and electron-impact[28] methods are somewhat susceptible to the formation of ions in various excited states.

A comparison of the Fe^+–alkane reactions with the Co^+–alkane reactions reported by Armentrout and Beauchamp[3] shows few qualitative differences in the exothermic products formed by these two systems. Quantitatively, Co^+ releases more H_2 from the normal alkanes in all instances and suggests that Co^+ has more of a preference for C–H insertion than does Fe^+. Co^+ also loses CH_4 less often from these alkane collision complexes which indicates that Co^+ avoids C_1–C_2 insertion even more than does Fe^+. The branched alkane reactions show a better correlation between the product distributions for the two metals, but it is interesting to note that the unusual C_2H_6 loss from 2,3-dimethylbutane is not reported for the Co^+ reaction. That we do not see any of the iron analogues of the high energy products formed in the ion beam such as $CoCH_3^+$ or $CoC_2H_5^+$ is evidence that our trapped metal ions are predominantly of low kinetic energy. Dehydrogenation is the main reaction with cycloalkanes for both the Fe^+ and Co^+ systems.[18] However, the complete absence of any ring-cleavage products for cyclopentane with Fe^+ as opposed to the large ethylene loss in the Co^+–cyclopentane reaction stresses the fact that some differences do exist between the reactions of these metal ions with alkanes.

In gas-phase reactions between simple metal ions and organic molecules, the presence of polar functional groups can greatly influence the site of attack along the molecule. However, results from our laboratory have shown that in reactions of Fe^+ with ketones and ethers,[12] the loss of H_2 and small alkanes from the initial collision complexes becomes more dominant as the alkyl portions of these compounds increase. The results presented here provide a general description as to how these metal ions can react with various saturated hydrocarbon structures and demonstrate how the alkyl portion of a molecule can be as potentially influential in directing product formation as a particular functional group.

Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and to the Department of Energy (DE-AC02-80-ER10689).

Registry No. Fe^+, 14067-02-8; Ti^+, 14067-04-0; methane, 74-82-8; ethane, 74-84-0; propane, 74-98-6; butane, 106-97-8; pentane, 109-66-0; hexane, 110-54-3; isobutane, 75-28-5; 2,3-dimethylbutane, 79-29-8; 2,2-dimethylbutane, 75-83-2; cyclopropane, 75-19-4; cyclopentane, 287-92-3; cyclohexane, 110-82-7; methylcyclohexane, 108-87-2; 2,2-dimethylpropane, 463-82-1.

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The Mechanism of Specific Acylation of the Active Site of α-Chymotrypsin by *N*-Acylimidazoles

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Abstract: The rates of acylation of α-chymotrypsin by a series of *N*-acylimidazoles have been measured, and values of k_2/K_m have been determined in the pH range 5.0–9.0 at 30 °C. Acylation of the active site could be shown in all cases by (a) the lack of reactivity of the acylated enzyme at pH 5.0 toward *N-trans*-cinnamoylimidazole and (b) the rate of deacylation at pH 6.91, which was nearly identical in each case with that of the acyl-enzyme derivative prepared with the corresponding *p*-nitrophenyl ester. The rate of acylation increases with increasing chain length, *N*-hexanoylimidazole having $k_2/K_m = 5 \times 10^4$ M^{-1} s^{-1} and *N*-(β-phenylpropionyl)imidazole having $k_2/K_m = 1.2 \times 10^6$ M^{-1} s^{-1} at pH 7.5. Compounds with alkyl-group branching in the acyl group acylate the enzyme at rates intermediate between those of *N*-butyryl and *N*-acetyl derivatives. These results suggest that proper steric fit in the active site is important although K_m must be very large (plots of k vs. $k/(S)_0$ have infinite slopes). The values of k_2/K_m are nearly independent of pH except in the case of the *N*-(3,3-dimethylbutyryl)-*N'*-methyl imidazolium ion with which k_2/K_m increases with increasing pH until a maximum is reached ($pK_{app} = 6.6$). This difference reflects the fact that reaction of the N-methylated derivative is via a positively charged species at all pH values, whereas the unmethylated compounds react through both the neutral species and conjugate acids with similar rate constants. The rate constants for the corresponding methylated and unmethylated derivatives extrapolate to the same value at pH ~4, showing that the reaction at low pH involves acylation of the enzyme by the *N*-acylimidazole conjugate acid. The limiting value of k_2/K_m for the *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazolium ion is 3.1 times slower in D_2O than in H_2O. Thus, the histidine in the active site is participating in the reaction in the base form, and the reaction involves proton transfer in the transition state. It can be concluded that the nearly pH-independent reactions of the unmethylated derivatives at pH >7 must also represent neutral histidine-57 catalyzed acylation of serine-195 by the neutral *N*-acylimidazole. Thus, histidine-57 participates in the reactions as a general base most likely by partially abstracting a proton from the serine hydroxyl in the transition state. Kinetically equivalent mechanisms involving the histidine-57 conjugate acid acting as a general acid can be ruled out.

The hydrolysis of *N*-acylimidazoles having alkyl group branching in the acyl group is marked by steric effects that are highly abnormal for bimolecular reactions.[1-3] The rates of the general base, general acid, and hydroxide ion catalyzed reactions are not retarded by increased branching of the acyl group at the α-carbon but are actually accelerated.[1] Branching at the β-carbon does produce a rate decrease but to a lesser extent than found for nucleophilic reactions of esters. Similar relative-rate ratios have been found in the hydrolysis of *N*-acylimidazolium ions,[2,3] which rules out steric inhibition of resonance as the cause of the abnormal steric order.[2] In contrast, aminolysis of *N*-acylimidazoles by diethylamine gives rise to a normal steric order.[4] It was suggested that the unusual steric effects in hydrolysis could be due to relief of strain in a transition state in which the carbon–nitrogen bond

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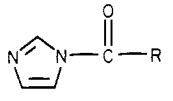
is breaking.[1] The determination of the pattern of steric effects in acylation reactions of these compounds with a proteolytic enzyme such as α-chymotrypsin is, therefore, of considerable mechanistic interest. Steric effects are normal in acylation of α-chymotrypsin by *p*-nitrophenyl esters[5] and in deacylation of the acyl-enzyme intermediate (δ = 1.0).[6]

Serine-195 is acylated in reactions of α-chymotrypsin. The enzyme also has a histidine residue at the active site which is active in the base form for usual ester substrates,[7,8] but which may also function as a general acid catalyst by assisting departure of the leaving group in acylation reactions of amide substrates.[8,9] Since reactions of *N*-acylimidazoles are subject to both general acid and general base catalysis,[1,10] the use of these compounds as substrates affords an excellent opportunity for determining whether mechanisms involving a general acid catalyzed step might be important for the enzyme under favorable circumstances. Furthermore, the N-methylated analogues permit assessment of the importance of protonation of the leaving group, i.e., kinetically equivalent possibilities can be distinguished.

Exchange of ^{18}O into the acylimidazole carbonyl is not observed in hydrolysis reactions of either the protonated[11] or neutral species[12] of *N*-acylimidazole derivatives of aliphatic carboxylic acids. Therefore, either nucleophilic attack is strictly rate limiting, or bond making and breaking are concerted as suggested by the abnormal steric effects.[1-3] If C–N bond breaking is part of the rate-determining step, then a stable tetrahedral intermediate cannot exist. The reactions of the trifluoroethoxide ion with *N*-acetylimidazole and the *N*-acetylimidazolium ion were also concluded to proceed by a concerted pathway or via an addition intermediate whose lifetime is too short for it to reach equilibrium with respect to proton transfer.[13] Consequently, an investigation of the acylation of α-chymotrypsin by such *N*-acylimidazoles would allow determination of whether histidine-57 will participate in the reactions of amides which do not form kinetically significant tetrahedral intermediates, i.e., such participation should only occur in the step in which nucleophilic attack occurs, unlike acylation reactions of conventional amide substrates. The rates of acylation of α-chymotrypsin by a series of *N*-acylimidazoles (I–VI) have therefore been measured, and in addition the acylation reactions of the N-methylated derivative of VI have been studied.



I, R = CH_3– IV, R = C_6H_5-CH_2CH_2–
II, R = $CH_3CH_2CH_2$– V, R = $(CH_3)_2CHCH_2$–
III, R = $CH_3(CH_2)_3CH_2$– VI, R = $(CH_3)_3CCH_2$–

When these studies were initiated it was not known whether the compounds would serve as efficient substrates. However, nonspecific substrates for α-chymotrypsin, which are characterized by a good leaving group so that the rate of acylation is much greater than that of deacylation, have been previously employed. Compounds such as acid chlorides and anhydrides of carboxylic acids,[14] sulfonyl halides,[15] and certain *N*-acylimidazoles[16-18] are effective acylation agents of chymotrypsin. It has indeed been found that the present series of compounds will specifically acylate the active site at rates greatly in excess of the nonenzymatic rates of hydrolysis.

Experimental Section

Materials. α-Chymotrypsin, crystallized three times, 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.[16] The *N*-acylimidazoles and *p*-nitrophenyl esters were the same as previously described[1,2] with exception of *N*-hexanoylimidazole which was prepared by the method of Staab,[4] mp 33–35 °C (lit.[4] mp 35 °C), and *N*-(β-phenylpropionyl)imidazole, mp 54–56 °C. Anal. Calcd for $C_{12}H_{12}N_2O$: C, 71.98; H, 6.04; N, 13.99. Found: C, 72.28; H, 6.30; N, 14.15. 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. *N*-(3,3-Dimethylbutyryl)-*N'*-methylimidazolium chloride was prepared according to the method of Wolfenden and Jencks,[19] mp 111–113 °C. The white solid, which was slightly hygroscopic, was thoroughly washed with dry ether and dried over P_2O_5. Stock solutions of proflavin hydrochloride (Aldrich) were routinely prepared with 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 of the solution with *N-trans*-cinnamoylimidazole at 310 nm (method A of Schonbaum et al.[16]). The reproducibility was found to be 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. Rate constants for acylation of α-chymotrypsin by *N*-acylimidazoles at 30 °C were determined in the presence of proflavin. The dye forms a 1:1 complex with the active site of α-chymotrypsin,[20] which results in a spectral shift. The maximum absorbance difference between complexed and uncomplexed dye occurs at 465 nm, thereby allowing reactions that by themselves may exhibit little spectral change to be conveniently followed spectrophotometrically.[21] Acylation of the enzyme results in displacement of proflavin, which consequently gives rise to a large absorbance change at 465 nm. All kinetic runs were in 0.1 M buffers (μ = 0.5 M) except with *N*-acetylimidazole and *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazolium chloride with which rate determinations were carried out in 0.05 M buffer (μ = 0.5 M) and in 0.3 M buffer (μ = 0.8 M), respectively. In most kinetic runs the concentrations of chymotrypsin and proflavin were 1.65×10^{-5} and 7.7×10^{-5} M, respectively. Substrate concentrations were varied in the range 1.0×10^{-4} to 8.0×10^{-3} M, with the exception of *N*-(β-phenylpropionyl)imidazole with which substrate concentrations were ca. tenfold less, $[E]_0 = 4 \times 10^{-6}$ M. Acetate, phosphate, Tris, and ammedol buffers were employed. The different buffers yielded consistent results when employed at the same pH. Acylation rates 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. Equations are given in ref 21a.

In a typical run employing the Beckman spectrophotometer 3.0 mL of buffer was added to a cuvette and aliquots of enzyme and proflavin stock solutions were added. After allowing approximately 10 min for the temperature to equilibrate, an aliquot of the substrate stock solution was added with a Hamilton syringe. In a typical run employing the stopped-flow spectrophotometer two separate solutions were prepared. One solution was a buffer which contained twice the final concentrations of enzyme and proflavin. This solution was introduced into one of two identical drive syringes. The other syringe contained water to which was added an aliquot of substrate stock solution to give a concentration equal to twice the desired final concentration. The length of time that a substrate was in the holding solution before an acylation rate was obtained was used to correct the pseudo-first-order rate constants obtained for acylation by dividing by $e^{-k_0 t}$, where k_0 is the first-order rate constant for hydrolysis, measured at 245 nm at 30 °C, and t is the time the substrate was in the holding solution before the acylation reaction was commenced. This correction was negligible in cases where spontaneous hydrolysis is slow. The drive syringes, mixing chamber, and cuvette were suspended

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in a water trough whose temperature was maintained at 30 ± 0.1 °C. Optical-density changes after mixing were recorded on a Hewlett-Packard storage oscilloscope (Model 1207B). With each buffer three to four reactions were tabulated. The reactions were followed to completion. Pseudo-first-order rate constants were calculated with an IBM-360 computer. Good first-order kinetics were obtained in all cases. Reaction pH values were obtained with a Radiometer Model 22 pH meter or a Beckman Model 3500 digital pH meter.

The rates of enzyme-catalyzed disappearance of the *N*-acylimidazoles could also be followed ($[S]_0$ less than $[E]_0$) at 245 nm with a Gilford Model 2000 recording spectrophotometer. Constant temperature was maintained by circulating water from a Precision Scientific Co. Temptrol Model 154 circulating bath around the cell compartment. The reaction cuvette contained 3.0 mL of buffer solution (0.1 M acetate or phosphate) and 100 μL of an enzyme solution. The final enzyme concentration was approximately 4×10^{-5} M and was known to ±1% in all runs. The reference cuvette contained an equal amount of enzyme solution plus 0.1 mL of acetonitrile. The substrate, in acetonitrile solution, was added by means of a Hamilton syringe. The recorder was started when the plunger of the syringe was fully depressed, the solution was stirred, and the first reading could be obtained after about 6 s. The disappearance of substrate was followed to completion. Second-order rate constants were obtained from the expression

$$[S]_0(n-1)k_A t + c = \ln\left[1.0 + (n-1.0)\frac{(A_0 - A_\infty)}{(A_t - A_\infty)}\right] \quad (1)$$

where $n = [E]_0/[S]_0$; A_0, A_t, and A_∞ are the absorbances at time zero, time t, and infinity; and k_A is the second-order rate constant.[22] Problems in obtaining kinetic data by this method are (a) the spontaneous hydrolysis of the substrate that occurs simultaneously with acylation, (b) deacylation of the acylated enzyme, and (c) difficulty in obtaining accurate absorbance readings at time zero by extrapolation. The first two problems were minimized at low substrate concentrations ($[S]_0 < [E]_0$) because of the much faster rates of acylation. The third difficulty was resolved by the use of a computer program designed to obtain the initial absorbance and the rate constant which best fit the observed data. The program was based on an iterative least-squares evaluation of the nonlinear integrated form of the second-order equation. An average reproducibility of about 4% was obtained. In all cases the calculated A_0 compared reasonably well with that obtained by inspection.

Acylation of the Active Site with *N*-Acylimidazoles. To ensure that the *N*-acylimidazoles were acylating the active site of α-chymotrypsin two experiments were carried out. The first experiment was to titrate with *N-trans*-cinnamoylimidazole using method B of Schonbaum et al.[16] after acylation by the appropriate *N*-acylimidazole ($[S]_0 = 1.5[E]_0$). The procedure was to add 100 μL of an enzyme solution of known concentration to 2.9 mL of pH 5.00 (0.1 M acetate) buffer in a cuvette. After equilibration of the cuvette in the cell compartment of the spectrophotometer 100 μL of an acetonitrile solution of *N*-acylimidazole was added. At a given time, depending on the substrate, 100 μL of an acetonitrile solution of *N-trans*-cinnamoylimidazole was introduced and the absorbance read at 310 nm. This reading corresponds to A_3 in the scheme of Schonbaum et al.[16] In the second experiment the pH was 6.91 (0.1 M phosphate buffer), and the *p*-nitrophenyl ester corresponding to the *N*-acylimidazole was added in excess after acylation of the enzyme with *N*-acylimidazole. The appearance of *p*-nitrophenolate anion was followed spectrophotometrically at 400 nm. 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 the *p*-nitrophenolate anion.[23] The fraction of anion present at pH 6.91 was calculated by using a pK_a of 7.15.

Results

Reactions catalyzed by α-chymotrypsin can be considered to involve breakdown of the enzyme–substrate complex to an acyl-enzyme intermediate which then hydrolyzes, as in eq 2.[8,24,25]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2 \quad (2)$$

This scheme yields eq 3,[24] where $K_m = (k_{-1} + k_2)/k_1$, and k is

$$k = \frac{(k_2 + k_3)[S]_0 + k_3 K_m}{[S]_0 + K_m} = \frac{k_2[S]_0}{[S]_0 + K_m} + k_3 \quad (3)$$

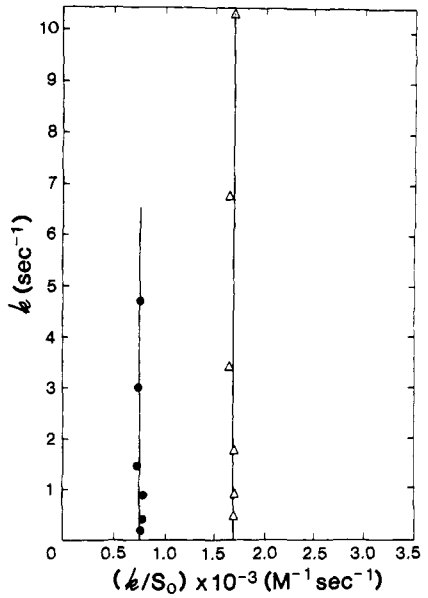

Figure 1. Plots of k vs. $k/[S]_0$ for acylation of α-chymotrypsin at pH 6.48 by *N*-isovalerylimidazole (●) and by *N*-butyrylimidazole at pH 5.98 (△) at 30 °C. The rate constants k are the pseudo-first-order rate constants for proflavin displacement from the active site (acylation) and $[S]_0$ is the initial substrate concentration.

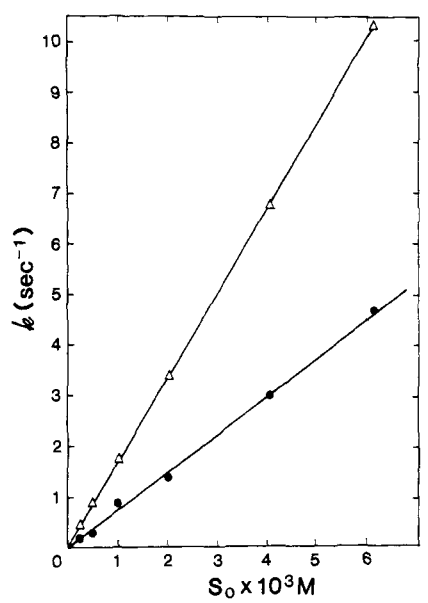

Figure 2. Plots of k vs. $[S]_0$ for acylation of α-chymotrypsin at pH 6.48 by *N*-isovalerylimidazole (●) and by *N*-butyrylimidazole at pH 5.98 (△) at 30 °C.

a first-order rate constant governing the pre-steady-state reaction. If $(k_2 + k_3)[S]_0 > k_3 K_m$ then eq 3 can be simplified to eq 4, and

$$k = \frac{(k_2 + k_3)[S]_0}{[S]_0 + K_m} \quad (4)$$

the data can be analyzed according to conventional Michaelis–Menten kinetics. At substrate concentrations which are small in comparison to K_m, it is not possible to determine the various constants by employing eq 4.[26] In that case the reaction will be experimentally second order.

Typical plots are shown in Figure 1 of k vs. $k/[S]_0$, where k is the pseudo-first-order rate constant for acylation of α-chymotrypsin by the *N*-acylimidazoles at 30 °C determined by proflavin displacement. The plots were in each case vertical as shown,

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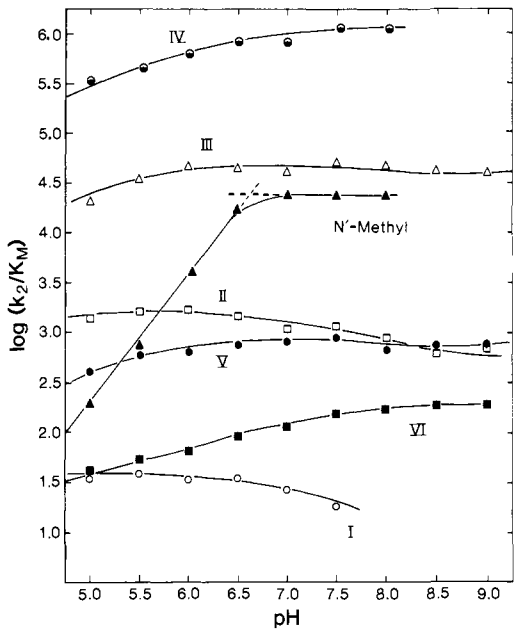

Figure 3. Plots of log (k_2/K_m) vs. pH for the acylation of α-chymotrypsin by *N*-acylimidazoles at 30 °C: *N*-acetyl (○); *N*-(3,3-dimethylbutyryl) (■); *N*-isovaleryl (●); *N*-butyryl (□); *N*-hexanoyl (△); *N*-(β-phenylpropionyl) (⊖); and *N*-(3,3-dimethylbutyryl)-*N'*-methyl (▲). The rate constants were obtained from the slopes of plots of k vs. $[S]_0$ for proflavin displacement from the active site.

indicating that the highest substrate concentration is still much less than K_m so that an enzyme–substrate complex is experimentally undetectable. Therefore, the reactions can be considered to be second order.

In the case of a second-order reaction, a plot of k vs. $[S]_0$ should have an intercept of zero, and the slope should be equal to the pH-dependent second-order acylation rate constant (k_2/K_m when $k_2 > k_3$). Typical plots are shown in Figure 2. The intercepts of the plots of k vs. $[S]_0$ 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.

The log (k_2/K_m)–pH profiles for acylation of α-chymotrypsin by *N*-acylimidazoles are shown in Figure 3. The pH dependence of the acylation reaction is not strongly influenced by the structure of the acyl group; acylation is essentially independent of pH. The compounds with straight-chain acyl groups have a maximum in their pH–rate constant profiles near pH 6.0. However, the increases in the rate constants with increasing pH at values below 6.0 are small. *N*-(3,3-Dimethylbutyryl)imidazole shows a small increase in rate constant with increasing pH to pH 8.0. As seen in Figure 3, the values of k_2/K_m are in the order IV > III > II > V > VI > I, i.e., the rate of acylation increases as the chain length of the acyl group increases. At pH 7.5 k_2/K_m has the following values: IV, 1.2×10^6 M^{-1} s^{-1}; III, 49 600 M^{-1} s^{-1}; II, 1120 M^{-1} s^{-1}; V, 870 M^{-1} s^{-1}; VI, 154 M^{-1} s^{-1}; and I, 18 M^{-1} s^{-1}. *N*-(3,3-Dimethylbutyryl)-*N'*-methylimidazolium ion shows an increasing rate constant with increasing pH below pH 7 and a pH-independent reaction at pH > 7. The limiting rate constant (k_2'/K_m) is 2.4×10^4 M^{-1} s^{-1}, and the value of pK_{app} is 6.6. The limiting rate constant for the *N*-methyl derivative was determined in D_2O at pD values of 7.5 and 8.0. The ratio $(k_2'/K_m)^{H_2O}/(k_2'/K_m)^{D_2O}$ is 3.1.

The acylation reactions could also be followed by monitoring the absorbance decrease at 245 nm due to disappearance of *N*-acylimidazole under second-order conditions ($[E]_0 > [S]_0$). In each case acylation took place much more rapidly than spontaneous hydrolysis, even though the $[S]_0/[E]_0$ ratio was low. For example, the ratio of half-times for acylation and spontaneous hydrolysis with *N*-butyrylimidazole at pH 6.91 is 120 with $[E]_0/[S]_0 = 1.37$. Differences in the rate constants obtained by direct observation at 245 nm and by the proflavin method are not

Table I. Titration of α-Chymotrypsin with *N*-Cinnamoylimidazole after Reaction with Various *N*-acylimidazoles at pH 5.00

acyl group	time, min	% acylation obsd[a]	theoretical % of reaction[b]
acetyl	7.0	35.0	91.1
	8.0	35.3	93.8
propionyl	2.8	62.1	92.1
	3.3	65.0	94.3
butyryl	1.5	94.3	>99
	1.75	98.1	>99
isobutyryl	3.8	65.7	97.4
	4.0	74.6	98.6
isovaleryl	5.5	89.0	>99
	6.3	88.6	>99
3,3-dimethylbutyryl	10.4	76.0	91.3
	13.0	77.2	94.4

[a] Initial concentration of acylimidazole is 1.5 times greater than the initial enzyme concentration; $[E]_0 = 4 \times 10^{-5}$ M. [b] Calculated from the rate expression where $[S]_0$ is greater than $[E]_0$, using a rate constant obtained from initial portions of the reaction. The equations are given in ref 22. With the employment of the rate constant and the time at which titration was commenced, the theoretical percent of reaction can be calculated.

Table II. Rate Constants for Deacylation at pH 6.91 and T = 25 °C of Various Acyl Chymotrypsins Formed from *N*-Acylimidazoles Compared to Those Formed from *p*-Nitrophenyl Esters

acyl group	k_3'(obsd) × 10^4, s^{-1}	k_3'(calcd) × 10^4,[a] s^{-1}
propionyl	36.0	31.3
butyryl	25.1	19.6
isobutyryl	13.0	9.88
isovaleryl	6.15	6.11[b]
3,3-dimethylbutyryl	0.92	1.01
β-phenylpropionyl	1420[c]	1440[c]

[a] Calculated from the data of Fife and Milstien,[6] where experimental conditions were slightly different; μ = 0.075 M. [b] Determined in this study with experimental conditions the same as in the reaction with the *N*-acylimidazoles; $[E]_0 = 4 \times 10^{-5}$ M. The *p*-nitrophenyl ester had an initial concentration of 1.8×10^{-4} M. [c] pH 7.05, 30 °C.

large considering the differences in calculation and the significant differences in reaction conditions (usually a factor of ~2 with a 5 °C difference in temperature), and the pH dependence is identical. Thus, the same factors are governing disappearance of substrate and formation of acyl enzyme. It has previously been found in reactions of ester substrates that the rate constants obtained by proflavin displacement are identical with those obtained by independent methods.[27] In view of the high accuracy of the rate constants obtained by the proflavin method and the problems associated with direct observation of the reaction at 245 nm (see Experimental Section), only rate constants obtained by the former method are employed in Figure 3.

That acylation of the enzyme is taking place at the active site was shown by first acylating the enzyme at pH 5.0 and then titrating with *N-trans*-cinnamoylimidazole in the manner described by Schonbaum et al.[16] Thus, the percent of activity remaining was determined and by difference the percentage of acylation which is reported in Table I. The nonenzymatic hydrolysis is relatively rapid at pH 5 since this pH lies on the ascending arm of the pH–rate profiles.[10] This factor possibly accounts for some of the difference between the observed acylation and the theoretical percent of acylation. It is also possible that at the low substrate concentrations employed in these experiments that the condition $[S]_0 > k_3K_m/(k_2 + k_3)$ is not fulfilled.[26] In the case of *N*-acetylimidazole k_3 is not insignificant in relation to k_2. Some nonspecific acylation of the protein could also be taking place at this pH with *N*-acetylimidazole.[28] Nonspecific acylation would,

(27) Himoe, A.; Brandt, K. G.; DeSa, R. J.; Hess, G. P. *J. Biol. Chem.* **1969**, *244*, 3483.

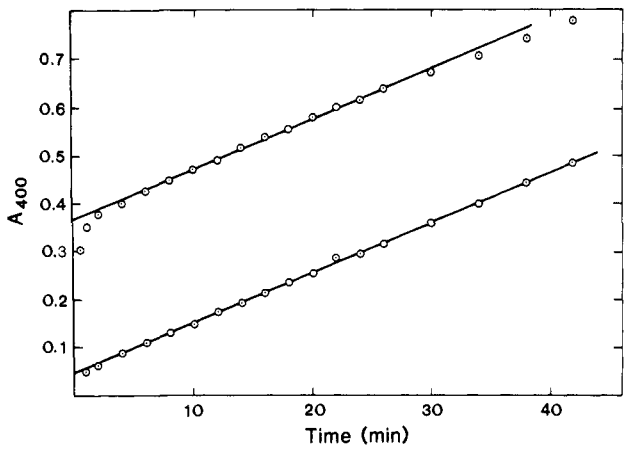

Figure 4. A plot of absorbance at 400 nm after injection of a solution of *p*-nitrophenyl isovalerate into a solution of acyl enzyme prepared by reaction with *N*-isovalerylimidazole at pH 6.91 (lower line). The top line represents hydrolysis of the same amount of *p*-nitrophenyl isovalerate by an enzyme solution to which the *N*-acylimidazole had not been previously added. $[E]_0 = 4 \times 10^{-5}$ M, [*N*-isovalerylimidazole]$_0 = 3.1 \times 10^{-5}$ M, and [*p*-nitrophenyl isovalerate]$_0 = 1.8 \times 10^{-4}$ M.

of course, influence the kinetic measurements when the absorbance decrease at 245 nm is followed, but would have little or no influence on measurements employing the proflavin method which directly monitors events at the active site.

Acylation of the active site was further confirmed by measuring the rates of deacylation of the acyl enzymes at pH 6.91. These data are reported in Table II. The observed rate constants are closely similar to those measured for deacylation of corresponding acyl enzymes prepared from *p*-nitrophenyl esters in an independent study with slightly different experimental conditions.[6] Thus, the acyl enzymes are deacylating normally. A typical plot of absorbance at 400 nm vs. time after injection of a solution of *p*-nitrophenyl ester into the solution of acyl enzyme is shown in Figure 4. Also shown in Figure 4 is a plot of absorbance vs. time for hydrolysis of the same amount of the corresponding *p*-nitrophenyl ester by the enzyme solution without prior addition of the *N*-acylimidazole. As can be seen there is only a very small initial rise in absorbance for the solution to which the *N*-acylimidazole had been added, but a large initial increase due to rapid acylation when the *p*-nitrophenyl ester is added alone. The *N*-acylimidazole has, therefore, acylated the active site almost completely. The rates of deacylation for the two solutions are, however, identical.

Discussion

Kinetics of Acylation. The hydrolysis of esters and amides catalyzed by α-chymotrypsin is thought to follow the scheme of eq 2 in which the substrate first binds to the enzyme and then acylates the active site to form an acyl-enzyme intermediate that subsequently solvolyzes.[8,24,25] It has been well established that an acyl-enzyme intermediate is formed during the hydrolysis of both specific and nonspecific ester and amide substrates.[7,8,29-32] This acyl-enzyme intermediate is an ester of serine-195.[7,8] If, in acylation reactions, K_m is much larger than the initial substrate concentration, then the concentration of ES will be so small as to be undetectable, and the reaction will be experimentally second order. The rate constant determined in this case from plots of $k - k_3$ vs. $[S]_0$, or k vs. $[S]_0$ if k_3 is negligible, is the ratio k_2/K_m.[26] It was found that the rates of acylation of α-chymotrypsin by the *N*-acylimidazoles were much greater than those of deacylation. In all cases, with the exception of *N*-acetyl, subtraction of k_3 from k (employing eq 3) produced no change in the second-order rate constant; i.e., plots of $k - k_3$ vs. $(k - k_3)/[S]_0$ and plots of $k - k_3$ vs. $[S]_0$ were identical with uncorrected plots. This is because k_3 is not significant in comparison to k. The exact slope of the plot of k vs. $[S]_0$ from eq 4 is, of course, $(k_2 + k_3)/K_m$, which will only be equal to k_2/K_m if $k_2 > k_3$. Since the second-order rate constants are identical whether determined from plots of k vs. $[S]_0$ (eq 4) or $k - k_3$ vs. $[S]_0$ (eq 3), it is clear that $k_2 \gg k_3$ and accordingly that $(k_2 + k_3)[S]_0 > k_3K_m$ is very likely.

Acylation of α-chymotrypsin by the present series of *N*-acylimidazoles is a second-order reaction even at very high ratios of $[S]_0/[E]_0$ (>300-fold), as seen in Figure 1 where k, the pseudo-first-order rate constant obtained from the proflavin displacement measurements corresponding to acylation, is plotted vs. $k/[S]_0$ (since k_3 is much smaller than k in these reactions it can be ignored except in the case of *N*-acetylimidazole with which k_3 must be substracted from k). Such a plot should be a straight line with slope equal to $-K_m$ and ordinate intercept equal to k_2.[33] However, in all cases such plots yielded infinite slopes showing 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. The highest substrate concentration is, of course, determined by the velocity limits of the stopped-flow instrument. Plots of k vs. $[S]_0$ were nicely linear as shown in Figure 2, giving k_2/K_m as the slope. The ratio k_2/K_m is not affected by any nonproductive binding of the substrate.[34]

Steric effects in acylation of α-chymotrypsin by *p*-nitrophenyl esters have been thoroughly studied[5] as have the subsequent deacylation reactions.[6] Plots of the logarithms of the rate constants vs. E_s, the Taft steric effects constants,[35] are linear ($\delta = 1.0$), except that the point for *n*-hexanoyl lies considerably above the line. Thus, with the exception of the *n*-hexanoyl acyl group, steric effects in the enzymatic reactions correspond with those in OH^--catalyzed ester hydrolysis.[35] The steric effects in the deacylation reaction are also in accord with those found in imidazole-catalyzed hydrolysis of esters of *N*-acetylserineamide,[36] where increasing steric bulk in the acyl group decreases the rate. However, the observed order of reactivity for acylation of α-chymotrypsin by *N*-acylimidazoles is not in accord with that in the general base or hydroxide ion catalyzed hydrolysis of these compounds.[1] *N*-Hexanoyl- and *N*-butyrylimidazole acylate the enzyme faster than the other compounds in the series, whereas *N*-acetylimidazole reacts more slowly than the other compounds. The derivatives with branched-chain acyl groups occupy intermediate positions in the series. Thus, increasing chain length accelerates the reaction greatly while chain branching retards the rate. Accordingly, *N*-isovalerylimidazole is more reactive than *N*-(3,3-dimethylbutyryl)imidazole but is less reactive than *N*-butyrylimidazole. The correlation of log (k_2/K_m) with the E_s constants is poor for acylation of the enzyme by the *N*-acylimidazoles. On such a plot (not shown) a reasonable line of slope 1.0 can be drawn through the points for II, V, and VI, the compounds with branched-chain acyl groups, but the point for *N*-acetylimidazole lies ~3 log units below the line and that for (β-phenylpropionyl)imidazole lies over 2 log units above the line. Increasing chain length in the acyl group of esters of hydroxybenzoic acids was previously found to give increased rates of α-chymotrypsin-catalyzed hydrolysis.[37]

The formation of an enzyme–substrate complex in α-chymotrypsin reactions has been generally assumed. If acylation by *N*-acylimidazoles was strictly a second-order reaction, as in eq 5, then the rate constants k_2/K_m would be true second-order

$$E + S \rightarrow ES' + P_1 \qquad (5)$$

constants. If, however, binding of substrate to the enzyme takes place, then the experimental values are complex constants, and

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(29) Bender, M. L.; Zerner, B. *J. Am. Chem. Soc.* **1962**, *84*, 2550.
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(31) Kezdy, F. J.; Clement, G. E.; Bender, M. L. *J. Am. Chem. Soc.* **1964**, *86*, 3690.
(32) Bender, M. L.; Clement, G. E.; Kezdy, F. J.; Heck, H. *J. Am. Chem. Soc.* **1964**, *86*, 3680.
(33) Eadie, G. S. *J. Biol. Chem.* **1942**, *146*, 85.
(34) Fersht, A. "Enzyme Structure and Mechanism"; W. H. Freeman and Co.: San Francisco, 1977; p 96.
(35) Taft, R. W., Jr. In "Steric Effects in Organic Chemistry", Newman, M. S., Ed.; Wiley: New York, 1956; Chapter 13, p 556.
(36) Milstien, J. B.; Fife, T. H. *J. Am. Chem. Soc.* **1968**, *90*, 2164.
(37) Hofstee, B. H. *Biochim. Biophys. Acta* **1957**, *24*, 211; **1959**, *32*, 182.

the observed order of reactivity becomes partially explicable in terms of ease of binding. It is therefore likely that both binding effects and kinetic effects are important in determining the steric order even though binding must be weak. Thus, acylation of α-chymotrypsin by *N*-acylimidazoles and *p*-nitrophenyl esters is affected quite differently by steric changes in the acyl group. Not only do the acyl imidazoles with long-chain acyl groups, e.g., hexanoyl, acylate the enzyme at a greater rate than the corresponding *p*-nitrophenyl ester, but *N*-acetylimidazole has a k_2/K_m at pH 7.5 that is almost 100-fold *less* than that of *p*-nitrophenyl acetate.

The nearly pH-independent acylation of α-chymotrypsin by the *N*-acylimidazoles could reflect several kinetically equivalent possibilities. Any interpretation is, of course, dependent on K_m being little affected by changes in pH, but this is a reasonable assumption since K_m for nonionizing substrates has been shown to be essentially pH independent in the range 5–8.[32,38-41] There is no doubt that serine-195 is being acylated in view of the identical rates of deacylation of acyl chymotrypsins prepared from the *N*-acylimidazoles and corresponding *p*-nitrophenyl esters. The simplest kinetic interpretation is that *N*-acylimidazoles acylate α-chymotrypsin via both the protonated and neutral species or kinetic equivalents. Thus, the scheme of eq 6, which takes into

$$\begin{array}{ccccc} E + S & \rightleftharpoons & ES & \xrightarrow{k_r} & \text{product} \\ {\scriptstyle H^+}\downarrow\uparrow{\scriptstyle K_1} & & {\scriptstyle H^+}\downarrow\uparrow{\scriptstyle K_2} & & {\scriptstyle H^+}\downarrow\uparrow{\scriptstyle K_3} \\ EH^+ + S & & ESH^+ & \xrightarrow{k_r'} & \text{product} \end{array} \qquad (6)$$

account ionization of both the enzyme and the substrate, is very likely being followed.[22] In view of the pK_a values of *N*-acylimidazoles (~4) and His-57 (~7), this scheme would explain the near pH independence of experimental rate constants in the range 5–9, if the reactions of the protonated species were of major significance below pH 6–6.5, and the reactions of the neutral species were of greater importance at higher pH values. The expression for k derived from the scheme of eq 6 is given in eq 7 considering that $K_2 > a_H$ and $K_m > [S]_0$. At $a_H > K_1$ eq 7

$$\frac{k}{S_T} = \frac{k_r K_1 K_3 + k_r' K_1 a_H}{K_1 K_3 K_m + K_3 K_m a_H} \qquad (7)$$

reduces to eq 8, whereas at $a_H < K_1$ eq 9 is obtained. Thus, at

$$\frac{k}{S_T} = \frac{k_r K_1}{K_m a_H} + \frac{k_r' K_1}{K_m K_3} \qquad (8)$$

$$\frac{k}{S_T} = \frac{k_r}{K_m} + \frac{k_r' a_H}{K_m K_3} \qquad (9)$$

pH <6 k_r'/K_m could 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. In the case of the 3,3-dimethylbutyryl derivative, k_r'/K_m is 100-fold larger than k_r/K_m (assuming correspondence of k_r'/K_m to the N-methylated compound). Therefore the reaction through the two species will be approximately equal 2 pH units above the pK_a of the *N*-acylimidazole, i.e., at pH 5.5–6. Above this pH the reaction of the neutral species will then predominate, and rate constants will increase slightly with increasing pH through the pK_a of histidine-57. This might vary with the other compounds, depending on the relative values of k_r and k_r'. For example, if the ratio of these rate constants (k_r'/k_r) is 10^3 or greater, then the experimental rate constants will decline as pH is raised beyond 7, as was observed with *N*-butyryl- and *N*-acetylimidazole.

Mechanism of Acylation. A method for determining the position of a proton in the transition state in reactions of *N*-acylimidazoles is to compare rate constants with those of the corresponding N-methylated derivatives.[19,42] It can be seen in Figure 3 in the log (k_2/K_m) vs. pH profile for acylation of α-chymotrypsin by the *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazolium ion that the rate increases with increasing pH until a maximum is reached. The apparent pK_a is 6.6 which is close to that expected for histidine-57.[8] The maximum rate constant at pH 7.5 is 2 orders of magnitude greater than for the unmethylated derivative, whereas the logarithms of rate constants for the two compounds extrapolate to the same value at pH 4. This pH must correspond with the pK_a of the *N*-(3,3-dimethylbutyryl)imidazolium ion (the pK_a of the *N*-acetylimidazolium ion is 3.6).[10] Thus at low pH the reaction involves the *N*-acylimidazolium ion rather than a kinetic equivalent. It is apparent, however, that at pH >5 the N-methylated compound is acylating the enzyme via a mechanistic pathway not available to the unmethylated derivative.

The downward bend in the log (k_2/K_m)–pH profile for acylation of the enzyme by the *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazolium ion at pH ~7 is most likely reflecting a critical ionization. A change in rate-determining step, which would also produce a downward bend in the profile, would not be expected in view of the N-methylated leaving group; a tetrahedral intermediate, if formed, should break down more rapidly to products than to reactants at all pH values. Thus, the profile indicates participation by the base form of histidine-57, i.e., the scheme of eq 10 is being followed from which eq 11 is obtained, where

$$EH^+ \overset{H^+}{\underset{K_1}{\rightleftharpoons}} E + SCH_3^+ \rightleftharpoons ESCH_3^+ \xrightarrow{k_r'} \text{products} \qquad (10)$$

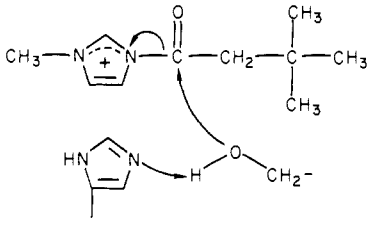
K_1 is the dissociation constant of His-57.

$$\frac{k}{S_T} = \frac{k_r'}{K_m}\left[\frac{K_1}{(K_1 + a_H)}\right] \qquad (11)$$

Histidine-57 could assist acylation of Ser-195 by partially abstracting a proton from the serine hydroxyl in the transition state or it could be acting as a nucleophile with subsequent rapid transfer of the acyl group to serine.[43] It has been suggested that His-57 might function as a nucleophile in acylation by reactive nitrophenyl esters,[48] and the leaving group pK_a of *N*-methylimidazole is ~7, i.e., comparable to that of *p*-nitrophenol. A classical method for distinguishing nucleophilic and general base mechanisms in simple chemical reactions is the D_2O solvent isotope effect.[8,49] A general base catalyzed reaction with proton transfer in the transition state will proceed more slowly in D_2O than in H_2O, while a nucleophilic reaction without proton transfer will occur at nearly the same rate. When applied to enzyme reactions this method is ambiguous.[50] However, it has been argued that the solvent isotope effect in both acylation and deacylation reactions of α-chymotrypsin allows a reliable mechanistic interpretation.[32,51] This viewpoint is strongly supported by the dea-

(38) Stewart, J. A.; Lee, H. S.; Dobson, J. E. *J. Am. Chem. Soc.* **1963**, *85*, 1537.
(39) Laidler, K. J.; Barnard, M. L. *Trans. Faraday Soc.* **1956**, *52*, 497.
(40) Hammond, B. R.; Gutfreund, H. *Biochem. J.* **1955**, *61*, 187.
(41) Cunningham, L. W.; Brown, C. S. *J. Biol. Chem.* **1956**, *221*, 287.
(42) Oakenfull, D. G.; Salvesen, K.; Jencks, W. P. *J. Am. Chem. Soc.* **1971**, *93*, 188.
(43) X-ray crystallographic analysis of α-chymotrypsin has shown that the carboxyl group of aspartic acid-102 is within hydrogen bonding distance of histidine-57.[44] On this basis mechanisms have been proposed involving the Asp-His-Ser triad. For a "charge relay" mechanism to be effective the pK_a of His-57 would of necessity be lower than that of Asp-102. Evidence obtained from NMR studies of bacterial serine proteases is contradictory on this point;[45,46] a study involving [^{15}N]-histidine[46] indicated that the pK_a was normal. A chemical model study has shown that Asp-102 should have little effect on the observed kinetics if the pK_a values are normal.[47] Therefore, the discussion is presented in terms of catalysis by histidine functioning without participation by Asp-102.
(44) Blow, D. M.; Birktoft, J. J.; Hartley, B. S. *Nature (London)* **1969**, *221*, 337.
(45) Hunkapiller, M. W.; Smallcombe, S. H.; Whitaker, D. R.; Richards, J. H. *Biochemistry* **1973**, *12*, 4732.
(46) Bachovchin, W. W.; Roberts, J. D. *J. Am. Chem. Soc.* **1978**, *100*, 8041.
(47) Rogers, G. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1974**, *96*, 2473.
(48) Hubbard, C. D.; Kirsch, J. F. *Biochemistry* **1972**, *11*, 2483.
(49) Bender, M. L.; Pollock, E. J.; Neveu, M. C. *J. Am. Chem. Soc.* **1962**, *84*, 595.
(50) Jencks, W. P. *Annu. Rev. Biochem.* **1963**, *32*, 603.

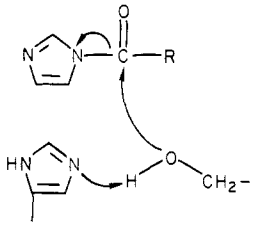
cylation of [(*p*-nitrophenoxy)carbonyl]chymotrypsin which proceeds in large part via nucleophilic attack by His-57 with a D_2O solvent isotope effect close to unity as predicted for a nucleophilic reaction.[21c] The plateau in the log (k_2/K_m) vs. pH profile for acylation by the *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazolium ion in D_2O occurs with a limiting rate constant that is 3.1-fold less than in H_2O, indicating that proton transfer is occurring in the transition state. It would reasonably be expected that $K_m^{D_2O}$ would be somewhat less than $K_m^{H_2O}$.[51] In this reaction the histidine conjugate acid cannot assist leaving group departure. Therefore, the most likely mechanism is VII, in which histidine partially



VII

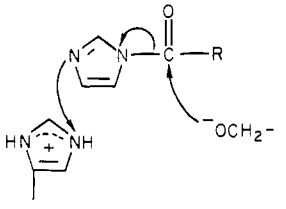
abstracts a proton from the serine hydroxyl in the transition state. A mechanism in which histidine-57 abstracts a proton from a tetrahedral intermediate formed in acylation of the neutral serine hydroxyl is unlikely; for breakdown of the intermediate to be rate determining, it would necessarily have to decompose to reactants much faster than to products. Mechanisms involving rate-determining breakdown of a tetrahedral intermediate are not in accord with the lack of ^{18}O exchange in hydrolysis of protonated *N*-acylimidazoles[11] or with the lack of evidence for stable tetrahedral intermediates in their bimolecular reactions with alcohols;[13] in nonenzymatic alcoholysis reactions of these compounds the transition state must resemble reactants.[13]

Since histidine must participate in the base form in acylation of α-chymotrypsin by N'-methylated *N*-acylimidazoles (VII), it must also do so in an analogous manner in reactions of the conjugate acids of the unmethylated derivatives. However, if that were the only pathway available, then the pH–log (rate constant) profiles would be pH independent at low pH (5–7), but the slope would be −1.0 at pH values greater than the pK_a of histidine-57. Therefore, a different mechanistic pathway is being utilized at pH >7, which must be a reaction of neutral *N*-acylimidazole (VIII) or a kinetic equivalent such as IX. Mechanism IX involves



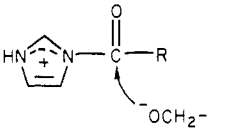
VIII

reaction of neutral *N*-acylimidazole with a zwitterionic active site, i.e., serine anion and histidinyl cation. Attack of un-ionized serine



IX

would also be pH independent without involvement of histidine-57, but such a reaction does not occur in acylation by the N-methylated acylimidazole (the reaction would be pH independent at all pH values). Since His-57 is involved in reactions of the methylated derivative (and by analogy *N*-acylimidazolium ions), it would also be expected to participate in reactions of the neutral species. Likewise, attack of the serine anion on the N-methylated compound does not occur,[52] thereby allowing X to be ruled out.



X

Mechanism VIII would require expulsion of the unstable imidazole anion, whereas IX would provide partial protonation of the leaving group in the transition state for product formation. The imidazole-catalyzed alcoholysis of *N*-acetylimidazole was considered to proceed by a mechanism similar to IX in which the leaving group is protonated by the conjugate acid of the catalyst.[42] Whether the transition state will more closely resemble VIII or IX will depend on the relative importance of increased basicity of the nucleophile and stabilization of the leaving group (IX) and increased concentration of the reactive species (VIII). The zwitterionic active site of IX would be present only at very low concentration. By taking the pK_a of the serine hydroxyl to be 13.6[53] and the pK_a of His-57 to be 6.6 it follows from eq 12 that the ratio of zwitterionic to neutral active site is 10^{-7}. The value

$$\frac{K_{ser}}{K_{His}} = \frac{[-CH_2O^-][His\ H^+]}{[-CH_2OH][His]} = \frac{2.51 \times 10^{-14}\ M}{2.51 \times 10^{-7}\ M} = 10^{-7} \qquad (12)$$

of k_2/K_m for *N*-hexanoylimidazole at high pH is $5 \times 10^4\ M^{-1}\ s^{-1}$. Thus reaction via mechanism IX would require a true second-order rate constant of $5 \times 10^{11}\ M^{-1}\ s^{-1}$, which is greater than that for a diffusion-controlled reaction ($10^{10}\ M^{-1}\ s^{-1}$). Thus mechanism IX demands an improbably high rate constant and can therefore be ruled out. This leaves VIII as the most likely mechanism for the acylation reaction.[54] It is clear that concentration factors outweigh the mechanistic advantages that would be obtained with IX or X.

The conclusion can be drawn that histidine-57 participates in the acylation reactions of *N*-acylimidazoles and *N*-acylimidazolium ions by partially abstracting a proton from the serine hydroxyl as it attacks the carbonyl. This is a mechanism that has been generally considered in acylation reactions.[7,8] However, in acylation reactions of specific amides a tetrahedral intermediate is probably formed.[56] 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.[57] If the rate-determining step in acylation by specific amide substrates is breakdown of a tetrahedral intermediate, then, since nucleophilic attack would be a preequilibrium step and therefore path independent, a mechanistic role for His-57 could not be specified in that step. However, the present results with *N*-acylimidazoles and *N*-acylimidazolium ions show that

(51) Bender, M. L.; Schonbaum, G. R.; Hamilton, G. A.; Zerner, B. *J. Am. Chem. Soc.* **1961**, *83*, 1255. Bender, M. L.; Hamilton, G. A. *Ibid.* **1962**, *84*, 2570.

(52) A slope of 1.0 in the log (k_2/K_m) vs. pH profile would be obtained for such a reaction of the N-methylated derivative. This might perhaps be detected at high pH where a sufficient concentration of serine anion is present (pK_a = 13.6)[53] if such observations were experimentally feasible.

(53) Bruice, T. C.; Fife, T. H.; Bruno, J. J.; Brandon, N. E. *Biochemistry* **1962**, *1*, 7.

(54) If a tetrahedral intermediate were formed, then proton transfer from serine and to the leaving group could occur in separate steps. This would be in accord with the small effect (100-fold) of N-methylation on the limiting value of k_2/K_m in that the leaving groups would be similarly stabilized,[55] but would require a tetrahedral intermediate that can reach equilibrium with respect to proton transfer.

(55) An alternative explanation is that the reactions involve little bond breaking in the transition state.

(56) Fersht, A. R.; Requena, Y. *J. Am. Chem. Soc.* **1971**, *93*, 7079. Fersht, A. R. *Ibid.* **1972**, *94*, 293.

(57) O'Leary, M. H.; Kluetz, M. D. *J. Am. Chem. Soc.* **1972**, *94*, 3585.

indeed general base abstraction of a proton from the serine hydroxyl can be an efficient mechanism in acylation of the enzyme by its substrates and is in fact required in reactions of the latter compounds.

Transition-State Stabilization. The lack of pronounced pH dependence in the acylation reaction of α-chymotrypsin by *N*-acylimidazoles at pH >7 is in marked contrast to the bell-shaped plots of $k_{cat.}/K_m$ vs. pH obtained with specific amide substrates.[32,58] The pH optimum is generally close to 8, and pK_2 is usually close to 9. The acidic group of $pK_a = 9$ may be the α-ammonium group of N-terminal isoleucine,[59] which has been considered to be important in the binding process.[60-62] Thus, the acylimidazoles may bind to the enzyme in a manner that is different from specific amide substrates even though binding is productive.[63]

It should be noted that values of k_2/K_m for the *N*-acylimidazole substrates with long-chain acyl groups are very large even though K_m must be quite high, e.g., k_2/K_m for the *N*-hexanoyl derivative is 50 000 M^{-1} s^{-1} at pH 7.5, and k_2/K_m for *N*-(β-phenylpropionyl)imidazole is 1.2×10^6 M^{-1} s^{-1} at pH 7.5, making it one of the kinetically most reactive substrates known for the enzyme. In comparison, $k_{cat.}/K_m$ (which is equal to k_2/K_s) for the typical amide substrate *N*-acetyl-L-phenylalanineamide is 1.5 M^{-1} s^{-1} at pH 7.9 (25 °C).[30] The large values of k_2/K_m for the *N*-acylimidazoles must be a reflection of the magnitude of k_2. These rate constants are in part understandable in view of the much better leaving group of an *N*-acylimidazole than a conventional amide (the pK_a of imidazole is 14.5 for ionization to the anionic species.[64] However, the relatively favorable leaving group of *N*-acylimidazoles cannot be totally responsible for the high k_2/K_m ratios. In Figure 3, it can be seen that k_2/K_m for *N*-hexanoylimidazole is considerably greater than the limiting k_2/K_m for the *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazolium ion even though the leaving group pK_a of the latter compound is ~7, i.e., 7 pK_a units more favorable. Likewise, the limiting k_2/K_m for acylation by *p*-nitrophenyl hexanoate (pK_a of the leaving group = 7) is only 14 000 M^{-1} s^{-1} (25 °C).[5] It is clear that the k_2 values are being profoundly influenced by the binding process. Certainly, the large variation in k_2/K_m within the series acetyl to hexanoyl ((2.5×10^3)-fold) would not be expected solely on the basis of changes in K_m considering the weak binding of all the compounds. Binding of the N-acylimidazoles must improve the steric fit of the substrate carbonyl and the participating groups in the active site. In fact, these substrates may represent cases in which binding in the transition state is greatly superior to binding of reactant. Thus the transition state will be stabilized while reactant binding is weak, thereby maximizing k_2 and values of k_2/K_m.

Acknowledgment. This work was supported by research grants from the National Institutes of Health.

Registry No. I, 2466-76-4; II, 4122-54-7; III, 60988-34-3; IV, 55628-83-6; V, 10364-92-8; VI, 4122-55-8; α-chymotrypsin, 9004-07-3; *N*-(3,3-dimethylbutyryl)-*N'*-methylimidazole, 81194-89-0; *N*-propionylimidazole, 4122-52-5; *N*-isobutyrylimidazole, 4122-53-6; *N*-*trans*-cinnamoylimidazole, 1138-15-4.

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Diffusive and Nondiffusive Time Scales in the Dissociation and Recombination of I_2 in Linear Alkanes

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Abstract: By measuring both the initial yield of I atoms and the cage escape probability of two caged I atoms, via picosecond spectroscopy, it is possible to distinguish two distinct regimes in liquid behavior. The initial yield is not dependent on bulk parameters such as viscosity but on a measure of compressibility. The cage escape probability is dependent on the diffusion constant calculated from the Stokes–Einstein relation. Thus, at early times the discrete molecular nature of the liquid is important, whereas at longer times the average behavior (diffusion) is the determining factor.

The subject of iodine photodissociation and recombination has been of interest to chemists for some time.[1] The interest is due to the apparent simplicity of the system, its ease of experimental investigation, and its applicability to studying fundamental processes in liquids. The earlier studies of I_2 recombination were done by scavenger techniques[1] where time resolution was not feasible. Recently, the first picosecond studies of this simple reaction were reported.[2,3] We have extended these results to a large number of solvents of the noninteractive type. A preliminary report of this work was presented last year.[4] We report here on one aspect of our more refined results. Future reports will consider further aspects of these studies.

The concern of this paper is with the initial dissociation process. We will offer evidence to suggest that the dissociation is controlled by the discrete, molecular nature of the solvent and not by macroscopic value measures of the solvent such as viscosity. On the other hand, the longtime yield of iodine atoms does seem to be bulk (viscosity) dependent. The distinction between these two limits seems to begin to define the limits of applicability of continuum theory to molecular motion in liquids.

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