*ON THE MECHANISM OF PEPSIN ACTION**

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Abstract.—The problem of the mechanism of pepsin action is considered in relation to recent data on the kinetics and specificity of the enzyme, as well as the finding, reported here, that pepsin exhibits a deuterium isotope effect in the cleavage of a peptide bond.

The kinetic parameters for the hydrolysis of the Phe(NO_2)-Phe bond of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe by pepsin have been determined in H_2O and in D_2O. The finding of a significant deuterium isotope effect (k_{H_2O}/k_{D_2O} = *ca.* 2) supports the hypothesis that the catalytic mechanism of pepsin involves the participation, in the rate-limiting step, of a proton donor (probably an enzymic carboxyl group) in addition to an enzymic carboxylate group acting as a nucleophile.

In a previous communication from this laboratory,[1] it was suggested that the mechanism of pepsin action at a peptide bond (RCO-NHR′) involves the attack of an enzymic carboxylate group (ECOO$^-$) at the carbonyl-carbon of the protonated amide group to form reversibly a tetrahedral intermediate which undergoes a reversible four-center exchange reaction leading to the expulsion of RCOOH and the formation of ECO-NHR′ (an imino-enzyme). The latter intermediate, on reaction with water (or a carboxylic acid), would yield NH_2R' (or lead to transamidation by imino-transfer) with the regeneration of ECOO$^-$. This hypothesis was based largely on the following experimental findings: (1) it was known that pepsin catalyzes transamidation reactions (Neumann *et al.*[2]) and exchange with labeled cleavage products (Fruton *et al.*[3]) in a manner consistent with an imino-transfer mechanism; (2) studies on the chemical modification of pepsin (Herriott,[4] Erlanger *et al.*,[5] and Delpierre and Fruton[1]) supported the view that one or more carboxyl groups of pepsin were involved in the catalytic mechanism; (3) enzymic attack at the carbonyl group of the amide was indicated by the finding (Sharon *et al.*,[6] Kozlov *et al.*[7]) that pepsin catalyzes O^{18} exchange between H_2O^{18} and "virtual substrates" such as Z-Phe or Ac-Phe.[8]

An essential part of the proposed mechanism was the protonation of the amide bond either before, or concurrently with, the formation of the tetrahedral intermediate assumed to be associated with the transition state in the catalytic process. Whatever the source of the proton, if its transfer to the amide bond of the substrate is part of the rate-limiting reaction in the action of pepsin, it would be expected that the rate of enzymic action on amide substrates should show a deuterium isotope effect. Thus, when Clement and Snyder[9] reported that the maximal velocity of the hydrolysis of Ac-Phe-Tyr by pepsin is the same in D_2O as in H_2O, a rate-limiting process involving proton transfer appeared to be excluded, and later hypotheses about the mechanism of pepsin action (see Clement *et al.*[10]) were based on this conclusion.

In this communication, we report that the cleavage, by pepsin, of the substrate

Gly-Gly-Gly-Phe(NO_2)-Phe-OMe at the Phe(NO_2)-Phe linkage is characterized by a significant deuterium isotope effect. This substrate is one of an extensive series of cationic peptide derivatives recently prepared in this laboratory in connection with the study of the specificity of pepsin (Inouye *et al.*,[11] Inouye and Fruton,[12] and Hollands *et al.*[13]). The cleavage of the Phe(NO_2)-Phe bond at pH values near 4, where pepsin exerts its optimal effect on many of these substrates, can be followed by spectrophotometry.[12]

Materials and Methods.—Swine pepsin (twice crystallized, lot PM708) was obtained from the Worthington Biochemical Corporation. When assayed in the usual manner,[1] with hemoglobin as the substrate, the proteinase activity of this enzyme preparation was 2,900 units/mg of protein. The enzyme concentration was estimated spectrophotometrically at 278 mμ, with the assumption that pepsin has a molar absorptivity of 50,900 and a molecular weight of 34,000. All the enzyme experiments were conducted in the presence of 0.04 *M* formate buffers and at 37°, as described by Hollands and Fruton.[13]

The sample of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe hydrobromide used in this work was kindly provided by Miss I. M. Voynick; the synthesis of this compound has been described previously.[13] Its hydrolysis at the Phe(NO_2)-Phe linkage was followed spectrophotometrically by a modification of the method described by Inouye and Fruton[12] for the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by pepsin. In the present study, the increase in absorbance accompanying the cleavage of the Phe(NO_2)-Phe bond of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe was measured at 326 mμ, to permit kinetic measurements at higher initial substrate concentrations than those used with Z-His-Phe(NO_2)-Phe-OMe. At this wavelength, the change in molar absorptivity ($\Delta\epsilon$) for 100% cleavage in the pH range 3.6 to 5.0 was not constant over the range of initial substrate concentration (S_0) used in the enzyme experiments; thus, 100% cleavage at S_0 = 0.2 mM was accompanied by a $\Delta\epsilon_{326}$ of 432 $\pm$ 8; at S_0 = 0.5 mM, it was 385 $\pm$ 5; and at S_0 = 1.3 mM, it was 331 $\pm$ 12.

For the enzymic experiments in D_2O, all the reaction components were dissolved in 99.8% D_2O (Stuart Oxygen Company), and the formate buffers were prepared from sodium formate and 0.1 *N* DCl. The pD of the buffer solutions was determined by means of a Corning expanded-scale pH meter (model 12), and the relationship pD = meter pH + 0.4 was assumed to hold (Glason and Long[15]). It was found that the values of $\Delta\epsilon_{326}$ for the complete cleavage of the Phe(NO_2)-Phe bond in D_2O over the pD range 4.0 to 5.0 were lower than those given above; they were for S_0 = 0.2 mM, 383; for S_0 = 0.6 mM, 358; for S_0 = 1.3 mM, 292.

In the kinetic runs, the initial rates (10–15% hydrolysis) were calculated from the changes in absorbance with the appropriate value of $\Delta\epsilon_{326}$ estimated from calibration curves based on the above values. At each pH or pD value, replicate determinations of the initial velocity (v) were made for each value of S_0, and satisfactory linear plots of v versus v/S_0 were obtained, from which values of V_m (maximal velocity) and K_m (Michaelis constant) were estimated. The data were subjected to computer analysis (Hollands and Fruton[14]), and the precision of the kinetic parameters is given in Table 1 in terms of 95% confidence limits. The values of k_{cat} are equal to V_m/enzyme concentration.

Results.—To determine the pH (or pD) value at which the initial rate of hydrolysis of the Phe(NO_2)-Phe bond of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe by pepsin was maximal under the conditions given above, separate experiments were conducted over the pH range 3.6 to 5.0 at a single substrate concentration, S_0 = 1.2 mM. Earlier experiments[13] had shown that substrates of this type (e.g., Gly-Gly-Phe-Phe-OEt) exhibit relatively flat pH dependence curves over the pH range 2.5 to 4.5, with an apparent maximum of k_{cat}/K_m near pH 4. The substrate Gly-Gly-Gly-Phe(NO_2)-Phe-OMe behaves in an analogous manner,

with optimal cleavage by pepsin (1 μM) near pH 4.0. Similar experiments in D_2O indicated that the maximal initial velocity in this solvent is near pD 4.4. Accordingly, the kinetic parameters k_{cat} and K_m were determined at pH 4.0 (in H_2O) and at pD 4.4 (in D_2O); the results are presented in Table 1. It will be seen that, within the precision of the data, the values of K_m are the same in H_2O and D_2O, and that the value of k_{cat} in H_2O is about twice that in D_2O. The values of K_m and k_{cat} in H_2O, found in the present study, are in satisfactory agreement with the values of $K_m = 1.5 \pm 0.1$ mM and $k_{cat} = 0.09 \pm 0.01$ sec^{-1} reported previously.[13]

It may be added that studies on the effect of D_2O on the initial rate of cleavage of Z-His-Phe(NO_2)-Phe-OMe (0.25 mM) at the Phe(NO_2)-Phe linkage also indicated a deuterium isotope effect (v_{H_2O}/v_{D_2O} = *ca.* 1.5), but the limited solubility of this substrate at pH values above 4.0 made it difficult to obtain acceptable data for k_{cat} and K_m at the apparent optimal values of pH (4.3) and of pD (4.7). Earlier work[13] had assigned values of $k_{cat} = 0.29$ sec^{-1} and $K_m = 0.46$ mM to the hydrolysis of Z-His-Phe(NO_2)-Phe-OMe by pepsin at pH 4.0 and 37°.

Discussion.—A satisfactory explanation of the discrepancy between our finding and the absence of a deuterium isotope effect in the hydrolysis of Ac-Phe-Tyr-OEt (Clement and Snyder,[9] Clement *et al.*[10]) is not readily apparent, and further

TABLE 1. *Kinetics of cleavage of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe by pepsin in H_2O and in D_2O.**

pH or pD	S_0† (mM)	k_{cat} (10^2 sec^{-1})	K_m (mM)	k_{cat}/K_m (10^3 sec^{-1} mM^{-1})
pH 4.0	0.2–1.7 (11)	7.9 ± 0.7	1.6 ± 0.2	4.8
pD 4.4	0.2–1.3 (5)	4.0 ± 0.7	1.4 ± 0.4	2.8

* Pepsin concentration, 1.1 μM; formate buffers; 37°.

† The number in parentheses denotes the number of runs in the determination of the kinetic parameters.

work is needed to resolve this problem. The appearance of a significant isotope effect in the peptic hydrolysis of Gly-Gly-Gly-Phe(NO_2)-Phe-OMe supports the view, however, that a proton (deuteron) transfer is involved in the catalytic mechanism of pepsin. Furthermore, it seems likely that a true kinetic isotope effect of D_2O on pepsin catalysis is involved, and that the lower catalytic activity in D_2O is not a consequence of alteration in the structure of pepsin. It should be added that Reid and Fahrney[16] have found that the cleavage of methylphenyl sulfite by pepsin shows no deuterium isotope effect, but the catalytic mechanism in this process may be different from that operative in the hydrolysis of peptide substrates.

In addition to studies on the effect of D_2O on pepsin catalysis, recent work has provided several other items of evidence relevant to the hypothesis that pepsin action may involve both acid catalysis and nucleophilic attack: (1) the pH dependence of the hydrolysis of neutral substrates (e.g., Ac-Phe-Tyr-NH_2) is consistent with the participation, in the catalytic mechanism, of a carboxylate group (derived from an acid of pK_a *ca.* 1) and a carboxyl group of pK_a *ca.* 4 (Lutsenko *et al.*,[17] Denburg *et al.*,[18] Clement *et al.*,[10] Cornish-Bowden and Knowles[19]); (2) the involvement of one or more enzymic carboxyl groups has been demonstrated by numerous recent studies on the chemical modification of

pepsin (Delpierre and Fruton,[1, 20] Erlanger *et al.*,[21] Rajagopalan *et al.*,[22] Hamilton *et al.*,[23] Kozlov *et al.*[24]), and an active-site aspartyl residue has been identified in the sequence Ile-Val-Asp-Thr-Gly-Thr-Ser (Knowles and Wybrandt,[25] Fry *et al.*[26]). These findings are consistent with the view that the catalytic mechanism of pepsin involves the participation of at least two enzymic carboxyl groups, one of which acts as a proton donor and the other (in its dissociated form) as a nucleophile. A mechanism of this type is formally analogous to the one proposed by Wang and Parker[27] for the cleavage of amide substrates by chymotrypsin, with an imidazolium group as the proton donor and the alkoxide ion derived from a serine hydroxyl group as the nucleophile. Wang[28] has emphasized the importance for catalysis of facilitated proton transfer along hydrogen bonds whose positioning is influenced by the interaction of the specificity groups of the substrate with chymotrypsin. In the case of pepsin, the specific interactions of its substrates on both sides of the sensitive peptide bond[11–13] may play an equal (if not greater) role in positioning directed hydrogen bonds involved in the rate-limiting protonation of the amide group.

The available data on the competitive inhibition of pepsin action by relatively resistant substrates or by substrate analogues (Denburg *et al.*,[18] Inouye and Fruton,[29] Zeffren and Kaiser,[30] Hollands *et al.*,[13] Knowles *et al.*[31]) strongly suggest that, for the hydrolysis of peptide substrates, K_m approximates the dissociation constant (K_S) of the enzyme-substrate complex, and that the release of the first reaction product occurs after the rate-limiting step in the over-all process. That there is an ordered release (Cleland[32]) of the two products of the cleavage of RCO-NHR′, with RCOOH departing first, is indicated by the finding that Ac-Phe is a noncompetitive inhibitor (Greenwell *et al.*[33]) and that Phe-OMe inhibits pepsin competitively (Inouye and Fruton[29]). However, attempts to observe a "burst" release of the RCOOH product have been unsuccessful thus far (Inouye and Fruton,[12] Cornish-Bowden *et al.*[34]), and the existence of a covalently linked imino-enzyme of the type postulated previously[2, 3] has not been demonstrated. It should be noted that the assumption of such an intermediate is not obligatory, in view of the ordered release of products and the ready reversibility of peptide hydrolysis at acid pH values (Kozlov *et al.*[35]). The importance for specificity of the side chain of the amino acid residue contributing the NH group to the sensitive peptide bond[11–13] raises the possibility that noncovalent interactions may be involved in holding the $R'NH_2$ product so as to produce the kinetic equivalent of an imino-enzyme.

In a recent critical discussion of the problem of the mechanism of pepsin action, Knowles[36] has developed more fully the proposal outlined by Delpierre and Fruton[1] and has offered additional evidence in its support. He has noted that the ratio of the rates of hydrolysis of the amide substrate Z-His-Phe(NO_2)-Phe-OMe and the ester substrate Z-His-Phe(NO_2)-Pla-OMe (Inouye and Fruton[12]) is consistent with a mechanism involving acid catalysis. Knowles has reported the failure of experiments[34] to trap an acyl-enzyme intermediate by methods successful for chymotrypsin and other proteinases; this result, while not disproving the possible formation of such an intermediate in pepsin catalysis, renders hypotheses that invoke its formation (Bender and Kezdy,[37] Clement

et al.[10]) less attractive. Knowles has also suggested that the pepsin-catalyzed O^{18} exchange between H_2O^{18} and acylamino acids may be explained without the assumption of an acyl-enzyme intermediate, on the basis of the observation of Shkarenkova *et al.*[38] that at least one active-site carboxyl group of pepsin can incorporate O^{18} rapidly from H_2O^{18}.

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