# Influence of Hydrophobic and Steric Effects in the Acyl Group on Acylation of α-Chymotrypsin by *N*-Acylimidazoles†

Robert L. Kogan and Thomas H. Fife*

*Department of Biochemistry, University of Southern California, Los Angeles, California 90033*

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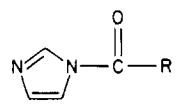
ABSTRACT: The second-order rate constants $k_2/K_m$ for acylation of α-chymotrypsin by a series of *N*-acylimidazole derivatives of aliphatic carboxylic acids have been determined at 30 °C by proflavin displacement from the active site. With cyclohexyl-substituted *N*-acylimidazoles, the rate constants increase with increasing chain length of the acyl group; i.e., $k_2/K_m$ is in the order cyclohexylcarbonyl < cyclohexylacetyl < β-cyclohexylpropionyl. The latter substrate has $k_2/K_m = 1.2 \times 10^6$ $M^{-1}$ $s^{-1}$ at pH 8.0, which appears to be a maximum value for *N*-acylimidazole substrates. A further increase in the chain length of the acyl group with (γ-cyclohexylbutyryl)imidazole results in a decrease in $k_2/K_m$. Hydrophobic effects of the hydrocarbon acyl groups are of predominant importance with regard to the relative values of $k_2/K_m$ for aliphatic *N*-acylimidazole substrates. There is a linear correlation of the logarithms of the rate constants at pH 8.0 with the hydrophobic substituent constants, π, having a slope of 1.71 ($r = 0.90$). On the other hand, there is little apparent correlation with the Taft steric effect constants, $E_s$. A four-parameter equation including both π and $E_s$ improved the correlation only slightly [$\log (k_2/K_m) = 1.88\pi + 1.01E_s + C$]. In contrast, steric effects as reflected in the $E_s$ constants are the major influence in acylation of the enzyme by corresponding *p*-nitrophenyl esters. There are very likely significant differences in transition-state structure with the two types of substrates.

The 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 (Bruice &

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

Benkovic, 1966; Bender & Kezdy, 1964; 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.

*N*-Acylimidazoles will acylate the active site of α-chymotrypsin quite readily (Schonbaum et al., 1961; Bender et al., 1962; Kogan et al., 1982). Binding of these substrates to the enzyme must be weak (an ES complex cannot be experimentally detected), but nevertheless, the second-order rate constants are quite large at 30 °C when there is a long hydrocarbon chain in the acyl group; $k_2/K_m$ for *N*-hexanoylimidazole at pH 7.5 is $5 \times 10^4$ $M^{-1}$ $s^{-1}$ while that for *N*-(β-phenylpropionyl)imidazole is $1.2 \times 10^6$ $M^{-1}$ $s^{-1}$. These rate constants are much larger than usually encountered in reactions of amides with α-chymotrypsin; e.g., $k_2/K_m$ at pH 7.9 for acylation by *N*-acetyl-L-phenylalaninamide is 1.5 $M^{-1}$ $s^{-1}$ (Zerner et al., 1964). There is clearly a rate-enhancing effect due to the hydrophobic nature of the acyl group of *N*-acylimidazoles (Kogan et al., 1982). Such an effect has been noted previously with other types of substrates (Hofstee, 1957, 1959; Jones et al., 1965; Milstien & Fife, 1969; Dupaiz, 1973). An understanding of this effect could provide considerable insight into the structure of the transition state in the acylation reaction. We have therefore attempted to maximize hydrophobic effects in the acylation reactions and to ascertain the limits of these effects by employing a series of cyclohexyl-substituted *N*-acylimidazole substrates (I–IV) with varying acyl group chain length.



I, R = $C_6H_{11}$
II, R = $CH_2C_6H_{11}$
III, R = $CH_2CH_2C_6H_{11}$
IV, R = $CH_2CH_2CH_2C_6H_{11}$
V, R = $C(CH_3)_3$

A formidable problem in a study of hydrophobic effects in enzymatic acylation reactions is that changes in the acyl group will also alter steric hindrance to approach of a nucleophile. We have therefore also studied the trimethylacetyl derivative (V), which combines a high degree of hydrocarbon surface with large steric bulk. Compounds I–V in combination with the *N*-acylimidazoles previously studied (Kogan et al., 1982) provide a series that allows the quantitative separation of hydrophobic and steric effects through the use of the π hydrophobicity constants (Fujita et al., 1964) and the Taft steric effect constants, $E_s$ (Taft, 1956).

## 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. 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*-acylimidazoles I–IV were prepared by mixing the appropriate carboxylic acid and imidazole with *N*,*N*-dicyclohexylcarbodiimide in a 1:1:1 molar ratio in methylene dichloride. The reaction was complete in several hours. The

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mixture was filtered to remove the *N*,*N*-dicyclohexylurea precipitate. The solvent was then removed by rotary evaporation. The residual material was either distilled or recrystallized from an appropriate solvent. *N*-(Cyclohexylcarbonyl)imidazole (I) had an mp of 87–88 °C after recrystallization from petroleum ether. Anal. Calcd for $C_{10}H_{14}N_2O$: C, 67.39; H, 7.92; N, 15.72. Found: C, 67.52; H, 7.85; N, 15.73. *N*-(Cyclohexylacetyl)imidazole (II) had an mp of 71–72 °C after recrystallization from petroleum ether. Anal. Calcd for $C_{11}H_{16}N_2O$: C, 68.72; H, 8.39; N, 14.57. Found: C, 68.96; H, 8.38; N, 14.95. *N*-(β-Cyclohexylpropionyl)-imidazole (III) had a bp of 135–137 °C at 0.02 mmHg and an mp of 47–49 °C. Anal. Calcd for $C_{12}H_{18}N_2O$: C, 69.87; H, 8.79; N, 13.58. Found: C, 69.89; H, 8.89; N, 13.34. *N*-(γ-Cyclohexylbutyryl)imidazole (IV) had an mp of 75.5–77.5 °C after recrystallization from cyclohexane. Anal. Calcd for $C_{13}H_{20}N_2O$: C, 70.87; H, 9.15; N, 12.72. Found: C, 70.49; H, 9.04; N, 12.61. *N*-(Trimethylacetyl)imidazole (V) was prepared as reported by Fife (1965) and had an mp of 54–56 °C [lit. (Staab, 1956) mp 55–56 °C].

*p*-Nitrophenyl esters corresponding to the *N*-acylimidazoles were prepared from the appropriate carboxylic 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 ethanol. *p*-Nitrophenyl cyclohexylcarboxylate had an mp of 50–52 °C [lit. (Silver, 1966) mp 49–51 °C]. *p*-Nitrophenyl cyclohexylacetate had an mp 25.5–26.5 °C (Murakami et al., 1977). *p*-Nitrophenyl β-cyclohexylpropionate had an mp of 80–82 °C. Anal. Calcd for $C_{15}H_{19}NO_4$: C, 64.97; H, 6.91; N, 5.05. Found: C, 64.67; H, 6.95; N, 4.98.

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, $\mu$ = 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*-acylimidazoles 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. 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 [Brandt et. al., 1967; Kogan et al. (1982) and references cited therein]. All kinetic runs were in 0.1 M buffers ($\mu$ = 0.5 M with NaCl). The concentrations of α-chymotrypsin and proflavin were at 0.3 to 1.65 × $10^{-5}$ M and 7.7 × $10^{-5}$ M, respectively. Substrate concentrations were varied in the range 2.5 × $10^{-5}$ to 5.4 × $10^{-3}$ M. Acetate, phosphate, Tris, and 2-amino-2-methyl-1,3-propanediol (ammediol) 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 with either a Beckman Model 25 spectrophotometer or a Durrum Model D-110 stopped-flow spectrophotometer. Absorbance changes after the mixing in the stopped-flow determinations were recorded on a Hewlett-Packard storage oscilloscope. Good first-order kinetics were obtained in all cases, and pseudo-first-order rate constants were calculated with an IBM-370 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).

The rates of deacylation of the acyl enzymes formed from the *N*-acylimidazoles 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). Similar measurements were described in Kogan et al. (1982).

## 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). Values of the first-order rate constant $k$ for acylation of α-chymotrypsin by the *N*-acylimidazoles (I–V) were determined at 30 °C by proflavin displacement from the active site. Plots of $k$ vs. $k/[S]_0$ were in each case vertical even at very high substrate concentrations (5 × $10^{-3}$ M). The vertical plots indicate that the highest substrate concentration is still much less than $K_m$. As a consequence, the reaction should be experimentally second order. A plot of $k - k_3$ vs. $[S]_0$ or $k$ vs. $[S]_0$ if $k_3$ is negligible should then have an intercept of zero, and the slope will be equal to the pH-dependent second-order acylation rate constant $k_2/K_m$. It was found that plots of $k$ vs. $[S]_0$ were invariably linear. Typical examples of such plots for acylation of the enzyme by *N*-acylimidazoles have been shown previously (Kogan et al., 1982; Kogan & Fife, 1984). 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 cases, the rates of acylation of α-chymotrypsin by the *N*-acylimidazoles were much greater than 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 constant $k_2/K_m$ because $k_3$ is not significant in comparison with $k$.

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

The log $(k_2/K_m)$–pH profiles for acylation of α-chymotrypsin by the *N*-acylimidazoles are shown in Figure 1. With the exception of I, acylation is essentially independent of pH at pH >7. As seen in Figure 1, the values of $k_2/K_m$ increase in the order V < I < II < IV < III; i.e., the rate of acylation in general increases as chain length in the acyl group increases to a maximum value with III. There is, however, a rate-retarding effect due to chain branching as shown by the relatively slow rate of acylation of the enzyme by V. At pH 8.0, $k_2/K_m$ values are as follows: I, 4800 $M^{-1}$ $s^{-1}$; II, 96 500 $M^{-1}$ $s^{-1}$; III, 1.2 × $10^6$ $M^{-1}$ $s^{-1}$; IV, 4.6 × $10^5$ $M^{-1}$ $s^{-1}$; V, 350 $M^{-1}$ $s^{-1}$. There is little apparent correlation between log $(k_2/K_m)$ and $E_s$, the Taft steric effect constant, as seen in Figure 2 where the

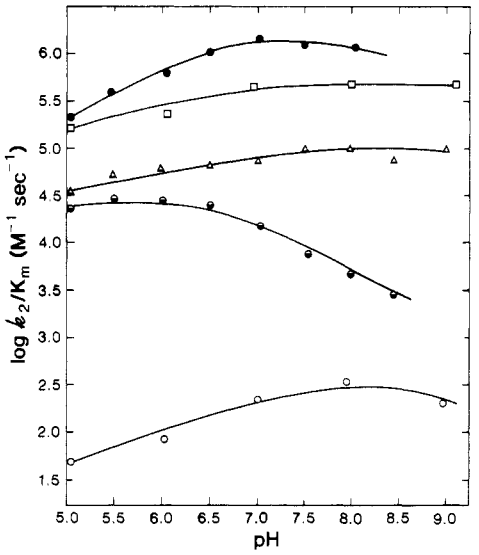


FIGURE 1: Plots of log ($k_2/K_m$) vs. pH for acylation of $\alpha$-chymotrypsin at 30 °C: *N*-(cyclohexylcarbonyl)imidazole (◒), *N*-(cyclohexylacetyl)imidazole (△), *N*-($\beta$-cyclohexylpropionyl)imidazole (●), *N*-($\gamma$-cyclohexylbutyryl)imidazole (□), and *N*-(trimethylacetyl)imidazole (○).

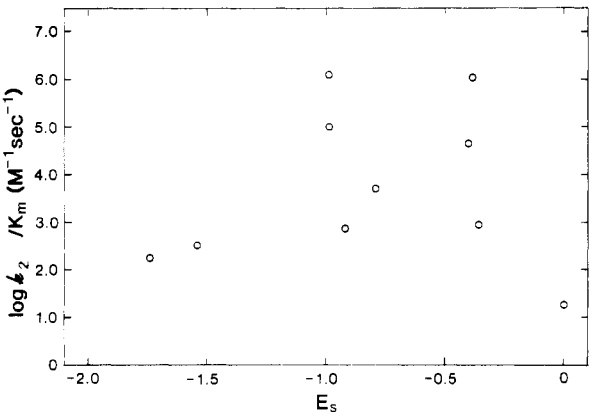


FIGURE 2: Plot of log ($k_2/K_m$) for acylation of $\alpha$-chymotrypsin at 30 °C and pH 8.0 vs. the Taft steric effects constant $E_s$ (Taft, 1956). The values of $k_2/K_m$ are those determined for reaction of compounds I–V in this study and aliphatic *N*-acylimidazoles reported in Kogan et al. (1982).

logarithms of the rate constants for I–V and those obtained previously with other aliphatic *N*-acylimidazoles are plotted vs. $E_s$. Plots of log ($k_2/K_m$) at pH 8.0 vs. $\pi$, the Hansch hydrophobicity constant (Fujita et al., 1964), are shown in Figure 3.[1] The slope of this plot is 1.71 ($r$ = 0.90). Inclusion of the $E_s$ constants in a four-parameter eq (eq 3) improves the

$$\log (k_2/K_m) = \gamma\pi + \delta E_s + C \qquad (3)$$

fit only slightly. While the value of $\gamma$ calculated as the slope of Figure 3 is 1.71 ± 0.25, that calculated from eq 3 is 1.88 ± 0.16. The value of $\delta$ in eq 3 is 1.01 ± 0.23. Polar effects in these reactions are negligible due to the small range of $\sigma^*$ values for the aliphatic acyl groups and the small influence of polar effects in acylation reactions of *N*-acylimidazoles (Kogan & Fife, 1984). It should be noted in Figures 2 and 3 that the values of $k_2/K_m$ extend over a $10^5$ range. In Figures 2 and 3, all substrates were compared at the same pH (8.0).

[1] The $\pi$ values either were those of Fujita et al. (1964) (phenoxyacetic acids) or, in view of the additive nature of the constants, were calculated for larger alkyl derivatives by allowing 0.5 for each additional $CH_3$ or $CH_2$ group.

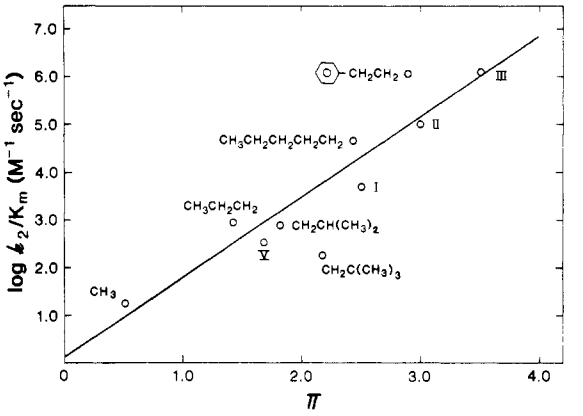


FIGURE 3: Plot of log ($k_2/K_m$) for acylation of $\alpha$-chymotrypsin at 30 °C and pH 8.0 vs. the Hansch hydrophobicity constant $\pi$ (Fujita et al., 1964). The values of $k_2/K_m$ are those determined for reaction of compounds I–V in this study and aliphatic *N*-acylimidazoles reported in Kogan et al. (1982).

Employing limiting values of $k_2/K_m$ or omitting the point for I, with which the rate constants decline slightly with increasing pH, does not significantly affect the plots.

The rate constants for deacylation of acyl-$\alpha$-chymotrypsins obtained by reaction of the enzyme with the *N*-acylimidazoles were closely similar to those measured for deacylation of corresponding acyl enzymes prepared from *p*-nitrophenyl esters independently. The limiting values of $k_3$ (pH >8) were as follows: (cyclohexylcarbonyl)-$\alpha$-chymotrypsin, $3.3 \times 10^{-2}$ $s^{-1}$; (cyclohexylacetyl)-$\alpha$-chymotrypsin, $1.29 \times 10^{-2}$ $s^{-1}$; ($\beta$-cyclohexylpropionyl)-$\alpha$-chymotrypsin, $5.15 \times 10^{-1}$ $s^{-1}$; (cyclohexylbutyryl)-$\alpha$-chymotrypsin, $2.44 \times 10^{-1}$ $s^{-1}$. The identical rate constants for deacylation of acylchymotrypsins, prepared from the *N*-acylimidazoles and the corresponding *p*-nitrophenyl esters show that the same group (Ser-195) is being acylated by the two types of compounds.

## Discussion

The acylation of $\alpha$-chymotrypsin by *N*-acylimidazoles is experimentally a second-order reaction [see Kogan et al. (1982)]. In each case the plots of $k$ vs. $k/[S]_0$ have infinite slopes. Therefore, the $K_m$ values are larger than the highest substrate concentrations studied ($10^{-2}$–$10^{-3}$ M) so that an enzyme–substrate complex is experimentally undetectable. The slopes of the linear plots of $k$ vs. $[S]_0$ are the second-order rate constants ($k_2/K_m$) for reaction of the free enzyme with substrate. It must be emphasized that the ratio $k_2/K_m$ is not affected by any nonproductive binding of the substrate and that $k_2/K_m$ is therefore the most reliable parameter to employ in structure–reactivity relationship studies (Brot & Bender, 1969; Fersht, 1977).

The rate constants $k_2/K_m$ for acylation of the enzyme by *N*-acylimidazoles are in general nearly pH independent. With the exception of I, there is in all cases a small increase in $k_2/K_m$ with increasing pH to approximately pH 7, and at higher pH values the reactions are pH independent. 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–log rate constant profiles must then primarily reflect the influence of pH on $k_2$. The simplest kinetic scheme is one in which the *N*-acylimidazoles acylate $\alpha$-chymotrypsin via both the protonated and neutral species or kinetic equivalents with rate constants that are not greatly different for the two species. Equations for such a scheme have

been given in Kogan et al. (1982). In the case of the *N*-(3,3-dimethylbutyryl) derivative the rate constant of the protonated species ($k_r'/K_m$) is 150-fold larger than that of the neutral species ($k_r/K_m$) (assuming correspondence of $k_r'/K_m$ to the rate constant of the N′-methylated compound) (Kogan et al., 1982). The reactions through the two species are therefore equal approximately 2 pH units above the p$K_a$ of the *N*-acylimidazole (~4), i.e., near pH 6. The reaction of the neutral species then predominates at higher pH, and at pH 8 the values of $k_2/K_m$ are strictly those of the neutral species. Note, however, that the rate constants obtained with I decrease with increasing pH at pH greater than 6.5. This very likely reflects a larger ratio of $k_r'/K_m$ to $k_r/K_m$ than with the other compounds in the series.

The rate constants for acylation of α-chymotrypsin increase with increasing chain length of the acyl group in the order I < II < III. The value of $k_2/K_m$ for the latter compound at pH 8.0 is $1.2 \times 10^6$ $M^{-1}$ $s^{-1}$, identical with that for acylation by *N*-(β-phenylpropionyl)imidazole (Kogan et al., 1982). An increase in rate with increasing chain length of the acyl group has been previously observed in reaction of the enzyme with ester substrates (Hofstee, 1957, 1959; Jones et al., 1965; Milstien & Fife, 1969). This effect must be associated with the hydrophobic nature of the active site region in which the acyl group of the substrate is bound. An upper limit on the size of the acyl group is apparently reached with III; $k_2/K_m$ for IV, with which the acyl group chain has been increased by an additional methylene group, is several fold less than that of III. Thus, folding of the hydrocarbon chain may have an unfavorable effect on the rate.

The importance of hydrophobic effects is clearly demonstrated by the linear relationship between log ($k_2/K_m$) and the substituent constants π shown in Figure 3. These constants were obtained as the ratio of the partitioning of substituted compounds in *n*-octanol and water, $\pi = \log (P_X/P_H)$ (Fujita et al., 1964), and presumably provide a quantitative relative measure of the substituents' ability to interact hydrophobically with a nonpolar environment. The linear plot with the large slope of 1.71 can be taken as evidence for binding of the *N*-acylimidazoles to the enzyme, even though kinetic evidence for the formation of an ES complex cannot be obtained.

It would reasonably be expected that steric hindrance in the acyl group toward approach of a nucleophile would be important with regard to the magnitude of $k_2/K_m$. However, as seen in Figure 2, there is little apparent correlation between log ($k_2/K_m$) for acylation of α-chymotrypsin by *N*-acylimidazoles and the Taft steric effects constant $E_s$. This is in contrast to acylation by corresponding *p*-nitrophenyl esters with which the plot of log ($k_2/K_m$) vs. $E_s$ had a slope of 0.95 ($r$ = 0.98) excluding points for long-chain acyl groups, e.g., hexanoyl (Milstien & Fife, 1969). Hansch & Coats (1970) reported that inclusion of the π constants with $E_s$ in a four-parameter equation improved the correlation when all of the data was employed. At pH 7.99, the best fit values of δ and γ were calculated to be 1.51 and 0.63. Thus, while hydrophobic interactions do have an influence on acylation by *p*-nitrophenyl esters, steric effects are definitely of predominant importance. On the other hand, employing the four-parameter equation (eq 3) for acylation by *N*-acylimidazoles gives values of δ and γ of 1.01 and 1.88, and the correlation is only moderately improved by inclusion of the $E_s$ constants. In the plot of log $k_2/K_m$ vs. π (Figure 3), γ is 1.71 ± 0.25, while from eq 3 γ is calculated to be 1.88 ± 0.16. As a consequence, the importance of steric effects is less in the acylation reactions of *N*-acylimidazoles in comparison with those of *p*-nitrophenyl esters, and it is clear that the hydrophobic nature of the acyl group is of much greater importance in the reactions of the *N*-acylimidazoles.

When the acyl group has little steric bulk, e.g., acetyl, the rates of acylation by the *N*-acylimidazole are slower than those of the nitrophenyl ester. However, with increasing chain length and hydrophobic character of the acyl group, the rate constants for the *N*-acylimidazoles become relatively large. The long-chain acylimidazoles have values of $k_2/K_m$ for reaction of the neutral species that are larger than those of the respective nitrophenyl esters, even though the p$K_a$ of the leaving group of an *N*-acylimidazole is 14.5 (Walba & Isensee, 1961), over 7 p$K_a$ units less favorable than *p*-nitrophenol. These differences in reactivity are then a reflection of the greater dependence of the acylation reaction on the π constants with the *N*-acylimidazoles and the smaller susceptibility of the reaction to steric influences with those substrates, as indicated by the relative magnitudes of γ and δ.

The $\rho_2$ subsite in which the hydrocarbon chain of the acyl groups is very likely bound is a hydrophobic pocket whose dimensions have been estimated to be 10–12 Å by 5.5–6.5 Å by 3.5–4.0 Å from X-ray crystallographic analysis (Steitz et al., 1969). Hydrophobic interactions in this site may provide much of the attractive force between the enzyme and its most effective substrates. On the other hand, the leaving group would be expected to reside in subsite $\rho_3$, which contains the Ser-195 residue. Significant binding interactions in this subsite may not occur with most substrates prior to acylation of Ser-195 (Zeffren & Hall, 1973). The $\rho_3$ subsite appears to be weakly hydrophobic; variation of the leaving group of ester substrates has shown that log ($1/K_m$) varies as 0.29π (Hansch & Coats, 1970). Therefore, since the acyl groups are the same for the *N*-acylimidazoles and *p*-nitrophenyl esters being compared, it is quite unlikely that the different susceptibilities of their acylation reactions to the hydrophobic nature of the acyl group are strictly due to differences in the strength of binding.

It is probable that the differences in steric and hydrophobic effects are due in part to differences in transition-state structure for the two types of compounds. A hydrophobic region in the active site would be most favorable in a reaction having a relatively nonpolar transition state. An early transition state in which there is only moderate bond making with the nucleophile (Ser-195) is indicated by the small Hammett ρ values for $k_2/K_m$ in acylation by substituted *N*-benzoylimidazoles (Kogan & Fife, 1984).[2] Mechanisms involving general acid catalysis by the His-57 conjugate acid can be ruled out in acylation of the enzyme by *N*-acylimidazoles (Kogan et al., 1982; Kogan & Fife, 1984). Therefore, differences in transition-state structure must relate directly to the nucleophilic attack by Ser-195; i.e., there is very likely less bond formation with the nucleophile in the transition state in the acylation reactions of the *N*-acylimidazoles than those of *p*-nitrophenyl esters in spite of the poorer leaving group of the former compounds.

In view of the large second-order rate constants and the apparent weak binding of both *N*-acylimidazoles and *p*-nitrophenyl esters to α-chymotrypsin (Kogan, et al., 1982; Milstien & Fife, 1969), it is likely that the binding energy

[2] The ρ value for reaction of the neutral species is only 0.9 while that for reaction of the protonated species at pH <5 is near zero. The logarithms of $k_2/K_m$ for acylation of α-chymotrypsin by the substituted *N*-benzoylimidazoles at pH 7.5 display little correlation with the hydrophobicity constants π. Inclusion of both σ and π in a four-parameter equation does not improve the fit in comparison with that from the two-parameter equation employing only σ. Thus, the Hammett ρ values are reflecting only electronic effects.

resulting from hydrophobic interactions with the acyl group is being expended to maximize the rate of the ensuing reaction. The possibility that hydrophobic interactions of substrates with the enzyme may have an important influence on the rate constants as well as the binding constants has been previously considered (Jones et al., 1965; Knowles, 1965; Ingles & Knowles, 1967). This could result from the freezing of the substrate in proper position for nucleophilic attack by Ser-195, thereby maximizing the steric fit to the nucleophile. If the substrate were forced into a reactive high-energy conformation, this would also necessarily be achieved through the use of binding energy. Such a process could have an especially large effect on the ease of nucleophilic attack on *N*-acylimidazoles due to activation of the carbonyl by disruption of its resonance interaction with the imidazole ring. This would in turn lead to a transition state resembling reactants.

**Registry No.** I, 60718-45-8; II, 93383-50-7; III, 93383-51-8; IV, 93383-52-9; V, 4195-19-1; imidazole, 288-32-4; *N,N*-dicyclohexylcarbodiimide, 538-75-0; chymotrypsin, 9004-07-3.

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