Mechanisms of Acylation of Chymotrypsin by Phenyl Esters of Benzoic Acid and Acetic Acid*

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COLIN D. HUBBARD AND T. SCOTT SHOUPE†
From the Department of Chemistry, University of New Hampshire, Durham, New Hampshire 03824

The kinetics of the acylation of α-chymotrypsin by a series of substituted phenyl p-nitrobenzoates have been studied by stopped flow and conventional spectrophotometry. Electron withdrawal in the leaving group accelerates the rate of acylation, and the p value obtained for eight esters is +1.96. The pH- and pD-independent acylation rate constants are, respectively, $1.40 \times 10^4$ M$^{-1}$s$^{-1}$ and $1.25 \times 10^4$ M$^{-1}$s$^{-1}$ for p-nitrophenyl p-nitrobenzoate, and, respectively, $2.19 \times 10^2$ M$^{-1}$s$^{-1}$ and $1.68 \times 10^2$ M$^{-1}$s$^{-1}$ for p-nitrophenyl benzoate at 25°C. An analysis of structure-reactivity results and kinetic solvent isotope effects indicates a mechanism for acylation by phenyl benzoates in which initial reaction is a nucleophilic attack by an imidazole of the enzyme (His 57). Subsequently, there is rapid transfer of the acylating group to the serine 195 from the acylimidazole species.

The kinetic solvent isotope effects for acylation by p-nitrophenyl phenyl acetate and p-nitrophenyl hydrocinnamate, in 5%, v/v, acetonitrile, are 1.3 and 2.0, respectively. The latter ester is inhibited more than is p-nitrophenyl benzene when 5%, v/v, dioxane is substituted for 5%, v/v, acetonitrile as co-solvent. In the presence of 5%, v/v, dioxane a change in the kinetic solvent isotope effect to 1.7 is found for p-nitrophenyl benzoate and p-nitrophenyl phenyl acetate while that for the analogous hydrocinnamate ester is unaffected. The results for the latter substrate are in accord with a general base-catalyzed mechanism.

Electron-withdrawing groups in the phenyl ring of phenyl acetates accelerate the enzyme acylation yielding a leaving group e of 2.05. The kinetic solvent isotope effects for acylation by p-nitrophenyl phenyl acetate and by p-nitrophenyl acetate are close to 2.0. The mechanism of acylation of chymotrypsin by phenyl acetates is not unambiguously defined using these data.

The chymotrypsin-catalyzed hydrolysis of ester substrates proceeds by the minimal mechanism outlined in Equation 1. This overall mechanism consists of two fundamental processes, acylation and subsequent deacylation of the enzyme. The deacylation reaction has been the subject of extensive study, and it has been concluded from structure-reactivity correlations (2–5) and solvent deuterium isotope effects (2, 6) that the reaction for non-specific ester substrates proceeds by imidazole-catalyzed attack of water on the acyl-enzyme. The mechanistic path of the acylation step is less clear (7–9).

In the past it has been implied and generally accepted, that acylation is the symmetrical reverse of deacylation (10) and proceeds by general base activation of Ser 195, which in turn acts as a nucleophile (11) (Scheme 1). It was concluded from a comparison of the magnitude of p for reactions of various nucleophiles and of chymotrypsin with substituted p-nitrophenyl and 2,4-dinitrophenyl benzoates that chymotrypsin acts as a neutral rather than anionic nucleophile toward these substrates. Kinetic solvent isotope ratios and other data together with the p analysis led to the suggestion that in the case of phenyl benzoates acylation may occur by direct nucleophilic attack by the imidazole of His 57, yielding a transient acylimidazole which rapidly collapses to the Ser 195 acylated enzyme (7) (Scheme 2). Since the substrates studied were non-specific, it does not seem unreasonable to expect that the geometry of their interaction with the active site of the enzyme might be sufficiently different from that for physiological substrates to provide for an alternate mechanism of acylation.

Because Scheme 2 seems to apply only to phenyl benzoates (although earlier it was thought that in addition phenyl acetates would react in an analogous manner) and because of the reports (8, 9) that acylation can only proceed through the general base-catalyzed pathway, it was appropriate to conduct further experiments which would shed light upon the pathway for the acylation of chymotrypsin by these phenyl esters.

The effect of leaving group substituents upon the rate of acylation by a series of substituted phenyl p-nitrobenzoates has been studied. A very good correlation of the polarity of the substituent in the leaving group with the rate constant for acylation has been obtained. This result, supported by further solvent deuterium isotope data, and when examined in con-
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**EXPERIMENTAL PROCEDURES**

**Materials.** Three times crystallized α-chymotrypsin was obtained from Worthington Biochemical Corp. N-trans-Cinnamoylimidazole (Eastman) was recrystallized from sodium-dried n-hexane yielding a purified product with a melting point of 132.0 to 133.7 (literature m.p. 133.0 to 133.5 (Ref. 13)). Acetonitrile was redistilled from P2O5. Dioxane, spectrophotometric grade, was obtained from Aldrich as was deuterium oxide, 99.6%. Deuterium chloride, a 50% solution in 99% D2O, was supplied by Stohler Isotope Chemicals.

**Esters**—The p-nitrophenyl benzoate esters (p-H and p-NO2) were those previously used (7). The following esters were prepared by conventional methods (12): p-cyanophenyl p-nitrobenzoate, p-bromo phenyl p-nitrobenzoate, p-chlorophenyl p-nitrobenzoate, m-nitrophenyl p-nitrobenzoate, m-acetylphenyl p-nitrobenzoate, p-chlorophenyl p-nitrobenzoate, p-chlorophenyl acetate, p-chlorophenyl phenylacetate, and p-nitrophenyl acetic acid. Acetonitrile as co-solvent, and were maintained at 25°C. All solutions were buffered (0.1 M acetate buffer), contained 5% (v/v) acetonitrile as co-solvent, and were maintained at 25°C. The reactant concentrations were usually 5 μM for each substrate and 50 μM for the chymotrypsin solutions.

**Kinetics**—Acylation rates for the phenyl benzoate esters and p-nitrophenyl benzoate at pH 5.05, are collected in Table I. A low pH value was chosen to reduce the rates of reaction in order that those which must be monitored in the ultraviolet range could be easily followed.

**RESULTS**

Acylation by the remaining phenyl p-nitrobenzoates were followed in a Cary 14 recording spectrophotometer fitted with a thermostated cell holder and a 0.0 to 0.2 absorbance unit slide wire. Reactions were initiated by rapid addition of microliter quantities of substrate in acetonitrile to the thermostated solution of enzyme in buffer in the reaction cuvette, and the disappearance of substrate was monitored at the appropriate wavelength. Blanks of enzyme solution of identical initial concentrations were used. The resulting absorbance versus time tracings were tested for first order kinetics by linear regression analysis using the program STATPACK from the University of New Hampshire computer center.

Acylation reactions of substituted phenyl acetates were studied under second order conditions by monitoring the appearance of the phenolic product in the Cary 14. Second order rate constants for acylation by p-nitrophenyl acetate and p-cyanophenyl acetate were determined by the method of Bender et al. (15), but because of competing decayslation, the rate constants for p-chlorophenyl acetate and phenyl acetate were determined from the initial rates of reactions. Pseudo-first order and second order conditions gave identical second order rate constants for p-nitrophenyl acetate, as did application of an earlier method (15) and the initial rate procedure for the second order acylation by p-cyanophenyl acetate.

All reactions were carried out at 25.0 ± 0.2°C. Hammett plots were constructed by linear regression analysis using STATPACK.
assure that both procedures yield the same kinetic data, the acylation of chymotrypsin by p-nitrophenyl benzoate was monitored by both methods at pH 5.05 (0.1 M acetate; 5% v/v, CH₃CN). The resulting second order rate constants derived from the disappearance of substrate and the appearance of product are 346 ± 10 m⁻¹ s⁻¹ and 363 ± 11 m⁻¹ s⁻¹, respectively. These are statistically equivalent; therefore, it is justifiable to compare kinetic parameters obtained by both methods.

Acylation of Chymotrypsin by Phenyl Acetates. The second order acylation rate constants are determined for a series of four p-substituted phenyl acetates at pH 7.47 (0.1 M acetate; 5% v/v, CH₃CN). The rate constant obtained for p-nitrophenyl acetate, 1425 m⁻¹ s⁻¹, is comparable to the rate constant obtained previously at pH 7.50 in 4.68% CH₃CN, 1370 m⁻¹ s⁻¹ (20). These kinetic parameters are compiled in Table II along with values for a different series of phenyl acetates presented in a previous study (19). Since the experiments of this previous study were carried out at pH 7.92 in 10% CH₃CN, their values, for the purpose of comparison, were normalized to give the same rate constant for p-nitrophenyl acetate as was found in this study.

Acylation Rates as Functions of pH and pD. The rates of the acylation reactions of p-nitrophenyl p-nitrobenzoate, BzONp, and p-nitrophenyl thiocinnamate were determined as a function of pH and pD. The values of pK₁, pK₂, and the pH(pD)-independent rate constant, kₘₐₓ, were determined from a nonlinear regression analysis of Equation 2 (21) and are reported in Table III.

The resulting solvent deuterium isotope effects (k[D]/k[H]) on acylation are 1.14, 1.30, and 2.10, for p-nitrophenyl p-nitrobenzoate, BzONp, and p-nitrophenyl thiocinnamate, respectively.

The dependence of the acylation rate constants on pH and pD for acylation by PAONp and HCONp in the pH range of 7.50 to 8.30 and the pD range 7.93 to 8.66 was determined. The pH- and pD-independent rate constants were estimated from the graphs of pH and pD versus kₜ/Kₘₓ. The solvent deuterium isotope effects for PAONp and HCONp were 1.3 and 2.0, respectively. Rate constants for acylation by PAONp and HCONp were determined in water at pH 8.0, and in deuterium oxide at pD 8.4, in the presence of 5% (v/v) dioxane. The results are compiled in Table III.

**DISCUSSION**

**Reactivity-Substituent Polarity Correlation for Leaving Group**—Application of structure-reactivity correlations to the mechanistic probing of reactions of enzymes with substrates can be of great value as long as the unique character of enzymatic reactions is taken into consideration (5, 22). The acylation of chymotrypsin involves two processes (Equation 1): the initial enzyme-substrate equilibrium to form the noncovalent Michaelis complex, and the actual acylation step which involves redistribution of covalent bonds. Alteration of the structure of the substrate could be manifested in either process. It is therefore important to know the potential effect of structure variation on each step before mechanistic conclusions are drawn since the second order rate constants in this study are composites of the enzyme-substrate equilibrium constant, Kₘₓ, and the first order rate constant for acylation, kₜ. Previous work has shown that variation of polar substituents on the phenolic leaving groups of ester substrates would be unlikely to affect Kₘₓ (23, 24). Therefore, a structure-reactivity correlation constructed by plotting the logarithm of kₜ/Kₘₓ versus the appropriate substituent parameter will reflect the effect of the electronic nature of the substituent on the transition state for acylation as manifested in the first order rate constant, kₜ.

Construction of a Hammett plot for the acylation by substituted phenyl p-nitrobenzoates using the σ⁻ substituent parameter yielded a ρ of 1.96 ± 0.30 (correlation coefficient, r = 0.934). The σ⁻ substituent charge on the phenolic oxygen generated in the transition state will be stabilized by resonance. A better correlation is obtained for the p-CN and p-NO₂ substituents using this procedure in line with the proposition that σ⁻ gives better linearity than σ for many reactions of phenols and phenolic esters (16). A plot for this correlation is shown in Fig. 1.

While leaving group ρ values and Brønsted β values are formally correlated with the relative nucleophilicities of the attacking and leaving groups, it can be seen by comparison of the leaving group ρ values presented in Table IV that a ρ value of 1.96 is consistent with imidazole being the primary nucleophile for acylation by phenyl p-nitrobenzoate esters.

A composite Hammett plot for the acylation by phenyl acetates including rate constants from this study and normalized rate constants from the study of Bender and Nakamura (19) is shown in Fig. 2. A ρ value of 2.05 ± 0.32 is obtained. Again, this value appears to be consistent with imidazole being the nucleophile, but in light of solvent deuterium isotope effects of approximately two for acylation by p-nitrophenyl acetate² and p-nitrophenyl thiocinnamate, the mechanism of acylation by phenyl acetates is unclear and will be discussed below.

In order to provide insight into the significance of the ρ obtained for nonspecific phenyl p-nitrobenzoates, it is of interest to compare this value with those obtained for substi-

² V. Zannis and J. F. Kirsch, unpublished results.
The acylation rate constants, $k''$, were obtained from use of Equation 2 when a complete pH (pD) dependence profile for acylation was obtained. The $pK_a$ values, characteristic of acylation, are also reported. Where $pK_a$ values are not reported, the $k''$ values were estimated from acylation rate constants in the pH (pD)-independent region of the profiles. The co-solvent was either 5% (v/v) acetonitrile or 5% (v/v) dioxane, and the buffer either 0.1 M acetate, 0.1 M phosphate, or 0.1 M pyrophosphate.

<table>
<thead>
<tr>
<th>Ester</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
<th>$k'' \times 10^{-3}$</th>
<th>Solvent</th>
<th>$k_{H_2O}/k_{D_2O}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Nitrophenyl $p$-nitrobenzoate</td>
<td>6.80</td>
<td>9.39</td>
<td>14.0</td>
<td>$H_2O$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.31</td>
<td>9.79</td>
<td>12.3</td>
<td>$D_2O$</td>
<td>1.14</td>
</tr>
<tr>
<td>$p$-Nitrophenyl benzoate</td>
<td>7.03</td>
<td>9.14</td>
<td>2.19</td>
<td>$H_2O$</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>7.30</td>
<td>9.72</td>
<td>1.68</td>
<td>$D_2O$</td>
<td>0.82</td>
</tr>
<tr>
<td>$p$-Nitrophenyl thiolacetate</td>
<td>6.97</td>
<td>8.20</td>
<td>2.02</td>
<td>$H_2O$</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>7.24</td>
<td>10.20</td>
<td>0.96</td>
<td>$D_2O$</td>
<td>2.10</td>
</tr>
<tr>
<td>$p$-Nitrophenyl phenylacetate</td>
<td>57</td>
<td>43</td>
<td>1.30</td>
<td>$H_2O$</td>
<td>18.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$D_2O$</td>
<td>10.9</td>
</tr>
<tr>
<td>$p$-Nitrophenyl hydrocinnamate</td>
<td>510</td>
<td>260</td>
<td>2.0</td>
<td>$H_2O$</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$D_2O$</td>
<td>101</td>
</tr>
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</table>

### Table IV

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>$\rho$</th>
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<tbody>
<tr>
<td>Phenyl $p$-Nitrobenzoates</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>1.96 (0.30)</td>
</tr>
<tr>
<td>OH$^-$</td>
<td>0.87 (0.06)</td>
</tr>
<tr>
<td>NH$_3^+$</td>
<td>2.6</td>
</tr>
<tr>
<td>Phenyl acetates</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>2.05 (0.32)</td>
</tr>
<tr>
<td>OH$^-$</td>
<td>0.8</td>
</tr>
<tr>
<td>(HOCH$_2$)CH$_2$O$^-$</td>
<td>0.38</td>
</tr>
<tr>
<td>Imidazole$^b$</td>
<td>1.8</td>
</tr>
<tr>
<td>NH$_3^+$</td>
<td>2.1</td>
</tr>
</tbody>
</table>

$^a$ Values in parentheses are standard deviation of mean.
$^b$ Results obtained in this study.
$^c$ From Ref. 25.
$^d$ From Ref. 26. The $\rho$ value is based upon only two esters, $p$-nitrophenyl $p$-nitrobenzoate and $p$-chlorophenyl $p$-nitrobenzoate, and therefore represents only an approximate value.
$^e$ From Ref. 19. The data were re-evaluated using a value of 1.0 for $\sigma^*$ for the $p$-$NO_2$ substituent.
$^f$ From Ref. 27.
$^g$ From Ref. 28.
$^h$ From Ref. 17.

A summary of the extrathermodynamic relationships for the acylation of chymotrypsin by a variety of ester substrates is of interest. In this study and a previous one (7) Hammett $\rho$ values for the variation of substituents on the acyl and phenolic positions of substituted phenyl benzoates were determined to be 0.97 ± 0.11 and 1.96 ± 0.30, respectively. These $\rho$ values are internally consistent in approximating model reactions of neutral nitrogen nucleophiles rather than strong anionic oxygen nucleophiles. Leaving group substituent effects for three series of specific phenyl esters yield $\rho$ values of less than 1, which are similar to values obtained for oxygen nucleophiles such as the hydroxide ion. Recently the acylation by a series of acyl-substituted aliphatic $p$-nitrophenyl esters was examined (30), and the substituent effects upon the second order acylation rate constants were analyzed using the Taft-Ingold relationship. Contributions of polar, steric, and specific effects were separated and a $\rho^*$, representing the polar contributions of the substituents, was found to be 2.18 ± 0.17, a value consistent with nucleophilic attack by anionic oxygen. Assuming that aliphatic nitrophenyl esters acylate by the conventional general base mech-
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Comparison of $\mu$ values for acylation of chymotrypsin by phenyl benzoates, specific phenyl esters, and aliphatic $p$-nitrophenyl esters

The $\mu$ values are derived from results reported here or are values from the literature as indicated.

<table>
<thead>
<tr>
<th>Ester series</th>
<th>$\mu$ acyl</th>
<th>$\mu$ leaving group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyl benzoates</td>
<td>0.97 ± 0.11</td>
<td>1.96 ± 0.39</td>
</tr>
<tr>
<td>Specific phenyl esters</td>
<td>0.45 ± 0.63</td>
<td></td>
</tr>
<tr>
<td>Aliphatic $p$-nitrophenyl esters</td>
<td>2.18 ± 0.17</td>
<td></td>
</tr>
</tbody>
</table>

* From Ref. 7.
* From results in Table I.
* From Ref. 24 and 23.
* From Ref. 29.

Significance of Perturbed $pK_a$ of Ser 195—Recently there has been some discussion of the identity of the ionizing group with a $pK_a$ of 7 in the catalytic triad of Asp 102, His 57, and Ser 195 (35–38). One implication of this proposed system is the possibility that the apparent $pK_a$ of Ser 195 is perturbed from near 13 for the free amino acid to approximately 7. Calculations on the active site of chymotrypsin have predicted a serine $pK_a$ of approximately 8 (39), and there is precedent for large perturbations of the $pK_a$ of amino acid residues in enzymes (40, 41). An operational $pK_a$ of approximately 7 would render the Ser 195 moiety the better leaving group in the transition state on the general base-activated path to the serine-acylated substrates. This situation would be reflected in a large positive $\rho$ since $\sigma^+$ substituent constants for phenols parallel their relative basicities.

A relatively low $pK_a$ for serine can provide an attractive explanation for the observed leaving group $\mu$ values for the $\rho$ of $p$-nitrobenzoates and phenyl acetates without having to invoke an acylimidazole intermediate, but it leaves unexplained the $\rho$ of 0.97 found for the variation in the acyl portion of a series of $p$-nitrobenzoates and the observed solvent deuterium isotope effects. The above alternate explanation of the magnitude of the leaving group $\mu$ values for two series of nonspecific ester substrates entails speculation and some contradiction, but until a more detailed and direct determination of the $pK_a$ values of the active site components is made it cannot be ignored.

Solvent Deuterium Isotope Effects Upon Acylation of Chymotrypsin by Homologous Series of $p$-Nitrophenyl Esters—The efficiency of binding in the “tosyl hole” of the chymotrypsin active site increases in the series BzONp, PAONp, and HCONp (24). Isotope effects of 1.30, 1.3, and 2.0 (Table III) were found for acylation by these three esters, respectively. An isotope effect of approximately 2 for acylation by HCONp is consistent with all or part of the hydrocinnamoyl side chain being optimally bound in the “tosyl hole,” presumably orienting the ester carbonyl for attack by the general base-activated hydroxyl of Ser 195. The much lower isotope effect for acylation by BzONp has been explained earlier. These two extremes in isotope effect would seem to be consistent with a unique binding of the benzote ester, with little dependence on tosyl hole binding, leading to initial acylation at His 57, and with a strong specific binding of the hydrocinnamate ester firmly in the tosyl hole leading to acylation at Ser 195. An examination of a model of the active site of chymotrypsin shows that if the benzoyl moiety of BzONp is placed in the tosyl hole, the ester
carbonyl is not in position for nucleophilic attack by the imidazole of His 57. The isotope effect of 1.3 for acylation by PAONp indicates that the acyl moiety is not in the correct binding mode to facilitate the general base-catalyzed mechanism of reaction.

When the tosyl hole is occupied by dioxane (42, 43), acylation of the enzyme by BzONp is retarded by a factor of 1.6 which is much less than the corresponding factors for acylation by PAONp (3.0) and HCONp (2.6) (Table III). This difference may speak for a different mode of binding for BzONp with less dependence upon binding in the tosyl hole. In a similar vein it has been suggested that dioxane binds to the active site of acylated papain and reorients the reactive moieties resulting in acceleration of the rate of deacylation (44). It has been shown that in this series HCONp, with a side chain length of 9.7 Å, acylates most rapidly as a consequence of efficient hydrophobic binding (20). Side chain length of the acyl moiety is not itself the only criterion for most efficient binding since acylation by HCONp is about 30 times more rapid than is acylation by p-nitrophenyl p-ethylbenzoate (7) which is of hydrophobic binding (20). Side chain length of the acyl moiety is not itself the only criterion for most efficient binding since acylation by HCONp is about 30 times more rapid than is acylation by p-nitrophenyl p-ethylbenzoate (7) which is of approximately the same acyl chain length. Clearly, the presumed binding in the tosyl hole is much more favored when the terminal group is a phenyl ring.

Approximate solvent deuterium isotope effects of 1.7, 1.7, and 2.0 result from measurements in the pH/pD independent region for acylation by BzONp, PAONp, and HCONp, respectively, in the presence of dioxane. A possible interpretation is that for phenyl benzoates there is a multiplicity of potential acylating mechanisms with one involving rate-determining proton transfer becoming more predominant in the dioxane-inhibited system. 

Conclusion — The intermediacy of a transient acylimidazole species on the path to the acylation of chymotrypsin for a limited series of nonspecific ester substrates is supported by the analysis of the results. This postulation has previously been criticized in light of the presumed symmetry of the acylation and deacylation mechanisms (8, 9, 31, 45, 46). While alternative explanations might be offered for individual fragments of evidence, a consistent interpretation of the preponderance of the data presented yields a strong argument for the existence of a transient acylimidazole intermediate in the acylation of chymotrypsin by phenyl benzoates.

Acknowledgment — We greatly appreciate valuable discussions with Dr. Andrew Williams.

REFERENCES
Mechanisms of acylation of chymotrypsin by phenyl esters of benzoic acid and acetic acid.
C D Hubbard and T S Shoupe


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