Acetylation of α- and δ-Chymotrypsins by p-Nitrophenyl Acetate

ENZYME-SUBSTRATE COMPLEX FORMATION AND pH DEPENDENCE*

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SUMMARY

The acylation step of the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate has been studied at pH 7.1 and 25° in 2-propanol-water mixtures. By the use of higher substrate concentrations than those used previously under the same conditions, it was shown that, contrary to a previous report, the reaction of acylation does follow saturation kinetics. The binding constant, \( K_a \), and the acylation rate constant, \( k_2 \), were determined.

The acylation step of the δ-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate in acetonitrile-water was also shown to follow saturation kinetics. The pH dependences of \( k_2 \) and \( K_a \) are similar to those reported for α-chymotrypsin, although the decrease in \( k_2 \) at high pH is less for δ-chymotrypsin.

Thus, the pH dependence of acylation by p-nitrophenyl acetate is qualitatively different from that of specific substrates where \( k_2 \) is pH independent and \( K_a \) increases with pH. An explanation for this peculiar pH dependence of chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate is offered.

In the hydrolysis of p-nitrophenyl acetate (S) catalyzed by α-chymotrypsin (E), a rapid initial liberation of p-nitrophenol, a "burst," was observed (1), followed by a slower, zero order release. The "burst" was shown to be due to the formation of an acyl-enzyme intermediate (ES'). From a kinetic study of the reaction, Gutfreund and Sturtevant (2) concluded that the scheme of Equation 1 was the simplest capable of explaining their results. This pathway involves the formation of a Michaelis-Menten complex (ES), followed by acylation of the enzyme and then deacylation of the acyl-enzyme intermediate. This mechanism is considered to apply also to the hydrolysis of specific substrates. The validity of Gutfreund and Sturtevant's kinetic interpretation has been confirmed in this laboratory (3), at lower concentrations of organic solvent and different pH values. However, the original data have later been shown to be somewhat ambiguous due to the restricted range of concentration of the substrate (4).

The catalytic properties of α-chymotrypsin are severely diminished in the alkaline pH region (5, 6); the second order rate constant, \( k_{cat}/K_m \), for the α-chymotrypsin-catalyzed hydrolysis shows a sharp decrease above pH 8.5, with an apparent dependence on an acid group of pK \( _a \) about 8.8 (7). For specific amide substrates this decrease is due to a change in the binding ability of the enzyme, appearing as an increase in the dissociation constant.

**TABLE I**

Solubilities of p-nitrophenyl acetate in 2-propanol-water mixtures

| Solubility | [Tris] + [Tris-HCl] | [2-Propanol] | % v/v |
|------------|---------------------|-------------|-------|
| NaCl       | 0.1 M               | 0.4         | 1.0   |
|            | 0.1 M               | 0.4         | 5.0   |

**TABLE II**

Kinetic data for hydrolysis of p-nitrophenyl acetate catalyzed by α-chymotrypsin

The hydrolysis utilized 0.4 M Tris-HCl buffer, pH 7.1, containing 0.1 M NaCl, 25.0°. \([S] = 2 \times 10^{-4} \text{ to } 1.35 \times 10^{-3} \text{ M at } 1\%\) 2-propanol; \(2 \times 10^{-4} \text{ to } 3 \times 10^{-3} \text{ M at } 5\%\) 2-propanol. \([E] = 3.2 \times 10^{-5} \text{ M}\). Twelve experiments were done at each 2-propanol concentration. Standard deviations were calculated according to the method of Youden (18).

| [2-Propanol] | 10^4 X k | k | K_a |
|--------------|----------|---|-----|
| % v/v        | sec⁻¹    | sec⁻¹ | nmol |
| 1.0          | 1.2      | 5.27 ± 0.28 | 1.85 ± 0.13 |
| 5.0          | 3.5      | 4.43 ± 0.22 | 3.16 ± 0.23 |
FIG. 1. The rate of acylation of $\alpha$-chymotrypsin as a function of substrate concentration. $A$, rate constant versus substrate concentration; $B$, Eadie plot of the same data. Conditions: 1% 2-propanol, 0.4 M Tris-HCl buffer, pH 7.1, 0.1 M NaCl, 25°. [E], $3.2 \times 10^{-4}$ M. O, this research; △, data from Faller and Sturtevant (4).

The validity of this difference in behavior between $p$-nitrophenyl acetate and specific amides has been questioned (9) since at higher buffer and organic solvent concentrations than those previously used (3, 7), Faller and Sturtevant (4) failed to confirm that the acylation step of the $\alpha$-chymotrypsin-catalyzed hydrolysis of $p$-nitrophenyl acetate follows Michaelis-Menten kinetics (Equation 1). The latter authors pointed out that they were unable to separate the observed rate constants into $k_2$, $K_s$, and thereby confirm that an enzyme-substrate complex is formed in the $\alpha$-chymotrypsin-catalyzed hydrolysis of $p$-nitrophenyl acetate, even under the conditions used by these authors.

By studying the pH dependence of binding constants of specific substrates (12), and inhibitors (13), we have established the existence of a significant difference between the alkaline pH dependences of $\alpha$- and $\delta$-chymotrypsin-catalyzed hydrolyses: the second order rate constants and substrate binding ability decrease much less at high pH for $\delta$-chymotrypsin than for $\alpha$-chymotrypsin. Therefore, in the present study we have also investigated the acylation of $\delta$-chymotrypsin by $p$-nitrophenyl acetate in order to determine whether the $\delta$-chymotrypsin-cata-
Fig. 2. The rate of acylation of α-chymotrypsin as a function of substrate concentration. A, rate constant versus substrate concentration; B, Eadie plot of the same data. Conditions: 5% 2-propanol, 0.4 M Tris-HCl buffer, pH 7.1, 0.1 M NaCl, 25°. [E], 3.2 × 10^{-3} M. O, this research, Δ, data from Faller and Sturtevant (4).

Experimental Procedure

Materials—α-Chymotrypsin (lot CDI-7KH) and β-chymotrypsin (lot CDD-6032) were three times crystallized, salt-free products from Worthington. Spectrophotometric titrations with N-trans-cinnamoylimidazole (15) at 335 nm indicated that the enzymes had activities corresponding to 80% and 83% purity, respectively. p-Nitrophenyl acetate (Aldrich) was recrystallized from absolute ethanol and chloroform-hexane, m.p. 80°. Stock solutions of the substrate were freshly prepared in 2-propanol (Baker, analyzed spectrophotometric grade) and acetonitrile (Mallinckrodt nanograde). Buffer solutions were prepared according to standard procedures (16) from doubly distilled water and analytical grade reagents. For the experiments with α-chymotrypsin the Tris buffers contained 0.1 M NaCl.

Methods—The pH of each buffer solution was measured before and after reaction using a Corning 12 research meter or a Radiometer 4e with type B glass electrode. Buffer solutions for pre-steady state measurements were made up twice the required final concentration and the pH quoted refers to that after mixing.

The solubilities of p-nitrophenyl acetate in 2-propanol-water and 2-propanol-buffer mixtures were determined from the concentration of saturated solutions in equilibrium with the solid phase. Ester concentrations were measured spectrophotometrically after alkaline hydrolysis.

The rates of the pre-steady state hydrolysis of p-nitrophenyl acetate catalyzed by α- and β-chymotrypsin were determined...
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Fig. 3. The rate of acylation of δ-chymotrypsin as a function of substrate concentration. A, rate constant versus substrate concentration; B, Eadie plot of the same data. Conditions: 1.6% acetonitrile, 0.1 M Tris-HCl buffer, pH 7.91, 25°. [E] 3.2 × 10⁻⁴ M

spectrophotometrically on a Durrum-Gibson stopped flow spectrophotometer at 400 nm, 25.0°. Equal volumes of enzyme in buffer and substrate in 2-propanol-water or acetonitrile-water (double final organic solvent concentration) were mixed in the instrument producing a solution of the required concentrations and pH. Mixing effects were shown to be absent by carrying out blanks in the absence of substrate and enzyme. “Burst” rate constants were determined by extrapolating the steady state portion of each reaction to zero time and plotting the logarithm of the difference between the measured absorbance and extrapolated absorbance against time (17). Deacylation rate constants were determined from measurements on a Cary 14 PM recording spectrophotometer at 400 nm, 25.0°, using concentrations of substrate and enzyme similar to those produced after mixing in the rapid reactions. For both methods, rate constants were calculated by an unweighted least squares analysis using a CDC 6400 computer.

RESULTS AND DISCUSSION

Solubility of p-Nitrophenyl Acetate—The solubility of p-nitrophenyl acetate in 2-propanol-water and 2-propanol-buffer mixtures was measured in order to determine the maximum substrate concentration that could be used for kinetic experiments. The results are shown in Table I. The controlling factor was found to be the solubility in the 2-propanol-water solution before mixing. The highest concentrations used are therefore less than half these solubilities.

Kinetics of Deacylation of Acetyl Chymotrypsins—Deacylation rate constants, k₉, were determined from the steady state rate constants (kₒₛₛ) at various substrate concentrations. The
FIG. 4. pH dependence of the acylation rate constant $k_a$ for the $\alpha$-chymotrypsin-catalyzed hydrolysis of $p$-nitrophenyl acetate. --- corresponds to the $k_a$ versus pH curve for the acylation of $\alpha$-chymotrypsin by the same substrate obtained previously by Bender et al. (7). Conditions: 1.6% acetonitrile, ionic strength 0.1 M at 25°C.

Observed steady state rate constants were corrected for incomplete saturation by the method of Faller and Sturtevant (4), Equation 2, and extrapolated to zero substrate concentration to allow for substrate activation (3).

$$\frac{1}{k_a} = \frac{1}{k_{obs}} - \frac{[S] + K_s}{k_a[S]}$$

(2)

The values of $k_a$ determined at two solvent concentrations are given in Table II. The error in $k_a$ is estimated to be less than 10% and this is insignificant in the subtraction of $k_a$ from the burst rate constant which is 7 to 200 times greater.

**Kinetics of Aylation of $\alpha$-Chymotrypsin by $p$-Nitrophenyl Acetate**—Values of the first-order rate constant, $k_b$, for the burst were determined from measurements at various substrate concentrations, as described under “Experimental Procedure.” Plots of $k_b - k_a$ against $[S]$ (4) for mixtures containing 1% and 5% 2-propanol are shown in Figs. 1A and 2A, respectively, while Eadie plots (14) of $k_b - k_a$ against $(k_b - k_a)/[S]$ are shown in Figs. 1B and 2B, respectively, with the computer calculated least squares lines. From these lines the values of the acylation rate constant, $k_a$, and the binding constant, $K_s$, were determined and are given together with their standard deviations in Table II. The theoretical curves shown in Figs. 1A and 2A were calculated from these values using Equation 3.

$$k_b - k_a = \frac{k_b[S]}{K_a + [S]}$$

(3)

In Figs. 1A and 2A the values of $k_b - k_a$ clearly fit much better to the curves shown than to a straight line: if there were no saturation of the enzyme, the values of $k_b - k_a$ would be expected to lie on a straight line through the origin, of slope equal to $k_b/K_a$ (19). From inspection of Figs. 1A and 2A it can be seen that there is little correlation between the points and such a line. The Eadie plots obtained at each 2-propanol concentration have slopes and intercepts which are finite, indicating values of $K_s$ not much higher than the highest substrate concentration used. The values of $k_a$ and $K_s$ obtained from these plots have standard deviations of less than 10%; they can therefore be taken to be reliable indicators of the saturation of the enzyme.

The data previously obtained in this system by Faller and Sturtevant (4) are plotted in Figs. 1A and 2A after correction to allow for the small difference in pH so that they can be compared with the present work. These points clearly agree with our data and fit well with the calculated curves. These data are therefore also consistent with saturation kinetics, although their limited range of substrate concentrations would not prove the validity of Equation 1 in this system.

The present work was carried out at high buffer concentration at which the deacylation rate constant, $k_a$, and the binding constant, $K_s$, are higher than under the conditions used in previous studies (3, 7, 10). At lower buffer concentration, the substrate concentration could be made greater than $K_s$, and, in addition, $k_b$, which must be subtracted from the observed burst rate con-

\[1\] This small correction (<10%) was calculated using an unpublished procedure (J. J. Brubacher and F. J. Kezdy) based on a generalized plot of log ($k_b/k_b(\text{lim})$) versus pH–$pK_a$ and a $pK_a$ of 7.0.
Experiments were done at each pH. Standard deviations were calculated according to the method of Youden (18).

TABLE III
Acylation of 6-chymotrypsin by p-nitrophenyl acetate. pH dependence of \( k_3 \) and \( K_s \).

| pH    | Buffer   | \( K_s \) (M) | \( k_3 \) (sec\(^{-1}\)) | 95% Confidence limits for \( k_3 \) (sec\(^{-1}\)) |
|-------|----------|---------------|--------------------------|-----------------------------------------------|
| 6.15  | Phosphate| 1.21 ± 0.35   | 1.38 ± 0.20              | 0.16                                          |
| 6.65  | Phosphate| 1.21 ± 0.19   | 2.74 ± 0.23              | 0.17                                          |
| 7.12  | Phosphate| 1.11 ± 0.17   | 3.46 ± 0.26              | 0.20                                          |
| 7.60  | Phosphate| 1.42 ± 0.28   | 4.20 ± 0.46              | 0.35                                          |
| 7.91  | Tris-HCl | 1.36 ± 0.13   | 4.73 ± 0.78              | 0.91                                          |
| 8.52  | Tris-HCl | 1.85 ± 0.21   | 4.72 ± 0.26              | 0.20                                          |
| 9.07  | Carbonate| 1.94 ± 0.26   | 4.96 ± 0.42              | 0.32                                          |
| 9.47  | Carbonate| 1.85 ± 0.21   | 4.29 ± 0.30              | 0.23                                          |

constant, was much smaller and generally insignificant. Therefore the values of \( k_2 \) and \( K_s \) obtained under these conditions are at least as reliable as those in the present study.

Kinetics of Acylation of \( \delta \)-Chymotrypsin by p-Nitrophenyl Acetate—Values of the acylation and deacylation rate constants, \( k_2 \) and \( k_3 \), respectively, were determined as described for \( \alpha \)-chymotrypsin. Plots of \( k_3 \) versus substrate concentration and \( k_3 - k_2 \) against substrate concentration at pH 7.91 are shown in Fig. 3. From the latter plot and similar plots at other pH values, values of \( k_2 \) and \( K_s \) were calculated using a least squares program and are given with their standard deviations in Table III. The curvature of the plot of \( k_3 - k_2 \) versus substrate concentration and the finite values of \( k_2 \) and \( K_s \) show that saturation kinetics is followed in the acylation of \( \delta \)-chymotrypsin by p-nitrophenyl acetate.

A \( k_3 \) versus pH profile from the data of Table III is shown in Fig. 4; included for comparison is the \( k_3 \) versus pH curve for the acylation of \( \alpha \)-chymotrypsin by p-nitrophenyl acetate (7). For \( \delta \)-chymotrypsin, \( K_s \) is almost pH independent. \( k_3 \) decreases at high pH, although this decrease is considerably less than that observed for \( \alpha \)-chymotrypsin. This difference between the two enzymes in the high pH range is in agreement with previous studies using specific substrates and inhibitors (9, 12, 13), namely that the activity of \( \delta \)-chymotrypsin decreases less at high pH than does that of \( \alpha \)-chymotrypsin. Possible explanations and significance of the enhanced stability of \( \delta \)-chymotrypsin over \( \alpha \)-chymotrypsin at alkaline pH have been discussed previously (12, 13).

Hypothesis Regarding pH Dependence of Acylation of Chymotrypsins by p-Nitrophenyl Acetate—The pH dependence of the binding constant, \( K_s \), of p-nitrophenyl acetate to \( \alpha \)-chymotrypsin differs greatly from that of a specific substrate or inhibitor; in addition the pH dependence of the acylation rate constant, \( k_3 \), differs from that of a specific amide substrate. These differences will be related to the different size and structure of p-nitrophenyl acetate.

The binding of specific substrates, such as N-acetylated tyrosine methyl ester, is considered to be controlled (20, 21) principally by the interaction of the aromatic side chain with its binding site, the \( \alpha \) site of Cohen et al. (21). The interactions of the other functionalities of the substrate with their binding sites ensure that the L substrate has one predominant form of binding. At high pH, a conformational change (9, 22) removes the interaction of the side chain with the \( \alpha \) site and \( K_s \) increases greatly; binding of a specific substrate or inhibitor displaces the conformational equilibrium, with proton uptake (13, 22-24), producing the active conformation. Therefore \( k_2 \) remains constant at high pH while \( K_s \) increases.

p-Nitrophenyl acetate is much smaller than a specific substrate and lacks the \( \beta \)-aryl functionality and is therefore likely to have more than one binding mode. It may also be possible for more than one molecule of the substrate to bind to the enzyme at the same time. We suggest that the conformational change in the enzyme which disrupts the aromatic binding site does not affect the binding of p-nitrophenyl acetate; the molecule is able to bind equally well to the active (EH\(^+\)) and inactive (E\(^*\)) forms of the enzyme. Consequently \( K_s \) does not exhibit a pH dependence at high pH. In addition the binding of p-nitrophenyl acetate would not cause a shift in the conformational equilibrium since the equality of \( K_{EB} \) and \( K_{ES} \) requires that the pK\(_a\) values of E and ES be the same. Therefore the observed acylation rate constant at high pH will be that of the inactive form.

The pH dependence of \( k_3 \) requires that this be less than that of the low pH form and this is consistent with the suggested movement (23) of the carboxylate group of aspartate-194 which is held in an ion-pair with the protonated amino terminal of the isoleucine-16. Such a movement would disturb the sensitive arrangement of serine-195 and histidine-57 needed for catalysis (20). Thus, the pH dependence of the acylation of chymotrypsins can be represented by the scheme of Equation 4 (see Scheme I).
where \( K_{\text{rat}}^{\text{exp}} = K_{\text{rat}} \). If \( k_2^* = 0 \), the experimental “burst” rate constant is given by

\[
k_2^{\text{exp}} = \frac{k_2}{[S]} \frac{K_{E\text{H}}}{1 + \frac{K_{E\text{H}}}{[H^+]}} [5]
\]

so that the observed acylation and binding constants are given by

\[
k_2^{\text{obs}} = \frac{k_2}{[S]} \frac{K_{E\text{H}}}{1 + \frac{K_{E\text{H}}}{[H^+]}} [6]
\]

and

\[
K_s^{\text{obs}} = K_s^E = K_s^{E\text{H}} [7]
\]

Our assumption that p-nitrophenyl acetate is able to bind equally well to the high and low pH forms of the enzyme is supported by a recent study of the a-chymotrypsin-catalyzed hydrolysis of methyl hippurate (27) which showed that the pH dependence of \( K_s \) for this substrate was much less pronounced than that of a specific substrate. The thermodynamics of binding indicated that the interaction of the benzoyl group with the \( ar \) site made only a small contribution to the total binding energy and that the loss in binding energy upon conversion to the high pH form was small. A small decrease in \( k_2 \) at high pH was also observed for this substrate.

Acetylation of the amino group of isoleucine-16 in chymotrypsin has been shown to lead to a partial loss of activity towards p-nitrophenyl acetate (26). The decreases in the size of the burst and the steady state rate were consistent with a considerable decrease in \( k_2 \) without a change in \( K_s \). This behavior agrees with our suggestion that p-nitrophenyl acetate binds equally well to the two forms of the enzyme, since acetylation of isoleucine-16 produces a form of the enzyme similar to that observed at high pH (26).

REFERENCES

1. Hartley, B. S., and Kilby, B. A., Biochem. J. 56, 288 (1954).
2. Guttmann, H., and Sturtevant, J. M., Biochem. J. 63, 656 (1956); Proc. Nat. Acad. Sci. U. S. A., 42, 719 (1956).
3. Keddy, F. J., and Bender, M. L., Biochemistry, 1, 1097 (1962).
4. Faller, L., and Sturtevant, J. M., J. Biol. Chem., 241, 4825 (1966).
5. Huang, H. T., and Niemann, C., J. Amer. Chem. Soc., 73, 1541 (1951).
6. Bender, M. L., Clement, G. E., Keddy, F. J., and Zerner, B., J. Amer. Chem. Soc., 85, 358 (1963).
7. Bender, M. L., Clement, G. E., Keddy, F. J., and Hell, H. D', J. Amer. Chem. Soc., 86, 3650 (1964).
8. Himoe, A., and Hess, G. P., Biochem. Biophys. Res. Commun., 23, 234 (1966).
9. Himoe, A., Parks, P. C., and Hess, G. P., J. Biol. Chem., 242, 919 (1967).
10. Bender, M. L., Clement, G. E., and Wedler, F. C., J. Biol. Chem., 241, 4826 (1966).
11. Johnson, C. H., and Knowles, J. R., Biochem. J., 103, 428 (1967).
12. Valenzuela, P., and Bender, M. L., Proc. Nat. Acad. Sci. U. S. A., 63, 1214 (1969).
13. Valenzuela, P., and Bender, M. L., Biochemistry, 9, 2440 (1970).
14. Eady, G. S., J. Biol. Chem., 146, 85 (1942).
15. Schonbaum, G. R., Zerner, B., and Bender, M. L., J. Biol. Chem., 236, 2930 (1961).
16. Long, C. (Editor), Biochemists' handbook, D. Van Nostrand Company, New York, 1961, p. 19.
17. Bender, M. L., Keddy, F. J., and Wedler, F. C., J. Chem. Educ., 44, 94 (1967).
18. Youden, W. J., Statistical methods for chemists, John Wiley, New York, 1961, p. 12.
19. Linek, H., and Burk, D., J. Amer. Chem. Soc., 56, 658 (1934).
20. Hein, G. F., and Niemann, C., J. Amer. Chem. Soc., 84, 4405 (1962).
21. Cohen, S. G., Milovanović, A., Schultz, R. M., and Weinstein, S. Y., J. Biol. Chem., 244, 2664 (1969).
22. Bender, M. L., and Wedler, F. C., J. Amer. Chem. Soc., 89, 3052 (1967).
23. Wedler, F. C. and Bender, M. L., J. Amer. Chem. Soc., 91, 3894 (1969).
24. McCollum, J., Ku, E., Ozell, C., Czerlinski, G., and Hess, G. P., Science, 151, 274 (1968).
25. Sigler, P. B., Blow, D. M., Matthews, B. W., and Henderson, R., J. Mol. Biol., 35, 143 (1968).
26. Ghelli, C., Garrel, J. R., and Labousse, J., Biochemistry, 9, 3902 (1970).
27. Cuppett, C. C., and Canady, W. J., J. Biol. Chem., 245, 1009 (1970).
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