INHIBITION OF THE ESTERASE ACTION OF CARBOXYPEPTIDASE A

E. T. Kaiser and F. W. Carson

Department of Chemistry, University of Chicago

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Recent studies on carboxypeptidase A have shown that the chemical modification of this enzyme with certain reagents leads to an increase in its esterase activity and a decrease in its peptidase activity. (Vallee and coworkers, 1963, 1964.) In addition, the pH rate profile for the hydrolysis of an ester substrate, hippuryl-DL-β-phenyllactic acid, was reported to be different from that observed in the hydrolysis of peptide substrates. These kinetic results led to the postulation of different mechanisms for the hydrolysis of esters and of peptides by carboxypeptidase A. This would seem to be the first case in which the esterolytic and proteolytic mechanisms of catalysis by an enzyme do not parallel one another.

In order to examine in more detail these apparent hydrolytic dissimilarities we have studied the esterase action of carboxypeptidase A both in the presence and in the absence of compounds known to competitively inhibit the peptidase action of this enzyme. O-hippuryl-L-mandelic acid was found to be an excellent ester substrate for use in this investigation. The ester was prepared in 94% yield by heating an equimolar mixture of 2-phenyl-5-oxazolone (Cornforth, 1949; Carter, Harrison, and Shapiro, 1953) and L-mandelic acid in benzene under reflux for 1 1/2 hours. The product, m.p. 166-167.5° (uncorr.), formed as an insoluble precipitate in the reaction mixture; recrystallization from 8:1 v/v 1,2-dichloroethane - acetonitrile raised the m.p. to 167.0-168.1°

(uncorr.). The pure ester had $[\alpha]_D^{24}$ + 208 (c 3.135, dimethylformamide) and gave both a satisfactory elemental analysis and the expected acid equivalent. The ester linkage was hydrolyzed to the extent of 100 ± 2 % by carboxypeptidase A in those kinetic runs for which end-points were obtained.

The kinetics of the carboxypeptidase A - catalyzed hydrolysis of O-hippuryl-L-mandelate were followed by the pH-Stat titration procedure described by us elsewhere. (Kaiser and Carson, 1964.) The initial velocity of reaction of 0.01 M O-hippuryl-L-mandelate at pH 7.5 and 25.0° is about 440 times faster than the rate for 0.01 M O-acetyl-L-mandelate, the substrate examined in our previous investigation, allowing much lower enzyme concentrations (ca. 10^{-7} M) to be used for the present study. A plot of $[E]_o/v$ vs. $1/[S]$ (Fig. 1) (Lineweaver and Burk, 1934.) shows a deviation from linear

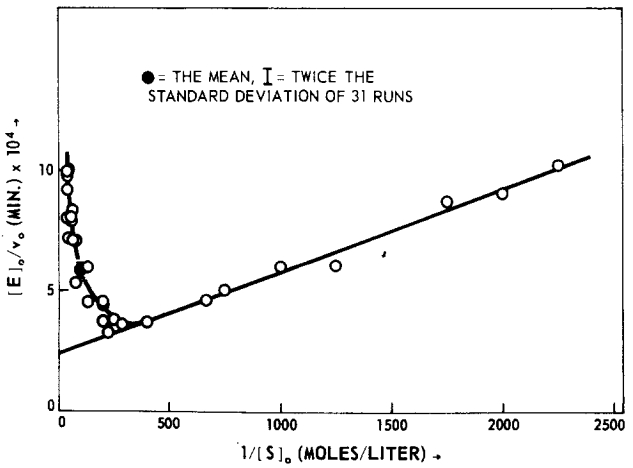

Fig. 1. Lineweaver-Burk plot for the Hydrolysis of Hippuryl-L-Mandelic Acid by Carboxypeptidase at 25° in 0.5 M Sodium Chloride, 2.5 x 10^{-4} M Tris, pH 7.5.

ity because of substrate inhibition at concentrations of O-hippuryl-L-mandelate above 3 x 10^{-3} M. The value of the ratio K_m/k_2 (where

k_2 is the rate constant for the breakdown of the enzyme-substrate complex to products) obtained as the slope of the linear part of the plot at low substrate concentrations is 3.41×10^{-7} min.-moles/l. (See Table I).

TABLE I

Inhibitor	[I] range in ester studies M x 10^3	$K_{i,est.}$* M x 10^3	$K_{i,pep.}$** M x 10^3	$K_{i,est.}/K_{i,pep.}$
2-phenylacetate	1 - 6	0.41	0.39	1.05
3-phenylpropionate	0.8 - 2.4	0.152	0.062	2.45
4-phenylbutyrate	6 - 30	1.37	1.13	1.21

* Determined for solutions containing 0.01 M hippuryl-L-mandelate, 0.5 M sodium chloride, 2.5×10^{-4} M Tris, and 9.35×10^{-8} M carboxypeptidase A, pH 7.5 at 25.0°; about 0.77 % v/v of dimethylformamide was in each 5.00 ml. reaction mixture. For O-hippuryl-L-mandelate K_m/k_2 was 3.41×10^{-7} min.-moles/l.

** Determined for solutions containing about 0.01 to 0.05 M carbobenzyloxyglycyl-L-phenylalanine, 0.1 M lithium chloride, 0.04 M phosphate buffer, and enough carboxypeptidase A to cause more than 60 % hydrolysis within 120 min., pH 7.5 at 25.0°.

With the ratio K_m/k_2 in hand, inhibitor constants K_i were determined by means of the equation

$$\frac{v}{v_i} = 1 + \frac{1}{K_i} \left(\frac{k_m}{k_2[E]_o}\right) \left(\frac{v[I]}{[S]_o}\right) \qquad (1)$$

where v represents the uninhibited velocity at any point and v_i is the velocity under the same conditions but in the presence of a competitive inhibitor. In typical experiments the uninhibited hydrolysis of O-hippuryl-L-mandelate was allowed to proceed for a time, and the inhibitor was then injected. Velocities were found by the calculation of the slopes of the

experimental traces of titrant consumed vs. time at the time of injection of the inhibitor. Fig. 2 shows that the data for the inhibition of the hydrolysis of the ester by 2-phenylacetate, 3-phenylpropionate, and 4-phenylbutyrate are well represented by equation 1.

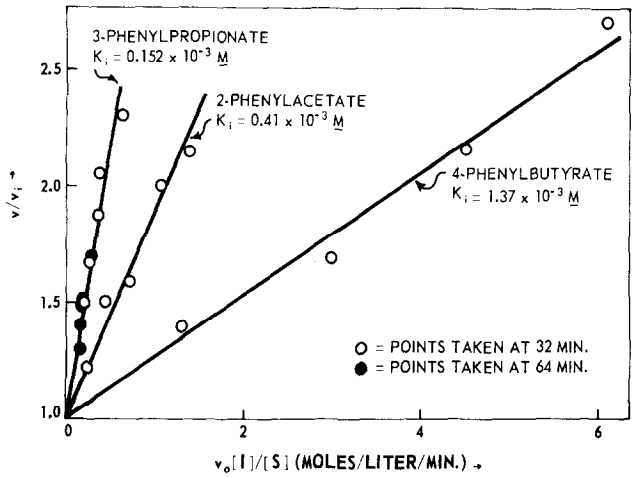

Fig. 2. Plot to test for competitive inhibition in the Hydrolysis of Hippuryl-L-Mandelate by Carboxypeptidase A.

In Table I values of $K_{i,est.}$, the inhibitor constants obtained in this study for the inhibition of esterase action by the carboxylate anions, are compared with the values of $K_{i,pep.}$, the inhibitor constants found by previous investigators (Elkins-Kaufman and Neurath, 1948; Neurath and Schwert, 1950) for the competitive inhibition by these species of the hydrolysis of the dipeptide, carbobenzyloxyglycyl-L-phenylalanine. The ratio $K_{i,est.}/K_{i,pep.}$ is near to unity for 2-phenylacetate and 4-phenyl butyrate although the agreement between the inhibitor constants

for 3-phenylpropionate is not quite as good.

The close correspondence which we have found between the values of $K_{i,est.}$ and $K_{i,pep.}$ strongly suggests that similar, and perhaps identical, enzyme-inhibitor complexes are involved in the inhibition of the esterase and of the peptidase action of carboxypeptidase A. The relationship between the enzyme-substrate complexes formed during the carboxypeptidase-catalyzed hydrolyses of esters and of peptides is now being examined in our laboratory.

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References-

(1) B. L. Vallee, J. F. Riordan, and J. E. Coleman, Proc. Nat. Acad. Sci. U. S., 49, 109 (1963).

(2) R. T. Simpson, J. F. Riordan, and B. L. Vallee, Biochemistry, 2, 616 (1963).

(3) J. F. Riordan and B. L. Vallee, ibid., 2, 1460 (1963).

(4) B. L. Vallee, Federation Proceedings, 23, 8 (1964).

(5) J. W. Cornforth in "The Chemistry of Penicillin", (H. T. Clarke, J. R. Johnson, and Sir R. Robinson, Eds.), Princeton University Press, Princeton, N. J., 1949, p.778. In our work the oxazolone was prepared by the modification of Cornforth's procedure described by Carter et al in ref. 6.

(6) H. E. Carter, J. B. Harrison, and D. Shapiro, J. Am. Chem. Soc., 75, 4705 (1953).

(7) E. T. Kaiser and F. W. Carson, ibid., 86, 2922 (1964).

(8) H. Lineweaver and D. Burk, ibid., 56, 658 (1934).

(9) E. Elkins-Kaufman and H. Neurath, J. Biol. Chem., 178, 645 (1948).

(10) H. Neurath and G. W. Schwert, Chem. Rev., 46, 69 (1950).