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Electronic Effects in the Acylation of α-Chymotrypsin by Substituted *N*-Benzoylimidazoles†

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ABSTRACT: Rate constants for the acylation of α-chymotrypsin by a series of acyl-substituted *N*-benzoylimidazoles have been determined by proflavin displacement from the active site. The second-order acylation rate constants k_2/K_m are large [e.g., that for *N*-(*m*-nitrobenzoyl)imidazole is 1.7×10^4 M^{-1} s^{-1} at pH 7.5], even though K_m must be quite large (plots of k vs. $k/[S]_0$ have infinite slopes). The values of k_2/K_m are nearly independent of pH in the range 5.0–9.0 when the substituent group is electron donating. Electron-withdrawing substituents produce an increase in k_2/K_m with increasing pH until a maximum is reached near pH 7. This is also the case in acylation by the *N*-[*p*-(dimethylamino)benzoyl]-*N*′-methylimidazolium ion (pK_{app} = 6.5). While the reaction of the N′-methylated derivative is via a positively charged species at all pH values, the unmethylated compounds react through both the neutral species and the conjugate acids, with the observed pH dependence depending on the relative values of the rate constants. The limiting value of k_2/K_m for the *N*-[*p*-(dimethylamino)benzoyl]-*N*′-methylimidazolium ion is 2.1 times less in D_2O than in H_2O. Thus, His-57 must be participating in the acylation reaction as a general base. The limiting values of k_2/K_m for the corresponding N′-methylated and unmethylated derivatives differ by a factor of only 150, which is similar to the difference in the second-order rate constants for nonenzymatic OH^--catalyzed hydrolysis. A plot of log k_2/K_m vs. the Hammett substituent constant σ is linear with a slope ρ of 0.9 for rate constants obtained at pH 7.5 and nearly zero for rate constants determined at pH <5.0. These ρ values are considerably smaller than those in nonenzymatic hydrolysis reactions of *N*-benzoylimidazoles. As a consequence, in the enzymatic acylation reactions, there must be less bond making with the nucleophile (Ser-195) in the transition state than in the hydrolysis reactions. The transition state for acylation must approximate the reactants. This is especially the case in acylation reactions of the *N*-benzoylimidazole conjugate acids.

Reactions catalyzed by α-chymotrypsin follow the scheme of eq 1, where ES′ is an acyl-enzyme intermediate. It has been

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

well established that an acyl-enzyme intermediate is formed during the hydrolysis of both specific and nonspecific ester and amide substrates (Bender & Kezdy, 1964; Gutfreund & Sturtevant, 1956; Bender & Zerner, 1962; Zerner & Bender, 1964; Zerner et al., 1964; Kezdy et al., 1964). The acyl-enzyme is undoubtedly an ester of serine-195 (Bruice & Benkovic, 1966; Bender & Kezdy, 1964). Histidine-57 is also located at the active site and participates in both acylation and deacylation. Electronic substituent effects have been determined in deacylation of substituted benzoylchymotrypsins (Caplow & Jencks, 1962) and in acylation by substituted phenolic esters (Bender & Nakamura, 1962; Hubbard & Kirsch, 1972) and anilides (Inagami et al., 1965). Acylation of the enzyme by ester substrates is characterized by Hammett ρ values (Hammett, 1940) that are large and positive (Bender & Nakamura, 1962; Hubbard & Kirsch, 1972). In contrast, substituted anilides give a highly negative ρ value (−2.0) in acylation (Inagami et al., 1965), although nonproductive binding of the anilide substrates may be important (Fastrez & Fersht, 1973). A nitrogen isotope effect in the α-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide requires that the C–N bond of the amide is broken in the rate-determining step (O'Leary & Kleutz, 1972). Breakdown of a tetrahedral intermediate formed in acylation reactions of amide substrates might therefore involve general acid catalysis by the His-57 conjugate acid since protonation of the leaving group is a requirement in hydrolysis reactions of amides to avoid expulsion of an amine anion. If the rate-determining step is breakdown of a tetrahedral intermediate, then a mechanistic role for His-57 in assisting nucleophilic attack by Ser-195 cannot be specified since formation of the tetrahedral intermediate will be an equilibrium step.

N-Acylimidazoles are kinetically favorable amide substrates for α-chymotrypsin (Schonbaum et al., 1961; Bender et al., 1962; Kogan et al., 1982). The rate constants k_2/K_m for acylation can be very large (Kogan et al., 1982) [e.g., the value of k_2/K_m for acylation by *N*-(β-phenylpropionyl)imidazole at pH 7.5 is 1.2×10^6 M^{-1} s^{-1} (30 °C)], even though binding to the enzyme must be weak (an ES complex cannot be experimentally detected). In these reactions, mechanisms involving the His-57 conjugate acid acting as a general acid can be ruled out (Kogan et al., 1982). Furthermore, it is unlikely that a kinetically significant tetrahedral intermediate is formed in nonenzymatic hydrolysis (Fife, 1965; Fee & Fife, 1966a,b; Fee, 1967) and alcoholysis (Oakenfull & Jencks, 1971) reactions of these compounds, and it is reasonable that this would

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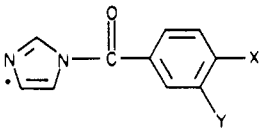
Chart I



I, X = *p*-$N(CH_3)_2$; Y = H
II, X = *p*-OCH_3; Y = H
III, X = H; Y = H
IV, X = *p*-CH_3; Y = H
V, X = *p*-Cl; Y = H
VI, X = *p*-CN; Y = H
VII, X = H; Y = NO_2

also be the case in acylation of Ser-195. Thus, the determination of substituent effects in acylation reactions of these amides will give mechanistic information that is directly applicable to the nucleophilic attack step uncomplicated by other functional group interactions. We have therefore determined the rate constants for acylation of α-chymotrypsin by a series of *N*-benzoylimidazoles (I–VII, see Chart I). In addition, the acylation reactions of the *N'*-methylated derivative of I have been studied.

Experimental Procedures

Materials. α-Chymotrypsin, 3 times crystallized, was obtained from Worthington Biochemical Corp. Acetonitrile was Eastman-Kodak Spectro-Grade. All other chemicals were reagent grade. The water employed was deionized and distilled. Acid chlorides were either obtained commercially (Aldrich, Mallinckrodt) or prepared from commercially obtained carboxylic acids (Sigma) by reaction with thionyl chloride. Physical constants were consistent with those previously reported. Imidazole was obtained commercially (Aldrich) and sublimed prior to use. *N-trans*-Cinnamoylimidazole was prepared according to the method of Schonbaum et al. (1961). The *N*-benzoylimidazoles were the same as previously described (Fee & Fife, 1966a; Caplow & Jencks, 1962). *N*-[*p*-(Dimethylamino)benzoyl]imidazole was carefully recrystallized from cyclohexane and had an mp of 109–111 °C [lit. (Staab et al., 1962) mp 108–109 °C]. *N*-(*p*-cyanobenzoyl)imidazole had an mp of 111–113 °C. Anal. Calcd for $C_{11}H_7N_3O$: C, 67.00; H, 3.58; N, 21.31. Found: C, 66.80; H, 3.93; N, 20.86. *N*-[*p*-(Dimethylamino)benzoyl]-*N'*-methylimidazolium chloride (VIII) was prepared according to the methods of Wolfenden & Jencks (1961) and had an mp of 138–140 °C. The white solid, which was slightly hygroscopic, was thoroughly washed with dry ether and dried over P_2O_5. Anal. Calcd for $C_{13}H_{16}ClN_3O$: C, 58.76; H, 6.07; N, 15.81. Found: C, 58.57; H, 6.25; N, 15.53.

p-Nitrophenyl 4-(dimethylamino)benzoate was prepared from *p*-(dimethylamino)benzoic acid and *p*-nitrophenol by stirring equivalent amounts in methylene dichloride in the presence of 1 equiv of dicyclohexylcarbodiimide at room temperature. The mixture was filtered, and the solvent was removed by rotary evaporation. The residual material was then recrystallized from acetone and had an mp of 191–193 °C [lit. (Kirsch et al., 1968) mp 195–196.5 °C]. *p*-Nitrophenyl 4-cyanobenzoate was prepared by refluxing *p*-nitrophenol and *p*-cyanobenzoyl chloride in dry benzene in the presence of 1 equiv of pyridine. After filtration and removal of the solvent by rotary evaporation, the material was recrystallized from acetone, mp 193–194 °C [lit. (Hubbard & Kirsch, 1972) mp 195–196 °C].

Stock solutions of proflavin hydrochloride (Aldrich) were routinely prepared in distilled water. Stock solutions of α-chymotrypsin were prepared in pH 5.0 acetate buffer (0.1 M, μ = 0.5 M). The normality of active sites in the stock enzyme solutions was determined by titration with *N-trans*-cinnamoylimidazole at 310 nm [method A of Schonbaum et al. (1961)], with a reproducibility of about 1%. Titration values of the stock solutions were stable for several weeks at 5 °C at pH 5.0 (0.1 M acetate buffer). Buffers were prepared from reagent-grade chemicals.

Kinetic Methods. Acylation of α-chymotrypsin by the *N*-benzoylimidazoles at 30 °C was monitored in the presence of proflavin by methods previously described (Kogan et al., 1982). Proflavin forms a 1:1 complex with the active site of α-chymotrypsin (Bernhard et al., 1966). The maximum absorbance difference between complexed and uncomplexed dye occurs at 465 nm. Acylation of the enzyme results in displacement of proflavin, which consequently gives rise to a large absorbance change at 465 nm and thereby allows reactions to be conveniently followed spectrophotometrically [Brandt et al., 1967; Kogan et al. (1982) and references cited therein]. All kinetic runs were in 0.1 M buffers (μ = 0.5 M NaCl). The concentrations of α-chymotrypsin and proflavin were generally 1.65×10^{-5} M and 7.7×10^{-5} M, respectively. Substrate concentrations were varied in the range 1.0×10^{-4} to 9.0×10^{-3} M. Acetate, phosphate, Tris [tris(hydroxymethyl)-aminomethane], and ammediol buffers were employed. The different buffers yielded consistent results when employed at the same pH. Acylation reactions were followed between 465 and 490 nm at 30 °C by employing either a Beckman Model 25 spectrophotometer or a Durrum Model D-110 stopped-flow spectrophotometer. Absorbance changes after mixing in the stopped-flow determinations were recorded on a Hewlett-Packard storage oscilloscope (Model 1207B). The reactions were followed to completion. Good first-order kinetics were obtained in all cases, and pseudo-first-order rate constants were calculated with an IBM 370-158 computer. Blank runs in the absence of substrate produced no significant absorbance change. Reaction pH values were obtained on a Radiometer Model 22 pH meter or a Beckman Model 3500 digital pH meter. Acylation reactions of *N*-acylimidazoles derived from aliphatic carboxylic acids give second-order rate constants that are similar when determined by either proflavin displacement or disappearance of substrate (Kogan et al., 1982). It has also been previously found in reactions of ester substrates that the rate constants obtained by proflavin displacement are identical with those obtained by independent methods (Himoe et al., 1969).

The nonenzymatic hydrolysis reactions of I and VIII were followed by measuring the absorbance decrease at 245 nm with a Beckman Model 25 spectrophotometer at 30 °C. The reactions were followed to completion, and pseudo-first-order rate constants (k_{obsd}) were computer calculated by using a linear regression program. The value of K_w was taken to be 1.47×10^{-14}.

Results

The scheme of eq 1 yields eq 2 for reactions of α-chymotrypsin, where $K_m = (k_{-1} + k_2)/k_1$ and k is a first-order rate constant governing the pre-steady-state reaction (Gutfreund & Sturtevant, 1956). Plots of k vs. $k/[S]_0$, where k is the pseudo-first-order rate constant for acylation of α-chymotrypsin by the *N*-benzoylimidazoles at 30 °C determined by proflavin displacement, were in each case vertical even at very high substrate concentrations (ratios of $[S]_0/[E]_0$ greater than 300-fold). The vertical plots indicate that the highest substrate concentration is still much less than K_m so that an enzyme–substrate complex is experimentally undetectable. In the case

$$k = \frac{(k_2 + k_3)[S]_0 + k_3K_m}{[S]_0 + K_m} = \frac{k_2[S]_0}{[S]_0 + K_m} + k_3 \quad (2)$$

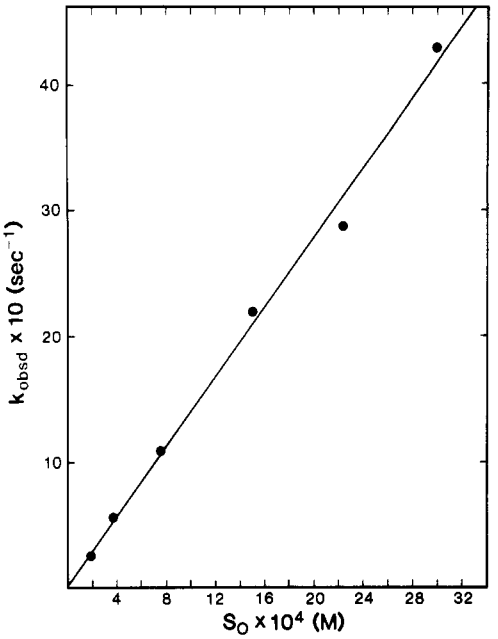


FIGURE 1: Plot of k vs. $[S]_0$ for acylation of α-chymotrypsin at pH 7.0 by *N*-(*p*-methoxybenzoyl)imidazole at 30 °C.

of a second-order reaction, a plot of $k - k_3$ vs. $[S]_0$ (eq 2) or k vs. $[S]_0$ if k_3 is negligible should have an intercept of zero, and the slope will be equal to the pH-dependent second-order acylation rate constant k_2/K_m. A representative plot of k vs. $[S]_0$ is shown in Figure 1. The intercepts of such plots were generally zero within experimental error. The values of $k/[S]_0$ determined from the abscissa intercepts of plots of k vs. $k/[S]_0$ should be equal to k_2/K_m and were closely similar in each case. In all reactions, the rates of acylation of α-chymotrypsin by the *N*-benzoylimidazoles were much greater than those of deacylation. Subtraction of k_3 from k, employing eq 2 and the values of k_3 determined by Caplow & Jencks (1962) or in this study for deacylation of substituted benzoyl-α-chymotrypsins, produced no change in the second-order rate constants k_2/K_m because k_3 is not significant in comparison to k.

The log (k_2/K_m)–pH profiles for acylation of α-chymotrypsin by the *N*-benzoylimidazoles are shown in Figure 2. The pH dependence of the acylation reaction is influenced by the nature of the para substituent; acylation is essentially independent of pH when the substituent is electron donating (I–IV). Electron withdrawing-substituents (V–VII) produce an increase in k_2/K_m from pH 5.0 to approximately pH 7; thereafter, the rate constants become nearly pH independent. The log (k_2/K_m) vs. pH profile for *N*-[*p*-(dimethylamino)-benzoyl]-*N'*-methylimidazolium ion follows a theoretical curve for dissociation of a group with a pK_a of 6.5. The limiting value of k_2/K_m at high pH is 6.3×10^4 M^{-1} s^{-1}. The limiting rate constant for the *N'*-methyl derivative was determined in D_2O at a pD value of 7.5. The ratio $(k_2'/K_m)^{H_2O}/(k_2'/K_m)^{D_2O}$ is 2.1.

As seen in Figure 2, the values of k_2/K_m at pH >7 in general increase as electron withdrawal in the acyl group increases, although differences are not large. At pH 7.5, k_2/K_m is (I) 420 M^{-1} s^{-1}, (III) 960 M^{-1} s^{-1}, (II) 1700 M^{-1} s^{-1}, (IV) 3300 M^{-1} s^{-1}, (V) 5600 M^{-1} s^{-1}, (VI) 1.2×10^4 M^{-1} s^{-1}, and (VII) 1.7×10^4 M^{-1} s^{-1}. Plots of log (k_2/K_m) at pH 5 and 7.5 vs. σ, the Hammett substituent constant (Hammett, 1940), are shown in Figure 3. The slopes of these plots (ρ) are 0.9 (r = 0.93) for rate constants obtained at pH 7.5 and near zero for rate constants determined at pH 5.0. Rate constants

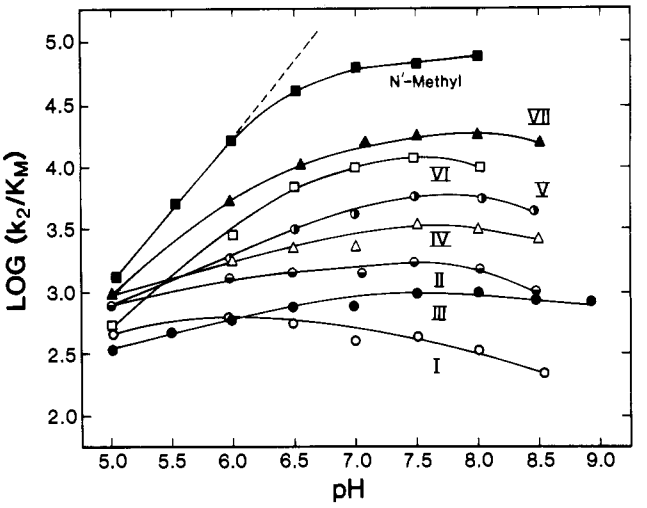


FIGURE 2: Plots of log (k_2/K_m) vs. pH for acylation of α-chymotrypsin by *N*-benzoylimidazoles at 30 °C: *N*-[*p*-(dimethylamino)benzoyl] (○); *N*-benzoyl (●); *N*-(*p*-methoxybenzoyl) (◒); *N*-(*p*-methylbenzoyl) (△); *N*-(*p*-chlorobenzoyl) (◑); *N*-(*m*-nitrobenzoyl) (▲); *N*-(*p*-cyanobenzoyl) (□); *N*-[*p*-(dimethylamino)benzoyl]-*N'*-methyl (■).

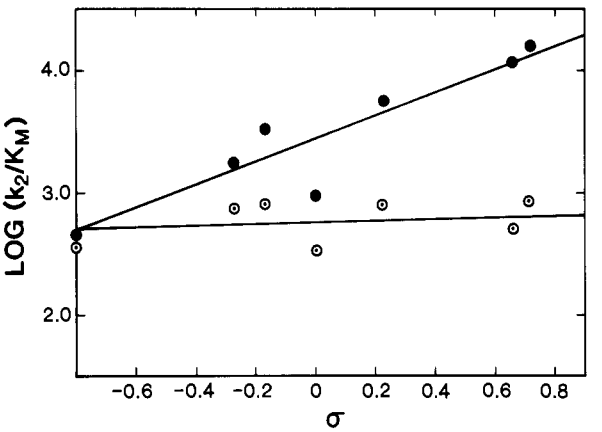


FIGURE 3: Plots of log (k_2/K_m) at 30 °C for acylation of α-chymotrypsin by substituted *N*-benzoylimidazoles vs. σ, the Hammett substituent constant, at pH 5.0 (⊙) and at pH 7.5 (●).

(k_2/K_m) were also determined for some of the compounds at pH 4.01. These constants had the values (I) 100 M^{-1} s^{-1}, (II) 240 M^{-1} s^{-1}, (V) 180 M^{-1} s^{-1}, and (VII) 250 M^{-1} s^{-1}. As at pH 5, the Hammett ρ value at pH 4 is near zero. There is little apparent correlation between log (k_2/K_m) for the neutral species reaction at pH 7.5 and the hydrophobicity constant π (Fujita et al., 1964).

The rates of deacylation of the acyl-enzymes formed from the *N*-benzoylimidazoles were measured by following the increase in absorbance at 400 nm after injection of a solution of the analogous *p*-nitrophenyl ester into the solution of acyl-enzyme. The first-order rate constant for deacylation was calculated from the linear portion of the tracing, the initial concentration of enzyme, and the extinction coefficient of *p*-nitrophenolate anion (Doub & Vandenbelt, 1947). The observed rate constants were closely similar to those measured for deacylation of corresponding acyl-enzymes prepared from *p*-nitrophenyl esters independently. In plots of absorbance vs. time for hydrolysis of the corresponding *p*-nitrophenyl ester by the enzyme solution, there is only a small initial rise in absorbance for the solution to which the *N*-acylimidazole had been previously added but a much larger initial increase due to rapid acylation when the same amount of the *p*-nitrophenyl ester is added alone. The difference in absorbance at time zero in the two cases, obtained by extrapolation, corresponded to

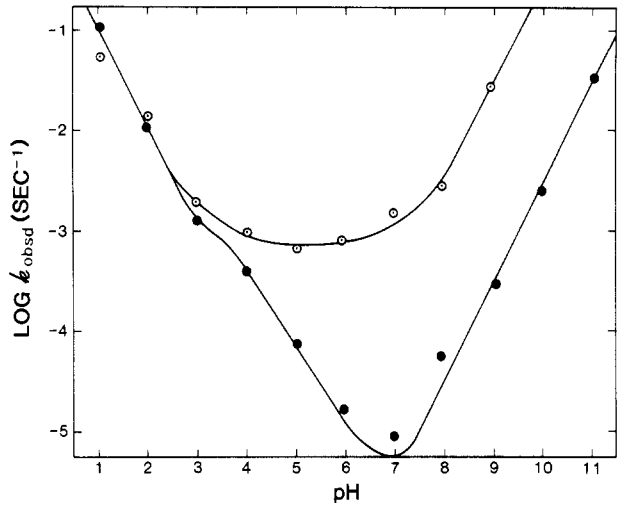


FIGURE 4: Plots of log k_{obsd} vs. pH for hydrolysis of *N*-[*p*-(dimethylamino)benzoyl]-*N'*-methylimidazolium ion (⊙) and *N*-[*p*-(dimethylamino)benzoyl]imidazole (●) in H_2O at 30 °C, μ = 0.1 M.

a concentration of *p*-nitrophenol equal to the enzyme concentration. The *N*-acylimidazoles have therefore acylated the active site almost completely. The rates of deacylation for the two solutions were, however, identical. The limiting value of k_3 (pH >8) for (*p*-cyanobenzoyl)-α-chymotrypsin prepared from the acylimidazole was 3.89×10^{-3} s^{-1}, whereas that obtained with the *p*-nitrophenyl ester was 3.92×10^{-3} s^{-1}. The corresponding rate constants for deacylation of *p*-(dimethylamino)benzoyl-α-chymotrypsin were 9.98×10^{-6} s^{-1} and 9.14×10^{-6} s^{-1}, respectively.

It may be noted in Figure 2 that values of the rate constants (k_2/K_m) at pH 7.5 for I and the analogous N'-methylated derivative differ by only a factor of 150. This is also true in the nonenzymatic OH^--catalyzed hydrolysis reactions. Plots of log k_{obsd} for nonenzymatic hydrolysis at 30 °C and μ = 0.1 M vs. pH are shown in Figure 4. Rate constants were determined in HCl solutions or in 0.01 M buffers (acetate, cacodylate, and Tris). There is buffer catalysis; however, it was shown that such low concentrations of buffer do not have an experimentally significant effect on the observed rate constants. The equation for k_{obsd} in hydrolysis of *N*-[*p*-(dimethylamino)benzoyl]-*N'*-methylimidazolium ion is given in eq 3

$$k_{obsd} = \frac{k_1 a_H^2 + k_2 K_a a_H + k_{OH} K_a K_w}{a_H^2 + K_a a_H} \quad (3)$$

while that for hydrolysis of the unmethylated derivative I is given in eq 4, where k_1 and k_2 are rate constants for attack

$$k_{obsd} = \frac{k_1 a_H^3 + k_2 K_a a_H^2 + k_{OH} K_w K_a a_H + k_{OH}' K_w K_a' K_a}{a_H^3 + K_a a_H^2 + K_a K_a' a_H} \quad (4)$$

of H_2O on the species with the *p*-(dimethylamino) group protonated and unprotonated, k_{OH} is the second-order rate constant for attack of OH^- on the N'-methylated or protonated species of I having the *p*-(dimethylamino) group in the neutral, free-base form, k_{OH}' is the second-order rate constant for attack of OH^- on the neutral species of I, and K_a is the dissociation constant for the *p*-(dimethylamino) group conjugate acid. The second-order rate constants k_{OH}' and k_{OH} are 21.5 and 2150 M^{-1} s^{-1}. A pH-independent reaction is encountered at pH 4–7 in hydrolysis of the *N'*-methyl derivative with $k_2 = 7.1 \times 10^{-4}$ s^{-1}. At low pH, hydronium ion catalysis is observed with both compounds, which must be due to protonation of the *p*-(dimethylamino) group; k_H = 1 M^{-1} s^{-1} ($k_H = k_1/K_a$). Rate measurements were not carried out at sufficiently high acidities to permit determination of K_a; i.e., the dissociation constant is larger than 10^{-1} M. This is reasonable considering that a positive charge in the molecule will increase the value greatly.

Discussion

The acylation of α-chymotrypsin by *N*-benzoylimidazoles is experimentally a second-order reaction. Qualitatively similar second-order acylation reactions were also observed with aliphatic acylimidazole substrates (Kogan et al., 1982). In all cases, plots of k vs. $k/[S]_0$ had infinite slopes, which shows that if binding is occurring, then K_m is much larger than the highest substrate concentration studied (10^{-2}–10^{-3} M) so that ES is experimentally undetectable. Plots of k vs. $[S]_0$ were linear as shown in Figure 1 and give k_2/K_m as the slope. The constant k_2/K_m is the second-order rate constant for reaction of the free enzyme with substrate. The ratio k_2/K_m is not affected by any nonproductive binding of the substrate (Fersht, 1977) and is, therefore, the most reliable parameter to employ in structure–reactivity relationship studies (Brot & Bender, 1969).

The acylation of the enzyme by the substituted *N*-benzoylimidazoles with electron-donating substituents is nearly pH independent. Serine-195 is undoubtedly being acylated in view of the identical rates of deacylation of acylchymotrypsins prepared from the *N*-acylimidazoles and the corresponding *p*-nitrophenyl esters. It is a reasonable assumption that K_m is little affected by changes in pH since K_m for nonionizing substrates has been shown to be essentially pH independent in the range 5–9 (Bender et al., 1964; Laidler & Barnard, 1956; Hammond & Gutfreund, 1955; Cunningham & Brown, 1956). The pH–rate constant profiles of Figure 2 must then primarily reflect the influence of pH on k_2. The simplest kinetic scheme is one in which the *N*-acylimidazoles acylate α-chymotrypsin via both the protonated and neutral species or kinetic equivalents with rate constants that are not greatly different (eq 5) (Kogan et al., 1982). The pK_a values

$$\begin{array}{ccccccc} E & + & S & \rightleftharpoons & ES & \xrightarrow{k_r} & \text{products} \\ {\scriptstyle H^+}\big\uparrow{\scriptstyle K_1} & & {\scriptstyle H^+}\big\uparrow{\scriptstyle K_2} & & {\scriptstyle H^+}\big\uparrow{\scriptstyle K_3} & & \\ EH^+ & & SH^+ & & ESH^+ & \xrightarrow{k_r'} & \text{products} \end{array} \quad (5)$$

of *N*-acylimidazoles and His-57 are approximately 4 and 7, respectively. Consequently, the scheme of eq 5 would explain the near pH independence of the experimental rate constants for I–IV in the pH range of 5–9 if the reactions of the protonated species are of major significance below pH 6 and the reactions of the neutral species are of greater importance at higher pH values.[1] In the case of the *p*-(dimethylamino) derivative, k_r'/K_m is 150-fold larger than k_r/K_m (assuming correspondence of k_r'/K_m to the rate constant of the N'-methylated compound). The reactions through the two species will therefore be approximately equal 2 pH units above the pK_a of the *N*-acylimidazole, i.e., at pH 6. Above this pH, the reaction of the neutral species will then predominate. The pH dependence of the rate constants will, of course, vary depending on the relative values of k_r and k_r'. It will be noted in Figure

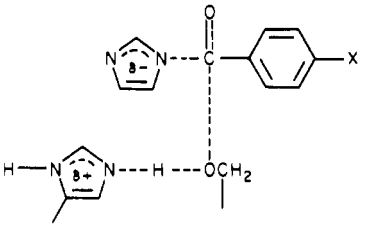
[1] The expression for k derived from the scheme of eq 5 is given in eq

$$k/[S]_T = (k_r K_1 K_3 + k_r' K_1 a_H)/(K_1 K_3 K_m + K_3 K_m a_H) \quad (6)$$

6, considering that $K_2 > a_H$ and $K_m > [S]_0$. Therefore at pH <6, k_r'/K_m would be the predominant influence on k, and at pH >7, k_r/K_m would be of greatest importance if these terms are not greatly different [see the equations and discussion in Kogan et al. (1982)].

2 that when electron-withdrawing substituents are present, the rate constants increase with increasing pH to a maximum value near pH 7. This very likely reflects the increased difficulty of protonation and the increased reactivity of the neutral species.

Electronic Effects in Acylation. The ρ value obtained from the plot of log (k_2/K_m) vs. σ for acylation of α-chymotrypsin by the series of *N*-benzoylimidazoles at pH 7.5 (Figure 3) is only slightly positive (0.9). It should be noted that in view of the near pH independence of k_2/K_m, the ρ value is still 0.9 at higher pH, e.g., 8–8.5, where there can be no contribution from the reaction of the protonated species. The second-order rate constant for acylation is a composite constant, and substituent groups might therefore produce changes in either k_2 or K_m. However, the effects of substituents in the acyl group on the binding (K_s) of substituted benzamides is quite small (ρ = 0 to −0.3) (Marini & Caplow, 1971). Rate constants (k_2/K_m) for acylation of the enzyme by substituted benzoate esters having polar meta and para substituents were found to give reasonably good regression lines in Hammett plots, although there was marked positive deviation produced by nonpolar substituents, presumably due to nonpolar binding effects (Hubbard & Kirsch, 1972). With the present series of substituted *N*-benzoylimidazoles, *p*-CH_3 is the only nonpolar substituent employed, and it will be noted in Figure 3 that it gives only a small deviation on the plots. The rate constants for the unsubstituted derivative III deviate slightly in a negative manner, which suggests that any steric interactions of the substituent groups with the active site are not appreciably increasing K_m in comparison with that of III. Even *m*-NO_2 gives a good fit on the plots established with the para substituents. Thus, it is probable that variations in K_m are not significantly altering the slopes. The ρ value at pH 7.5 very likely indicates that increased electron withdrawal in the acyl group of the *N*-benzoylimidazoles increases the rate constant k_2 for acylation. However, this ρ is considerably less positive than that in hydroxide ion catalyzed hydrolysis (ρ = 1.4) (Fee & Fife, 1966a). The small value of ρ indicates only moderate charge development in the transition state (IX); it is probable



IX

that neither bond making nor bond breaking has progressed to a great extent. The data are also in accord with a mechanism in which there is *no* C–N bond breaking in the transition state. In acylation reactions of substituted phenyl acetates (Bender & Nakamura, 1962) ρ is +1.8 for k_2/K_m, but the correlation was better with σ^- than with σ. Hubbard & Kirsch (1972) determined a ρ of 0.97 (k_2/K_m) for acylation of the enzyme by a series of acyl-substituted *p*-nitrophenyl benzoates and 1.6 for the analogous 2,4-dinitrophenyl benzoates.

The ρ for OH^--catalyzed hydrolysis of ethyl benzoates (Bruice & Benkovic, 1966) is large and positive (2.48), possibly reflecting the influence of electron withdrawal on nucleophilic attack by OH^- at the deactivated carbonyl of the benzoate esters. The ρ for OH^--catalyzed hydrolysis of *p*-nitrophenyl benzoates (Hubbard & Kirsch, 1972) is +2.0. The ρ for alkaline hydrolysis of substituted *N*-benzoylimidazoles is only 1.4, even though the imidazole anion leaving group has a pK_a of 14.5 (Walba & Isensee, 1961), i.e., only slightly less than that of ethanol (Ballinger & Long, 1960). Likewise, the ρ value for water catalysis is 1.35 (Choi & Thornton, 1974). The small ρ values for hydrolysis of *N*-benzoylimidazoles must reflect transition-state structures that are quite different than those in comparable reactions of esters.

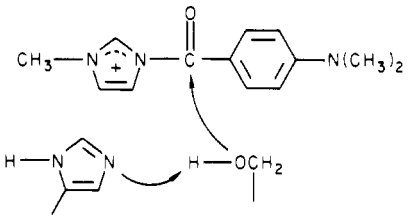
General acid catalysis by the His-57 conjugate acid does not occur in the nucleophilic attack of Ser-195 on neutral *N*-acylimidazoles; a reaction involving a zwitterionic active site, i.e., His-57 conjugate acid and Ser-195 anion, can be ruled out with *N*-acylimidazoles having aliphatic acyl groups (Kogan et al., 1982) and also the *N*-benzoylimidazoles of the present study, because such a mechanism would require true second-order rate constants greater than those for a diffusion-controlled reaction (10^{10} M^{-1} s^{-1}).[2] A mechanism involving attack of the serine anion on the *N*-acylimidazole conjugate acid can be ruled out for the same reason and also because attack of the serine anion does not occur in the reactions of the N′-methylated species at the pH values employed. General acid catalysis by the His-57 conjugate acid could occur in breakdown of a tetrahedral intermediate, but such a stepwise reaction would require that the tetrahedral intermediate be sufficiently stable to reach equilibrium with respect to proton transfer. A mechanism of that type is not in accord with a transition-state resembling reactants. It is probable that in the neutral species reactions His-57 is acting as a general base by partially abstracting a proton from Ser-195 in the transition state (IX). A general-base mechanism is required in acylation by the N′-methylated compound (VIII).

The position of a proton in the transition state of reactions of *N*-acylimidazoles can be determined by comparison of rate constants for reactions of exactly analogous N′-methylated derivatives and unsubstituted compounds (Wolfenden & Jencks, 1961; Oakenfull et al., 1971). As with *N*-(3,3-dimethylbutyryl)-*N*′-methylimidazolium ion (Kogan et al., 1982) and in contrast to the nearly pH-independent acylation reactions of I–IV, the log (k_2K_m)–pH profile for acylation by *N*-[*p*-(dimethylamino)benzoyl]-*N*′-methylimidazolium ion (VIII) shows a rising arm with increasing pH with a slope of 1.0 and a maximum in rate at pH >7. The pK_{app} of 6.5 is near that expected for the conjugate acid of His-57. Thus, the k_2/K_m vs. pH profile indicates participation by the base form of histidine-57.

Histidine-57 is most likely participating in the acylation reaction as a proton-transfer agent. The D_2O solvent isotope effect on k_2/K_m in the reaction of VIII is 2.1 while that in acylation of the enzyme by *N*-(3,3-dimethylbutyryl)-*N*′-methylimidazolium ion is 3.1 (Kogan et al., 1982). Interpretation of D_2O solvent isotope effects in enzyme reactions is difficult (Jencks, 1963), but it has been argued that the solvent isotope effect in both acylation and deacylation reactions of α-chymotrypsin allows a reliable mechanistic interpretation (Bender et al., 1964; Bender & Hamilton, 1962). This viewpoint is strongly supported by the deacylation of [(*p*-nitrophenoxy)carbonyl]chymotrypsin, which proceeds in large part via nucleophilic attack by His-57 with a D_2O solvent isotope effect close to unity as predicted for a nucleophilic reaction (Hutchins & Fife, 1972). It would reasonably be

[2] The ratio of zwitterionic to neutral active site would be approximately 10^{-7} [see the equations in Kogan & Fife (1982)]. Therefore in view of values of k_2/K_m greater than 10^3 M^{-1} s^{-1} for reaction of substituted *N*-benzoylimidazoles (II–VII), true second-order rate constants greater than 10^{10} M^{-1} s^{-1} would be required for a reaction involving a zwitterionic active site in which Ser-195 is ionized and His-57 is present as the conjugate acid.

expected that $K_m^{D_2O}$ would be somewhat less than $K_m^{H_2O}$ (Bender et al., 1964; Bender & Hamilton, 1962). A smaller k_2/K_m in D_2O than in H_2O therefore indicates that a proton is being transferred in the transition state. Since general-acid catalysis is precluded by the N′-methylated leaving group, this indicates that His-57 is participating by partially abstracting a proton from the Ser-195 hydroxyl group in the transition state (X).



X

The log (k_2/K_m) vs. pH profiles for the N′-methylated and unmethylated analogues I and VIII extrapolate to the same pH at approximately pH 4. Thus, since the N′-methylated derivative is a good model, the reactions of the unmethylated *N*-benzoylimidazoles at low pH must involve the conjugate acids in which nitrogen is protonated. The values of k_2/K_m for the series of *N*-benzoylimidazoles are closely similar at pH 5, i.e., in contrast to the positive ρ at pH 7.5 that at pH 5 is nearly zero. At pH 5, the *N*-benzoylimidazoles will be significantly protonated (~10%). Substituent effects on the pK_a would be reasonably small (Choi & Thornton, 1974). It can be seen in Figure 4 that the pK_a' for the dimethylamino-substituted compound is 4; a pK_{app} of 3.4 was determined in nonenzymatic hydrolysis reactions of the *m*-nitro derivative. Therefore, ρ for the protonation (K_a') is approximately 0.4. The ρ values near zero that have been found previously in hydronium ion catalyzed ester and amide hydrolysis (Bruice & Benkovic, 1966) are due to compensating effects of electron withdrawal on protonation and nucleophilic attack by water. However, the ρ value for hydronium ion catalyzed hydrolysis of substituted *N*-benzoylimidazoles at pH 4.7–6 is 0.99 (Choi & Thornton, 1974), which indicates that the ease of nucleophilic attack outweighs basicity considerations in the nonenzymatic hydrolysis of these compounds.

The ρ near zero at pH 4–5 in the acylation reaction can be considered to reflect improvement of the leaving group by protonation, thereby reducing ρ by shifting the position of the transition state toward reactant. The ρ near zero is in accord with a transition state in which there is little difference in charge in comparison with the reactant. Thus, ρ implies a transition state with very little bond making with the nucleophile and little or no C–N bond breaking, with the transition state more closely resembling reactants than that for the reaction of the neutral species at pH 7.5.

The values of k_2/K_m for I and VIII at pH 7.5 differ by a factor of only 150 even though the pK_a of the leaving group of VIII is over 7 pK_a units more favorable (the pK_a of *N*-methylimidazole is 7). Likewise, there is only a difference of 155 in k_2/K_m values for *N*-(3,3-dimethylbutyryl)-*N*′-methylimidazolium ion and *N*-(3,3-dimethylbutyryl)imidazole (Kogan & Fife, 1982). The small rate ratio in these reactions is in accord with leaving groups that are similarly stabilized in the transition state and/or with reactions in which there is little bond breaking. It may be noted that the ratio of the rate constants k_{OH}' and k_{OH} is also similar in nonenzymatic OH^--catalyzed hydrolysis of the unmethylated and N′-methylated *N*-benzoylimidazoles I and VIII. The values of k_{OH} for OH^--catalyzed hydrolysis differ by a factor of 470 with the methylated and unmethylated *N*-acetyl derivatives (Wolfenden & Jencks, 1961; Jencks & Carriuolo, 1959).

The similar rate constant ratios suggest that the same chemical factors are involved in the enzymatic acylation reactions and the nonenzymatic OH^--catalyzed hydrolysis reactions of the *N*-benzoylimidazoles and the corresponding N′-methylated compounds. However, the smaller ρ values in the enzymatic reactions show that there is very likely less bond formation in the transition state than in the hydrolysis reactions. An early transition state would result if the nucleophilic attack by Ser-195 is strongly facilitated by the enzyme. The ability of serine to act as a nucleophile must be due in part to the intracomplex nature of the attack step and to the highly efficient general-base catalysis by His-57 that is experimentally demonstrated in the acylation reactions of the N′-methylated derivative VIII.

Registry No. I, 87970-46-5; II, 10364-93-9; III, 10364-94-0; IV, 10347-11-2; V, 10364-95-1; VI, 87235-62-9; VII, 61652-82-2; VIII, 89165-28-6; α-chymotrypsin, 9004-07-3.

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Low-Temperature Reactions of Trypsin with *p*-Nitroanilide Substrates: Tetrahedral Intermediate Formation or Enzyme Isomerization†

Paul D. Compton and Anthony L. Fink*

ABSTRACT: The reactions of trypsin with the *p*-nitroanilides of N^{α}-carbobenzoxy-L-lysine, N^{α}-carbobenzoxy-L-arginine, and N^{α}-benzoyl-L-arginine have been studied in the 0 to −30 °C temperature region, over a range of pH* values, using 65% (v/v) aqueous dimethyl sulfoxide cryosolvent. At alkaline pH*, −30 °C, the catalytic reaction appears as a slow "burst" of product (or intermediate) followed by turnover. For all three substrates, the *rate* of the burst phase is identical. Preincubation of the enzyme at −30 °C abolishes the burst. On addition of trypsin to the cryosolvent at −30 °C, a time-dependent decrease in fluorescence emission is observed with the same rate as that of the burst with the anilides. The burst phase is thus interpreted as reflecting a temperature/solvent-induced isomerization of trypsin to a less catalytically efficient form, rather than the previously suggested formation of a tetrahedral intermediate [Compton, P. D., & Fink, A. L. (1980) *Biochem. Biophys. Res. Commun. 93*, 427–431]. The isomerization is not observed at high temperature (≥0 °C) or at neutral pH*. The burst phase was not observed with aqueous methanol cryosolvent, indicating that it is sensitive to both cosolvent and temperature.

There is considerable interest in the question of whether tetrahedral adducts exist as discrete intermediates or as transition states in protease catalysis. We have investigated the trypsin-catalyzed hydrolysis of *p*-nitroanilide substrates by using cryoenzymology in order to detect such putative intermediates. In an earlier study (Compton & Fink, 1980), we reported that a "burst" of absorbance in the 350–410-nm region could be observed prior to turnover in the reaction with N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide (ZLyspNA)[1] and attributed the reaction to the formation of a tetrahedral intermediate. Previous investigations (Fink, 1974) have shown that neither the catalytic nor the structural properties of trypsin are perturbed by exposure at 0 °C to 65% (v/v) dimethyl sulfoxide. However, the present structural (protein fluorescence emission) and catalytic (anilide hydrolysis) studies indicate that at lower temperatures in this cryosolvent a structural transition occurs resulting in reduced reactivity toward anilide substrates. Trypsin has a long history of temperature- and ligand-induced transitions (Otero et al., 1980; Mares-Guia et al., 1981) to which the present isomerization must be added.

Experimental Procedures

Materials. N^{α}-Carbobenzoxy-L-lysine *p*-nitroanilide, lot F877, purified by preparative TLC, and the arginine analogue, lot 63797P, were obtained from Vega Biochemicals. The former was also obtained as a generous gift from Prof. Tesser, Organic Chemistry Department, Nijmegen, Holland. N^{α}-Benzoyl-L-arginine *p*-nitroanilide, lot 18C0224, was from Sigma; 3 times crystallized trypsin from Worthington, lot TRL 3 30D727, was used without further purification. Stock solutions of enzyme were prepared in 1 mM HCl and stored at 4 °C. The activity was checked daily by burst titration with

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[1] Abbreviations: TI, tetrahedral intermediate; ZLyspNA, N^{α}-carbobenzoxy-L-lysine *p*-nitroanilide; ZArgpNA, N^{α}-carbobenzoxy-L-arginine *p*-nitroanilide; ZLyspNP, N^{α}-carbobenzoxy-L-lysine *p*-nitrophenyl ester; BzArgpNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; TLC, thin-layer chromatography.

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