BIOORGANIC CHEMISTRY 11, 19–23 (1982)

# Kinetic Studies of Mammalian and Microbial Cholesterol Esterases in Homogeneous Aqueous Solutions[1]

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*Received March 23, 1981*

The kinetics of one microbial and two mammalian cholesterol esterases have been examined using a variety of aryl acetates in homogeneous solution. The mammalian enzymes behaved identically but differed somewhat from that of microbial origin. The reactions of all three were not affected by either electronic or hydrophobic characteristics. Taurocholic acid was without effect on the microbial enzyme; at low concentrations it inhibited the mammalian system, but when present in millimolar amounts notable increases in rate were discerned, attributable to the detergent effect on the enzyme.

## INTRODUCTION

The report by Erlanson (*1*) that cholesterol esterase (EC 3.1.1.13) catalyzes the hydrolysis of *p*-nitrophenyl acetate in aqueous solution has made possible the investigation of some of the reaction characteristics of that enzyme under homogeneous conditions, which are reported here. Previous work was, of necessity, restricted by the water insolubility of the various long-chain cholesterol esters used as substrates, which were examined in various hetergeneous ("microcrystalline," emulsified, micellar) states (*2*).

We here report work with a wide range of aryl acetates which are substrates for cholesterol esterase. With these the effects of the electronic nature of the substituents, and of the hydrophobicity of the substrates on the reactivity of the enzymes, obtained from two mammalian and one microbial source, have been examined and compared. The effects of some dispersal agents on the enzyme have also been examined.

## EXPERIMENTAL

*Reagents.* Unless otherwise noted all reagents used were of analytical grade. The taurocholate used was supplied by Calbiochem.

The acetates employed were synthesized from the respective phenols (Aldrich Chemical Co., Pfalz and Bauer Inc., Eastman Kodak Co.) by the method of Chattaway (*3*). The products were distilled under vacuum or recrystallized until the experimental and theoretical elemental analyses were in accord.

[1] NRCC 20004.



0045-2068/82/010019-05$02.00/0

TABLE 1

EXPERIMENTAL $K_m$, $V_{max}$, AND $k_\psi$ VALUES OBTAINED WITH VARIOUS ARYL ACETATES IN 0.05 *M* TRIS/HCl, pH 7.0, 25°C USING CHOLESTEROL ESTERASES FROM THREE SOURCES[a]

| Substrate | Microbial | | | Bovine | | | Porcine | | |
|---|---|---|---|---|---|---|---|---|---|
| | $K_m$ | $V_{max}$ | $k_\psi$ | $K_m$ | $V_{max}$ | $k_\psi$ | $K_m$ | $V_{max}$ | $k_\psi$ |
| *p*-$NO_2$ phenyl | 1.43 | 4.17 | 2.67 | 0.69 | 2.78 | 5.37 | 0.78 | 2.72 | 2.70 |
| *m*-$NO_2$ phenyl | — | — | 2.00 | 0.43 | 0.83 | 2.07 | 2.13 | 2.03 | 0.70 |
| *p*-Br phenyl | 0.20 | 37.88 | 136.3 | 0.78 | 10.20 | 17.6 | 1.22 | 10.6 | 8.33 |
| *m*-Br phenyl | — | — | — | 0.34 | 6.83 | 34.6 | 0.25 | 4.38 | 16.9 |
| *p*-Cl phenyl | — | — | — | 0.68 | 9.37 | 12.08 | 0.80 | 7.76 | 8.2 |
| *m*-Cl phenyl | 0.70 | 20.00 | 37.30 | 0.81 | 7.63 | 7.5 | — | — | — |
| *p*-$CH_3$ phenyl | — | — | 52.5 | 0.61 | 6.33 | 9.82 | 0.45 | 5.83 | 10.8 |
| *m*-$CH_3$ phenyl | 2.04 | 58.82 | 28.14 | 0.63 | 7.52 | 11.11 | — | — | — |
| *p*-Phenylphenyl | 0.028 | 9.09 | 300 | 0.35 | 35.1 | 29.4 | 0.40 | 27.0 | 21.8 |
| *p*-*tert*.-Butylphenyl | 0.35 | 3.0 | 8.8 | 0.33 | 8.33 | 24.6 | 0.32 | 10.5 | 45.3 |
| 1-Naphthyl | — | — | 1.70 | 0.025 | 1.44 | 14.25 | 0.045 | 2.13 | 19.5 |

[a] Values of $K_m$ are $\times 10^3$ $M^{-1}$; those of $V_{max}$, $\times$ $10^7$ $M^{-1}$ sec; those of $k_\psi$ are $\times 10^4$ sec.

*Enzymes*. The microbiol cholesterol esterase was purchased from Boehringer–Mannheim; that from bovine pancreas was from the Sigma Chemical Company. Before use in this work these enzymes were passed through a column of agarose lima bean trypsin inhibitor (P & L Laboratories) which removed any contaminating trypsin.

The porcine cholesterol esterase was obtained from the pancreas of freshly slaughtered pigs following the method of Momsen and Brockman (*4*).

*Kinetics*. Reactions were followed spectrophotometrically (Beckman Model 26; Unicam SP-800) measuring, commonly, the rate of product formation. Measurements with *p*-nitrophenyl acetate were used for standardizing the enzyme solutions. Pseudo-first-order rate constants ($k_\psi$) were obtained from data collected at subsaturating concentrations of substrates ($10^{-5}$–$10^{-4}$ *M*); reactions were fol-

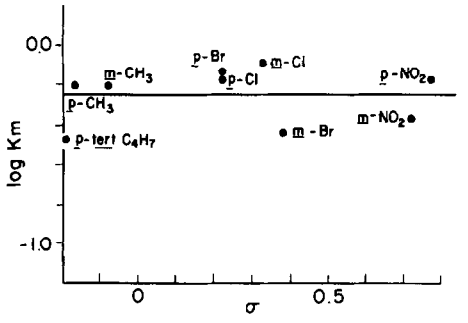


FIG. 1. $K_m$ values for various substituted aryl acetates, hydrolyzed in the presence of bovine pancreatic cholesterol esterase, vs Hammett $\sigma$ functions. The buffer system was 0.05 *M* Tris/HCl, pH 7.0, 25°C.

TABLE 2

THE EFFECT OF TAUROCHOLATE ON THE ACTIVITY OF BACTERIAL CHOLESTEROL ESTERASE[a]

| Substrate phenyl acetates | Taurocholate concentration ($M$) | | | | |
|---|---|---|---|---|---|
| | 0 | $1 \times 10^{-6}$ | $5 \times 10^{-6}$ | $1 \times 10^{-5}$ | $1 \times 10^{-3}$ |
| $p$-$NO_2$ | 13.1 | 11.5 | 13.9 | 11.5 | 6.4 |
| $p$-Br | 25.2 | 27.8 | 27.4 | 24.9 | 23.0 |
| $p$-$C_6H_5$ | 165.8 | 200.7 | 187.0 | 164.1 | 183.5 |

[a] In 0.05 $M$ Tris/HCl buffer, pH 7.0, at 25°C. Values of $k_\psi \times 10^4$ sec.

lowed over two or three half-lives. Michaelis–Menten parameters were determined from conventional double-reciprocal plots. As it may readily be shown that $k_\psi = V_{max}/K_m$, a check on the values obtained was available. When this equivalence was not found, independent experiments demonstrated the occurrence of product inhibition.

Inhibition constants ($K_i$) were estimated by the method of Niemann and coworkers (*5*, *6*).

Reactions were commonly followed in 0.05 $M$ Tris/HCl buffer of pH 7.0 (in the region of maximum activity for cholesterol esterase (*2*)) at 25°C in thermostated cells. Rate parameters reported are averages of at least two separate experiments; Michaelis–Menten kinetics were studied with eight concentrations to define the double-reciprocol plots used for estimating $K_m$ and $V_{max}$.

## RESULTS AND DISCUSSION

Values of $K_m$, $V_{max}$, and $k_\psi$ are collected in Table 1 for the enzymes from the three sources employed in this work and the range of aryl acetates studied. It

TABLE 3

EFFECT OF TAUROCHOLATE ON BOVINE PANCREATIC CHOLESTEROL ESTERASE[a]

| Taurocholate ($M$) | $k_\psi$ ($sec^{-1} \times 10^4$) |
|---|---|
| 0 | 7.7 |
| $1 \times 10^{-6}$ | 4.8 |
| $5 \times 10^{-6}$ | 5.5 |
| $1 \times 10^{-5}$ | 6.4 |
| $1 \times 10^{-3}$ | 40.8 |

[a] In 0.05 $M$ Tris/HCl, pH 7.0, 25°C, $p$-nitrophenyl acetate substrate.

seems evident from those data that the microbiol enzyme behaves differently from the two esterases of mammalian origin for which the results are, in turn, comparable. The only substrate for which results are similar is *p*-nitrophenyl acetate.

When the data of Table 1 are examined for the effects of the electronic character of the substrate substituents on the several kinetics parameters measured, using the Hammett $\sigma$ function, no evidence of such effects was found. A typical plot is that of Fig. 1, where the line best fitting the data is of zero slope, though some deviations from it are evident. This observation is valid for the cholesterol esterases from each of the three sources examined. It should be noted, as is clear in Table 1, that the two mammalian enzymes used reacted in effectively identical manners with the variety of aryl acetates studied, and that neither Michaelis parameter responded in a consistent fashion to the electronic nature of the substituents. Similarly, with the bacterial enzyme no influence of electronic effects from the substrate substituents was discovered. Hydrophobicity values (*7–9*) for the substrates employed again showed no clear correlation with the Michaelis–Menten values reported.

Examination of the data of Table 1 shows, however, that two substrates behave anomalously with the mammalian cholesterol esterases, namely, *p*-phenylphenyl and naphthyl acetates. This is clear when values of $V_{max}/K_m$ and $k_\psi$ for these acetates are compared, and the comparison suggests the possibility of product (phenol) inhibition playing a role in the reactions examined.

By means of the method of Niemann (*5, 6*) an inhibition constant ($K_i$) was calculated for *p*-phenylphenyl acetate under the conditions employed in the original measurements. As $K_i = 4 \times 10^{-5}$ *M*, competitive product inhibition was probably the cause of the anomaly noted above. Comparison with results obtained with *p-tert.*-butylphenyl acetate suggests that the bulk of the substrate, alone, is not significant, and the inhibition may be a result of the aromatic character of the substrates where it was observed.

The role of bile salts in the reactions of cholesterol esterase has been the subject of some discussion (*2*). While there is little doubt that they can act protectively (*2*) on cholesterol esterase, whether they may directly activate the enzyme is not unambiguously clear. Working initially with the microbial enzyme, we found, for the three substrates studied, no evidence of activation; at the millimolar concentration employed there was some inhibition when *p*-nitrophenyl acetate was the substrate. The results are collected in Table 2.

However, the data from a mammalian (bovine) cholesterol esterase are of greater interest. Thus, in Table 3 can be seen an inhibition of the reaction when micromolar taurocholate was employed ($K_i = 2 \times 10^{-6}$ *M*). As the concentration of the taurocholate was increased the inhibitory effect was overcome, and at a concentration of millimolar taurocholate a sixfold increase in reactivity was observed. Comparable activations at this and higher taurocholate concentrations have been reported by other workers (*2, 10*) and suggest that the effect may not be on the enzyme directly, because of the large molar excess of bile acid required, but on the solution parameters, and attributable to the detergent action by the taurocholate. To examine this hypothesis we have measured $k_\psi$ for hydrolysis of *p*-nitrophenyl acetate in the presence of bovine pancreatic esterase with three

concentrations of Triton X-100. There was a consistent increase in rate with that of the concentration of detergent so that, at 3 m*M* detergent, $k_\psi = 43.5 \times 10^{-4}$ $sec^{-1}$ compared with $5.4 \times 10^{-4}$ $sec^{-1}$ with no Triton X-100 present. These data, which are in contrast with those of Erlanson (*10*), suggest that at least for the hydrolytic reaction of cholesterol esterase, taurocholate is stimulatory because of its detergent action, presumably so modifying the structure of the enzyme.

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