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*THE MECHANISM OF PEPSIN ACTION**

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The initial studies on the action of crystalline swine pepsin on synthetic substrates showed that this enzyme hydrolyzes preferentially peptide bonds involving the imino group of L-tyrosine or of L-phenylalanine,[1] in contrast to the action of crystalline beef chymotrypsin, which hydrolyzes preferentially bonds involving the carbonyl group of an aromatic amino acid residue. The subsequent demonstration that chymotrypsin[2] and other proteinases[3, 4] catalyze transamidation reactions in which an acyl group is transferred from a substrate to an acceptor amine was consistent with the formulation of the mechanism of their action as a "carbonyl transfer,"[5] in which an intermediate "acyl-enzyme" is involved.[6] Recent studies[7] have provided strong evidence for the view that, in the case of chymotrypsin, the acyl group is attached to the β-hydroxyl group of a serine residue in the enzyme protein.

In a recent publication, Neumann *et al.*[8] have shown that when pepsin is incubated with a synthetic substrate such as carbobenzoxy-L-tyrosyl-L-tyrosine, there is formed L-tyrosyl-L-tyrosine in amounts that can be detected by means of paper chromatography or of ionophoresis. These investigators have interpreted their results as indicating the occurrence of "imino transfer" in the catalytic action of pepsin:

(1) $\mathrm{RCO\text{-}NHR' + Pepsin\text{-}OH \rightleftarrows RCOOH + Pepsin\text{-}NHR'}$
(2) $\mathrm{Pepsin\text{-}NHR' + H_2O + H^+ \rightarrow R'NH_3^+ + Pepsin\text{-}OH}$ (Hydrolysis)
(3) $\mathrm{Pepsin\text{-}NHR' + R''COOH \rightleftarrows R''CO\text{-}NHR' + Pepsin\text{-}OH}$ (Transfer)

The present communication reports additional evidence, based on an isotope exchange experiment, in favor of the occurrence of reaction (1) in the pepsin-catalyzed cleavage of peptide bonds. The experiment involved three incubation solutions whose composition was as follows: Solution A (10 ml) contained 50 micromoles each of carbobenzoxy-L-tyrosyl-L-tyrosine,[9] of carbobenzoxy-L-tyrosine,[10] and of C^{14}-L-tyrosine (2700 cpm per micromole; uniformly labeled C^{14}-L-tyrosine from Nuclear-Chicago diluted with unlabeled tyrosine), 10 mg of crystalline swine pepsin (Armour), and 0.1 *M* acetate buffer (pH 4.0). The composition of solution B was similar to that of solution A, except that the carbobenzoxy-L-tyrosine was labeled (2,700 cpm per micromole) and the L-tyrosine was not. Solution C was a nonenzymic control for solution B, and the pepsin was omitted from the incubation mixture.

The three solutions were kept at 37.5°C for 22 hours. A separate experiment, using unlabeled compounds, showed that during this time period, the enzyme caused an increase in ninhydrin-reactive material[11] corresponding to 17 per cent hydrolysis of carbobenzoxy-L-tyrosyl-L-tyrosine at one amide bond.

After the incubation period, 150 ml of ethanol were added to each of the three solutions, the mixtures were chilled overnight, and filtered. The three filtrates were worked up in identical manner; each of them was concentrated to dryness under reduced pressure, and the residue was extracted with methanol. The carbobenzoxy compounds present in the methanolic solution were subjected to catalytic hydrogenolysis with palladium black in the usual manner; about 60 minutes were required. After removal of the catalyst by filtration, the methanolic solution was concentrated to dryness under reduced pressure, and the residue was dissolved in a small volume of water. Paper chromatography (Whatman No. 1 paper) with *n*-butanol-acetic acid-water (4 : 1 : 5) as the solvent showed the presence of two ninhydrin-positive components, having R_F values of 0.40–0.42 and 0.60–0.64, identical with those given by authentic samples of L-tyrosine and of L-tyrosyl-L-tyrosine respectively. The component having R_F 0.60–0.64 (L-tyrosyl-L-tyrosine) was eluted, and purified by subjecting it twice to large-scale paper chromatography, followed by elution with water. To an aqueous solution of each of the three samples of L-tyrosyl-L-tyrosine obtained in this manner, there was added 50 mg of an authentic sample of the unlabeled dipeptide, the solution was concentrated to dryness, and the residue was dissolved in ethanol. Upon the addition of ether, the tyrosyltyrosine crystallized, and each sample was recrystallized once more. Their specific radioactivity was determined by means of gas-flow counter (background 10 ± 1 cpm) with 1.8–2.2 mg of sample per cm^2 on the planchet. The dipeptide samples from solution A (with C^{14}-labeled tyrosine) and from solution C (no pepsin, with C^{14}-labeled carbobenzoxy-L-tyrosine) gave no counts over background, whereas the dipeptide sample from solution B (complete system, with labeled carbobenzoxy-L-tyrosine) had a specific radioactivity of 22 cpm per mg. The last value was unchanged (within the precision of the counting procedure) after an additional recrystallization of the L-tyrosyl-L-tyrosine from Solution B. Since 50 mg of unlabeled carrier had been added after chromatographic separation of the dipeptide from the hydrogenated incubated mixture, it may be estimated that there were about 1,100 cpm of C^{14} in labeled L-tyrosyl-L-tyrosine. This amount of exchange of labeled carbobenzoxy-L-tyrosine into the carbobenzoxy dipeptide is clearly a

minimal value, since losses of the labeled dipeptide must have been incurred during the purification by paper chromatography.

The L-tyrosyl-L-tyrosine from solution B was treated with 1-fluoro-2,4-dinitrobenzene to yield the DNP-peptide, which was subjected to hydrolysis with 6 *N* HCl for 12 hours at 110°C. Extraction of the hydrolysate with ether gave a fraction that retained about 90 per cent of the radioactivity of the dipeptide, indicating that the dipeptide had been labeled in the amino-terminal tyrosyl residue, and that the carboxyl-terminal residue was unlabeled.

It should be noted that any L-tyrosyl-L-tyrosine formed by the reaction of the two molecules of the carbobenzoxy dipeptide to give the carbobenzoxy tripeptide, followed by the hydrolysis of the last named compound, would be expected to yield only unlabeled dipeptide. Furthermore, since carbobenzoxy-L-tyrosine is resistant to the action of pepsin under the conditions of the above experiment, the appearance of C^{14} in the L-tyrosyl-L-tyrosine from solution B must involve the reaction of carbobenzoxy-L-tyrosine as a unit. The simplest explanation is the occurrence of the exchange reaction (1). The fact that the dipeptide from solution A was not radioactive makes an acyl-transfer reaction unlikely in the case of pepsin. The results of the isotope exchange experiment described above are therefore in accord with the specificity studies[1] and transfer experiments[8] in favoring a mechanism of pepsin action that involves activation of the imino group of the sensitive peptide bond.

The unique properties of pepsin—its very acid isoelectric point, its ready denaturation at pH values above 6, its relatively high content of the residues of dicarboxylic amino acids, β-hydroxy amino acids, aromatic amino acids, and proline—invite speculation about the nature of the group (or groups) in the protein that are involved in its catalytic action. It has been suggested[12] that a protein carboxyl group of abnormally low pK may accept the NH group of the sensitive linkage to form an amide bond, which is cleaved either by water (hydrolysis) or by a carboxylic acid (transfer). The report[13] that the single phosphoryl group (per unit of about 35,000) of pepsin may be removed enzymically without affecting pepsin activity appears to rule out such a group as a participant in the catalytic action. Clearly, further studies, both with pepsin itself and with model compounds that may mimic its action, are needed to clarify the mechanism of pepsin action.

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