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

Jean-Jacques Béchet, Alain Dupaix,* and Colette Roucous

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

† From the Laboratoire d'Enzymologie Physico-chimique et Moléculaire, Groupe de Recherches du CNRS associé à l'Université de Paris-Sud, Orsay, 91405, France. *Received December 19, 1972.* This work was supported by grants from CNRS (Groupe de Recherches No. 13) and Délégation Générale à la Recherche Scientifique et Technique (Convention No. 71-7-2627). This work constitutes part of the thesis submitted by A. Dupaix in partial fulfillment of the requirements for the Doctorat ès Sciences.

TABLE I: Analytical and Physical Properties of Some *N*-Acylamino Acid *p*-Nitrophenyl Esters.

N-Acylamino Acid	Mp (uncorr, °C)	General Formula	Anal. (%)				$[\alpha]_D^{24}$ (deg) (*c* 2, *N*,*N*-Dimethylformamide)
				C	H	N	
HCO-L-Ala[a]	69–71	$C_{10}H_{10}N_2O_5$	Calcd	50.42	4.23	11.76	−31.2
			Found	51.04	4.49	11.65	
HCO-D-Ala[b]	70–71	$C_{10}H_{10}N_2O_5$	Calcd	50.42	4.23	11.76	
			Found	50.44	4.29	11.88	+23.4 ± 1.2
Z-L-Abu	59–60	$C_{18}H_{18}N_2O_6$	Calcd	60.33	5.06	7.82	
			Found	60.62	5.06	7.82	−34.4 ± 0.1
Z-D-Abu	56.5–59	$C_{18}H_{18}N_2O_6$	Calcd	60.33	5.06	7.82	
			Found	60.47	5.15	7.83	+35.5 ± 0.35
Z-L-Nva	68–68.5	$C_{19}H_{20}N_2O_6$	Calcd	61.28	5.41	7.52	−31.5 ± 0.0
			Found	61.20	5.48	7.60	
Z-D-Nva	68.5–69.5	$C_{19}H_{20}N_2O_6$	Calcd	61.28	5.41	7.52	+32.3 ± 2
			Found	61.39	5.30	7.77	
Z-D-Leu[c]	90.5–91.5	$C_{20}H_{22}N_2O_6$	Calcd	62.16	5.74	7.25	+32.8 ± 0.15
			Found	62.12	5.55	7.17	
Z-L-Nle[d]	68–68.5	$C_{20}H_{22}N_2O_6$	Calcd	62.16	5.74	7.25	
			Found	62.30	5.81	7.21	−28 ± 0.5
Z-D-Asn[e]	148–150	$C_{18}H_{17}N_3O_7$	Calcd	55.81	4.42	10.84	
			Found	55.60	4.44	10.62	+32.9 ± 0.8

[a] This compound was pure to 94% as measured by the total amount of *p*-nitrophenol released in 0.1 N NaOH. [b] 98% purity. [c] The properties of the L derivative are: mp 95°; $[\alpha]_D^{20}$ −33.5° (*c* 2, dimethylformamide) (Bodanszky and Du Vigneaud, 1959). [d] Mp 70–71.5° (Williams *et al.*, 1972). [e] The properties of the L derivative are: mp 165–166°; $[\alpha]_D^{20}$ −31.5° (*c* 2, dimethylformamide) (Bodanszky and Du Vigneaud, 1959).

and Du Vigneaud, 1959) and were recrystallized from ether–petroleum ether or ethanol. General properties of these derivatives are reported in Table I.

The preparation of some *N*-acylamino acids which were commercially unavailable and the preparation of particular esters are described below.

N-Carbobenzoxy-D-norvaline and *N*-carbobenzoxy-L-norleucine were prepared by the action of carbobenzoxy chloride on amino acids as described by Greenstein and Winitz (1961). The oils formed were used directly for the synthesis of corresponding *p*-nitrophenyl esters.

Formyl-D-alanine and formyl-L-alanine were synthesized according to Sheehan and Yang (1958), by adding acetic anhydride to alanine in 98% formic acid at 5°. After recrystallizations from ethanol, the melting points and optical rotations of these compounds were in good agreement with those from the literature (Lefrancier and Bricas, 1965).

Formyl-D-alanine and formyl-L-alanine *p*-nitrophenyl esters were prepared by the dicyclohexylcarbodiimide method. Recrystallizations from chloroform–hexane were difficult and gave products with poor yields.

Benzyloxycarbonyl-D-asparagine *p*-nitrophenyl ester was obtained as described by Stewart and Young (1969) by adding 4 mM carbobenzoxy-D-asparagine to a fourfold excess of *p*-nitrophenol in the presence of 4.4 mM dicyclohexylcarbodiimide in *N*,*N*-dimethylformamide at 0°. The product was crystallized from ether; yield 50%.

Kinetic Measurements. Kinetic measurements and calculations of kinetic constants were carried out in the same way as described previously (Dupaix *et al.*, 1973).

N-Carbobenzoxyamino acid *p*-nitrophenyl esters are relatively stable in aqueous solution at neutral pH, in comparison with other *N*-acylamino acid *p*-nitrophenyl esters whose hydrolysis proceeds through an oxazolinone intermediate (De Jersey *et al.*, 1969). In the pH range 6–8, the rates of their spontaneous hydrolyses are at most equal to 5–10% of the rates of the enzymatic hydrolyses. However, some of them are more unstable in water at neutral pH; thus the rates of the spontaneous hydrolyses of *N*-carbobenzoxy-L (or D) -asparagine *p*-nitrophenyl ester and *N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester are at least equal to 15–30% of the rates of their enzymatic hydrolyses at pH 7.2.

The spontaneous hydrolysis of *N*-carbobenzoxy-L-asparagine *p*-nitrophenyl ester likely involves the formation of an imide intermediate (Bernhard *et al.*, 1962). Indeed, the potentiometric titration of the reaction products indicates that these products are weak acids with pK_a values equal to about 7.7 (*p*-nitrophenol) and 9.5 (benzyloxycarbonyl-L-aminosuccinimide) in dioxane–water (2:3, v/v). In the opposite way, the α-chymotrypsin-catalyzed hydrolysis of this ester leads to the formation of *p*-nitrophenol (pK_a = 7.7) and *N*-carbobenzoxy-L-asparagine (pK = 4.2) as shown by potentiometric titrations of the reaction products in dioxane–water (2:3, v/v).

Optical Rotation. Measurements of optical rotation were performed on an electronic quick polarimeter (Roussel-Jouan).

Estimation of Steric Substituent Constants for the Amino Acid Derivatives. The steric substituent constants E_s for the amino acid derivatives are not found in the literature; thus, they have been calculated in the following way.

From E_s values (Taft, 1956) for carboxylic derivatives RCOOR′ which are disubstituted on their α carbon (*i.e.*, R = $R_1(R_2)CH$) or monosubstituted (*i.e.*, R = R_1CH_2 or R_2CH_2), we have tried to correlate $E_{s,R_1(R_2)CH}$ with the E_s values for each substituent, E_{s,R_1CH_2} and E_{s,R_2CH_2}. Thus, the E_s values for the following ten substituents [$(CH_3)_2CH$, $(C_2H_5)_2CH$, n-$(C_3H_7)_2CH$, $(C_6H_5)_2CH$, $(C_2H_5)(CH_3)CH$, $(C_6H_5)(CH_3)CH$, $(C_6H_5)(C_2H_5)CH$, Br_2CH, Cl_2CH, and F_2CH] have

TABLE II: σ^* and E_s Values for the Substituent $R_1CONHCH(R_2)$– in *N*-Acylamino Acid *p*-Nitrophenyl Esters. pK Values of Some *N*-Acylamino Acids in Aqueous Solution at 25°.

No.	*N*-Acylamino Acids	pK[a]	σ^* [b]	E_s [c]
1	HCO-Gly		0.582[d]	−0.36 ± 0.1
2	Ac-Gly		0.56[d]	−0.39 ± 0.1
3	Pro-Gly		0.56[d]	−0.40 ± 0.1
4	But-Gly		0.56[d]	−0.40 ± 0.1
5	Z-Gly	3.70 ± 0.02	0.57	−0.45 ± 0.1
6	HCO-L-Ala	3.50 ± 0.05	0.68	−1.1 ± 0.2
7	HCO-D-Ala		0.68	−1.1 ± 0.2
8	Z-L-Ala	3.67 ± 0.07	0.586	−1.3 ± 0.2
9	Z-D-Ala		0.586	−1.3 ± 0.2
10	Z-L-Abu	3.83 ± 0.12	0.497	−2.06 ± 0.3
11	Z-D-Abu		0.497	−2.06 ± 0.3
12	Z-L-Val	3.92[e]	0.45	−3.5 ± 0.5
13	Z-D-Val		0.45	−3.5 ± 0.5
14	Z-L-Nva	3.81 ± 0.01	0.503	−2.13 ± 0.3
15	Z-D-Nva		0.503	−2.13 ± 0.3
16	Z-L-Leu	3.86[e]	0.48	−2.03 ± 0.3
17	Z-D-Leu		0.48	−2.03 ± 0.3
18	Z-L-Nle		0.503[f]	−2.16 ± 0.3
19	Z-L-Asn	3.56 ± 0.01	0.647	−2.20 ± 0.3
20	Z-D-Asn		0.647	−2.20 ± 0.3
21	Z-L-Phe	3.69 ± 0.2	0.575	−2.11 ± 0.3
22	Z-D-Phe		0.575	−2.11 ± 0.3
23	Z-L-Trp		0.575[g]	−2.79 ± 0.3
24	Z-L-Lys		0.50[f]	−2.03 ± 0.3

[a] The pK values for both enantiomers of *N*-acylamino acids were assumed to be identical. [b] The polar substituent constants σ^* were calculated from σ_i values according to eq 3; these σ_i values were obtained from the pK values according to eq 2 (Charton, 1964). [c] E_s values were calculated according to eq 1. [d] These σ^* values were calculated from σ_i values given by Charton (1964) by using eq 3. [e] pK values given by Khurgin and Dmitrieva (1965), but corrected from the solvent effect; a mean shift of the pK values equal to 0.56 was found in the measurements made in dioxane–water (1:4, v/v) (Khurgin and Dmitrieva, 1965) and in aqueous solution (our determinations) for Z-Gly, Z-Ala, and Z-Phe. [f] This σ^* was assumed to be identical with the σ^* value for Z-L-Nva. [g] This σ^* value was assumed to be identical with the σ^* value for Z-L-Phe.

been compared with those of their simpler components (CH_3-CH_2, $C_2H_5CH_2$, ...). We have found eq 1, with a correlation

$$E_{s,R_1(R_2)CH} = (2.54 \pm 0.17)(E_{s,R_1CH_2} + E_{s,R_2CH_2}) \quad (1)$$

coefficient r equal to 0.831.

As a matter of fact, the more important deviations from this relationship are observed for the dihalogeno substituents. If they are omitted, we obtain a similar equation with a more satisfactory correlation coefficient, $r = 0.948$. Moreover, we have assumed large enough standard deviations for the E_s values of *N*-acylamino acids calculated by means of eq 1 in order to be sure that these E_s values allow significant evaluations for the steric properties of the substituents (see Table II).

Estimation of Polar Substituent Constants σ^* for the Amino Acid Derivatives. Charton (1964) has defined a relationship between the "inductive" substituent constant $\sigma_{i,x}$ and the p$K_{a,x}$ of the corresponding substituted acetic acid XCH_2COOH

$$\sigma_{i,x} = b(pK_{a,x}) + d \quad (2)$$

with $b = -0.2515$ and $d = 1.186$ at 25°.

On the other hand, from σ^* values given by Taft (1956) and corresponding σ_i values given by Charton (1964), the correlation eq 3 may be established, with $n = 18$ substituents

$$\sigma^* = (2.22 \pm 0.03)\sigma_i \quad (3)$$

whose electron-withdrawing powers are very different (FCH_2, $HOCH_2$, CH_3, t-$C_4H_9CH_2$, ...); $r = 0.9974$. Thus, measurements of the pK_a values for *N*-acylamino acids ($R_1CONHCH(R_2)COOH$) allow the calculation of σ_i values for the substituents $R_1CONHCH(R_2)$ using eq 2; from these σ_i values eq 3 leads to the corresponding σ^* values.

The pK values of some *N*-acylamino acids were determined potentiometrically, using a Vibron pH meter Model 33 B (Electronic Instruments Ltd.) with a glass electrode GFH 33 and standardized against standard pH 4.0 buffer solution. All titrations were performed in aqueous solutions containing different NaCl concentrations (0.1, 0.05, 0.01 M NaCl). The amino acid derivatives (5×10^{-3} M) were neutralized with 2.0 N NaOH and titrated with 1.0 N HCl, in a thermostated cell at 25°. The pK values were extrapolated to an ionic strength I equal to zero (see Table II).

Results

Experimental values of the kinetic constants k_3, K_1'', and K_m for the α-chymotrypsin-catalyzed hydrolysis of *N*-acylamino acid *p*-nitrophenyl esters are listed in Table III. These constants are defined from the classical reaction scheme given in the preceding paper (Dupaix *et al.*, 1973).

Using the Taft–Ingold relationship established for *p*-nitrophenyl esters of carboxylic acids (see the accompanying paper) we have separated the polar, steric, and specific effects occurring during the deacylation and acylation steps in the enzymatic hydrolysis of these amino acid derivatives. We have expressed the specific effects by the deacylation specificity constant S_d and the acylation specificity constant S_a, respectively. The different values of S_d and S_a are reported in Table IV.

The S_d values for the deacylation of *N*-α-carbobenzoxyaminoacyl-enzymes ($C_6H_5CH_2OCONHCH(R_2)CO$-enzyme) and the S_a values for the acylation of α-chymotrypsin by the corresponding *p*-nitrophenyl esters have been therefore plotted *vs.* the length of the acyl part R_2CHCO (Figures 1 and 3) and *vs.* the "hydrophobic bonding" constant π characteristic of R_2H (Figures 2 and 4).

Discussion

Specificity and Stereospecificity of α-Chymotrypsin in the Deacylation Step. When considering Z-L-aminoacyl-enzymes[1]

[1] Abbreviations used are: HCO, formyl; Ac, acetyl; Pro, propionyl; But, butyryl; Z, benzyloxycarbonyl; Bz, benzoyl; OMe, methyl ester; ONp, *p*-nitrophenyl ester. The abbreviated designation of derivatives of amino acids corresponds to the proposals of the Joint IUPAC–IUB Commission on Biochemical Nomenclature.

TABLE III: Kinetic Parameters for the α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acylamino Acid *p*-Nitrophenyl Esters.[a]

No.	*N*-Acylamino Acid	pK_1''	10^6K_m (M)	10^2k_3 (sec^{-1})	$10^{-4}(k_{cat}/K_m)$ (M^{-1} sec^{-1})
1	HCO-Gly[b]	6.95 ± 0.03	15.7 ± 0.8	24.5 ± 2.9	1.56 ± 0.18
2	Ac-Gly[b]	6.87 ± 0.01	138 ± 11	75.2 ± 2.3	0.54 ± 0.02
3	Pro-Gly[b]	7.00 ± 0.015	113 ± 9	55.95 ± 3.3	0.495 ± 0.03
4	But-Gly[b]	6.936 ± 0.014	136 ± 22	87.1 ± 6.2	0.64 ± 0.045
5	Z-Gly[b]	7.01 ± 0.035	4.9 ± 0.5	41.1 ± 2.0	8.38 ± 0.40
6	HCO-L-Ala	6.93 ± 0.02	13.3 ± 0.9	24.6 ± 0.5	1.85 ± 0.04
7	HCO-D-Ala	7.08 ± 0.01	6.1 ± 0.5	7.2 ± 0.1	1.18 ± 0.02
8	Z-L-Ala	7.21 ± 0.02	20 ± 1	304 ± 25	15.2 ± 1.25
9	Z-D-Ala	7.43 ± 0.04	8.3 ± 0.9	8.99 ± 0.25	1.08 ± 0.03
10	Z-L-Abu	7.48 ± 0.02	1.8 ± 0.1	271 ± 15	150 ± 8
11	Z-D-Abu	7.26 ± 0.01	1.97 ± 0.02	3.53 ± 0.07	1.79 ± 0.03
12	Z-L-Val	7.27 ± 0.02	17.8 ± 1.4	11.85 ± 0.4	0.66 ± 0.02
13	Z-D-Val	7.16 ± 0.01	7.36 ± 0.84	0.79 ± 0.03	0.108 ± 0.004
14	Z-L-Nva	7.57 ± 0.02	2.67 ± 0.21	818 ± 57	306 ± 21
15	Z-D-Nva	7.45 ± 0.03	3.15 ± 0.20	14.94 ± 0.50	4.74 ± 0.16
16	Z-L-Leu	7.59 ± 0.04	4.8 ± 0.4	834 ± 45	174 ± 9
17	Z-D-Leu	7.22 ± 0.015	2.42 ± 0.29	11.05 ± 0.30	4.57 ± 0.12
18	Z-L-Nle	7.45 ± 0.06	5.8 ± 2.5	1230 ± 200	212 ± 34
19	Z-L-Asn	7.25 ± 0.04	13.6 ± 1.3[c]	3370 ± 530	248 ± 39
20	Z-D-Asn	6.76 ± 0.03	1.46 ± 0.14[c]	2.25 ± 0.05	1.54 ± 0.04
21	Z-L-Phe	7.53 ± 0.06	0.48[d]	3915 ± 100	8156 ± 210
22	Z-D-Phe	7.65 ± 0.04	0.38[d]	18.1 ± 1	47.76 ± 2.6
23	Z-L-Trp	7.51 ± 0.07	<0.5	1782 ± 110	>3500
24	Z-L-Lys	6.91 ± 0.04	43 ± 3[c]	294 ± 12	6.79 ± 0.28

[a] Experimental conditions: temperature, 25°; 0.5 M NaCl; 4.5% (v/v) acetonitrile–water. Unless otherwise noted, 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] Values of kinetic parameters from Dupaix *et al.* (1973). [c] Temperature, 25°; 0.5 M NaCl; 4.5% (v/v) acetonitrile–water; pH 7.0. [d] These K_m values were estimated from total enzymatic hydrolysis.

of the general formula $C_6H_5CH_2OCONHCH(R_2)CO$-enzyme, the structural requirements for specificity can be characterized as follows: the longer or the more hydrophobic the side-chain R_2, the higher the S_d value (Figures 1 and 2). Optimal R_2 length is found for derivatives of phenylalanine (**21**) or tryptophan (**23**), *i.e.*, for a side-chain length of about 10 Å. Jones *et al.* (1965) have previously indicated that in the α-chymotrypsin-catalyzed hydrolysis of a series of *N*-acetyl-L-amino acid methyl esters of the general formula $CH_3CONHCH[(CH_2)_nH]COOCH_3$, k_{cat} reaches a maximum when $n = 5$. Moreover, the increase in S_d values for Z-L-aminoacyl-enzymes is not linearly related to the apolar character of the R_2 substituent and the curve S_d *vs.* π is similar to a saturation curve[2] (Figure 2).

A particular point in Figures 1 and 2 is the enhanced reactivity of Z-L-alanyl-α-chymotrypsin (**8**) in comparison with that of Z-glycyl- and Z-D-alanyl-α-chymotrypsin (**5** and **9**). When the *N*-acylamino substituent R_1CONH– does not carry an apolar part like in the formylamino derivatives, the intrinsic reactivity of the L enantiomer of formylalanyl-

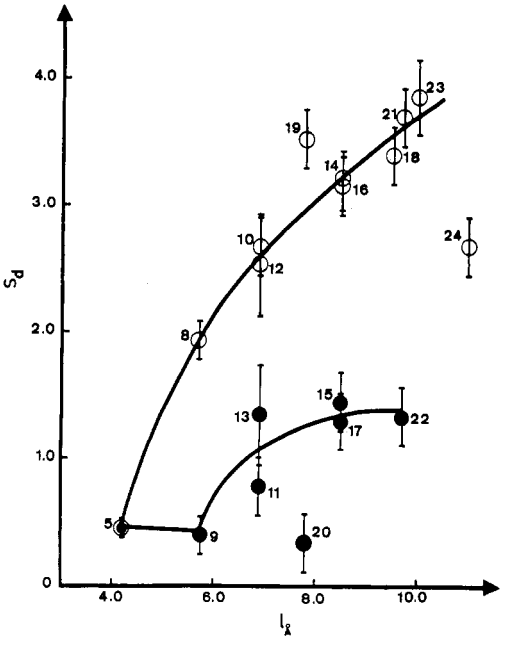

FIGURE 1: Plot of the deacylation specificity constants S_d for Z-L-aminoacyl-enzymes and Z-D-aminoacyl-enzymes ($C_6H_5CH_2OCONHCH(R_2)CO$-enzyme) *vs.* the length *l* of the acyl part R_2CHCO (*l* was calculated from the carbonyl carbon assuming fully extended models): (○) Z-L-aminoacyl-chymotrypsins; (●) Z-D-aminoacyl-chymotrypsins; (◉) Z-glycyl-enzyme. The numbers pertain to the compounds listed in Table IV.

[2] Z-L-Asparaginyl-enzyme (**19**), whose S_d value (equal to 3.52) *vs.* π (equal to −1.21) falls outside the graph of Figure 2, deacylates more rapidly than expected from the polar character of its side-chain R_2, while *N*-α-Z-L-lysyl-enzyme (**24**) has a normal behavior (see Figure 2). The reasons for such a phenomenon are not clear. However, Cohen *et al.* (1970) have previously reported that the presence of amide groups in different regions of the substrate molecule might facilitate the chymotryptic attack by allowing the desolvation of the reactant groups at the active site of the enzyme.

TABLE IV: α-Chymotrypsin-Catalyzed Hydrolysis of *N*-Acylamino Acid *p*-Nitrophenyl Esters: Deacylation (S_d) and Acylation (S_a) Specificity Constants. Values of the Hydrophobic Bonding Constant π for the Side-Chain R_2H of *N*-Acylamino Acid Derivatives $R_1CONHCH(R_2)COOC_6H_4$-*p*-NO_2.

No.	*N*-Acylamino Acid	S_d	S_a	π^a
1	HCO-Gly	0.14 ± 0.06	−0.44 ± 0.05	0
2	Ac-Gly	0.70 ± 0.08	−0.84 ± 0.05	0
3	Pro-Gly	0.575 ± 0.08	−0.87 ± 0.05	0
4	But-Gly	0.77 ± 0.08	−0.76 ± 0.05	0
5	Z-Gly	0.455 ± 0.08	0.36 ± 0.06	0
6	HCO-L-Ala	0.464 ± 0.15	−0.196 ± 0.010	0.5
7	HCO-D-Ala	−0.07 ± 0.15	−0.39 ± 0.10	0.5
8	Z-L-Ala	1.94 ± 0.15	1.03 ± 0.10	0.5
9	Z-D-Ala	0.405 ± 0.15	−0.12 ± 0.10	0.5
10	Z-L-Abu	2.68 ± 0.23	2.62 ± 0.16	1.0
11	Z-D-Abu	0.79 ± 0.23	0.70 ± 0.16	1.0
12	Z-L-Val	2.53 ± 0.40	1.12 ± 0.26	1.3
13	Z-D-Val	1.35 ± 0.40	0.34 ± 0.26	1.3
14	Z-L-Nva	3.20 ± 0.23	2.95 ± 0.16	1.5
15	Z-D-Nva	1.46 ± 0.23	1.14 ± 0.16	1.5
16	Z-L-Leu	3.18 ± 0.23	2.70 ± 0.16	1.8
17	Z-D-Leu	1.30 ± 0.23	1.12 ± 0.16	1.8
18	Z-L-Nle	3.40 ± 0.23	2.81 ± 0.16	2.0
19	Z-L-Asn	3.52 ± 0.23	2.58 ± 0.16	−1.21
20	Z-D-Asn	0.34 ± 0.23	0.37 ± 0.16	−1.21
21	Z-L-Phe	3.70 ± 0.23	4.21 ± 0.15	2.63
22	Z-D-Phe	1.35 ± 0.23	1.98 ± 0.15	2.63
23	Z-L-Trp	3.86 ± 0.30	>4.2	2.64
24	Z-L-Lys	2.68 ± 0.23	1.26 ± 0.15	0.84[b]

[a] Values were calculated from data of Fujita *et al.* (1964) and Hansch *et al.* (1968). [b] From Leo *et al.* (1971).

enzyme (**6**) is comparable to that of formylglycyl-enzyme (**1**) (see Table IV) and the stereospecificity is reduced. Thus, the ratios k_{cat}^L/k_{cat}^D for *N*-formyl- and *N*-carbobenzoxyalanyl-enzymes are, respectively, 3.4 and 34. Hein and Niemann (1962b) have compared the enzymatic hydrolyses of formyl- and acetylphenylalanine methyl esters whose side-chain R_2 is longer than that of alanine derivatives and they have indicated that the hydrolysis of formyl derivatives was less stereospecific than that of acetyl ones. The size of the acylamino group attached to the asymmetrical α carbon is therefore an important factor for a good stereorecognition of amino acid derivatives by the enzyme.

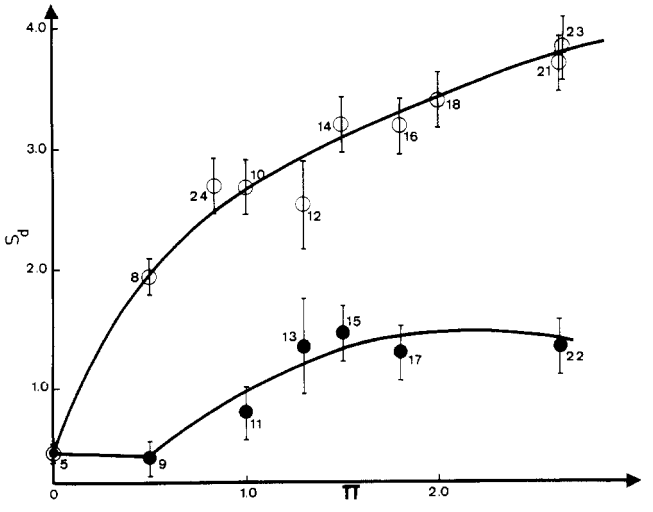

FIGURE 2: Plot of the deacylation specificity constants S_d for Z-L-aminoacyl- and Z-D-aminoacyl-enzymes ($C_6H_5CH_2OCONHCH(R_2)CO$-enzyme) *vs.* π, the hydrophobic bonding constant of the side chain: (○) Z-L-aminoacyl-chymotrypsins; (●) Z-D-aminoacyl-chymotrypsins; (◉) Z-glycyl-enzyme. The numbers pertain to the compounds listed in Table IV. Z-L-Asn- and Z-D-Asn-enzyme are not included in the figure for their S_d values as a function of π are outside the graph.

As shown by their higher S_d values, the *N*-benzyloxycarbonyl-L-aminoacyl-enzymes deacylate more rapidly than the acyl-enzymes lacking an acylamino substituent (Dupaix *et al.*, 1973). For example, the S_d value for Z-L-phenylalanyl-enzyme is equal to 3.70, while the S_d value for phenylpropionyl-enzyme is equal to 1.23; likewise, for Z-L-tryptophanyl-enzyme, S_d = 3.86 and for 3-(3-indolyl)propionyl-enzyme, S_d = 2.05. Comparison of the S_d values for the whole Z-L-aminoacyl-enzymes and acyl-enzymes lacking an acylamino substituent shows that the positive contribution of the –OCONH– group to the deacylation rate constant varies over a range between 1 and ~300. This contribution is therefore limited and not so important as previously assumed (Hein and Niemann, 1962a,b; Ingles and Knowles, 1968). Silver *et al.* (1970) and Pattabiraman and Lawson (1972) have also recently shown that the role of the acylamino substituent was overestimated.

However, the double presence of this acylamino group and the apolar side-chain R_2 in any *N*-acylamino acid derivative ($R_1CONHCH(R_2)CO$–) having the L configuration confers to the latter a better reactivity in the presence of α-chymotrypsin than that observed with the corresponding compounds having a simple structural element (for instance, $R_1CONHCH_2CO$– or R_2CH_2CO–), Therefore, it is likely that in these *N*-acyl-L-aminoacyl-enzymes the acylamino group

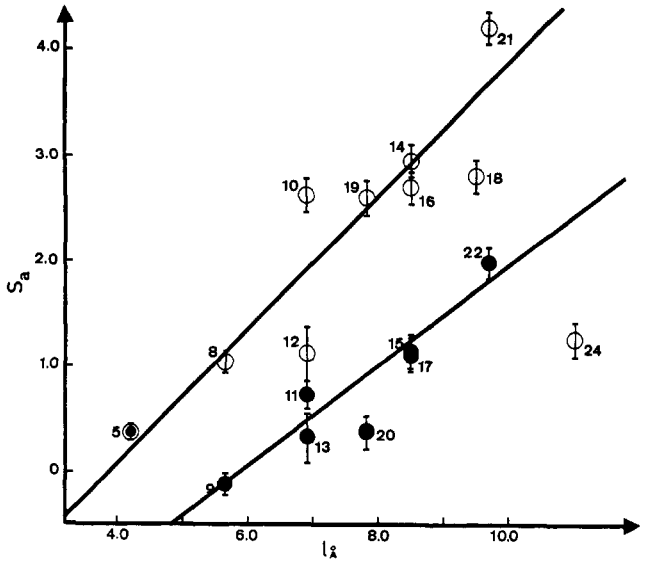

FIGURE 3: Plot of the acylation specificity constants S_a for Z-L-amino acid *p*-nitrophenyl esters and Z-D-amino acid *p*-nitrophenyl esters ($C_6H_5CH_2OCONHCH(R_2)COOC_6H_4$-*p*$NO_2$) *vs.* the length *l* of the acyl part R_2CHCO (*l* was calculated from the carbonyl carbon assuming fully extended models): (○) Z-L-amino acid-ONp; (●) Z-D-amino acid-ONp; (◉) Z-Gly-ONp. The numbers pertain to the compounds listed in Table IV. The value of S_a for Z-L-Trp-ONp is not plotted because of the uncertainty in the S_a value (too low a K_m value to be estimated accurately).

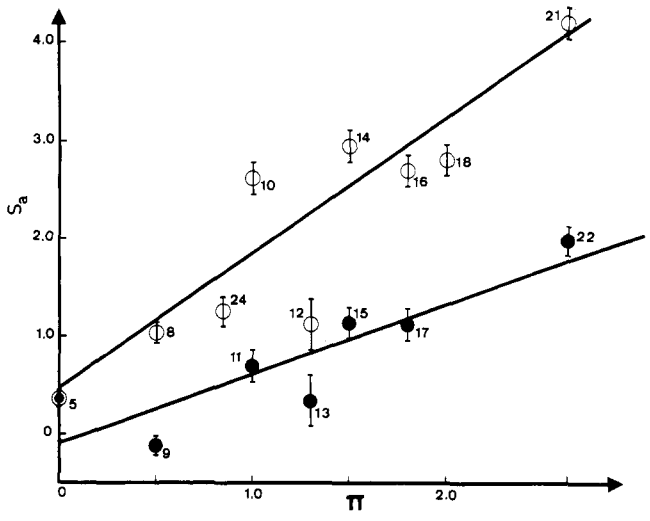

FIGURE 4: Plot of the acylation specificity constants S_a for Z-L-amino acid *p*-nitrophenyl esters and Z-D-amino acid *p*-nitrophenyl esters ($C_6H_5CH_2OCONHCH(R_2)COOC_6H_4$-*p*-$NO_2$) *vs.* π, the hydrophobic bonding constant of the side chain: (○) Z-L-amino acid-ONp; (●) Z-D-amino acid-ONp; (◉) Z-Gly-ONp. The numbers pertain to the compounds listed in Table IV. Z-L-Asn-ONp and Z-D-Asn-ONp are not included in the figure for their S_a values *vs.* π are outside the graph. Z-L-Trp-ONp is also not included because of the uncertainty in the S_a value (too low a K_m value to be estimated).

could interact with a specific locus of the enzyme, probably a hydrogen bond acceptor site (Henderson, 1970) that would orient the apolar side-chain R_2 in the direction of the specificity pocket allowing its binding. Consequently, the bond being hydrolyzed would be precisely positioned relative to the attacking water molecule.

In the case of Z-D-aminoacyl-enzymes, an attenuated positive relation between S_d values and the length or the apolar character of the side-chain R_2 is observed (Figures 1 and 2). Comparison of the S_d values for such D-aminoacyl-enzymes and unsubstituted derivatives indicates that the presence of the asymmetrical α carbon has neither a marked positive nor a detrimental effect. Thus, the S_d values for Z-D-phenylalanyl-enzyme (S_d = 1.35) and phenylpropionyl-enzyme (S_d = 1.27) (Dupaix *et al.*, 1973) are comparable. This behavior of Z-D-aminoacyl-enzymes is different from that observed in the case of acetyl-D-aminoacyl-enzymes which deacylate progressively more slowly than acetylglycyl-enzyme as the size of the amino acid side chain increases (Ingles and Knowles, 1967). For such derivatives, the concept of negative specificity was introduced. The authors suggested that a D-amino acid derivative was bound on the enzyme at three loci like an L-amino acid derivative but that its susceptible carbonyl pointed the opposite way from that of an L-amino acid (presumably nonideally with respect to the catalytic functionalities of the enzyme). This model may not account for the behavior of Z-D-aminoacyl-enzymes whose *N*-acyl substituent is more bulky. In this last case, the binding could not be very different from that of derivatives like Z-glycyl-enzyme or phenylpropionyl-enzyme and the possible positive interaction of the peptide link of the acylamino group with a hydrogen bond acceptor locus, as in the case of the L series, would be absent. It is also possible that the substituents R_1 and R_2 enter into different productive binding modes in the Z-D-aminoacyl-enzymes.

Effects of Substituents on the pK_1'' for Deacylation. Relatively large changes in pK_1'' values (around 0.9 pH unit) are observed for the different *N*-acylaminoacyl-enzymes studied (see Table III). This phenomenon is unexpected on the basis of the comparable electron-withdrawing properties of the substituents and the relationships found by Hansch (1972) and Bernhard *et al.* (1966) between pK_1'' and σ* values for various acyl-enzymes in which the acyl group does not carry an asymmetrical α carbon. The pK_1'' value for any *N*-acyl-L-aminoacyl-enzyme is generally superior to that of the corresponding D derivative with the exception of *N*-acylalanyl-enzymes (and less significantly Z-Phe-enzyme). It is interesting that the more hydrophobic the side-chain R_2 of *N*-acyl-L- (or D)-aminoacyl-enzymes, the higher the pK_1'' value. Henderson (1970) has shown that the carbonyl oxygen of the acyl group of indoleacryloyl-α-chymotrypsin was hydrogen bonded to the imidazole of His-57 with a water molecule as an intermediate at acidic pH. It may be assumed that this interaction would be stronger as the side-chain R_2 of *N*-acylaminoacyl-enzymes becomes more hydrophobic, maybe because of a better orientation of the interacting groups.

Specificity and Stereospecificity of α-Chymotrypsin in the Acylation Step. The plots of the acylation specificity constants S_a for *N*-α-benzyloxycarbonylamino acid derivatives ($C_6H_5CH_2OCONHCH(R_2)COOC_6H_4pNO_2$) *vs.* the length of the acyl part R_2CHCO (Figure 3) or *vs.* the "hydrophobic bonding" constant π characteristic of R_2H (Figure 4) lead to some different results when compared with the same plots for the deacylation step. Indeed, the main feature of Figures 3 and 4 is a tendency toward linear behavior although the correlation coefficients are poor. Thus, for the Z-L-amino acid *p*-nitrophenyl esters, except valine and asparagine derivatives, the correlation equation between S_a and π is found equal to

$$S_a = (1.38 \pm 0.17)\pi + (0.47 \pm 0.29) \quad (4)$$

with n = 8 compounds; r = 0.945.

For the corresponding D isomers, the relationship between S_a and π is found equal to

$$S_a = (0.70 \pm 0.15)\pi + (-0.09 \pm 0.26) \quad (5)$$

with $n = 7$ compounds; $r = 0.875$.

Therefore, the apolar character of the side-chain R_2 is essential for the specific recognition of any substrate by the α-chymotrypsin in the acylation step.

Furthermore, most substrate features which favored the deacylation step also favor the acylation. Thus, the size of the *N*-acyl part is an important requirement for a good enzymatic "stereorecognition" of the *N*-acylamino acid derivatives. The S_a values for formyl-L-alanine ($S_a = -0.196$) and formylglycine ($S_a = -0.44$) *p*-nitrophenyl esters are close, while those of Z-L-alanine and Z-glycine *p*-nitrophenyl esters are more different and equal to 1.03 and 0.36. Likewise, the ratio of the k_{cat}/K_m values for formyl-L-alanine and formyl-D-alanine *p*-nitrophenyl esters (equal to 1.57) is smaller than that observed for Z-L-alanine and Z-D-alanine *p*-nitrophenyl esters (equal to 14.1).

N-Benzyloxycarbonyl-L-amino acid *p*-nitrophenyl esters acylate α-chymotrypsin more rapidly than their corresponding D derivatives and *p*-nitrophenyl esters for which the acyl group has no asymmetrical α carbon. However, the contribution of the acylamino substituent in the reactivity of these L derivatives in the presence of enzyme is not so important as previously assumed (Hein and Niemann, 1962a,b; Ingles and Knowles, 1968) and varies over a range between 1 and about 100. As discussed for the deacylation step, specific interaction of this acylamino group with an enzymatic locus must be responsible for the enhanced reactivity of L compounds. The corresponding D enantiomers or the undisubstituted *p*-nitrophenyl esters would interact only by their apolar side chain with the enzyme as shown by the comparable S_a values for related derivatives and the value of the slope in the representation of S_a *vs.* π (compare Figure 4 to Figure 6 of the accompanying article).

The separation of the effects of substituents on the hydrolysis of *p*-nitrophenyl esters of *N*-acylamino acids by α-chymotrypsin leads to the conclusion that the enzyme exerts its specificity and stereospecificity, during the acylation step as well as during the decylation step, in a progressive way. Each structural feature of the substrates bring about its own discrete contribution necessary for a good enzymatic hydrolysis. It is their association which makes a specific substrate.

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