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The Kinetic Consequences of the Acyl–Enzyme Mechanism for the Reactions of Specific Substrates with Chymotrypsin[1,2]

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Indirect evidence for the formation of an acyl–enzyme intermediate in the α-chymotrypsin-catalyzed hydrolysis of specific substrates is presented, as are many of the implications of the acyl–enzyme mechanism. The dichotomy of meaning of the apparent Michaelis constant, K_m(app), and the catalytic rate constant, k_{cat}, in these reactions, because of the two bond-breaking steps, is discussed. Literature data indicate that while K_m(app) of specific amide substrates is a true binding constant, K_m(app) of specific ester substrates is a combination of an equilibrium constant and rate constants. Furthermore, k_{cat} of specific amide substrates is controlled by the acylation step, k_2, while k_{cat} of specific ester substrates is controlled by the deacylation step, k_3. A family of substrates containing the same acylamino acid moiety but having differing inherent reactivity toward nucleophiles may show identical k_{cat}'s in α-chymotrypsin reactions; such a phenomenon may be used as a strong argument for the postulation of an acyl–enzyme intermediate. The determination of k_2/k_3 ratios and of the individual constants k_2 and k_3 from k_{cat} and K_m(app) is discussed. Examples of an inverse relationship between k_{cat} and K_m(app) in a given family of reactions are noted. The partitioning of the acyl–enzyme intermediate by water and an added nucleophile can account for various transfer reactions. The identical partitioning from two different substrates of a common acid moiety may be used as a strong argument to postulate acyl–enzyme intermediate formation, if it is shown that the two substrates give different partitionings in nonenzymatic reactions. The presence of the acyl–enzyme intermediate leads to the possibility of an additional (noncompetitive) inhibition through combination of the inhibitor with the acyl–enzyme. Both the activation parameters and the pH dependence of the catalytic rate constant may be complex because of the complex nature of the catalytic rate constant. Most of these considerations will be tested in the accompanying papers.

Introduction

Previous papers from this laboratory on the mechanism of α-chymotrypsin-catalyzed hydrolyses have concentrated on the observation and description of the individual steps of the reaction through the use of nonspecific (kinetically poor) substrates which possess two characteristics: (1) a good leaving group so that the rate constant of acylation is greater than that of deacylation, and (2) a highly conjugated structure so that the extinction coefficient of the substrate is large and is perturbed significantly by attachment to the enzyme.[3] Using this approach it was possible to observe and characterize the acylation and deacylation steps and also the acyl–enzyme intermediate in a number of α-chymotrypsin-catalyzed hydrolyses.

In the present series of papers investigations of the α-chymotrypsin-catalyzed hydrolyses of specific (kinetically good) substrates are described. In general, it is not possible to observe the individual steps of acylation and deacylation in the hydrolyses of specific substrates because the two requirements mentioned above are not met; furthermore, the reactions of specific substrates are so exceedingly fast that, in contrast to the slow reactions of nonspecific substrates, instrumentation is not available to detect the individual steps of the reaction. Therefore, it is often necessary to resort to indirect arguments to detect and characterize the individual steps of the reaction.

One of the primary goals of these investigations is to determine whether an acyl–enzyme intermediate is formed in the α-chymotrypsin-catalyzed hydrolysis of specific substrates, as was demonstrated earlier for nonspecific substrates. The garnering of evidence for intermediate formation may be approached in many ways.[4] The most satisfying method is the isolation of the intermediate from the reaction mixture and proof of its subsequent transformation to give the reaction products. The next most desirable method is the detection of transient intermediates by the observation of some physical property associated with the intermediate. But most evidence for transient intermediates is completely indirect in nature, stemming from the chemical properties of the system such as the kinetics, the effect of structure on reactivity, or various chemical consequences such as the product distribution, the stereochemistry of the products, or isotopic results.[4] In the present paper the basis of the indirect kinetic evidence for acyl–enzyme intermediate formation will be presented and analyzed.

The Acyl–Enzyme Hypothesis.—In 1950, Wilson, Bergmann, and Nachmansohn proposed that an acyl–enzyme intermediate was formed in acetylcholinesterase-catalyzed hydrolyses.[5] This suggestion was followed by similar suggestions for α-chymotrypsin, which have been discussed in detail previously.[3] The equation for an enzyme-catalyzed hydrolysis involving an acyl–enzyme intermediate is shown in eq. 1. This

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

formulation includes ES, the enzyme–substrate complex; ES′, the acyl–enzyme; and P_1 and P_2, the alcohol and acid portions of an ester substrate, respectively.[6]

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

(2) Paper XXVII in the series: The Mechanism of Action of Proteolytic Enzymes.

(3) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962).

(4) M. L. Bender in "Techniques of Organic Chemistry," 2nd Ed., A. Weissberger, Ed., Vol. VIII, Part 2, John Wiley and Sons, Inc., New York, N. Y., 1963, Chapter XXV.

(5) I. B. Wilson, F. Bergmann, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950).

(6) F. J. Kézdy, G. E. Clement, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3690 (1964).

In this and subsequent discussions, the substrate binding constant, K_s, of α-chymotrypsin-catalyzed reactions is treated as a true equilibrium constant. All investigations so far indicate that for α-chymotrypsin reactions this is a true equilibrium binding constant (k_{-1}/k_1) rather than a steady state constant $(k_2 + k_{-1})/k_1)$.[7]

Measurements of the reaction of α-chymotrypsin with benzoyl-L-tyrosine ethyl ester indicate that k_1 is $\geq 2 \times 10^6\ M^{-1}$ sec.$^{-1}$ [8]; furthermore, k_1 for the formation of enzyme–substrate complexes is generally between 10^6 and $10^8\ M^{-1}$ sec.$^{-1}$.[9] For α-chymotrypsin reactions, K_s's for amides vary from 10^{-2} to $10^{-3}\ M$. If in α-chymotrypsin reactions, $k_1 = 10^8\ M^{-1}$ sec.$^{-1}$, and if K_s is a steady-state constant as defined above, $k_2 + k_{-1}$ must be equal to 10^5–10^6 sec.$^{-1}$ which is higher than any recorded rate constant for k_2 by several orders of magnitude[10]; thus one can be certain that K_s is indeed an equilibrium constant. Several experiments in our laboratory confirm this conclusion: (1) the presteady state (k_2 step) does not exhibit an induction period which must exist if a steady state is achieved after a finite period of time; (2) the values of true (reversible) inhibitor constants are neither pH dependent nor time dependent; and (3) K_s is essentially independent of pH whereas k_2 is highly pH dependent.

The formulation of the acyl–enzyme mechanism (eq. 1) should be compared with the usual Michaelis–Menten scheme of an enzymatic process shown in eq. 2. In the latter equation, ES, P_1, and P_2 have the same

$$E + S \underset{}{\overset{K_m(app)}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (2)$$

meanings as above, but K_m(app) is not identical with K_s nor, of course, is k_{cat} identical with k_2 or k_3. The relationship between the constants of eq. 1 and 2 has been pointed out before.[11–13]

The relationship between K_s of eq. 1 and K_m(app) of equation 2 is

$$K_m(app) = (k_3/(k_2 + k_3))K_s \qquad (3)$$

The relationship between the rate constants, k_2 and k_3, of eq. 1 and the catalytic rate constant, k_{cat}, of eq. 2 is

$$k_{cat} = k_2k_3/(k_2 + k_3) \qquad (4)$$

Equations 1–4 lead to a considerable number of kinetic consequences which may be used to test the fit of the reactions of specific substrates to the acyl–enzyme hypothesis. Different kinetic results may be obtained depending on the rate-determining step of the reaction. Two limiting cases may be defined. If $k_2 \gg k_3$, then

$$K_m(app) = (k_3/k_2)K_s \qquad (5)$$

and

$$k_{cat} = k_3 \qquad (6)$$

whereas if $k_3 \gg k_2$, then

$$K_m(app) = K_s \qquad (7)$$

and

$$k_{cat} = k_2 \qquad (8)$$

This dichotomy of meaning of the kinetic constants can and does have far reaching meaning in the interpretation of mechanism of α-chymotrypsin-catalyzed reactions.

Kinetic Consequences of the Acyl–Enzyme Hypothesis. Michaelis Constants.—Let us first consider the comparison shown in Table I of the inhibition constants of D-esters and corresponding D-amides, which of course must be true binding constants. The K_i's of the D-ester and D-amide inhibitors are quite similar to one another. The ratio K_i (amide)/K_i (ester) is, furthermore, constant to within a factor of two for all three pairs of inhibitors. It thus appears that the leaving group of the molecule contributes relatively little to binding and that the over-all binding is largely determined by the amino acid moiety.

TABLE I

INHIBITION CONSTANTS OF SOME D-INHIBITORS OF α-CHYMOTRYPSIN

Inhibitor, N-acetyl-	K_i of amide, mM	K_i of ester, mM	K_i^A/K_i^E	Ref.
D-Tyrosine	12 ± 2	5 ± 1[a]	2.4	14, 15
D-Phenylalanine	12 ± 3	2.4 ± 0.4[c]	5.0	14, 15
D-Tryptophan	2.3 ± 0.4	0.8 ± 0.2[b]	2.9	14, 15

[a] Ethyl ester. [b] Isopropyl ester. [c] Methyl ester.

In contrast to the similarity of the inhibition constants of D-amide and D-ester pairs, the K_m(app) of a specific L-amide substrate is quite different from that of the corresponding specific L-ester substrate (noted in Table II).

TABLE II

K_m(APP) OF SPECIFIC AMIDE AND ETHYL ESTER SUBSTRATE PAIRS IN α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS

Substrate, N-acetyl-	K_m(app) of amide, mM	K_m(app) of ester, mM	K_m^A/K_m^E	Ref.
L-Tyrosine	34	0.7	46	16, 17
L-Phenylalanine	31	1.2	26	16, 18
L-Tryptophan	5	0.09	55	16, 17

These large differences in K_m(app) of the L-ethyl ester and L-amide components of a pair cannot be explained in terms of differing inherent binding ability of the pair, since the specificity is reasonably well defined in terms of the interaction of the common acid portion of the molecule with the enzyme surface, and since the ethyl ester and amide groups both contain similar electronegative atoms, as indicated by the similarities shown by the D-amide and D-ester pairs discussed above.[19]

If binding differences are not the explanation, what is the explanation of the differences in Table II? As noted in the previous section, the acyl–enzyme hypothesis predicts that K_m(app) of a specific substrate may have an entirely different meaning, depending on whether k_2 or k_3 is the rate-determining step of the particular reaction. On this basis the most straightforward explanation of the data of Table II is that the (real) K_s's of the L-ester and L-amide differ by only a

(7) H. Gutfriend, *Discussions Faraday Soc.*, **15**, 167 (1955).
(8) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).
(9) M. Eigen, *Pure Appl. Chem.*, **6**, 97 (1963).
(10) B. Zerner, R. P. M. Bond, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3674 (1964).
(11) I. B. Wilson and E. Cabib, *J. Am. Chem. Soc.*, **78**, 202 (1956).
(12) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).
(13) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, **81**, 5907 (1959).
(14) R. J. Foster, H. J. Shine, and C. Niemann, *ibid.*, **77**, 2378 (1955).
(15) R. J. Foster and C. Niemann, *ibid.*, **77**, 3370 (1955).
(16) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).
(17) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).
(18) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1956).
(19) The differences in K_m(app) of an ethyl ester and amide pair have been attributed to differences in the true binding constant of the ethyl ester and amide due to the added interaction of the C_1H_3 group of the former compound; G. E. Hein and C. Niemann, *J. Am. Chem. Soc.*, **84**, 4487 (1962).

small factor, and that the values of K_m(app) of the ester and its corresponding amide are different because of differing ratios ($k_3/(k_2 + k_3)$) in the two reactions.

The analogy between binding of D-substrates and of L-substrates, for the amides at least, is supported by experimental evidence: the binding is of the same order of magnitude, and the changes from one family of compounds to another are similar. Thus it is surprising that K_i(D-ester)/K_m(app)(L-ester) = 7 whereas K_i(D-amide)/K_m(app)(L-amide) = 0.42. These considerations strongly suggest a similar two-point binding for D- and L-substrates and further that the observed K_m(app) values are best interpretable in terms of possible perturbation of the true K_s by the ratio of rate constants shown in eq. 3.

One implication of the previous discussion is that the observed K_m(app) of a specific amide substrate is the real K_s, that is, a true binding constant. Certainly the concordance between the K_i's of the D-amides (which are true binding constants) and the K_m(app)'s of the L-amides is an indication that the latter are also true binding constants. Furthermore, the fact that the catalytic rate constant of a specific amide substrate is approximately one-thousandth that of the corresponding specific ester substrate means that the inherent reactivity of an amide toward nucleophilic attack is being seen, implying that for a specific amide substrate the decomposition of ES is the rate-determining step of the reaction and thus that K_m(app) = K_s.[20] Finally, on the basis of eq. 1, of the fact that for an amide substrate ES is an amide, and of the hypothesis that the intermediate ES' is an ester for all substrates,[3] it may be predicted that k_2 must be much smaller than k_3 (because of the relative reactivity of amides and esters), and thus reactions of specific amide substrates can be characterized by eq. 7, K_m(app) = K_s, and eq. 8, $k_{cat} = k_2$.

As noted above, the observed k_m(app)'s of specific ester and amide substrate pairs differ greatly from one another, although one would expect that the binding of such a pair should be similar. Furthermore, the K_m(app) of a specific amide substrate was postulated above to be the real K_s. These two arguments then require that the observed K_m(app)'s of specific ester substrates are not true binding constants, but are in fact binding constants which are perturbed by rate constants according to eq. 3. The ratios of K_m^A(app)/K_m^E(app) for specific substrates of chymotrypsin vary from 26 to 55 (Table I); it is suggested that a goodly part of these ratios is due to the perturbation of the real K_s by the ratio of rate constants ($k_3/(k_2 + k_3)$). This ratio must then be less than one for specific ester substrates, for only then can K_m^E(app) be less than K_s^E which must approximate K_s^A. If, in fact, the ratio ($k_3/(k_2 + k_3)$) is less than one-half, then k_3 must be less than k_2; in other words, deacylation must be the rate-determining step for specific ester substrates, and thus the reactions of specific ester substrates must be characterized kinetically by eq. 5 and 6.[21]

Relative Rate Constants.—Rate effects brought about by remote substituents on discrete acylation and deacylation steps of chymotrypsin reactions lead to a description of both steps as involving nucleophilic attack on a carboxylic acid derivative substrate.[22,23] One then might superficially expect that the catalytic rate constants (k_{cat}) for specific substrates of chymotrypsin would reflect the relative reactivity of the substrates toward nucleophiles such as hydroxide ion. This result is sometimes seen, but there are many exceptions to this conclusion—some very flagrant exceptions.

The rigorous view, of course, must be derived from a consideration of eq. 4, with the aid of eq. 6 and 8, which describe various ramifications of the catalytic rate constant. If acylation is rate determining, the catalytic rate constant will describe k_2 and the relative reactivity of the original substrate toward nucleophiles will be measured. However, if deacylation is rate determining, the catalytic rate constant will described k_3; this constant will no longer measure the reactivity of the original substrate, but rather that of the acyl–enzyme intermediate. Thus when the catalytic rate constant of a series of related substrates containing the same amino acid moiety is observed, two limiting results can be obtained: (1) the relative reactivity of the series of substrates toward nucleophiles will be completely evident, indicating that the reactions of at least most of the series are controlled by the acylation step, k_2; and (2) the relative reactivity of the series of substrates toward nucleophiles will be totally absent, indicating that the reactions of the series are controlled by the deacylation step, k_3. The latter situation is of great importance since it can be used as a strong argument to postulate intermediate formation.[10]

Calculation of the Ratio k_2/k_3.—Ordinarily the information available from the kinetics of an enzymatic hydrolysis of a specific substrate consists solely of the catalytic rate constant, k_{cat}, and the apparent Michaelis constant, K_m(app). From this information alone it is not possible to determine the ratio k_2/k_3. One rearrangement of eq. 3 and 4 in terms of the ratio k_2/k_3 leads to

$$k_2/k_3 = (K_s/K_m(\text{app})) - 1 \qquad (9)$$

indicating that the ratio k_2/k_3 may be calculated using the observables, k_{cat} and K_m(app), together with *one* additional piece of information, K_s.

Let us first consider the application of eq. 9 in the calculation of k_2/k_3 for specific ester substrates. K_s of the ester may be estimated by using the hypothesis that for an amide substrate K_s = K_m(app) (*vide supra*) and further that the K_s's of an amide and ester substrate pair differ by a constant close to unity; that is, $K_s^E = cK_s^A$ The value of c may be evaluated from the data of Table I, using the corresponding D-compounds. It appears from Table I that the K_i's of a D-amide–ester pair differ by a factor of 2.4 to 5. As a first approximation, then, which is probably correct to within a factor of two, we shall assume that the relationship found for D-inhibitors of a given amino acid derivative applies as well to the L-substrates, *e.g.*, $K_s^E = K_s^A/2.4$ for the derivatives of N-acetyl-L-tyrosine. Using eq. 9 and the ratios of K_s^A/K_s^E taken from the respective inhibitor ratios of Table I, the results shown in Table III were calculated. A considerable variation in the estimated K_s will still lead to a result

(20) Gutfreund and Sturtevant (ref. 8) have made a similar argument.

(21) *Cf.* I. B. Wilson in "The Enzymes," 2nd Ed., Vol. 4, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, Inc., New York, N. Y., 1960, p. 511.

(22) M. L. Bender and K. Nakamura, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

(23) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962).

which indicates that acylation is considerably faster than deacylation for these three specific substrates. This is perhaps a surprising result, and certainly is one that contradicts a widely held conclusion.[24]

TABLE III

CALCULATED k_2/k_3 RATIOS FOR SPECIFIC ESTER SUBSTRATES OF α-CHYMOTRYPSIN

Substrate, N-acetyl-	K_m(app), mM	$K_s = K_s^A/c$,[a] mM	k_2/k_3
L-Tyrosine ethyl ester	0.7	14.2	18
L-Phenylalanine ethyl ester	1.2	6.2	4
L-Tryptophan ethyl ester	0.09	1.73	19

[a] K_s^A is taken from Table II and c is taken from Table I.

TABLE IV

KINETICS OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF DERIVATIVES OF N-ACETYLGLYCINE[a]

Substrate, ester	$k_{cat.}$, sec.$^{-1}$	K_m(app), mM	k_2, sec.$^{-1}$	k_3, sec.$^{-1}$	K_s, mM	Ref.
Ethyl	0.013	96	*0.013*[b]	*2.29*	*96*	25
Methyl	0.013	30.7	*0.013*	*2.29*	*30.7*	26
p-Nitrophenyl	1.88	0.396	10.6	2.29	2.23	27

[a] In aqueous solutions at 25.0 ± 0.1°, pH 7.90, and 0.50 M with respect to sodium chloride. [b] Calculated values are shown in italics.

the nonenzymatic nucleophilic reactions of these substrates. Considering reactions of two such compounds, A and B, a set of three equations in three unknowns will then be applicable. These three equations consist of: (1) $k_{cat}^A = k_2^A k_3/(k_2^A + k_3)$; (2) $k_{cat}^B = k_2^B k_3/(k_2^B + k_3)$; and (3) $k_2^A/k_2^B = c$ where c is the ratio of rate constants of the respective nonenzymatic nucleophilic reactions of A and B.

If one k_3 can be experimentally determined or estimated for such a set of reactions, that k_3 must of course be applicable to all members of the set. By knowing the k_3, it is then possible to determine k_2 for all reactions using eq. 4. One method to determine k_3 is to use a labile substrate such as a *p*-nitrophenyl ester, making it possible to separate kinetically the two steps of acylation and deacylation or alternatively to observe solely the (slower) deacylation step.

The kinetics of a series of α-chymotrypsin-catalyzed hydrolyses of derivatives of N-acetylglycine, shown in Table IV, has been completely analyzed in terms of the rate constants of individual steps, because it is possible to determine experimentally k_2 and k_3 of N-acetylglycine *p*-nitrophenyl ester using a stopped-flow mixing device.

TABLE V

KINETICS OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF DERIVATIVES OF HIPPURIC ACID[a]

Derivative	$k_{cat.}$, sec.$^{-1}$	K_m(app), mM	k_3, sec.$^{-1}$	Rel. k_{OH^-} of[b] acetate, M^{-1} sec.$^{-1}$	k_{OH^-} of benzoate × 10^3,[c] M^{-1} sec.$^{-1}$	K_s, M	Ref.
Amide		12.5				12.5	31
Methyl ester	0.143	2.40	0.192	1.0	9.01	3.22	29
Methyl ester	.125	3.23	.160	1.0	9.01	4.14	28
Ethyl ester	.098	2.31	.118	0.601	2.87	2.78	28
Propyl ester	.127	1.90	.163	.549	1.93	2.44	28
Isopropyl ester	.050	2.3	.055	.146	4.64	2.52	29
Isopropyl ester	.048	2.05	.052	.146	4.64	2.24	28
Butyl ester	.173	1.11	.248			1.60	28
Isobutyl ester	.166	2.4	0.234		1.43	3.38	29
Choline bromide ester	.535	0.60	~7.7			~8.7	29
Homocholine bromide ester	.433	1.2	1.8			4.98	29
4-Pyridinylmethyl ester	.567	0.092	∞				29
p-Nitrophenyl ester	~ .500[d]	0.088					30

[a] In aqueous solution at pH 7.0 and 25.0 ± 0.1°. [b] L. P. Hammett "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 211. [c] "Tables of Chemical Kinetics. Homogeneous Reactions," National Bureau of Standards Circular 510; 25°, 56% acetone–water. [d] Extrapolated to pH 7.0 and aqueous solution from pH 7.8 and 15% acetone–water.

Calculation of Individual Rate Constants.—A consequence of the previous discussion is that it is possible to calculate individual acylation and deacylation rate constants from over-all catalytic constants under certain conditions. Two approaches will be presented.

If one determines a set of two or more catalytic rate constants for enzymatic hydrolyses using related substrates which contain the same acyl amino acid moiety but different leaving groups, the compounds must possess identical deacylation, k_3, constants but differing acylation, k_2, constants. The k_2's should be related to one another according to the known relative rates of

The interesting conclusion of Table IV is that acylation is the rate-determining step in the hydrolysis of the methyl and ethyl esters of N-acetylglycine whereas previous consideration of the hydrolysis of the ethyl esters of N-acetyl-L-tyrosine, phenylalanine, and tryptophan indicated that deacylation is the rate-controlling step.

A more extensive set of experimental data concerning the kinetic constants of hippuramide and ten esters of hippuric acid is available from the literature and this laboratory as shown in Table V. An inspection of the catalytic rate constants indicates a spread of about tenfold with the choline bromide, 4-pyridinylmethyl, and *p*-nitrophenyl esters exhibiting almost identical and maximal rate constants in this series. On the basis of this identity of maximal values, it is assumed that the latter three compounds proceed with the deacylation step rate-controlling. Of these three compounds, the 4-pyridinylmethyl ester was selected as that compound whose k_3 was most nearly rate controlling, and it was assumed that k_3 for this entire series of compounds

(24) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959), concluded that both acylation and deacylation were equally rate controlling in the α-chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosine ethyl ester.

(25) J. P. Wolf, III, and C. Niemann, *Biochemistry*, **2**, 493 (1963).

(26) J. P. Wolf, III, and C. Niemann, *ibid.*, **2**, 82 (1963).

(27) Unpublished observations of Dr. R. J. Thomas in this laboratory.

(28) G. H. Nelson, J. L. Miles, and W. J. Canady, *Arch. Biochem. Biophys.*, **96**, 545 (1962).

(29) R. M. Epand and I. B. Wilson, *J. Biol. Chem.*, **238**, 1718 (1963).

(30) Unpublished observations of Dr. B. Zerner.

(31) R. J. Foster and C. Niemann, *J. Am. Chem. Soc.*, **77**, 3371 (1955).

corresponded to the catalytic rate constant of this compound, 0.567 sec.$^{-1}$. Using this one assumption, the acylation rate constants, k_2, and the Michaelis constants, K_s, were calculated for the entire set of compounds. One interesting result of these calculations is that the calculated K_s's of the simple alkyl esters, such as the methyl ester, ethyl ester, and propyl ester, are invariably less than the (experimentally measured) K_s of the amide. In a crude way, it can be seen that K_s(amide) = 3 to 5 K_s(ester). This observation is an important corroboration of the premise made earlier that K_s(amide) = 2.4 to 5.0 K_s (ester) for various specific substrates of α-chymotrypsin, on the basis of a comparison of the K_i's of D-inhibitors. Another result of these calculations is that the k_2's of the simple alkyl esters parallel the saponification rates of the corresponding acetates or benzoates in a crude way, except for the butyl esters.

The Relationship between k_{cat} and K_m(app).—Equation 3 predicts an inverse relationship between k_{cat} and K_m(app) for a series of substrates of the same family. This relationship can be seen in the rearranged form of eq. 3

$$K_m(\text{app}) = K_s/((k_2/k_3) + 1)$$

In a given family of substrates (in which the amino acid moiety is constant), previous experience indicates that K_s is essentially invariant, and of course in a given family having a common acyl–enzyme, k_3 is invariant. Under these circumstances an increase in k_2 will produce an increase in k_{cat} but will produce a decrease in K_m(app) leading to an inverse relationship between k_{cat} and K_m(app) in many families of reactions of chymotrypsin, such as those shown in Tables IV, V, and VI. This argument is restricted to conditions in which neither k_2/k_3 nor k_3/k_2 is negligible with respect to 1.

TABLE VI

THE RELATIONSHIP BETWEEN k_{cat} AND K_m(APP) IN THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF A FAMILY OF N-ACETYL-L-VALINE COMPOUNDS[32]

Substrate	K_m(app), mM	k_{cat}, sec.$^{-1}$
Amide	250	Too slow
Isopropyl ester	177	0.081
Ethyl ester	110	.13
Methyl ester	117	.15
β-Chloroethyl ester	19	.22

The Ratio k_{cat}/K_m(app).—The division of eq. 4 by 3 leads to eq. 10.

$$k_{cat}/K_m(\text{app}) = k_2/K_s \qquad (10)$$

This equation has interesting implications for reactions in which k_3 is rate controlling. For such reactions, it is possible to determine the observable k_{cat} and K_m(app) and then to use these quantities to calculate k_2/K_s, which under certain circumstances can be used in place of k_2. For example, suppose one wishes to know the effect of some external variable, such as pH or an added nucleophile, on k_3 and k_2. The effect of such a variable on k_3 can be determined directly from its effect on k_{cat} if indeed k_3 is the rate-determining step of the reaction. The effect of the variable on k_2 of the same reaction can then be found by observing its effect on k_{cat}/K_m(app) = k_2/K_s, assuming that its effect on K_s is nil.

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Partitioning of the Acyl–Enzyme Intermediate.—Equation 1 tacitly omits an important factor in the chymotrypsin reaction, namely that every hydrolytic reaction involves a molecule of water. Since it is impossible to vary the concentration of water in water for kinetic purposes, it is convenient to add an extra nucleophile. The introduction of a second nucleophile such as alcohol or an amine will result in a system following eq. 11, which indicates a partitioning of the acyl–enzyme intermediate by water and the added nucleophile

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \begin{matrix} ES' \\ + \\ P_1 \end{matrix} \begin{matrix} \xrightarrow{k_3(H_2O)} E + P_2 \\ \xrightarrow{k_4(N)} E + P_3 \end{matrix} \qquad (11)$$

One implication of the partitioning of an acyl–enzyme intermediate by water and an added nucleophile is that an identical partitioning ratio should be observed for two substrates which may be quite different in inherent reactivity but which produce the same acyl–enzyme. For example, the acetylcholinesterase-catalyzed reactions of both ethyl acetate and acetylcholine in the presence of 0.5 M hydroxylamine produced 10% acetohydroxamic acid and 90% acetic acid.[33] Furthermore, in the α-chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of ten hippuric acid esters of varying reactivity, the same fraction of hydroxamic acid was formed.[34] These results are consistent with the acyl–enzyme hypothesis, and in particular with eq. 11.

A necessary and sufficient condition for the exclusion of the intermediacy of an acyl–enzyme is the lack of adherence to the rule of identical partitionings discussed above. It was suggested that such a situation exists in the α-chymotrypsin-catalyzed hydrolysis and hydroxylaminolysis of N-acetyl-L-tyrosine hydroxamic acid.[35]

Equation 11 leads to kinetic equations describing dP_1/dt, dP_2/dt, and dP_3/dt. These equations may be analyzed in terms of the effect of the concentration of the added nucleophile on the catalytic rate constant, which has a different meaning, depending on whether P_1, P_2, or P_3 is being observed, and whether k_2 or k_3 is the rate-determining step of the reaction.[36]

Inhibition.—The postulation of an acyl–enzyme intermediate in the reaction increases the possibilities of inhibition of the reaction. The simplest inhibition of course is a competition between inhibitor and substrate for the native enzyme, resulting in what might be called inhibition at the enzyme–substrate level. However, with the addition of an acyl–enzyme to the mechanism, the inhibitor may conceivably combine with the acyl–enzyme and depress the rate of the deacylation step. This is especially true in acetylcholinesterase-catalyzed reactions since this acyl–enzyme has lost the specificity of the quaternary nitrogen group in the expelled leaving group. In acetylcholinesterase-catalyzed reactions, the noncompetitive component noted in the reversible inhibition produced by substituted ammonium ions has been attributed to combination of the inhibitor with the acyl–enzyme, leading

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to the inhibition of the deacylation step.[37,38] Furthermore, the substrate inhibition seen in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine has also been attributed to the same phenomenon.[11,39] Although these phenomena are also theoretically possible with α-chymotrypsin, they will not likely be observed because the acyl–chymotrypsin intermediate derived from a specific substrate preserves essentially all of the original specificity, resulting in blockage of the active site toward a foreign inhibitor.

Activation Parameters.—Since the catalytic rate constant is a complex constant, the activation energy of the catalytic rate constant may not be a constant independent of temperature. If, for example, the temperature dependencies of k_2 and k_3 are different from one another, a change in rate-determining step with temperature may occur. Such a phenomenon has apparently been observed in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine,[11] and in the α-amylase-catalyzed hydrolysis of amylose.[40] No report of such a phenomenon in an α-chymotrypsin-catalyzed hydrolysis is known.

pH Dependence.—If indeed the catalytic rate constant is a complex constant, the pH dependence of this quantity may also be a complex function. This is especially true if the pH dependence of k_2 is different from that of k_3. If this is the case, the pH dependence of the reaction of a specific substrate whose rate-determining step is k_2 will differ from that of a specific substrate whose rate-determining step is k_3. A perusal of the literature indicates this may indeed be the case. For example, the pH–k_{cat} profiles of the specific ester substrates, N-acetyl-L-tryptophan ethyl ester and N-acetyl-L-tyrosine ethyl ester, whose rate-determining steps are postulated above to be deacylation, k_3, are sigmoid curves[41]; on the other hand, the pH-rate profiles (at a S_0/K_s ratio of 2.0 to 3.7 which approaches conditions of maximal velocity and therefore k_{cat}) of the specific amide substrates N-acetyl-L-tryptophan amide and N-nicotinyl-L-tryptophan amide, whose rate-determining steps are postulated above to be acylation, k_2, are bell-shaped curves.[42] Furthermore, if the pH dependencies of the two steps are different from one another, a change in rate-determining step with pH might be found at some point. Finally, if the pH dependencies of k_2 and k_3 are different from one another, and if the catalytic rate constant of specific substrates is a complex function of both steps, the pH dependence of the catalytic rate constant will consist of a family of curves determined by the relative contribution of each step in the over-all catalytic rate constant.[43]

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Kinetic Evidence for the Formation of Acyl–Enzyme Intermediates in the α-Chymotrypsin-Catalyzed Hydrolyses of Specific Substrates[1,2]

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The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl, methyl, and *p*-nitrophenyl esters and of N-acetyl-L-phenylalanine ethyl, methyl, and *p*-nitrophenyl esters was determined. The catalytic rate constants of the three tryptophan reactions are practically identical with one another, as are the catalytic rate constants of the three phenylalanine reactions. Ethyl, methyl, and *p*-nitrophenyl esters would be expected to show widely different rates in chymotrypsin reactions which are nucleophilic in character. The identity of each set of catalytic rate constants may therefore only be explained in terms of a rate-determining decomposition of a common intermediate. This intermediate may be most simply identified as N-acetyl-L-tryptophanyl-α-chymotrypsin in the first set of reactions and N-acetyl-L-phenylalanyl-α-chymotrypsin in the second set of reactions. Using the hypothesis that the *p*-nitrophenyl ester reaction proceeds with deacylation completely rate controlling, or using a relationship between the relative K_m(app)'s of these and other compounds, individual rate constants for acylation and deacylation are calculated. The relative k_2's (acylation) are in good agreement with the nucleophilic order of the corresponding carboxylic acid derivative as measured with hydroxide ion.

Introduction

One of the predictions of the kinetic analysis of the previous paper[4] concerns the relative catalytic rate constants of the α-chymotrypsin-catalyzed hydrolysis of a family of compounds having a common acylamino acid moiety but different leaving groups. This prediction is based on the postulate of an acyl–enzyme intermediate in α-chymotrypsin reactions[3,4] (eq. 1) and the nucleophilic character of both the acylation and deacylation reactions.[5,6] The combination of these two hypotheses

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

predicts that the catalytic rate constants of those reactions whose rate-determining step is acylation, k_2, will exhibit the full relative nucleophilic reactivity of the various compounds, whereas the catalytic rate constants

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