STEREOSPECIFIC ACTIVATION OF CHYMOTRYPSIN CATALYZED HYDROLYSES*

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Received August 23, 1966

We wish to report that the optical antipodes of (1-carbomethoxy-2-methylpropyl)-trimethylammonium iodide, $[(CH_3)_2CHCH_2CH(CO_2CH_3)N(CH_3)_3]^+ I^-$ (CaMP) are modifiers of chymotrypsin catalyzed hydrolyses of a variety of substrates, and that the L-isomer, but not the D-isomer acts as an activator of the hydrolysis of both benzoyl-D- and L-alanine methyl ester and of the hydrolysis of N^{α}-benzoyl-L-arginine methyl ester. The type of behavior observed for both modifiers depends dramatically on the substrate. The enzyme displays distinct stereospecificity towards the two isomers of the quaternary ammonium salt: significant activation is only observed with the L-antipode.

In general, it has been assumed that the enzyme chymotrypsin possesses but a single binding site. This belief is supported by a great deal of evidence ranging from equilibrium dialysis studies (Loewus and Briggs, 1952) to a kinetic analysis of the observed velocity of the enzyme catalyzed hydrolysis of a typical substrate in the presence of pairs of inhibitors (Rapp, 1964). Studies in which reversible inhibitors "protect" the enzyme from denaturation, the action of irreversible inhibitors and similar phenomena can also be cited to support the view that chymotrypsin has but a single binding cavity.

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* We gratefully acknowledge the support of this investigation by the Public Health Service (Grant GM 1011-03) and the National Science Foundation (Grant GB-795).

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Yet, a certain amount of evidence suggests that chymotrypsin can, under certain circumstances, accommodate at least two small organic molecules. Original reports concerning activation by excess substrate (Wolff and Niemann, 1959; Wolff and Niemann, 1963) have been questioned (Trowbridge, et al., 1963; Ingles and Knowles, 1965) and defended (Wallace, et al., 1964). Less ambiguous is the reported substrate activation of the chymotrypsin catalyzed hydrolysis of N^{α}-toluenesulfonyl-L-arginine methyl ester (Trowbridge, et al., 1963) and the activation of the hydrolysis of bifunctional substrates by 9-aminoacridine (Wallace, et al., 1966).

When one considers that the natural substrates of chymotrypsin are polypeptides, it would not be surprising if the enzyme were capable of interacting with a multi-amino acid segment of such a chain, and there is some indirect evidence to support this view (Neil, et al., 1966). Translated into terms of small substrates or modifiers, it is at least reasonable to suggest that the enzyme can bind two or more of these simultaneously.

MATERIALS AND METHODS

Substrates were prepared in the usual manner by acylation of the corresponding amino acids followed by esterification (Applewhite, et al., 1958). Kinetic studies were carried out on a pH-stat in the manner which has been described in the literature (Applewhite, et al., 1958). The kinetic data were analyzed by the method of Booman and Niemann (1956) with the aid of an IBM 1620 digital computer and a program written as described by Abrash, et al., (1960). Modifiers were prepared from the corresponding D- and L-amino acids by esterification with thionyl chloride in methanol (Brenner and Huber, 1953): The L-ester hydrochloride (53 g., 0.29 mole) was dissolved in 300 ml. of dry acetonitrile and refluxed in a dry system containing 86 g., 0.60 moles of potassium carbonate and 186 g., 1.31 moles of methyl iodide for 60 hours. Additional amounts of methyl

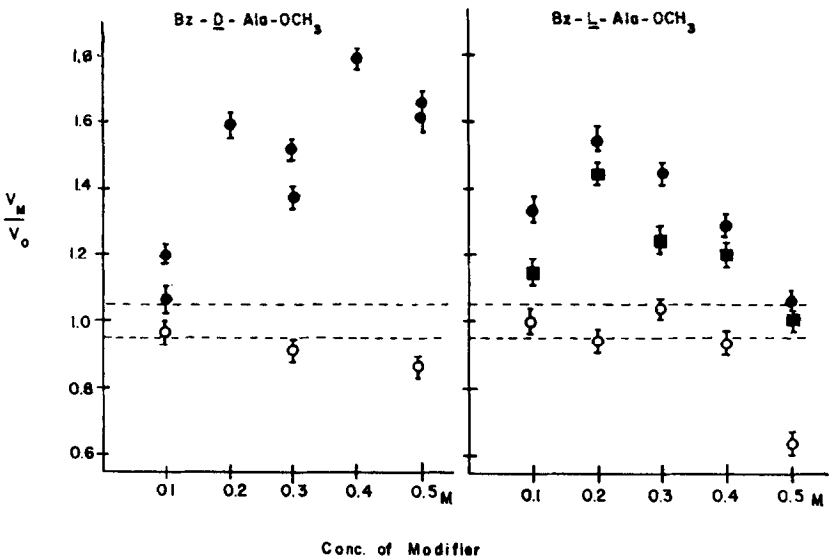
iodide (40 ml., 0.60 moles each time) were added at 12 hour intervals. The mixture was filtered and the solution evaporated to leave a crude product which was crystallized three times from methanol. The yield of pure quaternary salt was 49 g. (54%), m.p. 187-189 dec., $[\alpha]_D$ + 12.0° (c, 2% H_2O). Anal. Calcd: for $C_{10}H_{22}NO_2I$ (315): C, 38.1; H, 7.1; I, 40.3; Found: C, 38.0; H, 6.8; I, 40.3. The D-isomer was prepared similarly in 36% yield, m.p. 185-187 dec., $[\alpha]_D$ - 11.8° (c, 2% H_2O). Anal. Found: C, 37.7; H, 7.1; I, 40.2.

In the experiments reported here, substrate concentrations were approximately equal to K_0 values observed for these compounds in our system (0.5 M KI). All reactions were carried out at 25.0 ± 0.1, at pH 7.9 and total ionic strength 0.5 M consisting of modifier and sodium iodide.

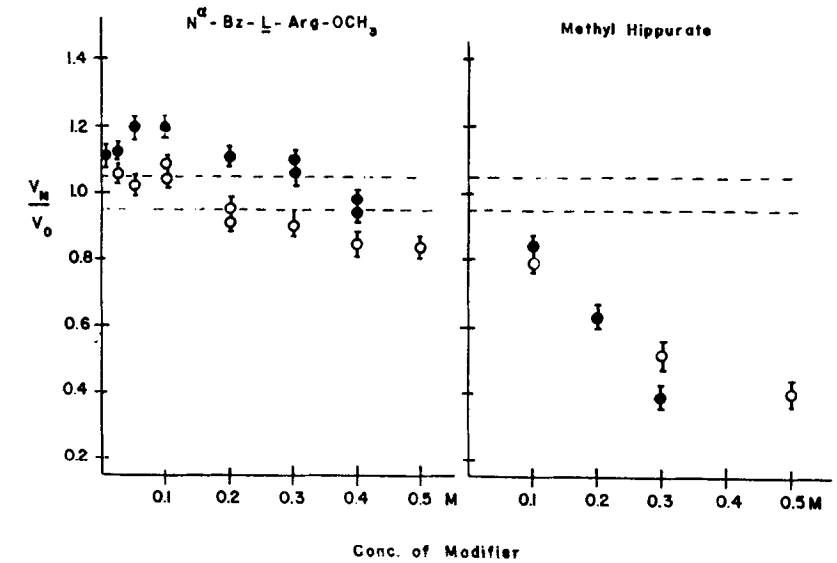
RESULTS AND DISCUSSION

Evidence that both the quaternary ammonium compounds interact with the enzyme could be obtained from the results with methyl hippurate. Both isomers proved to be inhibitors of the reaction in the concentration range studied. The degree of inhibition varied with substrate concentration, but did not follow any simple inhibition type. The representative data are shown in Fig. 3. The effect can be attributed neither to iodide ion nor to quaternary groups in general since the concentration of iodide ion was 0.5 molar in all experiments and independent determinations showed that tetramethylammonium iodide had no effect on the reactions studied up to the limit of its solubility (0.25 M) at constant ionic strength. The hydrolysis of neither optical antipode of CaMP was catalyzed by the enzyme.

Despite the evidence that both modifiers reversibly interact with the enzyme, only the L-antipode showed significant modification of the rate of hydrolysis of benzoyl-L- and D-alanine methyl esters. In both cases activation was observed, and for the L- compound a maximum was



Figures 1 and 2. Effect of L- and D- CaMP on the chymotrypsin catalyzed hydrolyses of benzoyl-D-alanine methyl ester and benzoyl-L-alanine methyl ester. All reactions at 25.0°, pH 7.9, total ionic strength 0.5 M by addition of sodium iodide. Fig. 1: ● L-CaMP, [S] = 2.39 X 10^{-3}M; o D-CaMP [S] = 2.39 X 10^{-3}M. Fig. 2: ● L-CaMP, [S] = 0.912 X 10^{-3}M; ■ L-CaMP, [S] = 4.44 X 10^{-3}M; o D-CaMP, [S] = 2.38 X 10^{-3}M.



Figures 3 and 4. Effect of L- and D-CaMP on the chymotrypsin catalyzed hydrolyses of N^{α}-benzoyl-L-arginine methyl ester and methyl hippurate. All reactions at 25.0°, pH 7.9, total ionic strength 0.5M by addition of sodium iodide. Fig. 3: ● L-CaMP, [S] = 14.6 X 10^{-3}M; o D-CaMP, 12.3 X 10^{-3}M. Fig. 4: ● L-CaMP, [S] = 1.91 X 10^{-3}M; o D-CaMP, [S] 2 2.36 X 10^{-3}M.

noted at approximately 0.2 M. (Fig. 1 and 2).

As might be expected, the degree of activation was dependent both on the concentration of substrate and of modifier. Finally, similar results were obtained when the two compounds were tested as modifiers of the chymotrypsin catalyzed hydrolysis of N^{α}-benzoyl-L-arginine methyl ester. A qualitative summary of the results is presented in Table I.

Table I

Behavior of D- and L-(1-carbomethoxy-2-methylpropyl)-trimethylammonium iodide as modifiers of the chymotrypsin catalyzed hydrolyses of acylated amino acid esters.[a]

	Effect of ammonium salt	
Substrate	L-isomer	D-isomer
methyl hippurate	mixed inhibition	mixed inhibition
Benzoyl-L-alanine methyl ester	activation	slight inhibition
Benzoyl-D-alanine methyl ester	activation	slight inhibition
N^{α}-Benzoyl-L-arginine methyl ester	activation	inhibition

[a] All reactions at 25.0°C, pH 7.9, total ionic strength 0.5 M, consisting of modifier and NaI.

The substrate dependent behavior of L-CaMP and the different type of behavior of its antipode, D-CaMP, adds a new dimension to the study of chymotrypsin specificity. The observation of activation of catalytic activity with some compounds is *prima facie* evidence for the formation of a ternary enzyme-substrate-modifier complex. The secondary site occupied by the modifier shows stereospecificity and also at least some structural specificity since other organic molecules, e.g., indole, inhibit the enzyme catalyzed hydrolyses of the alanine derivatives (Hein and Niemann, 1962).

The type of explanation suggested by Inagami and Murachi (1964) for trypsin does not appear to be applicable, since the alanine deriva-

tives and N^{α}-benzoyl-L-arginine methyl ester must be considered as "trifunctional" substrates. It may be significant that no activation is observed with better chymotrypsin substrates. Both L- and D-CaMP have a slight inhibitory effect on the enzyme catalyzed hydrolyses of acetyl-L-valine and -phenylalanine methyl esters.

It may also be significant that L-CaMP, N^{α}-toluenesulfonyl-L-arginine methyl ester and 9-aminoacridine, the compounds which activate chymotrypsin towards some substrates, all possess a positive charge. It is conceivable that chymotrypsin contains a "secondary", anionic binding site, and even that this site plays a functional role in the secondary specificity of the enzyme towards polypeptide substrates (Neil, et al., 1966). However, such speculations must await further experiments, possibly using suitable enzyme modifiers.

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