BBA 66156

THE NATURE OF THE MODIFIER SITE OF PIG LIVER ESTERASE*

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(Received March 31st, 1970)

SUMMARY

The velocity of hydrolysis of ethyl butyrate by pig liver esterase (carboxylic ester hydrolase, EC 3.1.1.1) is greater at high substrate concentrations than is demanded by extrapolation of low substrate data assuming Michaelis–Menten kinetics. Two sites are proposed; an esterolytic site and a modifier site. The control site is shown to be hydrophobic by the use of selective inhibitors. At high substrate concentration, the control site acquires a substrate molecule and a probable modification of the reaction pathway results in the observed increase in velocity. Butanol and butanone exclude substrate from this site in addition to competing for the esterolytic site.

INTRODUCTION

It has been reported that pig liver esterase (carboxyl esterase hydrolase, EC 3.1.1.1) fails to follow Michaelis–Menten kinetics[1,2]. The esterolytic activity is accelerated at high substrate concentration to an extent greater than demanded by extrapolation of low substrate data in accord with simple Michaelis–Menten kinetics. This behavior has been attributed to the presence of two active sites per molecule of enzyme[2]. Indeed BARKER AND JENCKS[3] demonstrated monomer and dimer form (mol. wt. 86 000 and 168 000). Both species hydrolyzed ethyl butyrate. KRISH[4] has reported a minimal molecular weight for the enzyme of 96 000. HORGAN *et al.*[5] report finding one active site serine per mol. wt. 78 000. One hypothesis is that the observed kinetics are not a function of the monomer–dimer transition and can be described by some other mechanism. If this is true, (1) the sites on the bivalent enzyme should have unique functions, and (2) the sites should be different in nature. A number of esters and inhibitors were synthesized and examined for interaction with the enzyme.

* A preliminary report appeared in *Federation Proc.*, 26 (1967) abst. 3122, p. 816. The work is taken from the thesis submitted by P.R.O. in partial fulfillment for the degree of Ph.D.

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The results confirm the hypothesis that the enzyme has two sites; one a hydrophobic binding and control site, the other an esterolytic site. The actions of these sites can be separated by selective inhibitors.

MATERIALS AND METHODS

Pig liver esterase

The pig liver esterase was prepared as previously reported[6]. All substrates were prepared in this laboratory from spectro-quality reagents to ensure purity. The aliphatic esters were synthesized by refluxing 0.5 mole of the corresponding acid in 300 ml ethanol with 2 ml conc. H_2SO_4 as a catalyst. The reaction was stopped in 6 h with the removal of heat. The H_2SO_4 was neutralized by K_2CO_3 and the salts filtered. The solution was then vacuum distilled at 20 mm Hg. All liquid esters were redistilled until boiling points and index of refraction were satisfactory. Solids were recrystallized until melting points corresponded to literature values.

The final criterion of purity for the substrates after boiling point, melting point, and refractive index was analytical thin-layer chromatography on silica gel.

Kinetic measurements

The rates were measured by a pH-stat technique using a Radiometer Titrator Type $TTTb_1$, coupled to a Titragraph Type SBR2C and burette SBU-2. The acid formed during the hydrolysis of the ester was continuously titrated with standard alkali (free of CO_2) to maintain a constant pH. An external calomel reference electrode with a KCl bridge was employed to permit the use of small vessels. All runs were made in buffer-free 0.05 M KCl solutions under a blanket of N_2 maintained at 25° by a water bath.

In a typical run, an aliquot of enzyme stock solution was delivered to the titration vessel and the volume allowed to come to temperature by immersion in the thermostated water bath and brought to the desired pH. A stream of CO_2-free N_2 was continuously blown over the solution. The substrate was added with a constriction pipette and allowed to hydrolyze completely for all runs. In this manner the exact concentration of the substrate was always known. Only the initial rates were taken in the treatment of the data, all points were determined in five individual experiments. The velocity constants are reported as mM/sec per mg enzyme at an ethyl butyrate concentration of 23 mM. The minimal molecular weight of 90 000 (refs. 3, 4) was used in the determination of the kinetic constants, $k$cat.

RESULTS

The effect of substrate concentration upon the kinetic behavior of pig liver esterase was examined with ethyl butyrate as the substrate. A Lineweaver–Burk plot, typical of our data, is shown in Fig. 1 (Curve 1). The non-linearity of the plot indicates that the reaction pathway fails to follow the Michaelis–Menten scheme. The curve shows a marked increase in reaction velocity at high substrate concentration. Two K_m and two V_m values are apparent; they are designated K_H, V_H for high substrate concentration and K_L, V_L for low substrate concentration. The constants are given in Table I.

TABLE I

KINETIC CONSTANTS FOR HYDROLYSES OF ETHYL BUTYRATE BY PIG LIVER ESTERASE

Constants were determined graphically at pH 8.0 and 25°.

K_H	1.31 mM
V_H	$14.3 \cdot 10^{-2}$ mM/sec per mg enzyme
$k_{cat\ H}$	12.52 sec^{-1}
K_L	0.36 mM
V_L	$5.7 \cdot 10^{-2}$ mM/sec per mg enzyme
$k_{cat\ L}$	5.10 sec^{-1}

Since all of the reaction mechanisms that result in the observed kinetics give the same general rate expression;

$$v = \frac{a[S]^2 + b[S] + C}{g[S]^2 + h[S] + M} \tag{1}$$

steady state kinetics do not permit a choice of mechanism. However, through biochemical methods and selective kinetic studies a reaction pathway can be chosen that best fits the existing evidence. We are in complete agreement with ADLER AND KISTIAKOWSKY[2] that the most likely mechanisms are: (1) two active sites in the enzyme, and (2) a modifier (which may be a substrate molecule).

Rate expression

The general scheme which gives expressions that satisfy the kinetic data assumes that the enzyme has two sites of interaction with substrate which in turn has two sites of interaction with the enzyme. These are labeled 1 and 2 in the formation on the enzyme, symbolized by E and unspecified on the substrate (S).

$$E_2^1 + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat\ L}} E_2^1 + P \tag{2}$$

$$ES + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{-S} \xrightarrow{k_{cat\ H}} ES + P \tag{3}$$

It is assumed that the various complexes are in equilibrium with each other (*i.e.* that $k_{cat\ L}$ and $k_{cat\ H}$ are much smaller than k_{-1} and k_{-2}, respectively. We can define Michaelis-type constants,

$$K_L = \frac{k_{-1} + k_{catL}}{k_1} \text{ and } K_H = \frac{k_{-2} + k_{catH}}{k_2}$$

and

$$\frac{1}{v} = \frac{K_H K_L + K_H S + S^2}{K_H V_L S + V_H S^2}$$

When $S \gg K_H$ (K_L) we are in the region of high substrate concentration and the limiting relationship of Eqn. 3 is:

$$\frac{1}{v} = \frac{K_H}{V_H}\left(\frac{1}{S}\right) + \frac{1}{V_H}$$

When $S \ll K_L$ (K_H) we are in the region of low substrate concentration and the limiting relationship is:

$$\frac{1}{v} = \frac{K_L}{V_L}\left(\frac{1}{S}\right) + \frac{1}{V_L}$$

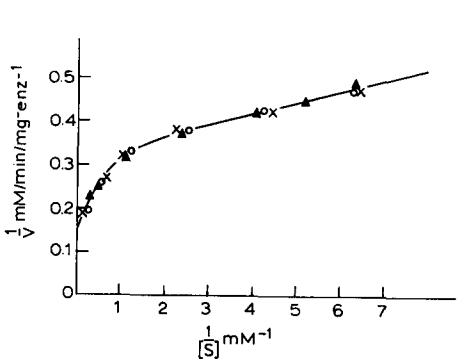
Thus linear extrapolation of the two branches of the experimental curves (Fig. 1) allows the determination of the characterizing parameters K_L, K_H, V_L and V_H.

Effect of added reaction products

Fig. 1 is a plot of pig liver esterase action at pH 8.0 on ethyl butyrate as substrate ($1/[S]$ *vs.* $1/v$ with added reaction products. Curve 2 is ethyl butyrate with 100 mM butyric acid, Curve 3 with 174 mM ethyl alcohol, and Curve 1 is ethyl butyrate with no added products. Since the reaction products have no influence on the kinetic behavior of pig liver esterase even at concentration far above substrate concentration, the deviation from Michaelis–Menten kinetics cannot be attributed to product interaction with enzyme at pH 8.0.

As previously described[6], the enzyme hydrolyzes only one ester group in dicarboxylic diesters. We considered the possible explanations of this limitation. (1) The enzyme may not recognize a molecule with a free charged carboxyl as a substrate although the molecule contains an ester group. This may be attributed to a negatively charged group at the active site, creating like-charge repulsion, and (2) the half-ester, half-acid may be bound in some manner to the enzyme preventing further hydrolysis. The second alternative can be tested by using ethyl potassium succinate in the reaction mixture of ethyl butyrate and enzyme. A Lineweaver–Burk plot of ethyl butyrate with 0.15 M ethyl potassium succinate added to the reaction mixture is identical to Fig. 1; the enzyme is not inhibited by the monoethyl succinate ion. The alternative of like-charge repulsion of the half-ester, half-acid due to a carboxyl at the active site can be examined by a measure of the velocity as a function of pH. The velocity was examined at substrate concentrations that fall in the V_H or V_L portion of the Lineweaver–Burk plot (Fig. 2). They represent the kinetics at the extremes. Curve A is at 51 mM ethyl butyrate and Curve B is at 0.12 mM ethyl butyrate.

The curves indicate that different groupings in the enzyme are operating at high and low substrate concentrations. At high substrate the midpoint of the activity



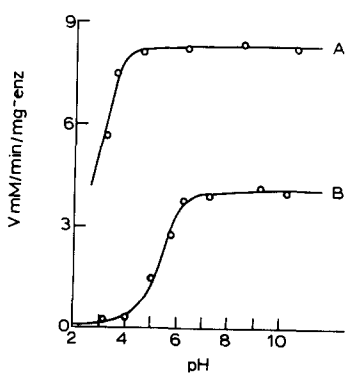


Fig. 1. $1/[S]$ *vs.* $1/v$ plot of liver esterase with ethyl butyrate as substrate (Curve 1, ▲—▲). Curve 2 (×—×) with 100 mM butyric acid added and Curve 3 (○—○) with 174 mM ethyl alcohol added, pH 8.0 and 25°.

Fig. 2. pH dependence of the hydrolysis of ethyl butyrate by liver esterase. Curve A is at 51 mM ethyl butyrate and Curve B at 0.12 mM ethyl butyrate. These concentrations fall at the extremes of the bivalent Lineweaver–Burk plots. Values are corrected to pK 4.8 for butyric acid. Two different dependencies are apparent, pK 3.8 for high substrate and pK 5.8 for low substrate concentration.

curve falls at pH 3.8, which is in the range of the pK of a free carboxyl group in a protein[7] and at low substrate the pK is 5.8, which may indicate an imidazole at the active site. The difference in pH dependence demonstrated here was previously shown by ADLER AND KISTIAKOWSKY.[2] The 3.8 pK unit becomes dominant at high substrate and the 5.8 pK unit dominates at low concentration. The analysis of the pH dependence of pig liver esterase demonstrates that the enzyme is divalent (has two sites) but it does not distinguish whether both sites are esterolytic as proposed by ADLER AND KISTIAKOWSKY[2] or if one site is a binding site and the other is a hydrolytic site.

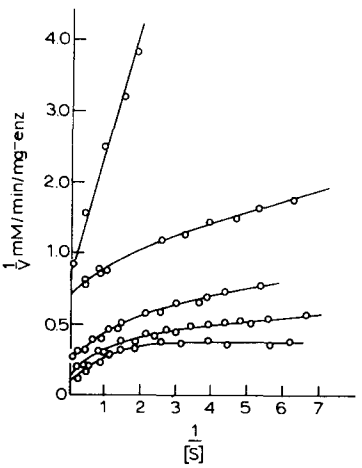


Fig. 3. The $1/[S]$ *vs* $1/v$ plot of liver esterase with a series of aliphatic esters as substrates. In descending order: ethyl formate, ethyl acetate, ethyl propionate, ethyl butyrate and ethyl valerate. All curves are obtained at pH 8.0 and 25°.

Aliphatic esters

The aliphatic series of esters: ethyl formate, ethyl acetate, ethyl propionate, ethyl butyrate, and ethyl valerate were examined with respect to their steady state kinetics. Fig. 3 is a plot of $1/[S]$ *vs.* $1/v$ obtained for the hydrolysis of these compounds. Ethyl formate shows no deviation from Michaelis–Menten kinetics and the acceleration of ethyl acetate hydrolysis at high substrate concentration is small. The constants obtained from the dissections of the curves into two linear functions demonstrate that k_{cat} at high and low substrate concentrations is an increasing function of acyl carbon length. At high substrate concentration, ethyl butyrate; ethyl propionate; and ethyl valerate demonstrate a larger $k_{cat\ H}$ as the K_H increases. The constancy of k_H/K_H for these compounds indicates that the rate and Michaelis constants vary directly with each other as a function of the number of carbons in the acyl group. HOFSTEE[9,10] has shown that the V for the hydrolysis of fatty acid esters of *o*-hydroxybenzoic acid by horse liver esterase is a monotonically increasing function of chain length and that K_m is dependent on chain length. This is not the case for the binding at high substrate with pig liver esterase but may hold true at low substrate concentration. The appearance of the loose binding site which accelerates the reaction enforces the concept of a two-"site" enzyme, one for binding and control, the other for esterolysis.

TABLE II

KINETIC CONSTANTS FOR HYDROLYSIS OF A SERIES OF ALIPHATIC ESTERS BY PIG LIVER ESTERASE

All values determined graphically at pH 8.0 and 25°. K_H and V_H represent the mean values of fiv outside limits of ±0.05 for K_H and ±0.10 for V_H.

Aliphatic ester	$k_{cat\ H}$ (sec^{-1})	K_H (*mM*)	V_H (*mM/sec per mg enzyme*) × 10^2	$k_{cat\ H}/k_H$ ($sec^{-1} \cdot mmole^{-1}$)	$k_{cat\ L}$ (sec^{-1})	K_L (*mM*)	V_L (*mM/sec per mg enzyme*) × 10^2
Ethyl formate	2.79	3.66	3.10	0.763	—	—	—
Ethyl acetate	3.55	1.09	4.40	3.26	0.9	0.055	1.0
Ethyl propionate	8.38	0.71	9.50	11.70	4.41	0.12	4.91
Ethyl butyrate	12.52	1.30	14.30	9.50	5.10	0.036	5.7
Ethyl valerate	19.98	1.75	22.20	11.4	5.23	0.042	5.81

Separation of sites

A solution to the problem of whether both sites are esterolytic or whether site is a binding and an internal accelerator and the other "site" is for hydrolysis r lie in the selective inhibition of the binding sites. Previous experimentation monstrated that compounds such as amides, half-esters, half-acids, free acids, met alcohol, ethyl alcohol and amino compounds have no inhibitory affect[6]. The incre in K_m and k_{cat} with the longer-chain fatty acid esters indicates a preference for hydrophobic species. Thus by analogy an inhibitor must be hydrophobic and : compatible with water. The first species to be tried was 2-butanone. Fig. 4 is a L weaver–Burk plot of liver esterase with ethyl butyrate as substrate and with ad

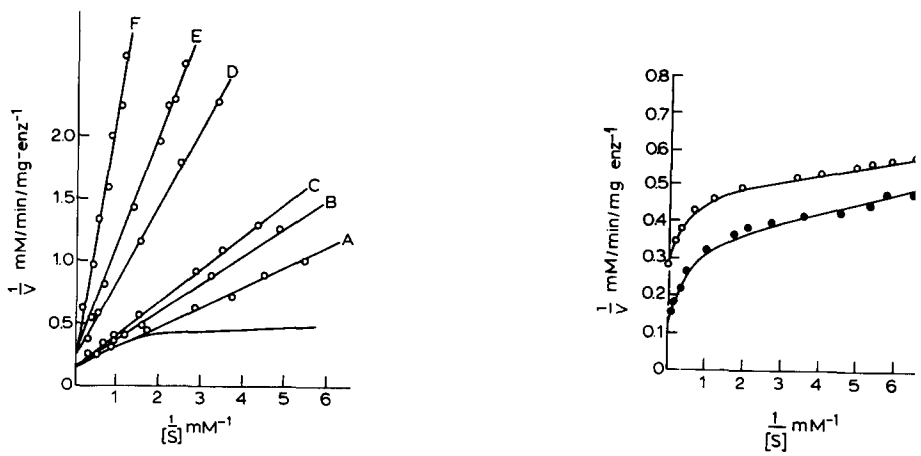


Fig. 4. A plot of ethyl butyrate hydrolysis $1/(S)$ *vs.* $1/v$) with added inhibitors. Curve A is \ 100 mM 2-butanone. Curves B–F is with increasing concentrations of *n*-butanol; B, 27.4 r C, 41.1 mM; D, 87.6 mM; E, 109 mM; F, 164 mM. At the lower concentration of hydroph inhibitor the V approximates V_H. At 109 mM and 164 mM *n*-butanol V_H is completely inhibi V_L is $5.7 \cdot 10^{-2}$ mM/sec per mg protein which is the V reported for the maximum velocity ex polated to low substrate concentration. The heavy line is the reaction without added inhibi

Fig. 5. Plot ($1/[S]$ *vs.* $1/v$) of the hydrolysis of ethyl butyrate (●—●) and *sec.*-butyl buty (○—○) by pig liver esterase at pH 8.0 and 25°.

2-butanone at 100 mM. Curve A: The reciprocal velocity is now linear with respect to reciprocal substrate concentration. Since 2-butanone is a ketone, arguments might be presented that the molecule inhibits because the enzyme recognizes the carbonyl function rather than the carbon–hydrogen skeleton. 1-Butanol was then tried. Previously methanol and ethanol had been shown to have no effect on the kinetics even at very low substrate concentrations. 1-Butanol is less polar than 2-butanone (dielectric constant 17.1 and 18.3, respectively). Curves B, C, D, E and F of Fig. 4 is a plot of ethyl butyrate hydrolysis at increasing concentrations of 1-butanol. The $1/[S]$ *vs.* $1/v$ plot is linear. Apparently, the inhibitor selectively binds with one of the sites on the enzyme and has only an indirect inhibitory effect on the other. If both sites were identical and esterolytic, the curve should look like the uninhibited reaction with different dimensions. At higher 1-butanol concentrations (109 and 164 mM) the V of the reaction is $5.7 \cdot 10^{-2}$ mM /sec per mg protein, k_{cat} 5.1 sec^{-1}, which is the v reported for the velocity extrapolated at low substrate concentration. Thus the reaction characterized by $k_{cat\ H}$ is completely inhibited. It appears that the inhibition is competitive to $k_{cat\ L}$ and noncompetitive to $k_{cat\ H}$. The inhibitor replaces substrate on the binding site affecting $k_{cat\ H}$ and acceleration mechanism is thereby quenched. The previously derived constants now become more meaningful. The unaccelerated kinetic constants K_L and V_L and $k_{cat\ L}$ are those extrapolated to low substrate concentration data. At high substrate concentration, the accelerated mechanism becomes active and can now be described by K_e, (acceleration constant, a revised definition of K_H). The accelerator constants are: ethyl propionate, 0.715 mM; ethyl butyrate, 1.31 mM; ethyl valerate, 1.75 mM.

Acyl–enzyme complex

Since pig liver esterase is a true esterase it was important to examine the catalysis with respect to the acyl–enzyme complex mechanism[11–15]. This mechanism proposes a two-step reaction with the rate-controlling step the hydrolysis of the acyl–enzyme complex. If the K_w of two esters is similar (that is, both leaving groups are essentially the same), then regardless of K_m differences the rate constant will be the same. The rate of hydrolysis of ethyl butyrate and *sec.*-butyl butyrate by pig liver esterase were compared. Fig. 5 shows the great difference in the V_L and V_H of these two substrates. If the hydrolysis of the butyryl–enzyme were the rate-determining step, the velocities would be similar. It is seen that nowhere along the curve do the two substrates appear similar. Hence, we propose that the rate-determining step is not the deacylation of the enzyme, but an earlier acylation step that is controlled by the modifier site. This observation does not negate an acyl–enzyme complex in the mechanism for pig liver esterase catalysis but merely states that hydrolysis of the acyl–enzyme is not rate determining.

CONCLUSION

The kinetic behavior of pig liver esterase has been shown to be similar to that observed for enzymes which are recognized to be allosteric. Evidence has been presented to support the hypothesis that there are binding and acceleration sites for substrates and/or modifiers in the liver esterase molecule in addition of the catalytic

hydrolytic site. The acceleration phenomenom found in the reaction of pig liver esterase is probably associated with a monomeric form of the enzyme.

ACKNOWLEDGEMENT

This investigation was supported by a National Science Foundation Grant GB 4134.

P.R.O. is a National Institute of Dental Research Predoctoral Trainee (DT-27), U.S. Public Health Service.

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