Specificity of α-Chymotrypsin. Separation of Polar, Steric, and Specific Effects in the α-Chymotrypsin-Catalyzed Hydrolysis of Acyl-Substituted *p*-Nitrophenyl Esters†

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ABSTRACT: The α-chymotrypsin-catalyzed hydrolysis of various acyl-substituted *p*-nitrophenyl esters has been studied at 25°, between pH 6 and 8. The substituent effects on the second-order acylation rate constants ($k_{\mathrm{cat}}/K_{\mathrm{m}}$) and on the deacylation rate constants (k_3) have been analyzed using the Taft–Ingold relationship, and contributions of polar, steric, and specific effects have been separated. A specificity constant S has been defined for each step and for each substrate, and its value is discussed in relation to the structure of the substrates. We find that (1) deacylation is favored when the acyl-enzyme has the following structural properties on its acyl function: an optimal length of the acyl chain (10–12 Å); hydrophobic character of the chain end; (2) in a general way, the same structural properties of the acyl part of the substrate favor acylation and deacylation steps. The observed differences are mostly due to changes in the dissociation constant (K_s) with the structure of the substrates. In the acylation step, the enzymatic reactivity follows almost strictly an extraction process.

Many studies on the specificity of α-chymotrypsin are found in the literature (Niemann, 1964; Ingles and Knowles, 1967, 1968; Cohen *et al.*, 1969; Hayashi and Lawson, 1969; Silver *et al.*, 1970; Berezin *et al.*, 1970, 1971), but the reasons for such a specificity are not yet well understood.

Due to their high reactivity, the *p*-nitrophenyl esters are good substrates for the analysis of this specificity, since the nature of their acyl group may be modified without changing the rate-limiting step of the enzymatic process. α-Chymotrypsin catalyzes the hydrolysis of carboxylic compounds according to a mechanism of general base catalysis (Bender and Kezdy, 1965) so that the substituent effects on α-chymotrypsin-catalyzed hydrolysis may be compared with those observed in the alkaline hydrolysis. Taft (1956) succeeded in separating steric and polar effects of nonconjugated substituents R on the acidic and alkaline hydrolyses of ethyl esters with the general formula $\mathrm{RCOOC_2H_5}$. We have applied his analysis to the α-chymotrypsin-catalyzed hydrolysis of acyl-substituted *p*-nitrophenyl esters ($\mathrm{RCOOC_6H_4}$-*p*-$\mathrm{NO_2}$) for the purpose of characterizing the specific effects of the substituents R on the enzymatic hydrolysis of these esters. A preliminary report of the results presented in this paper has been published (Dupaix *et al.*, 1970).

Experimental Procedure

Materials. α-Chymotrypsin was a salt-free, three times crystallized product (batch no. CDI 61738) purchased from Worthington Biochemical Corp.

Carboxylic acids and *p*-nitrophenol used to prepare the *p*-nitrophenyl esters were commercial products obtained from Schuchardt Chemische Fabrik, Fluka Chemische Fabrik, Aldrich Chemical Co., K and K Laboratories. They were used without further purification.

Acetonitrile was purified according to Coetzee (1967) and other solvents were purified according to Fieser (1955).

All other chemicals including *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid (Hepes)[1] and 2-(*N*-morpholino)-ethanesulfonic acid (Mes) purchased from Calbiochem were reagent grade and were employed without further purification.

Preparation of Compounds. Melting points were determined on a Büchi apparatus and were uncorrected. Elemental analyses were performed by the Laboratoire de Microanalyse, C.N.R.S., Gif-sur-Yvette, 91, France.

Propionylglycine and butyrylglycine were synthesized as described by Bondi and Eissler (1910) and were recrystallized from ether. Propionylglycine had mp 121–124° (lit. 125.5–127°, Katz *et al.*, 1953) and butyrylglycine had mp 63–65° (lit. mp 65–70°, Bondi and Eissler, 1910).

Most of *p*-nitrophenyl esters were prepared by the dicyclohexylcarbodiimide method (Bodanszky and Du Vigneaud, 1959; Buzas *et al.*, 1963) and were recrystallized twice or more from ethanol. General yields were 80% in dicyclohexylurea and 40% in ester after one recrystallization. We did not attempt to obtain better yields.

p-Nitrophenyl methoxyacetate was synthesized by adding methoxyacetyl chloride purchased form Aldrich Chemical Co. to *p*-nitrophenol dissolved in anhydrous ether in the presence of 1 equiv of pyridine (Fife, 1965). It was recrystallized from ether. The yield was 35%.

The different synthesized *p*-nitrophenyl esters have been characterized by their melting points, their ultraviolet absorption spectra in ethanol, the spectra of their hydrolysis products in 0.1 N NaOH (the molar extinction coefficient of *p*-nitrophenolate ion is 18,300 at 400 nm), and elementary analyses for compounds not previously described. The general properties of these esters were in good agreement with those from the literature. In Table I are reported the properties of the

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

TABLE I: General Properties of Some *p*-Nitrophenyl Esters, $RCOOC_6H_4\text{-}p\text{-}NO_2$.

R	Mp (°C, uncorrected)	General Formula		*Anal.* (%)			
				C	H	Cl	N
CH_3OCH_2	64–65	$C_9H_9NO_5$	Calcd	51.19	4.29		6.63
			Found	51.38	4.19		6.88
$Cl(CH_2)_2$	34–35[a]	$C_9H_8NO_4Cl$	Calcd	47.07	3.51	15.44	6.10
			Found	48.11	3.76	15.23	6.42
$Cl(CH_2)_3$	63.5–64.5	$C_{10}H_{10}NO_4Cl$	Calcd	49.29	4.13	14.55	5.75
			Found	49.25	4.06	14.51	6.00
$Cl(CH_2)_4$	31–31.5	$C_{11}H_{12}NO_4Cl$	Calcd	51.27	4.69	13.76	5.43
			Found	51.44	4.57	13.62	5.53
$C_6H_5(CH_2)_3$	37	$C_{16}H_{15}NO_4$	Calcd	67.36	5.30		4.91
			Found	67.13	5.17		5.01
$C_6H_5(CH_2)_4$	62.5–63	$C_{17}H_{17}NO_4$	Calcd	68.21	5.72		4.68
			Found	68.14	5.35		4.87
β-$(C_8H_6N)CH_2$	85–86.5[b]	$C_{16}H_{12}N_2O_4$	Calcd	64.86	4.08		9.45
			Found	64.79	4.10		9.41
β-$(C_8H_6N)(CH_2)_2$	107–109.5[b,c]	$C_{17}H_{14}N_2O_4$	Calcd	65.80	4.55		9.03
			Found	66.02	4.64		8.83
β-$(C_8H_6N)(CH_2)_3$	99–101[b]	$C_{18}H_{16}N_2O_4$	Calcd	66.66	4.97		8.64
			Found	66.44	4.96		8.37
$CH_3CH_2CONHCH_2$	113–114	$C_{11}H_{12}N_2O_5$	Calcd	52.38	4.79		11.10
			Found	52.17	4.79		11.01
$CH_3(CH_2)_2CONHCH_2$	116–122	$C_{12}H_{14}N_2O_5$	Calcd	54.13	5.30		10.55
			Found	54.11	5.21		10.39
$CH_3CONH(CH_2)_3$	71-5–72.5[d]	$C_{12}H_{14}N_2O_5$	Calcd	54.13	5.30		10.52
			Found	54.02	5.26		10.24

[a] This compound had 92% purity based on the amount of *p*-nitrophenol released on complete alkaline hydrolysis of a known weight of ester (spectrophotometric measurement at 400 nm). Other preparations have led to an oil which decomposed with halogen liberation after distillation under reduced pressure. [b] Yellow crystals. [c] Mp 111–112° (Williams and Salvadori, 1971). [d] Recrystallized from ether–petroleum ether (bp 30–60°).

compounds which to our knowledge are not described elsewhere.

The *p*-nitrophenyl esters of acetic acid (Eastman Kodak), *N*-acetylglycine (Cyclo Chemical Corp.), and *N*-carbobenzoxyglycine (Pierce Chemical Co.) were commercial products, the purity of which was checked.

Kinetic Measurements. Enzyme stock solutions were prepared by dissolving the crystals in 0.001 N HCl at 4°. The protein concentration was estimated at 280 nm with a Cary 16 recording spectrophotometer using a molar extinction coefficient of 5×10^4 (Dixon and Neurath, 1957) and a mol wt of 2.5×10^4 (Wilcox *et al.*, 1957). The absolute concentration of active sites was obtained by measuring the burst of *p*-nitrophenol in the enzymatic hydrolysis of *p*-nitrophenyl acetate (Bender *et al.*, 1966); the per cent of active sites was found equal to 92–94.

Kinetic experiments were carried out at 25 ± 0.1°, between pH 6 and 8 in buffered aqueous solutions (0.5 M NaCl; 4.5% acetonitrile–water, v/v). Buffers employed at a concentration of 0.025 M were Mes–NaOH and Hepes–NaOH (Good *et al.*, 1966).

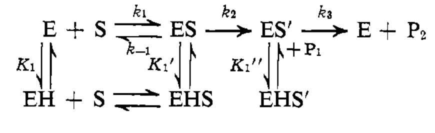
Kinetic measurements were made with a Cary 16 spectrophotometer equipped with a thermostated cell compartment and coupled to a Sefram Graphispot recorder. The α-chymotrypsin-catalyzed hydrolyses of the different *p*-nitrophenyl esters were followed by recording the liberation of *p*-nitrophenolate ion at 400 nm. The molar extinction coefficient of *p*-nitrophenoxide ion was found equal to 18,300 and the pK_a of *p*-nitrophenol was 7.03 at 25° under our experimental conditions. The nonenzymatic hydrolysis rates for each ester were also measured and the necessary corrections were made. Before and after each run, measurements of the pH of the solution were made using a Tacussel pH meter (TS-60/N), standardized against standard buffer solutions (Electronic Instrumented Limited). In a typical run, 2.5 ml of buffer, 50 μl of α-chymotrypsin in 0.001 N HCl, and 70 μl of acetonitrile were mixed in a quartz cell and allowed to come to temperature equilibrium (25°) for approximately 5 min. Then 50 μl of substrate stock solution in acetonitrile was introduced and the course of hydrolysis was monitored.

An iterative fitting of the initial rates to the Michaelis equation for various substrate concentrations gave the kinetic constants k_3 and K_m, at a given pH value. Determinations of the pK of His-57 in the acyl-enzyme were performed according to the same procedure at constant substrate concentration ($S > K_m$), and at various pH values between 6 and 8. Values of pK_1'' and k_3 (at optimum pH) were also obtained by an iterative fitting of the initial rates to eq 2 (see Results). These calculations were made using a Wang Model 373 electronic calculator.

Results

Between pH 6 and 8, in the pH range where the His-57 of the active site ionizes, the mechanism of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl esters may be represented by Scheme 1 (Bender *et al.*, 1964; Philipp and Bender, 1973), where S is the substrate, E and EH are the

SCHEME I



active and inactive forms of the enzyme, ES and EHS are the corresponding forms of the enzyme–substrate complex, ES′ and EHS′ are the different forms of the acyl-enzyme intermediate, and P_1 and P_2 are the hydrolysis products, respectively, *p*-nitrophenol and carboxylic acid.

Under steady state and conditions where $S_0 \gg E_0$, the rate of appearance of the P_1 product is given by eq 1

$$\frac{d[P_1]}{dt} = \frac{k_{cat}E_0S_0}{K_m + S_0} \qquad (1)$$

in which k_{cat} and K_m are pH dependent and equal to

$$k_{cat} = \frac{k_2k_3}{k_2(1 + (H^+)/K_1'') + k_3(1 + (H^+)/K_1')}$$

and

$$K_m = \frac{k_{-1} + k_2}{k_1}\,\frac{k_3(1 + (H^+)/K_1)}{k_2(1 + (H^+)/K_1'') + k_3(1 + (H^+)/K_1')}$$

In these equations, (H^+) is hydrogen ion activity and the various kinetic and equilibrium constants (k_1, K_1, . . .) are implicitly defined in Scheme I.

For the enzymatic hydrolysis of various *p*-nitrophenyl esters it has been shown that the rate-limiting step of the reaction is deacylation (Zerner and Bender, 1964; Ingles and Knowles, 1967). Then the expressions of k_{cat} and K_m simplify and become equal to

$$k_{cat} = \frac{k_3}{1 + (H^+)/K_1''} \qquad (2)$$

and

$$K_m = \frac{k_{-1} + k_2}{k_1}\,\frac{k_3(1 + (H^+)/K_1)}{k_2(1 + (H^+)/K_1'')} \qquad (3)$$

The constant K_m may also be written in the form

$$K_m = \frac{K_sk_3(1 + (H^+)/K_1)}{k_2(1 + (H^+)/K_1'')}$$

in which K_s is equal to $(k_{-1} + k_2)/k_1$. The latter is a true equilibrium constant (k_{-1}/k_1) in the case where k_2 is much smaller than k_{-1}.

The experimental values of k_3, K_1'', and K_m for the α-chymotrypsin-catalyzed hydrolysis of various acyl-substituted *p*-nitrophenyl esters ($RCOOC_6H_4$-*p*-NO_2) are given in Table II. We did not attempt to study the possible variations of K_m with pH for it is known that K_m does not vary in the pH range 6–8 (Bender *et al.*, 1964; McConn *et al.*, 1971).

The Taft–Ingold relationship has been used to separate the different effects of substituents R on the deacylation rate constant k_3 and also on the second-order acylation rate constant $k_{cat}/K_m = k_2/K_s$ defined by Zerner and Bender (1964) and Brot and Bender (1969).

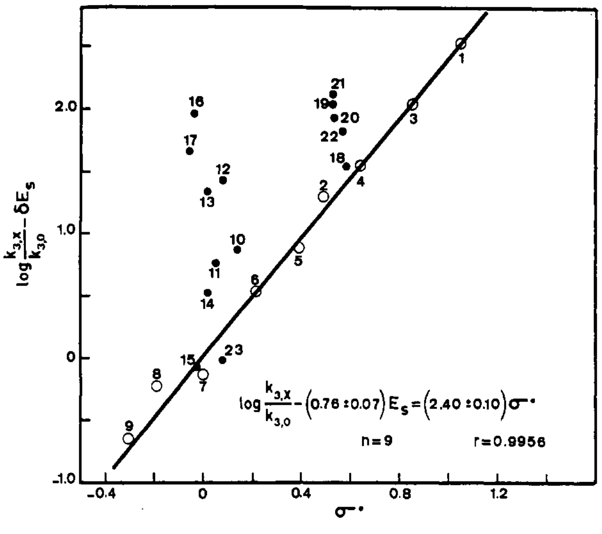


FIGURE 1: Deacylation step; representation of the Taft–Ingold relationship for the α-chymotrypsin-catalyzed hydrolysis of acyl-substituted *p*-nitrophenyl esters. The correlation line was calculated from the points represented by open circles by an iterative fitting of the data to the Taft–Ingold relationship. We used for calculation only the compounds for which σ* and E_s values were given by Taft (1956). The numbers refer to the compounds in Table II.

Effect of Substituents on the Deacylation Rate Constant. The best correlation (correlation coefficient (*r*) = 0.9956) to the Taft–Ingold relationship (Taft, 1956), for the largest possible number (*n*) of compounds (compounds **1–9** in Table II) has been found equal to

$$\log k_{3,x}/k_{3,0} = (2.40 \pm 0.10)\sigma^* + (0.76 \pm 0.07)E_s \qquad (4)$$

In eq 4 the kinetic constant $k_{3,x}$ is the deacylation rate constant for any acyl-enzyme; $k_{3,0}$ is the standard of comparison for R = CH_3. The polar and steric substituent constants σ* and E_s are given in Taft's tables (Taft, 1956) or are estimated as indicated in Table III. The values of 2.4 and 0.76 refer to the polar and steric reaction constants ρ* and δ. A number of points deviate significantly from the correlation line (Figure 1) and the Taft–Ingold equation may be modified to include a specificity constant[2] S_d according to eq 5. S_d is then defined as the distance between the experimental points and the correlation line. Values of this constant S_d for the different studied compounds are reported in Table III.

$$\log k_{3,x}/k_{3,0} - \delta E_s - S_d = \rho^*\sigma^* \qquad (5)$$

Effect of Substituents on the Second-Order Acylation Rate Constant. In the same way as for deacylation, we have been interested in finding a linear relationship between the second-order acylation rate constant k_{cat}/K_m and Taft's parameters σ* and E_s. When considering the same nine compounds as previously we obtain eq 6, comparable to the preceding equation (4)

$$\log \frac{(k_{cat}/K_m)_x}{(k_{cat}/K_m)_0} = (2.18 \pm 0.17)\sigma^* + (0.53 \pm 0.10)E_s \qquad (6)$$

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[2] The specificity constant *S* previously used (Dupaix *et al.*, 1970) is now written S_d for the deacylation step and will be written S_a for the acylation step.

TABLE II: Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of Acyl-Substituted *p*-Nitrophenyl Esters, $RCOOC_6H_4$-*p*-NO_2.[a]

No.	R	pK_1''	10^6K_m (M)	10^2k_3 (sec^{-1})	10^{-4} (k_{cat}/K_m) (M^{-1} sec^{-1})
1	$ClCH_2$	6.90 ± 0.04	5.16 ± 0.28	295 ± 4	57.1 ± 2.6
2	H	7.60 ± 0.09	12.5 ± 1.0	243 ± 85	19.4 ± 6.8
3	ICH_2	6.95 ± 0.05	5.82 ± 0.35	79.0 ± 6.5	13.6 ± 1.1
4	CH_3OCH_2	7.16 ± 0.02	3.06 ± 0.15	35.0 ± 4.8	11.4 ± 1.6
5	$Cl(CH_2)_2$	7.13 ± 0.01	2.39 ± 0.20	2.16 ± 0.16	0.904 ± 0.07
6	$C_6H_5CH_2$[b]	7.41 ± 0.02	0.394 ± 0.079	2.23 ± 0.33	5.6 ± 0.8
7	CH_3	7.40 ± 0.01	3.46 ± 0.64	0.996 ± 0.015	0.288 ± 0.004
8	$(CH_3)_2CH$	7.34 ± 0.02	2.12 ± 0.15	0.357 ± 0.018	0.168 ± 0.008
9	$(CH_3)_3C$	7.04 ± 0.02	1.8 ± 0.9	0.0205 ± 0.002	0.0114 ± 0.001
10	$Cl(CH_2)_3$	7.33 ± 0.02	3.57 ± 0.16	5.35 ± 0.10	1.50 ± 0.03
11	$Cl(CH_2)_4$	7.41 ± 0.06	3.03 ± 0.24	4.26 ± 0.26	1.40 ± 0.09
12	$C_6H_5(CH_2)_2$[b]	7.26 ± 0.02	0.37 ± 0.02	18.60 ± 0.77	50 ± 2
13	$C_6H_5(CH_2)_3$[b]	7.33 ± 0.05	0.99 ± 0.013	13.4 ± 1.4	13.5 ± 1.4
14	$C_6H_5(CH_2)_4$	7.73 ± 0.05	2.79 ± 0.02	2.07 ± 0.16	0.74 ± 0.06
15	$\beta(C_8H_6N)CH_2$	7.55 ± 0.04	<0.1	0.304 ± 0.014	>3
16	$\beta(C_8H_6N)(CH_2)_2$[b]	7.40 ± 0.02	0.30 ± 0.05	33.6 ± 0.8	112 ± 3
17	$\beta(C_8H_6N)(CH_2)_3$[b]	7.64 ± 0.02	0.80 ± 0.03	14.9 ± 3.6	18.6 ± 4.5
18	$HCONHCH_2$	6.95 ± 0.03	15.7 ± 0.8	24.5 ± 2.9	1.56 ± 0.18
19	$CH_3CONHCH_2$	6.87 ± 0.01	138 ± 11	75.2 ± 2.3	0.54 ± 0.02
20	$CH_3CH_2CONHCH_2$	6.99 ± 0.015	113 ± 9	55.95 ± 3.3	0.495 ± 0.03
21	$CH_3(CH_2)_2CONHCH_2$	6.94 ± 0.015	136 ± 22	87.1 ± 6.2	0.64 ± 0.045
22	$C_6H_5CH_2OCONHCH_2$	7.01 ± 0.03	4.9 ± 0.5	41.1 ± 2.0	8.38 ± 0.40
23	$CH_3CONH(CH_2)_3$	7.30 ± 0.05	<1	0.62 ± 0.05	>0.6

[a] Experimental conditions: temperature, 25°; 0.5 M NaCl; 4.5% (v/v) acetonitrile–water. Values of K_m were obtained by measuring the rates of hydrolysis at a constant pH value equal to 7.56. Values of pK_1'' and k_3 were obtained by measuring the rates of hydrolysis at various pH values and at constant substrate concentration ($S > K_m$). By assuming that K_m remained constant with pH, the k_3 values were corrected from K_m values determined at pH 7.56. All data were treated with a Wang electronic calculator. [b] These values of K_m were obtained from total enzymatic hydrolysis.

with $n = 9$ and $r = 0.948$. The experimental value of k_{cat}/K_m for compound 6 is superior by a factor of about 10 to that calculated from correlation eq 6; this deviation is surely significant. If compound 6 is omitted to calculate the correlation equation, the values of ρ^* and δ do not change very much but their variances are better as well as the correlation coefficient leading to eq 7, with $n = 8$ and $r = 0.990$ (see Figure

$$\log \frac{(k_{cat}/K_m)_x}{(k_{cat}/K_m)_0} = (2.19 \pm 0.12)\sigma^* + (0.53 \pm 0.07)E_s \quad (7)$$

2).

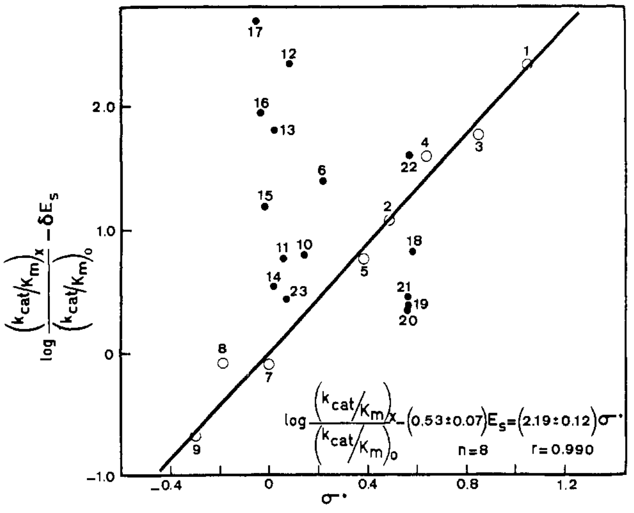

FIGURE 2: Acylation step; representation of the Taft–Ingold relationship for the α-chymotrypsin-catalyzed hydrolysis of acyl-substituted *p*-nitrophenyl esters. The correlation line was calculated from the points represented by open circles by an iterative fitting of the data to the Taft–Ingold relationship. We used for calculation only the compounds for which σ^* and E_s values were given by Taft (1956). The numbers refer to the compounds in Table II.

Positive and negative deviations occur for many compounds leading us to write eq 8 which is similar to eq 5.

$$\log \frac{(k_{cat}/K_m)_x}{(k_{cat}/K_m)_0} - \delta E_s - S_a = \rho^*\sigma^* \quad (8)$$

The values of the specificity constant S_a for the studied *p*-nitrophenyl esters are reported in Table III.

Therefore, these deacylation and acylation specificity constants (S_d and S_a) have been analyzed in terms of chain length (Figures 3 and 4) and hydrophobicity (Figures 5 and 6) of the different substituents. In these illustrations, the hydrophobic bonding powers of the substituents R are defined by their "hydrophobic bonding" constants π which are related to partition coefficients of compounds RH between 1-octanol and water (Fujita *et al.*, 1964; Hansch and Anderson, 1967).

TABLE III: Deacylation and Acylation Specificity Constants for the α-Chymotrypsin-Catalyzed Hydrolysis of Acyl-Substituted *p*-Nitrophenyl Esters $RCOOC_6H_4$-*p*-NO_2. Polar, Steric, and "Hydrophobic Bonding" Constants for the Substituents R.

No.	R	σ^*	E_s	π	S_d	S_a
1	$ClCH_2$	1.05	−0.24		0	0
2	H	0.49	1.24		0	0
3	ICH_2	0.85	−0.37		0	0
4	CH_3OCH_2	0.64^a	−0.19		0	0
5	$Cl(CH_2)_2$	0.385	−0.9	1.39	0	0
6	$C_6H_5CH_2$	0.215	−0.38	2.63	0	0.93
7	CH_3	0.0	0.0		0	0
8	$(CH_3)_2CH$	−0.19	−0.47		0	0
9	$(CH_3)_3C$	−0.3	−1.54		0	0
10	$Cl(CH_2)_3$	0.14^b	-0.35^d	1.89	0.526	0.50
11	$Cl(CH_2)_4$	0.051^b	-0.35^d	2.39	0.64	0.67
12	$C_6H_5(CH_2)_2$	0.08	−0.38	3.13	1.234	2.17
13	$C_6H_5(CH_2)_3$	0.02	−0.45	3.63	1.289	1.77
14	$C_6H_5(CH_2)_4$	0.02^b	-0.45^d	4.13	0.477	0.51
15	$\beta(C_8H_6N)CH_2$	-0.022^b	-0.75 ± 0.1^d	2.64	-0.03 ± 0.08	>1.4
16	$\beta(C_8H_6N)(CH_2)_2$	-0.037^b	-0.75 ± 0.1^d	3.14^e	2.05 ± 0.15	2.97 ± 0.1
17	$\beta(C_8H_6N)(CH_2)_3$	-0.052^b	-0.82 ± 0.1^d	$3.64^{e,f}$	1.79 ± 0.15	2.26 ± 0.1
18	$HCONHCH_2$	0.582^b	-0.36 ± 0.1^d	$-1.21^{e,f}$	0.132 ± 0.077	-0.45 ± 0.05
19	$CH_3CONHCH_2$	0.56^b	-0.39 ± 0.1^d	$-0.71^{e,f}$	0.70 ± 0.08	-0.84 ± 0.05
20	$CH_3CH_2CONHCH_2$	0.56^b	-0.40 ± 0.1^d	$-0.21^{e,f}$	0.58 ± 0.08	-0.87 ± 0.05
21	$CH_3(CH_2)_2CONHCH_2$	0.56^b	-0.40 ± 0.1^d	$0.29^{e,f}$	0.77 ± 0.08	-0.76 ± 0.05
22	$C_6H_5CH_2OCONHCH_2$	0.57^b	-0.45 ± 0.1^d	$1.74^{f,g}$	0.45 ± 0.08	0.36 ± 0.05
23	$CH_3CONH(CH_2)_3$	0.075^c	-0.40 ± 0.1^d	0.29^e	-0.22 ± 0.08	>0.3

[a] From Ballinger and Long (1960). [b] These σ^* values were calculated from p*K* values of the conjugated acids and corresponding σ_i (Charton, 1964). [c] This value was calculated by using a factor of 2.73 for the reduction of the inductive effect of a substituent resulting from the interposition of a methylene group (Wells, 1962). [d] The E_s values were estimated after comparison with known E_s values of compounds with similar size (Taft, 1956). [e] These values were calculated according to Hansch's rules (Fujita *et al.*, 1964; Hansch and Anderson, 1967). [f] When considering only the aliphatic part of these compounds for the calculation of π values no improvement was observed in the correlation between S_d or S_a and π values. [g] This value was calculated from log *P* for *N*-methyl phenylcarbamate (Hansch and Anderson, 1967).

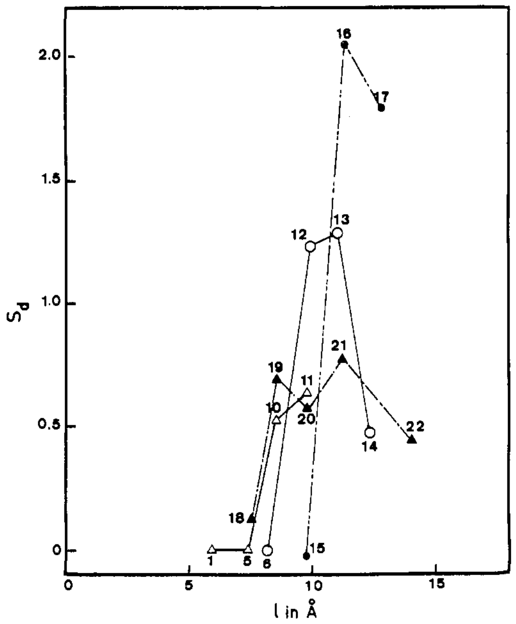

FIGURE 3: Deacylation specificity constant S_d *vs.* the length (*l*) of the acyl group (*l* in ångströms was calculated from the carbonyl carbon assuming fully extended models). The numbers refer to the compounds in Table II: (△) R = $Cl(CH_2)_n$ with *n* = 1, 2, 3, 4; (○) R = $C_6H_5(CH_2)_n$ with *n* = 1, 2, 3, 4; (●) R = $\beta(C_8H_6N)(CH_2)_n$ with *n* = 1, 2, 3; (▲) glycine derivatives.

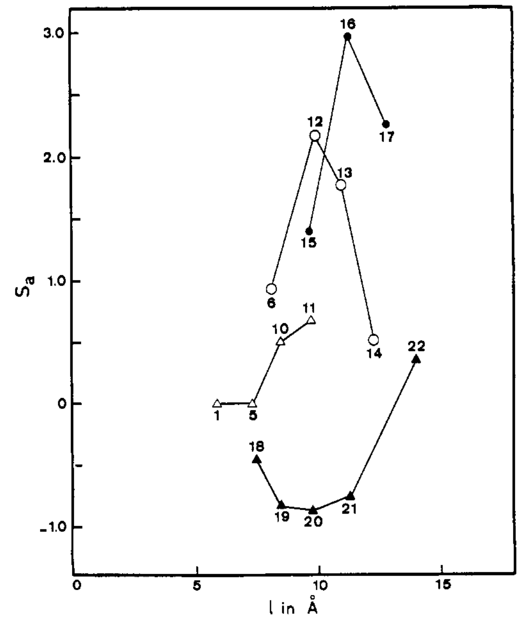

FIGURE 4: Acylation specificity constant S_a *vs.* the length (*l*) of the acyl group (*l* in ångströms was calculated from the carbonyl carbon assuming fully extended models). The numbers refer to the compounds in Table II: (△) R = $Cl(CH_2)_n$ with *n* = 1, 2, 3, 4; (○) R = $C_6H_5(CH_2)_n$ with *n* = 1, 2, 3, 4; (●) R = β-$(C_8H_6N)(CH_2)_n$ with *n* = 1, 2, 3; (▲) glycine derivatives.

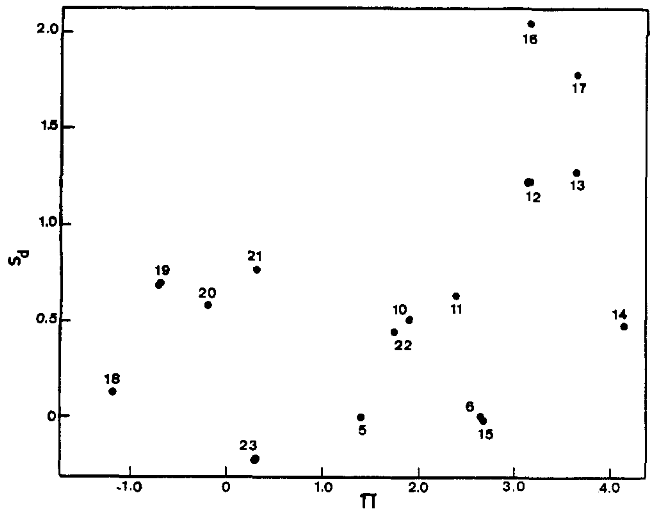

FIGURE 5: Deacylation specificity constant S_d *vs.* π, the hydrophobic constant of the substituents. The numbers refer to the compounds in Table II. S_d and π values are given in Table III.

Discussion

Specificity of α-Chymotrypsin in the Deacylation Step. The deacylation step is an intramolecular reaction and in such reactions steric effects may be complex and may induce strong increases in the rates of reactions (Kirby and Lancaster, 1970). However, as a first approach, Taft's analysis may be used to normalize the kinetic data and allow a separation between nonspecific and specific effects of substituents by comparison with a nonenzymatic reaction. Some authors have recently followed an analog approach for the study of the chymotryptic hydrolysis of various acyl-substituted *p*-nitrophenyl esters (Fife and Milstien, 1967; Enriquez and Gerig, 1969).

The values of polar and steric reaction constants ρ^* and δ are comparable to values obtained in the hydroxide ion catalyzed hydrolysis of acyl-substituted ethyl (Taft, 1956) or phenyl (Holmquist and Bruice, 1969a,b; Bruice *et al.*, 1970) esters. A positive value of ρ^* indicates the involvement of some form of base catalysis in the deacylation mechanism as previously shown by Caplow and Jencks (1962) and a positive value of δ shows that the differences in steric hindrance of the substituent R between the ground state and the transition state of the reaction are comparable in enzymatic and hydroxide ion catalyzed hydrolyses.

The specificity of α-chymotrypsin in the deacylation step is mainly a "size specificity." Indeed, as shown in Figure 3, the specificity constant S_d for different series of acyl-enzymes becomes positive beyond some particular length of the acyl part and passes through a maximum. This optimum length is similar for each series of compounds, *i.e.*, 10–12 Å; it corresponds to that of specific substrates like phenylalanine or tryptophan derivatives.

However, bulky substituents like phenylacetyl (**6**) and (3-indolyl)acetyl (**15**) are not recognized by the enzyme in contrast with their higher homologs, 3-phenylpropionyl (**12**) and 3-(3-indolyl)propionyl (**16**) which have only one more methylene group.[3] Specific recognition of any substrate is therefore strongly dependent on the geometry of the active site.

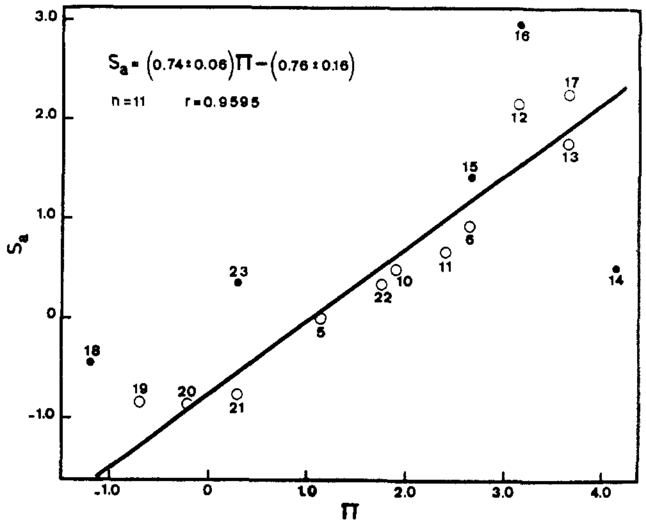

FIGURE 6: Acylation specificity constant S_a *vs.* π, the hydrophobic constant of the substituents. The correlation line was calculated from the points represented by open circles. Substituent **15** is not included in the calculation because of the uncertainty in the S_a value (too low a K_m value to be estimated accurately). The numbers refer to the compounds in Table II. S_a and π values are given in Table III.

Recently Hansch (1972) has evaluated the electronic, steric, and hydrophobic effects of substituents R upon the deacylation rate constants using our earlier results (Dupaix *et al.*, 1970) and has found that hydrophobic forces played a positive role in the deacylation step. If Figure 5 (established with a larger number of compounds) is considered, it appears that there is no simple correlation between the S_d values for acyl-enzymes having a bulky substituent in their acyl part and the corresponding substituent constants π. However, for derivatives whose chain length is optimal (*i.e.*, 10–12 Å), the more hydrophobic the chain extremity, the higher the S_d value. This point is particularly shown by the comparison of the S_d values for chlorovaleryl-enzyme (substituent **11**; S_d = 0.64), butyrylglycyl-enzyme (substituent **21**; S_d = 0.77), and 4-acetamidobutyryl-enzme (substituent **23**; S_d = −0.22) whose chain lengths are closely related.

Henderson (1970) has recently given the structure of indoleacryloyl-α-chymotrypsin at pH 4, based on crystallographic studies at 2.5-Å resolution. He finds that the indolyl part of the acyl-enzyme binds deeply in a pocket which is embedded in the surface of the enzyme and which has the dimensions 10–12 Å × 5.5–6.5 Å × 3.5–4.0 Å (Steitz *et al.*, 1969).

Therefore, as a consequence of the covalent bond between Ser-195 and the carbonyl group of the substrate in the acyl-enzyme, several structural requirements of the acyl part will be required for a good deacylation. If the chain of the acyl function is too short (shorter than 8 Å) or too long (longer than 13 Å) or if, in spite of an optimal length, its extremity is not apolar, the acyl group will bind poorly in this hydrophobic pocket; consequently, the hydrolyzing group will not be precisely positioned relative to the attacking water molecule. Likewise, a particular structure as that found in derivatives **6** and **15**, where the susceptible carbonyl carbon is not separated by two methylene groups from a bulky aromatic ring, will not interact with the specificity site and will not be recognized.

Specificity of α-Chymotrypsin in the Acylation Step. The

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[3] Pattabiraman and Lawson (1972) have also observed the same phenomenon for the α-chymotrypsin-catalyzed hydrolyses of methyl 2-phenylacetate and methyl L-2-acetamido-2-phenylacetate in comparison with those of methyl 3-phenylpropionate and methyl L-2-acetamido-3-phenylpropionate, respectively.

effects of the substituents R on the second-order acylation rate constant $k_{cat}/K_m = k_2/K_s$ may be complex, for both constants k_2 and K_s may be differently affected by the nature of R. Thus, it is not easy to separate unambiguously the substituent effects acting on the acylation rate constant k_2 and the dissociation constant K_s.

However, calculated values of the reaction constants σ^* and δ in the acylation step are comparable to those found in the deacylation step and therefore characterize the acylation process itself rather than the Michaelis complex formation. Indeed, acylation (k_2) and deacylation (k_3) are known to be mechanistically similar to one another (Bender and Kezdy, 1964). The value of $\rho^* = 2.19$ is reminiscent of the reaction of acyl-substituted *o*-nitrophenyl esters with oxygen bases where $\rho^* = 2.35$ rather than with nitrogen bases where $\rho^* = 1.44$ (Holmquist and Bruice, 1969b). Also, the δ value of 0.53 is far from the δ value found in the imidazole nucleophile catalyzed hydrolysis of *p*-nitrophenyl esters where $\delta = 1.4$ (Milstien and Fife, 1968). Thus, the polar and steric effects due to the substituents R in the acylation step indicate that a general base catalyzed nucleophile attack by Ser-195 upon these activated esters is more probable than a nucleophilic attack by the imidazole ring of His-57. This result is different from that obtained by Hubbard and Kirsch (1970, 1972) with acyl-substituted *p*-nitrophenyl benzoates. For these conjugated derivatives the Hammett ρ value is equal to 1.0 in the acylation step, while it is equal to 2.1 in the deacylation step as shown by Caplow and Jencks (1962). Hubbard and Kirsch have then suggested that the imidazole side chain of His-57 is the primary acyl acceptor in the reaction of α-chymotrypsin with *p*-nitrophenyl benzoates. The reasons for such a different behavior in both these series of *p*-nitrophenyl esters are not clear.

A priori, it may be thought that the same structural properties of the acyl part of the substrates will favor acylation and deacylation steps. As shown in Figure 4, the acylation specificity constant S_a is optimal for compounds having a chain length of about 10 Å. However, in the case of glycine derivatives, an inverse profile of S_a *vs.* the chain length is found. Moreover a rather good correlation is found between S_a and π for different bulky substituents including glycine derivatives (Figure 6) while such a relationship is not apparent in the deacylation step (Figure 5). Thus, the nature of forces responsible for the specificity in the acylation step is mainly hydrophobic.

Berezin *et al.* (1970) have found a linear relationship between the binding constants of aliphatic alcohols or aromatic compounds with α-chymotrypsin active center and their respective octanol–water partition coefficients, just as we find when plotting the acylation specificity constant S_a *vs.* π (Figure 6). Therefore, it seems likely that this last correlation is due to the changes of K_s values in the second-order acylation rate constants k_2/K_s with the apolar nature of the substituents R. The high values of the second-order acylation rate constants for aromatic derivatives (for instance *p*-nitrophenyl esters of 3-phenylpropionic acid (**12**) or of 3-(3-indolyl)-propionic acid (**16**)) would be due to low K_s values while the glycine derivatives (with the significant exception of carbobenzoxyglycine *p*-nitrophenyl ester (**22**)) would be poorly hydrolyzed because of unfavorable K_s values related to the difficulty of the acylamino group transfer from water to a nonpolar enzymatic medium.

In conclusion, the use of Taft's equation has allowed a separation of nonspecific and specific effects of substituents on the enzymatic hydrolysis of a large set of substrates; therefore, the specific recognition of any substrate by the enzyme is shown on a quantitative basis to be dependent on some structural properties of substrates.

Acknowledgments

We wish to thank Professor J. Yon for helpful suggestions and criticisms.

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Specificity of α-Chymotrypsin. The α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acylamino Acid *p*-Nitrophenyl Esters†

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ABSTRACT: The α-chymotrypsin-catalyzed hydrolysis of various *N*-acylamino acid *p*-nitrophenyl esters has been studied at 25°, between pH 6 and 8. The deacylation step is characterized by: rates increasing with the length of the amino acid side chain and no apparent linear behavior between the deacylation specificity constant (S_d) and the hydrophobic bonding constant (π) which characterizes the amino acid side chain. Deacylations of *N*-acyl-L- and -D-aminoacyl-enzymes show similar patterns but the magnitude of the effects is less for D derivatives. No negative specificity is observed for the deacylation of *N*-benzyloxycarbonyl-D-aminoacyl-enzymes in comparison with that of L isomers. The stereospecificity is not absolute and depends on the nature of the *N*-acyl substituent which must have a sufficient length to allow good "stereorecognition" of the substrate by the enzyme. Although there are many common features between the deacylation and the acylation steps, some of the results are different. Thus, the acylation specificity constant S_a increases linearly with the hydrophobic bonding constant π for the side chain of the amino acid.

In the preceding paper (Dupaix *et al.*, 1973) the action of α-chymotrypsin on a series of *p*-nitrophenyl esters of carboxylic acids has been compared for the acylation and deacylation steps of the enzymatic reaction. Concerning this kind of substrate the requirements of the enzymatic specificity in both steps have been defined. In order to extend these observations to other classes of substrates, we have been interested in studying the enzymatic hydrolyses of amino acid derivatives. Though many studies on such compounds are available in the literature (Jones *et al.*, 1965; Ingles and Knowles, 1967, 1968; Berezin *et al.*, 1971a,b) most of them have been carried out on substrates for which the rate-determining step of the enzymatic hydrolysis was not well defined; this complicated the analysis of enzymatic specificity. Therefore, we have studied the α-chymotrypsin-catalyzed hydrolysis of *N*-acylamino acid *p*-nitrophenyl esters, whose rate-determining step is deacylation (Zerner and Bender, 1964; Ingles and Knowles, 1967).

Experimental Procedures

The experimental procedures and materials, except those described below, were the same as those given in the preceding paper (Dupaix *et al.*, 1973).

Preparation of Compounds. Most *N*-acylamino acids and their corresponding *p*-nitrophenyl esters were obtained from commercial sources (Cyclo Chemical Co.; Pierce Chemical Co.; Sigma Chemical Co.; K & K Laboratories) and were used without further purification. The esters were characterized by their melting points, their specific rotatory powers, their ultraviolet spectra in ethanol, and the spectra of their hydrolysis products in 0.1 N NaOH. The experimental data were generally in good agreement with those from the literature (Fletcher and Jones, 1972).

Other *N*-acylamino acid *p*-nitrophenyl esters were synthesized by the dicyclohexylcarbodiimide method (Bodanszky

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