IS HISTIDINE INVOLVED IN THE CATALYTIC MECHANISM OF UNSPECIFIC CARBOXYLESTERASES ?

Eberhard Heymann and Gerda Marcussen-Wulff

Biochemisches Institut im Fachbereich Medizin,

D-23 Kiel, Neue Universität, Otto-Meyerhof-Haus

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Summary: The reaction between the liver carboxylesterases from ox and pig and the inhibitor α-bromoacetophenone was studied by amino acid analysis. A significant modification of histidine in pig liver esterase was not found, but there was a slight loss of some other residues. In ox liver esterase the total inhibition correlated with the loss of about 1.7 histidine residues. However, in contrast to previous results with chicken and ox esterases the specific active-site-directed inhibitor E 600 did not prevent the modification of the reactive histidine. It is concluded that an earlier report on the involvement of histidine in the action of liver esterases (1) is partly incorrect or perhaps applicable only to chicken liver esterase.

Willadsen et al. (1) reported on the modification of a single histidine residue in the active center of chicken and ox liver esterases (EC 3.1.1.1) by α-bromoacetophenone. This was concluded from kinetic evidence, from qualitative autoradiographic studies of the hydrolysate of enzyme labeled with [^{14}C]-bromoacetophenone, and from quantitative measurement of the incorporation of this radioactive inhibitor. However, in preliminary investigations (2) with alkylating agents we did not find any similarity in the behaviour of the basic group of the active site between pig liver esterase and proteases like chymotrypsin or trypsin, which are known to bear a histidine residue at the active site. Furthermore, the results of Willadsen et al. (1) are in disagreement

Abbreviation: E 600 = diethyl-4-nitrophenyl phosphate

with our previous finding (2) that the inhibition of pig liver esterase by α-bromoacetophenone was not delayed in the presence of substrate. Since it was not clear whether this discrepancy was due to the use of different animals, we have re-examined the reaction of pig and ox liver esterase with α-bromoacetophenone.

EXPERIMENTAL

The liver carboxylesterases were prepared according to our new methods (3). The preparations used here were of high purity, because their equivalent weights, estimated with E 600, were 60 500 (pig liver esterase) and 60 000 (ox liver esterase), respectively (4). α-Bromoacetophenone was purchased from Fluka AG (Buchs, Switzerland). E 600 was kindly provided by Bayer (Leverkusen/GFR).

Inhibition experiments: The esterases ($\approx 5 \times 10^{-5}$ M) were dissolved in 0,01 M ammonium carbonate (pH 7). Only freshly prepared solutions of the inhibitors α-bromoacetophenone ($\approx 10^{-3}$ M) and E 600 ($\approx 10^{-3}$ M) in water were used. 50 - 70 nmoles of native esterase were treated with several 500 nmole portions of α-bromoacetophenone at 30° C until the residual activity (measured with methyl butyrate (3)) had lowered to 10 - 15 % (about 3 - 4 h). The excess of inhibitor was then removed by gel filtration with Sephadex G 50 (Pharmacia, Sweden). - In control experiments the same amount of esterase was pretreated with 100 nmoles of E 600 so that the residual activity was 0 within 15 min. The pre-inhibited esterase was then treated with α-bromoacetophenone and desalted under exactly the same conditions.

Amino acid analyses: The estimation of tryptophane was performed chemically (5) and spectrophotometrically (6). In both cases the presence of acetophenone-residues caused irregular results. It was impossible, therefore, to estimate the tryptophane contents in the modified enzymes. Free SH-groups were estimated according to Ellman (7). All other amino acids were determined from the acid hydrolysate with an automatic amino acid analyzer (NC 1,

Table 1 - Number of amino acids in native and in chemically modified pig liver esterase.

residues per mole of esterase [a]				
	24-h-hydrolysis			fully corrected values in native esterase [b]
	without inhibitor	with BA	with E 600 and BA	
Val	40.0 ± 0.2	39.7 ± 0.5	38.3 ± 0.2	43.7
Cys	3.0 ± 0.1	2.2 ± 0.2	3.0 ± 0.2	4.0
Met	13.2 ± 0.3	13.0 ± 0.1	12.5 ± 0.4	12.1
Ile	15.9 ± 0.2	15.7 ± 0.3	15.7 ± 0.05	16.1
Leu	55.5 ± 0.7	55.8 ± 0.3	55.7 ± 0.1	54.7
Tyr	13.0 ± 0.1	12.5 ± 0.1	12.8 ± 0.1	12.9
Phe	26.6 ± 0.5	26.6 ± 0.5	26.7 ± 0.3	25.8
Lys	32.8 ± 0.5	31.9 ± 0.3	32.5 ± 0.5	32.2
His	11.1 ± 0.2	10.9 ± 0.2	10.7 ± 0.2	11.0
Arg	16.9 ± 0.2	17.0 ± 0.2	17.1 ± 0.2	16.0

BA: α-Bromoacetophenone

a) moles of amino acid per 60 000 g of esterase ± standard deviation of the mean, n = 3.

b) from a series of three hydrolyses (n = 9); the complete analysis will be published elsewhere.

Technicon, Frankfurt/GFR). The desalted samples of native and inhibited esterase were hydrolysed in vacuo (after repeated flushing with argon) with carefully degassed 6 M HCl (reagent grade; E. Merck, Darmstadt/GFR) at 105° C for a period of 24 h.

RESULTS AND DISCUSSION

The discrepancy between the results of Willadsen et al. (1) and ours (2) might have been due to the difference in the administration of the inhibitor α-bromoacetophenone or to the choice of the animal. Therefore, in our re-examination experiments we used conditions very similar to those described by the other group, and we examined the liver esterases from both ox and pig. Three series of amino acid analyses of the same charge of highly purified liver esterase were performed under exactly the same conditions. Besides native enzyme and enzyme treated with α-bromoacetophenone we analyzed a sample of esterase which had been inhibited by E 600 prior to the modification with bromoacetophenone. According to Willadsen et al. (1) such a preincubation with E 600 should prevent the modification of the reactive histidine.

In a preliminary experiment with pig liver esterase, we found no significant decrease of histidine. This was confirmed in the main experiment (Table 1) with a larger amount of enzyme. If the slight loss of histidine residues from 11.1 to 10.9 is significant, it is not measurably delayed by E 600. In ox liver esterase, which contains more histidine than the enzyme from pig, the treatment with α-bromoacetophenone causes a loss of 1.7 histidine residues (Table 2). This is in agreement with the results of Willadsen et al. (1). However, in contrast we find a modification of the same order of magnitude after blocking the

Table 2 - Number of amino acids in native and in chemically modified ox liver esterase.

residues per mole of esterase [a]				
	24-h-hydrolysis			corrected values from Klapp et al. (8)
	without inhibitor	with BA	with E 600 and BA	
Val	38.8 ± 0.3	39.0 ± 0.1	37.0 ± 0.05	39.2
Cys	3.4 ± 0.2	3.0 ± 0.1	2.8 ± 0.1	4.0
Met	10.5 ± 0.2	11.2 ± 0.1	11.2 ± 0.2	9.2
Ile	19.9 ± 0.1	20.5 ± 0.2	19.1 ± 0.1	20.7
Leu	58.6 ± 0.4	58.3 ± 0.2	59.2 ± 0.7	54.6
Tyr	14.3 ± 0.1	14.0 ± 0.1	13.9 ± 0.1	12.4
Phe	26.8 ± 0.2	26.6 ± 0.2	26.6 ± 0.2	25.3
Lys	33.9 ± 0.2	32.3 ± 0.5	32.6 ± 0.3	31.8
His	13.9 ± 0.1	12.2 ± 0.1	11.9 ± 0.1	14.4
Arg	18.5 ± 0.2	18.7 ± 0.3	19.0 ± 0.1	16.7

BA: α-Bromoacetophenone

a) moles of amino acid per 60 000 g of esterase ± standard deviation of the mean, n = 3.

active site with E 600. That means that the histidine residue, modified in this case, is not located near the serine of the active center. In addition it is unlikely that the catalytic

mechanism of pig liver esterase is different from the very similar enzymes from ox and chicken. Nevertheless, we are convinced that at least one basic residue is involved in the catalytic mechanism of unspecific carboxyl esterases. Whether or not it is histidine cannot be concluded from such experiments with α-bromoacetophenone. From earlier results (2,9) we have some evidence that a primary amino group might be involved in the catalysis.

Only three other amino acids, namely cystine, tyrosine, and lysine were significantly modified by bromoacetophenone. (The amino acids not listed in the Tables showed no significant changes.) However, even if the modification of these residues is delayed to a small extent by preincubation with E 600, this is not sufficient experimental evidence for an involvement of these residues in the catalytic mechanism. Since no free SH-groups are detectable in ox (10) and pig liver esterase, α-bromoacetophenone must have reacted with SS-bridges. The methionine residues, which are known to be modified by bromoacetophenone in α-chymotrypsin (11), remain unchanged in the liver esterases.

The loss of valine (Table 1 and 2) and isoleucine (Table 2) after pretreatment with E 600 and bromoacetophenone may be interpreted as due to retardation of the hydrolysis of some lipophilic residues caused by the lipophilic diisopropyl phosphate residue.

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