The Labelling of a Catalytic Group of Pepsin; Evidence for an Acyl Intermediate

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Summary When pepsin is incubated with Z-Tyr or Z-Tyr-Tyr in the presence of CT_3OH, most of the radioactivity incorporated into the protein is covalently bound, apparently by an ester type of linkage.

THE proteolytic enzyme pepsin catalyses the hydrolysis of amide linkages of synthetic dipeptides[1,2] such as benzyloxycarbonyltyrosyl-tyrosine (I) and also catalyses an exchange[3,4] of the carboxylic oxygen atoms of benzyloxylcarbonyltyrosine (II) with the oxygen atoms of $H_2{}^{18}O$. We report that the incubation of pepsin with either Z-Tyr-Tyr (I) or Z-Tyr (II) in the presence of CT_3OH resulted in the incorporation of radioactivity into the protein moiety The present results and previous evidence from this[5] and other laboratories[2-4,6] strongly suggest that the crucial event in the pepsin-catalysed hydrolysis involves the concerted transfer of the acyl† as well as the amino-fragments of the amide linkage to the enzyme. A number of hypothetical mechanisms for the reaction have been considered.[5,7,8]

Z-Tyr (II) (5 mg.) was incubated with pepsin (30 mg.) in 0·2M-sodium acetate buffer (3·9 ml., pH 4·6) in the presence of CT_3OH (0·1 ml., $1{\cdot}36 \times 10^8$ c.p.m./mmole) at 37° for 24 hr. The incubation was terminated with trichloroacetic acid and the precipitated protein was washed four times with ethanol—water (1 : 1). Measurement of radioactivity showed that 4×10^4 c.p.m. had been incorporated in the protein fraction. Assuming a molecular weight of 36,500 for pepsin this represents an incorporation of 0·36 mole of methanol per mole of pepsin.‡ In a control experiment it was shown that the incubation of pepsin with radioactive methanol, but without Z-Tyr resulted in no incorporation of radioactivity in the precipitated protein. When the radioactive protein obtained above was dialysed against 8M-urea for 24 hr. at least 90% of the original radioactivity was retained in the protein fraction, thus showing that most of the radioactivity was covalently bound to the protein. When, however, the radioactive protein was dialysed against 8M-urea which was also 1M in hydroxylamine at pH 9, practically all the radioactivity was removed from the protein fraction. This observation may be interpreted as suggesting that the radioactivity was bound to the protein by an ester type of linkage.

The incorporation of radioactivity in the above experiment is best explained if one assumes that the initial reaction of pepsin with Z-Tyr (II) results in the formation of the intermediate (III) which is then hydrolysed in such a way that the overall process corresponds to a nucleophilic attack at the enzyme carbonyl group (Scheme). If the formation of the intermediate (III) is due to the property of pepsin that is also utilized in the hydrolysis of amide linkages then the present work provides support for the involvement of an acyl intermediate in pepsin catalysis.

$$\text{Pepsin} + \underset{\text{(II)}}{\text{Z-Tyr-CO}_2\text{H}} \longrightarrow \underset{\text{(III)}}{\text{Z-Tyr-CO·O·CO·Pepsin}}$$

$$\xrightarrow{\text{CH}_3\text{OH}} \text{(II)} + \text{Pepsin-CO}_2\text{CH}_3$$

SCHEME

When the incubation was performed with Z-Tyr-Tyr (I) instead of Z-Tyr (II), once again radioactivity was incorporated into the precipitated protein (0·46 mole of methanol per mole of pepsin). In principle the incorporation of radioactivity into the precipitated protein in the experiment can either be due to the hydrolysis of an acyl intermediate, or an amino-intermediate,[5] or both.

The work reported above provides a convenient method for labelling a catalytic group of pepsin. The work also suggests that the hydrolysis which is catalysed by pepsin may possibly take place through the formation of an intermediate where the substrate is bound to the enzyme through an anhydride linkage, and this intermediate may then be further hydrolysed by nucleophilic attack on the enzyme carbonyl group. The latter conclusion is further supported by a recent report,[9] which has been confirmed by us, that the pepsin-catalysed hydrolysis of dipeptides

† The term "acyl-intermediate" in this communication simply implies that the acyl moiety of the amide group of the substrate has been transferred to a suitable group on the enzyme; for this and other terminology used here, see ref. 5.

‡ The pH-dependence of the incorporation of radioactivity in the precipitated protein gave a bell-shaped curve with a maximum at pH 4·0.

in the presence of methanol did not give the methyl esters of the corresponding hydrolysis products, which would be expected if the hydrolysis of the acyl-enzyme occured by attack on the substrate carbonyl group[10] [see structure (III)].

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[10] See also T. P. Stein and D. Fahrney, *Chem. Comm.*, 1968, 555, and compare with the results mentioned in ref. 17 of this paper.