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Influence of Leaving-Group Electronic Effect on α-Chymotrypsin: Catalytic Constants of Specific Substrates

(enzyme/protease/kinetics/intermediates)

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ABSTRACT **Rate constants and binding constants for the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyltyrosine, tryptophan, and phenylalanine anilides are presented. Both k_{cat} and K_m are independent of electronic effects in the substrate over a range of 9.8 orders of magnitude (as measured by pK of the leaving group). Similarly, K_m is independent of charge and orientation about the α-carbon for various substrates and pseudo-substrates. These results are not consistent with the pretransition state protonation hypothesis; instead, they are discussed in terms of a tetrahedral intermediate that is thermodynamically less stable than the Michaelis complex.**

Several workers have studied the substituent dependence of α-chymotrypsin-catalyzed hydrolysis of anilides, but the results are inconclusive. It has been shown (1, 2) that for *N*-acetyltyrosine anilides substituted in the aniline ring, electron-donating substituents enhance k_{cat}, the rate constant for acylation ($\rho = -2.0$). Binding of the anilide to the enzyme, however, is inhibited by electron-withdrawing groups, although a plot of K_m versus σ gives only a fair correlation (ρ = about −2)

$$E + S \overset{K_m}{\rightleftharpoons} ES \overset{k_{cat}}{\rightarrow} ES' \rightarrow E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

In order to account for these results, it was proposed that protonation of the leaving aniline occurs in a rapid preequilibrium before the rate-determining step of the reaction (2–4). The pretransition state protonation theory accounts for the accelerating effect of electron-donating groups in the ring by the formation of a positive charge on the amide nitrogen adjacent to the ring. This explanation, however, is based on work that covered only a small range of substituents in the ring (*p*-CH_3O, *p*-CH_3, *m*-CH_3O, *p*-Cl, and *m*-Cl). Bundy and Moore (5), have found that for hydrolysis of substituted *N*-benzoyltyrosine anilides by α-chymotrypsin, the *p*-nitroanilide has a k_{cat} twice that of the *m*-nitroanilide. Furthermore, the K_m for the *p*-nitro compound is about three times that for the *m*-nitro. These results are not in accord with the pretransition state protonation theory, since a *p*-nitro group is more electron-withdrawing than is a *m*-nitro substituent. In addition, Inagami, Patchornik and York, (3) found that, in the *N*-acetyltyrosine system, the *p*-nitroanilide showed a large positive deviation from the correlation for both k_{cat} and K_m.

Several explanations may be advanced for the apparent deviation of the nitro-substituted compounds. The first is that the nitro substituent may be anomalous, as suggested by Inagami (3). The second is that there may be a change in mechanism so that the leaving group in the case of the nitroanilides is not the neutral aniline molecule, but is rather the negatively-charged anilide ion. A model for this process may be seen in previous work from these laboratories in which it was found that the hydroxide ion-catalyzed hydrolysis of ring-substituted acetanilides occurs through a pathway involving protonation of the amide nitrogen for all compounds except *p*-nitroacetanilide and *p*-formylacetanilide. For these two compounds, the leaving group is the negative anilide ion (6, 7). A third possibility is that there are specific interactions other than electronic effects between the anilides and chymotrypsin that cause these compounds to give different kinetic constants and that the correlation is fortuitous.

The purpose of this publication is to point out that literature data, extended here, can resolve the difficulties introduced by the previous results. Two sets of data are compiled. The first is a tabulation of chymotrypsin kinetic constants for various *N*-acetyl-L-tyrosine substrates. These constants were obtained under conditions similar enough so that any effects caused by differences in conditions should not affect the overall results. These substrates (various amides and one ester) vary greatly but continuously in the pK of the leaving group. To extend literature data so that the pK values corresponding to the amides and the ethyl esters approach each other, two new compounds were prepared and used as substrates.

The second set of data is a compilation of binding constants for various specific substrates and pseudo-substrates. These constants were also obtained under very similar conditions. They allow comparisons to be made showing the effect of very large pK changes in the leaving group and the effect of charge and configurational changes at the alpha-carbon atom. The trends observed in these two sets of data are then compared with those predicted from kinetic relationships derived assuming the existence of a tetrahedral intermediate on the reaction course.

MATERIALS AND METHODS

α-Chymotrypsin (three times recrystallized) was purchased from Worthington Biochemicals. Its concentration in solution was determined by the active-site titration method with *N-trans*-cinnamoylimidazole (8).

N-Acetyl-L-tyrosine *m*-nitroanilide was prepared by dissolving 2.40 g of *N*-acetyl-L-tyrosine and 1.2 ml of *N*-methylmorpholine in 100 ml of tetrahydrofuran at 0°. Ethyl chloro-

TABLE 1. *Kinetic constants for chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine derivatives*

Substrate	k_{cat}, k_2 (sec^{-1})	K_m, (M) × 10^2	k_{cat}/K_m (M^{-1} sec^{-1})	pK of the leaving group
Ethyl ester (25)	5.33 × 10^3	1.8	2.76 × 10^5	−0.553 (21)
p-Nitroanilide*	0.0442	0.0595	74.3	0.98, 1.11 (22)
m-Nitroanilide*	0.0921	0.435	21.2	2.45 (22)
m-Chloroanilide (1)	0.011	0.082	13.4	3.46 (22)
p-Chloroanilide (1)	0.014	0.067	20.9	3.81 (22)
m-Methoxyanilide (1)	0.047	0.60	7.83	4.20 (22)
p-Methylanilide (1)	0.087	1.3	6.69	5.07 (22)
p-Methoxyanilide (1)	0.21	1.2	17.5	5.29 (22)
Hydrazide (26)	0.059	3.0	1.97	7.94 (23)
Hydroxamide (27)	1.88	4.3	43.7	7.99 (23)
Glycinamide (10, 27)	0.64, 0.44	2.3	27.5, 19.1	8.07 (10)
Alanineamide (10)	7.5	1.72	436	8.15 (10)
Amide (10, 27)	0.14, 0.17	3.2	4.4, 5.3	9.26 (10)
Methylamide (26)	0.0006	6.1	0.00984	10.72 (24)

* This work.

formate (1.12 ml) was added and, after 1 min, 1.52 g of *m*-nitroaniline was added in 10 ml of tetrahydrofuran. The mixture was stirred for 3 hr at room temperature and the tetrahydrofuran was evaporated under reduced pressure. The remaining solids were dissolved in ethyl acetate and the solution was washed three times with dilute hydrochloric acid and one time with aqueous sodium bicarbonate. The ethyl acetate layer was removed and evaporated to dryness under reduced pressure. The remaining solid was recrystallized from ethanol–water and ethyl acetate–hexane. The compound melted at 213° and was subjected to C H N analysis: C: 59.13 (59.47 theoretical), H: 4.98 (4.99 theoretical), N: 12.15 (12.24 theoretical). Comparison of the extent of chymotryptic reaction with the extent of alkaline hydrolysis indicated the absence of *D* isomer.

N-Acetyl-L-tyrosine *p*-cyanoanilide was also prepared by the method given above, using 1.3 g of *p*-cyanoaniline. The product, after undergoing purification as for the *m*-nitroanilide, gave 450 mg of white crystals: C: 66.95 (66.86 theory), H: 5.41 (5.30 theory), and N: 12.86 (13.00 theory).

N-Acetyl-L-tyrosine *p*-nitroanilide was prepared by the method given above. The product obtained after washing with dilute hydrochloric acid and sodium bicarbonate solutions was recrystallized twice from ethanol–water mp 250° (lit. 248.9°) (5). Comparison of the extent of chymotryptic and alkaline hydrolyses indicated the presence of 10% *D* isomer.

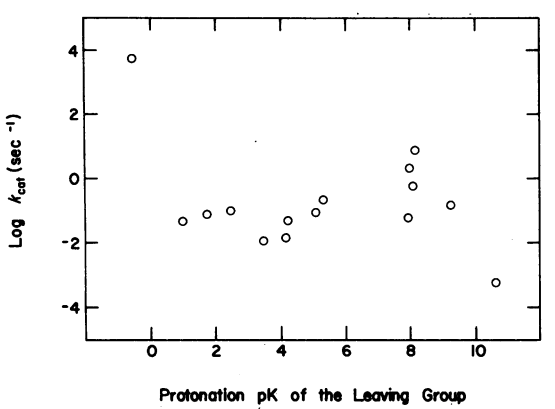

FIG. 1. Dependence of log k_{cat} on leaving-group pK in the chymotryptic hydrolysis of *N*-acetyl-L-tyrosine derivatives. Data of Table 1.

The α-chymotrypsin-catalyzed hydrolysis of these compounds was observed in pH 7.94 (I = 0.1 M) Tris·HCl buffer (3.1% dimethylsulfoxide). The hydrolysis of the *m*-nitroanilide was observed spectrally at 390 nm ($\Delta\epsilon$ 935 abs. M^{-1}), the *p*-nitroanilide at 380 nm ($\Delta\epsilon$ 11,550 abs. M^{-1}), and the *p*-cyanoanilide by pH-stat titration. The kinetics were analyzed according to the method of Eadie (9) from initial-rate data.

RESULTS AND DISCUSSION

Compiled data for the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tyrosine amides and the ethyl ester are presented in Table 1. Despite large variations in the pK of the leaving group, changes in kinetic constants are small and show no trend. This scatter, perhaps resulting from interactions due to leaving-group specificity sites on the enzyme (10, 11), is sufficient to account for the results (1, 2) that led to the formulation of the pretransition state protonation theory (2–4). This theory was based upon the apparent correlation between basicity of the leaving group and log k_{cat}. However, the experimental results covered only a small range of basicity of the leaving groups (pK 3–6). The hydrolysis rates of these anilides show a trend that is not observed in other anilides (pK 0–3) or in amides (pK 7–11). The failure of the less-basic anilides to conform to his plot was explained (5) by the postulation that they do not hydrolyze by a pretransition state protonation mechanism, but the deviation of amides from this correlation cannot be explained in this manner. Consideration of the values of k_{cat} for the entire range of pK′ values (Fig. 1) shows that k_{cat} is independent of the pK of the leaving group. Consequently, it appears that the negative ρ observed previously (1, 2) is an artifact due to the use of substrates covering only a narrow range of pK values.

This anomaly may be due to nonproductive conformations, since the trend seen in the pK 3–6 region in Fig. 1 (log k_{cat} versus pK) is not seen in a plot of log k_{cat}/K_m versus pK (Fig. 2). It is known that nonproductive binding modes, while decreasing k_{cat} and K_m, do not affect the ratio k_{cat}/K_m (12).

Evidence supporting this explanation is the fact that the K_m values for these anilides (pK 3–6) are lower than K_m values for esters, amides, and other anilides (Table 1).

Our results, together with theoretical criticisms mentioned by others (13–15), show that the experimental data do not support the pretransition state protonation hypothesis, but are compatible with the intervention of a tetrahedral intermediate.

The independence of rate on pK of the leaving group can also give insight into the question of whether a tetrahedral intermediate accumulates in substantial concentration along the reaction pathway. Evidence for the existence of a tetrahedral intermediate in chymotrypsin-catalyzed hydrolysis of amides has been given by both Caplow (15, 16) and Fersht (13, 17). In addition, Caplow (15) has interpreted his data to indicate that the tetrahedral intermediate is formed in significant concentrations, and that the observed value of K_m (for some anilides) is actually composed of the true binding constant *and* the equilibrium constant for formation of the tetrahedral intermediate. Fersht, on the other hand, argues that the tetrahedral intermediate is present only in steady-state concentrations (17).

The proposed enzymatic reaction pathway is given in Eq. **1**, with ES being the noncovalent Michaelis complex, ET the tetrahedral reaction intermediate, and EP_2 the acyl-enzyme.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ET \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EP_2 \xrightarrow{k_7} E + \underset{+}{P_2} \underset{P_1}{} \qquad [1]$$

It is necessary to see how the data (Table I) are consistent with derivations for k_{cat} and K_m for this mechanism. The general derivation for k_{cat} and K_m for this type of enzymatic reaction scheme has been given by Dixon and Webb (18), and their numerical nomenclature is used. This expression may be simplified (under initial rate conditions where k_6 is neglected) as in Table 2.

In order for the binding constant, K_m, to reflect the formation of the tetrahedral intermediate, it is necessary that it be in equilibrium with the Michaelis complex and that the breakdown of the tetrahedral intermediate be rate-determining (i.e., $k_4 \gg k_5$) (5). Evidence for the interpretation $k_4 > k_5$ may be found in the work of O'Leary and Kluetz (19), in which they found a nitrogen isotope effect in the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide.

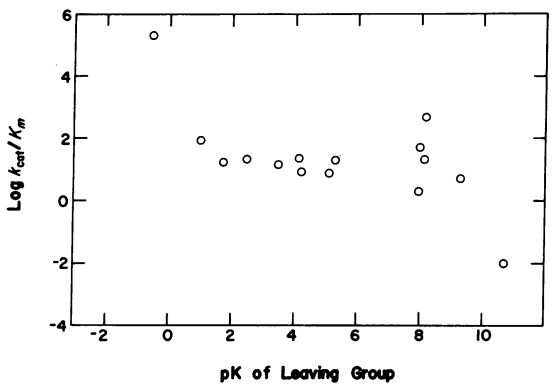

FIG. 2. Dependence of log k_{cat}/K_m on leaving-group pK in the chymotryptic hydrolysis of *N*-acetyl-L-tyrosine derivatives. Data of Table 1.

TABLE 2. *Constants for chymotrypsin reaction, assuming the formation of a tetrahedral intermediate followed by an acylenzyme (Eq. 1)*

Condition	K_m	k_{cat}	k_{cat}/K_m
$k_2, k_3, k_4, k_7 \gg k_5$	$\frac{k_2k_4 + k_3k_5}{k_1(k_3 + k_4)}$	$\frac{k_3k_5}{k_3 + k_4}$	$\frac{k_1k_3k_5}{k_2k_4 + k_3k_5}$
$k_2, k_3, k_4, k_7 \gg k_5$ $k_3 \gg k_4$	$\frac{k_2k_4 + k_3k_5}{k_1k_3}$	k_5	$\frac{k_1k_3k_5}{k_2k_4 + k_3k_5}$
$k_2, k_3, k_4, k_7 \gg k_5$ $k_4 \gg k_3$	$\frac{k_2}{k_1}$	$\frac{k_3k_5}{k_4}$	$\frac{k_1k_3k_5}{k_2k_4}$
$k_2, k_3, k_4, k_7 \gg k_5$ $k_3 = k_4$	$\frac{k_2}{2k_1}$	$1/2k_5$	$\frac{k_1k_5}{k_2}$

However, variation of this isotope effect with pH suggests that the values of k_4 and k_5 are not too dissimilar. It can be readily seen from Table 2 that K_m will be dependent on k_3, and k_4 if the tetrahedral intermediate is more stable than the Michaelis complex ($k_3 > k_4$), but K_m will be independent of k_3 and k_4 if the tetrahedral intermediate is not formed in significant amounts ($k_4 > k_3$). Since large electronic effects or configurational changes in the substrate should be expected to affect k_3 and k_4, it is only necessary to see whether these changes have any effect on K_m.

Table 3 presents comparable values of *N*-acetyl-L-tryptophan, *N*-acetyl-L-tyrosine, and *N*-acetyl-L-phenylalanine compounds at pH values near those of strongest binding. [Table 2 shows that existence of a tetrahedral intermediate can only improve (lower) binding constants, so comparisons must be made at the smallest values of K]. The data show that the binding constants are independent of orientation, charge, and pK of the leaving group, as well as being independent of the method of observation. We conclude from this fact that K_m is independent of constants involving the tetrahedral intermediate (k_3 and k_4). This result requires that the tetrahedral intermediate be less stable than the Michaelis complex and, therefore, not be present in significant amounts.

TABLE 3. *Binding constants of specific substrates and pseudo-substrates to α-chymotrypsin*

Compound	Method*	Binding constant × 10^3	pH	Ref.
N-Acetyltryptophan				
D-amide	*D*	2 ± 1	4–5	28
L-amide	*K*	3.3 ± 0.3	5.73	29
L-ethyl ester	*K*	2.3	5.0	31
L-anion	*D*	2 ± 1	4–5	28
D-anion	*D*	2 ± 1	4–5	28
L-amide	*K*	6.3	8	30
L-amide	*I*	6.7	8	30
N-Acetyltyrosine				
L-ethyl ester	*K*	18	5.0	25
L-amide	*K*	34	8	32
N-Acetylphenylalanine				
L-ethyl ester	*K*	22 ± 5	3–4	32
L-amide	*K*	31	7.9	27

* Methods are *K* (kinetic determination); *I* (inhibition constant); *D* (equilibrium dialysis).

These results indicate that the K_m–pH dependencies observed previously for some (but not all) anilides (3, 16) must result from interactions with the active-site imidazole or other leaving-group specificity sites (10, 11), and not from accumulation of a tetrahedral intermediate. Perturbations of the pK value for k_{cat}, as seen by several workers (3, 11), are to be expected if binding (K_m) becomes pH-dependent near neutrality, possibly due to imidazole–anilide (aniline ring) interactions. Similar effects may be present in compounds with formylhydrazide leaving-groups (13, 17). In this view, these k_{cat}–pK shifts do not have any mechanistic significance, but are only artifacts. This independence of k_{cat}/K_m on pK for amides is surprising in view of the results of Jencks *et al.* (20). They showed that the ability of a nucleophile to accelerate deacylation of furoyl-chymotrypsin is independent of nucleophile pK over a wide range, but that there is a substantial effect ($\beta = 0.6$) of pK on equilibrium constants for acylation of an alcohol by amides. These results strongly suggest that in the acylation of chymotrypsin, k_{cat}/K_m should show a substantial negative β, i.e., rate increasing with decreasing pK. In this regard it should be noted that a negative β for k_{cat} or k_{cat}/K_m could result if the effects of pK on k_3/k_4 and k_5 differ. This discrepancy indicates the necessary direction of future work.

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