COMPARISON OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ESTERS WITH DIFFERENT LEAVING GROUPS

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1. Introduction

Most studies on α-chymotrypsin (α-CT)* specificity and stereospecificity refer to esters which carry poor leaving groups like methyl or ethyl alcohol [1–6]. Recently some studies have been published on the α-CT-catalyzed hydrolysis of substrates having a good leaving group [7–12], and it may be asked whether the specificity of α-CT towards activated esters is similar to that observed with methyl or ethyl esters. In order to answer this question, we compare in this paper the different results of the literature with our own data on *p*-nitrophenyl esters [10–12].

At optimum pH, under steady state conditions, the α-CT-catalyzed hydrolysis of esters RCOOR′ may be described according to the classical scheme [13]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow[+\,P_1]{k_3} ES' \xrightarrow{k_4} E + P_2$$

where E is the enzyme, S is the substrate, ES is the enzyme–substrate complex, ES′ is the acyl-enzyme, P_1 and P_2 are alcohol and acid hydrolysis products, respectively. The subsequent reaction rate and the kinetic parameters are given in equations 1–5:

$$v_o = k_{cat}\,[E_o]\,[S_o]/(K_m + S_o) \quad (1)$$

$$K_s = (k_{-1} + k_2)/k_1 \quad (2)$$

$$K_m = [k_3/(k_2 + k_3)]\,K_s \quad (3)$$

$$k_{cat} = k_2 k_3/(k_2 + k_3) \quad (4)$$

$$k_{cat}/K_m = k_2/K_s \quad (5)$$

The rate-determining step changes with the nature of the leaving group so that comparison of α-CT-catalyzed hydrolyses of different substrates must be carried out by means of the second-order rate constant $k_{cat}/K_m = k_2/K_s$. Bender et al. [13, 14] have indeed shown that, of all kinetic constants, k_{cat}/K_m most accurately reflects the specificity of substrates to α-CT.

2. Materials and methods

The physical properties of substrates, their synthesis and their enzymatic hydrolyses have been described already [11, 12].

3. Results and discussion

In table 1, the values of k_{cat}/K_m for a series of acyl-substituted methyl and nitrophenyl esters are reported. It is found that the ratio of k_{cat}/K_m for *p*-nitrophenyl and methyl esters is high and varies in the range $7 \times 10^2 - 6.8 \times 10^5$, depending on the nature of the acyl group R. Such high ratios have been also reported by Williams [15] for the *p*-nitrophenyl and ethyl esters of cinnamic acid and acetylglycine. These ratios are larger than those observed in the alkaline hydrolysis of such esters (ratio ~ 50–100) or in the

* *Abbreviations:*
α-CT, α-chymotrypsin; OMe, methyl esters; ONp, paranitrophenyl esters; Ac, acetyl; Bz, benzoyl; For formyl; Z, benzyloxycarbonyl. The abbreviated designation of derivatives of amino acids corresponds to the proposals of the Joint IUPAC–IUB Commission on Biochemical Nomenclature.

Table 1

Comparison of k_{cat}/K_m values for the α-chymotrypsin-catalyzed hydrolysis of acyl-substituted esters RCOOR'.

R	k_{cat}/K_m (M^{-1} sec^{-1})		Ratio
	Methyl[a]	*p*-Nitrophenyl	
H	0.023[b]	1.56×10^4[c]	6.8×10^5[c]
CH_3OCH_2	0.02[b]	7.3×10^3[c]	3.65×10^5[c]
$C_6H_5CH_2$	1.37 – 1.76	5.6×10^4	$3.2 - 4.1 \times 10^4$
$C_6H_5(CH_2)_2$	73.4 – 97.7	5.0×10^5	$5.1 - 6.8 \times 10^3$
$C_6H_5(CH_2)_3$	144 – 192	1.35×10^5	$7.0 - 9.4 \times 10^2$
$C_6H_5(CH_2)_4$	5.8 – 7.7	0.74×10^4	$9.6 - 12.8 \times 10^2$
$\beta(C_8H_6N)(CH_2)_2$	181 – 242	1.12×10^6	$4.65 - 6.2 \times 10^3$

[a] Pattabiraman and Lawson [6]. Their experimental values were obtained at pH 8 and 25°C, in aq. 41.7% dimethylsulfoxide. The values which are reported here are different from those measured experimentally, by a factor 15–20 in order to correct the solvent effect [6].

[b] The second-order rate constants for the α-chymotrypsin-catalyzed hydrolysis of ethyl esters of formic acid and methoxyacetic acid were measured potentiometrically at pH 7.0, 25°C and 0.5 M NaCl. The concentrations of enzyme ($6.9 \times 10^{-5} - 2.2 \times 10^{-4}$ M) and substrate ($4.8 \times 10^{-2} - 2.4 \times 10^{-2}$ M) were varied over a limited range.

[c] At pH 7.0, 25°C, 0.5 M NaCl.

Table 2

Comparison of the second-order rate constants for acylation of α-chymotrypsin by *N*-Ac-amino acid OMe, N-Bz-amino acid OMe and N-Z-amino acid ONp, at 25°C.

Amino acid	k_{cat}/K_m (M^{-1} sec^{-1})			Ratios of k_{cat}/K_m	
	N-Ac-amino acid OMe	N-Bz-amino acid OMe	N-Z-amino acid ONp[f]	N-Z-amino acid ONp / N-Ac-amino acid OMe	N-Z-amino acid ONp / N-Bz-amino acid OMe
Gly	0.42[a]	73.1[b]	8.38×10^4	2.0×10^5	1.14×10^3
	0.1265[b]	26.5[c]		6.6×10^5	3.16×10^3
Ala	1.72[a]	24[d]	15.2×10^4	8.85×10^4	6.35×10^3
Val	1.35[a]	15.35[b]	0.66×10^4	4.9×10^3	4.3×10^2
	1.97[b]	9.8[e]		3.35×10^3	6.75×10^2
Abu	19.6[a]	227[b]	150×10^4	7.65×10^4	6.6×10^3
	21.1[b]			7.1×10^4	
Nva	264[a]	2882[b]	306×10^4	1.16×10^4	1.06×10^2
	356[b]			0.86×10^4	
Nle	1 250[a]		212×10^4	1.7×10^3	
	3 000[b]			0.7×10^3	
Leu	1 325[a]		174×10^4	1.315×10^3	
Phe	42 000[a]	8.79×10^5[c]	$8\,156 \times 10^4$	1.94×10^3	0.93×10^2
	104 000[b]			0.785×10^3	

[a] Jones et al. [3], pH 7.9, 0.1 M NaCl. [b] Berezin et al. [5], pH 7.8, 0.1 M KCl. [c] Wolf, III, et al. [2], pH 7.9, 0.5 M NaCl. [d] Rapp et al. [4], pH 7.9, 0.2 M NaCl. [e] Jones and Niemann [1], pH 7.9, 0.1 M NaCl. [f] Values from [12].

Table 3
Stereospecificity in the α-chymotrypsin-catalyzed hydrolysis of *N*-acylamino acid esters.

N-acylamino acid	ONp[a] $\frac{(k_{cat}/K_m)_L}{(k_{cat}/K_m)_D}$	OMe[b] $\frac{(k_{cat}/K_m)_L}{(k_{cat}/K_m)_D}$
Z-Gly	1	1
For-Ala	1.57	–
Z-Ala	14.1	≥ 17[c]
Z-Val	6.11	≥ 14
Z-Abu	83.8	≥ 200[d]
Z-Nva	64.6	≥ 2 650[d]
Z-Leu	38.05	≥ 16 000
Z-Phe	171	≥ 420 000

[a] Ratio of values given in [12]. [b] Ratio of values for *N*-acetyl-amino acid methyl esters, according to Silver and Matta [9]. [c] For the α-chymotrypsin-catalyzed hydrolysis of *N*-benzoylal-amine methyl esters, the ratio $\frac{(k_{cat}/K_m)_L}{(k_{cat}/K_m)_D}$ is equal to about 8, at pH 7.9; 25°C; 0.1 M NaCl, after Rapp et al. [4]. [d] Calculated from values given by Jones et al. [3], according to Silver and Matta [9].

α-CT-catalyzed hydrolysis of specific esters [16], like acetyl-L-phenylalaninate or acetyl-L-tryptophanate (ratio ~ 50).

These high ratios might be at least *partly* due to a more favourable value of K_s in the k_{cat}/K_m value (i.e. k_2/K_s) for *p*-nitrophenyl esters compared with methyl esters. Thus the interaction of the aryloxy group of *p*-nitrophenyl esters with the enzyme surface might lower the K_s values. Purdie and Benoiton [17] have compared the activity of α-CT towards *p*-nitrobenzyl and methyl esters of L-phenylalaninate (ratio of k_{cat}/K_m ~ 100), and have suggested that an aryl function in the leaving group could contribute substantially to binding. Williams and Udris [18] have also recently compared the ratio k_{cat}/K_m for different possible substrates of α-CT and indicated that the leaving group of these substrates could bind productively on the enzyme and particularly in the specificity pocket.

In table 2, the k_{cat}/K_m values for the acylation of α-CT by Z-L-amino acid *p*-nitrophenyl esters are compared with those obtained for the acylation by *N*-acetyl-L-amino acid methyl esters [3, 5] and *N*-benzoyl-L-amino acid methyl esters [1, 2, 4, 5]. For the acylation of α-CT by these three series of compounds, a comparable structure-reactivity correlation is observed. However, the ratio $(k_{cat}/K_m)_{ONp}/(k_{cat}/K_m)_{OMe}$ varies with the nature of the amino acid, particularly when methyl esters are derivatives of *N*-acetyl amino acids; the less specific the amino acid side chain, higher is this ratio. So the acylation of α-CT by methyl esters is more sensitive to structural changes in the lateral chain of the substrates than that by *p*-nitrophenyl esters.

Moreover, as shown by Silver et al. [8, 9], this sensitivity to the nature of the amino acid is still more important if the amino acid is a D derivative. The ratio $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D$ for the α-CT-catalyzed hydrolysis of enantiomers of Z-Phe-ONp is equal to 171 while it is estimated to be higher than 4.2×10^5 for the enzymatic hydrolysis of Ac-Phe-OMe (see table 3). Silver et al. [8, 9] have attributed this phenomenon to the particular reactivity of *p*-nitrophenyl esters due to the apolar character of the leaving group, *p*-nitrophenol, and have suggested that *p*-nitrophenyl esters were able to enter into multiple productive binding modes.

Nevertheless, this is not the only possible explanation. Thus some evidence has been given recently for the formation of a tetrahedral intermediate in the α-CT-catalyzed hydrolysis of phenyl esters [19], amides [20] and anilides [21–23]. The formation of this tetrahedral intermediate is a likely rate-limiting step in reactions of esters, but it may be assumed, in the acylation of α-CT by esters having a poor leaving group and a nonspecific acyl part, that the breakdown of this tetrahedral intermediate becomes the rate-limiting step; consequently, the rate of the enzymatic hydrolysis of such compounds is strongly reduced. Thus for the α-CT-catalyzed hydrolysis of Z-L-(or C)-amino acid *p*-nitrophenyl esters and for that of *N*-acetyl- or *N*-benzoyl-L-amino acid methyl esters having a specific amino acid side chain, the formation of this tetrahedral intermediate would be the rate-limiting step, while for that of less specific substrates such as *N*-acetyl-glycine methyl ester or methyl formate, its breakdown would become partly the rate-limiting step. Polgar [24] has suggested that this tetrahedral intermediate interacted with imidazolium ion of the histidine 57, and that this interaction implicated the formation of a bifurcated hydrogen bond. It may be assumed then that this interaction is less efficient

with substrates having a poor leaving group and a non-specific acyl part; consequently, the breakdown of this intermediate is more difficult.

Caplow and Harper [25] have recently compared the rates of hydrolysis of *N*-acetyl-L-tyrosine methyl ester and *N*-acetyl-*N*-methyl-L-tyrosine methyl ester in the presence of α-CT; they have interpreted the varying effects of substrate structure in terms of the stability of the transition states leading to and from the tetrahedral intermediate.

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