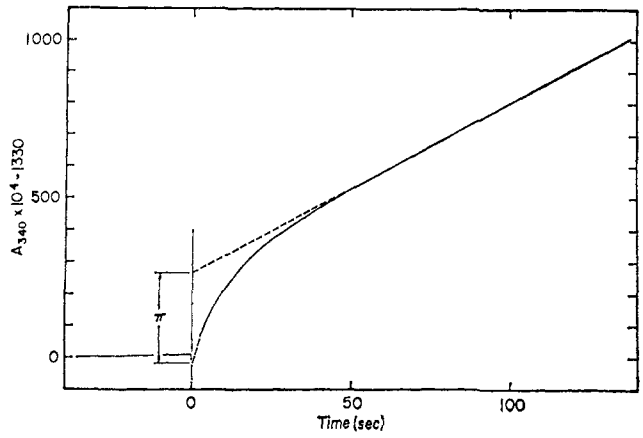


Figure 1. The trypsin-catalyzed hydrolysis of *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate at pH 2.66, 0.05 *M* citrate buffer; $E_0 = 7.40 \times 10^{-6}$ *M*; $S_0 = 2.03 \times 10^{-4}$ *M*, 1.29% (v./v.) acetonitrile–water, 25°. Cary Model 14 spectrophotometer, 8 in./min. recording chart speed, noise level = $5 \times 10^{-4}$ absorbance unit.

fied in this system. Therefore, for the calculation of the individual rate constants, we have used a combination of the presteady-state data together with data from the steady state, determined under identical conditions.

Table I. The Presteady State of the Trypsin-Catalyzed Hydrolysis of *p*-Nitrophenyl α-N-Benzyloxycarbonyl-L-lysinate Hydrochloride[a]

| $S_0 \times 10^5$, *M* | $b \times 10^2$, sec.$^{-1}$ | $\pi \times 10^6$, *M* |
|---|---|---|
| 56.5 | 18.2 | 5.36 |
| 38.5 | 13.4 | 4.74 |
| 20.3 | 9.44 | 4.57 |
| 13.7 | 7.88 | 4.03 |
| 9.78 | 5.71 | 3.62 |

[a] $E_0 = 7.40 \times 10^{-6}$ *M*. For other conditions, see Table II.

From the Lineweaver–Burk plot for the steady state, $k_{cat}E_0$ and $K_s/k_2E_0$ were determined from the intercept and the slope, respectively. Equation 2 may be rearranged to eq. 3. The quotient of $K_s/k_2E_0$ divided by

$$\left(\frac{S_0}{k_{cat}E_0} + \frac{K_s}{k_2E_0}\right)/b = \frac{K_s}{k_2k_3E_0} + \frac{S_0}{k_2k_3E_0} \qquad (3)$$

the intercept of a plot of the left side of eq. 3 *vs.* $S_0$, $K_s/k_2k_3E_0$, yields $k_3$. The product of the slope of this plot ($1/k_2k_3E_0$) and $k_{cat}E_0$ ($=k_2k_3E_0/(k_2 + k_3)$) yields ($k_2 + k_3$) and thus $k_2$. Knowledge of the $k_2/k_3$ ratio (27.6) then permits the calculation of $K_s$ from $K_m(app) = K_s/(1 + (k_2/k_3))$. The rate constants are summarized in Table II.

Table II. The Trypsin-Catalyzed Hydrolysis of *p*-Nitrophenyl α-N-Benzyloxycarbonyl-L-lysinate Hydrochloride[a-c]

| | |
|---|---|
| $k_2$ | 0.395 sec.$^{-1}$ |
| $k_3$ | $1.43 \times 10^{-2}$ sec.$^{-1}$ |
| $K_s$ | $7.95 \times 10^{-4}$ *M* |

[a] 25.0°, 1.29% (v./v.) acetonitrile–water, pH 2.66, 0.05 *M* citrate. [b] Worthington 2× crystallized, lyophilized bovine trypsin (TRL 6256); age of solution: >20 min. and <4 hr. [c] The substrate was a Cyclo Chemical Corp. product, m.p. 151°, $[\alpha]^{20}D$ 21.6° (*c* 2, dimethylformamide).[6]

Knowledge of the rate and equilibrium constants of the reaction permits the calculation of the enzyme concentration from $\pi$, the "initial burst" of *p*-nitrophenol, as a function of the substrate concentration. Values of $\pi$ were determined by graphical extrapolation, to $t = 0$, of the differences between the steady-state extrapolated lines and the absorbances at time *t* (Table I). By plotting $1/\sqrt{\pi}$ as a function of $1/S_0$ we obtain a straight line (using the value of $1/K_m(app)$ from the steady-state kinetics as an additional point). The intercept of this plot on the ordinate gives[6] $\pi_0 = E_0/(1 + (k_3/k_2))^2$, and thus $E_0 = 6.45 \times 10^{-6}$ *M*, indicating a purity of the enzyme, by weight, of 50%. Since our system of equations is redundant, we can calculate $E_0$ in an independent way: from $k_2$ and $k_3$, we can calculate $k_{cat}$, and then from the experimental value of $k_{cat}E_0$, $E_0 = 7.40 \times 10^{-6}$ *M* (57% purity by weight). The agreement of the two $E_0$ values seems reasonable, considering the experimental difficulties and the complexity of the calculations.[7]

The observation of "initial burst" of *p*-nitrophenol and of the kinetics of both the presteady-state and steady-state reactions are satisfactorily described by the three-step mechanism of eq. 1. These observations are consistent with the conclusion that, within experimental error, the totality of the tryptic hydrolysis of *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate hydrochloride involves a single reaction pathway with the formation of an α-N-benzyloxycarbonyl-L-lysyl-trypsin intermediate. This substrate is the most specific substrate of trypsin, based on its $k_{cat}(lim) = 170$ sec.$^{-1}$, the fastest known trypsin catalysis. Studies of the pH dependence of this reaction show that $k_{cat}$ is dependent, as usual, on a single basic group of $pK_a$ 6.80 ($I = 0.05$) from pH 2 to pH 7.4, indicating that the results obtained here at pH 2.66 may be reasonably extrapolated to neutral pH. Thus, the present observations must be pertinent to the pathway of trypsin catalysis.

(6) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, **19**, 161 (1965).

(7) The titration procedure for trypsin utilizing *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate hydrochloride previously reported[6] must be modified to include the $k_2/k_3$ ratio of 27.6. This modification will have the effect of raising the $E_0$ reported in that paper by 7.4%.

(8) National Institutes of Health Postdoctoral Research Fellow.

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## The Trypsin-Catalyzed Hydrolysis of the *p*-Nitrophenyl, Methyl, and Benzyl Esters of α-N-Benzyloxycarbonyl-L-lysine[1]

*Sir:*

The first step in the elucidation of the mechanism of any reaction is the establishment of the reaction pathway, that is, the characterization of intermediates formed in the reaction. Several pieces of evidence indicate that trypsin-catalyzed reactions proceed through an acyl-enzyme intermediate. The most important experimental indications are: (1) the methyl, ethyl, isopropyl, benzyl, and cyclohexyl esters of α-N-benzoyl-L-arginine are hydrolyzed with identical rate constants by trypsin[2]; (2) acetyl-trypsin is formed in

(1) This research was supported by grants from the National Institutes of Health.

(2) G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, 179, 665 (1949).

the trypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate[3]; *trans*-cinnamoyl-trypsin is seen in the trypsin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole[4]; a fast stoichiometric release of *p*-nitrophenol occurs in the trypsin-catalyzed hydrolysis of *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate hydrochloride[5] and the kinetics of the presteady-state and steady-state reactions in this system are satisfactorily described by eq. 1 involving an acyl-trypsin intermediate.[6]

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

However, a recent report presents data which tend to disprove the compulsory formation of an acyl-enzyme intermediate in the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester.[7] Moreover, the evidence enumerated above may not be general if one postulates that: (1) the slowly reacting acetyl and cinnamoyl derivatives do not necessarily proceed *via* the same pathway as the fast arginine and lysine derivatives; (2) "activated" *p*-nitrophenyl esters do not necessarily proceed by the same pathway as the "nonactivated" methyl or ethyl esters and the amide; and (3) the small spread of reactivities expected in the series of esters of argument 1 above casts doubt on the validity of the argument. Therefore experiments were designed to determine whether an acyl-trypsin is formed in the trypsin-catalyzed hydrolysis of α-N-benzyloxycarbonyl-L-lysine methyl and benzyl esters.

**Table I.** The Kinetics of the Trypsin-Catalyzed Hydrolysis of the Methyl, Benzyl, and *p*-Nitrophenyl Esters of α-N-Benzyloxycarbonyl-L-lysine[a]

| Ester | pH | $k_{cat}$, sec.$^{-1}$ | $K_m$(app), *M* |
|---|---|---|---|
| *p*-Nitrophenyl[b] | 5.80 | 6.79 | $1.00 \times 10^{-5}$ |
| | 5.84 | 6.93 | $0.93 \times 10^{-5}$ |
| | 5.85 | 7.02 | $0.94 \times 10^{-5}$ |
| Methyl[c] | 5.80 | 6.40 | $2.98 \times 10^{-4}$ |
| | 5.80 | 6.55 | $2.72 \times 10^{-4}$ |
| Benzyl[d] | 5.80 | 6.18 | $\sim 1 \times 10^{-4}$ |

[a] 25.0°, phosphate buffer, $I = 0.5$; $E_0 = (2.5\text{–}6) \times 10^{-7}$ *M*; 1.29% (v./v.) acetonitrile–water. [b] This compound was described previously.[6] [c] Hydrochloride purchased from Cyclo Chemical Co. as an oil; after two recrystallizations from methanol–diethyl ether, m.p. 75–76°. *Anal.* Calcd. for $C_{15}H_{13}N_2O_4Cl$: C, 54.46; H, 7.01; N, 8.47; Cl, 10.72. Found: C, 54.49; H, 7.19; N, 8.93; Cl, 10.80. The amount of D isomer must be less than 10% from its behavior with trypsin. [d] Tosylate salt, Cyclo Chemical Co. product, recrystallized from ethanol–diethyl ether, m.p. 111–112°. *Anal.* Calcd. for $C_{28}H_{34}N_2O_7S$: C, 61.98; H, 6.32; N, 5.16; S, 5.91. Found: C, 61.90; H, 6.36; N, 5.41; S, 5.76. No detectable amount of the D isomer was observed in its reaction with trypsin. [e] pH 5.8 was chosen since: (1) at this pH $K_m$ of this reaction does not yet show the marked pH dependence occurring at lower pH values[8]; (2) the enzyme concentration is in a convenient range; (3) the rate of the reaction is not too fast and can be measured accurately; and (4) the nonenzymatic hydrolysis of the *p*-nitrophenyl ester is slow. The pH dependence of $k_{cat}$ of the methyl and *p*-nitrophenyl ester reactions is identical from pH 3 to 7.5, and thus the equivalence seen at pH 5.8 is valid over this range.

(3) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, 37, 751 (1959).
(4) M. L. Bender and E. T. Kaiser, *J. Am. Chem. Soc.*, 84, 2556 (1962).
(5) M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, 19, 161 (1965).
(6) M. L. Bender, F. J. Kézdy, and J. Feder, *J. Am. Chem. Soc.*, 87, 4953 (1965).
(7) S. A. Bernhard and H. Gutfreund, *Proc. Natl. Acad. Sci. U. S.*, 53, 1238 (1965); T. E. Barman and H. Gutfreund, *ibid.*, 53, 1243 (1965).
(8) *Cf.* J. A. Stewart and J. E. Dobson, *Biochemistry*, 4, 1086 (1965).

The steady-state kinetics of the trypsin-catalyzed hydrolysis of the methyl, benzyl, and *p*-nitrophenyl esters of α-N-benzyloxycarbonyl-L-lysine were determined at pH 5.8, using Lineweaver–Burk plots of single experiments. The kinetic comparison shown in Table I indicates that the catalytic rate constants of the three esters are identical within ±6%, whereas the $K_m$(app) values are markedly different. These data are quite similar to data on the α-chymotrypsin-catalyzed hydrolysis of the ethyl, methyl, and *p*-nitrophenyl esters of N-acetyl-L-tryptophan.[6] Like the chymotrypsin data, the trypsin data are inconsistent with the nucleophilic character of trypsin reactions since methyl, benzyl, and *p*-nitrophenyl esters have relative rates of 1, 1.1, and 50 toward the nucleophile hydroxide ion.[9,10] On the other hand, these data are completely consistent with the three-step mechanism (eq. 1) for all substrates, involving the rate-determining decomposition ($k_3$) of a common intermediate, α-N-benzyloxycarbonyl-L-lysyl-trypsin, $k_2$ values which reflect the reactivity of these esters toward nucleophiles, and closely similar $K_s$ values. The slight trend in $k_{cat}$ values ($k^{NPE} > k^{BE} = k^{ME}$) is in the expected nucleophilic order and indicates that deacylation is not completely rate controlling; the $K_m$(app) values are also in the expected order ($K_m^{NPE} \ll K_m^{BE} \sim K_m^{ME}$). However, the limited accuracy of the data and the lack of an estimated $K_S$ prevents the calculation of $k_2$, as was done for chymotrypsin-catalyzed reactions.

The combination of the observation of the presteady state for the *p*-nitrophenyl ester[6] of α-N-benzyloxycarbonyl-L-lysine and the identity of the $k_{cat}$ values for the methyl, benzyl, and *p*-nitrophenyl esters of α-N-benzyloxycarbonyl-L-lysine proves beyond any reasonable doubt that all three esters are hydrolyzed by trypsin *via* an acyl-enzyme intermediate.

(9) B. Zerner, R. P. M. Bond, and M. L. Bender, *J. Am. Chem. Soc.*, 86, 3674 (1964).
(10) The nucleophilic character of the reaction is seen in the relation between the $k_{cat}$ values of α-N-benzoyl-L-arginine ethyl ester and amide which have values of 16 and 0.04 sec.$^{-1}$,[11] respectively, and in the positive Hammett ρ constant of the deacylation of a series of *para*-substituted benzoyl-trypsins.[12]
(11) S. A. Bernhard, *Biochem. J.*, 59, 506 (1955).
(12) C. R. Gunter, unpublished experiments in this laboratory.

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## Transient Formation of an Inactive Intermediate in the Reaction with α-N-Benzoyl-L-arginine Ethyl Ester and α-N-Benzoyl-L-arginine[1]

*Sir:*

In previous communications evidence that the trypsin-catalyzed hydrolysis of *p*-nitrophenyl, benzyl, and methyl esters of α-N-benzyloxycarbonyl-L-lysine proceed through an α-N-benzyloxycarbonyl-L-lysyl-trypsin intermediate was presented.[2,3] In this communication we present evidence that an enzymatically inactive but regenerable intermediate is formed in the trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl

(1) This research was supported by grants from the National Institutes of Health.
(2) M. L. Bender, F. J. Kézdy, and J. Feder, *J. Am. Chem. Soc.*, 87, 4953 (1965).
(3) M. L. Bender and F. J. Kézdy, *ibid.*, 87, 4954 (1965).