The α-Chymotrypsin–Catalyzed Hydrolysis of a Series of Acylated-L-Valine Esters*

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Kinetic data are presented for the α-chymotrypsin-catalyzed hydrolysis of the methyl esters of acetyl-, chloroacetyl-, and benzoyl-L-valine and of the ethyl, β-chloroethyl, and isopropyl esters of acetyl-L-valine. These data have been interpreted as providing evidence for inhibition of the enzyme-catalyzed reaction by a structural feature of the substrate, in this instance associated with secondary carbon atoms in the 4-position from the carbonyl oxygen atom of the hydrolyzable carboalkoxy group. They have also been interpreted as indicating participation of the carboalkoxy group in determining the dissociation constant of the enzyme-substrate complex.

Applewhite *et al.* (1958b), in describing a search for ester-type substrates of α-chymotrypsin suitable for kinetic studies at enzyme concentrations of the order of 10^{-5} M, noted that the rates of hydrolysis of a limited series of acylated α-amino acid esters appeared to be related to their "six numbers" (Newman, 1950). For the methyl esters of acetyl-L-valine, acetyl-L-isoleucine, and acetyl-L-leucine, with "six numbers" of 8, 8, and 5, respectively, the ratio of their rates of hydrolysis is approximately 4:5:800.

The "rule of six" (Newman, 1950, 1956) is a substitute for more precise methods of estimating steric effects. For enzyme-catalyzed reactions it must be applied with recognition of the other factors determining substrate reactivity (Hein and Niemann, 1961). In this communication we shall be concerned with the interpretation of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of a series of acylated L-valine esters in terms of steric factors influencing the formation of enzyme-substrate complexes and their rate of decomposition into enzyme and reaction products.

In the earlier study (Applewhite *et al.*, 1958b) it was found that the initial rates of hydrolysis of the methyl esters of acetyl-, chloroacetyl-, and benzoyl-L-valine, in aqueous solutions at 25.0°, *p*H 7.90 and 0.10 M with respect to sodium chloride, were described by equation (1).

$$d[P]/dt = k_0[E][S]/(K_0 + [S]) \qquad (1)$$

Two sets of values of the constants K_0 and k_0 for the above three substrates are given in Table I. One set are those reported previously (Applewhite *et al.*, 1958b) based upon the assumption that the rate of production of acid from the enzyme blank reaction is not influenced by the presence of substrate. Martin and Niemann (1957) have shown that this practice is questionable because the enzyme blank reaction may be substantially suppressed in the presence of substrate. Therefore, a second set of values was calculated from the original data without correction for the enzyme blank reaction. These values are designated as revised values in Table I. Both sets were corrected for the non-enzyme-catalyzed hydrolysis of the substrates.

Independent evaluation of the constants for the methyl ester of acetyl-L-valine (Martin and Niemann, 1958; Kurtz and Niemann, 1961a) and the methyl ester of chloroacetyl-L-valine (Kurtz and Niemann, 1961b), with correction for the enzyme blank reaction as described by Martin and Niemann (1957), has resulted in values of K_0 = 108 ± 9 and 111 ± 6 × 10^{-3} M and k_0 = 2.2 ± 0.1 × 10^{-3} M/minute/mg protein-nitrogen per ml for the former substrate and K_0 = 46.9 ± 2.9 × 10^{-3} M and k_0 = 1.65 ± 0.09 × 10^{-3} M/minute/mg protein-nitrogen per ml for the latter. The most probable values of K_0 and k_0 for the methyl esters of acetyl-, chloroacetyl-, and benzyl-L-valine are given in Table II along with those for the corresponding alanine and leucine derivatives insofar as they are known. The analogous glycine derivatives have not been included because it is now apparent that the presence of a side-chain other than hydrogen on the α-amino acid derivative leads to relations between structure and substrate reactivity that are substantially different from those encountered with the bifunctional substrates (Wolf, 1959).

The data summarized in Table II reveal several trends. First, for substrates with a common side-chain values of K_0 and k_0 appear to be dependent upon the nature of the α-acylamino component, the magnitude of both constants decreasing with increasing size of this constituent. Second, the data for the methyl esters of acetyl-L-alanine, acetyl-L-valine, and acetyl-L-leucine indicate that within a series containing a common α-acylamino component the magnitude of K_0 is also dependent upon the nature of the α-amino acid side-chain, the former generally decreasing as the size of the latter increases. However, within this series the value of k_0 for the methyl ester of acetyl-L-valine contravenes the proposition, suggested by the behavior of the alanine and leucine derivatives, that with increasing size of the side-chain values of k_0 increase

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TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF THE METHYL ESTERS OF ACETYL-, CHLOROACETYL-, AND BENZOYL-L-VALINE[a]

Acyl Component	K_0[b]		k_0[d]		k_0/K_0[f]	
	Original[c]	Revised	Original[e]	Revised	Original[g]	Revised
Acetyl	125.6 ± 3.4	116.7 ± 3.5	2.33 ± 0.06	2.26 ± 0.05	0.185 ± 0.009	0.195 ± 0.009
Chloroacetyl	47.1 ± 0.8	39.3 ± 0.7	1.755 ± 0.028	1.61 ± 0.03	0.373 ± 0.007	0.409 ± 0.014
Benzoyl	4.9 ± 0.3	4.3 ± 0.3	0.696 ± 0.036	0.65 ± 0.05	1.43 ± 0.16	1.52 ± 0.22

[a] In aqueous solutions at 25.0°, *p*H 7.90 and 0.10 M with respect to sodium chloride. [b] In units of 10^{-3} M. [c] Originally designated as K_S. [d] In units of 10^{-3} M/min./mg protein-nitrogen per ml. [e] Originally designated as k_3. [f] In units of min.$^{-1}$ (mg protein-nitrogen/ml)$^{-1}$ × 10. [g] Originally designated as k_3/K_S.

with decreasing values of K_0. These observations, and the consistent data for the methyl esters of benzoyl-L-alanine and benzoyl-L-valine, support the view that when the substrates listed in Table II occupy the active site of the enzyme, access to the carbonyl group of the carbomethoxy constituent is sterically limited to a greater degree by the isopropyl side-chain of the valine derivatives than by the methyl or isobutyl side-chains of the alanine or leucine derivatives. Having arrived at the preceding interpretation, we judged it desirable to seek other evidence to confirm or refute the tacit assumption that for a substrate in an enzyme-substrate complex access to the carbonyl group of the reactive carboalkoxy function of an ester-type substrate can be sterically limited by structural elements of the substrate. Therefore, a study of the kinetics of the α-chymotrypsin-catalyzed hydrolysis of the ethyl, β-chloroethyl, and isopropyl esters of acetyl-L-valine was undertaken.

TABLE II

KINETIC CONSTANTS FOR A SERIES OF ACYLATED L-α-AMINO ACID METHYL ESTERS[a]

Acyl Component	Side-Chain	K_0[b]	k_0[c]
CH_3CO	CH_3	611 ± 10[d,e]	18.8 ± 0.3[d,e]
	$CH(CH_3)_2$	112 ± 12	2.2 ± 0.1
	$CH_2CH(CH_3)_2$	2.9[f,g]	67[f,g]
$ClCH_2CO$	$CH(CH_3)_2$	43 ± 4	1.60 ± 0.05
C_6H_5CO	CH_3	9.75 ± 0.86[f,g]	3.80 ± 0.16[f,g]
	$CH(CH_3)_2$	4.6 ± 0.3	0.65 ± 0.05

[a] In aqueous solutions at 25.0°, *p*H 7.90 and 0.10 M with respect to sodium chloride unless otherwise noted. [b] In units of 10^{-3} M. [c] In units of 10^{-3} M/min./mg protein-nitrogen per ml. [d] Reaction system 0.50 M with respect to sodium chloride. [e] Wolf (1959). [f] Reaction system 0.20 M with respect to sodium chloride. [g] Unpublished data obtained in these laboratories.

EXPERIMENTAL

Acetyl-L-valine Ethyl Ester.—A solution of 10 g of L-valine in 30 ml of anhydrous ethanol was cooled in an ice-salt bath, saturated with anhydrous hydrogen chloride, and then heated on a steam bath under refluxing conditions for 1 hour. The excess hydrogen chloride and solvent was removed *in vacuo* and the oily residue, containing unreacted L-valine hydrochloride, was reesterified. Following a second reesterification the oily ester hydrochloride was stirred for 15 hours with 10 g of anhydrous sodium acetate and 50 ml of acetic anhydride and the acetyl-L-valine ethyl ester isolated as described for the analogous methyl ester (Applewhite *et al.*, 1958b). The crude ester, a solid with a low melting point, was recrystallized from pentane by preparing a saturated solution at room temperature, seeding, and storing overnight at 4°. Three recrystallizations and desiccation *in vacuo* over solid sodium hydroxide gave 5.1 g (32%) of acetyl-L-valine ethyl ester, large colorless needles, m.p. 32.5–34.2, $[\alpha]_D^{25} = -50.3°$ (*c*, 4.5% in water).

Anal. Calcd. for $C_9H_{17}O_3N$ (187.2): C, 57.7; H, 9.2. Found: C, 57.7; H, 9.2.

Acetyl-L-valine β-Chloroethyl Ester.—A suspension of 15 g of L-valine in 50 ml of anhydrous ethylene chlorohydrin was esterified with hydrogen chloride as described for the ethyl ester. As before, the esterification procedure was twice repeated to obtain the crude ester hydrochloride free of L-valine hydrochloride. This intermediate was acetylated with 15 g of sodium acetate and 100 ml of acetic anhydride, and the product was isolated as for the methyl ester (Applewhite *et al.*, 1958b). The oily product, which resisted all attempts at crystallization, was decolorized with Norite in a hexane-chloroform solution, the solvent removed *in vacuo*, and the viscous residue distilled to give 5 g of forerun, b.p. 95–105°/2 mm, 2 g of forerun b.p. 105–160°/2 mm, and 15 g of product, b.p. 160–170°/2 mm. Trituration of the oily product with hexane induced crystallization. The solid was collected, recrystallized three times from a mixture of 95% hexane and 5% chloroform at −10°, and dried *in vacuo* over solid sodium hydroxide to give 5.0 g (19%) of the β-chloroethyl ester of acetyl-L-valine, clusters of large colorless needles, m.p. 44.5–45.5°.

Anal. Calcd. for $C_9H_{16}O_3NCl$ (221.7): C, 48.8; H, 7.3. Found: C, 49.0; H, 7.4.

Acetyl-L-valine Isopropyl Ester.—L-Valine, 5 g, was esterified with 100 ml of isopropyl alcohol as described for the ethyl ester and the oily ester hydrochloride acetylated with 5 g of sodium acetate and 25 ml of acetic anhydride as for the ethyl and β-chloroethyl esters. The crude isopropyl ester was recrystallized from hexane and dried *in vacuo* over solid sodium hydroxide and

TABLE III

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF A SERIES OF ESTERS OF ACETYL-L-VALINE[a]

Ester	No. of Expers.	$[S]_0$[b]	v_0[c]	Extent of Reaction (%)	K_0[b,d]	k_0[d,e]
Ethyl	8	19.8 –59.6	4.44– 9.93	1.33– 1.80	110 ± 5	1.93 ± 0.05
β-Chloroethyl	13	2.16–16.97	5.03–23.40	11.0 –18.6	18.8 ± 1.1	3.32 ± 0.19
Isopropyl	9	11.05–80.5	1.09– 5.60	0.56– 0.79	177 ± 20	1.22 ± 0.10

[a] In aqueous solutions at 25.0°, *p*H 7.90 ± 0.01 and 0.10 M with respect to sodium chloride, with $[E]$ = 0.1467 mg protein-nitrogen per ml. [b] In units of 10^{-3} M. [c] In units of 10^{-5}/M min. [d] Evaluated by a least-squares fit to the equation $[S]_0[E]/v_0 = K_0/k_0 + [S]_0/k_0$. [e] In units of 10^{-3} M/min./mg protein-nitrogen per ml.

paraffin to give 2.3 g (27%) of acetyl-L-valine isopropyl ester, colorless needles, m.p. 49.0–50.8°.

Anal. Calcd. for $C_{10}H_{19}O_3N$ (201.3): C, 59.7; H, 9.5. Found: C, 60.0; H, 9.7.

Kinetic Studies.—The general procedure was identical with that employed by Applewhite *et al.* (1958b) and described earlier by Applewhite *et al.*, (1958a). All experiments were conducted with crystalline, salt-free, bovine α-chymotrypsin, Armour lot No. 283. In every experiment the reaction time was 8 minutes. Other pertinent experimental details are given in Table III. The initial velocities and kinetic constants were evaluated with the aid of a Datatron 205 digital computer as described by Abrash *et al.* (1960) except that no correction was made for the enzyme blank reaction. A series of experiments was performed with the β-chloroethyl ester of acetyl-L-valine, in aqueous solutions at 25.0° and 0.10 M with respect to sodium chloride, with $[E]$ = 0.1467 mg protein-nitrogen per ml and $[S] = 14.71 \times 10^{-3}$ M. A maximum value for v_0, *i.e.*, 6.02×10^{-4} M/minute, was observed at *p*H 7.80 ± 0.10 for a range from *p*H 7.30 to 8.30.

DISCUSSION

It was noted previously that for the methyl esters of acetyl-L-valine, acetyl-L-isoleucine, and acetyl-L-leucine, with "six numbers" of 8, 8, and 5, respectively, the ratio of their rates of hydrolysis by α-chymotrypsin is approximately 4:5:800. These data suggest that the critical structural features responsible for the markedly lower rates of hydrolysis of the valine and isoleucine derivatives are their isopropyl and *sec*-butyl side-chains. Therefore, for the enzyme-catalyzed reactions steric inhibition is more reasonably associated with the presence of a secondary, and possibly a tertiary, carbon atom in the fourth atomic position from the carbonyl oxygen atom of the reactive carboalkoxy group of an ester type substrate rather than with its "six number." This modification of Newman's "rule of six" (Newman, 1950, 1956) is supported by data given in Table IV. The rates of hydrolysis of the methyl and ethyl esters of acetyl-L-valine, with "six numbers" of 8 and 11, respectively, are almost indistinguishable. However, both substrates contain but a single secondary carbon atom in the four position. In contrast, the isopropyl ester of acetyl-L-valine, with two secondary carbon atoms in the four position, one in the amino acid side-chain, and the other in the alcohol constituent, is hydrolyzed at a significantly slower rate than the corresponding methyl or ethyl esters. These observations support the reality of steric inhibition of α-chymotrypsin-catalyzed reactions by structural features of the substrate and in particular by those arising from secondary carbon atoms in the 4 position.

For reasons summarized elsewhere (Neurath and Hartley, 1959) values of K_0 of Tables II and IV may be interpreted as dissociation constants of enzyme-substrate complexes and those of k_0 as rates of their decomposition into enzyme and reaction products. For the methyl, ethyl, β-chloroethyl, and isopropyl esters of acetyl-L-valine values of k_0 are linearly related to those of K_0 by the relation $k_0 = 3.55–0.013\ K_0$, which suggests that within this series values of K_0 and k_0 are determined by a common factor. Hein and Niemann (1961) have identified this factor as the interaction of the potentially hydrolyzable carboalkoxy group with its complementary locus at the active site of the enzyme and have concluded that for the acylated α-amino acid esters this interaction, and in particular the contribution of the component carbonyl group, is critical in determining the magnitude of the dissociation constant of the enzyme-substrate complex as well as its rate of decomposition into enzyme and reaction products. Within the preceding series and relative to the methyl and ethyl esters it is probable that the greater value of K_0 and lesser value of k_0 for the isopropyl ester is a consequence of a greater steric shielding of the reactive carbonyl group, whereas for the β-chloroethyl ester the lesser value of K_0 and the greater value of k_0 arises, at least in part, from an increased polarization of the same carbonyl group.

In the series including the methyl esters of acetyl-L-alanine, acetyl-L-valine, and acetyl-L-leucine the only evidence for steric shielding of the reactive carbonyl group is the anomalously low value of k_0 for the valine derivative. In this series the uniform decrease in values of K_0 is not surprising in view of the role of the size of the side-chain in determining the magnitude of K_0 (Hein and Niemann, 1961). However, it is expected 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.

For acylated α-amino acid ester substrates represented by the formula $R_1CHR_2COR_3$, where $R_1 = R_1'CONH$ and $R_2 \neq H$, two limit types have

TABLE IV

KINETIC CONSTANTS FOR A SERIES OF ACYLATED L-VALINE ESTERS[a]

Acyl Component	Ester Component	K_0[b]	k_0[c]	k_0/K_0[d]
CH_3CO	CH_3	112 ± 12	2.2 ± 0.1	0.0195 ± 0.0009
	C_2H_5	110 ± 5	1.93 ± 0.05	0.0176 ± 0.0013
	CH_2CH_2Cl	18.8 ± 1.1	3.32 ± 0.19	0.178 ± 0.020
	$CH(CH_3)_2$	177 ± 20	1.22 ± 0.10	0.0073 ± 0.0011
CH_2ClCO	CH_3	43 ± 4	1.60 ± 0.05	0.0409 ± 0.0014
C_6H_5CO	CH_3	4.6 ± 0.3	0.65 ± 0.05	0.152 ± 0.022

[a] In aqueous solutions at 25.0°, *p*H 7.90 and 0.10 M with respect to sodium chloride. [b] In units of 10^{-3} M. [c] In units of 10^{-3} M/min./mg protein-nitrogen per ml. [d] In units of min.^{-1} $(\text{mg protein-nitrogen/ml})^{-1}$.

been recognized (Hein and Niemann, 1961). They are $S^{3E}_{R_2R_3}$, where the enzyme-substrate dissociation constants are determined by R_2-ρ_2 and COR_3-ρ_3 interactions, and $S^{3E}_{R_1R_3}$, where R_1-ρ_1 and COR_3-ρ_3 interactions are similarly determining. It was noted previously (Hein and Niemann, 1961) that for substrates of the $S^{3E}_{R_2R_3}$ limit type a decrease in the enzyme-substrate dissociation constant caused by an enhanced R_2-ρ_2 interaction leads to an increase in k_0. In contrast, with those of the $S^{3E}_{R_1R_3}$ limit type a decrease in the enzyme-substrate dissociation constant caused by an enhanced R_1-ρ_1 interaction leads to a decrease in k_0. While it is likely that the acylated-L-valine esters are intermediate between the preceding two limit types, the constants observed for the methyl ester of chloroacetyl-L-valine and the β-chloroethyl ester of acetyl-L-valine provide support for and permit extension of the above generalizations. With the former substrate replacement of a hydrogen atom of R_1' by a chlorine atom causes a decrease in K_0 and a decrease in k_0. With the latter, replacement of a hydrogen atom of R_3 by a chlorine atom causes a decrease in K_0 but an increase in k_0. From these data and those considered previously (Hein and Niemann, 1961) we may generalize as follows. When enzyme-substrate dissociation constants are determined by interaction of COR_3 with ρ_3 and R_1 with ρ_1 and/or R_2 with ρ_2 an increase in the COR_3-ρ_3 or R_2-ρ_2 interaction, as indicated by a decrease in K_0, leads to an increase in k_0. In contrast, an increase in the R_1-ρ_1 interaction, associated with a decrease in K_0, leads to a decrease in k_0. This generalization predicts that the substrates most rapidly hydrolyzed are those in which the R_2-ρ_2 and COR_3-ρ_3 interactions are maximized and that of R_1-ρ_1 minimized, but not beyond the point where it begins to lose its orienting function.

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