absence of oxalate. In the absence of oxalate, values of 2.2 and 1.6 are obtained for $^{D}(V/K_{malate})$ and $^{D}V$, respectively, with $Mn^{2+}$ as the divalent metal ion (Gavva, 1988). Deuterium isotope effects in the presence of 5 $\mu$M oxalate at pH 5.5 measured for the noncompetitive region with $Mn^{2+}$ as the divalent metal ion are $^{D}(V/K_{malate}) = 2.4 \pm 0.3$ and $^{D}V = 1.35 \pm 0.05$. The isotope effect on $V/K_{malate}$ is identical with that obtained at zero oxalate while the isotope effect on $V$ is lower than the value of 1.6 as predicted by the mechanism shown in Scheme I. The decreased effect on $V$ indicates a slowing down of a step other than hydride transfer and after release of the first product (likely to be $CO_2$) since the value of $^{D}(V/K_{malate})$ does not change. This step is almost certainly NADH release.

The deuterium isotope effect of 1.6 on $V$ is the value obtained at infinite malate concentration including the substrate activation region. The value of $^{D}(V/K_{malate})$ obtained for the $C_{asym}$ region, however, is $1.34 \pm 0.10$, much lower than the value obtained for $^{D}(V/K_{malate})$ for the noncompetitive region. The lower value is expected from a consideration of the expressions for $C_f$ derived for the competitive (eq A25) and noncompetitive (eq A28, where $C_f$ is $k_5/k_4$) regions of the curve. Since data were collected at pH 5.5, the first term in the numerator of eq A25 will not increase $C_f$ substantially. The $1 + H/K_1$ term is calculated to be only 0.25 while the $I/L_{i1}$ and $I/L_{i2}$ terms are estimated at 10 and 0.25, respectively, assuming $L_{i1}$ and $L_{i2}$ are equal to $K_{i1}$ and $K_{i2}$, a reasonable assumption as shown above. It is the contribution of the induced substrate activation pathway that contributes most to the increase in $C_f$ at this pH. This is expected since the $V/K$ for the substrate activation region is not really a $V/K$ in the true sense, i.e., the rate under conditions where malate is limiting for the overall reaction and all other reactants are saturating. It is instead an apparent $V/K$ for substrate activation obtained under conditions in which the addition of malate to the E·NADH·$M^{2+}$ complex is limiting, i.e., $V_{max}$ conditions.

Thus, the data are qualitatively and quantitatively consistent with a mechanism in which malate and oxalate bind optimally to forms of the E·NADH·Mn complex unprotonated and protonated, respectively, at a general base with a p$K$ of 4.9. Protonation of this general base has been shown (Kiick et al., 1986) to result in a decrease in the off-rate for NADH with oxalate stabilizing the protonated form. Malate binds to the unprotonated form of the complex so that at infinite malate concentration the unprotonated form is stabilized. The off-rate for NADH is not affected by bound malate, and thus when the malate concentration is very high in the presence of oxalate, the off-rate for NADH is effectively increased as a result of a shift in the equilibrium between unprotonated and protonated forms of the E·NADH·Mn complex. The result is substrate activation by malate.

**Registry No.** NAD–malic enzyme, 9028-46-0; malate, 97-67-6; deuterium, 7782-39-0; oxalate, 144-62-7; L-malate-2-*d*, 71655-88-4.

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## Appendix: Derivation of the Rate Equation for Substrate Activation by Malate Induced by Oxalate

Under conditions in which NAD and $Mn^{2+}$ (or $Mg^{2+}$) are saturating and malate is varied at different fixed levels of oxalate, the mechanism depicted in Scheme I applies where A, B, C, P, Q, R, and I are NAD, $Mn^{2+}$ (or $Mg^{2+}$), L-malate, pyruvate, $CO_2$, NADH, and oxalate, respectively. The segments X and Y are assumed to be in rapid equilibrium. An equation based on a mechanism similar to that given above was derived previously by Dalziel and Dickinson (1966) to describe the substrate activation observed by cyclohexanol in the equine liver alcohol dehydrogenase reaction. The difference between the two treatments is that the present mechanism allows for inhibitor binding and also for different protonation states of enzyme–reactant (or inhibitor) complexes.

Using the method of King and Altman (1956) and applying the rapid equilibrium assumption of Cha (1968) to segments X and Y, we derived the rate equation describing Scheme I:

$$[E_t]/v = \{f_9k_9(k_2 + k_{12})(k_4k_6 + k_4k_7 + k_5k_7)/[C]^2 + [f_3k_3(k_5k_7 + f_9k_5k_9 + f_9k_6k_9 + f_9k_7k_9)(k_2 + k_{12}) + f_{11}k_2k_4k_{11}(k_6 + k_7)]/[C] + f_3f_{11}k_3k_{11}(k_2k_5 + k_2k_6 + k_2k_7 + k_5k_7)\}/\{f_3f_9k_3k_5k_7k_9(k_2 + k_{12})/[C] + f_3f_{11}k_2k_3k_5k_7k_{11}\} \quad (A1)$$

where $f_3$, $f_9$, and $f_{11}$ are defined as

$$f_3 = \frac{1}{1 + [I]/K_{i2} + [H]/K_1 + [H][I]/K_1K_{i1}} \quad (A2)$$

$$f_9 = f_{11} = \frac{1}{1 + [I]/L_{i2} + [H]/L_1 + [H][I]/L_1L_{i1}} \quad (A3)$$

Equation A1 can be rewritten generally as

$$\frac{[E_t]}{v} = \frac{DZ^2 + EZ + F}{GZ + H} \quad (A4)$$

where $Z = 1/C$ and the coefficients $D$, $E$, $F$, $G$, and $H$ are $f_9k_9(k_2 + k_{12})(k_4k_6 + k_4k_7 + k_5k_7)$, $f_3k_3(k_5k_7 + f_9k_5k_9 + f_9k_6k_9 + f_9k_7k_9)(k_2 + k_{12}) + f_{11}k_2k_4k_{11}(k_6 + k_7)$, $f_3f_{11}k_3k_{11}(k_2k_5 + k_2k_6 + k_2k_7 + k_5k_7)$, $f_3f_9k_3k_5k_7k_9(k_2 + k_{12})$, and $f_3f_{11}k_2k_3k_5k_7k_{11}$, respectively. Equation A4 describes a function such as that shown in Figure 4. For Figure 4, two asymptotes can be defined, one at high values of $1/C$ ($NC_{asym}$) and a second at $1/C$ near zero ($C_{asym}$). Equations for the $C$ asymptotes can be obtained by differentiating $[E_t]/V$ with respect to $1/C$, giving

$$\frac{d([E_t]/V)}{dZ} = \frac{DGZ^2 + 2DHZ + (EH - FG)}{G^2Z^2 + 2GHZ + H^2} \quad (A5)$$

The slope of $C_{asym}$ is obtained at $Z = 0$ and is given as

$$\text{slope of } C_{asym} = (E/H) - (FG/H^2) \quad (A6)$$

Substituting the expressions for $E$, $F$, $G$, and $H$ gives

$$\text{slope of } C_{asym} = k_4(k_6 + k_7)/f_3k_3k_5k_7 + (k_2 + k_{12})/k_2k_{11}f_{11} - k_9(k_2 + k_{12})/k_2^2k_{11} \quad (A7)$$

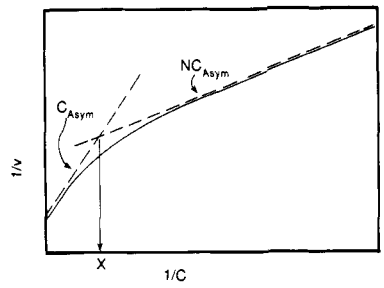


FIGURE 4: Double-reciprocal plot exhibiting substrate activation by $C$. $C_{asym}$ and $NC_{asym}$ are the asymptotes as $1/C$ approaches infinity and zero, respectively. X is the value of the $x$ coordinate of the intersection point of the asymptotes.

The intercept is obtained from the limit of eq A1 at $1/C$ equal to zero:

$$\text{intercept} = 1/k_2 + 1/k_7 + (k_6 + k_7)/k_5k_7 \quad \text{(A8)}$$

The full equation for $C_{asym}$ is given in eq A9. The term $C_{asym}$ (and also $NC_{asym}$) refers to the asymptote of the double-reciprocal form of eq A1 so that eq A9 is in double-reciprocal

$$C_{asym} = \{k_4(k_6 + k_7)/f_3k_3k_5k_7 + (k_2 + k_{12})/k_2k_{11}f_{11} - k_9(k_2 + k_{12})/k_2^2k_{11}\}/[C] + 1/k_2 + 1/k_7 + (k_6 + k_7)/k_5k_7 \quad \text{(A9)}$$

form. Another term that could be substituted for $C_{asym}$ is $[E_t]/vC_{asym}$. We prefer to use $C_{asym}$ since it already refers to the asymptote of the double-reciprocal form of Figure 4. To obtain $NC_{asym}$, the limit of eq A4 as $Z$ goes to infinity is taken. Dividing out eq A4 gives

$$\frac{DZ^2 + EZ + F}{GZ + H} = DZ/G + 1/G(E - DH/G) + \{F - H/G(E - DH/G)\}/(GZ + H) \quad \text{(A10)}$$

At infinite $Z$, the last term becomes zero, and $NC_{asym} = DZ/G + 1/G(E - DH/G)$. Substituting the expressions for $D$, $Z$, $G$, $E$, and $H$ gives

$$NC_{asym} = \{k_4(k_6 + k_7)/k_3k_5k_7 + 1/k_3\}/f_3[C] + 1/k_9f_9 - k_2k_{11}/[f_3k_3k_9(k_2 + k_{12})] + 1/k_7 + (k_6 + k_7)/k_5k_7 \quad \text{(A11)}$$

The $x$ coordinates to the intersection point of $C_{asym}$ and $NC_{asym}$ are obtained from a solution of the equation obtained when $C_{asym}$ and $NC_{asym}$ are set equal under conditions when [I] is equal to 0 and solving for $1/C$. This expression is given in eq A12. The $y$ coordinate is then obtained by substituting the

$$x \text{ coordinate} = \frac{k_{11}k_2}{(k_2 + k_{12})k_9} = 1/[C] \quad \text{(A12)}$$

expression for the $x$ coordinate into the expression for either $C_{asym}$ or $NC_{asym}$ and simplifying. The expression for the $y$ coordinate is given in eq A13. Equation A11 is in the form

$$y \text{ coordinate} = \frac{k_4(k_6 + k_7)k_{11}k_2(1 + [H]/K_1)}{k_3k_5k_7(k_2 + k_{12})k_9} + \frac{[I]k_4(k_6 + k_7)k_{11}k_2(1/K_{i2} + [H]/K_1K_{i1})}{k_3k_5k_7(k_2 + k_{12})k_9} + \frac{1 + [H]/L_1}{k_9} + \frac{[I](1/L_{i2} + [H]/L_1L_{i1})}{k_9} + \frac{1}{k_7} + \frac{k_6 + k_7}{k_5k_7} \quad \text{(A13)}$$

$y = mx + b$ where $x$ is $1/C$. The app$K_{is}$ for the $NC_{asym}$ is obtained by solving for [I] a secondary expression for the slope of $NC_{asym}$ vs [I] under conditions where the slope of $NC_{asym}$ is equal to zero. The expression for the slope of $NC_{asym}$ is given by

$$\text{slope of } NC_{asym} = [k_4(k_6 + k_7)/k_3k_5k_7 + 1/k_3]/f_3 = 0 \quad \text{(A14)}$$

Substituting $1 + [I]/K_{i2} + [H]/K_1 + [H][I]/K_1K_{i1}$ for $1/f_3$ and solving for [I] give

$$[I] = -\frac{1 + [H]/K_1}{1/K_{i2} + [H]/K_1K_{i1}} = -\frac{K_{i1}(1 + [H]/K_1)}{K_2/K_1 + [H]/K_1} = \text{app}K_{is} \quad \text{(A15)}$$

When $[H] \gg K_1 > K_2$, app $K_{is}$ for $NC_{asym} = K_{i1}$ and when $[H] \ll K_2 < K_1$, app$K_{is}$ for $NC_{asym} = K_{i1}K_1/K_2 = K_{i2}$. As a result, intrinsic $K_i$ values can be obtained. As discussed for the app$K_{is}$, the app$K_{ii}$ for $NC_{asym}$ is obtained by solving for [I] a secondary expression for the intercept of $NC_{asym}$ vs [I] under conditions where the intercept of $NC_{asym}$ is equal to zero. The expression for the intercept of $NC_{asym}$ is given by

$$\text{intercept of } NC_{asym} = 1/k_9f_9 - k_2k_{11}/[f_3k_3k_9(k_2 + k_{12})] + 1/k_7 + (k_6 + k_7)/k_5k_7 = 0 \quad \text{(A16)}$$

Substituting for $f_3$ and $f_9$ and factoring out [I] give

$$\text{intercept of } NC_{asym} = [I]\{(1/L_{i2} + [H]/L_1L_{i1})/k_9 - k_2k_{11}(1/K_{i2} + [H]/K_1K_{i1})/[k_3(k_2 + k_{12})k_9]\} + (1 + [H]/L_1)/k_9 - k_2k_{11}(1 + [H]/K_1)/[k_3(k_2 + k_{12})k_9] + 1/k_7 + (k_6 + k_7)/k_5k_7 = 0 \quad \text{(A17)}$$

Solving for [I] gives

$$[I] = -\{(1 + [H]/L_1)/k_9 - (1/k_3)[k_{11}k_2(1 + [H]/K_1)]/[(k_2 + k_{12})k_9] + 1/k_7 + (k_6 + k_7)/k_5k_7\}/\{(L_2/L_1 + [H]/L_1)/k_9L_{i1} - [k_{11}k_2(K_2/K_1 + [H]/K_1)]/[k_3(k_2 + k_{12})k_9K_{i1}]\} = \text{app}K_{ii} \quad \text{(A18)}$$

When $[H] \gg L_1 > K_1$, the app$K_{ii}$ for $NC_{asym}$ is

$$\text{app}K_{ii} = \frac{H/L_1k_9 - k_{11}k_2H/[K_1k_3k_9(k_2 + k_{12})]}{H/L_{i1}L_1k_9 - k_{11}k_2H/[K_1K_{i1}k_3k_9(k_2 + k_{12})]} \quad \text{(A19)}$$

Thus, if $L_{i1} = K_{i1}$, app$K_{ii} = L_{i1}$.

As above, the app$K_{is}$ for $C_{asym}$ is obtained by solving for [I] a secondary expression for the slope of $C_{asym}$ vs [I] under conditions where the slope of $C_{asym}$ is equal to zero. The expression for the slope of $C_{asym}$ is given by

$$\text{slope of } C_{asym} = k_4(k_6 + k_7)/k_3k_5k_7f_3 + (k_2 + k_{12})/k_2k_{11}f_{11} - k_9(k_2 + k_{12})/k_2^2k_{11} = 0 \quad \text{(A20)}$$

Substituting for $f_3$ and $f_{11}$ and solving for [I] when the slope is zero give

$$[I] = \{[k_4(k_6 + k_7)(1 + [H]/K_1)]/k_3k_5k_7 + [(k_2 + k_{12})(1 + [H]/L_1)]/k_2k_{11} - [(k_2 + k_{12})k_9/k_{11}k_2](1/k_2)\}/\{[k_4(k_6 + k_7)(K_2/K_1 + [H]/K_1)]/k_3k_5k_7K_{i1} + [(k_2 + k_{12})(L_2/L_1 + [H]/L_1)]/k_2k_{11}L_{i1}\} = \text{app}K_{is} \quad \text{(A21)}$$

When $[H] \gg K_1 > K_2$ and $[H] \gg L_1 > L_2$:

$$\text{app}K_{is} = \{[k_4(k_6 + k_7)([H]/K_1)]/k_3k_5k_7 + [(k_2 + k_{12}) \times ([H]/L_1)]/k_2k_{11}\}/\{[k_4(k_6 + k_7)([H]/K_1)]/k_3k_5k_7K_{i1} + [(k_2 + k_{12})([H]/L_1)]/k_2k_{11}L_{i1}\} \quad \text{(A22)}$$

Equations for the deuterium isotope effects are given below where the rate constants, $k_5$ and $k_6$, are isotope sensitive. In the case of $C_{asym}$, the isotope effect on $V$ is given by

$$^DV = \frac{^Dk_5 + k_5(1/k_7 + 1/k_2) + (k_6/k_7)^DK_{eq}}{1 + k_5(1/k_7 + 1/k_2) + k_6/k_7} \quad \text{(A23)}$$

while the isotope effect on $V/K$ is given by

$$^{D}(V/K) = \frac{^{D}k_5 + C_f + C_r{}^{D}K_{eq}}{1 + C_f + C_r} \quad (A24)$$

where

$$C_f = \{(k_5/k_4)(1 + [H]/K_1) + (k_5/k_4)[I] \times [[k_3(k_2 + k_{12})]/k_2k_{11}](1/L_{i2} + [H]/L_1L_{i1})\}/\{(1 + [H]/K_1) + [I](1/K_{i2} + [H]/K_1K_{i1})\} \quad (A25)$$

and $C_r = k_6/k_7$.

In the case of $NC_{asym}$, the expressions for the isotope effects are given as

$$^{D}V = \frac{^{D}k + C_{vf} + C_r(^{D}K_{eq})}{1 + C_{vf} + C_r} \quad (A26)$$

where

$$C_{vf} = 1 + \frac{k_5}{k_2} + \frac{k_5}{k_9} + [I](1/L_{i2} + [H]/L_1L_{i1}) - [I]\frac{k_5k_{11}k_2}{k_3k_9(k_2 + k_{12})}(1/K_{i2} + [H]/K_1K_{i1}) \quad (A27)$$

and $C_r = k_6/k_7$ while

$$^{D}(V/K) = \frac{^{D}k + k_5/k_4 + (k_6/k_7)^{D}K_{eq}}{1 + k_5/k_4 + k_6/k_7} \quad (A28)$$

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# Interface-Mediated Inactivation of Pancreatic Lipase by a Water-Reactive Compound: 2-Sulfobenzoic Cyclic Anhydride†

André Moulin, Jean-Dominique Fourneron, Gérard Piéroni, and Robert Verger*

*Centre de Biochimie et de Biologie Moléculaire du CNRS, 31 Chemin Joseph-Aiguier, 13402 Marseille Cedex 9, France*

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ABSTRACT: 2-Sulfobenzoic cyclic anhydride (SBA) rapidly and selectively inactivates porcine pancreatic lipase (PPL) *only* when added during the hydrolysis of an emulsified ester such as tributyrin or dodecyl acetate. The present data suggest that the inactivation of PPL occurs preferentially at the oil/water interface and not in the aqueous phase, since colipase and bile salt were found to adversely affect the inhibition process. Moreover, it is shown that at a molar ratio of SBA to pure PPL of 1, 40% of the lipase activity was already irreversibly lost. Complete inactivation was observed at SBA to pure PPL molar ratios of 120. A 60% inactivation occurred when 0.5 mol of $^{3}H$-labeled SBA was attached per mole of PPL. The SBA-inactivated PPL competes for binding to the dodecyl acetate/water interface as efficiently as the native enzyme. Larger SBA concentrations are required when crude lipase preparations are used as well as with pure PPL in the presence of bile salts and colipase. Lipases were found to have variable sensitivites to SBA inactivation, depending on their origin. In the presence of bile salts and tributyrin at pH 6.0, human gastric lipase activity was not affected by the presence of a $10^6$ molar excess of SBA.

Achieving specific and covalent inactivation of lipolytic enzymes is a difficult task, because of nonmutually exclusive processes such as interfacial denaturation, changes in "interfacial quality", and surface dilution phenomena (Verger & de Haas, 1976; Dennis, 1987). Furthermore, the interfacial enzyme binding and/or the catalytic turnover can be diversely affected by the presence of potential amphipatic inhibitors (Verger, 1984; Kurganov et al., 1985).

A large number of chemical reagents has been used for modifying several "essential" amino acid residues in porcine pancreatic lipase (Verger, 1984; Desnuelle, 1986). Pancreatic lipase is not inactivated by classical water-dispersed serine esterase inhibitors such as diisopropyl fluorophosphate, benzamidine, or phenylmethanesulfonyl fluoride; in fact, these serine protease inhibitors are currently used in a millimolar concentration range to prevent pancreatic lipase proteolysis from occurring during the purification procedures (Verger, 1984). However, some other hydrophobic aromatic sulfonyl halides such as 4-iodobenzenesulfonyl chloride and 1-(dimethylamino)naphthalene-5-sulfonyl chloride (dansyl chloride) inactivate porcine pancreatic lipase by reacting with several residues, probably including a serine (Verger, 1970; Maylié et al., 1972).

Desnuelle et al. (1960) first showed that gum arabic emulsified diethyl *p*-nitrophenyl phosphate irreversibly inactivates porcine pancreatic lipase, in sharp contrast with aqueous solutions of this organophosphorus compound. Maylié et al. (1972) and Rouard et al. (1978) described the covalent modification of a serine residue of pancreatic lipase induced by mixed micelles of diethyl *p*-nitrophenyl phosphate and bile salts. The finding that colipase played an essential role in this inactivation process confirms that the first step in the inactivation is an interaction between lipase and bile salt containing micelles. The requirements of lipase as far as specific sub-

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