THE INVOLVEMENT OF HISTIDINE IN THE ACTION OF LIVER CARBOXYLESTERASES

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Summary:

Chicken and ox liver carboxylesterases are inhibited irreversibly by α-bromoacetophenone. A kinetic analysis of the process indicates that inhibition occurs at the active site by reaction with a group of pK_a' 6.15 which is reactive in the basic form. Using [^{14}C]bromoacetophenone, it has been shown that loss of activity is accompanied by incorporation of one molecule of inhibitor per active site, together with some non-specific reaction. Acid hydrolysis of the inhibited enzyme leads to a number of radioactive products, including 1- and 3-phenacylhistidines. Chicken liver carboxylesterase is irreversibly inhibited by methyl *p*-nitrobenzenesulfonate. This inhibition is prevented by the presence of the competitive inhibitor, n-propanol.

Carboxylesterases (E.C. 3.1.1.1) bear a marked family resemblance to two important groups of enzymes, the cholinesterases and the serine proteinases. Notable similarities include the sensitivity to organophosphates and carbamates, the existence of a uniquely reactive serine residue in the protein molecule, and the similarity of the amino acid sequences adjacent to that serine residue (Augusteyn *et al.*, 1969). It has been shown that a histidine residue is a component of the catalytic mechanism of α-chymotrypsin and other serine proteinases. To date, however, there has been little evidence that histidine is important in the action of carboxylesterases. Photo-oxidation of pig liver carboxylesterase causes loss of both activity and histidine, but the gross and non-specific effects accompanying the reaction make the result of doubtful significance (Pahlich *et al.*, 1969). Heymann and Krisch (1972) studied the effect of a number of alkylating agents, including α-bromoacetophenone, on pig-liver carboxylesterase. They concluded, on tenuous grounds, that the observed inhibition by α-bromoacetophenone was due to non-specific modification. We now report some experiments with the alkylating agents α-bromoacetophenone and methyl *p*-nitrobenzenesulfonate which demonstrate the importance of histidine in the action of carboxylesterases.

Experimental Section

[^{14}C] Bromoacetophenone was synthesized from acetophenone-7-^{14}C (0.019 Ci/mole; I.C.N.) by the method of Cowper and Davidson (1943), and purified by sublimation and recrystallization from ether-petroleum

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ether: mp 50.5-51°, lit. mp 49-51° (Cowper and Davidson, 1943). Chicken and ox liver carboxylesterases were purified according to procedures developed in this laboratory. Carboxylesterase activity was determined spectrophotometrically at 25° using a Cary 14 recording spectrophotometer. *o*-Nitrophenyl butyrate and *p*-nitrophenyl acetate were both used as substrates, the release of the nitrophenols being followed at 416 nm and 400 nm respectively. Assays were performed in phosphate buffer at pH 7.4. The concentrations of the carboxylesterases were determined by titration of the active sites with paraoxon (Horgan *et al.*, 1969).

Results and Discussion

Kinetics of the Inactivation Reaction

The effect of α-bromoacetophenone (BAP) on the carboxylesterases was determined by incubation of enzyme (~3 x 10^{-7} N) with BAP at 25° in 0.05 M Tris-HCl buffer, pH 8.34, 3.1% v/v acetonitrile, and assaying 100-μl aliquots in the standard assay with *o*-nitrophenyl butyrate or *p*-nitrophenyl acetate. Activity was lost rapidly in a reaction which was first-order for 2-3 half-lives. Eventually, inactivation was complete (~1% activity after 9 hours with $[BAP]_0$ = 4.42 x 10^{-5} M) and was not reversed by exhaustive dialysis at 4° against 0.05 M Tris-HCl buffer, pH 7.56.

Table I shows the effect of BAP concentration on the first-order rate constant for the inactivation, k_{obs}, at pH 8.34. Since BAP is a substrate analogue, it may be proposed that the enzyme-inhibitor complex is an intermediate in the reaction leading to irreversible loss of activity, according to the kinetic scheme, $E + I \overset{K_i'}{\rightleftharpoons} EI \xrightarrow{k_1} E^*$, where E* is the irreversibly inhibited enzyme. If this scheme is operative, a plot of k_{obs}^{-1} against $[I]^{-1}$ should be linear, and yield values of K_i' and k_1. Double reciprocal plots of data obtained for the chicken enzyme at pH 8.34 (Table I) and at a number of other pH's were linear, giving values for k_1 at a number of pH's. Figure 1 shows the effect of pH on the ratio $k_{1\ meas.}/k_{1\ pH\ 8.34}$. These data clearly show the dependence of the inhibition on the ionization of a single residue of pK_a' 6.15 (histidine ?), active in the basic form.

The value of K_i' at pH 8.34 was found to be 3.2 x 10^{-5} M. As the *irreversible* inhibition has a half-life of about 50 min at pH 8.34, it is possible to examine BAP as a *reversible* inhibitor. BAP was found to be a competitive inhibitor of the hydrolysis of *o*-nitrophenyl butyrate, with a K_i at pH 8.34 of 3.3 x 10^{-5} M. The agreement between K_i and K_i' is excellent evidence that the inhibition is caused by modification of a residue at the active site of the enzyme.

Stoichiometry of the Reaction with BAP

Chicken liver carboxylesterase (8.39 x 10^{-6} N) in 0.05 M Tris-HCl buffer, pH 8.34, was incubated with 1.06 x 10^{-4} M [^{14}C]BAP. Samples were withdrawn periodically and dialysed exhaustively against Tris-HCl buffer, pH 7.56, to remove inhibitor. The specific activity and amount of enzyme-bound carbon-14 were then determined

TABLE I

Effect of α-Bromoacetophenone Concentration on the Inhibition of Liver Carboxylesterases[a]

Species	10^5 $[BAP]_0$ (M)	10^4 k_{obs} (sec^{-1})
Chicken	12.8	1.92
	9.83	1.71
	6.39	1.58
	4.42	1.36
Ox	9.85	0.43
	4.92	0.28

[a] In 0.05 M Tris-HCl buffer, pH 8.34.

(Table II). Extrapolation of these results indicates that complete inhibition corresponds to the incorporation of 1.45 molecules of inhibitor per enzyme active site. This in itself argues for the modification of one essential amino acid residue, accompanied by some non-specific modification. This interpretation is supported by the behaviour of the diethylphosphoryl-enzyme. The presence of a diethylphosphoryl group at the active site is likely to prevent alkylation of an adjacent residue. Hence the label incorporated in this experiment gives an estimate of the non-specific reaction, and so the incorporation of 0.84 moles of inhibitor (1.21-0.37) results in 78% inhibition, which is close to a stoichiometric reaction.

Identification of the Products of Reaction of Carboxylesterase with BAP

Chicken liver carboxylesterase was reacted with [^{14}C]BAP (1.26×10^{-4} M) for 1.5 h before dialysis and acid hydrolysis (5.7 M HCl, 110°, 24 h). The hydrolysate was subjected to paper chromatography in n-butanol-acetic acid-water (40:6:15 by vol), and the radioactive materials located by counting 1-cm strips. Similarly, N-acetyl-L-histidine (0.1 mole) and [^{14}C]BAP (0.1 mole) were reacted in 0.5 ml of acetonitrile and 1 ml of water for 24 hours. The reaction mixture was dried, and subjected to acid hydrolysis and paper chromatography as above. R_f values of the radioactive species present in the hydrolysates are listed in Table III.

After acid hydrolysis, about 60% of the radioactivity originally bound to the inhibited enzyme was

TABLE II

Correlation of the Extent of Inhibition of Chicken Liver Carboxylesterase by [^{14}C]BAP with the Amount of Inhibitor Bound

Sample	Reaction time (min)	Moles inhibitor[a]/moles enzyme	% Inhibition[b]
Control[c]	0	0	0
Paraoxon-treated enzyme[d]	120	0.37	–
Inhibited esterase	37	0.80	56
" "	65	0.95	63
" "	122	1.21	78
" "	184	1.32	87

[a] Samples after dialysis were taken to dryness, dissolved in Soluene solubilizer, and counted in toluene-Liquiflor scintillator, using [^{14}C] BAP as internal standard.

[b] % Inhibition is obtained by comparing the specific activities of inhibited and control enzyme after dialysis.

[c] Enzyme subjected to the same treatment as inhibited samples, but in the absence of BAP.

[d] Paraoxon (50 μl of 2.93 mM solution in CH_3CN) was added to 2 ml of enzyme (8.39×10^{-6} N). When the rapid release of *p*-nitrophenol was complete, an aliquot of BAP was added.

TABLE III

	R_f Values of Radioactive Products				
Carboxylesterase	0.29	0.54	0.69	0.88	
N-Acetyl-L-histidine	0.32	0.53	0.66	0.89	0.97

lost during the removal of the HCl. A similar loss of radioactivity occurred in the N-acetyl-L-histidine model reaction. In both cases, the major volatile radioactive material after hydrolysis was found to be acetophenone, which was identified by conversion to the dinitrophenylhydrazone and comparison with authentic acetophenone dinitrophenylhydrazone by chromatography in two solvent systems.

To characterize further the non-volatile products of acid hydrolysis (Table III), 10 mmoles of both N-acetyl-L-histidine and BAP were reacted in 20 ml of a 50% aqueous solution of acetonitrile for 5 h at pH 9, the

TABLE IV

Inhibition of Chicken Liver Carboxylesterase by Methyl *p*-Nitrobenzenesulfonate[a]

$[MNBS]_0$ (mM)	% Inhibition
3.59	77
1.79	55
0.90	34
0	0

[a] $[E]_0 = 1.49 \times 10^{-6}$ N; 0.05 M Tris-HCl buffer, pH 7.50; 9.1% v/v CH_3CN. Reaction mixtures were incubated for 24 h at 25°, then 20-μl aliquots were assayed against *p*-nitrophenyl acetate.

solvent removed, and the residue hydrolysed in 5 M HCl for 6 h at 105°. After removal of the HCl, the products were subjected to ion-exchange chromatography on Amberlite IRC-50, resulting in the isolation of two main fractions. These compounds (as the hydrochlorides) had R_f values of 0.32 and 0.66 (*cf.* Table III), gave a positive test for a ketone function, and had virtually identical NMR spectra (100 mg/ml, D_2O), which were consistent with their identification as 1- and 3-phenacylhistidines.

Inhibition of Chicken Liver Carboxylesterase by Methyl p-*Nitrobenzenesulfonate*

Methyl *p*-nitrobenzenesulfonate (MNBS) has been shown by Nakagawa and Bender (1970) to react specifically with histidine-57 in α-chymotrypsin, causing almost complete inhibition. Acid hydrolysis of the inhibited enzyme showed the presence of 1 mole of 3-methylhistidine per mole of enzyme. Table IV shows the effects of different concentrations of MNBS on the residual activity of chicken liver carboxylesterase. Studies of the kinetics of the inhibition are made difficult by the spontaneous hydrolysis of the ester ($t_{1/2}$ = 100 min in 0.05 M Tris-HCl, pH 7.50, 25°), which occurs on the same time scale as the inhibition of the enzyme. n-Pentanol, a competitive inhibitor of chicken liver carboxylesterase ($K_i = 8.2 \times 10^{-4}$ M), at a concentration of 40 mM protects the enzyme completely against inhibition by MNBS (3.59 mM). Lower concentrations of pentanol afford partial protection.

More complete inhibition of the enzyme (2.9×10^{-6} N) was achieved by successive treatments with MNBS (initially 3.59 mM), with dialysis after each treatment (4 h at 25°) to remove acetonitrile and excess reagents and products. After four treatments, the specific activity had fallen to 4.1% of that of a control subjected

to the same treatment without MNBS. Acid hydrolysis and amino acid analysis of this inhibited enzyme showed the presence of 1- and 3-methylhistidines in the hydrolysate. It seems probable that the histidine residue being modified by MNBS is the same residue that reacts with BAP.

Conclusion

The present data show the presence of an essential histidine residue in the active site of chicken and ox liver carboxylesterases. Future experiments will be directed towards:

(i) establishing the amino acid sequence around the essential histidine residue;

(ii) determining whether carboxylesterase inhibited by MNBS possesses residual activity, as does methylated α-chymotrypsin (Henderson, 1971);

(iii) extending these observations to include acetylcholinesterase, since preliminary experiments show that acetylcholinesterase is inactivated by BAP and MNBS, as well as other histidine-directed reagents.

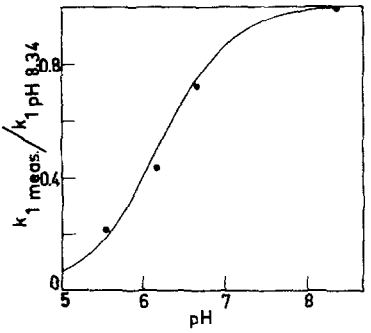

Figure 1. The effect of pH on the rate constant (k_1) for the inactivation of chicken liver carboxylesterase by BAP. Buffers: 0.05 M 2-(N-morpholino)-ethanesulfonic acid, pH 5.54, 6.14 and 6.65, and 0.05 M Tris-HCl, pH 8.34. The curve is calculated for pK'_a 6.15.

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