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CATALYSIS AND LEAVING GROUP BINDING IN ANILIDE HYDROLYSIS BY CHYMOTRYPSIN

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Summary

The influence of the leaving group on the reactivity of specific anilides in α-chymotrypsin-catalyzed hydrolysis (chymotrypsin, EC 3.4.21.2) involves both its binding to the enzyme (steric effect) and electronic nature (electronic effect). These effects are considered in terms of the stereoelectronic theory for the formation and cleavage of the tetrahedral intermediate in acyltransfer reactions. The application of this theory to the enzyme hydrolysis leads to the conclusion that the nature of the reaction products and the effectiveness of the catalysis are controlled by the orientation of the leaving group nitrogen lone pair orbital. The leaving group binding affects the formation of a reactive conformation of the enzyme tetrahedral intermediate that is presumed to intervene between the Michaelis complex and the acylenzyme. The steric and electronic effects could be separated in a straightforward fashion only in the case of equal binding of the leaving groups to the leaving-group-binding site of α-chymotrypsin.

Introduction

Although α-chymotrypsin-catalyzed hydrolysis of specific anilides has been extensively studied, the results are still inconclusive. The electronic effects of the substituents in the aniline ring on the steady-state parameters are the basic mechanistic information in these studies. It is, however, difficult to separate these effects from the steric effects of the substituents. Assuming that the steric effects are small compared to the electronic effects, positive [1—3], negative [4] and no [5,6] substituent dependences have been found. These contradictory data and the compelling evidence for the presence of a leaving group

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Abbreviation: K_{EF}, enzyme-proflavin binding constant.

binding site contiguous with the active site region [7—9] suggest that the neglecting of the steric effects is a too rough approximation. In the present study, we reinvestigate the problem using acetyl-L-phenylalanine ring-activated anilides as substrates. The electronic effects of the electron-attracting substituents (ring-activating groups) are known to be strongly negative in the model nonenzymatic reactions [10,11]. We have determined directly the specificity constant k_{cat}/K_m of these anilides and, in separate experiments, their binding to the enzyme using the proflavin displacement method [12]. The results clearly suggest that the influence of the leaving group on the specificity constant involves both its binding (steric effect) to the enzyme leaving-group-binding site and its electronic nature (electronic effect). The two effects could be separated in a straightforward fashion only in the case of equal binding of the leaving groups to the enzyme leaving-group-binding site.

Materials and Methods

α-Chymotrypsin

3 times crystallized, salt-free, lyophilized bovine α-chymotrypsin was purchased from Worthington. Stock solutions were made in 10^{-3} M HCl. Protein concentration was determined spectrophotometrically using $\epsilon_{280} = 50\ mM^{-1} \cdot cm^{-1}$ as a molar absorption coefficient and molecular weight of 24 800. The functional molarity of the enzyme stock solutions was determined by active-site titration with *N-trans*-cinnamoylimidasole [13]. The enzyme was found to be 95—97% active.

Substrates and inhibitors

Acetyl-L-phenylalanine anilides. These were prepared by the method of Galat and Elion [14]. An equimolar mixture of crystalline acetyl-L-phenylalanine amide prepared according to the method of Huang et al. [15] and the corresponding aniline hydrochlorides were heated in an oil bath to 155°C. The resulting oils were cooled and the melts subjects to fractional crystallization from ethanol/water using the large difference in the solubility of acetyl-L-phenylalanine and acetyl-DL-phenylalanine anilides. As is indicated by the extent of the α-chymotrypsin hydrolysis, the first fraction is pure racemate and the last one the optically active L-compound. The melting point and specific rotation of the *p*-nitroanilide coincide with the corresponding values for the optically homogeneous L-compound obtained by Ramenskii et al. [16]. The *p*-nitroanilide synthesized and used by Fink [17] is not optically homogeneous as the melting point reported (262—264°C) is between that of the racemate (270—272°C) and the L-compound (243—245°C).

Acetyl-L-phenilalanine N-methylanilides. These were prepared by selective methylation of the corresponding anilides according to the method of Pachter and Kloetzel [18]. The presence of water of crystallisation in the solid acetyl-L-phenylalanine *N*-methyl-*p*-nitroanilide has been proved by infrared spectroscopy. The direct coupling of acetyl-L-phenylalanine amide and *N*-methyl-*p*-nitroaniline hydrochloride gives an anilide with no specific rotation, probably acetyl-DL-phenylalanine *N*-methyl-*p*-nitroanilide. The basic hydrolysis of the

N-methylanilides gives quantitatively acetyl-phenylalanine and the corresponding *N*-methylanilines.

The physical constants and elemental analyses of the synthesized compounds are summarized in Table I.

Kinetic studies

The α-chymotrypsin-catalyzed hydrolysis of the anilides studied was followed both spectrophotometrically using a Cary 118 Spectrophotometer and titrimetrically using a Radiometer pH-stat assembly (TTT11/PHM26 titrator + SBU1a syringe burette assembly + SRBR2c titrigraph). The reactions were carried out under pseudo-first-order conditions, $K_s >> [S] >> [E]$, in 0.05 M Tris-HCl buffer pH 7.6, 5% (v/v) *N,N*-dimethylformamide (spectrophotometric experiments) and 0.1 M KCl, pH 7.6, 5% (v/v) *N,N*-dimethylformamide (pH-stat experiments), at 25 ± 0.1°C. The wavelengths monitored were 410 nm for *p*-nitro-, 294 nm for *p*-methylsulfonyl-, 296 nm for *p*-cyano-, 340 nm for *p*-acetyl- and 400 nm for *p*-nitro-*o*-methylanilide. The automatic end-point titration with $2.0 \cdot 10^{-4}$ M KOH (freed of CO_2) of acetyl-L-phenylalanine released during the hydrolysis was carried out in the pH-stat cell under N_2. The second-order rate constant k_{cat}/K_m was calculated from the pseudo-first-order rate constant $k_{cat} \cdot [E]_0/K_m$ determined both from the first-order absorbance changes due to the aniline release (spectrophotometric experiments) and from the first-order changes in the base consumption (pH-stat experiments) due to acetyl-L-phenylalanine release.

The stopped-flow direct search for the accumulation of intermediates was performed as described by Fastrez and Fersht [5] using a Durrum D-10 Stopped-Flow Analyser. 1 mM α-chymotrypsin Tris-HCl buffer pH 7.6 was

TABLE I

PHYSICAL CONSTANTS AND ELEMENTAL ANALYSIS OF ACETYL-L-PHENYLALANINE DERIVATIVES (ACETYL-L-Phe-X)

X	Formula	M.P. (°C)	$(\alpha)_D^{25}$ (0.5 acetone) (degrees)	Analysis		
				C	H	N
p-$NO_2C_6H_4NH$	$C_{17}H_{17}N_3O_4$	243-5	77.6	Calcd. 62.38	5.20	12.84
				Found 62.29	5.32	12.50
p-$CH_3SO_2C_6H_4NH$	$C_{18}H_{20}N_2O_4S$	226-7	57.6	Calcd. 60.00	5.55	7.78
				Found 59.80	5.63	7.76
p-CNC_6H_4NH	$C_{18}H_{17}N_3O_2$	227-8	54.0	Calcd. 70.36	5.53	13.89
				Found 70.59	5.69	13.68
p-$CH_3COC_6H_4NH$	$C_{19}H_{20}N_2O_3$	222-3	58.0	Calcd. 70.37	6.17	8.64
				Found 69.95	6.40	8.86
p-NO_2,*o*-$CH_3C_6H_3NH$	$C_{18}H_{19}N_3O_4$	170-1	8.5	Calcd. 63.34	5.57	12.32
				Found 63.50	5.70	11.98
p-$NO_2C_6N_4NCH_3 \cdot H_2O$	$C_{18}H_{21}N_3O_5$	72-6	227.7	Calcd. 60.20	5.85	11.70
				Found 60.24	6.01	11.89
p-$NO_2C_6H_4NCH_3$	$C_{18}H_{19}N_3O_4$	173-5	0.0	Calcd. 63.34	5.57	12.32
				Found 63.78	5.81	12.05
p-$CH_3COC_6H_4NCH_3$	$C_{20}H_{22}N_2O_3$	141-3	175.0	Calcd. 70.98	6.55	8.28
				Found 71.13	7.07	8.21

mixed with 5—20 mM anilide in pH 7.6 Tris-HCl buffer containing 10% *N,N*-dimethylformamide and the transmittance at the corresponding analytical wavelength was monitored. No relaxations were observed in the stopped-flow time range.

Binding studies

The binding studies were performed under the conditions used in the kinetic studies using the proflavin-displacement method as described by Brandt et al. [12]. The enzyme-substrate binding constants K_s were calculated from the equation

$$K_s = \frac{[EF]_s}{[F]} K_{EF}[S_0]\left[[E_0] - [EF]_s\left(1 + \frac{K_{EF}}{[F]}\right)\right]^{-1} \quad (1)$$

where E, S and F refer to the enzyme, substrate and proflavin. The required value for the concentration of the α-chymotrypsin-proflavin complex in the presence of substrate $[EF]_s$ has been calculated from the difference in the absorption at 469 nm of the enzyme-proflavin complex in the presence and absence of the substrate. The total absorbance changes measured with a Cary 118 Spectrophotometer ranged from 0.003 to 0.03 absorbance units depending on the substrate and its concentration. The α-chymotrypsin-proflavin binding constant K_{EF} has been measured from the α-chymotrypsin-proflavin binding data at 469 nm. The value obtained and used in the calculations is $3.3 \pm 0.3 \cdot 10^{-4}$ M. It is higher than that reported in the literature [12] due to the effect of the *N,N*-dimethylformamide (5% v/v) on the proflavin binding.

Results and Discussion

Table II summarizes the kinetic and binding data for the α-chymotrypsin-catalysed hydrolysis of acetyl-L-phenylalanine anilides obtained in this work

TABLE II

KINETIC AND BINDING PARAMETERS FOR THE CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-PHENYLALANINE DERIVATIVES (Acetyl-L-Phe-X) AT 25°C AND pH 7.6—8.0

The quantity k_{cat}/K_m was determined from the first-order plots of the kinetic data obtained spectrophotometrically (A) and titrimetrically (B). Enzyme-substrate binding constants K_s were determined using the proflavin-displacement method [12]. The acylation rate constants k_{+2} were calculated using the equation $(k_{cat}/K_m) = (k_{+2}/K_s)$ [19]. The values for σ^- are taken from [25].

X	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)		K_s (mM)	k_{+2} (s^{-1})	σ^-	ref.
	A	B				
p-$NO_2C_6H_4NH$	25.0 ± 2.1	19.3	0.40 ± 0.02	0.01 ± 0.001	1.27	This study
p-$CH_3SO_2C_6H_4NH$	71.5 ± 6.0	66.0	3.63 ± 0.32	0.26 ± 0.045	1.05	This study
p-CNC_6H_4NH	20.8 ± 2.4	21.6	1.84 ± 0.20	0.04 ± 0.005	1.00	This study
p-$CH_3COC_6H_4NH$	38.5 ± 3.2	33.0	3.40 ± 0.32	0.14 ± 0.023	0.87	This study
p-NO_2,*o*-$CH_3C_6H_4NH$	3.8 ± 0.3		2.53 ± 0.30	0.01 ± 0.001		This study
p-$NO_2C_6H_4NCH_3$			>2.50			This study
p-$CH_3COC_6H_4NCH_3$			124.50 ± 15.0			This study
$NH_2COCH(CH_3)NH$		114.0	25.00 ± 1.20	2.80 ± 0.200		7
NH_2	1.5		30.00	0.05		33
NH_2			28.00 ± 7.00			12
OCH_3		10^5	7.63	796.00		21

together with the data from the literature for the corresponding amides and ester. The determined kinetic parameter is the second-order rate constant k_{cat}/K_m (the specificity constant). The determination of the catalytic constant k_{cat} and the Michaelis constant K_m is not precise enough because the K_s values of the specific anilides studies are quite high, necessitating the use of substrate concentrations approaching or far beyond their solubility limit in buffer solutions. Moreover, the specificity constant has a definite physical meaning being 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:

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_{+2}}{\longrightarrow} EA + P_1 \overset{k_{+3}}{\longrightarrow} E + P_2 \qquad (2)$$

It is equal to k_{+2}/K_s and being independent of the non-productive binding of the substrate [19] is the most logical and reliable parameter to use for the structure-reactivity relationships. In order to measure k_{cat}/K_m as accurately as possible, the reaction should be carried out under pseudo-first-order condition, $K_s >> [S] >> [E]$. As the K_s values of the anilides studied are around 10^{-3} M (Table II), a reaction of 10^{-5} M α-chymotrypsin and 10^{-4} M anilide was accordingly found to be first-order in substrate following it both spectrophotometrically or titrimetrically. The specificity constants were found by conventional analysis of the corresponding pseudo-first order traces. The results from the two methods of observation are included in Table II. They are in very good agreement suggesting that the values obtained can be considered reliable.

An examination of the values of k_{cat}/K_m for the acetyl-L-phenylalanine anilides studies in Table II shows that there are no trends in their changes with the electron-withdrawing poor power of the para-substituents. Similar behaviour has been observed in the α-chymotrypsin hydrolysis of acetyl-L-tyrosine anilides [5,6] and attributed [5] to the effects of the substituents on the ratio of productive (aniline moiety in the leaving group site) and non-productive (aniline moiety in the amino acid side chain site) modes of binding. However, it was subsequently demonstrated [20] that this explanation could not be substantiated: the corresponding D-isomer that is suitable for non-productive binding, binds even more weakly than the L-isomer. Moreover, after suitable corrections for the non-productive binding, Fastrez and Fersht [5] have again found no correlation.

Since non-productive binding is not important in the chymotrypsin anilide hydrolysis, the binding constants determined in studies using the proflavin-displacement method could be considered as enzyme-substrate binding constants, K_s. Indeed, after adding equal volumes of chymotrypsin stock solution to the reference cell containing proflavin and substrate, and to the sample cell containing only proflavin, a positive absorbance difference, disappearing in time, was observed (Fig. 1). On the other hand, the stopped-flow experiments show no accumulation of a tetrahedral intermediate; there is no accumulation of the acylenzyme, acetyl-L-phenylalaninyl-chymotrypsin, since its deacylation is too fast ($k_{+3} = 111\ s^{-1}$ [21]) to be considered rate-limiting. Therefore, the observed increase in absorbance at 469 nm reflects the dissociation of the enzyme-proflavin complex brought about by the formation of a Michaelis complex, ES.

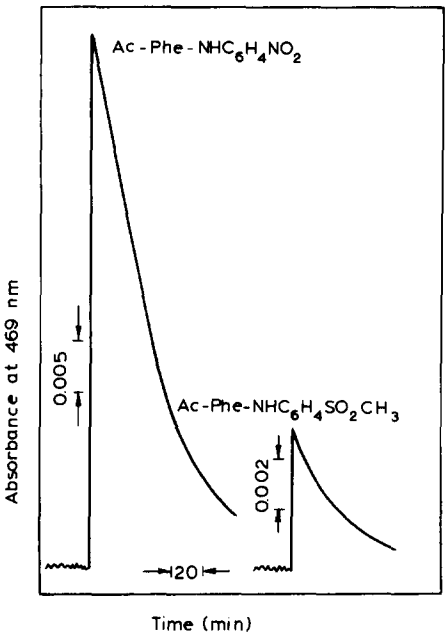

Fig. 1. Absorbance changes at 469 nm, measured by Cary 118 Spectrophotometer, after adding of equal volumes of chymotrypsin to the sample cell containing proflavin and to reference cell containing proflavin plus acetyl-L-phenylalanine *p*-nitroanilide or *p*-methylsulfonylanilide. Final concentrations of the reagents: $[E_0] = 5.0 \cdot 10^{-5}$ M, $[F_0] = 2.5 \cdot 10^{-5}$ M and $[S_0] = 5.0 \cdot 10^{-4}$ M in Tris-HCl buffer pH 7.6, 5% (v/v) *N*,*N*-dimethylformamide, 25°C.

The K_s values of the acetyl-L-phenylalanine anilides studied (Table II) are in the range of 0.4—3.6 mM whereas the corresponding value for acetyl-L-phenylalanine amide is 32 mM. On the other hand, the anilides with the smaller and more hydrophobic nitro- and cyano- groups bind more strongly then the anilides with larger and more hydrophilic methylsulfonyl and acetyl groups. The enhanced binding of the anilides compared to the amide and that of hydrophobic compared to hydrophilic anilides suggests an additional hydrophobic interaction of the aniline moieties with the presumed hydrophobic leaving group site, contiguous with the chymotrypsin active site region [7,8]. These results support the inferences from the model binding studies made by Farmer and Hageman [9]. They found the presence of a leaving group hydrophobic binding pocket in the chymotrypsin lined by Ala-55, Gly-196, Ile-212, Gly-216, Ile-99 and Val-213, and also that large substituents do not fit sterically in this pocket. The last conclusion is further substantiated by the effect of the *o*-methyl substitution decreasing binding to about 1/8 (Table II). The effect is more significant in the case of *N*-methylation of *p*-nitro- and *p*-acetylanilides, where more than an 8-fold and about 36-fold decrease in binding, respectively, were observed (Table II) as compared to the corresponding unsubstituted anilides. The *N*-methyl-*p*-acetylanilide binds even more weakly than the corresponding amide indicating a perturbation of all enzyme-substrate interactions. These effects strongly suggest the importance of the steric effects in the

chymotrypsin anilide hydrolysis and show that their ignorance in the interpretation of the substituent effects would lead to wrong conclusions.

It worth noting that the relationships as discussed above could be found by examining the K_m-values of acetyl-L-tyrosine anilides and amides summarized by Fastrez and Fersht [5]. This coincidence could be interpreted as providing evidence that K_m is a measure of K_s in the enzyme hydrolysis of these anilides.

p-Nitroanilides are the most reactive anilides in the nonenzymatic (alkaline) hydrolysis regardless of the catalyst nature (general acid, general base of general acid-base) [10,11]. Under conditions that suffice to hydrolyse the anilide completely, the acetyl-glycine amide bond in the acetylglycine *p*-nitroanilide was unaffected (Petkov, D. and Pojarlieff, I., manuscript in preparation). The ninhidrin test shows that only 2% of the anilide has undergone an acetyl-glycine amide bond cleavage reaction. This has not been found in the enzyme hydrolysis: acetyl-L-phenylalanine *p*-nitroanilide (Table II) and acetyl-L-tyrosine *p*-nitroanilide [5] are among the least reactive anilide substrates of chymotrypsin. The former hydrolyses even more slowly than the non-activated chymotrypsin substrate acetyl-L-phenylalaninyl-L-alanine amide (Table II). Therefore, the interactions of the aniline moieties with the leaving group site greatly affect the reactivity of the anilide substrates in the enzyme hydrolysis. This effect is more pronounced in the case of *o*- and *N*-methylation. The last modification gives rise to the complete loss of the reactivity of *p*-nitro- and *p*-acetylanilides: under the conditions used with the unmodified anilides no chymotrypsin hydrolysis has been observed. Such a loss in reactivity has been reported for acetyl-L-phenylalaninyl-L-alanine amide as well: the corresponding acetyl-L-phenylalaninyl-sarcosine amide is inactive in chymotrypsin hydrolysis [22].

It is generally accepted that the most common mechanism for the nonenzymatic acyl-transfer reactions proceeds through the formation of a tetrahedral intermediate. By anology with the non-enzymatic mechanism, an intermediate ET with a tetrahedral configuration of the attacked carbonyl carbon is presumed [3,5,11] to intervene between the Michaelis complex ES and the acylenzyme EA in the enzyme hydrolysis of specific anilides:

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

Quite recently, the importance of the conformation of the tetrahedral intermediate for the hydrolysis has been pointed out [23]. It has been deduced empirically [23] and substantiated theoretically [24] that the expelling of the leaving group from the tetrahedral intermediate requires the assistance of two antiperiplanar lone pair (non-bonding) orbitals on the remaining heteroatoms. An examination of the conformation of the tetrahedral intermediate (Fig. 2) crystallographically derived from the bovine trypsin-pancreatic trypsin inhibitor complex [25] shows that the C^{β}-O bond of Ser-195 lies in a plane bisecting the angle formed by the former carbonyl O, former carbonyl C and the inhibitor Ala-16 N. In this conformation O^{γ} of Ser-195 has a lone pair orbital oriented antiperiplanar to the inhibitor Lys-15 C-Ala-16 N bond (Fig. 3). On the other hand, the orientations of the three equivalent lone pair orbitals of the former carbonyl O are fixed by hydrogen bonding (Fig. 2) of this oxyion with the "oxyion hole" formed by the backbone NH of Gly-193 and Ser-195 [26].

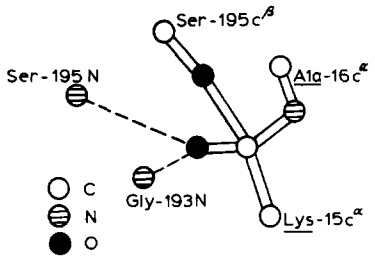

Fig. 2. Structural model of the enzyme tetrahedral intermediate formed by bovine trypsin and pancreatic trypsin inhibitor [25]. Inhibitor residues are underlined.

A similar conformation of the tetrahedral intermediate could be derived examining the crystal structure of the porcine trypsin-soybean trypsin inhibitor complex [27,28]. Therefore, if we suppose that hydrogen bonding fixation of the oxyion lone pair orbitals is as predicted by stereoelectronic theory, the nature of the products and the effectiveness of the enzyme catalysis depend on the orientation of the leaving nitrogen lone pair orbital. If it is antiperiplanar to the Ser-195 O^{γ}-C bond (Fig. 3a), the reaction goes both to the acylenzyme EA and to Michaelis complex ES; if it is synclinal to the Ser-195 O^{γ}-C bond (Fig. 3b), the enzyme tetrahedral intermediate ET breaks down exclusively to an acylenzyme (Eqn. 3). Due to the structural similarities of trypsin and chymotrypsin, there is little doubt that their conformations of ET are closely similar.

From the conclusion that the orientation of the leaving nitrogen lone pair orbital controls the nature of the reaction products and the effectiveness of the enzyme catalysis follows the importance of the leaving group conformation. Since it could be greatly affected by the leaving group binding, the kinetic behaviour of the specific anilides studied in a chymotrypsin hydrolysis should be in concurrence with the above corollaries from the stereoelectronic theory.

As follows from the principle of the microscopic reversibility, the stereo-

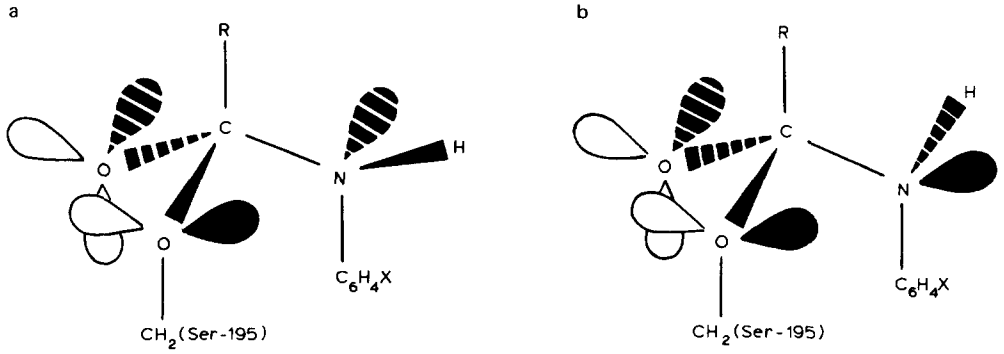

Fig. 3. Conformation of the enzyme tetrahedral intermediate and the orientation of the lone pair orbitals of the heteroatoms as derived from the crystal structure of bovine trypsin-pancreatic trypsin inhibitor complex (Fig. 2). The orientation of the leaving group nitrogen lone pair orbital could be antiperiplanar (a) or synclinal (b) to the Ser-195 O^{γ}-C bond.

electronic requirements for the forward reaction should be valid for the reverse reaction as well: the formation of the tetrahedral intermediate [23]. This means that the incipient lone pair orbitals on the carbonyl oxygen and on the leaving group nitrogen should be antiperiplanar to the incipient Ser-195 O^{γ}-C bond. Therefore the nonreactivity of the N-methylated anilides in chymotrypsin hydrolysis could be accounted for by the unfavourable steric effect of the methyl group on the formation of a productive conformation of ET. The same effect of the methyl group has been accepted by Bizzozero and Zweifel [22] to be the cause for the non-reactivity of acetyl-L-phenylalaninyl-sarcosine amide in chymotrypsin hydrolysis.

The formation of a conformation of ET with an antiperiplanar orientation of the leaving group nitrogen lone pair orbital to the Ser-195 O^{γ}-C bond is a necessary but not sufficient condition for an effective catalysis. It is evident (Fig. 3) that the protonation of the leaving group eliminates the nitrogen lone pair orbital assistance in the cleavage of the Ser-195 O^{γ}-C bond. As a result, the protonated ET breaks down exclusively to an acylenzyme EA. This is the role of the general acid catalysis in terms of the stereoelectronic theory. The importance of this point to our present discussion is that if ET forms with the *N*-methylated anilides, the *N*-methyl group prevents the subsequent protonation of the leaving group. The model building studies of Bizzozero and Zweifel [22] have shown that the direct protonation of the leaving group nitrogen form His-57 is possible only in the case of a synclinal orientation of the leaving group nitrogen lone pair orbital to the Ser-195 O^{γ}-C bond (Fig. 3b). As the formation of this conformer can not be achieved by an orbital assisted mechanism, its formation from the conformer with an antiperiplanar orientation of the leaving group nitrogen lone pair orbital (Fig. 3a) requires a pyramidal inversion at the leaving nitrogen. This inversion could be hampered by the methyl group in the *N*-methyl anilides and an accumulation of ET and a change in the spectrum due to the difference in spectra of an anilide and its ET [5] should be observed. There is no change in the transmittance, however, after mixing solutions of an *N*-methylated anilide and chymotrypsin in the stopped-flow cell. This favours the assumption of steric hindrance preventing the formation of a productive conformation of ET as a reasonable explanation of the *N*-methyl anilides nonreactivity in chymotrypsin hydrolysis.

The fact that acetyl-L-phenylalanine anilides undergo enzyme hydrolysis indicates the realization of a conformation of ET with an antiperiplanar orientation of the leaving nitrogen lone pair orbital to the Ser-195 O^{γ}-C bond (Fig. 3a). The development of this conformation, however, is hampered because the interactions of the aniline moiety with the leaving group binding site in ES state have to be overcome. This effect is well pronounced in the calculated first-order rate constant k_{+2} (Table II), because it is related to the free energy difference between the Michaelis complex ES and the transition state ES* (Eqn. 2). In terms of this interpretation it is clear that the steric and electronic affects could be separated only in the case of equal binding (equal steric effect). This condition is fulfilled for acetyl-L-phenylalanine *p*-methylsulfonyl- and *p*-acetylanilides (Table II) and for acetyl-L-tyrosine *p*-nitro- and *p*-chloroanilides [5]. The small negative substituent dependence of k_{cat}/K_m or k_{+2} with the anilide of these pairs ($\rho \simeq 1$) is in concurrence with the observed substi-

tuent effect on the formation of a tetrahedral intermediate in nonenzymatic anilide hydrolysis [10,11]. The formation of ET being rate-limiting in the tetrahedral mechanism (Eqn 3) is strongly supported by the observed very low or absent nitrogen isotope effect in the chymotrypsin amide hydrolysis [29, 30]. This conclusion is also consistent with the observed absence of accumulation of ET with the anilides studied in stopped-flow experiments.

An alternative explanation of the kinetic behaviour of the specific anilides in chymotrypsin hydrolysis would attribute an unfavourable effect of the leaving group binding on the pyramidal inversion at the leaving group N. The pyramidal inversion is hampered when the nitrogen has substituents involved in cycles [31]. This isomerization should be important when the energy barrier for the supposed pyramidal inversion is smaller than the energy barrier for the breakdown of the preceding ET conformer. Since the rate-limiting step in the breakdown of the tetrahedral intermediate with ring-activated anilides is not the proton transfer but the heavy-atom reorganization [10], the proposed rate-limiting pyramidal inversion at the leaving group nitrogen is not tenable.

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