HYDROPHOBIC INTERACTION IN THERMOLYSIN SPECIFICITY

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1. Introduction

Neutral metalloendopeptidase thermolysin (EC 3.4.24.4) catalyzes the hydrolysis of such peptide bonds where the amino side of the bond is contributed preferably by an amino acid with a large hydrophobic residue, e.g., Phe or Leu [1–3], which points to the presence of a corresponding hydrophobic pocket in the active site of the enzyme. This conclusion is in accordance with X-ray crystallography data [4]. However, studies of the binding of low-M_r inhibitors, have shown that structurally similar inhibitors can bind differently in the enzyme active site [5–8], which complicates the evaluation of the rôle of the hydrophobic pocket in thermolysin specificity. Since the substrate specificity constant k_2/K_s includes only the productive mode of binding, kinetic studies of the reactions of the enzyme with representative series of substrates are necessary for obtaining unambiguous information about the topography of the enzyme active site and the nature of interactions between enzyme and substrate during hydrolysis.

Here, we have used a series of dipeptide substrates FA–Gly–X–NH_2 (for X see table 1) for quantitative evaluation of the role of the hydrophobic pocket in the active site of thermolysin in its substrate specificity.

2. Materials and methods

Crystalline thermolysins (*B. thermoproteolyticus* Rokko) were the products of Daiwa Kasei (Japan) and Calbiochem-Behring (USA) and were used without purification.

Synthesis of furylacryloyl dipeptide amides, FA–Gly–X–NH_2, has been described in [9]. FA–Gly–Acp–NH_2 was synthesized and analyzed analogously.

Other materials were of analytical grade.

Enzyme stock solutions were made in 20 mM Tris–acid maleate–NaOH buffer (pH 8.6) containing 5 mM $CaCl_2$. The hydrolysis of the Gly–X bond in the substrates was monitored spectrophotometrically at 322 nm on a Varian Techtron 635 recording spectrophotometer at 25°C. Initial concentrations of the substrates and the rates of their hydrolysis were calculated using ϵ_{322} 13 400 and $\Delta\epsilon_{322}$ 2300 [10] for all substrates except FA–Gly–Tyr–NH_2 for which ϵ_{322} was 15 800 and $\Delta\epsilon_{322}$ 4700. Operational normalities of the enzyme solutions were calculated from the initial rates of enzymic hydrolysis of FA–Gly–Leu–NH_2 (0.1 mM) measured at pH 7.2 in 0.11 M Tris–HCl at 322 nm, using $k_{cat}/K_m = 94.6 \times 10^2\ M^{-1} . s^{-1}$ from [3]. The rate of FA–Gly–Leu–NH_2 hydrolysis at pH 7.2 in 0.17 M Tris–acid maleate–NaOH buffer containing 5 mM $CaCl_2$ (table 1) was 1.5-times higher.

Amino acid sidechain hydrophobicity constants π_R were calculated from the aliphatic π values [11–13].

3. Results and discussion

Dependences of the initial rate of thermolysin-catalyzed hydrolysis upon substrate concentration have been measured over 0.03–0.11 mM substrate. Straight lines in the coordinates v *vs* $[S]_0$ were obtained for all the substrates used. In accordance with the accepted reaction scheme:

Abbreviations: FA, furylacryloyl; Z, benzyloxycarbonyl; Abu, L-α-aminobutyryl; Nva, L-norvalyl; Nle, L-norleucyl; Acp, D,L-α-aminocapryl

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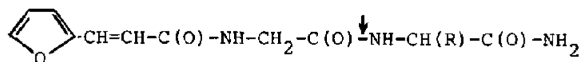
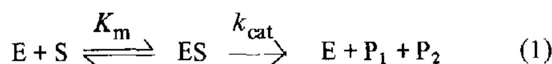
Table 1
Thermolysin-catalyzed hydrolysis of furylacryloylglycyl amino acid amides



(↓ indicates scissile bond) in 0.17 M Tris–acid maleate–NaOH buffer, pH 7.2 at 25°C; $[S]_0$ = 0.03–0.11 mM, $[E]_0$ = 21–280 nM

No.	Variable amino acid (X)	R	$(k_{cat}/K_m) \times 10^{-3}$ $(M^{-1} . s^{-1})$	π_R [11–13]
1	Ala	CH_3-	0.48 ± 0.02	0.5
2	Abu	CH_3-CH_2-	3.6 ± 0.3	1.0
3	Val	$(CH_3)_2CH-$	11.0 ± 0.6	1.3
4	Nva	$CH_3(CH_2)_2-$	28.1 ± 0.9	1.5
5	Leu	$(CH_3)_2CH-CH_2-$	142 ± 8	1.8
6	Nle	$CH_3(CH_2)_3-$	16.7 ± 0.9	2.0
7	Acp	$CH_3(CH_2)_5-$	0.18 ± 0.04[a]	3.0
8	Phe	$C_6H_5-CH_2-$	110 ± 10	2.26
9	Tyr	$HO-C_6H_4-CH_2-$	0.39 ± 0.06	1.59

[a] Effective concentration of the racemic substrate has been taken as 0.5 of the concentration calculated by ϵ_{322}. The possible competitive inhibition by D-isomer should not appear in v under pseudo first-order conditions, $[S]_0 << K_m$. This was experimentally confirmed in the case of FA–Gly–Phe–NH_2 by adding equimolar concentrations of the D-isomer into the reaction mixture



$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \quad (1)$$

the second-order rate constants k_{cat}/K_m were calculated from the slopes of these lines using the enzyme molarity, and are presented in table 1. The constants vary ~800-fold, depending upon substrate structure.

For the substrates with aliphatic X the plot of $\log(k_{cat}/K_m)$ *vs* π_R, which gives the hydrophobicity of the sidechain R in X, is presented in fig.1. For the substrates 1–5 a straight line is observed which is described by:

$$\log(k_{cat}/K_m) = (1.9 \pm 0.3)\pi_R + (0.7 \pm 0.1) \quad (2)$$

with r = 0.997 and s = 0.082. The $\log(k_{cat}/K_m)$ for FA–Gly–Ile–NH_2 from [3] also fits the straight line. Thus, the rate of enzymic hydrolysis of these substrates appears to be controlled by the hydrophobicity of the X sidechain, which suggests that this sidechain is bound in a hydrophobic pocket in the active site of thermolysin. The depth of the pocket seems to correspond to the length of n-propyl group in the sidechain of X (Nva). The $\log(k_{cat}/K_m)$ for

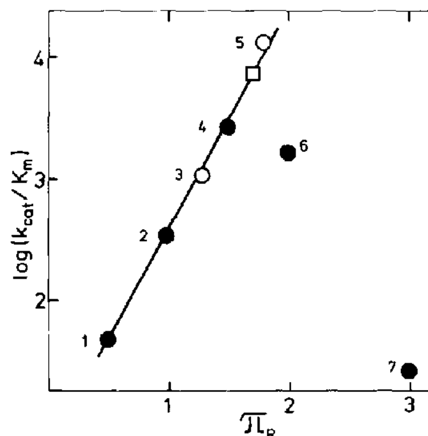


Fig.1. Plot of $\log(k_{cat}/K_m)$ against π_R for the thermolysin-catalyzed hydrolysis of furylacryloylglycyl amino acid amides. The numbers refer to the substrates listed in table 1: (•) normal hydrocarbon substituents; (○) branched hydrocarbon substituents; (□) $\log(k_{cat}/K_m)$ for FA–Gly–Ile–NH_2 from [3], with an increment 0.18 added to account for the difference in buffer systems; π_R for R = $-CH(CH_3)CH_2CH_3$ is 1.7 [11–13].

substrates with the longer *n*-butyl (Nle) and *n*-hexyl (Acp) sidechains show increasing negative deviations from the $\log(k_{cat}/K_m)$ *vs* π_R straight line, while the points for Leu and Ile derivatives with branched and the more hydrophobic than *n*-propyl sidechains, fit the line excellently since their total lengths do not exceed that of *n*-propyl.

The decrease in $\log(k_{cat}/K_m)$ for substrates with the amino acid X sidechains longer than *n*-propyl may be caused by an unfavourable shift in the location of the sensitive peptide bond of bound substrate from the catalytic 'point' in the enzyme active site. In accordance with it, rather good linear correlation between $\Delta\log(k_2/K_m)$ and Δl(l = length of amino acid sidechain R in Å) is observed in this series including FA–Gly–Phe–NH_2, FA–Gly–Nle–NH_2, FA–Gly–Tyr–NH_2 and FA–Gly–Acp–NH_2, if FA–Gly–Nva–NH_2, with R = $-(CH_2)_2CH_3$, is taken as the substrate with the optimum length of the X sidechain.

In chymotrypsin [14,15] and acetylcholinesterase [16] the hydrophobic interaction reveals itself in both non-covalent binding and bond-breaking acylation steps. In terms of eq. (3) and (4):

$$pK_s = C + \varphi_b\pi \quad (3)$$

$$\log k_2 = C' + \varphi_a\pi \quad (4)$$

φ_b and φ_a have been observed to be equal for these serine enzymes, which has been interpreted as an indication of a special mechanism in enzyme specificity, consisting in shutting of the active site hydrophobic slit in the activated complex [15,16].

In the pioneering study of the hydrophobicity effects in the specificity of metalloproteases, Klyosov and Vallee [17] have shown that for carboxypeptidase A, an enzyme with the active site similar to that of thermolysin, hydrophobicity of the amino acid X sidechains in Z–Gly–X and Z–X–Phe is revealed only at the binding stage, whereas k_{cat} in the hydrolysis step remains constant within the series of X = Ala, Abu, Nva, Nle, Phe [17]. In the binding step, φ_b in eq. (3) is ~1, which points to extraction process as the basis of carboxypeptidase substrate-selectivity, in accordance with the data of the hydrophobic substituents distribution in the octanol–water model system.

For thermolysin, no data on individual k_2 and K_s are available, but the slope of $\varphi' = 1.9$ (from eq. (3) and (4), $\varphi' = \varphi_b + \varphi_a$) of the correlation plot in the $\log(k_{cat}/K_m)$ (presumably, $\log(k_2/K_s)$ [18]) against π_R coordinates points to a hydrophobic selectivity mechanism different from that of carboxypeptidase A. As in the above serine enzymes [15,16], the hydrophobic selectivity enhancement in thermolysin may include shutting of the hydrophobic slit in the activated complex of substrate hydrolysis, providing an additional negative-sign (in the free energy scale) contribution of hydrophobic interaction in the transition state which reveals itself as $\varphi_a > 0$, thus allowing for $\varphi_b = \varphi_a$ and $\varphi' \sim 2$. The data in [8] appear to confirm this possibility as they have shown that the inhibition of thermolysin activity (pK_i) by *N*-β-phenylpropionyl aliphatic amino acids correlates with the hydrophobicity parameter π for amino acid sidechain substituents with a slope $\varphi_b = 0.74$, i.e., ~1/2 of φ' for FA–Gly–X–NH_2.

It is interesting to note that data [19] on the hydrolysis of an analogous substrate series, Z–Gly–X–NH_2, in the presence of a *Bacillus subtilis* neutral protease, if presented as a $\log(k/[E]_o)$ *vs* π_R relationship, as well as our unpublished data on the hydrolysis of FA–Gly–X–NH_2 by a neutral protease from a thermoactinomycet, also give straight lines with the slope $\varphi' \sim 2$. This suggests that the regularities observed in this study of thermolysin may be quite general for neutral metalloendopeptidases from microorganisms. A metalloprotease from *Aeromonas proteolytica* [20] seems to be an exception.

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