The α-Chymotrypsin Catalyzed Hydrolysis of Several Esters of Acetyl-L-Norvaline*

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Received June 4, 1962

The kinetics of the α-chymotrypsin catalyzed hydrolysis of the methyl, β-chloroethyl, and isopropyl esters of acetyl-L-norvaline (α-acetamido-*n*-valeric acid) have been determined in aqueous solutions at 25.0°, pH 7.90 and 0.1 M in sodium chloride. Under these conditions the reaction follows the rate equation $-d[S]/dt = k_0[E][S]/(K_0 + [S])$. Comparison of the constants k_0 and K_0 obtained with those for the corresponding valine derivatives leads to the conclusion that interaction of the carboalkoxy group of the substrate with its complementary locus at the active site of the enzyme is important in the binding process and that this interaction is subject to steric hindrance by *beta*-branching of either the side-chain or ester component.

In an earlier communication (Waite and Niemann, 1962) it was shown that the rate of the α-chymotrypsin catalyzed hydrolysis of a series of acylated-L-valine esters was described by equation 1

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S])$$

(1)

when these reactions were examined in aqueous solutions at 25.0°, pH 7.90 and 0.10 M in sodium chloride. However, when the constants k_0 and K_0 evaluated for each substrate were compared with those for the corresponding alanine and leucine derivatives, *cf.* Table I, it was concluded that the hydrolysis of the valine derivatives was being hindered by a structural feature of the substrate molecule, *viz.*, the isopropyl side-chain. The data indicated that a secondary carbon atom in the four position from the carbonyl oxygen atom of the ester group was instrumental in determining the magnitude of the dissociation constant of the enzyme-substrate complex as well as its rate of decomposition into products. It was predicted that, "when the kinetic constants for the methyl ester of acetyl-L-norvaline are determined, the value of K_0 will be found to be smaller and that of k_0 larger than those of the corresponding valine derivative" (Waite and Niemann, 1962). It is the purpose of this communication to describe a set of experiments which confirm the above prediction and provide further information about the structural specificity of α-chymotrypsin.

EXPERIMENTAL

The methyl, β-chloroethyl, and isopropyl esters

of acetyl-L-norvaline (α-acetamido-*n*-valeric acid) were prepared. The kinetics of their hydrolysis in the presence of α-chymotrypsin were determined in aqueous solutions at 25.0°, pH 7.90 and 0.10 M in sodium chloride.

Acetyl-L-norvaline Methyl Ester.—To a well-stirred solution of 2.88 g of acetyl-L-norvaline in 50 ml of absolute methanol cooled in an ice-salt bath was added dropwise 2.62 g of redistilled thionyl chloride at such a rate that the temperature remained below 5° (Brenner and Huber, 1953). When the addition was complete (30 minutes) the mixture was heated at 50° for 2 hours and the excess solvent removed by vacuum distillation. The oily residue was dissolved in 100 ml of ethyl acetate, and the solution was washed with 30 ml of 1 M aqueous potassium carbonate, then with 30 ml of water, and dried over anhydrous magnesium sulfate. Evaporation of the solvent gave 2.0 g of an oil which slowly crystallized. This product was recrystallized from isopropyl ether to give 1.55 g (45%) of the methyl ester, colorless needles, m.p. 48.0 − 48.5°, $[\alpha]_D^{25}$ − 57.5 ± 1.5° (*c*, 2% in water).

Anal. Calcd. for $C_8H_{15}O_3N$ (173.2): C, 55.5; H, 8.7; N, 8.1. Found: C, 55.5, 55.6; H, 8.7, 8.7; N, 8.1, 8.1.

Acetyl-L-norvaline β-Chloroethyl Ester.—A solution of 3.0 g of acetyl-L-norvaline in 50 ml of β-chloroethanol was cooled to 0° and saturated with anhydrous hydrogen chloride. The solution was then heated at its refluxing temperature for 1 hour prior to its evaporation *in vacuo* to an oily residue. This residue was twice esterified as described above and the final product dissolved in 100 ml of ethyl acetate. The ethyl acetate solution was washed with 50 ml of 1 M aqueous potassium carbonate, then with 50 ml of water, and dried over anhydrous magnesium sulfate. Removal of solvent from the dried solution by evaporation *in vacuo* gave 3.0 g of an oil which was crystallized from a mixture of isopropyl ether and hexane to give 2.7 g (59%) of the β-

TABLE I

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF α-*N*-ACYLATED ALIPHATIC L-α-AMINO ACID ESTERS[a]

Acyl Component	Side-Chain Component	Ester Component	K_0 (mM)	$k_0{}^b$ (sec. $^{-1}$)	Ref.
CH_3CO	CH_3	CH_4	611 ± 10	1.29 ± 0.02	c, d
C_6H_5CO	CH_3	CH_3	9.75 ± 0.86	0.261 ± 0.011	e, f
CH_3CO	$CH(CH_3)_2$	CH_3	112 ± 12	0.151 ± 0.007	g
CH_3CO	$CH(CH_3)_2$	C_2H_5	110 ± 5	0.133 ± 0.003	g
CH_3CO	$CH(CH_3)_2$	CH_2CH_2Cl	18.8 ± 1.1	0.228 ± 0.013	g
CH_3CO	$CH(CH_3)_2$	$CH(CH_3)_2$	177 ± 20	0.084 ± 0.007	g
CH_2ClCO	$CH(CH_3)_2$	CH_3	43 ± 4	0.111 ± 0.003	g
C_6H_5CO	$CH(CH_3)_2$	CH_3	4.6 ± 0.3	0.045 ± 0.003	g
CH_3CO	$CH_2CH(CH_3)_2$	CH_3	3.14 ± 0.98	4.41 ± 0.02	h

[a] In aqueous solutions at 25.0°, pH 7.90 and 0.10 M in sodium chloride unless otherwise noted. [b] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5%. [c] Wolf (1959). [d] Reaction system 0.5 M in sodium chloride. [e] Hein and Niemann (1962). [f] Reaction system 0.20 M in sodium chloride. [g] Waite and Niemann (1962). [h] Unpublished experiments of J. B. Jones.

chloroethyl ester, feathery needles, m.p. 52.0 – 52.5°, [α]D²⁵ – 55.3 ± 1.7° (c, 2.2% in water).

Anal. Calcd. for $C_9H_{16}O_3NCl$ (221.7): C, 48.8; H, 7.3; N, 6.3. Found: C, 49.0; H, 7.3; N, 6.4

Acetyl-L-norvaline Isopropyl Ester.—A solution of 2.0 g of acetyl-L-norvaline in 50 ml of isopropyl alcohol was cooled to 0°; saturated with anhydrous hydrogen chloride, and treated as described for the β-cholorethyl ester. The oily residue remaining after the third esterification was taken up in 100 ml of ethyl acetate, and the solution was washed with 50 ml of 1 M aqueous potassium carbonate, then with 30 ml of water, and dried over anhydrous magnesium sulfate. Removal of solvent from the dried solution by evaporation *in vacuo* afforded 2.1 g of an oil which was crystallized from a mixture of isopropyl ether and hexane to give 1.6 g (57%) of the isopropyl ester, colorless needles, m.p. 35.5–36.5°, [α]D²⁵ −56.5 ± 2.0° (c, 1.7% in water).

Anal. Calcd. for $C_{10}H_{19}O_3N$ (201.3): C, 59.7; H, 9.5; N, 7.0. Found: C, 59.8, 59.9; H, 9.6, 9.4; N, 7.1, 7.2.

Kinetic Studies.—The kinetic studies were carried out with a pH-stat (Neilands and Cannon, 1955) following the procedure described by Applewhite *et al.* (1958a,b). A 10.0-ml reaction volume was employed, containing 1.0 ml of enzyme stock solution, 1.0 ml of 1.0 M aqueous sodium chloride, and from 1.0 to 8.0 ml of substrate stock solution, the volume being adjusted to 10.0 ml, when necessary, with carbon dioxide–free distilled water. The reactions were conducted in an atmosphere of nitrogen at 25.0 ± 0.1° and pH 7.90 ± 0.05, the latter value being maintained within the limits specified by the automatic addition of standardized 0.001 to 0.1 N aqueous sodium hydroxide. All stock solutions were prepared from carbon dioxide–free distilled water. The substrate stock solutions were in the concentration range from 0.01 to 0.07 M and the enzyme stock solutions contained 1.0 or 0.10 mg enzyme per ml. The α-chymotrypsin was a bovine, salt-free preparation, Armour Lot No. T-97207, and

contained 14.26% nitrogen on an "as is" basis. All calculations were carried out on a Datatron 220 digital computer, programmed as described by Abrash *et al.* (1960). The rates of the enzyme and substrate blank reactions were insignificant relative to those of the enzyme-catalyzed reactions. Therefore there was no need to correct the latter for either enzyme or substrate blank reactions.

RESULTS

The results of the preceding experiments are summarized in Table II. The data contained in this table include values of pertinent experimental parameters and those of the constants k_0 and K_0 of equation (1) for each of the substrates investigated. Implicit in the latter values is the condition that, under the conditions specified, the rates of the α-chymotrypsin catalyzed hydrolysis of the methyl, β-chloroethyl, and isopropyl esters of acetyl-L-norvaline are described by equation (1).

DISCUSSION

The behavior of the three common esters of acetyl-L-valine and acetyl-L-norvaline as substrates of α-chymotrypsin may be compared in terms of their constants k_0, K_0, and the ratio k_0/K_0. Values of these constants for the three pairs of substrates are given in Table III.

If the ratio k_0/K_0 is taken as an index of reactivity of a given substrate it is seen that the difference between the least and most reactive substrate is *ca.* 10⁴, a large difference considering the relatively limited variation in structure. Furthermore, for each of the three pairs of esters the acetyl-L-norvaline derivative is at least several hundred times more reactive than the comparable ester of acetyl-L-valine. This order of reactivity is that expected from the observations of Waite and Niemann (1962) and supports the proposition of these investigators that a secondary carbon

TABLE II

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF THREE ESTERS OF ACETYL-L-NORVALINE[a]

Ester	Expts.[b]	$[E]^c$ (μM)	$[S_0]$ (mM)	K_0 (mM)	$k_0{}^c$ (sec.$^{-1}$)	k_0/K_0 (M^{-1} sec.$^{-1}$)
CH_3	11-0	3.456	0.61– 9.79	10.2 ± 0.90	2.70 ± 0.17	265.3
CH_2CH_2Cl	11-0	0.346	0.27– 2.14	0.79 ± 0.07	3.72 ± 0.14	4693
$CH(CH_3)_2$	11-0	3.456	2.38–52.0	31.5 ± 0.8	2.92 ± 0.04	92.6

[a] In aqueous solutions at 25.0°, pH 7.90 ± 0.05 and 0.10 M in sodium chloride. [b] First number denotes number of experiments carried out to determine K_0 and k_0, second number gives number rejected after application of statistical reiterative procedure (Abrash et al., 1960). [c] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin.

TABLE III

COMPARISON OF L-VALINE AND L-NORVALINE DERIVATIVES[a]

Side-Chain Component	Ester Component	K_0 (mM)	$k_0{}^b$ (sec.$^{-1}$)	$k_0/K_0{}^b$ (M^{-1} sec.$^{-1}$)
n-C_3H_7	CH_3	10.2 ± 0.9	2.70 ± 0.17	265.3
i-C_3H_7	CH_3	112 ± 12	0.151 ± 0.007	1.35
n-C_3H_7	CH_2CH_2Cl	0.79 ± 0.07	3.72 ± 0.12	4693
i-C_3H_7	CH_2CH_2Cl	18.8 ± 1.1	0.228 ± 0.013	12.1
n-C_3H_7	i-C_3H_7	31.5 ± 0.8	2.92 ± 0.04	92.6
i-C_3H_7	i-C_3H_7	177 ± 20	0.084 ± 0.007	0.47

[a] On the basis of their hydrolysis by α-chymotrypsin in aqueous solutions at 25.0°, pH 7.90 and 0.10 M in sodium chloride and in terms of the constants k_0 and K_0 of equation (1). [b] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin.

atom in the four position from the carbonyl oxygen atom of the ester group, when present in the side-chain or ester component, will diminish the reactivity of a substrate through shielding of the potentially reactive carbonyl group.

A more detailed analysis of the data given in Table III is possible, based on the treatment presented by Hein and Niemann (1961, 1962). The present results substantiate some of the conclusions arrived at earlier and permit further definition of the active site of α-chymotrypsin.

Two features of the present data become immediately apparent. Within the limited structural range studied, there is a large variation in the extremes of the values of K_0, and also, this variation for K_0 is five times that of the difference between the extremes of k_0. These facts provide support for the thesis (Hein and Niemann, 1962) that the values of K_0 for this series of compounds approximate the dissociation constants of the respective productive enzyme-substrate complexes, and that the carboalkoxy groups of these substrates participate in the binding of substrate to the active site of the enzyme. If this were not so, and if K_0 approximated a substration constant, containing a rate constant for the breakdown of the enzyme-substrate complex to products, then the relative changes in K_0 and k_0 would not be so divergent, and the values for such similar compounds should tend to parallel each other. If K_0 were principally an index of non-productive binding, then the k_0 values would be expected to

be small, and to show relatively little variation from one substrate to another.

It has already been suggested (Waite and Niemann, 1962) that beta-branching with respect to the ester carbonyl group affects the magnitude of both K_0 and k_0. The present compounds illustrate this effect for both sides of the carbonyl group. For both ester series the variation in K_0 with change in the ester component is i-C_3H_7 > CH_3 > C_2H_4Cl. Comparison of the three pairs of esters shows that the ratio K_0 valine/K_0 norvaline is greater than one in each case and that there is a constant increase in the ratio as the magnitude of K_0 decreases. As the COR_3 – ρ_3 binding (Hein and Niemann, 1961) becomes more effective, the influence of beta-branching becomes more significant. This conclusion is a specific instance of the more general proposition that the contribution of a particular structural feature to orientation and binding of substrate at the active site is not invariant but may change as the over-all binding energy varies.

Implicit in the discussion above is the concept of specific and variable binding of the alcohol component R_3 to the ρ_3 locus. Independent evidence for this binding mode is also available from the observation by Isaacs and Niemann (1960) that acetyl-α-chymotrypsin stereospecifically acetylates butan-2-ol and from the observations by Balls and his co-workers (Balls and Aldrich, 1955; Balls and Wood, 1956; McDonald and Balls, 1956) on the effect of alcohols on the

hydrolysis of p-nitrophenyl acetate by α-chymotrypsin. The quantitative results of the latter workers are in agreement with the order of binding constants deduced for the aliphatic esters of acetyl-L-valine and acetyl-L-norvaline.

Comparison of k_0 values for the two series reveals two major trends. First, the values for the norvaline derivatives are uniformly more than ten-fold greater than those for the corresponding valine derivatives. Second, the ratio k_0 norvaline / k_0 valine is significantly greater for the isopropyl esters than for the other two cases. Both these results indicate that, within these series, values of K_0 and k_0 are influenced in the same direction, although not to the same degree, by similar factors.

However, closer examination reveals an anomaly in the k_0 values. While the effect of *beta*-branching on both sides of the carbonyl group is to increase the value of K_0, the k_0 values do not obey an analogous rule: for the norvaline series, values of k_0 for the isopropyl and methyl esters are essentially equal. There may be several reasons for this behavior which is a second order effect. First, with methyl acetyl-L-norvalinate non-productive combination (Hein and Niemann, 1961) may be more important than with the other substrates, a situation that would lead to lower values of k_0 and K_0 if the dissociation constants of the non-productive enzyme-substrate complexes were comparable to or smaller than that of the productive complex. While this is a possibility, there is no independent evidence to support such an interpretation with the possible exception of the observations of Balls and his co-workers (Balls and Aldrich, 1955; Balls and Wood, 1956; McDonald and Balls, 1956) of the greater effectiveness of normal relative to branched chain alcohols in the α-chymotrypsin catalyzed transesterification of p-nitrophenyl acetate. Second, the dependence of the constants k_0 and K_0 upon the concentration of sodium chloride (Martin and Niemann, 1958) may not be the same for all substrates, with the result that comparisons are being made under conditions that are not completely comparable. Finally, it is conceivable that the values of k_0 and K_0 are being determined, at least in part, by factors that are still indeterminate. One interesting possibility is that *beta*-branching in the ester component does not interfere with hydrolysis provided the substrate is sufficiently tightly bound in a productive mode. The resolution of this problem is dependent upon

the acquisition of additional information on the kinetic behavior of the above and similar acylated α-amino acid esters. Such studies are in progress.

The prediction of Waite and Niemann (1962) that the value of K_0 for methyl acetyl-L-norvalinate will be smaller and that of k_0 larger than those for the corresponding valine derivative has been substantiated. In addition, the experiments reported in this communication are part of the supporting evidence for two of the postulates proposed by Hein and Niemann (1962) in their explanation of the steric and structural specificity of α-chymotrypsin. These are: one, only orientations including $COR_3 - \rho_3$ interactions can lead to substrate activity, that is, to successful binding leading to reaction products; and, two, branching of the side-chain β to the potentially reactive carbonyl group results in a steric hindrance to binding and hydrolysis of the carbonyl function.

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