SEPARATION OF POLAR, STERIC AND SPECIFIC EFFECTS IN THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACYL-SUBSTITUTED p-NITROPHENYL ESTERS.

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SUMMARY.

The α-chymotrypsin-catalyzed hydrolysis of various acyl-substituted p-nitrophenyl esters has been studied at 25°C, between pH 6 and 8. The substituent effects on the deacylation rates of corresponding acyl-chymotrypsins have been analyzed using the Taft-Ingold relationship and contributions of polar, steric and specific effects, separated. A specificity constant S has been defined and its value discussed in relation to the structure of the substrate.

Use of linear free energy relationships in enzymatic catalysis is complicated by the specificity of enzymes and has been often limited to some series of substrates. So, the α-chymotrypsin-catalyzed hydrolysis of *meta*- and *para*- substituted phenylacetates (1), benzoylimidazoles (2) and anilides of N-acetyl-L-tyrosine (3,4) obeys to the Hammett relationship. For these reactions, experimental values of the reaction constant ρ are variable, depending on the nature of the substrate and the studied step of the enzymatic reaction. The α-chymotrypsin-catalyzed hydrolysis of acyl-substituted p-nitrophenyl esters with various alkyl groups on the α-C, shows a normal steric order of reactivity (5,6) with the exception of derivatives with a long chain (5,6,7).

By use of the Taft-Ingold relationship (8), we have tried to separate the polar, steric and specific effects of different non-conjugated substituents R on the rates of deacylation[§] in the α-chymotrypsin-catalyzed hydrolysis of

[§]Further work on the acylation step is now in progress in our laboratory.

acyl-substituted p-nitrophenyl esters, $R\text{-}COOC_6H_4pNO_2$, and to analyze particularly specific deviations in the linear free energy relationship.

TABLE I

α-chymotrypsin-catalyzed hydrolysis of acyl-substituted paranitrophenylesters

$R\text{-}COOC_6H_4pNO_2$

R	pK_1''	$k_{3,x}$ (a): $10^2 sec^{-1}$ expl.	$k_{3,x}$ (b): $10^2 sec^{-1}$ calcul.	E_s	σ^*	S
$Cl\text{-}CH_2$	6.90 ± 0.04	262 ± 12	238	-0.24	1.05	0
H	7.60 ± 0.09	150 ± 25	140	+1.24	0.49	0
$I\text{-}CH_2$	6.95 ± 0.05	57 ± 3	64	-0.37	0.85	0
$CH_3\text{-}O\text{-}CH_2$	7.16 ± 0.02	34 ± 9	28	-0.19	0.64	0
$Cl\text{-}(CH_2)_2$	7.13 ± 0.01	2.09 ± 0.03	2.14	-0.9	0.385	0
$C_6H_4\text{-}CH_2$	7.41 ± 0.02	1.88 ± 0.05	2.05	-0.38	0.215	0
CH_3	7.40 ± 0.01	0.99 ± 0.015	1.21	0	0	0
$(CH_3)_2CH$	7.34 ± 0.02	0.34 ± 0.08	0.196	-0.47	-0.19	0
$(CH_3)_3C$	7.04 ± 0.02	0.018 ± 0.002	0.018	-1.54	-0.3	0
$Cl\text{-}(CH_2)_3$	7.33 ± 0.02	5.18 ± 0.15		-0.35[c]	0.14[d]	0.555
$Cl\text{-}(CH_2)_4$	7.41 ± 0.06	4.45 ± 0.37		-0.35[c]	0.051[d]	0.69
$C_6H_4\text{-}(CH_2)_2$	7.26 ± 0.02	17.8 ± 0.5		-0.38	0.08	1.26
$C_6H_4\text{-}(CH_2)_3$	7.33 ± 0.05	12.0 ± 0.6		-0.45	0.02	1.275
$C_6H_4\text{-}(CH_2)_4$	7.73 ± 0.05	1.85 ± 0.14		-0.45[c]	0.02[d]	0.465
Z-Gly	7.01 ± 0.03	35 ± 1		-0.45±0.1[c]	0.57[d]	0.46
Z-L-Ala	7.21 ± 0.02	189 ± 4		-1.25±0.2[c]	0.46[d]	2.02
Z-L-Leu	7.59 ± 0.04	670 ± 40		-2.3 ±0.3[c]	0.42[d]	3.44
Z-L-Phe	7.53 ± 0.06	3700 ± 300		-2.3 ±0.3[c]	0.515[d]	3.965

Conditions : T = 25° C, I = 0.5 (NaCl), 4.5 % (v/v) acetonitrile-water.

(a) An iterative fitting of the data to equation 1 gave the deacylation constants $k_{3,x}$, at optimum pH, and K_1''.

(b) The theoretical $k_{3,x}$ values on the correlation line were recalculated from the values of $\rho^* = 2.35$ and $\delta = 0.73$.

(c) The E_s values were estimated after comparison with known E_s values of compounds with similar size (8).

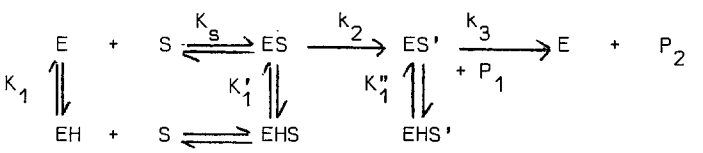
(d) The σ^* values were calculated from pK values of the conjugated acids and corresponding σ_i (15).

MATERIAL AND METHODS

The esters of p-nitrophenol, given in Table 1, have been prepared by the dicyclohexylcarbodiimide method (9) and recrystallyzed before use. They have been characterized by their melting point, their U.V. spectrum, the U.V. spectrum of their hydrolysis products, and elementary analysis in the case of previously non described compounds. The p-nitrophenyl esters of N-α-C-carbobenzoxy-amino acids are products from the Pierce Chemical Company.Chymotrypsin is a Worthington, 3 x crystallyzed, salt free product (batch n° CDI 61378). Its concentration in solution was determined spectrophotometrically at 280 nm, using a molar extinction coefficient of 50,000 (10) and molecular weight of $2.5\ 10^4$ (11). The absolute concentration of active sites was titrated by measurements of the burst of p-nitrophenolate ion, using p-nitrophenylacetate (12) : the percent of active site was found equal to 92-94 %. Kinetic measurements were carried out at 25°C, in buffered aqueous solutions, I = 0.5 (NaCl), 4.5 % (v/v) acetonitrile-water. Employed buffers were 2-(N-morpholino)ethane-sulfonic acid between pH 6 and 7, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid between pH 7 and 8, at the concentration of 0.025 M (13). The hydrolysis reactions were studied spectrophotometrically by following the appearance of p-nitrophenolate ion at 400 nm, with a Cary 16 spectrophotometer coupled to a Sefram Graphispot enregistror. The molar absorptivity of p-nitrophenolate ion was measured and found equal to 18,300 ; pK of p-nitrophenol was determined equal to 7.03 under our experimental conditions.

RESULTS.

Between pH 6 and 8, in the pH range where the histidine 57 of the active site ionizes, the mechanism of the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl esters may be represented by the scheme I (14) :



Scheme I

where S is the substrate, E and EH are the active and inactive forms of the enzyme, ES and EHS the corresponding ones of the enzyme-substrate complex ; ES' and EHS' the different forms of the acyl-enzyme intermediate. P_1 and P_2 are the hydrolysis products, respectively p-nitrophenol and carboxylic acid.

In the steady state, under conditions where $S_o >> E_o$ and $S_o > K_{m\ app}$ [§] the appearance rate of product P_1 is given by the equation (1) :

$$\frac{dP_1}{dt} = \frac{k_3 E_o}{1 + H/K''_1} \qquad (1)$$

The experimental values of k_3 and K''_1 are given in Table 1.

The Taft-Ingold relationship has been used to evaluate the different effects of substituents R on the rate of α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl esters $R\text{-}COOC_6H_4pNO_2$:

$$\log \frac{k_{3,x}}{k_{3,0}} = \rho^* \sigma^* + \delta E_s \qquad (2)$$

In equation (2) the kinetic constant $k_{3,x}$ is the deacylation rate for any acyl-enzyme ; $k_{3,0}$ is the standard of comparison for $R = CH_3$; σ^* and E_s are polar and steric substituent constants, ρ^* and δ, the polar and steric reaction constants. The substituent constants σ^* and E_s are given in Taft's tables (8), but for some substituents, these constants were estimated approximatively by comparison with similar ones.[Δ]

The best correlation has been calculated for nine compounds with a Wang model 373 electronic calculator and has the form :

[§] The apparent Michaelis constant $K_{m\ app}$ was determined < 5.10^{-6} M for all studied substrates, and S_o was varied between 10^{-4} M and 10^{-5} M.

[Δ] In order to obtain precise constants, the alkaline hydrolysis of these esters in water and in 70 % (by volume) aqueous acetone is under investigation in our laboratory.

$\log k_{3,x}/k_{3,0} - (0.73 \pm 0.05)E_s = (2.35 \pm 0.06)\sigma^*$, or the equivalent one, $\log k_{3,x}/k_{3,0} - (0.76 \pm 0.04)E_s = (5.5 \pm 0.2)\sigma_i$, in which σ_i is the polar substituent constant defined by Charton (15). In the figure 1, the substituents on the correlation line vary over a wide range of polar and steric requirements leading to a spread of reactivity of $1.5\ 10^4$. Experimental values of ρ^* (or ρ_i) and δ are comparable to values obtained in the hydroxide ion-catalyzed hydrolysis of acyl-substituted ethyl or phenyl esters (8, 16, 17), and suggest the involvement of some form of base catalysis in the deacylation mechanism (2).

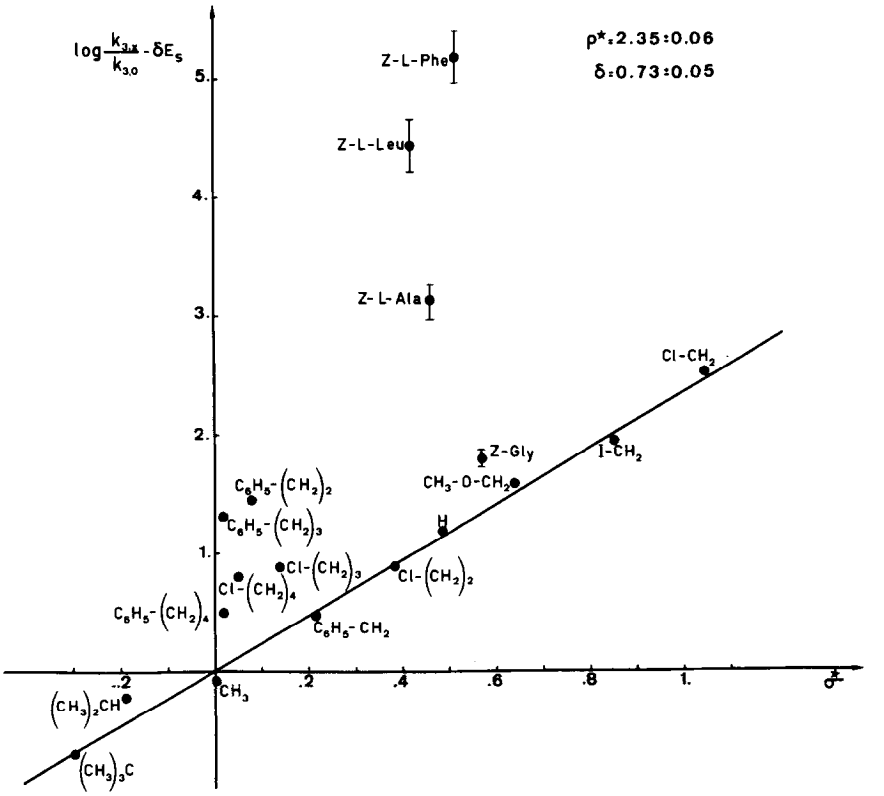


Figure 1 : Representation of the Taft-Ingold relationship for the α-chymotrypsin-catalyzed hydrolysis of acyl-substituted paranitrophenyl-esters, $R\text{-}COOC_6H_4pNO_2$.

Conditions : T = 25°C, I= 0.5 (NaCl), 4,5 % (v/v) acetonitrile-water.

$k_{3,x}$ are the rate constants for deacylation at optimum pH. The correlation line was obtained by an iterative fitting of the data to equation 2.

A number of points deviate significantly from the correlation line, and the reactivity of corresponding compounds implies that factors other than polar and steric effects are important. The Taft-Ingold equation has to be modified to include a specificity constant S, following equation (3) :

$\log k_{3,x}/k_{3,0} - \delta E_s + S = \rho^* \sigma^*$ (3).

The distance between the experimental points and the correlation line gives a measurement of the constant S (see table 1).

So, in the case of linear alkyl or phenylalkyl derivatives, the specificity constant S becomes greater than 0 beyond some particular length of the alkyl chain (8 Å approximatively assuming a fully extended model), increases to an optimal value for a chain length of 10-11 Å, then decreases beyond. A similar phenomenon has been previously observed with analogous compounds by different authors (18, 5-7, 19). Moreover, the more hydrophobic character of the benzene ring may explain the larger value of S for compounds with $R = C_6H_5(CH_2)_2$ or $C_6H_5(CH_2)_3$ in comparison with aliphatic compounds where R is $Cl(CH_2)_3$ or $Cl(CH_2)_4$.

No specific effect of the acyl-amino group -O-CONH-, in the α-chymotrypsin-catalyzed hydrolysis of N-acyl-glycinate of p-nitrophenol is apparent : the S value for $R = C_6H_5CH_2OCONHCH_2$ is similar to that observed for $R = C_6H_5(CH_2)_4$, which has a comparable chain length.

However, when the alpha carbon atom becomes asymmetric as in N-α-C-carbobenzoxy-L-aminoacids derivatives, important deviations occur. The values of S for these derivatives are much larger than those for linear long chain compounds ; so, in the case of N-α-C-carbobenzoxy-L-phenylalanine, S is near to 4. Numerous authors (20-25) have previously emphasized the following structure requirements for α-chymotrypsin specific substrates : asymmetric alpha carbon atom, acylaminogroup and alkyl or phenylalkyl chain branched on it.

In order to make the meaning of the specificity constant S precise further work is now in progress in our laboratory upon D-aminoacids derivatives and asymmetric compounds which don't carry an acylaminogroup.

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