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The Effect of the Leaving Group in the Acylation of α-Chymotrypsin by *N*-Acylimidazoles: The Reaction of *N*-(3,3-Dimethylbutyryl)-4(5)-nitroimidazole

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The kinetic and mechanistic effects of substitution in the leaving group of *N*-acylimidazoles in α-chymotrypsin-catalyzed hydrolysis reactions have been investigated. The values of k_2/K_m have been determined for acylation of the enzyme by a series of *N*-(3,3-dimethylbutyryl)-4(5)-substituted imidazoles at 30°C. The plot of log k_2/K_m vs pH for acylation by *N*-(3,3-dimethylbutyryl)-4(5)-nitroimidazole has regions of slope 1.0 and 0 with a pK_{app} of 6.5, in contrast with the nearly pH-independent profiles for *N*-acylimidazoles without electron-withdrawing substituents in the leaving group. The effect of electron withdrawal is to lower the pK_a of the conjugate acid of the *N*-acylimidazole, which thereby restricts the acylation reaction of that species. The pH independence of the acylation reactions of *N*-acylimidazoles without electron withdrawal in the leaving group must reflect reactions of the neutral and protonated species that proceed with similar rate constants. The pK_{app} of 6.5 is constant through wide structural variations and is therefore not due to the kinetics of the reaction, but is very likely the pK_a of the conjugate acid of His-57. There is only a small effect of the pK_a of the leaving group in the acylation reactions with $\beta_{1g} = -0.3$. Therefore, there can only be moderate C–N bond breaking in the critical transition state. A plot of log k_2/K_m vs σ_m, the Hammett substituent constant for *meta* substituents, is linear with a slope, ρ, of 2.6 ($r = 0.98$). When *para* substituent constants are employed the ρ value is 2.1. The effect of substitution in the leaving group is nearly identical in the enzymatic acylation reaction and in the OH^--catalyzed hydrolysis of these compounds in regard to the values of ρ and β_{1g}. Thus, the transition states in the two reactions must be similar in regard to the extent of C–N bond breaking and C–O bond formation with the nucleophile. The transition state very likely resembles the reactants.

INTRODUCTION

α-Chymotrypsin-catalyzed reactions of both specific and nonspecific ester and amide substrates follow the scheme of Eq. [1], where ES′ is an acyl enzyme intermediate (*1–5*).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{\displaystyle P_1}{\underset{+}{ES'}} \xrightarrow{k_3} E + P_2 \qquad [1]$$

The acyl enzyme is an ester of serine-195 (*1, 6*). Histidine-57 is also located at the active site and presumably participates in both acylation and deacylation. Crystallo-

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graphic studies of serine proteases have suggested that the enzymes might stabilize an anionic tetrahedral intermediate by hydrogen bonding in the "oxyanion hole" (*7–12*).[1] With α-chymotrypsin this stabilization would involve the peptide NH groups of Ser-195 and Gly-193 as the proton donors.

N-Acylimidazoles are excellent acylating agents of the active site of α-chymotrypsin (*14–19*). Binding of these substrates to the enzyme is weak (an ES complex cannot be experimentally detected), but nevertheless the second-order rate constants at 30°C are very large with compounds having a long hydrocarbon chain in the acyl group; k_2/K_m for acylation of the enzyme by *N*-(β-phenylpropionyl)imidazole (*17*) and *N*-(β-cyclohexylpropionyl)imidazole (*19*) is 2×10^6 M^{-1} s^{-1}, at 30°C, making these compounds the best amide substrates known for α-chymotrypsin.

Major steric rate retardations due to increased alkyl group branching in the acyl group are absent in nonenzymatic hydrolysis of *N*-acylimidazoles (*20*) and *N*-acylimidazolium ions (*21*), which indicates that attack by a nucleophile has not progressed to a great extent in the critical transition state. Thus, there cannot be large charge development on the carbonyl oxygen in the transition state. The same considerations apply in the enzymatic acylation reactions of these compounds (*17*, *19*). The very rapid rates of acylation of α-chymotrypsin by *N*-acylimidazoles cannot then be attributed to oxyanion hole interactions, but must be due to other mechanistic factors, which should be more easily identified if the former effects can be neglected.

The nonenzymatic alkaline and pH-independent hydrolysis reactions of *N*-acyl-4(5)-substituted imidazoles have been investigated (*22*). Determination of the second-order rate constants k_2/K_m for acylation of α-chymotrypsin by *N*-acyl-4(5)-substituted imidazole substrates would allow the effect of the leaving group in these reactions to be ascertained and would permit a direct comparison of the enzymatic reaction with OH^--catalyzed hydrolysis. Since in the latter reaction the transition state structure has been reasonably well established (*16*, *23*), such a comparison should provide considerable insight into the mechanism of the enzymatic reaction. Consequently, we have now studied acylation of the enzyme by the 4(5)-nitro-substituted compound **I** and a series of corresponding 4(5)-substituted *N*-3,3-dimethylbutyrylimidazoles (Scheme 1).

EXPERIMENTAL SECTION

Materials. α-Chymotrypsin, 3× 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. Imidazole was obtained from Aldrich and was sublimed prior to use. *N-trans*-Cinnamoylimidazole was prepared according to the method of Schonbaum *et al.* (*15*). The

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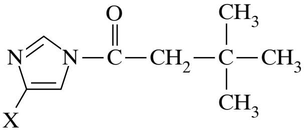
[1] Postulations of rate enhancements in enzymatic reactions where stabilization of an intermediate occurs should be viewed with caution. Stabilization of a tetrahedral intermediate would lower the rate of reversion to reactants, but would also decrease the rate of breakdown to products. A central postulate in enzymology has been that an enzyme will stabilize the *transition state* of the catalyzed reaction (*13*).



X
I, NO_2
II, Br
III, H
IV, CH_3

SCHEME 1

N-acylimidazoles **I–IV** were prepared in the same manner as previously described (*22*).

Stock solutions of proflavin hydrochloride (Aldrich) were prepared in distilled water. Stock solutions of α-chymotrypsin were prepared in pH 5.0 acetate buffer (0.1 M, μ = 0.1 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.* (*15*)], with a reproducibility of about 1%. Titration values of the stock solutions are stable for several weeks at 5°C and pH 5.0 (0.1 M acetate buffer). Fresh solutions were routinely prepared. Buffers were prepared from reagent grade chemicals.

Kinetic methods. Acylation of α-chymotrypsin by the *N*-acylimidazoles at 30°C was monitored in the presence of proflavin by methods previously described (*17*). Proflavin forms a 1:1 complex with the active site of α-chymotrypsin. There is a large difference in absorbance at 465 nm between the complexed and the uncomplexed species. Acylation of the enzyme results in the displacement of proflavin with a consequent large absorbance change at 465 nm. Therefore, acylation reactions can be conveniently followed spectrophotometrically in the presence of proflavin (*17*, *24*, and references cited therein). Second-order rate constants for the acylation reactions of *N*-acylimidazoles are quite similar when determined by either proflavin displacement or disappearance of substrate (*17*). All kinetic runs were in 0.1 M buffers (μ = 0.1 M with NaCl). The substrate concentrations were varied in the range 1.0×10^{-4} to 2×10^{-3} M with **I** and **II** and 2×10^{-4} to 8×10^{-3} M with **III** and **IV.** The enzyme concentration was generally 2.08×10^{-5} M. The proflavin concentration was 7.2×10^{-5} M. Acetate, phosphate, MES, *N*-ethylmorpholine, Tris, and morpholine buffers were employed. The different buffers yielded consistent results when employed at the same pH. Acylation reactions were followed to completion between 465 and 490 nm at 30°C, employing either a Beckman Model 25 spectrophotometer or a Durrum Model D-110 stopped-flow spectrophotometer. Absorbance changes in the stopped-flow determinations, after mixing the enzyme–proflavin and substrate solutions contained in the two drive syringes, were recorded on a Hewlett–Packard storage oscilloscope (Model 1207B). Good first-order kinetics were obtained in all cases, and pseudo-first-order rate constants were computer calculated. Corrections were made for the very slow spontaneous hydrolysis of the substrate in the aqueous holding solution ($\mu = 0.1$ M). Reaction pH values were obtained using a Radiometer Model 22 pH meter or a Beckman Model 3500 digital pH meter.

The rates of deacylation of the acyl enzymes formed from the substituted *N*-3,3-dimethylbutyryl imidazoles can be measured by following the increase in absorbance at 400 nm after injection of a solution of *p*-nitrophenyl 3,3-dimethylbutyrate into the solution of acyl enzyme (*17*). The rate constant for deacylation of the acyl enzyme obtained with *N*-3,3-dimethylbutyrylimidazole is $9.2 \times 10^{-5}\ \mathrm{s}^{-1}$ at 25°C and pH 6.9, compared with $1.01 \times 10^{-4}\ \mathrm{s}^{-1}$ for that formed from the corresponding *p*-nitrophenyl ester. 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 (*25*). 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 ($[\mathrm{S}]_0 > [\mathrm{E}]_0$; see Ref. *17* for an illustration of this effect). The difference in absorbance at time zero in the two cases, obtained by extrapolation, invariably corresponds to a concentration of *p*-nitrophenol equal to the enzyme concentration. The *N*-acylimidazoles, therefore, acylate the active site almost completely.

RESULTS

The scheme of Eq. [1] yields Eq. [2] for reactions of α-chymotrypsin,

$$k = \frac{(k_2 + k_3)[\mathrm{S}]_0 + k_3 K_m}{[\mathrm{S}]_0 + K_m} = \frac{k_2[\mathrm{S}]_0}{[\mathrm{S}]_0 + K_m} + k_3, \qquad [2]$$

where $K_m = (k_{-1} + k_2)/k_1$ and k is the first-order rate constant governing the pre-steady-state reaction (*26*). The first-order rate constants k for acylation of α-chymotrypsin by the *N*-acylimidazoles (**I–IV**) were determined at 30°C by proflavin displacement from the active site. Plots of k vs $k/[\mathrm{S}]_0$ were in each case vertical even at very high substrate concentrations (8×10^{-3} M with **IV**), which indicates that the highest substrate concentration is still much less than K_m. Consequently, the reactions are experimentally second order. A plot of $k - k_3$ vs $[\mathrm{S}]_0$, or k vs $[\mathrm{S}]_0$ if k_3 is negligible, as is the case when the acyl group is 3,3-dimethylbutyryl (*27*), should then have an intercept of zero, and the slope will be equal to the pH-dependent second-order acylation rate constant k_2/K_m. Linear plots of k vs $[\mathrm{S}]_0$ were invariably found. A typical example in acylation of the enzyme by *N*-(3,3-dimethylbutyryl)-4(5)-nitroimidazole is shown in Fig. 1. The intercepts of such plots are generally zero within experimental error. The values of $k/[\mathrm{S}]_0$ determined from the abscissa intercepts of plots of k vs $k/[\mathrm{S}]_0$ should be equal to k_2/K_m and were closely similar to the values determined from the plots of k vs $[\mathrm{S}]_0$. The rates of acylation of α-chymotrypsin by the *N*-acylimidazoles **I–IV** are much greater than

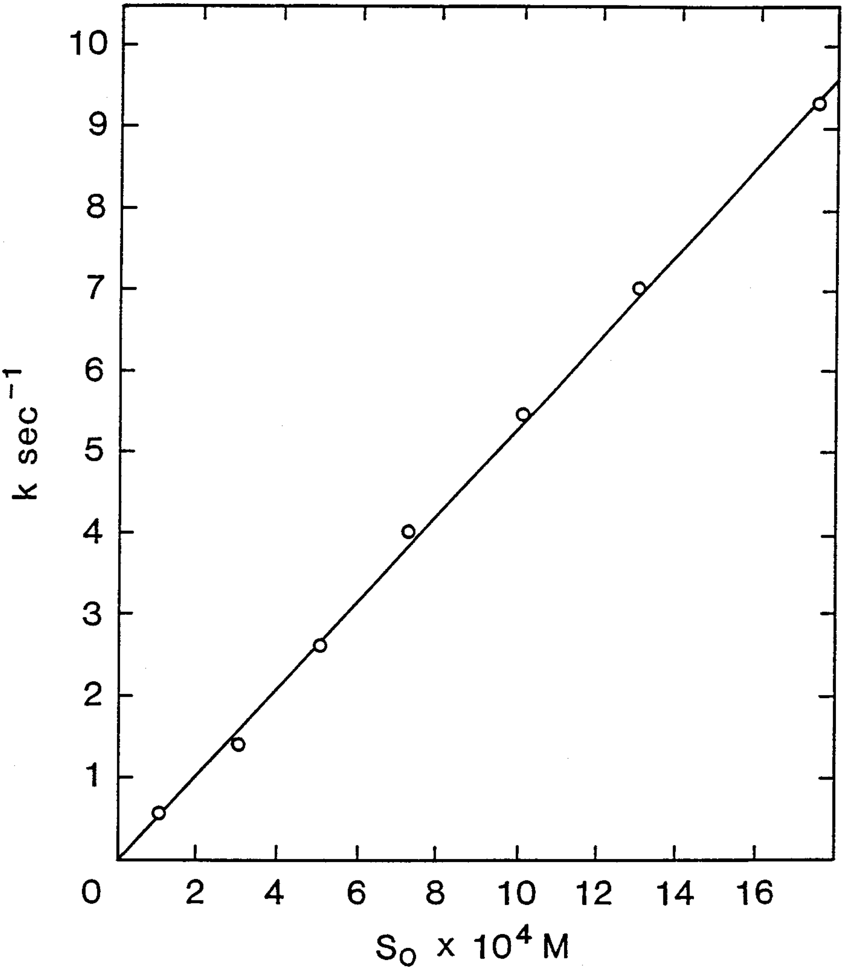


FIG. 1. Plot of k vs $[S]_0$ for acylation of α-chymotrypsin by *N*-(3,3-dimethylbutyryl)-4(5)-nitroimidazole at 30°C and pH 7.22 (*N*-ethylmorpholine buffer).

those of deacylation. Subtraction of k_3 from k employing Eq. [2] and the values of k_3 determined for deacylation produced no change in the second-order rate constants k_2/K_m.

The log k_2/K_m vs pH profiles for acylation of α-chymotrypsin by the *N*-acylimidazoles **I** and **IV** are shown in Fig. 2. The acylation reaction of **IV** is essentially independent of pH. However, in the case of the nitro-substituted derivative **I** the profile has regions with the limiting slopes of 1.0 and 0 with a pK_{app} of 6.5. The limiting values of k_2/K_m at high pH increase in the order **IV** < **III** < **II** < **I.** At pH 8.0 k_2/K_m is **I,** 6700 M^{-1} s^{-1}; **II,** 2850 M^{-1} s^{-1}; **III,** 150 M^{-1} s^{-1}; and **IV,** 66 M^{-1} s^{-1}.

There is a reasonable linear correlation between log k_2/K_m at pH 8.0 and σ_p, the

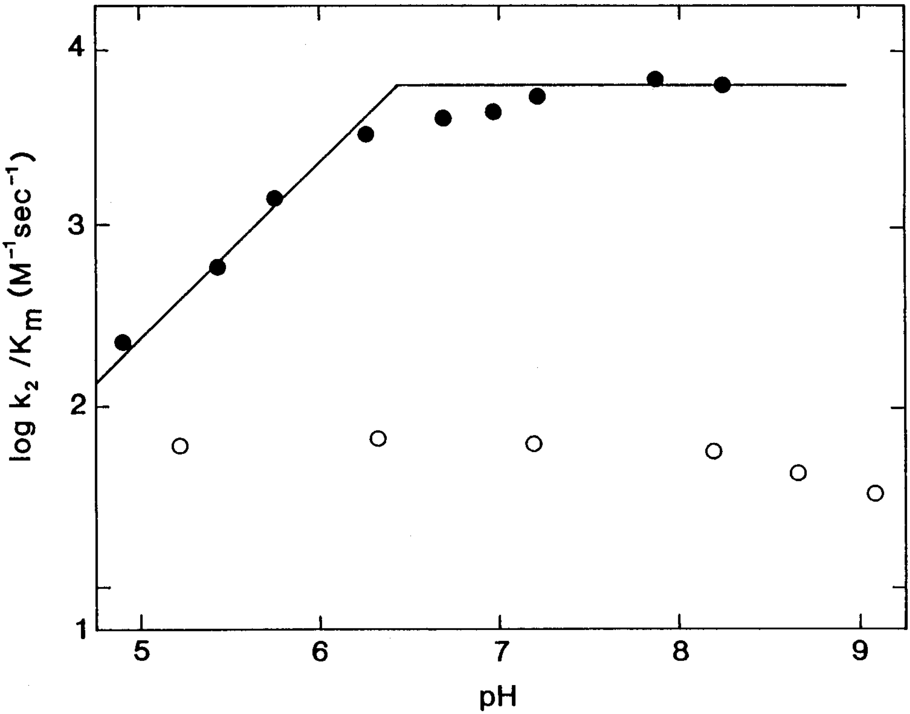


FIG. 2. Plots of log k_2/K_m vs pH for the acylation of α-chymotrypsin by *N*-(3,3-dimethylbutyryl)-4(5)-nitroimidazole (●) and *N*-(3,3-dimethylbutyryl)-4(5)-methylimidazole (○) at 30°C.

Hammett substituent constant (*28*) for *para* substituents (σ_p for NO_2 is 0.778). The slope ρ is 2.15 with a correlation coefficient of 0.92. The point for the bromo-substituted compound deviates positively from the line by 0.6 log units. This deviation by Br may indicate that hydrophobic interactions are also of some importance. The use of the Hansch hydrophobicity constants π (*29*) in a 4-parameter equation (Eq. [3]),

$$\log k_2/K_m = \sigma\rho + \gamma\pi + C, \qquad [3]$$

improves the fit of the experimental data, but does not appreciably alter the ρ value. The ρ calculated from Eq. [3] is 2.3 and $\gamma = 0.6$. The *meta* substituent constants provide a better linear correlation with log k_2/K_m (σ_m for Br is 0.391) with $\rho = 2.6$ ($r = 0.98$), and this plot is shown in Fig. 3. Again the point for the bromo-substituted compound deviates positively but by only 0.2 log units.

DISCUSSION

The acylation of α-chymotrypsin by *N*-acylimidazoles is experimentally a second-order reaction (see also Refs. *17–19*). In each case K_m must be much larger than the highest substrate concentration studied (10^{-3} to 10^{-2} M) so that an enzyme