MECHANISM OF PEPSIN CATALYSIS: GENERAL BASE CATALYSIS BY THE ACTIVE-SITE CARBOXYLATE ION

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1. Introduction

Significant progress has been made in the past few years in studying pepsin (EC 3.4.23.1) and other acid proteinases [1]. These enzymes were investigated by high resolution X-ray analysis [2–4] and the data thus obtained used to postulate a mechanism of action of acid proteinases [5]. However, the evidence about the most important stages of pepsin catalysis in solution has not been available hitherto.

At least two carboxyl groups operate in the active site of pepsin; those of Asp-32 and Asp-215 having pK_a values about 1.4 and 4.5, respectively [6]. The enzyme forms relatively stable intermediates with the C- and N-terminal fragments of a peptide substrate [7,8], which are called 'amino enzyme' and 'acyl enzyme' and whose chemical nature is still unknown. Interaction of these intermediates with the corresponding acceptors yields transpeptidation products.

The character of the primary attack by the catalytically active groups on a substrate is of crucial importance for elucidating the structure of intermediate compounds in pepsin catalysis. The carboxylate ion of the active site may act either as a nucleophilic (covalent) catalyst, or as a general base catalyst which splits a proton off a water molecule.

We have shown that the carboxylate ion of Asp-32 functions as a general base catalyst. The evidence for this rests upon the following data reported here:

(i) The L-leucyl-L-leucine product of the pepsin-catalysed acyl-transfer type transpeptidation of L-leucyl-L-tyrosine amide incorporates ^{18}O into its peptide bond, when transpeptidation is carried out in $H_2\,^{18}O$.

(ii) The residual, non-reacted substrate partly exchanges the oxygen of the peptide bond for ^{18}O in the same conditions.

(iii) Pepsin-catalysed oxygen exchange of the acetyl-L-phenylalanine carboxyl group with water is accelerated in the presence of a tripeptide, the methyl ester of L-phenylalanyl-L-alanyl-L-alanine.

These data, as well as other evidence about pepsin catalysis, make it possible to formulate a mechanism of pepsin action.

2. Materials and methods

A commercial preparation of porcine pepsin (Olaina Plant of Chemical Reagents, USSR) was purified by chromatography on DEAE-cellulose as in [9].

L-[2-^{14}C]Leucyl-L-tyrosine amide was prepared as in [10]. It had spec. radioact. 140×10^6 cpm.

N-([2-^{14}C]Acetyl)-L-phenylalanine with spec. radioact. 111×10^6 cpm was prepared as in [11].

The methyl ester of L-phenylalanyl-L-alanyl-L-alanine was synthesized with 30% yield by a stepwise procedure from methyl L-alaninate and the corresponding benzyloxycarbonyl amino acids using a technique of mixed anhydrides. Melting point 210°C, $[\alpha]_D^{20}$ −3.9 (*C* 0.65, DMF).

The methyl ester of D-phenylalanyl-L-alanyl-L-alanine was prepared in a similar way; $[\alpha]_D^{20}$ −39.3 (*C* 1.15, DMF). 85% and 50% enriched $H_2\,^{18}O$ was used in experiments.

In transpeptidation experiments, 10.5 mg

L-[2-^{14}C]leucyl-L-tyrosine amide was dissolved in 5 ml 0.2 M Na-citrate buffer (pH 3.6) prepared with 85% H_2 ^{18}O, and 6.5 mg pepsin added. The mixture was kept at 20°C for 72 h and then freeze-dried. The residue was dissolved in water, the pH was adjusted to 9.0 and then again acidified to 1.0. The protein precipitate was separated by centrifugation and the supernatant evaporated. The residue was subjected to paper electrophoresis (5000 V, 2 h, 0.15 M bicarbonate–ammonium buffer, pH 8.15). Zones corresponding to the original substrate and leucyl-leucine were eluted and subjected to paper chromatography in butanol/water/acetic acid (4:5:1) and then to chromatography on cellulose MN300 plates in BuOH/Py/AcOH/H_2O (60:40:12:48). The yield of the transpeptidation product was approx. 30%. The purity of the preparations was checked by amino acid analysis and determination of N-terminal amino acids.

For mass-spectrometric analysis aliquots of the HLeu–LeuOH (0.2 mg) were converted into Tfa/Leu–LeuOMe. The transpeptidation product was also converted to the phenylthiohydantoin (PTH) of leucine by a modified Edman procedure [12]. For activation analysis a sample of HLeu–LeuOH was acetylated and hydrolyzed by carboxypeptidase A at pH 8.5 (5–6 min), to eliminate the C-terminal leucine. The acetyl-L-leucine thus obtained was extracted with ethylacetate from acidified solutions.

When studying the rate of the carboxyl group oxygen exchange, 4 mg *N*-([2-^{14}C]acetyl)-L-phenylalanine was dissolved in 1 ml 50% H_2 ^{18}O, and 0.05 ml 2 M acetate buffer (pH 4.5) and 1 mg pepsin were added thereupon. The mixture was allowed to stand at 20°C for 6 h. The reaction was terminated by adjusting to pH 10. The mixture was acidified to pH 2.0, the precipitated protein was separated, and the solution extracted with ethylacetate. Acetyl-L-phenylalanine was isolated from the extract and purified by chromatography. In parallel experiments, 10.7 mg methyl ester of L-phenylalanyl-L-alanyl-alanine, or an equal amount of the D-phenylalanyl tripeptide was added to the mixture. Following incubation, the tripeptide was extracted with ethylacetate from an alkaline solution (pH 10), whereas acetyl-L-phenylalanine was extracted from an acidified solution (pH 2).

The content of ^{18}O in samples was assayed by chromato-mass-spectrometry with an LKB 9000 instrument or by α-particle irradiation as in [13].

3. Results and discussion

As found [14], leucyltyrosine amide is converted with high yield, under the action of penicillopepsin, into leucyl-leucine, the product of the acyl-transfer type transpeptidation. The same reaction proceeds under the action of porcine pepsin catalysis. Here, any resynthesis of the original substrate from leucine and tyrosine amide is hardly observed.

One might expect that ^{18}O is not incorporated from H_2 ^{18}O into the transpeptidation product if it is formed from the covalent 'acyl enzyme', a corresponding anhydride produced by the carboxyls of the enzyme and leucine, since water is not involved in the reaction. However, if water participates in the first stage of splitting a substrate, and the 'acyl enzyme' is a non-covalent complex between the enzyme and leucine, heavy oxygen must be incorporated into the peptide bond of the transpeptidation product. Moreover, in this case, the original substrate can incorporate ^{18}O into its peptide bond due to degradation of a tetrahedral intermediate yielding initial substances.

We have conducted the transpeptidation reaction catalyzed by pepsin in H_2 ^{18}O using labeled H[^{14}C]Leu–TyrNH_2, and isolated the reaction product HLeu–LeuOH, and the non-reacted substrate. For measuring the ^{18}O content in samples, the transpeptidation product was converted into methyl *N*-trifluoroacetyl-L-leucyl-L-leucinate and the latter was subjected to chromato-mass-spectrometry. The ^{18}O content was also measured by α-particle activation analysis [13]. For this purpose, the transpeptidation product was acetylated and hydrolyzed by carboxypeptidase A in order to eliminate the C-terminal leucine containing ^{18}O in its carboxyl. A control experiment using AcLeuLeuOH enriched with ^{18}O has shown that hydrolysis by carboxypeptidase does not result in a loss of ^{18}O from the peptide bond being hydrolyzed. The HLeu–LeuOH was also converted into leucyl-phenylthiohydantoin (PTH) in which the ^{18}O content was measured by both chromato-mass-spectrometric and activation analysis methods.

Table 1
The content of ^{18}O in the samples

Sample	Method[a]	% ^{18}O in $H_2{}^{18}O$	^{18}O found[b]	Theoretical ^{18}O content upon complete exchange of one O atom[b]	Proportion of exchange (%)
AcLeuOH	A	55	4.6 ± 0.2	5.65	81 ± 4
PTH–Leu	A	85	7.0 ± 0.3	7.20	97 ± 5
PTH–Leu	M(194)	87	0.9 ± 0.15	0.87	104 ± 6
TfaLeu–LeuOMe	M(212)	54	0.45 ± 0.1	0.54	83 ± 20
HLeu–$TyrNH_2$	A	87	0.9 ± 0.1	5.2	17.5 ± 2
AcPheOH[c]	A	50	0.02 ± 0.005	4.3	0.46 ± 0.1
AcPheOH (+ tripeptide)[c]	A	50	0.19 ± 0.01	4.3	4.4 ± 0.3

[a] A, activation analysis [13]; M, chromato-mass-spectrometry; in parentheses, *m/e* of the ^{18}O containing peaks measured

[b] In μg ^{18}O/100 μg sample in the case of method A, and as the intensity of the ^{18}O containing peak divided by a sum of the intensities of the ^{18}O and ^{16}O containing peaks, in the case of method M. av. 3 exp.

[c] Incubation for 1 h

The results of determining the content of ^{18}O in the samples are presented in table 1. The transpeptidation product incorporates about one heavy oxygen atom in its peptide bond. The substrate, HLeu–$TyrNH_2$, is also capable of exchanging the oxygen of its peptide bond with ^{18}O, but the extent of exchange here does not exceed 20%.

We have studied also the rate of oxygen exchange in AcPheOH in the absence and in the presence of the tripeptide HPhe–Ala–AlaOMe, i.e., in the conditions when pepsin catalyzes synthesis–hydrolysis of the tetrapeptide AcPhe–Phe–Ala–AlaOMe. One might expect that when the covalent 'acyl enzyme' is formed during this process, the rate of ^{18}O exchange will not increase in the presence of the tripeptide. On the other hand, if synthesis–hydrolysis of the tetrapeptide involves the non-covalent 'acyl enzyme' and the rate of this interconversion is high as compared with the rate of the 'genuine' pepsin-catalyzed ^{18}O exchange in AcPheOH [15,16], the rate of oxygen exchange in the latter will be considerably accelerated in the presence of tripeptide. Indeed, the data in table 1 show that such an acceleration does take place. The exchange is not accelerated if D-HPhe–Ala–AlaOMe is added.

As we have shown [17], the enzyme does not exchange the oxygen atoms of its Asp-32 and Asp-215 carboxyls in the course of the substrate hydrolysis in $H_2{}^{18}O$. It is evident therefore that, in the course of catalysis, pepsin forms neither the covalent 'acyl enzyme' nor the covalent 'amino enzyme', and the carboxylate ion of Asp-32 acts as a general base catalyst.

Analogous conclusions were drawn recently [5] for the penicillopepsin mechanism on the basis of X-ray analysis.

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