Kinetics of Acyl Transfer by Beef Liver Esterase*

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ABSTRACT: A study of the rate of hydrolysis, by beef liver esterase, of several substrates (methyl β-phenylpropionate, methyl β-phenyl-L-lactate, and methyl β-phenyl-D-lactate) in the presence of amine nucleophiles (glycine methyl ester, L-isoleucine methyl ester) has given data consistent with the assumption that these nucleophiles compete with water for an intermediate acyl-enzyme. The data indicate that with methyl β-phenylpropionate and methyl β-phenyl-D-lactate, the rate-limiting process is the acylation of the enzyme, whereas the hydrolysis of methyl β-phenyl-L-lactate is characterized by a rate-limiting deacylation of the acyl-enzyme. Consistent with this conclusion is the finding that with the first two substrates the inhibition of their hydrolysis by the amine nucleophiles is linear noncompetitive in nature, whereas with methyl β-phenyl-L-lactate, the inhibition is linear competitive in nature. Estimates of the ratio of the rates of transfer of an acyl group to amine and to water indicate that Ile-OMe is about three times more effective as an acyl acceptor than is Gly-OMe, despite their similar pK_a values, and it may be inferred that Ile-OMe is bound more tightly to the enzyme than is Gly-OMe. The acyl-Ile-OMe and acyl-Gly-OMe products are resistant to enzyme action, whereas with L-phenylalanine methyl ester as the acceptor, the resulting acyl-Phe-OMe is hydrolyzed to acyl-Phe. Despite this complication, an estimate has been made of the ratio of the rates of acyl transfer to Phe-OMe and to water in a system in which Phe-OMe serves both as the acyl donor and as the acceptor amine. The data presented in this paper are discussed in relation to the hypothesis that the acyl donor and acceptor amine are bound at separate sites that are similar in their affinity for a given ester.

Previous work in this laboratory has shown that beef liver esterase is an efficient catalyst in the transfer of acyl groups from the methyl esters of suitable amino acids (*e.g.*, L-phenylalanine, L-leucine, and L-tryptophan) and fatty acids (*e.g.*, β-phenylpropionic acid and hexanoic acid) to the α-amino group of L-phenylalanine methyl ester and of other amino acid esters at pH 7.2–8.5. This transfer reaction competes with the enzyme-catalyzed hydrolysis of the acyl donor, and exhibits stereochemical specificity with respect to the acceptor amine. Thus, whereas L-phenylalanine methyl ester is extensively converted into Phe-Phe-OMe,[1] D-phenylalanine methyl ester is largely hydrolyzed by the enzyme and cannot function as an efficient acceptor of acyl groups. With L-phenylalanine methyl ester as the acceptor amine, the acylamino acid ester initially formed (Phe-Phe-OMe, Ppr-Phe-OMe) is hydrolyzed to the acylamino acid (Phe-Phe, Ppr-Phe), in accordance with the side-chain specificity of the enzyme with respect to the acyl donor in hydrolytic or transfer reactions. On the other hand, with L-isoleucine methyl ester or glycine methyl ester as the acceptor amine, the acylamino acid ester that is formed is not converted into a measurable extent to the corresponding acylamino acid; this result is consistent with the relative resistance of these two amino acid esters to the hydrolytic action of beef liver esterase (Krenitsky and Fruton, 1966; Goldberg and Fruton, 1969).

Considerable evidence has accumulated in favor of the view that beef liver esterase, like other esterases (including chymotrypsin, trypsin, elastase, and subtilisin), is a "serine-enzyme" and that an acyl-enzyme is formed as an intermediate in the catalytic process (Janz *et al.*, 1959; Blakely *et al.*, 1967). It has also been shown that the transferase action of the enzyme is abolished by reagents (*e.g.*, DFP) that block the reactive serine residue (Krenitsky and Fruton, 1966; Benöhr and Krisch, 1967), indicating that the transfer process involves the reaction of an amine nucleophile with the same acyl-enzyme that reacts with water in the hydrolytic process. In the discussion to follow, it will be assumed, therefore, that the overall process may be written as

$$\text{X–OMe} + \text{EH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{EH (X–OMe)} \xrightarrow{k_2} \text{E–X} + \text{MeOH}$$

$$\text{E–X} + \text{H}_2\text{O} \xrightarrow{k_3} \text{EH} + \text{XOH}$$

$$\text{E–X} + \text{HY} \xrightarrow{k_4} \text{EH} + \text{X–Y}$$

In the above reactions, X denotes the acyl portion of the ester substrate, HY the amine nucleophile, XOH the carboxylic acid derived from the ester substrate, and X–Y the product of the transfer reaction. For this process, the initial concentration of X–OMe (s_0) for half-maximal initial velocity is defined as $K_M = K_S(k_3 + k_4 n_0)/(k_2 + k_3 + k_4 n_0)$, where $K_S = k_{-1}/k_1$ and n_0 is the initial concentration of the

* From the Kline Biology Tower, Yale University, New Haven, Connecticut 06520. *Received March 30, 1970.* These studies were aided by grants from the National Institutes of Health (GM-06452) and from the National Science Foundation (GB-5212X).

† Predoctoral trainee under Biochemistry Training Grant GM-53 of the National Institutes of Health. The work reported in this paper is included in the dissertation submitted by Michael I. Goldberg in partial fulfillment of the requirements for the Ph.D. degree from Yale University.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 2485 (1966), are: Ppr, β-phenylpropionyl; Pla, β-phenyl-L-lactyl; D-Pla, β-phenyl-D-lactyl. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

TABLE I: Relative Retention Times of Acylamino Acid Esters.

Compound	Isothermal Temp (°C)[a]	Rel Retention Time[b]	Pla-X-OMe / Ppr-X-OMe
Pla-OMe	166	0.028	
Ppr-OMe	166	0.021	
Pla-Gly-OMe	200	0.31	1.2
Ppr-Gly-OMe	200	0.25	
Pla-Ile-OMe	200	0.60	1.2
Ppr-Ile-OMe	200	0.49	
Pla-Phe-OMe	230	1.93	1.0
Ppr-Phe-OMe	230	1.86	

[a] Other operating conditions (H_2 flow, He flow) during gas–liquid partition chromatography have been described previously (Goldberg and Fruton, 1969). [b] Retention time relative to ethyl stearate; at 166°, retention time for ethyl stearate is 170 min; at 200°, 31 min; at 230°, 9.5 min.

amine nucleophile. The maximal velocity (V_m) per unit of enzyme concentration (E) is denoted k_{cat}. For the rate of disappearance of X–OMe (or appearance of MeOH), k_{cat} is defined as $k_2(k_3 + k_4n_0)/(k_2 + k_3 + k_4n_0)$; for the rate of formation of XOH, $k_{cat} = k_2k_3/(k_2 + k_3 + k_4n_0)$; and for the rate of formation of X–Y, $k_{cat} = k_2k_4n_0/(k_2 + k_3 + k_4n_0)$ (Bender *et al.*, 1964).

The experiments reported in the present communication were intended to provide data on the relative rates of transfer and hydrolysis (k_4/k_3) in reactions catalyzed by beef liver esterase. Such data are needed for a better understanding of the factors that determine the relative efficiency of various amine nucleophile as acceptors, and as a basis for the study of the relation of the state of aggregation of this oligomeric enzyme (Benöhr and Krisch, 1967) to its transferase action (Goldberg and Fruton, 1969). In the present studies, two experimental approaches were employed. In the first, the pH-Stat technique was used to determine the effect of amine nucleophiles on the initial rates of hydrolysis of the methyl esters of β-phenylpropionic acid, β-phenyl-L-lactic acid, and β-phenyl-D-lactic acid; the amines chosen were Ile-OMe and Gly-OMe because of the resistance of the corresponding acylamino acid esters to enzymic hydrolysis. In the second approach employed in the present studies, the time course of acyl transfer was determined by measurement of the yields of products. With Ppr-OMe or Pla-OMe as the acyl donor, and with Ile-OMe or Gly-OMe as the acceptor amine, the analyses were performed by gas–liquid partition chromatography (Goldberg and Fruton, 1969); with Phe-OMe as both acyl donor and acceptor amine, the gel filtration method of analysis (Krenitsky and Fruton, 1966; Goldberg and Fruton, 1969) was employed.

Experimental Section

With the following exceptions, all the materials and methods used in the experiments reported in this paper have been described previously (Krenitsky and Fruton, 1966; Goldberg and Fruton, 1969). Methyl β-phenyl-D-lactate was prepared in the manner described by Inouye and Fruton (1967). In the calculation of the concentration of the amine nucleophiles at a given pH value, the following pK_a values were used: Gly-OMe, 7.34; Ile-OMe, 7.34; Phe-OMe, 6.70 (Edsall and Blanchard, 1933; Almond *et al.*, 1959). The beef liver esterase preparation had a specific activity of 30 units/mg of protein (Goldberg and Fruton, 1969).

Gas–Liquid Partition Chromatography of Methyl Esters of Fatty Acids and of Acylamino Acids. For those experiments in which Ppr-OMe was the donor and Gly-OMe or Ile-OMe was the acceptor, the amount of transfer product (Ppr-Gly-OMe or Ppr-Ile-OMe) was determined in the manner described by Goldberg and Fruton (1969). When Pla-OMe was the donor, and Gly-OMe, Ile-OMe, or Phe-OMe was the acceptor, the following method of analysis was adopted. The amount of Pla-OMe was determined from a standard curve, and the amounts of the transfer products, Pla-Gly-OMe, Pla-Ile-OMe, and Pla-Phe-OMe, were estimated using the same operating conditions and the same standard curves as in the determination of Ppr-Gly-OMe, Ppr-Ile-OMe, and Ppr-Phe-OMe, respectively. In Table I are given the relative retention times for the peaks expected for the transfer of products from the reaction in which Pla-OMe is the donor. It is evident the ratio of the relative retention time of each Pla derivative to that of the corresponding Ppr derivative is about 1.1.

When Pla-OMe and Gly-OMe were incubated with the enzyme, analysis of the neutral products at 200° gave two peaks, with relative retention times of 0.31 and of 1.4. Neither peak was due to Pla-OMe, nor did they appear when Pla-OMe and Gly-OMe were incubated in the absence of the enzyme. The peak having the smaller retention time was identified as Pla-Gly-OMe, by use of the relationship to the value for Ppr-Gly-OMe. The peak with the larger retention time (1.4) was found when Pla-OMe (50 mM) was incubated with the enzyme in the absence of Gly-OMe, indicating that this peak is derived from the action of the enzyme on Pla-OMe alone, and that it corresponds to the lactide methyl β-phenyl-L-lactyl-(β-phenyl)-L-lactate (Pla-Pla-OMe). Its amount was estimated from the standard curve for Pla-OMe, and the values obtained in this manner were divided by two to correct for the difference in response between Pla-OMe and Pla-Pla-OMe.

Transferase Action of Beef Liver Esterase. In the experiments with either Ppr-OMe or Pla-OMe as donor and Gly-OMe or Ile-OMe as acceptor, the procedure for the separation of the components of the reaction mixture (Goldberg and Fruton, 1969) was modified; the isolation sequence was terminated at the end of the first ether extraction so that values were obtained only for the amount of unreacted acyl donor and of the acylamino acid ester present in the incubation mixture, and the amount of acidic product was determined by difference. In the experiments with Pla-OMe as donor and Phe-OMe as acceptor, the complete separation procedure was followed. Control experiments with Pla-OMe and with β-phenyl-L-lactic acid, to determine the efficiency of their partition between water and ether under the conditions of the separation method, gave recoveries of about 96%; similar values had been obtained in previous control experiments with β-phenylpropionyl derivatives (Goldberg and Fruton, 1969).

TABLE II: Kinetics of Ester Hydrolysis by Beef Liver Esterase in the Presence of L-Isoleucine Methyl Ester.[a]

Substrate	Concn Range (mM)[b]	Concn of Ile-OMe (mM)[c]	k_{cat}[d]	K_M (mM)	k_{cat}/K_M[e]
Ppr-OMe	0.5–3.2 (5)	0	18.2 ± 0.6	1.6 ± 0.3	11.4
	1.0–4.9 (5)	4.65	16.7 ± 0.3	1.9 ± 0.3	8.8
	1.0–4.9 (5)	9.3	15.4 ± 0.1	1.8 ± 0.4	8.6
	1.0–4.9 (5)	23.2	12.3 ± 0.5	2.0 ± 0.2	6.2
	1.0–4.9 (5)	46.4	8.5 ± 0.4	1.8 ± 0.3	4.7
D-Pla-OMe	2.0–19.2 (5)	6.1	4.4 ± 0.2	5.3 ± 0.3	0.8
	2.0–19.2 (5)	18.2	3.7 ± 0.3	5.5 ± 0.4	0.7
	2.0–19.2 (5)	42.8	3.1 ± 0.4	5.5 ± 0.4	0.5
Pla-OMe	4.4–21.8 (5)	0	10.3 ± 0.4	7.3 ± 0.3	1.4
	6.0–30.0 (5)	8.55	9.9 ± 0.6	8.3 ± 0.6	1.2
	6.0–30.0 (5)	24.2	9.7 ± 0.7	11.1 ± 1.4	0.9
	6.0–30.0 (5)	47.4	10.4 ± 0.9	16.4 ± 1.9	0.6
	6.0–30.0 (5)	68.8	9.2 ± 0.9	19.9 ± 2.9	0.5

[a] Enzyme concentration, 0.04 unit/ml; pH 8.5; 37°. [b] Numbers in parentheses denote the number of runs. [c] Concentration of conjugate base. [d] mM/min per enzyme unit per ml. [e] l./min per enzyme unit per ml.

Results

In Table II are given some of the kinetic parameters obtained in the present studies for the hydrolysis of Ppr-OMe, D-Pla-OMe, and Pla-OMe in the presence of Ile-OMe as the amine nucleophile. These values were estimated from v *vs.* v/s_0 plots of pH-Stat data for the initial rate of acid production (less than 10% hydrolysis). It will be noted that, with Ppr-OMe as the substrate, K_M is independent of the concentration of added nucleophile (n_0) whereas k_{cat} decreases with increasing n_0; a similar conclusion appears to apply with D-Pla-OMe as the substrate, although fewer data are available in this case. On the other hand, with Pla-OMe as the substrate, an increase in the concentration of Ile-OMe leads to a significant increase in K_M, with no change in k_{cat} for acid formation.

Bender *et al.* (1964) and Seydoux and Yon (1967) have shown that the effect of nucleophilic competition on the kinetic parameters in the hydrolysis of esters by chymotrypsin or trypsin is related to the nature of the rate-limiting step in the hydrolytic process. When the rate-limiting step is acylation of the enzyme ($k_3 \gg k_2$), K_M is independent of the concentration of nucleophile and is equal to K_S, whereas k_{cat} for acid production is equal to $k_2k_3/(k_3 + k_4n_0)$. Thus, in this case, $(1/k_{cat}) = (1/k_2) + (k_4n_0/k_2k_3)$, and a plot of $1/k_{cat}$ for acid production against n_0 should give a straight line with a slope of k_4/k_2k_3 and an intercept of $1/k_2$. This permits an estimate to be made of the magnitude of k_2, for the rate-limiting acylation step, and of the ratio k_4/k_3, which describes the relative rate of reaction of the acyl-enzyme with the added nucleophile and with water. In Figure 1 is given a representative plot of data for the hydrolysis of Ppr-OMe by beef liver esterase in the presence of Ile-OMe at pH 7.2 and 8.5. The data for both pH values fall on the same straight line, in agreement with the assumption that only the conjugate base of Ile-OMe can act as an acceptor amine.

Similar linear plots were obtained for the hydrolysis of Ppr-OMe in the presence of Gly-OMe, and for the hydrolysis of D-Pla-OMe in the presence of Ile-OMe; in both cases, K_M was constant and k_{cat} (hydrolysis) decreased with increasing n_0, within the precision of the measurements. The estimated values of k_2 and k_4/k_3 are given in Table III. These data support the conclusion that, in the hydrolysis of Ppr-OMe and D-Pla-OMe by beef liver esterase over the pH range 7.2–8.5, acylation of the enzyme is the rate-limiting

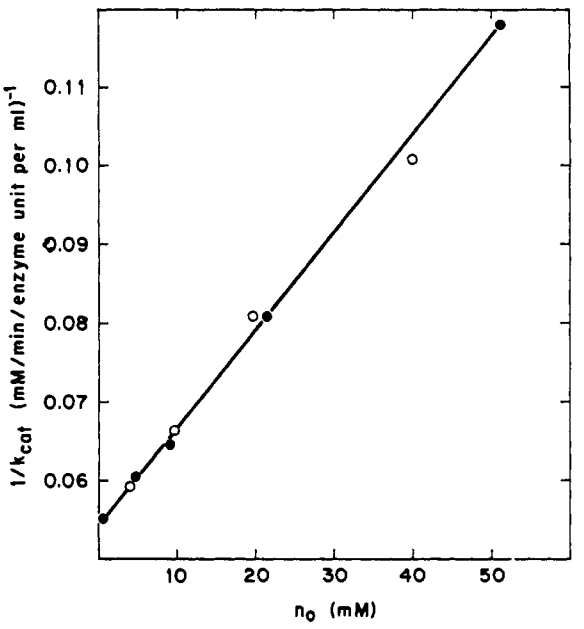

FIGURE 1: Plot of $(1/k_{cat})$ *vs.* n_0 for the hydrolysis of Ppr-OMe in the presence of Ile-OMe: enzyme concentration, 0.04 unit/ml; 37°. The initial concentration of Ile-OMe (n_0) given is that for the conjugate base: (●) pH 8.5; (○) pH 7.2.

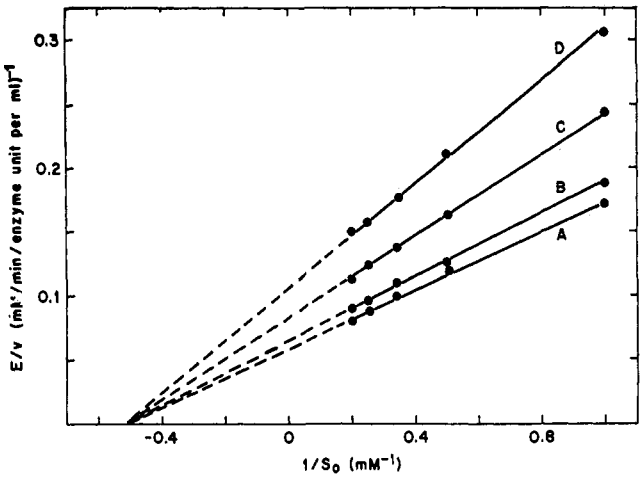

FIGURE 2: Inhibition of the hydrolysis of Ppr-OMe by Ile-OMe: enzyme concentration, 0.04 unit/ml; pH 8.5; 37°; concentration of Ile-OMe, curve A, 4.7 mM; curve B, 9.3 mM; curve C, 23.2 mM; curve D, 46.4 mM.

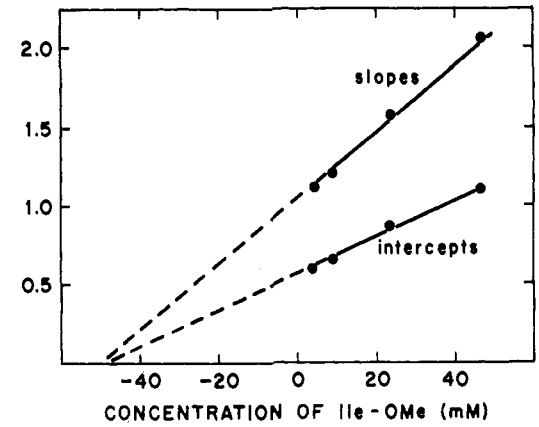

FIGURE 3: Replot of intercepts and slopes *vs.* concentration of Ile-OMe for data in Figure 2.

step. They also indicate that although Gly-OMe and Ile-OMe have the same pK_a value, the more hydrophobic amine gives a higher k_4/k_3 ratio, in agreement with earlier data (Goldberg and Fruton, 1969) that showed Ile-OMe to be a more effective acceptor of acyl groups than Gly-OMe. Since K_M is independent of nucleophile concentration, and k_{cat} for acid production varies inversely with n_0, the case in which acylation is rate limiting is formally identical with noncompetitive inhibition by the nucleophile, with $K_I = k_3/k_4$. Such K_I values are conveniently estimated by plotting E/v *vs.* $1/s_0$ and then replotting both the slopes and intercepts against the nucleophile concentration (Cleland, 1963); representative plots for the hydrolysis of Ppr-OMe in the presence of Ile-OMe are given in Figures 2 and 3. Similar plots, consistent with linear noncompetitive inhibition, were obtained for the hydrolysis of Ppr-OMe in the presence of Gly-OMe, and for the hydrolysis of D-Pla-OMe in the presence of Ile-OMe; the corresponding K_I values are given in the last column of Table III.

For the case in which acylation is rate limiting, the rate of disappearance of the ester substrate should be independent of the nucleophile concentration, and equal to k_2. Experiments to examine this point were performed at pH 8.5 with Ppr-OMe (4.9 mM) as the substrate and Ile-OMe (2.3, 4.6, 9.3, 23, or 46 mM) as the nucleophile. The time course of the reaction was followed up to about 60% completion by the determination of the residual Ppr-OMe (gas–liquid partition chromatography). The rate of Ppr-OMe disappearance accorded with pseudo-first-order kinetics, and the first-order rate constant (*ca.* 2 min^{-1} per enzyme unit per ml) was proportional to enzyme concentration over a tenfold range (0.009–0.09 unit per ml). Since the initial substrate concentration in these experiments was approximately 2.5 greater than the estimated value of K_M for Ppr-OMe (*ca.* 2 mM), data for the initial rate of disappearance of Ppr-OMe, as determined by analysis for residual substrate, may be compared to the data given in Table II for the initial rates of acid production. Although the precision of the data for the rate of Ppr-OMe disappearance is not so good as in the case of the pH-Stat data, it may be noted that, over the 20-fold range of concentration of Ile-OMe used, the value of k_{cat} for Ppr-OMe disappearance (16.4 ± 1.5 mM/min per enzyme unit per ml) was independent of Ile-OMe concentration, and was near the k_{cat} value for acid production in the absence of added nucleophile.

Under conditions where acylation is rate limiting, the rate of formation of the transfer product is given by the expression $k_{cat} = k_2k_4n_0/(k_3 + k_4n_0)$; thus, this rate increases hyperbolically with increasing n_0, tending toward a limiting value of k_2 for $k_4n_0 \gg k_3$ (Seydoux and Yon, 1967). Determinations of the rate of formation of Ppr-Ile-OMe by gas–liquid partition chromatography (from the same experiments in which the rate of disappearance of Ppr-OMe was measured) showed that, in the range 4.6–23 mM Ile-OMe, the rate of formation of the transfer product increased hyperbolically to a maximum near 1 mM/min per enzyme unit per ml, and dropped off at higher levels of Ile-OMe. This maximal value is much lower, however, than the estimated value of *ca.* 18 mM/min per enzyme unit per ml (Table III); this discrepancy may be tentatively attributed to inhibition of the transfer reaction, possibly through nonproductive binding of Ile-OMe (see Discussion).

It was noted in Table II that, with Pla-OMe as the substrate,

TABLE III: Estimated Kinetic Parameters for the Enzymic Hydrolysis of Esters in Presence of Amine Nucleophiles.[a]

Substrate	Nucleophile	k_2[b]	k_4/k_3 (M^{-1})[c]	K_I (mM)[d]
Ppr-OMe	Ile-OMe	18.6	24.6	44
	Gly-OMe	17.7	7.8	133
D-Pla-OMe	Ile-OMe	4.6	10.8	52

[a] Enzyme concentration, 0.04 unit/ml; pH 8.5; 37°. [b] mM/min per enzyme unit per ml. These values were obtained from the intercept on the ordinate in plots such as that given in Figure 1. [c] Calculated from values for slope and intercept in plots such as that given in Figure 1. [d] Estimated from E/v *vs.* $1/s_0$ plot at various concentrations of the nucleophile.

TABLE IV: Effect of pH on Hydrolysis of Enantiomeric Methyl β-Phenyllactates.[a]

Substrate	pH	Concentration Range (mM)[b]	k_{cat}[c]	K_M (mM)	k_{cat}/K_M
D-Pla-OMe	6.5	2.5–20 (5)	4.4 ± 0.4	11.3 ± 2.1	0.4
	7.2	2.5–25 (6)	4.4 ± 0.3	8.2 ± 1.5	0.5
	7.8	2.5–25 (6)	4.6 ± 0.2	6.6 ± 0.8	0.7
Pla-OMe	6.5	2.5–20 (5)	7.3 ± 0.5	11.0 ± 1.0	0.7
	7.2	5.6–56 (8)	16.5 ± 0.9	33.9 ± 0.6	0.5
	7.8	2.5–26 (6)	15.9 ± 0.6	21.9 ± 0.6	0.7

[a] Enzyme concentration, 0.1–0.2 unit/ml; 37°. [b] Numbers in parentheses denote the number of runs. [c] mM/min per enzyme unit per ml.

k_{cat} for acid production was independent of the concentration of Ile-OMe, whereas K_M increased with increasing values of n_0. This behavior is consistent with a rate-limiting deacylation, where k_{cat} for the disappearance of substrate should equal $k_3 + k_4 n_0$. A plot of the data for the rate of disappearance of 25 mM Pla-OMe (determined by gas–liquid partition chromatography) at pH 8.5 as a function of the initial concentration of Gly-OMe (46–230 mM) gave a straight line with a slope near 0.03 min^{-1}/enzyme unit per ml (the estimated value of k_4) and an intercept at 9.3 mM/min per enzyme unit per ml, a value near that found for k_{cat} (acid production) in the hydrolysis of Pla-OMe in the presence of Ile-OMe (Table II). These results are consistent with a rate-limiting deacylation reaction, for which the value of k_{cat} for acid production equals k_3. The k_4/k_3 ratio for the system in which Pla-OMe is the acyl donor and Gly-OMe is the amine nucleophile is therefore near 3 M^{-1}. As part of the above experiment, the Pla-Gly-OMe formed was also determined; a plot of the estimated rate of formation of the transfer product against Gly-OMe concentration gave a straight line passing through the origin, with a slope (k_4) of about 0.01 min^{-1}/enzyme unit per ml. The lack of good agreement between the two estimates of k_4 may be attributed to the relatively low yields of transfer product, and the limitations of the analytical method employed.

When deacylation is rate limiting, K_M should equal $(K_S/k_2) \times (k_3 + k_4 n_0)$. A plot of the K_M data for Pla-OMe in Table II against the concentration of Ile-OMe gave a straight line with an intercept at 6 mM for $K_S k_3/k_2$ and a slope of *ca.* 0.2. As further evidence for the conclusion that, in the hydrolysis of Pla-OMe by beef liver esterase, deacylation of the acylenzyme is rate limiting, it may be noted that a plot of E/v for acid production in the presence of Ile-OMe against the reciprocal of the concentration of Pla-OMe (Figure 4) accorded with linear competitive inhibition by Ile-OMe; a plot of the slopes against n_0 gave a straight line with an intercept on the abscissa corresponding to a K_I value of *ca.* 54 mM. This is the behavior to be expected when there is an ordered release of products, the acid leaving second (Cleland, 1963; Hsu *et al.*, 1966). The K_I value found is in reasonable agreement with those given in Table III for Ile-OMe as a noncompetitive inhibitor of the hydrolysis of Ppr-OMe or D-Pla-OMe.

In Table IV are given data for the effect of pH on the kinetic parameters for the hydrolysis of the enantiomers of methyl β-phenyllactate. It was noted previously (Goldberg and Fruton, 1969) that the enzymic hydrolysis of Ppr-OMe over the pH range 6.5–7.8 is characterized by a relatively constant value of k_{cat} and a decrease in the value of K_M from *ca.* 8 mM to *ca.* 3 mM. The data in Table IV indicate that whereas the hydrolysis of D-Pla-OMe resembles that of Ppr-OMe in this respect, the kinetic parameters for Pla-OMe exhibit a different variation with pH. Hofstee (1964, 1967) has suggested that the kind of independent variation of k_{cat} and K_M seen with Ppr-OMe and D-Pla-OMe is consistent with a rate-limiting acylation step. On the other hand, with Pla-OMe, where deacylation appears to be rate limiting,

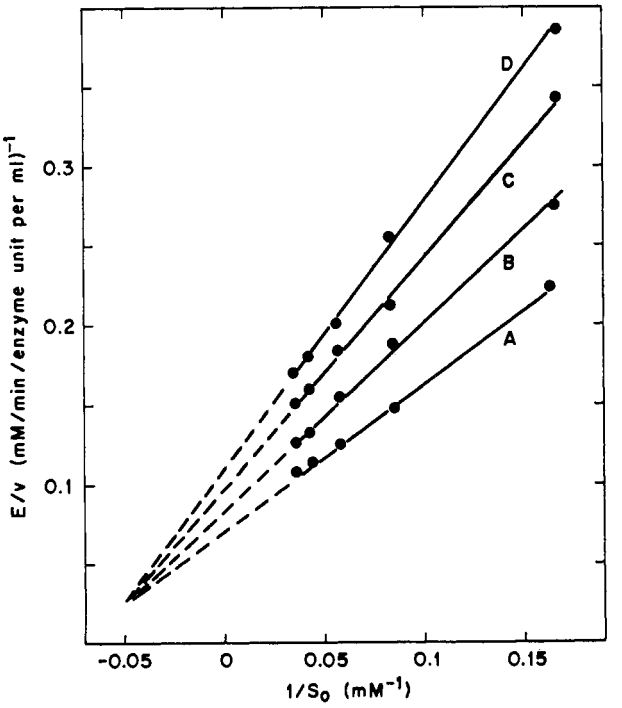

FIGURE 4: Inhibition of the hydrolysis of Pla-OMe by Ile-OMe: enzyme concentration, 0.10 unit/ml; pH 8.5; 37°; concentration of Ile-OMe, curve A, 8.6 mM; curve B, 24.2 mM; curve C, 47.4 mM; curve D, 68.8 mM.

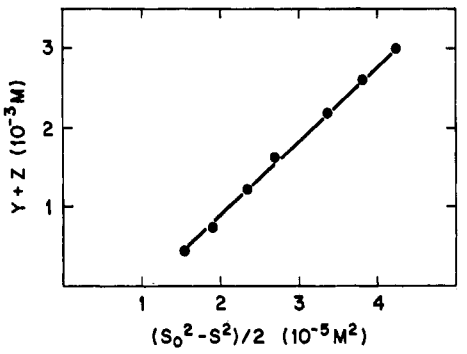

FIGURE 5: Determination of k_4/k_3 for process in which Phe-OMe is both acyl donor and acceptor amine. Enzyme concentration, 0.01 unit/ml; initial concentration of Phe-OMe, 10 mM; pH 8.5; 37°. The reaction was stopped at various times by the addition of HCl, and samples were analyzed by means of Sephadex G-10 columns as described by Goldberg and Fruton (1969). The amount (in microequivalents of Phe units) of Phe-Phe-OMe (y) and Phe-Phe (z) for each value of total Phe-OMe that had reacted was determined.

K_M should equal $K_S k_3/k_2$, and variations in $k_{cat}(k_3)$ would be expected. The relatively high values of K_M for Pla-OMe differ from those found for the two other esters, where K_M approximates K_S, and the value of K_S for Pla-OMe may be expected even higher than K_M, since the latter term would be multiplied by k_2/k_3.

An alternative method for estimating k_4/k_3 is based on the theoretical treatment of Durell and Fruton (1954), developed in connection with studies on transfer reactions catalyzed by papain and trypsin. For the cleavage of Ppr-OMe in the presence of Ile-OMe, when $s_0 \gg K_M$, the time course for the production of Ppr-Ile-OMe (concentration y) should be given by $dy/dt = -k_4 n(ds/dt)/(k_3 + k_4 n)$, which may be integrated to $\ln[(n_0 - y)/n_0] = (k_4/k_3)x$, where $x = s_0 - s - y$. Thus, a plot of the logarithmic term against x (concentration of Ppr-OH) for various reaction times should give a straight line passing through the origin and having a slope k_4/k_3. Because of the limited precision of the values of y and x at early stages of the reaction, reproducible linear plots could only be obtained for the points corresponding to longer reaction times (30–60% disappearance of Ppr-OMe). From the slope of such a plot, the k_4/k_3 ratio for the cleavage of Ppr-OMe (5 mM) in the presence of Gly-OMe (186 mM) at pH 8.5 was found to be about 8 M^{-1}. This value compares favorably to that given in Table III. At lower acceptor concentrations (19–46 mM), the extent of transfer could not be determined as accurately, and the value of k_4/k_3 fell in the range of 5–12 M^{-1}. Although these, and similar values obtained for the reaction in which Ile-OMe was the acceptor, cannot be assigned high accuracy, they are of the same order of magnitude as those calculated from data supplied by the pH-Stat method. They also serve to indicate that such a method of determining k_4/k_3 may be usefully applied in situations where hydrolysis data are not obtainable (*e.g.*, the reaction of Phe-OMe as donor and acceptor).

It would appear from the foregoing, therefore, that a significant difference exists for the k_4/k_3 ratios for the cleavage of Ppr-OMe in the presence of Ile-OMe and Gly-OMe. If it is assumed that k_3 (the rate of hydrolysis of the acyl-enzyme) is the same in both cases, it may be concluded that Ile-OMe is a more effective amine nucleophile than Gly-OMe because it is bound more tightly at the active site. As pointed out by Seydoux *et al.* (1969) in connection with the competition of alcohols with water for reaction with acyl-trypsins, the term k_4 may be considered to represent the product $k_4^*(K_W/K_N w)$, where w is the concentration of water and K_W and K_N are the dissociation constants of the complexes formed between the acyl-enzyme and water and the nucleophile, respectively. Thus, the difference in the values for k_4 for Ile-OMe and Gly-OMe may be considered to reflect differences in the value of K_N for these two amine nucleophiles.

As noted previously, Phe-OMe is a more effective amine acceptor in the esterase-catalyzed cleavage of Ppr-OMe than is Ile-OMe or Gly-OMe (Goldberg and Fruton. 1969). However, since both Phe-OMe and Ppr-Phe-OMe are substrates for the enzyme, the types of analysis employed to estimate k_4/k_3 in the presence of Gly-OMe or Ile-OMe could not be used. To gain an estimate of the partition of the acyl-enzyme when it reacts with water or Phe-OMe, the system in which Phe-OMe served both as acyl donor and as acceptor amine was examined. It was assumed, as an approximation, that the following processes were involved

$$\text{Phe-OMe} + \text{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{E(Phe-OMe)} \xrightarrow{k_2} \text{Phe-E} + \text{MeOH}$$

$$\text{Phe-E} + \text{H}_2\text{O} \xrightarrow{k_3} \text{Phe-OH} + \text{E}$$

$$\text{Phe-E} + \text{Phe-OMe} \xrightarrow{k_4} \text{Phe-Phe-OMe} + \text{E}$$

$$\text{Phe-Phe-OMe} + \text{E} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{E(Phe-Phe-OMe)} \xrightarrow{k_6} \text{Phe-Phe-E} + \text{MeOH}$$

$$\text{Phe-Phe-E} \xrightarrow{k_7} \text{Phe-Phe} + \text{E}$$

The application of the steady-state treatment to this system for the rate of formation of Phe-Phe-OMe (concentration y) and of Phe-Phe (concentration z), gave the expression

$$dy/dt + dz/dt = -k_4 s(ds/dt)/(k_3 + 2k_4 s)$$

which was integrated to

$$y + z = [(s_0 - s)/2] + \ln[(1 + 2(k_4/k_3)(s)/1 + 2(k_4/k_3)s_0]/4(k_4/k_3)$$

By use of the Taylor series expansion $[\ln(1 + x) = x - (x^2/2)]$ for the first two terms, and simplifying the resulting expression, the following equation was obtained.

$$y + z = (k_4/k_3)(s_0^2 + s^2)/2$$

If this equation is valid, then a plot of $y + z$ (the concentration in Phe microequivalents of Phe-Phe-OMe and Phe-Phe) against $(s_0^2 - s^2)/2$ should yield a straight line whose slope is k_4/k_3 and which passes through the origin. A representative plot is given in Figure 5. Although satisfactory straight lines were obtained, extrapolation to the abscissa did not yield the

origin as a point, suggesting that during the initial stages of the reaction, some Phe-OMe was hydrolyzed before Phe-Phe-OMe was formed. Further studies are needed to elucidate this behavior, but it is significant that, over a tenfold concentration range of enzyme (0.01–0.10 unit/ml), the value of k_4/k_3 = *ca.* 110 M^{-1}. Such a high ratio for k_4/k_3 indicates that Phe-OMe is a better acceptor than either Gly-OMe or Ile-OMe and is probably bound much more effectively to the acyl-enzyme.

Since it has been demonstrated that Phe-OMe can act as both donor and acceptor, it might be expected that other substrates which contain a nucleophilic group, such as Pla-OMe, might function in the same manner. Among the products of the reaction between Pla-OMe and Gly-OMe at pH 8.5 was Pla-Pla-OMe. The extent of synthesis of Pla-Pla-OMe was dependent upon the initial concentration of Pla-OMe and decreased in the presence of increasing concentrations of Gly-OMe. The reverse was true for the transfer product, Pla-Gly-OMe. Thus, with 50 mM Pla-OMe and 10 mM Gly-OMe (enzyme, 0.1 unit/ml; pH 8.5; 20 min), the yield of Pla-Pla-OMe was 0.7 mM and that of Pla-Gly-OMe was 0.1 mM. When the concentration of Pla-OMe was lowered to 10 mM, no Pla-Pla-OMe was detected and the concentration of Pla-Gly-OMe had risen to 0.3 mM.

Discussion

The data presented above appear to be consistent with the view that the action of beef liver esterase involves the formation of an acyl-enzyme intermediate at one site (D, donor site), and that this intermediate may react with a nucleophilic acceptor bound to a second site (A, acceptor site). The data are also consistent with the hypothesis that the acyl donor (*e.g.*, Ppr-OMe or Phe-OMe) as well as the acceptor amine (*e.g.*, Ile-OMe or Phe-OMe) may be bound either at the D site or at the A site. Thus, binding of Ile-OMe (which is not cleaved) in the D site or of Ppr-OMe (which cannot serve as acceptor) in the A site may inhibit the rate of appearance of the product Ppr-Ile-OMe formed upon the esterase-catalyzed reaction of Ppr-OMe with Ile-OMe. It appears likely that nonproductive binding of this type may be involved not only in the inhibition of the formation of Ppr-Ile-OMe, but also in the results reported above for the formation of Pla-Gly-OMe and of Phe-Phe-OMe.

The finding that the rate-limiting step in the hydrolysis of Ppr-OMe or of D-Pla-OMe appears to be the acylation of the enzyme, whereas the kinetics of the hydrolysis of L-Pla-OMe indicate a rate-limiting deacylation of the acyl-enzyme, suggests that the binding of the acyl donor in site D involves a stereospecific interaction with the substituent at the α-carbon of the substrate. It may be surmised that the α-hydroxyl group of L-Pla-OMe (and possibly also the α-amino group of L-Phe-OMe) can interact by hydrogen bonding to an enzymic group in a manner that accelerates the acylation reaction or retards the deacylation reaction by electron withdrawal from the carbonyl group of the acyl donor. It is clear from the lack of stereochemical specificity with respect to the hydrolysis of the enantiomeric Pla-OMe or Phe-OMe substrates, and the rapid hydrolysis of Ppr-OMe, that the acylation of the enzyme proceeds at a significant rate even when an α substituent (OH or NH_2) is absent or in an apparently unfavorable orientation in the enzyme–substrate complex. The principal contributions to productive interaction at the D site appear to be associated with the hydrophobic side-chain group and the COOR′ group of the acyl donor. Previous studies (Goldberg and Fruton, 1969) have shown that the action of beef liver esterase on substrates of the type RCOOR′ is favored by the presence of a hydrophobic R group (*e.g.*, benzyl, *n*-hexyl), and that branching at the β carbon of the RCO group inhibits hydrolysis.

The data on the inhibition, by Ile-OMe, of the initial rate of hydrolysis of Ppr-OMe, Pla-OMe, and D-Pla-OMe, support the conclusion that Ile-OMe can compete with these acyl donors for the D site. It is noteworthy that the estimated K_I value for Ile-OMe is the same (within the precision of the measurements) with the three donors, even though the rate-limiting step in the hydrolysis of Pla-OMe differs from that for the other two acyl donors.

It has been reported above that Gly-OMe is about 3 times less effective as a competitive inhibitor than is Ile-OMe in affecting the initial rate of hydrolysis of Ppr-OMe. A similar ratio is noted for the values of k_4/k_3 when these two amine nucleophiles act as acceptors in the transfer reaction. In the latter case, the nucleophiles are assumed to interact with site A, thus suggesting that they may be bound by sites D and A to an equal degree. As judged by the value for k_4/k_3, Phe-OMe is about 10 times more effective than is Ile-OMe as an acceptor. Although a reliable value of K_S for Phe-OMe could not be estimated, it may be assumed to approximate that of Ppr-OMe (*ca.* 5 mM at pH 8.5), or about 0.1 of the value of K_I for Ile-OMe. These conclusions strongly suggest the possibility that sites D and A may be similar in their ability to bind amino acid esters such as Ile-OMe and Phe-OMe, and that these sites may indeed be similar in structure. At the present state of our knowledge, it is not possible to decide whether sites D and A belong to the same monomeric active enzyme unit, or whether a single site per monomeric unit can act either as an A site or as a D site. In the latter case, efficient transfer of acyl groups from an acyl donor bound at one monomeric unit to an acceptor amine bound at another unit would require a tight and stereochemically favorable association of the two monomeric units. In view of the demonstration (Benöhr and Krisch, 1968) that beef liver esterase, like other liver carboxyl esterases, is a dimer of molecular weight *ca.* 170,000, it is possible that the efficiency of the transfer process (as measured by k_4/k_3) may be a function of the ratio of dimeric to monomeric forms of the enzyme. Thus, conditions that promote dissociation may be expected to decrease k_4/k_3, *i.e.*, to promote hydrolysis as compared to transfer to an acceptor amine.

It has been reported previously (Goldberg and Fruton, 1969) that 175-fold dilution of the enzyme is accompanied by a marked decrease (from 70 to 42%) in the fraction of Phe units of Phe-OMe that appear as Phe-Phe-OMe or Phe-Phe, as compared to the total Phe-OMe that has undergone reaction. Furthermore, the addition of 10% dioxane was found to cause a similar shift in favor of hydrolysis of Phe-OMe (Goldberg and Fruton, 1969). Such effects of dilution or of dioxane are consistent with the hypothesis that the transfer reaction involves the cooperative interaction of the subunits of a dimeric enzyme, but cannot be considered as proof of the correctness of this view.

It may be noted that an additional effect of dioxane was to decrease the rate of hydrolysis of Phe-Phe-OMe to Phe-Phe (Goldberg and Fruton, 1969), and a similar effect was noted

with chloramphenicol (Krenitsky and Fruton, 1966). These observations raise the possibility that the dipeptide ester is hydrolyzed most effectively when it is bound by the dimeric form of the enzyme.

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Mechanistic Studies of Beef Plasma Amine Oxidase*

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ABSTRACT: The initial velocity patterns for the deamination of benzylamine by beef plasma amine oxidase have been determined. The results suggest that the reaction proceeds through a Ping-Pong mechanism in which approximately 1 mole of benzaldehyde is formed anaerobically per mole of enzyme as reported by Reed and Swindell. The product inhibition patterns have been determined at saturating and nonsaturating concentrations of the two variable substrates.

From the results obtained a formal mechanism for the reaction has been proposed and the corresponding rate equation derived.

Beef plasma amine oxidase was initially purified by Tabor *et al.* (1954) and subsequently crystallized by Yamada and Yasunobu (1962a). The latter investigators have shown that this enzyme requires copper for activity (1962b) and in agreement with Tabor *et al.* (1954), that the enzyme contains a prosthetic group with an aldehyde function. Indirect evidence presented by Yamada and Yasunobu (1963a) indicates that the organic prosthetic group may be pyridoxal phosphate which is covalently attached to the enzyme. The molecular weight of the enzyme has been shown to be 170,000 (Achee *et al.*, 1968) and it has been shown that there are two identical subunits which are covalently attached through SS linkages (Achee *et al.*, 1968).

Little is known about the mechanism by which this enzyme catalyzes the oxidation of amines. Enzymes from different sources appear to have different substrate specificities and spectra and there is no evidence to indicate that all the copper–pyridoxal phosphate amine oxidases proceed by the same mechanism. Thus, it is necessary for the time being to consider these amine oxidases from different sources as separate entities. Reed and Swindell (1969) have reported that in the reaction catalyzed by the beef plasma amine oxidase, 1 mole of benzaldehyde is produced anaerobically and that the K_m for oxygen is dependent on the amine concentration. These studies suggest that a Ping-Pong mechanism describes the reaction catalyzed by beef plasma amine oxidase.

Recent advances in the theoretical treatment of multisubstrate–enzyme reactions, due to the pioneering work of Alberty (1953), Boyer (1959), Wong and Hanes (1962), Bloomfield *et al.* (1962), Dalziel (1967), Frieden (1957), and especially Cleland (1963a–c) and Fromm (1967), provide methods for elucidating the formal mechanism by kinetic studies. The present communication reports the results of the kinetic investigation of crystalline beef plasma amine oxidase.

Experimental Section

Materials. Crystalline beef plasma amine oxidase was isolated by a method which has been published previously

* From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii. *Received February 26, 1970.* This work was supported in part by the National Institutes of Health Grant MH 10280-05, the National Science Foundation Grant GB 18739, The American Heart Association Grant 68 769, and a grant from the Hawaii Heart Association.

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