A Further Comparison of the Behavior of Analogous Aromatic and Hydroaromatic Substrates of α-Chymotrypsin*

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The kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine methyl ester and acetyl-L-hexahydrophenylalanine methyl ester have been evaluated in aqueous solutions at 25.0°, *p*H 7.90 and 0.10 M with respect to sodium chloride. Michaelis-Menten kinetics were observed, and the values of both K_0 and k_0 were greater for the former substrate than for the latter. Three alternative explanations of this behavior were considered. The explanation adopted as a working hypothesis assumes attack on the carbomethoxy group of the substrate by a nucleophile and/or electrophile at the active site of the enzyme and assistance of this attack by deformation of a region of the site through interaction of the aromatic side-chain component of the substrate with its complementary locus at the site.

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalaninamide, in aqueous solutions at 25.0°, *p*H 7.90, and 0.02 M with respect to the tris(hydroxymethyl)aminomethane component of a buffer containing this base and hydrochloric acid, were examined by Huang *et al.* (1952) and those of acetyl-L-hexahydrophenylalaninamide, under the same conditions, by Jennings and Niemann (1953). For both substrates the rates of hydrolysis were described by equation (1). The data of these two groups of investi-

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

gators when reevaluated by Foster and Niemann (1955) gave for the aromatic substrate[1] $K_0 = 31 \pm 3$ mM, $k_0 = 0.055 \pm 0.014$ sec.$^{-1}$, and for the hexahydro-analog $K_0 = 27 \pm 4$ mM, $k_0 = 0.046 \pm 0.003$ sec.$^{-1}$. It was concluded from these data (Jennings and Niemann, 1953) that replacement of the benzyl group by a hexahydrobenzyl group has relatively little effect upon either the K_0 or the k_0 values.

In the course of developing a theory of the structural and stereochemical specificity of α-chymotrypsin (Hein and Niemann, 1961, 1962) it became evident that α-*N*-acylated α-amino acid amides were generally intermediate between possible limit-type substrates and that data obtained through use of these compounds could be ambiguous. Therefore, it was decided to re-examine the role of aromaticity in the side-chain component of a substrate by investigating a pair of substrates that offered a better chance of approximating the behavior of a hypothetical limit-type substrate. Since there was reason to believe that acetyl-L-phenylalanine methyl ester approximates the behavior of the hypothetical $S^{ar}_{R_2R_3}$ limit-type substrate, *i.e.*, a trifunctional ester-type substrate in which the enzyme-substrate dissociation constant is determined primarily by interaction of the side-chain and carboalkoxy group with their complementary loci at the active site (Hein and Niemann, 1961, 1962), we have in this investigation compared the behavior of this compound with that of acetyl-L-hexahydrophenylalanine methyl ester.

Experimental

Acetyl-L-phenylalanine Methyl Ester.– A cooled solution of 5.0 g of acetyl-L-phenylalanine in 50 ml of absolute methanol was saturated with hydrogen chloride. The reaction mixture was refluxed for 1 hour, the solvents were evaporated, and the above procedure was repeated a second and third time. The oily residue finally obtained was taken up in 100 ml of ethyl acetate and the solution washed with 50 ml of 1 M aqueous potassium carbonate solution and then with 50 ml of water. The ethyl acetate solution was dried over anhydrous magnesium sulfate and evaporated to give 3.0 g of sirup which soon crystallized. Recrystallization from diisopropyl ether gave 2.2 g of the desired ester, colorless needles, m.p. 90°, $[\alpha]_D^{25}$ 17.8 ± 1.2° (*c*, 2% in methanol). Huang *et al.* (1952) give m.p. 90–91°, $[\alpha]_D^{25}$ 19° (*c*, 2% in methanol).

L-Hexahydrophenylalanine.—This compound was prepared by hydrogenation and hydrogenolysis of L-tyrosine (Waser and Brauchli, 1924). A solution of 18.1 g of L-tyrosine in 300 ml of 2 N aqueous hydrochloric acid containing platinum black, prepared by prereduction of 1 g of platinic oxide, was hydrogenated at 25° and 30 p.s.i. until hydrogen uptake ceased (10 hours). The mixture was then warmed to dissolve the precipitated α-amino acid and filtered to remove the catalyst. The free acid was precipitated by addition of sodium acetate to the cooled filtrate. The crude product was recrystallized from aqueous ethanol to give 10.5 g of amino acid, microcrystals, which when dissolved in methanol gave an ultraviolet absorption spectrum free from benzenoid absorption bands.

Acetyl-L-hexahydrophenylalanine Methyl Ester.—Thionyl chloride, 6.6 g, was added dropwise with stirring over a 30-minute period to 50 ml of methanol at 0°. To this solution was added 8.75 g of L-hexahydrophenylalanine, and the mixture was heated at 50° with stirring for 2 hours. Evaporation of the solvent under reduced pressure gave the crystalline ester hydrochloride, which was suspended in 50 ml of ethyl acetate. To the stirred, cooled (0°) suspension was added 27.6 g of potassium carbonate in 50 ml of water, followed by 10.2 g of acetic anhydride. The mixture was allowed to warm to 25° and stirred for an additional 12 hours. The ethyl acetate layer was separated, the aqueous phase was extracted with two 50-ml portions of ethyl acetate, and the combined ethyl acetate extracts were dried over anhydrous magnesium sulfate. Removal of the solvent gave a residue which was taken up in 100 ml of chloroform and washed first with 1 N aqueous hydrochloric acid, then with 3% aqueous sodium carbonate, and finally with water. The chloro-

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[1] All values of k_0 are based upon an assumed molecular weight of 25,000 and a nitrogen content of 16.5%.

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-PHENYLALANINE METHYL ESTER AND ACETYL-L-HEXAHYDROPHENYLALANINE METHYL ESTER[a]

Substrate	Expts. No.[b]	[S] (mM)	K_0 (mM)	k_0[g] (sec.$^{-1}$)	k_0/K_0 (M^{-1} sec.$^{-1}$)
Ac-L-Phe-OCH_3[c]	7-0[d]	0.420–3.384	1.25 ± 0.95	52.5 ± 15.8	4.2×10^4
Ac-L-H_6Phe-OCH_3[e]	8-0[d]	0.103–0.824	0.18 ± 0.07	13.0 ± 1.3	7.2×10^4
	7-0[f]	0.110–0.880	0.19 ± 0.04	15.2 ± 0.8	8.0×10^4

[a] In aqueous solutions at 25.0°, pH 7.90 ± 0.10, and 0.10 M with respect to sodium chloride. [b] First digit, number of experiments; second digit, number of those rejected by computer program. [c] Acetyl-L-phenylalanine methyl ester. [d] $[E] = 3.456 \times 10^{-8}$ M. [e] Acetyl-L-hexahydrophenylalanine methyl ester. [f] $[E] = 6.912 \times 10^{-8}$ M. [g] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin.

form solution was dried over anhydrous magnesium sulfate, and the solvent was evaporated to give 6.0 g of a colorless solid which was recrystallized from a mixture of hexane and diisopropyl ether to give 5.4 g of the desired product, feathery needles, m.p. 106–107°, $[\alpha]_D^{25}$ −22.1 ± 0.4° (c, 2.4% in methanol).

Anal. Calcd. for $C_{12}H_{21}O_3N$ (227.3): C, 63.4; H, 9.3; N, 6.2. Found: C, 63.6; H, 9.1, N, 6.3.

Kinetic Studies.— The procedure was identical with that described previously (Applewhite *et al.*, 1958a,b). All experiments were conducted in aqueous solutions at 25.0°, pH 7.90 ± 0.10 and 0.10 M with respect to sodium chloride. The enzyme preparation was crystalline bovine salt-free α-chymotrypsin, Armour and Co. lot no. T-97207. The primary experimental data were analyzed with a Datatron 220 digital computer, programmed as described earlier (Abrash *et al.*, 1960).

RESULTS

The results obtained in the present investigation are summarized in Table I. It must be realized that with the two substrates under consideration, and particularly with acetyl-L-phenylalanine methyl ester, one is working very close to the limit of experimental capability of the pH-stat. Also, one is simultaneously confronted with the problems that arise when working with enzyme concentrations of the order of 10^{-8} M (Bixler and Niemann, 1959). At such low enzyme concentrations, adsorption of enzyme by the glass equipment reduces the amount of enzyme in solution. While little can be done to remedy this situation with respect to enzyme adsorbed on glass electrodes, an effort was made to minimize adsorptive losses during transfer operations by using small volumes of more concentrated enzyme stock solutions rather than larger volumes of more dilute solutions and avoiding extended serial dilutions. Even with these precautions considerable error was encountered in the evaluation of k_0 for acetyl-L-phenylalanine methyl ester.

Bender and Glasson (1960) examined the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-phenylalanine methyl ester in aqueous solutions at 25.2 ± 0.1°, pH 7.80 and 0.75 mM with respect to the Tris component of a buffer containing this base and hydrochloric acid with the ionic strength maintained at 0.042 M by the addition of sodium chloride. Their values of the constants of equation (1) are K_0 = 1.80 ± 0.45 mM, k_0 = 48.2 ± 12.1 sec.$^{-1}$. However, Bender and Glasson (1960) assume a molecular weight of 22,500 and a nitrogen content of 14% for α-chymotrypsin. This assumption leads to the relation, 1.0 mg protein-nitrogen per ml = 3.175×10^{-4} M for converting from the former to the latter units in arriving at a value of k_0 in units of sec.$^{-1}$. In our work we have assumed a molecular weight of 25,000 and a nitrogen content of 16.5%, which results in the conversion factor 1.0 mg protein-nitrogen per ml = 2.424×10^{-4} M. Thus, when converted to the latter scale Bender and Glasson's value of k_0 becomes 63.2 ± 15.9 sec.$^{-1}$, while ours is k_0 = 52.5 ± 15.8 sec.$^{-1}$. The values of K_0, 1.80 ± 0.45 and 1.25 ± 0.95, respectively, also show a comparable deviation. However, this agreement is all that can be expected in view of the experimental difficulties encountered with these substrates.

Of the two sets of values of K_0 and k_0 given in Table I for acetyl-L-hexahydrophenylalanine methyl ester, the latter set, K_0 = 0.19 ± 0.04 mM, k_0 = 15.2 ± 0.8 sec.$^{-1}$, is preferred because they were obtained at the higher enzyme concentrations and thus are probably more accurate.

DISCUSSION

In contrast to acetyl-L-phenylalaninamide and acetyl-L-hexahydrophenylalaninamide, where there is no significant difference in the two sets of values of K_0 and k_0, acetyl-L-phenylalanine methyl ester and acetyl-L-hexahydrophenylalanine methyl ester have significantly different kinetic constants. We believe that the difference in behavior of these amides and esters can be attributed to differences in the way analogous α-*N*-acetamido acid amides and esters combine with the active site of the enzyme (Hein and Niemann, 1961). With the above esters it appears that one is close to a $S^{3K}_{R_2R_1}$ limit-type substrate, whereas with the amides a relatively poor $COR_3 - p_3$ interaction leads to a higher incidence of nonproductive combination. In the case at hand this is associated with a lesser dependence of the values of the constants K_0 and k_0 upon the structures of the substrates. It will be recalled that a similar situation was encountered previously with a series of α-*N*-acylated-L-tyrosinamides and hydrazides (Lutwack *et al.*, 1957).

In a recent communication Doherty (1962) reported that the rate of the α-chymotrypsin-catalyzed hydrolysis of methyl β-(*m*-aminophenyl)propionate was greater than that of the analogous cyclohexyl derivative. However, values of K_0 and k_0 for these reactions were not given and a comparison of the behavior of these substrates and those which we have examined cannot be made.

The value of K_0 = 0.19 mM for acetyl-L-hexahydrophenylalanine methyl ester demonstrates that an aromatic structural component need not be present for a substrate to have a low value of K_0. Furthermore, the magnitudes of K_0 and k_0 for this substrate and those of its aromatic analog provide no support for the proposition that the magnitude of K_0 is depressed because of a large value of k_0, a situation that might arise if K_0 was not an apparent enzyme-substrate dissociation constant (Hein and Niemann, 1961). However, the data given in Table I suggest that the higher value of k_0 for acetyl-L-phenylalanine methyl ester may be associated with the presence of an aromatic nucleus in the side-chain of this substrate.

The observation that the values of K_0 and k_0 for acetyl-L-hexahydrophenylalanine methyl ester are lower than those for acetyl-L-phenylalanine methyl ester might lead one to the conclusion that nonproductive combination is being encountered with the first substrate but not with the second. The argument here would be identical with one developed earlier for the case of acetyl-L-phenylalanine methyl ester and acetyl-L-tryptophan methyl ester (Hein and Niemann, 1961). It would be based upon the proposition that the lower value of k_0 for the hexahydro compound could arise as a result of the formation of an energetically more favorable, but nonproductive, enzyme-substrate complex that is competitive with the productive complex. Specifically, if the value of k_0 for acetyl-L-phenylalanine methyl ester were assumed to be identical to the rate of decomposition of the productive complex of acetyl-L-hexahydrophenylalanine methyl ester, *i.e.*, k_2, then application of the relations shown in equations (2) and

$$K_0 = k_0 K_S / k_2 \quad (2)$$

$$K_0 = K_S K_{S_I} / (K_S + K_{S_I}) \quad (3)$$

(3), given by Hein and Niemann (1961), would give values of $K_S = 0.66$ mM and $K_{S_I} = 0.26$ mM for the dissociation constants of the productive and nonproductive complexes of acetyl-L-hexahydrophenylalanine methyl ester, respectively. While this interpretation may be reasonable, it lacks the force of similar arguments given previously(Hein and Niemann, 1961, 1962; Wolf and Niemann, 1963) because there is no independent evidence for nonproductive combination of cyclohexyl derivatives as there is, for example, with indole derivatives (Hein and Niemann, 1961).

An alternative, but related, explanation assigns a special role to the aromatic nucleus in acetyl-L-phenylalanine methyl ester. The aromatic nucleus could assist in achieving a better orientation of the carbomethoxy group with respect to the attacking nucleo- and/or electrophilic groups at the active site of the enzyme. Such orientation could be developed not only by positioning of the substrate molecule through interaction of the various structural components of the substrate molecule with the combining loci at the active site (Hein and Niemann, 1961, 1962) but also by alteration of the site itself.

An aromatic nucleus is both stiff and flat, and in an interaction such as encountered with acetyl-L-phenylalanine methyl ester, in which $-\Delta F^*_{298} \simeq 4$ kcal., the aromatic nucleus, in combining with its locus, *i.e.*, ρ_2, might cause a deformation in an adjacent region of the site and bring about a more favorable orientation of the attacking nucleo- and/or electrophilic groups of the site relative to the carbomethoxy group of the substrate, thus leading to an increase in the magnitude of k_0. This effect, which is a kind of steric assistance, presumably cannot arise with acetyl-L-hexahydrophenylalanine methyl ester, for which $-\Delta F^*_{298} \simeq 5$ kcal., and in which when combining with the ρ_2 locus the more deformable cyclohexyl group is itself deformed rather than effecting alteration of the conformation of the site. This explanation requires that the value of k_0 for a limit-type substrate containing an aromatic side-chain component always be greater than that of an otherwise structurally equivalent hydroaromatic analog and is thus amenable to experimental test.

A third explanation hinges on the stereochemical nonequivalence of aromatic and hydroaromatic nuclei. Steric hindrance has previously been encountered among substrates of α-chymotrypsin (Waite and Niemann, 1962; Jones and Niemann, 1962). However, in the earlier examples, which were concerned with the consequences of β-substitution, a decrease in the value of k_0 was associated with an increase in that of K_0. This situation does not obtain in the case at hand, and any explanation based on steric grounds must recognize that the value of K_0 for the hydroaromatic compound is substantially less than that of the aromatic compound. Thus, one is forced into the same dilemma encountered in the first explanation. There is no precedence for a kind of steric interaction that would cause a decrease in both k_0 and K_0, except that contemplated above, and in which structural components of the substrate interact with loci other than those presumed to be complementary. Thus, at present, the second explanation given above appears to be the most attractive, but only as a working hypothesis for the design of future experiments.

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