SPECIFICITY OF α-CHYMOTRYPSIN. DIPEPTIDE SUBSTRATES

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1. Introduction

The earlier investigations of α-chymotrypsin catalyzed hydrolysis of peptide substrates lead to the conclusion that the peptide bonds on the carboxyl side of the amino acid residues carrying aromatic side-chains, the so-called specific amino acids, are most susceptible to chymotryptic cleavage [1]. The role of aromatic and other hydrophobic side-chains in determining the rate of hydrolysis of model substrates such as acylated amino acid esters and amides was one of the main subjects of the work concerned with the specificity of this enzyme [2]. With regard to the hydrolysis of the natural substrates, the proteins, it became evident from a large number of chymotryptic digestions that the susceptibility for cleavage of peptide bonds next to specific amino acid residues depends also on the structure of adjacent amino acids [3]. Work carried out with small peptide substrates containing one specific amino acid only, provided evidence that at least two residues on the amino side and three residues on the carboxyl side give rise to interactions responsible for specificity [4]. An extended kinetic analysis of the complicated relationship between the structure of the specific amino acid residue L_{x1} as well as of other amino acid residues, L_x and L_y, and the reactivities in peptide substrates,

$$E_x\text{–}L_{xn} \ldots L_{x2}\text{–}L_{x1}\overset{\downarrow}{\text{–}}L_{y1}\text{–}L_{y2} \ldots L_{ym}\text{–}E_y$$

appear to provide a valuable method for studying the specificity of α-chymotrypsin.

In this communication we report our first results with substrates of the type $E_x\text{–}L_{x1}\text{–}L_{y1}\text{–}E_y$ using steady-state kinetic methods. The basic prerequisite for this kind of analysis, that only the bond between L_{x1} and L_{y1} is hydrolyzed to any observable extent during the measurements, is realized by using peptide substrates with one single specific amino acid residue (L_{x1}). Since α-chymotrypsin constitutes an endopeptidase, peptides with free amino acid residues at both ends are not suitable for such studies, particularly if small peptides are investigated. Furthermore, it seemed important to avoid end groups E_x and E_y which would give rise to disturbing interactions with the enzyme protein or would lead to side-reactions. Taking these factors into consideration, the following dipeptide derivatives were chosen as representative examples: Ac–Tyr–Gly–NH_2, Ac–Tyr–Ala–NH_2, Ac–Phe–Gly–NH_2 and Ac–Phe–Ala–NH_2.

2. Materials and methods

Sigma α-chymotrypsin (three times crystallyzed, salt free, Lot 86B-0470) was used without further purification. The operational normality of the enzyme solutions was determined by titration with *N-trans*-cinnamoylimidazole [5]. The synthesis of the dipeptide substrates will be described elsewhere [6].

The kinetic measurements were done with the aid of a pH-stat instrument (Radiometer) composed of a pH meter PHM 26c, a titrator TTT 11b, an automatic burette ABU 1 and a thermostated titration cell. The titrator TTT 11b was appropriately modified [6] to allow accurate measurements in the presence of varying amounts of buffering glycine amide or alanine amide produced during the reaction. The burette

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Table 2

Steady-state kinetic data. The values for k_{cat} and K_m of the dipeptide substrates were determined from initial rate measurements by changing the substrate concentration in the range indicated in the second column. Details of the reaction conditions and the data processing procedure are given in section 2.

Substrate	Enzyme concentration E (μM)	Range of initial substrate concentrations S_0 (mM)	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($sec^{-1} M^{-1}$)
Ac–Tyr–NH_2 *	–	–	0.17 ±0.02	32 ±4	5.3
Ac–Tyr–Gly–NH_2 **	22.0	9–37	0.64 ±0.10	23.3 ±2.8	27.5
Ac–Tyr–Ala–NH_2	4.9	4.6–35	7.5 ±0.5	17.2 ±0.9	436
Ac–Phe–NH_2 *	–	–	0.055 ±0.014	31 ±3	1.8
Ac–Phe–Gly–NH_2	38.0	4.9–31	0.140 ±0.006	14.6 ±0.3	9.6
Ac–Phe–Ala–NH_2	8.4	3–30	2.8 ±0.2	25.0 ±1.2	114

* The two amino acid substrates were measured by Foster and Niemann [11] using the same experimental conditions as described in Section 2.

** At pH 7.8 and 25°, Foster and Niemann [11] obtained: $k_{cat} = 0.52 \pm 0.07$ sec^{-1}, $K_m = 23 \pm 3$ mM.

and a similar ratio is noted for the corresponding substrates where L_{x1} = Phe. This finding can be interpreted to mean that either one or both of the following two effects are operative: (1) the replacement of a hydrogen at the amide nitrogen by the carboxamide methyl residue creates a pure substitution effect which changes the susceptibility of the bond for hydrolysis, and (2) additional specific interactions, such as those between the amide group in the dipeptide substrate and the enzyme protein, cause a more favorable orientation of the substrate within the active site, whereby the rate of hydrolysis can be enhanced. From the above experiments alone, it is not yet possible to decide which one of the two effects is more important, but they nevertheless allow us to conclude that the insertion of a glycine residue affects the k_{cat} value in much the same way whether L_{x1} is tyrosine or phenylalanine.

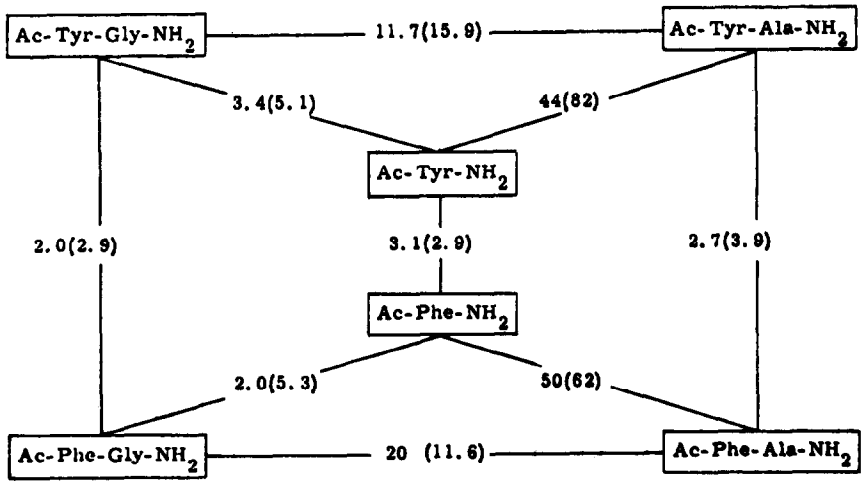

Fig. 1. Relationship between the structure of the substrates and their reactivity. The ratios of the k_{cat} values for pairs of substrates are indicated in the graph between the substrates to be compared. The numbers in brackets signify the corresponding k_{cat}/K_m ratios.

Table 1
Stoichiometry of the reaction. The equivalent amounts of substrate hydrolyzed were calculated according to eq. (1) from infinity readings of the base consumed by using the pK values determined for Gly–NH_2 or Ala–NH_2 as given in the third column.

Substrate	Amount of substrate subjected to hydrolysis (μmoles)	Equivalent amount of substrate hydrolyzed (μmoles)	pK of the corresponding L_{y1}–NH_2 species
Ac–Tyr–Gly–NH_2	49.0	48.5	8.07 ± 0.02
Ac–Phe–Gly–NH_2	75.2	75.7	
Ac–Tyr–Ala–NH_2	228.8	226.1	8.15 ± 0.02
Ac–Phe–Ala–NH_2	82.2	76.8	

ABU 1 was, in addition, equipped with a device providing the data in the form of pulses, the number of which is proportional to the base consumption. The pulses were counted by an electronic counter (Elesta CTP 50) at time intervals regulated by an electronic clock (Elesta CTP 51).

The data which were recorded simultaneously by a recorder (Radiometer SBR 2) mechanically coupled with the burette and by a teleprinter (Teletype 497/a) registering the pulses, represent the consumption of sodium hydroxide as a function of time. The corresponding amount of substrate hydrolyzed was calculated from the base consumption, taking into account the pK values pK_P of the amino acid derivatives produced, according to the equation

$$A_S = A_B[1 + 10^{(pK_P - pH)}] \quad (1)$$

where A_S is the amount of substrate hydrolyzed and A_B the amount of base consumed. The pK values of glycine amide and alanine amide as obtained by titration at 25° in 0.2 M NaCl solution are given in table 1.

The data were processed with a CDC 1604 A computer. The initial rates were calculated by linear regression of the data on a power expansion series. The kinetic parameters k_{cat} and K_m are the result of an iterative regression [7] on the Michaelis-Menten equation

$$\frac{v}{E} = \frac{k_{cat}}{1 + K_m/S_0} . \quad (2)$$

First approximations of the two parameters needed for this procedure were obtained from the reciprocal form of eq. (2) by the usual least square treatment.

3. Results and discussion

From table 1 where the stoichiometry of the reaction is demonstrated with a representative example of each dipeptide substrate, it becomes evident that the equivalent of one bond is being hydrolyzed, as is expected, since only the L_{x1}–L_{y1} bond will hydrolyze to an observable extent. The steady-state kinetic data listed in table 2 indicate that all structural modifications under discussion result in marked changes of the catalytic constant k_{cat} rather than the Michaelis constant K_m. This is a phenomenon which was also observed in the case of the pepsin-catalyzed hydrolysis of substrates of the type Z-His-L_{x1}–L_{y1}–OMe where the structure of both L_{x1} and L_{y1} were modified [8, 9]. Since it is assumed [10] that k_{cat} values are an appropriate measure for the rate determining bond breaking and bond making step, which, at least for the model substrates E_x–L_{x1}–NH_2 is thought to be the acylation step, we shall use these values to discuss the relationship between the structure of the substrates and their reactivity. Thus substrates of different structure are compared in pairs by using the ratios of the k_{cat} values; these ratios are indicated between the corresponding formulas in fig. 1.

We shall first compare the two dipeptide substrates where L_{y1} = Gly with the two amino acid amide substrates. As is seen in fig. 1, the k_{cat} value of Ac–Tyr–Gly–NH_2 is 3.4 times larger than that for Ac–Tyr–NH_2

A much larger k_{cat} ratio is found upon comparison of the two dipeptide substrates where L_{y1} = Ala with the two amino acid amide substrates. The k_{cat} value for Ac–Tyr–Ala–NH_2 is 44 times larger than that for Ac–Tyr–NH_2, while the corresponding substrates where L_{x1} = Phe yield a ratio which is only slightly larger. In this case, the methyl at the α-carbon of L_{y1} tends to create additional interactions with the enzyme protein. It is therefore necessary to also consider the k_{cat} ratios appearing between the dipeptide substrates where L_{y1} = Ala with those where L_{y1} = Gly. Here one can be sure that no substitution effect can explain the large k_{cat} ratio of 11.7 obtained by comparing Ac–Tyr–Ala–NH_2 with Ac–Tyr–Gly–NH_2, or, correspondingly at a somewhat larger ratio, Ac–Phe–Ala–NH_2 with Ac–Phe–Gly–NH_2. The increase in rate can only be the result of interactions between the methyl group and the enzyme protein which bring about a still more favorable orientation of the substrate within the active site. In view of this finding, it seems plausible that the rate differences observed upon inserting a glycine residue, as discussed above, can also largely be attributed to a similar effect, and that there the substitution effect either makes only a small contribution to k_{cat} or even acts to reduce it. These arguments become even more likely if one considers that the k_{cat} ratios due to interactions with the methyl group are much larger than those due to interactions with the amide group.

The k_{cat}/K_m values, which are widely used as a measure for reactivity [12], yield a picture that is quite similar to that obtained on the basis of the k_{cat} values alone. It is furthermore noteworthy that the k_{cat} as well as the k_{cat}/K_m ratios are nearly the same for pairs of substrates which contain either the same L_{x1} or the same L_{y1} residue. This means that the effects of the structural modifications in those two residues are, at least as a first approximation, independent of each other. Clearly a study of higher analogs as substrates, as well as further structural modifications on both sides of the bond to be hydrolyzed, will provide more insight into how the enzyme recognizes structural features of certain groups at the individual amino acid residues L_x and L_y. Work along these lines is in progress.

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