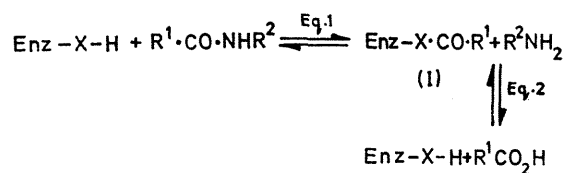# Studies on the Mechanism of Action of Pepsin

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Summary A covalent intermediate formed during the pepsin-catalysed hydrolysis of benzyloxycarbonyl-tyrosyl-tyrosine has been isolated.

It is now well established that a large number of proteolytic enzymes including chymotrypsin, trypsin, papain, and subtilisin catalyse the cleavage of an amide linkage through a sequence of reactions represented in a generalized form[1] as in Scheme 1.

The first event in this sequence is the transfer of the

$$\text{Enz}-X-H + R^1 \cdot CO \cdot NHR^2 \underset{}{\overset{Eq.1}{\rightleftharpoons}} \text{Enz}-X \cdot CO \cdot R^1 + R^2 NH_2$$

$$(1) \quad \Big\Updownarrow Eq.2$$
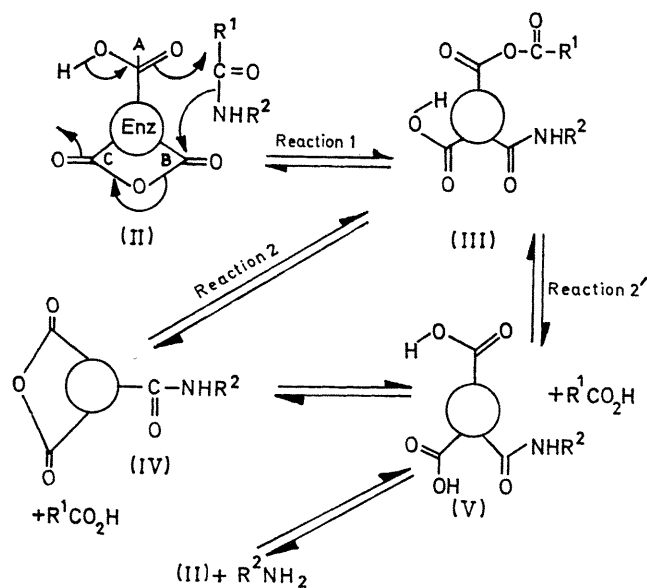
$$\text{Enz}-X-H + R^1 CO_2 H$$

Scheme 1

amide carbonyl group of the substrate to a suitable nucleophilic site on the enzyme thus giving 'the acyl enzyme

complex' (I); in this process (Equation 1, Scheme 1) the amide nitrogen is eliminated as an amine. The next step that involves the hydrolysis of the acyl intermediate (I) results in the liberation of the acid moiety and the regeneration of the enzyme.

The work on the mechanism of action of pepsin using synthetic substrates suggested that this enzyme may catalyse the hydrolysis of the peptide bond through a novel process involving the intermediacy of an 'amino-enzyme complex'.[2,3] Subsequent observations necessitated further elaboration of the idea and it has been suggested that the hydrolysis of a peptide bond by pepsin may occur through the transfer of both the acyl and the amino-moieties to the enzyme[4-6] [conversion (II) $\rightleftharpoons$ (III), Scheme 2], the involvement of such an amide intermediate is both intriguing and interesting.

The work of Newmann et al.[2] led us to predict that in the event an 'amino-enzyme' intermediate participates in the pepsin-catalysed hydrolysis of synthetic dipeptides, then such a species must be relatively long lived and hence amenable to isolation under favourable circumstances. We report the first successful isolation of a covalent intermediate formed during the pepsin-catalysed hydrolysis of a substrate, benzyloxycarbonyl-tyrosyl-tyrosine (VI). It is suggested that this intermediate contains the carboxy-terminal tyrosine residue of (VI) linked to the enzyme through an amide linkage. A hypothetical mechanism[†] for the hydrolysis of a peptide linkage by pepsin is outlined in Scheme 2.

(II)

Reaction 1

(III)

Reaction 2

Reaction 2′

(IV)

+R^{1}CO$_2$H

H—O

+R^{1}CO$_2$H

OH

(V)

(II)+ R^{2}NH$_2$

SCHEME 2

Z-[^{3}H]Tyr-[^{3}H]Tyr‡ (500 μg. in ethanol 0·1 ml.; 1·25 × 10^6 counts/min.) was incubated with pepsin (20 mg.) in pH 4 acetate buffer (1·9 ml.) at 37° for 1 hr. The incubation mixture was then terminated by the addition of trichloroacetic acid (TCA) and the precipitated protein was washed several times with ethanol. Measurement of radioactivity showed that 10,050 counts/min. [or 10^{-8} mole equivalent of (VI)] had been incorporated in the precipitated protein;§ we shall refer to the radioactive component of the precipitate as 'compound X'. When Z-[^{3}H]-Tyr-[^{3}H]-Tyr (500 μg.) was added immediately before the termination of the incubation, no significant amount of radioactivity was found in the precipitated protein. The incorporation of the radioactivity observed above was specific for Z-Tyr-Tyr (VI) and could not be demonstrated when either labelled tyrosine or labelled Z-Tyr were substituted for the compound (VI).

The presence of covalently bound radioactivity in the 'compound X' was demonstrated by dialysis at pH 8·5 in 8M-urea which was also 1M in hydroxylamine for 24 hr., when no loss of radioactivity from the protein fraction occurred. The inclusion of 1M-hydroxylamine in the above dialysis further suggested that the radioactive component was not linked to the precipitated protein via an ester or an anhydride linkage (such linkages are known to be cleaved[8,9] by hydroxylamine). It may therefore be inferred that the radioactive component is bound to the precipitated protein through an amide linkage. The following considerations suggest that the radioactive component precipitated with TCA was a 'dynamic intermediate' involved in pepsin catalysis.

(i) In the incubation mentioned above the radioactivity could only be precipitated when the reaction mixture was terminated with TCA, which rapidly denatures the protein. The termination of the incubation with either ethanol or heat-treatment resulted in a slow precipitation, and the protein thus obtained was practically nonradioactive. These experiments show that although the intermediate was labile in the native form, however, it could be stabilized by the spontaneous destruction of the tertiary structure of the enzyme.

(ii) The plotting of pH against the incorporation of radioactivity into the precipitated protein gave a curve which was similar to the pH–rate profile for the pepsin-catalysed hydrolysis of proteins.[8]

(iii) In a time course study, it was shown that longer incubation periods resulted in a corresponding decrease in the incorporation of radioactivity into the precipitated protein which approximately parallelled the increase in the release of tyrosine from Z-Tyr-Tyr.

(iv) When Z-[^{3}H]Tyr-Tyr, labelled only in one of the Tyr residues, was incubated with pepsin, no incorporation of

† It is suggested that the enzyme contains a potential anhydride linkage[1] and at least one catalytic carboxy-group 'A'. The binding of the substrate to the enzyme induces a conformational change thus bringing the OH group of 'A' in close proximity to the oxygen atom of the group 'C'; this facilitates the initial transfer reaction by making the group 'A' nucleophilic and the carbonyl group 'B' electrophilic. The intermediate (IV) is included to make the reaction 2 (Scheme 2) readily reversible in order to account for the transpeptidation reaction;[2] the latter reaction may occur with substrates where the decomposition of the intermediate (IV) or (V) is particularly slow, i.e. synthetic dipeptides.[2] The direct conversion (III) → (V) is an alternative possibility.

‡ Tritiated benzyloxycarbonyl-tyrosyl-tyrosine (VI, Z-[^{3}H]Tyr-[^{3}H]Tyr) was prepared by the extension of the equilibration method originally developed by Kirby et al.[7] The labelled Z-Tyr-Tyr thus prepared should contain tritium in the aromatic rings of both the tyrosine residues. Benzyloxycarbonyl-[^{3}H]tyrosine was prepared by the above method[7] and was then converted into Z-[^{3}H]Tyr-Tyr.

§ The incorporation of radioactivity into the precipitated protein was found to be linear when the concentration of Z-Tyr-Tyr was varified between the range 0·5—10 mg. Furthermore, the termination of the incubation mixture between 30—60 min. gave qualitatively a similar incorporation of radioactivity.

radioactivity in the precipitated protein occurred, showing that it is only the carboxy-terminal tyrosine residue of (VI) that is covalently bound in the 'compound X'.

The cumulative evidence presented above is consistent with the notion that the radioactivity in the precipitated protein is due to a compound that is an intermediate in the enzymic reaction. It is tentatively suggested that this intermediate has the structure of type (V)¶. These results highlight the role of a carboxy-group (or its equivalent) in pepsin catalysis. Previously a carboxy-group on the active site of pepsin has been suggested on the basis of alkylation[8–11] and ^{18}O exchange[12] studies. Mechanistic consideration would, however, require that the carboxy-function involved in the alkylation or ^{18}O exchange experiments is the one indicated by the suffix 'A' (II) whereas another carboxy-group or its equivalent (group 'B') participates in the binding of radioactivity.

¶ It is to be noted that whether the intermediate in the enzymic reaction has the structure (III), (IV), or (V), our method of isolation will yield only (V).

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