[CONTRIBUTION FROM THE DEPARTMENT OF COLLOID SCIENCE, UNIVERSITY OF CAMBRIDGE]

A Simple Model of Molecular Specificity in Enzyme–Substrate Systems. II. The Correlation of the Michaelis Constant with the Inhibition Constant

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The enzyme-catalyzed hydrolysis of two substrates RX and RX′ are described. The ratio of hydrolysis rates differ to the extent $k >> k'$, and it is found that RX′ competitively inhibits the hydrolysis of RX so that the inhibition constant K_I' can be measured. The constants $K_I' = [E][I]/[EI]$ and $K'_M = [E][S']/[ES']$ are found to be numerically equal for the case trypsin-benzoyl-L-argininamide (where I = S′). The latter constant (K'_M) necessarily includes the rate of decomposition of enzyme–substrate complex whereas the former does not. The fact that the two constants are numerically equal implies that k'_2 (the rate of dissociation of complex to free enzyme and reactant) is much greater than k'_3 (the rate of decomposition of complex) or else that the number of stable interactions between enzyme and substrate are very limited. Evidence for the former hypothesis is given.

Introduction

In a previous paper[2] the interaction of enzyme and substrate and subsequent degradation of the substrate was treated under the assumptions: (a) interactions can be separated exclusively into those involving the reactive center (chemical forces) and the non-reactive portion of the substrate (physical forces); (b) the so-called "biological specificity" is confined to the interaction step; (c) the constant $K_M = [E][S]/[ES]$ is a true thermodynamic equilibrium constant (*i.e.*, it is independent of the rate of decomposition of ES to products. An experimental investigation relating to assumption c has now been completed.

Consider the systems

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (1.1)$$

$$E + S' \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} ES'_1 \xrightarrow{k'_3} E + P' \qquad (1.2)$$

$$K'_M = [E][S']/[ES'_1] = (k'_2 + k'_3)/k'_1$$

$$E + S' \underset{k'_b}{\overset{k'_a}{\rightleftharpoons}} ES'_2 \qquad (1.3)$$

$$K'_{eq} = [E][S']/[ES'_2] = k'_b/k'_a$$

and the condition $k_3 >> k'_3$, it being assumed that steady-state conditions apply to eq. 1.1 and 1.2 and equilibrium conditions to eq. 1.3. If the partial degradation of S to P were followed in the presence of S′, the slowly reacting substrate might function as a competitive inhibitor. For the case of a competitive inhibitor of the first system we define

$$K_I' = [E][S']/[ES']$$

and the initial rate of degradation in the presence of S′ (v_I), is given by

$$k_3[E_0]/v_I = 1 + K_M/[S_0] + K_M[S']/K_I'[S_0] \qquad (1.4)$$

thus determining K_I. (Subscript zero refers to initial concentration.)

A priori, the conditions $K_I' \leqslant K'_M$ must hold. The only restriction for the binding of the less reactive substrate is that of stability. K'_M on the other hand is a measure of complexes capable of degradation.[2] The problem of interest is to determine the degree of inequality between the two parameters K_I' and K'_M.

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(2) S. A. Bernhard, THIS JOURNAL, 77, 1966 (1955).

2. Methods

The preparation and electrometric titration kinetic methods for following the hydrolysis of benzoyl-L-argininamide (BAA) and ethyl ester (BAEE) are described in detail elsewhere.[3,4] All runs were studied in 0.2 *M* NaCl and 0.01–0.002 *M* Na_2HPO_4–KH_2PO_4 buffer, at 25° and *p*H 7.60.

K_s values were determined by the method of Lineweaver and Burk[5] from rate-concentration plots, or as shown in Fig. 1.

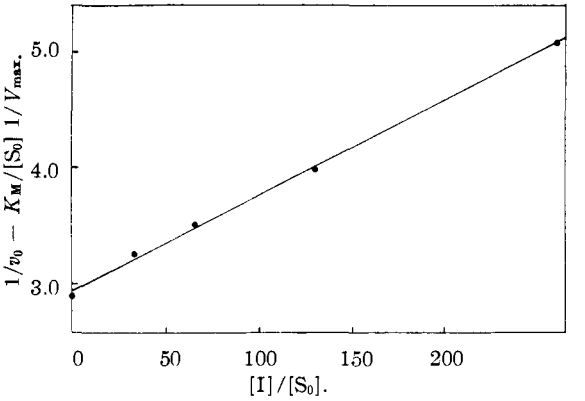

Fig. 1.—Competitive inhibition by benzoyl-L-argininamide of the trypsin-catalyzed hydrolysis of benzoyl-L-arginine ethyl ester. The plot is made on the basis of the equation

$$\frac{1}{v_0} - \frac{K_M}{V_{max.}[S_0]} = \frac{1}{V_{max.}} + \frac{1}{V_{max.}}\frac{K_M}{K'_I}\frac{[I]}{[S_0]}$$

Each point represents the average of three rate determinations. $[S_0] = 9.4, 9.4, 4.7, 2.35, 2.35 \times 10^{-4}$ *M*; [I] = 0, 3.2, 3.2, 3.2, 6.4×10^{-2} *M* (left to right): $K_I' = 3.1 \times 10^{-3}$ *M*.

3. Results and Discussion

K_I' for BAA was found to be $3.3 \pm 0.2 \times 10^{-3}$ *M*, the same value ($3.1 \pm 0.2 \times 10^{-3}$ *M*) as was obtained for K'_M.[3] $k_3^{ester} \sim 300\ k_3^{amide}$ and hence use of equation 1.4 was justifiable. We conclude (for this system) that the enzyme–substrate complex capable of degradation results from the energetically most favorable interaction. Since $K'_M = (k'_2 + k'_3)/k'_1$, the allowable alternatives are either that $k'_2 >> k'_3$ (equation 1.3) or that although the term k'_3/k'_1 contributes significantly to the value of K'_M (equation 1.2), increasing the value of K'_M and thus decreasing the relative stability of the poten-

(3) S. A. Bernhard, *Biochem. J.*, in press.

(4) S. A. Bernhard and H. Gutfreund, *Trans. Faraday Soc.*, in press.

(5) H. Lineweaver and D. Burk, THIS JOURNAL, 56, 658 (1934).

tially reactive enzyme–substrate complex, there is no other configuration of enzyme–substrate complex of greater stability. Strong arguments in favor of the former hypothesis are given below and elsewhere.[6] In any case, the possibility of a "strained" enzyme–substrate complex, or any other catalytic mechanisms such that an energetically less favorable complex leads to a more favorable decomposition rate, is excluded.

Preliminary evidence from this Laboratory has indicated that the same arguments apply for the systems α-chymotrypsin–acetyl-L-phenylalaninamide.

It has recently been brought to our attention by Dr. M. Morales[7] that K_M and K'_M for trypsin-benzoyl-L-arginine derivatives (ester and amide) must both be equilibrium constants (equal to k_2/k_1 and k'_2/k'_1, respectively). This conclusion follows from observations of the pH dependence of the hydrolysis rate (v).[8] The data correlate quantitatively with the equation

$$\frac{k_3[E_0]}{v} = \left(1 + \frac{K_M}{[S_0]}\right)\left(1 + \frac{[H^+]}{K_{EH}}\right) \qquad (3.1)$$

where K_{EH} is assumed to be the dissociation constant of EH. Morales[9] has shown that a necessary and sufficient condition for the above equation to hold is that $k_0/k_{-0} = k_2/k_1 = K_M$, where k_0 and k_{-0} refer to the process

$$EH + S \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} EHS$$

(6) H. Gutfreund, *Discs. Faraday Soc.*, "Symposium on Rapid Reaction Kinetics," in press.

(7) M. Morales, private communication.

(8) H. Gutfreund, THIS JOURNAL, in press.

(9) M. Morales, not yet published. See as well, J. Botts and M. Morales, *Trans. Faraday Soc.*, **49**, 1 (1953).

It is of interest to note the consistency in the conclusions of Morales and this paper. On the basis of apparently different criteria, the hypothesis K_M is equal to k_2/k_1 seems evident.

The above equation 3.1 appears to be valid for the system acetylcholinesterase–acetylcholine as well,[10–12] indicating that the equilibrium assumptions of the previous paper are valid.

The evidence suggests that a detailed study of the forces of interaction between the proteolytic enzymes and amino acid derivatives (as in Paper I of this series) might be fruitful. It is of interest to note that the differences in K_M among acetyl-L-phenylalaninamide, acetyl-L-tyrosinamide and acetyl-L-tryptophanamide or among the corresponding nicotinyl derivatives[13] can be correlated with the dispersion energy differences calculated by the method of the previous paper.

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(10) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **186**, 683 (1950).

(11) K. J. Laidler, *Trans. Faraday Soc.*, in press (1954).

(12) M. Dixon, *Biochem. J.*, **55**, 161 (1953).

(13) H. T. Huang, R. J. Foster and C. Niemann, THIS JOURNAL, **74**, 105 (1952).

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Magnetic Catalysis of Decarboxylation and Other Reactions[1]

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The effect of rare earth ions on the rate of decarboxylation of phenylmalonic acid in aqueous solution is reported. It is found that at 0.5 M, the highly paramagnetic dysprosium ion accelerates the rate by about 10% as compared to the results for the diamagnetic ions of lanthanum, yttrium and lutetium. Exploratory measurements indicate no magnetic catalysis for the non-enzymatic hydrolysis of urea but a possibly positive effect for the isomerization of maleic acid. Additional comments are included with respect to isotope effects on the rate of the decarboxylation reaction.

Recently we reported[2] preliminary results indicating a positive catalytic effect of dysprosium ion on the decarboxylation of phenylmalonic acid in aqueous solution. We are now presenting our final results on this reaction together with exploratory work on two other reactions.

Our interest was aroused in this subject by the peculiar results which had been reported on the effects of C^{13} and C^{14} on the rate of decarboxylation of malonic and related acids. This work has been the subject of several recent reviews.[3] The decrease in the rate on substitution of C^{14} for C^{12} in the carboxyl group is reported in most cases to be substantially greater than twice the decrease on C^{13} substitution. Such results cannot be explained as effects of change of mass alone. Since C^{13} has a nuclear spin and magnetic moment, while both C^{12} and C^{14} have zero values for these quantities, it seemed desirable to investigate the possible effects of the inhomogeneous magnetic field that such a magnetic moment would cause. Certain rare earth ions have

(1) This research was assisted by the American Petroleum Institute through Research Project 50. The dysprosium and lutetium were kindly made available to us by Dr. F. H. Spedding.

(2) K. S. Pitzer and E. Gelles, THIS JOURNAL, **75**, 5132 (1953).

(3) (a) P. E. Yankwich, *Ann. Rev. Nuclear Sci.*, **3**, 235 (1953); (b) H. G. Thode, *Ann. Rev. Phys. Chem.*, **4**, 95 (1953); (c) J. Bigeleisen, *J. Phys. Chem.*, **56**, 823 (1952).