DETERMINATIONS OF THE NORMALITY OF PIG LIVER CARBOXYLESTERASE SOLUTIONS

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Received February 23, 1966

Jansz et al. (1959) reported that the amino acid sequence around the active centre of horse liver carboxylesterase is

gly-glu-$\overset{*}{\text{ser}}$-ala

where the starred serine is the one which is phosphorylated with diisopropyl phosphofluoridate (DFP). This is very similar to the active centre sequence of the serine proteinases (Hartley, 1960, 1964; Keil, 1964; Walsh et al., 1964 a, b; Neurath, 1964; Walsh and Neurath, 1964; Mikeš et al., 1964),

gly-asp-$\overset{*}{\text{ser}}$-gly

where the starred serine is again the one which is phosphorylated with DFP (Cohen et al., 1959). Further, the other carboxylesterases are similarly inhibited by DFP (Jansz, 1959; Webb, 1948; Keay and Crook, 1965). These similarities suggest a possible similarity of mechanism for the two groups of enzymes. It is now well established that the hydrolysis of carboxylic esters catalyzed by α-chymotrypsin and trypsin proceeds via an acyl-enzyme intermediate (Bender et al., 1962; Bender and Zerner, 1962; Bender and Kaiser, 1962; Zerner and Bender, 1964; Zerner et al., 1964).

Therefore, we are investigating the mechanism of action of a number of liver esterases (pig, horse, ox and chicken). In this communication we report on the reaction of pig liver carboxylesterase (Horgan, Webb and Zerner, 1966) with p-nitrophenyl dimethylcarbamate (NPDMC)* and p-nitrophenyl diethyl phosphate (E 600)**. These reactions reveal an initial rapid release of p-nitrophenol followed by a much slower zero-order turnover of substrate, the kinetics of which are consistent with the scheme

$$E + S \overset{K_s}{\rightleftarrows} ES \overset{k_2}{\rightarrow} \underset{+\ P_1}{ES'} \overset{k_3}{\rightarrow} E + P_2 \qquad (1)$$

where E is the enzyme; S is NPDMC or E 600; ES' is dimethylcarbamyl- or diethylphosphoryl-enzyme; P_1 is p-nitrophenol; P_2 is the acid moiety of the substrate; k_2 and k_3 are the rate constants of carbamylation (phosphorylation) and decarbamylation (dephosphorylation), respectively, and where k_3 is rate-limiting with both substrates. The kinetics allow the first direct determination of the normality of this enzyme and hence the number of active sites per molecular weight of 163,000.***

The kinetics of hydrolysis of NPDMC catalyzed by pig liver carboxylesterase was followed at 400 mμ in the thermostatted cell compartment of a Cary 14 spectrophotometer fitted with a 0-0.1

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* Synthesized from dimethylcarbamoyl chloride (Aldrich) and p-nitrophenol in pyridine; m.p. 106°-107°; Anal. Calcd. for $C_9H_{10}N_2O_4$: C, 51.43; H, 4.80; N, 13.33. Found C, 51.90; H, 4.86; N, 12.85. For the use of dimethylcarbamoyl derivatives as enzyme reagents see Wilson et al. (1960, 1961) and Bender and Stoops (1965).

** We acknowledge with thanks the gift of the E 600 from Albright and Wilson, London.

*** Preliminary studies with p-nitrophenyl diphenylcarbamate reveal no "burst", but the substrate is slowly hydrolyzed by the enzyme.

absorbance slide wire, at 25° ± 0.1°. Extrapolation of the zero-order turnover of substrate to zero time gives the quantity A_{burst} of Table I, from which the normality of the enzyme solution may be calculated using the expression (Ouellet and Stewart, 1959)

$$[P_1]_{burst} = \left[\frac{k_2}{k_2 + k_3} \cdot \frac{[S]_o}{[S]_o + K_{m(app)}} \right]^2 \cdot [E]_o \qquad (2)$$

Representative data are shown in Table I. In addition, the following conditions have been shown to be satisfied by a variety of kinetic procedures (Kézdy and Bender, 1962; Bender et al., 1965). (a) $k_2 \sim 200\ k_3$; (b) $[S]_o \gg K_{m(app)}$ ($K_{m(app)} \sim 5 \times 10^{-6}$ M); (c) $[S]_o >> [E]_o$. The titration is reproducible to ± 2%.

A similar experiment with E 600 gives an identical burst (± 1%). The titration curve with E 600, however, differs in that phosphorylation is faster and dephosphorylation slower than the corresponding reactions with NPDMC. Nonetheless, there is real turnover of E 600 by this enzyme. This is in contrast to the results of Keay and Crook (1965).

That both of these inhibitions occur at the active site is shown by competitive assays with p-nitrophenyl butyrate which is an exceedingly good substrate for the enzyme.

The dry weight of the purified enzyme was determined after exhaustive dialysis against distilled water by lyophilization in a high vacuum system, until no trace of water could be detected by standard high vacuum procedures. The protein was then transferred under vacuum to a dry box and ca. 10 mg. accurately weighed out. It was found that a concentration of 1 mg. ml^{-1} has an absorbance of 1.305 at 280 mμ (0.15 M Tris, pH 8.16). This result, together with the normality of the

TABLE I

TITRATION OF PIG LIVER CARBOXYLESTERASE[a]

[NPDMC] x 10^4 M	A_{burst} [b]	[ENZYME] x 10^6 N [c]
0.522	0.0685	3.82
1.045	.0715	3.99
2.089	.0722	4.03
4.179	.0724	4.04
5.224	.0724	4.04

[a] 7.46% CH_3CN; 0.15 M Tris, pH 8.16; [b] at 400 mμ; [c] calculated taking account of the relevant constants.

enzyme, gives an equivalent weight of 78,000. It is clear, therefore, that the enzyme contains two active sites per 163,000 molecular weight. Whether the 163,000 unit is a dimer is not yet known, but fragmentation studies are being actively pursued in this Laboratory.

Dialysis of the dimethylcarbamyl enzyme against buffer regenerates full activity. Hydroxylamine causes an initial acceleration of decarbamylation but long exposure causes inactivation of the enzyme. This may be prevented by the addition of cysteine which suggests that the denaturation may be due to the oxidation of sulfhydryl groups (Fishbein and Carbone, 1965).

The determination of the normality of the enzyme has made possible the calculation of rate constants for the hydrolysis of the classical

substrates (butyrate and acetate esters) of this enzyme (Dixon and Webb, 1964). Preliminary studies show that the pig liver carboxylesterase hydrolyzes p-nitrophenyl acetate some 10^5 times as efficiently as α-chymotrypsin at pH 7.4! The function of these enzymes in the liver and their mechanism of action continue under active investigation in this Laboratory.

Acknowledgements

We acknowledge with thanks the indefinite loan of a Cary 14 spectrophotometer from the Wellcome Trust, London. This work was supported in part by the N.H.M.R.C. (Australia). It was communicated in part at the Melbourne Meeting of the Australian Biochemical Society in August, 1965.

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