsall, J. T. (1948), *in* Proteins, Amino Acids and Peptides, New York, Reinhold, p. 85.
Dixon, M., and Webb, E. C. (1959), Enzymes, New York, Academic, p. 25.
Du Vigneaud, V., and Irish, O. J. (1938), *J. Biol. Chem. 122*, 349.
Eigen, M., and Hammes, G. G. (1963), *Advan. Enzymol. 25*, 1.
Fruton, J. S., Bergman, M., and Anslow, W. P. (1939), *J. Biol. Chem. 127*, 627.
Fruton, J. S., Fujii, S., and Knappenberger, M. H. (1961), *Proc. Natl. Acad. Sci. U.S. 47*, 759.
Green, N. M., and Neurath, H. (1954), *Proteins 2*, 1127.
Herriott, R. M. (1954), *in* Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., ed., Baltimore, The Johns Hopkins Press, p. 41.
Herriott, R. M. (1962), *J. Gen. Physiol. 45*, 57.
Hirs, C. H. W. (1960), *Ann. N.Y. Acad. Sci. 88*, 611.
Jackson, W. T., Schlamowitz, M., and Shaw, A. (1964), Abstracts of the 148th Meeting of the American Chemical Society, Chicago, p. 60c.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 3157.
Koshland, D. E. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 473.
Li, C. H. (1956), *Advan. Protein Chem. 11*, 101.
Manning, D. T., and Niemann, C. J. (1958), *J. Am. Chem. Soc. 80*, 1478.
Myers, C. S. (1932), *J. Am. Chem. Soc. 54*, 3721.
Neumann, H., Levin, Y., Berger, A., and Katchalski, E. (1958), *Proc. Intern. Symp. Enzyme Chem., Tokyo Kyoto, 1957*, 129.
Neumann, H., Levin, Y., Berger, A., and Katchalski, E. (1959), *Biochem. J. 73*, 33.
Northrop, J. H. (1946), *J. Gen. Physiol. 30*, 177.
Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.
Schlamowitz, M., and Peterson, L. U. (1959), *J. Biol. Chem. 234*, 3137.
Schlamowitz, M., and Peterson, L. U. (1961), *Biochim. Biophys. Acta 46*, 381.
Schlamowitz, M., Shaw, A., and Jackson, W. T. (1964), *Biochemistry 3*, 636.
Sharon, N., Grisaro, V., and Neumann, H. (1962), *Arch. Biochem. Biophys. 97*, 219.
Steinhardt, J., and Beychok, S. (1964), *Proteins 2* (2nd ed.), 139.
Vallee, B. L. (1964), *Federation Proc. 23*, 8.
Webb, J. L. (1963), Enzymes and Metabolic Inhibitors, Vol. I, New York, Academic, pp. 58, 59, 163