Chymotrypsin-Catalyzed Hydrolysis of *m*-, *p*-, and *o*-Nitroanilides of *N*-Benzoyl-L-tyrosine*

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ABSTRACT: The kinetics of the chymotrypsin-catalyzed hydrolysis of *m*-, *p*-, and *o*-nitroanilides of *N*-benzoyl-L-tyrosine have been investigated. The rate of acylation of the enzyme is increased by withdrawal of electrons from the reaction site, indicating the importance of the approach of a nucleophile to the carbonyl carbon of the anilide. With the *p*-nitroanilide the rate of acylation in D_2O is less than in H_2O by a factor of 3.1, suggesting a proton-transfer mechanism. The results are consistent with the interpretation that the chymotrypsin-catalyzed hydrolysis of anilides is a general acid–general base catalyzed nucleophilic reaction.

A considerable body of evidence, much of it discussed by Bender (1962) and more recently by Bender and Kézdy (1965), indicates that chymotrypsin-catalyzed reactions proceed *via* a three-step pathway

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$
$$\downarrow$$
$$P_1$$

in which an acyl-enzyme, ES′, is formed as a reaction intermediate.

Studies of the electronic effects of structure on the reactivity of substrates have been undertaken in attempts to elucidate the mechanism of chymotrypsin catalysis. The results of these studies indicate a difference in the response of phenol ester and anilide substrates to changes in charge density at the reaction site. Bender and Nakamura (1962), in an investigation of substituent effects, have shown that electron withdrawal facilitates the acylation of chymotrypsin by phenol esters, while other investigators have found that electron-donating substituents facilitate the acylation of chymotrypsin by *N*-benzoyl- and *N*-acetyl-L-tyrosine anilides (Sager and Parks, 1963; Inagami *et al.*, 1965).

In a previous study it was found that *N*-acetyl- and *N*-benzoyl-L-tyrosine *p*-nitroanilides can be used to advantage in a reasonably rapid and sensitive spectrophotometric method for determining chymotrypsin activity (Bundy, 1963). In the present investigation we have extended this study to the electronic effect of the nitro substituent using *m*-, *p*-, and *o*-nitroanilides of *N*-benzoyl-L-tyrosine. As pointed out by Inagami *et al.* (1965), the use of anilides is advantageous in studying the acylation step (k_2) because acylation has been shown to be rate determining in the reaction of chymotrypsin with these substrates ($k_2 \ll k_3$).

Experimental Section

Materials. α-Chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp. Stock solutions were prepared by dissolving the enzyme in 0.002 N HCl which is 0.1 M in $CaCl_2$. Enzyme concentration was determined using a test for the operational normality of chymotrypsin solutions (Erlanger and Edel, 1964). The molarity ranged from 5 to 10% below that obtained from absorbancy at 280 mμ using the optical factor 0.486 and a molecular weight of 24,800.

The substrates listed in Table I were prepared as previously described for *N*-benzoyl-L-tyrosine *p*-nitroanilide (Bundy, 1963). For the *ortho* and *meta* derivatives the L form was obtained by fractional crystallization from ethanol–water. Stock solutions were prepared by dissolving the substrates in acetone or dimethylformamide (Eastman Kodak, spectral grade). For each substrate, hydrolysis by chymotrypsin accounted for

TABLE I: *N*-Benzoyl-L-tyrosine Nitroanilides.[a]

Substituent	% Found C	H	N	Mp (°C)
m-NO_2	65.09	4.74	10.13	218–219
p-NO_2	65.15	4.82	10.32	227–228
o-NO_2	64.96	4.81	10.20	195–196

[a] Calcd for $C_{22}H_{19}N_3O_5$: C, 65.17; H, 4.73; N, 10.37.

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TABLE II: Chymotrypsin-Catalyzed Hydrolysis of Substituted Anilides of *N*-Benzoyl-L-tyrosine.[a]

Substituent	$(S_0) \times 10^4$ (M)	k_2 (sec^{-1})	$K_m \times 10^4$ M
		Acetone	
m-NO_2	0.50 – 2.52	0.34 ± 0.06	10.9 ± 0.6
p-NO_2	0.42 – 2.10	1.03 ± 0.07	4.4 ± 0.15
o-NO_2	0.51 – 2.56	1.50 ± 0.15	61.2 ± 3.0
		Dimethylformamide	
m-NO_2	0.75 – 3.75	0.16 ± 0.02	5.1 ± 0.5
p-NO_2	0.67 – 3.50	0.34 ± 0.01	1.7 ± 0.2
o-NO_2	0.70 – 3.69	0.86 ± 0.2	35.9 ± 2.0

[a] Determined at 30° in 0.033 M Tris–HCl buffer, pH 7.95, 0.037 M $CaCl_2$, 10% (v/v) acetone or 6.67% (v/v) dimethylformamide. Five–seven substrate concentrations within the ranges specificed. Each kinetic constant given as the mean of four determinations ± standard deviation from the mean.

100 ± 2% of the substrate added under the conditions used.

Tris–HCl buffers were prepared by adjusting solutions of Tris (Trizma Base, Sigma Chemical Co.) to pH 7.95 with HCl. Stock solutions of buffer were 0.5 M in Tris and 0.5 M in $CaCl_2$. The pH was measured at the temperature at which the buffer was to be used.

Deuterium oxide was purchased from International Chemical and Nuclear Corp. and contained at least 99.75% D_2O. The Tris–DCl buffer was prepared at 25° as described by Bender and Hamilton (1962) with the same acid to base ratio as the corresponding Tris–HCl buffer.

Methods. Hydrolysis was followed by measuring the release of *m*-, *p*-, or *o*-nitroaniline at 400, 410, or 430 mμ, respectively, using a Perkin-Elmer Model 202 recording spectrophotometer with a thermostated cell compartment. Buffer (0.2 ml), substrate (0.3 ml or 0.45 ml in acetone; 0.2 ml in dimethylformamide), and sufficient water to bring the volume to 2.9 ml (2.95 ml in the D_2O experiments) were mixed and brought to temperature. The reaction was initiated by adding 0.1 ml of enzyme solution (50 μl in the D_2O experiments). The kinetic constants k_{cat} and K_m were determined from Lineweaver–Burk plots of the effect of substrate concentration on initial velocity (5–10% of completion). In chymotrypsin reactions following eq 1, where acylation is rate determining, $k_{cat} = k_2$, and $K_m = (k_{-1} + k_2)/k_1$ (Gutfreund and Sturtevant, 1956). Initial substrate concentrations were well in excess of enzyme concentrations which ranged between 1 and 19 × 10^{-7} M.

Results

Effect of Substituent. Experimentally determined k_2 and K_m values for the chymotrypsin-catalyzed hydrolysis of *m*-, *p*- and *o*-nitroanilides of *N*-benzoyl-L-tyrosine are given in Table II. The results indicate that acylation of the enzyme is facilitated by electron withdrawal from the reaction site. This is true in both solvent systems. However, there is a noticeable solvent effect, the rates being significantly decreased in the dimethylformamide solvent system. Acetone was employed in our initial investigations because acetone has been found to have little or no effect on the rate of acylation (Clement and Bender, 1963; Bundy, 1963). Results using dimethylformamide are included for better comparison with the results of Inagami *et al.* (1965) who employed 5% (v/v) dimethylformamide in studying the hydrolysis of *N*-acetyl-L-tyrosine anilides. In the acetone solvent, k_2 for *N*-benzoyl-L-tyrosine *p*-nitroanilide is about three times greater than k_2 for the *N*-acetyl derivative (Bundy, 1963). We think that it should be noted here that caution should be exercised in comparing chymotrypsin rate constants obtained using dimethylformamide solvents with those obtained using other solvent systems. We have found k_2 for the reactions studied here to vary with dimethylformamide concentration. The values given in Table III show the effect of dimethylformamide on the hydrolysis of the *p*-nitroanilide.

Effect of D_2O. Evidence in the literature suggests that nucleophilic catalysis is relatively unaffected by deu-

TABLE III: Effect of Dimethylformamide on the Chymotrypsin-Catalyzed Hydrolysis of *N*-Benzoyl-L-tyrosine *p*-Nitroanilide.[a]

Dimethylformamide (%, v/v)	k_2 (sec^{-1})	$K_m \times 10^4$ (M)
6.67	0.34 ± 0.01	1.7 ± 0.2
13.33	0.53 ± 0.05	5.4 ± 0.4
20.0	0.92 ± 0.09	18.9 ± 0.7

[a] Determined at 30° in 0.033 M Tris–HCl buffer, pH 7.95, 0.037 M $CaCl_2$. Five initial substrate concentrations within the range 0.67–3.50 × 10^{-4} M. Each kinetic constant is given as the mean of four determinations ± standard deviation from the mean.

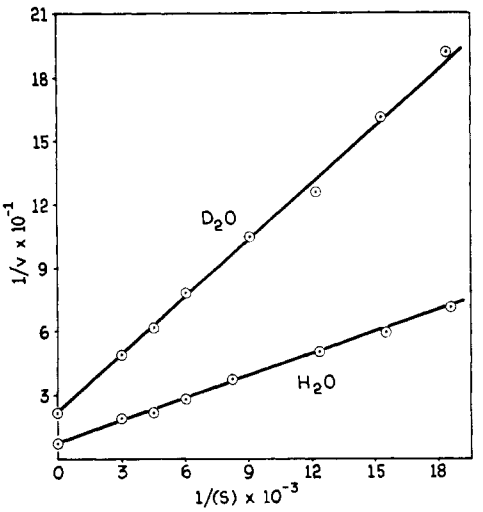

FIGURE 1: Lineweaver–Burk plots for the hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide in D_2O and H_2O (for experimental conditions see Table IV).

terium oxide whereas the rate of a reaction proceeding *via* a proton-transfer mechanism is decreased 2- to 3-fold in deuterium oxide (Bender *et al.*, 1962). In the present study the value of k_2 for the *p*-nitroanilide in D_2O is less than in H_2O by a factor of 3.1. The results are illustrated in Figure 1 and the kinetic constants are given in Table IV. This kinetic isotope effect, which

TABLE IV: The Chymotrypsin-Catalyzed Hydrolysis of *N*-Benzoyl-L-tyrosine *p*-Nitroanilide in H_2O and D_2O.[a]

Solvent	$E \times 10^7$ (M)	pH or pD	k_2 (sec^{-1})	$K_m \times 10^4$ (M)
H_2O	1.99	7.95	0.73 ± 0.07	4.65 ± 0.2
D_2O	2.10	8.54	0.24 ± 0.02	4.16 ± 0.3

[a] Determined at 25° in 0.033 M Tris–HCl or Tris–DCl buffer, 0.037 M $CaCl_2$, 15% (v/v) acetone. Each kinetic constant given as the mean of three determinations ± standard deviation from the mean.

is similar to that obtained by Bender and Hamilton (1962) using *N*-acetyl-L-tryptophan methyl ester and by Inagami *et al.* (1965) using *N*-acetyl-L-tyrosine *m*-chloroanilide, suggests that acylation proceeds *via* a proton-transfer mechanism.

Discussion

The observed kinetic isotope effect and the enhancement of acylation by both electron-withdrawing (present work) and electron-donating substituents (Sager and Parks, 1963; Inagami *et al.* 1965) indicate the importance in the acylation of chymotrypsin of both the approach of a nucleophile to the carbonyl carbon of the anilide and the transfer of a proton to the leaving group. It would seem that an electron-withdrawing substituent (nitro group) exerts its effect by increasing the electrophilicity of the carbonyl carbon of the anilide, making it more susceptible to nucleophilic attack, while electron-donating substituents enhance the rate by facilitating proton transfer in the removal of the aniline.[1] This is consistent with the minimal mechanism suggested by Inward and Jencks (1965) in which a conjugate acid of the catalyst (imidazolium cation) donates a proton to the leaving group, in this case nitroaniline. The results are also consistent with the more detailed mechanism proposed by Bender and Kézdy (1965) in which an imidazole group on the enzyme acts simultaneously as a general base and general acid.

The effect of *ortho* substitution is of some interest. The usual *ortho* effect, $k_p > k_o$, was not observed. The observed effect, $k_p < k_o$, may be due to internal hydrogen bonding between the *o*-nitro group and the anilide nitrogen which could facilitate anilide bond cleavage by increasing the ease of proton transfer to the anilide nitrogen. An alternative explanation might be the operation of a Bunnett effect (Reinheimer and Bunnett, 1959) in which the *o*-nitro group, in close proximity to the reaction site, increases the polarization of an incoming nucleophile, thus facilitating its attack.

As mentioned above (Experimental Section), in chymotrypsin reactions following eq 1, where acylation is rate determining, $K_m = (k_{-1} + k_2)/k_1$. In the present study K_m responded inversely as k_2 with *meta* and *para* substitution. In the case of *ortho* substitution the large increase in K_m cannot be accounted for by the much smaller increase in k_2. Thus the results indicate that the nitro substitution of anilides also has a significant, though at present unresolved, effect on the initial interaction of enzyme and substrate (k_{-1}/k_1).

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[1] This is, of course, a tentative reconciliation of the data. As pointed out by a referee, the present results, using electron-withdrawing substituents, are in contradiction to those of Sager and Parks (1963) and Inagami, *et al.*, (1965), and the reconciliation assumes a biphasic Hammett plot of log k_2 *vs.* the σ constant of the substituent.

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CORRECTION

In the paper by Sidney Harshman and Victor A. Najjar in Volume 4, No. 11, November 1965, on p 2531, Figure 2 should be corrected as follows: The autoradiograph of the separation of the peptide at pH 2.0 should be reversed to make it consistent with the others in which the positive electrode is at the left and the negative electrode at the right. The labels on the two peptides should be reversed.