KINETIC EVIDENCE FOR AN ACYL-ENZYME INTERMEDIATE IN THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF N-ACETYL-L-TRYPTOPHAN ETHYL ESTER*

Myron L. Bender** and Gerald E. Clement***
Department of Chemistry, Northwestern University
Evanston, Illinois

Received July 1, 1963

In a recent communication from this laboratory, the methyl, ethyl and p-nitrophenyl esters of N-acetyl-L-tryptophan were shown to be hydrolyzed at pH 7.0 by α-chymotrypsin with the same catalytic rate constant (maximal velocity). (Zerner and Bender, 1963). This behavior cannot be rationalized in terms of a one-step process, for nucleophilic reactions, to which chymotrypsin conforms, would demand that the three esters be hydrolyzed at a hundred-fold or greater difference in rates. This behavior can, however, be explained if one postulates the formation of a common intermediate (ES') (equation 1) whose subsequent decomposition (k_3) is rate-determining. For these three

---

*This research was supported by grants from the National Institute of Health. Paper XXIII in the series, the mechanism of action of proteolytic enzymes.
**Alfred P. Sloan Foundation Research Fellow.
***National Institutes of Health Postdoctoral Research Fellow.

substrates, the common intermediate is N-acetyl-L-tryptophanyl-α-chymotrypsin.

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

In a second communication, a mechanistic correlation was presented showing that the pH-rate profile of the hydrolysis of those substrates in which k_2 is rate-determining is a bell-shaped curve while those in which k_3 is rate-determining is a sigmoid curve. (Bender, et al., 1963). If indeed k_2 exhibits a bell-shaped pH-rate profile while k_3 shows a sigmoid pH-rate profile in the hydrolysis of N-acetyl-L-tryptophan ethyl ester, it would be expected that the rate-determining step which was shown to be k_3 at pH 7 would change to k_2 at some high pH, for at some high pH the right-hand "leg" of the bell must cross below the "flat" of the sigmoid curve. We have therefore determined the kinetic constants for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester over a wide range of pH from 2.5 to 11.6.*

Equation 1 has been shown to lead to equations 2 and 3 (Gutfreund and Sturtevant, 1956). The bell-shaped and sigmoid pH-rate profiles of acylation (k_2) and deacylation (k_3) can be analyzed in terms of equations 4 and 5 respectively. Using these equations together with the

*These experiments were possible since it was shown that α-chymotrypsin was stable over the entire pH range during the course of kinetic measurements. Denaturation studies were carried out by titration with N-cinnamoylimidazole.

$$K_m(app) = k_3/(k_2 + k_3)\ K_m \quad (2)$$

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

$$k_2 = k_2(lim)/(1 + (H^+)/K_1 + K_2/(H^+)) \quad (4)$$

$$k_3 = k_3(lim)/(1 + (H^+)/(K_1')) \quad (5)$$

conclusions of the previous communications, the following predictions can be made: (1) k_{cat} of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester will follow a sigmoid curve to some high pH (while k_3 is rate-determining), but above some pH, k_{cat} will decrease (as k_2 becomes rate-determining); and (2) $K_m(app)$ will increase (as the rate-determining step changes from k_3 to k_2), leading eventually to the limiting value which is the true K_m. The experiments shown in Fig. 1 bear out these predictions.

In Fig. 1, the change in rate-determining step from k_3 to k_2 may be seen both in curve B, k_{cat}, which changes from a sigmoid curve to a bell-shaped curve at about pH 10, and in curve C, $K_m(app)$ which changes at about the same pH from its low pH-independent value to its high value. Furthermore, Fig. 1 shows that $k_{cat}/K_m(app)$ (curve A) is a true bell-shaped curve as it should be, for from equations 2 and 3 this quotient is equal to k_2/K_m; since K_m from independent data on other systems is pH independent, the pH dependence of $k_{cat}/K_m(app) = k_2/K_m$ is simply the pH dependence of k_2, a bell-shaped curve.

The circles of Fig. 1 are experimental points. The solid curves of Fig. 1 are theoretical curves calculated in the following way. Equations 2 and 3 can be combined and rearranged to

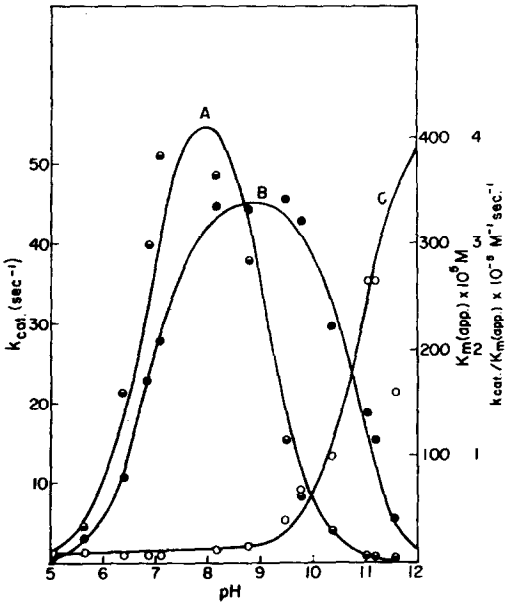

Fig. 1. The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester at 25.0° in 0.81% acetonitrile-water: A, $k_{cat}/K_m(app)$; B, k_{cat}; C, $K_m(app)$.

$$1/K_m(app) = 1/K_m + (k_{cat}/K_m(app))(1/k_3) \quad (6)$$

At pH values greater than 8, $k_3 = k_3(lim)$, a constant; therefore, above pH 8 a plot of $1/K_m(app)$ vs. $k_{cat}/K_m(app)$ should be linear with a slope of $1/k_3(lim)$ and an intercept $1/K_m$. The expected linearity was observed yielding a K_m of 4.1×10^{-3}M for N-acetyl-L-tryptophan ethyl ester. Knowing K_m, one may calculate $k_2(lim)$ and pK_2 (corresponding to the right leg of the bell) from eqs. 2 and 4. pK_1 and pK_1' can be calculated directly from plots of k_{cat}/K_m and k_{cat} respectively. The results of these calculations are shown in Table I. The theoretical curves of Fig. 1 were calculated using the data of Table I, together with the proper combination of equations 2-5. It is seen that the agreement

between experiment and theory is quite good, indicating that the bell-shaped and sigmoid dependencies for the acylation (k_2) and deacylation (k_3) reactions are

Table I

The α-Chymotrypsin-catalyzed Hydrolysis of N-acetyl-L-tryptophan Ethyl Ester[a]

Step	k(lim) sec.$^{-1}$	pK_a	$k_{calcd.}$[c] sec.$^{-1}$	k[b,c] sec.$^{-1}$
Acylation(k_2)	1818	6.77, 9.21	1100	480
Deacylation(k_3)	46.5	6.86	27	29

a. The kinetics were followed spectrophotometrically at 300 mμ measuring initial rates ($(E_o) \ll (S_o)$). The exact experimental techniques have been described by Bender et al. (1962).
b. Calculated by Zerner and Bender (1963) by an independent experimental approach.
c. pH 7.0.

verified and further that the stepwise nature of the reaction (equation 1) and the change in rate-determining step of reaction with pH are verified. The agreement between the rate constants of acylation and deacylation determined in the present experiments are in reasonable agreement with those calculated previously from an entirely different kinetic argument (see Table I).

In the low pH region of 2.5 to 5.04, an additional sigmoid dependence of k_{cat} may be observed, implying the kinetic importance of a base with an apparent pK_a of 4. The apparent limiting rate constant corresponding to this part of the reaction is much less (280 times) than the limiting rate constant at pH 7.9.

A pH dependence of K_m, corresponding to this, has been observed previously (Bender, et al., 1962; Stewart et al., 1963). A detailed report of the theoretical interpretation and mechanistic implications of this kinetic pH dependence will be the subject of a future publication.

The data for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl ester are thus seen to conform to the predictions of the two-step mechanism, equation 1, involving an acyl-enzyme intermediate. In general the overall pH dependence of a chymotrypsin reaction will consist of a family of curves, with curve A as one extreme, a sigmoid dependency at the other extreme, and with curve B as only one of number of intermediate examples.

REFERENCES

Bender, M. L., Clement, G. E., Kezdy, F. J. and Zerner, B., J. Am. Chem. Soc., 85, 357 (1963).
Bender, M. L., Schonbaum, G. R. and Zerner, B., J. Am. Chem. Soc., 84, 2562 (1962).
Clement, G. E. and Bender, M. L., Biochem., 2, 0000 (1963). Gutfreund, H. and Sturtevant, J. M., Biochem. J., 63, 656 (1956).
Stewart, J. A., Le, H. S. and Dobson, J. E., J. Am. Chem. Soc., 85, 1537 (1963).
Zerner, B. and Bender, M. L., J. Am. Chem. Soc., 85, 356 (1963).