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The Steric Effect of the Leaving Group in the α-Chymotrypsin-Catalyzed Hydrolysis of Acetyl-L-phenylalanine p-Alkoxycarbonyl Anilides

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The relative rate of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine p-alkoxycarbonyl anilides tends to a maximum with the increase of the leaving group bulkiness. This rate enhancement specificity appears to be entropy controlled: the bulky p-alkoxycarbonyl groups increase both enthalpy and entropy of activation. These kinetic and thermodynamic data are interpreted in terms of the stereoelectronic theory for the formation and cleavage of the tetrahedral intermediate in acyl-transfer reactions; the bulky p-alkoxycarbonyl groups favor the formation of a reactive conformation of the enzyme tetrahedral intermediate.

INTRODUCTION

We have recently shown (*1*, *2*) that the influence of the leaving group on the reactivity of specific anilides in enzyme hydrolysis involves its binding to the enzyme (steric effect) and electronic nature (electronic effect). Therefore, separate study of the steric effect is possible only in the case of equal electronic nature of the substituents in the aniline moiety. This feature is presented by acetyl-L-phenylalanine p-alkoxycarbonyl anilides. We have studied the rate-enhancement specificity of α-chymotrypsin toward these anilides and found that it is entropy controlled.

MATERIALS AND METHODS

Bovine α-chymotrypsin was obtained from Worthington, and the normality of the enzyme stock solution was determined by titration with *N-trans*-cinnamoylimidazole (*3*). Acetyl-L-phenylalanine p-alkoxycarbonyl anilides were synthesized by the general method previously described (*2*, *4*) from acetyl-L-phenylalanine amide and the corresponding p-alkoxycarbonyl aniline hydrochlorides. p-Alkoxycarbonyl anilines were obtained from commercial sources except p-pentoxycarbonyl, p-benzyloxycarbonyl, and p-4-(2,2,6,6-tetramethyl-piperidoxy)carbonyl anilines. The latter compounds were prepared by the method of King (*5*) from nitrobenzoic acid and the appropriate alkohol. p-4-(2,2,6,6-Tetramethyl-1-oxyl-piperidoxy)carbonyl anilide was obtained from the corre-

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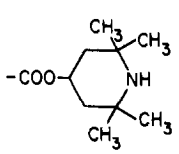
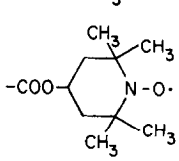
sponding *p*-4-(2,2,6,6-tetramethyl-piperidoxy)carbonyl anilide by the general oxygenation procedure used by Rozantzev (*6*). As a free radical, this anilide displays an ESP triplet in water and is stable for months. Details of the above synthesises have been described elsewhere (*7*).

The anilides were all chromatographically homogeneous and had satisfactory uv and ir spectra. Their physical constants and elemental analyses are shown in Table 1. As indicated by the extent of the chymotrypsin hydrolysis, the optical purity was in all cases greater than 96%.

The α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine *p*-carboxy and *p*-alkoxycarbonyl anilides was observed as pseudo-first-order ultraviolet absorption changes (ranged from 0.2 to 0.5 *A*) at 295 and 305 nm, respectively. The second-order rate constants k_{cat}/K_m (specificity constants) was calculated from the pseudo-first-order rate constants $k_{cat}(E)_0/K_m$, determined by least-

TABLE 1

PHYSICAL CONSTANTS AND ELEMENTAL ANALYSIS OF ACETYL-L-PHENYLALANINE *p*-ALKOXYCARBONYL ANILIDES

p-substituent	Formula	Melting point (°C)	$(\alpha)_D^{25a}$ (degrees)	Analysis	C	H	N
-COOH	$C_{18}H_{18}N_2O_4$	241-2	42.0	Calcd	66.25	5.52	8.58
				Found	66.18	5.84	8.20
$-COOCH_3$	$C_{19}H_{20}N_2O_4$	228-9	54.4	Calcd	67.20	5.89	8.25
				Found	67.55	6.13	8.04
$-COO(CH_2)_1CH_3$	$C_{20}H_{22}N_2O_4$	220-1	44.0	Calcd	67.90	6.21	7.90
				Found	67.64	6.50	8.08
$-COO(CH_2)_2CH_3$	$C_{21}H_{24}N_2O_4$	172-4	33.0	Calcd	68.50	6.52	7.61
				Found	68.36	6.64	8.05
$-COO(CH_2)_3CH_3$	$C_{22}H_{26}N_2O_4$	196-7	40.0	Calcd	69.10	6.80	7.33
				Found	69.10	7.02	7.06
$-COO(CH_2)_4CH_3$	$C_{23}H_{28}N_2O_4$	192-4	39.0	Calcd	69.69	7.07	7.07
				Found	69.73	7.02	6.85
$-COO(CH_2)_5CH_3$	$C_{24}H_{30}N_2O_4$	178-9	33.5	Calcd	70.50	7.31	6.82
				Found	71.00	7.14	6.83
$-COO(CH_2)_1C_6H_5$	$C_{25}H_{24}N_2O_4$	177-9	34.0	Calcd	72.00	5.75	6.73
				Found	72.03	5.95	6.82
	$C_{27}H_{35}N_3O_4$	125-9	37.5	Calcd	69.67	7.52	9.03
				Found	69.49	7.74	8.96
	$C_{27}H_{34}N_3O_5$	208-9	37.5	Calcd	67.50	7.08	8.45
				Found	67.22	7.03	8.50

[a] c 0.4, acetone.

squares analysis of the pseudo-first-order traces. The specificity constant increases with deprotonation of an amino acid side chain group with a $pK_a \sim 7.0$ (imidazole group of His-57) and protonation of the α-amino group of Ile-16 (*8*, *9*). That is why the kinetic measurements at pH 8.0 provide almost pH-independent values for k_{cat}/K_m during the thermodynamic study. The activation parameters ΔH^* and ΔS^* were calculated by least-squares treatment of $\log(k_{cat}/K_m)$ versus $1/T$ plot (Arrhenius plot) and $\log(k_{cat}/K_m)$ versus $(1/T - 1/T(\text{hm}))$ plot (Krug *et al.* (*10*) plot). $\Delta G^*_{T(\text{hm})}$, the activation free-energy changes at the harmonic mean of the experimental temperatures, $T(\text{hm})$, were calculated from the equation

$$\Delta G^*_{T(\text{hm})} = RT(a - \ln(kT(\text{hm})e/h) - 1),$$

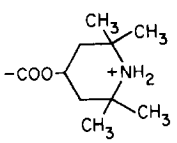
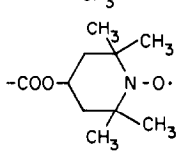
where a refers to the intercept estimate at $1/T = 1/T(\text{hm})$ of the Krug *et al.* plot (*10*).

RESULTS AND DISCUSSION

The specificity constants k_{cat}/K_m of the *p*-alkoxycarbonyl anilides studied are

TABLE 2

KINETIC AND ACTIVATION PARAMETERS OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-PHENYLALANINE *p*-ALKOXYCARBONYL ANILIDES

p substituents	k_{cat}/K_m[a] (M^{-1} sec^{-1})	ΔH[b] (kcal)	ΔS[b] (e.u.)	$\Delta H_{T(\text{hm})}$[c] (kcal)	$\Delta S_{T(\text{hm})}$[c] (e.u.)	$\Delta G_{T(\text{hm})}$[c] (kcal)
$-COO^-$	1.9					
$-COOCH_3$	36.2	9.1	−40.1	9.6	−40.4	22.08
$-COO(CH_2)_1CH_3$	36.2	9.1	−40.1	9.6	−40.4	22.08
$-COO(CH_2)_2CH_3$	60.1	10.1	−37.0	9.8	−39.1	21.85
$-COO(CH_2)_3CH_3$	100.0	12.0	−29.4	10.8	−34.6	21.44
$-COO(CH_2)_4CH_3$	126.7	—[d]	—[d]			
$-COO(CH_2)_1C_6H_5$	106.8	11.4	−31.5	10.5	−35.7	21.52
	100.7	10.4	−34.9	10.3	−36.3	21.49
	96.8	10.4	−34.9	10.3	−36.3	21.49

[a] 0.1 *M* Tris–0.05 *M* NaCl buffer, pH 8.0, 4% $HCON(CH_3)_2$, 25 ± 0.1°C. SD does not exceed 3%.

[b] Calculated by least-squares treatment of the $\log(k_{cat}/K_m)$ vs $(1/T)$ plots. Rate constants were obtained in the temperature interval 28–40°C. SD does not exceed 5%.

[c] Calculated by least-squares treatment of the $\log(k_{cat}/K_m)$ vs $(1/T - 1/T(\text{hm}))$ plot. SD does not exceed 5% for $\Delta H_{T(\text{hm})}$ and $\Delta S_{T(\text{hm})}$, and 2% for $\Delta G_{T(\text{hm})}$.

[d] Substrate solubility is too low to obtain reliable values.

shown in Table 2. This second-order rate constant is related to the free energy difference between the initial state E + S and the rate-limiting transition state between the Michaelis complex ES and the acylenzyme EA. Its direct determination from the observed pseudo-first-order absorbance changes eliminates the errors arising from an unprecise determination of the substrate concentration, the use of optically unhomogeneous substrates and low substrate concentrations.

The variation of k_{cat}/K_m with the size of the alkyl substituents in the leaving group (Table 2) suggests a rate-enhancement specificity of α-chymotrypsin toward acetyl-L-phenylalanine *p*-alkoxycarbonyl anilides. There is a great difference between the (k_{cat}/K_m) values of *p*-carboxy and *p*-alkoxycarbonyl anilides. As the corresponding values of the substituent σ^- are 0.13 and 0.68 (*11*), the observed substituent effect could be due to the different electronic nature of the leaving group, provided they have equal or no steric effect. On the other hand, if *p*-alkoxycarbonyl groups have equal electronic effects, the variation of the specificity constants could be explained by the different steric effects of these substituents. Actually, there is no substituent dependence in the case of *p*-methoxy$^-$ and *p*-ethoxycarbonyl anilides (Table 2). Furthermore, previous studies of enzyme-catalyzed hydrolysis of specific ring-activated anilides (*1, 2, 4*) have shown that the catalytic rate increases with increase in the electron withdrawal (Hammett $\rho \sim 1.0$). Therefore, since with the increase of the alkyl group length the electron withdrawal power of the *p*-alkoxycarbonyl groups decreases (σ^- values of *p*-methoxycarbonyl and *p*-ethoxycarbonyl groups are 0.782 and 0.752, respectively (*12*)), a rate-decreasing specificity should be observed. This not being the case (Table 2) suggests strongly that steric effects dominate in the α-chymotrypsin hydrolysis of acetyl-L-phenylalanine *p*-alkoxycarbonyl anilides.

The activation parameters associated with the specificity constant are also included in Table 2. The bulky alkyl substituents in the leaving group appear to increase both the enthalpy and entropy of activation. This suggests that the observed rate enhancement specificity of α-chymotrypsin toward the specific anilides studied is entropy controlled: positive changes in both activation enthalpy and entropy accompany faster rates.

The $\Delta H^* - \Delta S^*$ plot (Fig. 1a) gives a straight line with a slope (isokinetic temperature) equal to 251.3°K. The 95% confidence interval for this quantity is (398.1, 104.5) and includes the harmonic mean of the experimental temperatures, $T(\text{hm}) = 308.5$°K. Therefore, according to Krug *et al.* (*10*) the observed compensation phenomenon involves both statistical and thermodynamic compensation effects. In order to discriminate between the statistical and chemical patterns the regression should be of enthalpy, $\Delta H^*_{T(\text{hm})}$, or free energy, $\Delta G^*_{T(\text{hm})}$, evaluated at the harmonic mean of the experimental temperatures (*13*). In this uncorrelated plane (Fig. 1b) there is no linear correlation, suggesting that the observed compensation effect in the $\Delta H^* - \Delta S^*$ plane (Fig. 1a) is a realization of the propagation of experimental error. Therefore, the observed entropy-controlled rate-enhancement specificity could not be attributed to the specific solvation processes accompanying enzyme–substrate interactions (*14*).

Compelling evidence for the presence of a hydrophobic leaving-group-binding pocket, contiguous with the active site region of α-chymotrypsin (*2, 15, 16*),

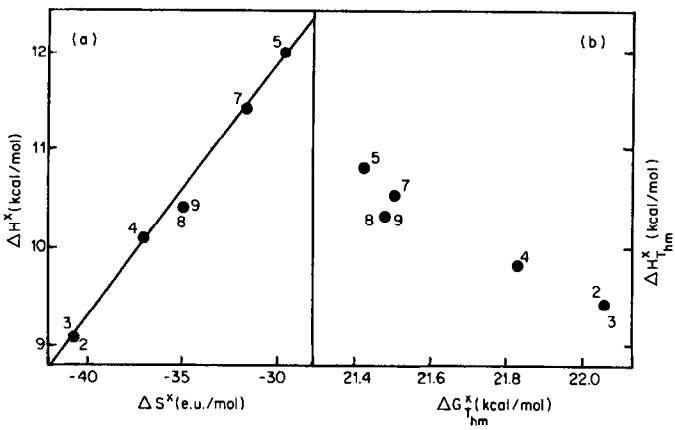


FIG. 1. Arrhenius (a) and Krug *et al.* (*10*) (b) plots for the specificity constant k_{cat}/K_m of α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine *p*-alkoxycarbonyl anilides. The figures are the numbers of the anilides in Table 2.

provides a basis for the interpretation of the observed rate-enhancement specificity. The model binding studies of Farmer and Hageman (*17*) have shown that specific anilides with large *p*-substituents were difficult to position in the pocket. Therefore, the bulky *p*-alkoxycarbonyl leaving groups of the anilides studied do not fit sterically in the leaving-group-binding pocket and are probably out of it in the transition state of the enzyme acylation. This conclusion is in concurrence with the observed lack of compensation effect (Fig. 1b), since the existence of a thermodynamic compensation effect is usually interpreted as indication of enzyme–substrate hydrophobic interaction (*14*). This is also supported by the fact that the elimination of the positive charge in acetyl-L-phenylalanine *p*-4-(4,2,6,6-tetramethyl-piperidoxy)carbonyl anilide (Table 2) does not practically affect the reactivity of the substrate, indicating that this bulky substituent does not interact with the hydrophobic leaving-group-binding pocket of the enzyme. On the other hand, the binding studies of the interactions of α-chymotrypsin and specific anilides using the proflavin-displacement method (*2*) revealed that better binding does not lead to better catalysis. Unfortunately, the low solubility of the anilides studied prevents the use of this method for the determination of enzyme–substrate binding constant K_s. The K_m value of acetyl-L-phenylalanine *p*-4-(2,2,6,6-tetramethyl-piperidoxy)carbonyl anilide, the only soluble in buffer solution *p*-alkoxycarbonyl anilide, is equal to 5.55 m*M* (*7*), whereas the corresponding value for acetyl-L-phenylalanine *p*-nitroanilide is 0.40 m*M* (*2*). The *p*-4-(2,2,6,6-tetramethyl-piperidoxy)carbonyl anilide, however, hydrolyzes more than 50 times faster (k_{cat} = 0.53 sec^{-1} (*7*)) than *p*-nitroanilide(k_{cat} = 0.01 sec^{-1} (*2*)). All these results could be interpreted in terms of the stereoelectronic theory for the formation and cleavage of the tetrahedral intermediate in acyltransfer reactions (*18*).

By analogy with the nonenzymatic reactions an enzyme tetrahedral intermediate ET is presumed to intervene between the Michaelis complex ES and the

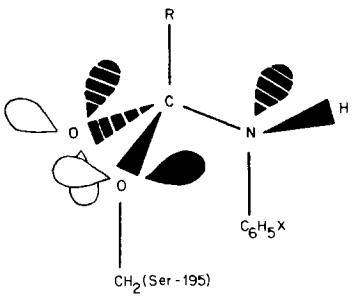


FIG. 2. Conformation of the enzyme tetrahedral intermediate and the orientation of the nonbonded electron pairs of the heteroatoms as derived from the crystal structure of bovine trypsin–pancreatic trypsin inhibitor complex (*2*). X denotes the *p*-alkoxycarbonyl group.

acylenzyme (*2, 4, 19*):

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} ET \xrightarrow{k'_{+2}} EA + P_1 \xrightarrow{k_{+3}} E + P_2 \qquad [2]$$

According to the stereoelectronic theory (*18*) as applied to enzyme hydrolysis (*2*), the effectiveness of the enzyme catalysis is controlled by the orientation of the leaving-group-nitrogen nonbonded electron pair of the enzyme tetrahedral intermediate ET (Fig. 2). The binding of the leaving group hampers the development of a reactive conformation of the enzyme tetrahedral intermediate. This is supported by the fact that the observed rate-enhancement specificity is entropy controlled: for anilides with bulky substituents the entropy of activation is up to 6–11 e.u. less negative than that of acetyl-L-phenylalanine *p*-methoxycarbonyl anilide (Table 2). The elimination of the leaving group binding makes the free rotation around the C–N bond (Fig. 2) possible in the transition state of the formation of enzyme tetrahedral intermediate. The calculated and observed changes in the activation entropy associated with freezing of the rotation of one bond are up to 7.5 e.u. (*20*). Thus, the favorable steric effect of bulky alkyl substituents could be interpreted as providing experimental evidence in favor of the validity of the stereoelectronic theory in enzyme-catalyzed hydrolysis.

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