# Steric Effects in the Acylation of α-Chymotrypsin*

Julie B. Milstien and Thomas H. Fife

ABSTRACT: The acylation of α-chymotrypsin by esters of *p*-nitrophenol has been investigated at various pH values at 25°. Esters studied included acetate, propionate, butyrate, hexanoate, isobutyrate, isovalerate, trimethylacetate, and 3,3-dimethylbutyrate. With the exception of *p*-nitrophenyl hexanoate, the reaction shows a normal steric order of reactivity, increased branching decreasing the rate of enzymatic acylation. Under the conditions of this study, the acylation reaction was experimentally second order.

A plot of log $(k_2/K_m)_{rel}$ *vs.* the Taft steric effect constants, $E_s$, was linear with a slope of 0.95 ± 0.22. This is similar to the slope of log $k_3$ *vs.* $E_s$ found in the deacylation reaction.

The kinetics of the acylation of α-chymotrypsin by *p*-nitrophenyl acetate have been extensively studied (Gutfreund and Sturtevant, 1956; Kézdy and Bender, 1962; Faller and Sturtevant, 1966). The reaction shows an initial "burst" of *p*-nitrophenol followed by a zero-order release of product. This behavior has been interpreted as indicating three steps including the formation of an enzyme–substrate complex, the rearrangement of this complex to form an acyl-enzyme, and the breakdown of the acyl-enzyme to products as in eq 1, where E is the

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

enzyme, S is the substrate, ES is the enzyme–substrate complex, ES′ is the acyl-enzyme, $P_1$ is *p*-nitrophenol, and $P_2$ is acetate ion. This formulation yields eq 2 (Gutfreund and Sturtevant, 1956),

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

where $K_m = (k_{-1} + k_2)/k_1$, and $k$ is a first-order rate constant governing the presteady-state reaction. If $(k_2 + k_3)(S)_0 \gg k_3K_m$, then eq 2 can be simplified to

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

and the data can be analyzed according to conventional Michaelis–Menten kinetics. Ideally, to determine $K_m$ it is necessary to vary substrate concentration over a range of $0.1K_m$ to $10K_m$.

Under conditions of substrate concentrations which are small in comparison to $K_m$, it is not possible to determine the various constants by employing eq 3. If, in fact, $K_m$ is much larger than the initial substrate concentration, 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 is the ratio $k_2/K_m$ (Faller and Sturtevant, 1966).

It was previously found (Fife and Milstien, 1967) that the deacylation of a series of acyl-α-chymotrypsins with varied steric bulk in the acyl group gave a linear plot of log $(k_3/k_0)$, where $k_0$ is the deacylation rate constant for the acetyl derivative, *vs.* $E_s$, the Taft steric effects constants (Taft, 1956), with a slope of 1.05 ± 0.21. In the present study, the effect of varying steric bulk in the acyl portion of the ester substrate on the acylation rate constant $k_2/K_m$ has been determined.

## Experimental Section

*Materials.* α-Chymotrypsin (three-times crystallized) was obtained from Worthington Biochemical Corp. Acetonitrile was Eastman-Kodak Spectro Grade which was twice distilled over $P_2O_5$ and once over $K_2CO_3$. The *p*-nitrophenyl esters were the same as previously studied (Fife and Milstien, 1967).

*Kinetic Measurements.* The rates of acylation by the *p*-nitrophenyl esters of α-chymotrypsin were followed on a Zeiss PMQ II spectrophotometer equipped with a Brush Model No. RD-5615-00 high-gain amplifier, a Brush Model No. RD-2321-00-S-2929 oscillograph, and a stopped-flow apparatus as described by French *et al.* (1965). Constant temperature to ±0.1° was maintained by circulating water from a Haake Model F circulating bath or a Precision Scientific Lo-Temptrol 154 circulating water bath through the brass thermostat block of the stopped-flow apparatus. Usually at least five determinations were made for each rate measured, and each rate constant is the average of at least three determinations. Substrate and enzyme solutions were made up in previously degassed buffer so that the final percentage of acetonitrile was always 4.68%, and the

* From the Department of Biochemistry, University of Southern California, Los Angeles, California. *Received July 23, 1968.* This study was supported by National Institutes of Health Research Grant GM-14357. This study represents a portion of the work to be submitted by J. B. M. in partial fulfillment of the requirements for the Ph.D. degree, University of Southern California.

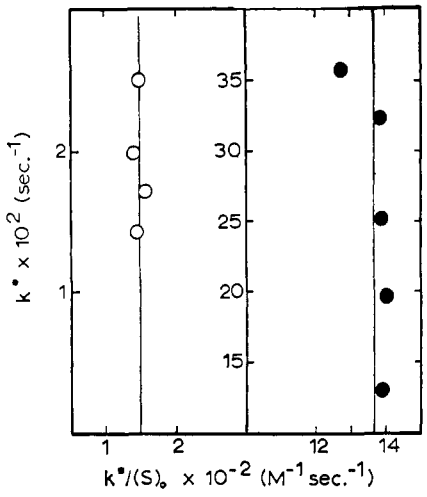


FIGURE 1: Plots of $k^*$ *vs.* $k^*/(S)_0$ for the acylation of α-chymotrypsin in 4.68% acetonitrile at 25° and $\mu$ = 0.075 M by *p*-nitrophenyl trimethylacetate at pH 7.99 (○) and *p*-nitrophenyl acetate at pH 7.58 (●).

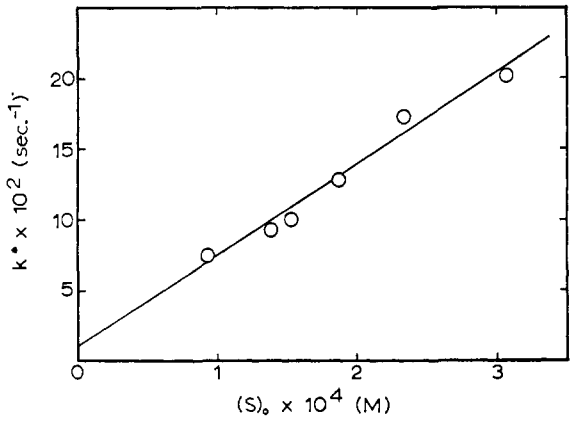


FIGURE 2: Plot of $k^*$ *vs.* $(S)_0$ for the acylation of α-chymotrypsin by *p*-nitrophenyl butyrate in 4.68% acetonitrile at 25°, and pH 5.92, $\mu$ = 0.075 M.

$(S)_0/(E)_0$ ratio was greater than 10. Operational normality of enzyme solutions was determined by the titrimetric procedure of Schonbaum *et al.* (1961). Phosphate buffers were used for kinetic measurements at pH values from 5.92 to 7.99, and Tris-HCl buffers from pH 7.99 to 8.90, $\mu$ = 0.075 M. The pH of buffers was measured on a Radiometer Model 22 pH meter. Reactions were followed by observation of *p*-nitrophenol at 330 mμ or of *p*-nitrophenolate ion at 400 mμ. Appropriate corrections were made for spontaneous hydrolysis of the esters.

Pseudo-first-order rate constants for the burst reaction were determined by the method of Faller (1964). From eq 1 the resulting expression for the release of *p*-nitrophenol when substrate is in large excess is that given in eq 4 (Gutfreund and Sturtevant, 1956). $A$ is

$$(P_1) = At + B(1 - e^{-kt}) \quad (4)$$

equal to the rate of production of *p*-nitrophenol when the burst rate is complete. When $AT$ is subtracted from eq 4, the remaining curve is described by

$$(P_1') = B(1 - e^{-kt}) \quad (5)$$

A plot of absorbance (which is proportional to the concentration of *p*-nitrophenol released) *vs.* time was made. The steady-state rate (zero-order portion) was extrapolated to zero time. This value was taken as $OD_\infty$ for the burst rate. The pseudo-first-order rate constants were then determined on an Olivetti-Underwood Programma 101 which was programmed to do regression and correlation analysis. The output of interest consisted of the regression coefficient (rate constant) of $\ln((OD_\infty - OD_i)/(OD_\infty - OD_t))$ *vs.* time, the intercept of the log function *vs.* time, the correlation coefficient, and the standard error of the estimate.

## Results

It can be seen from eq 2 that a plot of $k^*$, where $k^* = k - k_3$, *vs.* $k^*/(S)_0$ should give a straight line with slope equal to $-K_m$ and ordinate intercept equal to $k_2$. When this plot (Eadie, 1942) was made, the slope was infinite for each of the ester substrates, as shown in Figure 1 for typical examples. This implies that if binding is occurring then $K_m$ is much larger than the highest substrate concentration studied so that the concentration of ES is experimentally undetectable. The substrate concentration is, of course, limited by low solubility. Therefore the data were treated according to second-order kinetics.

In the case of a second-order reaction, a plot of $k^*$ *vs.* $(S)_0$ should have an ordinate intercept of zero, and the slope of this plot should be equal to the pH-dependent second-order acylation rate constant, which is termed $(k_2/K_m)'$. A typical plot is shown in Figure 2. The least-squares values of the slopes are presented in Table I. Also presented are the average values of $k^*/(S)_0$ determined from the abscissa intercepts of plots of $k^*$ *vs.* $k^*/(S)_0$. These values should from eq 2 be equal to $(k_2/K_m)'$. It can be seen that in most cases they are closely similar. Both sets of data are included since they were calculated by different methods. The intercepts of the plots of $k^*$ *vs.* $(S)_0$ were generally zero within the limits of experimental error as determined by the standard error of the estimate.

Theoretical curves have been fitted to the pH–rate data, using

$$\left(\frac{k_2}{K_m}\right)' = \left(\frac{k_2}{K_m}\right)\frac{1}{(1 + (H/K_1) + K_2/H)} \quad (6)$$

where $K_1$ and $K_2$ are the ionization constants of the essential groups necessary for enzymatic catalysis and $(k_2/K_m)$ is the pH-independent acylation rate constant. A pH–$(k_2/K_m)'$ maximum could be clearly observed for each ester although rate measurements were not made at pH values greater than 8.90. The method of Alberty and Massey (1954) was used to estimate $K_1$ and $K_2$. The calculated values for $pK_1$, $pK_2$, $pH_{optimum}$, and $k_2/K_m$ providing the best fit to the data are given in Table II. A broader pH–$(k_2/K_m)'$ maximum was found for *p*-nitrophenyl acetate than with the other esters in the series

TABLE I: Acylation of α-Chymotrypsin by Esters of *p*-Nitrophenol at 25.0° in 4.68% Acetonitrile, $\mu$ = 0.075 M.

| Ester | pH | Buffer | $(k_2/K_m)' \times 10^{-2}$ [a, c] | $k^*/(S)_0 \times 10^{-2}$ [b, c] |
|---|---|---|---|---|
| Acetate | 5.92 | Phosphate | 3.66 | 4.02 |
| | 6.19 | Phosphate | 3.77 | 5.23 |
| | 7.34 | Phosphate | 10.4 | 13.9 |
| | 7.58 | Phosphate | 12.4 | 13.7 |
| | 7.99 | Tris | 13.6 | 15.2 |
| | 8.12 | Tris | 13.6 | 16.2 |
| | 8.90 | Tris | 13.3 | 16.4 |
| Propionate | 5.92 | Phosphate | 6.32 | 5.75 |
| | 6.19 | Phosphate | 5.79 | 7.40 |
| | 7.34 | Phosphate | 23.0 | 26.7 |
| | 7.58 | Phosphate | 25.9 | 29.7 |
| | 7.99 | Tris | 34.2 | 32.6 |
| | 8.12 | Tris | 25.9 | 29.1 |
| Butyrate | 5.92 | Phosphate | 6.49 | 6.89 |
| | 6.19 | Phosphate | 8.32 | 11.3 |
| | 7.34 | Phosphate | 23.9 | 28.2 |
| | 7.58 | Phosphate | 19.8 | 25.0 |
| | 7.99 | Tris | 26.4 | 27.1 |
| | 8.12 | Tris | 28.2 | 25.8 |
| | 8.90 | Tris | 17.8 | 24.9 |
| Hexanoate | 5.92 | Phosphate | 35.6 | 32.2 |
| | 6.19 | Phosphate | 46.0 | 40.8 |
| | 7.58 | Phosphate | 123 | 102 |
| | 7.99 | Tris | 93.4 | 93.6 |
| | 8.12 | Tris | | 118 |
| | 8.90 | Tris | 63.1 | 101 |
| Isobutyrate | 5.92 | Phosphate | 3.10 | 4.38 |
| | 6.19 | Phosphate | 3.32 | 4.71 |
| | 7.34 | Phosphate | 17.2 | 10.7 |
| | 7.58 | Phosphate | 18.6 | 13.6 |
| | 7.99 | Phosphate | 20.7 | 15.7 |
| | 7.99 | Tris | 19.2 | 17.0 |
| | 8.12 | Tris | | 15.1 |
| | 8.90 | Tris | 6.25 | 9.86 |
| Isovalerate | 5.92 | Phosphate | 0.981 | 1.90 |
| | 6.19 | Phosphate | 2.31 | 2.84 |
| | 7.34 | Phosphate | 5.12 | 5.52 |
| | 7.58 | Phosphate | 7.53 | 6.00 |
| | 7.99 | Phosphate | 4.09 | 4.98 |
| | 7.99 | Tris | 4.00 | 7.31 |
| | 8.90 | Tris | 2.14 | 3.40 |
| Trimethylacetate | 5.92 | Phosphate | 0.24 | 0.31 |
| | 6.19 | Phosphate | 0.67 | 0.48 |
| | 7.34 | Phosphate | 1.50 | 1.38 |
| | 7.58 | Phosphate | 1.59 | 2.25 |
| | 7.99 | Tris | 1.45 | 1.48 |
| | 8.12 | Tris | 1.31 | 1.88 |
| | 8.90 | Tris | 0.73 | 0.77 |
| 3,3-Dimethylbutyrate | 5.92 | Phosphate | 0.067 | 0.11 |
| | 6.19 | Phosphate | 0.079 | 0.12 |
| | 7.34 | Phosphate | 0.46 | 0.40 |
| | 7.58 | Phosphate | 0.50 | 0.67 |
| | 7.99 | Tris | 0.49 | 0.61 |
| | 8.12 | Tris | 0.37 | 0.55 |
| | 8.90 | Tris | 0.23 | 0.22 |

[a] Values are the slopes of plots of $k^*$ *vs.* $(S)_0$. [b] Values determined from plots of $k^*$ *vs.* $k^*(S)_0$. [c] $M^{-1}$ sec $^{-1}$.

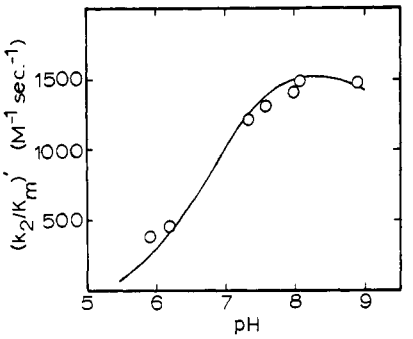


FIGURE 3: pH–rate profile for the acylation of α-chymotrypsin by *p*-nitrophenyl acetate in 4.68% acetonitrile at 25°; the circles are experimental points; the solid line was calculated using the constants listed in Table II.

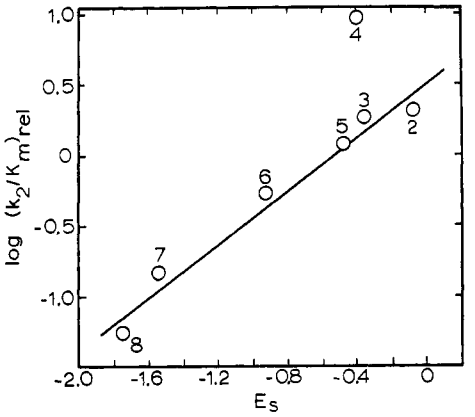


FIGURE 4: Plot of log $(k_2/K_m)_{rel}$ *vs.* $E_s$ for the acylation of α-chymotrypsin by esters of *p*-nitrophenol in 4.68% acetonitrile at 25° and $\mu$ = 0.075 *M*. Numbers correspond to those in Table III.

as shown by the profile in Figure 3. The line is theoretical employing the constants listed in Table II and the points represent an average of the $(k_2/K_m)'$ and $k^*/(S)_0$ values given in Table I.

The plot of log $(k_2/K_m)_{rel}$, where $(k_2/K_m)_{rel} = (k_2/K_m)/(k_2/K_m)^0$, and $(k_2/K_m)^0$ is the second-order acylation rate constant for acetate ester, *vs.* $E_s$ was made using the pH-independent values of $k_2/K_m$ given in Table II. The plot is shown in Figure 4. The point for the acetate ester has been omitted for comparison with the deacylation data (Fife and Milstien, 1967). All data on the plot except for the hexanoate ester are correlated with a slope of 0.95 ± 0.22 and a correlation coefficient of 0.98. If the point for the acetate ester is included, the slope is 0.82 ± 0.38, and the correlation coefficient is 0.94.

## Discussion

The slope of the plot of log $(k_2/K_m)_{rel}$ *vs.* $E_s$ shown in Figure 4 is 0.95, nearly identical with the slope of log $k_3$ *vs.* $E_s$ obtained for the deacylation of a series of acyl-α-chymotrypsins in which the acyl groups included those studied in the present work (Fife and Milstien, 1967). Steric influences on $k_2$ and $k_3$ would be expected to be nearly the same since acylation and deacylation must proceed by the same mechanism (Bender and Kézdy, 1964). As a consequence, any effect of steric bulk on the binding of substrate should give rise to a difference between steric influences on $k_2/K_m$ and on $k_3$. It can be seen in Table III that the ratios of the relative rates for acylation to those for deacylation are close to unity for the straight-chain compounds. With the branched compounds, there does seem to be slightly greater difference between acylation and deacylation. There is, of course, no experimental evidence for the existence of a Michaelis–Menten complex with the compounds studied. The relative rate ratios in Table III could therefore result

TABLE II: Theoretical p*K* Values and pH-Independent Rate Constants for the Acylation of α-Chymotrypsin by Esters of *p*-Nitrophenol.

| Ester | p$K_1$ | p$K_2$ | $pH_{optimum}$ | $k_2/K_m$ ($M^{-1}$ $sec^{-1}$) |
|---|---|---|---|---|
| 1. Acetate | 6.7 | 10.1 | 8.4 | 1,550 |
| 2. Propionate | 6.7 | 9.3 | 8.0 | 3,200 |
| 3. Butyrate | 6.6 | 9.4 | 8.0 | 2,825 |
| 4. Hexanoate | 6.8 | 9.1 | 8.0 | 14,000 |
| 5. Isobutyrate | 6.8 | 8.8 | 7.8 | 1,850 |
| 6. Isovalerate | 6.7 | 8.5 | 7.6 | 800 |
| 7. Trimethyl-acetate | 6.8 | 8.6 | 7.7 | 225 |
| 8. 3,3-Di-methyl-butyrate | 7.0 | 8.5 | 7.7 | 80 |

TABLE III: Comparison of the Relative Rate Ratios for the Acylation by Esters of *p*-Nitrophenol of α-Chymotrypsin with Those for the Deacylation of the Resulting Acyl-α-chymotrypsins at 25.0°, $\mu$ = 0.075 M.

| Ester | $(k_2/K_m)_{rel}$[a] | $k_{3rel}$[b] | $(k_2/K_m)_{rel}/k_{3rel}$ |
|---|---|---|---|
| 1. Acetate | 1.0 | 1.0 | 1.0 |
| 2. Propionate | 2.1 | 1.6 | 1.3 |
| 3. Butyrate | 1.8 | 1.2 | 1.5 |
| 4. Hexanoate | 9.0 | 7.4 | 1.2 |
| 5. Isobutyrate | 1.2 | 0.52 | 2.3 |
| 6. Isovalerate | 0.52 | 0.25 | 2.0 |
| 7. Trimethylacetate | 0.15 | 0.03 | 5.0 |
| 8. 3,3-Dimethylbutyrate | 0.05 | 0.03 | 1.7 |

[a] $(k_2/K_m)/(k_2/K_m)^0$ was determined in 4.68% acetonitrile. [b] $k_3/k_3^0$ (Fife and Milstien, 1967) was determined in 1.6% acetonitrile.

from the acylation rate constant being a simple second-order rate constant rather than a complex constant involving $K_m$. Faller and Sturtevant (1966) found that acylation of $\alpha$-chymotrypsin by *p*-nitrophenyl acetate was experimentally second order. However, rate measurements with $(S)_0 \ll (E)_0$ (Kézdy and Bender, 1962) can be interpreted as indicating Michaelis–Menten kinetics.

Hofstee (1959) studied the chymotrypsin-catalyzed hydrolysis of straight-chain fatty acid esters of hydroxybenzoic acids and found that while $V_{max}$ increased exponentially from $C_2$ to $C_7$, $K_m$ was essentially constant at a chain length of $C_5$–$C_6$. For chain lengths of $C_7$ and longer, $K_m$ decreased. It is likely that the relatively fast rate of acylation by *p*-nitrophenyl hexanoate is due to an effect of the long straight-chain acyl group on $k_2$ since a rate-enhancing effect of nearly identical magnitude occurs in the deacylation step ($k_3$). McDonald and Balls (1956) found that straight-chain alcohols accelerate the rate of transesterification of the acetyl group from acetylchymotrypsin. This they concluded was due to the existence of an alcohol binding site in which the longer chain alcohols could become better oriented. The faster hydrolysis of the hexanoate ester could then be due to an interaction of the aliphatic chain with the protein producing an orientation more favorable for the reaction. In accord with an orientation effect, the entropy of activation for deacylation of hexanoyl-$\alpha$-chymotrypsin is more positive than that for acetyl-$\alpha$-chymotrypsin, while there is little difference in the activation energies (Fife and Milstien, 1967).

The value of $k_2/K_m$ for the acylation reaction with *p*-nitrophenyl acetate in 4.68% acetonitrile (1550 $M^{-1}$ $sec^{-1}$) compares favorably with that determined by Kézdy and Bender (1962) in 4% acetonitrile (1960 $M^{-1}$ $sec^{-1}$) at pH 7.8. In 1.6% acetonitrile the value is 3530 $M^{-1}$ $sec^{-1}$. Thus there is a decrease in the rate constants with increasing percentages of acetonitrile in the solvent as can also be seen from the data of Clement and Bender (1963) who postulated that organic solvent affects mainly $K_m$. The effect of organic solvent appears to decrease as branching increases. The value of $k_2/K_m$ of 225 $M^{-1}$ $sec^{-1}$ for the trimethylacetate ester, determined in this study, is comparable with that previously determined by Bender and Hamilton (1962) in 1.6% acetonitrile; $k_2/K_m$ = 232 $M^{-1}$ $sec^{-1}$.

A comparison (Milstien and Fife, 1968) of steric effects in the imidazole general base catalyzed hydrolysis of esters of *N*-acetylserinamide with those in the imidazole nucleophile-catalyzed hydrolysis of esters of *p*-nitrophenol and those in the deacylation of acyl-$\alpha$-chymotrypsins (Fife and Milstien, 1967) indicated that the role of the imidazole group at the active site of $\alpha$-chymotrypsin is more likely that of a classical general base than a nucleophile, supporting interpretations made on the basis of the $D_2O$ solvent isotope effects (Bender and Kézdy, 1964). The slope of the Taft steric effects plot for the deacylation reaction was similar to that for the classical general base catalyzed reaction when comparisons were made at the same temperature, but was much smaller than that for the nucleophile-catalyzed reaction. The acylation reaction in the present study shows a pattern of steric effects similar to the deacylation reaction, in accord with a classical general base role also for histidine in the acylation reaction. The effect of varying steric bulk in the acyl group of substrates for $\alpha$-chymotrypsin appears to be mainly on the actual acylation process itself, and the observed effects on the rates of acylation are not for the most part reflections of differences in binding.

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