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SC 11016

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester*

For many years the specificity of α-chymotrypsin was associated with substrates containing an aromatic side chain. Even though the hydrolysis of esters of L-methionine was reported as early as 1950 (ref. 1), considerable stress on the specificity of α-chymotrypsin for aromatic α-amino acid derivatives is to be found in the current literature[2]. Recently HEIN AND NIEMANN[3,4] developed a detailed interpretation of the structural and stereospecificity of α-chymotrypsin by considering the significance of all possible interactions of any given asymmetric substrate with an active site of singular conformation. Through examination of the available kinetic data, it was possible to distinguish certain limit type substrates and it became apparent that unambiguous comparisons between substrates could only be made for those which belonged to the same class. Acylated-L-α-amino acid methyl esters represent one such class of substrates for which the principal binding mode between substrate and enzyme involves interaction of the α-amino acid side-chain and ester group of the substrate with their corresponding loci at the active site of the enzyme[3,4].

Typical of the preceding class of substrates is the acylated aromatic α-amino acid ester, acetyl-L-phenylalanine methyl ester[5], a good substrate of α-chymotrypsin with a substration constant, $K_0 = 1.8$ mM and a rate constant $k_0 = 63.1$ sec^{-1}*. However, acetyl-L-valine methyl ester[6], $K_0 = 112$ mM and $k_0 = 0.15$ sec^{-1}, and acetyl-L-alanine methyl ester[7], $K_0 = 611$ mM and $k_0 = 1.3$ sec^{-1} are also substrates.

From the observed values of both substration and rate constants the relative specificity for the aromatic α-amino acid derivative is readily apparent. However, it became of interest to investigate the possibility of finding a completely aliphatic substrate having values for one or both constants approximating those for the supposedly specific aromatic substrates. Acetyl-L-leucine methyl ester appeared to be a likely candidate. Although its hydrolysis by α-chymotrypsin had been reported[8], no kinetic data were available. We have prepared pure acetyl-L-leucine methyl ester and have determined its kinetic behavior with α-chymotrypsin in aqueous solutions at 25.0° pH 7.90 and 0.20 and 0.10 M in NaCl. The compound behaves as a normal trifunctional substrate and its hydrolysis by α-chymotrypsin is inhibited by several typical inhibitors in a fully competitive manner[9].

The kinetic constants obtained for acetyl-L-leucine methyl ester, $K_0 = 2.9$ mM and $k_0 = 4.6$ sec^{-1}, provide considerable information about the structural specificity

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** Based upon a mol. wt. of 25 000 and a nitrogen content of 16.5% for α-chymotrypsin.

of α-chymotrypsin when compared with the values of these constants for a representative series of acetylated-L-α-amino acid methyl esters[3,4]. From this comparison, it may be shown that the above value of K_0 for acetyl-L-leucine methyl ester is close to the upper limit of values of K_0 observed for comparable substrates having aromatic side chains. JENNINGS AND NIEMANN[10] previously demonstrated that replacement of a benzenoid nucleus by a cyclohexyl group had little effect on the kinetic constants of acetyl-L-phenylalaninamide. The present case, however, involves K_0 values which are an order of magnitude lower, reflecting the fact that structurally related esters and amides are not substrates of the same class despite their superficial resemblance.

While a comparison of the constants for acetyl-L-phenylalanine and acetyl-L-leucine methyl esters shows little change in K_0, the effect of replacement of the benzyl by an isobutyl group manifests itself in the values of the rate constants, k_0. The rate constant for the aromatic compound is almost 14 times greater than that of its aliphatic analog. This fact, as well as other data[3,4] suggests that, in addition to its function in binding substrate to enzyme, the side-chain component possibly affects the orientation, relative to the ester group, of either the nucleophile or electrophile presumably present at the active site of the enzyme[3,4]. Although the isobutyl side-chain functions almost as effectively as the benzyl group in a binding role, the aromatic nucleus appears to facilitate nucleophilic or electrophilic attack on the ester carbonyl group. Alternatively, the lower value of k_0 in the case of the aliphatic substrate may be due to a steric effect exerted by the isobutyl group in the neighborhood of the ester group.

The conclusion arrived at above is directly contrary to one reached by BERNHARD AND GUTFREUND[11], who report kinetic constants for acetyl-L-leucine ethyl ester, $K_0 = 21.8$ mM and $k_0 = 20$ sec^{-1} and state, "when the [side chain] is a hydrocarbon ring, binding is strong and catalysis is rapid (acetyl-L-tyrosine ethyl ester). If the [side chain] is replaced by a group with more internal degrees of freedom and less surface (acetyl-L-leucine ethyl ester) the binding affinity is greatly diminished and the specific rate is somewhat diminished." As no experimental data are provided, the results discussed by BERNHARD AND GUTFREUND[11] are difficult to evaluate. They present a table which compares both methyl and ethyl esters in the same series, as well as constants evaluated under different experimental conditions. For example, the kinetic constants for α-*N*-acetyl-L-tyrosine methyl ester, often quoted in the lite-

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-LEUCINE METHYL ESTER*

*No. Expts.***	*(NaCl)* *(M)*	*(S)* *(mM)*	*(E)**** *(μM)*	K_0 *(mM)*	k_0**** *(sec^{-1})*
20–0	0.20	0.204–8.45	0.906–1.47	2.88 ± 0.22	4.63 ± 0.15
7–0	0.20	0.534–3.74	1.32	4.07 ± 1.21	5.24 ± 1.06
8–0	0.10	0.630–5.04	3.46	3.14 ± 0.98	4.41 ± 0.71
				2.9 ± 0.3§	4.6 ± 0.3§

* In aqueous solutions at 25.0 ± 0.1° and pH 7.90 ± 0.10.

** Number of experiments performed for evaluation of K_0 and k_0. The second number refers to those rejected by the statistical reiterative procedure used for evaluation of K_0 and k_0.

*** Based on a mol. wt. of 25 000 and a nitrogen content of 16.5% for α-chymotrypsin.

§ Preferred values.

rature, were obtained[12] for a system 0.1 *M* in $CaCl_2$ and thus are not directly comparable with those obtained in systems containing only univalent ions. Ca^{2+} ion is known[13] to have a specific and still indeterminate effect on the kinetic constants.

N-Acetyl-L-leucine methyl ester was prepared essentially as described by APPLEWHITE *et al.*[8]. From 13.1 g of L-leucine there was obtained 10.4 g of a syrup which was twice recrystallized from a mixture of ethyl ether and pentane to give 5.9 g (32%) of acetyl-L-leucine methyl ester, large colorless cubes, m.p. 46–47°, $[\alpha]_D^{25} - 56.8 \pm 1.0°$ (*c*, 3% in water). Found: C, 57.8; H, 9.1; N, 7.4. Calcd. for $C_9H_{17}NO_3$: C, 57.7; H, 9.2; N, 7.5%. KARRER, ESCHER AND WIDMER[15] report a m.p. of 74–75° and an $[\alpha]_D^{16} - 17.2°$. However, it appears that the preparation obtained by these investigators was substantially racemized. The product obtained in the present studies was comparable to that obtained earlier[8].

The procedure employed for the kinetic studies was identical with that described previously[8,16]. All experiments were conducted in aqueous solutions at 25.0°, pH 7.90 ± 0.10 and 0.10 or 0.20 *M* in NaCl. The enzyme preparation was bovine, crystalline, salt-free α-chymotrypsin obtained from Armour and Co., Lot No. T-97207. The pertinent experimental parameters are summarized in Table I. The primary data were analyzed using the Datatron 220 digital-computer program described recently[16]. As for similar substrates, the data fit the rate equation $d[P]/dt = k_0[E][S]/(K_0 + [S])$. The values obtained for the constants k_0 and K_0 are given in Table I.

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Crystalline phosphoglycerate kinase from human erythrocytes

It is well-known that the main phosphorus compound in most mammalian erythrocytes is 2,3-diphosphoglycerate. Although RAPOPORT AND LUEBERING[1] have suggested that the first step in the enzymic formation of 2,3-diphosphoglycerate in erythrocyte