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KINETIC ANALYSIS OF THE COMPETITIVE INHIBITION OF PHOSPHOLIPASE A_2 IN TRITON X-100 MIXED MICELLES

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Abstract: A detailed kinetic scheme is proposed for the competitive inhibition of phospholipase A_2 in Triton X-100 mixed micelles. The water-soluble enzyme first binds to phospholipid in the lipid/water interface and then searches the 2D interface for substrate, binding it in the catalytic site. Phospholipid inhibitors act as substrates in the first binding step but as competitive inhibitors in the second step.

Phospholipase A_2 (PLA_2) is a lipolytic enzyme which acts on substrates that are part of a lipid/water interface.[1] This interface plays an important role in enzymatic activity.[2-6] It also complicates the study of enzyme kinetics, especially those involving enzyme inhibition. Several forms of the substrate have been employed in kinetic studies of PLA_2 inhibition including monomers,[7] micelles,[8,9] and vesicles.[10] We[11-13] have previously developed and evaluated substrate analogues that serve as competitive inhibitors of PLA_2. In the present study, we propose a general kinetic model and a method to determine the dissociation constants of water-insoluble competitive inhibitors of lipolytic enzymes in a mixed micellar system.

a. Bulk

$$\begin{array}{l} E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \\ \quad + \\ \quad I \underset{}{\overset{K_2}{\rightleftharpoons}} EI \end{array} \qquad [ES] + [EI] = [EM]$$

b. Surface

$$EM + S \overset{K_3}{\rightleftharpoons} EMS \xrightarrow{k_5} EM + P$$

$$EM + I \overset{K_4}{\rightleftharpoons} EMI$$

Scheme I

The hydrolysis of phospholipids catalyzed by PLA_2 in mixed micellar systems follows surface dilution kinetics.[14-16] This suggests that the enzyme undergoes two binding steps before catalysis occurs.[15,16] These two steps have been defined in the "dual phospholipid model".[16-19] The water-soluble PLA_2 initially binds to phospholipid in the micellar interface. Once bound to that surface, the enzyme binds a second phospholipid in the catalytic site. It searches for this molecule in the two-dimensional space of the interface. Therefore,

the activity of PLA_2 toward the micellar substrate depends not only on the bulk concentration of the substrate but also on its surface concentration. Based on this kinetic model and the observation that the enzyme binds to Triton X-100 micelles only if the micelles contain phospholipid,[17] a general kinetic scheme is proposed for the inhibition of PLA_2 in the micellar system (Scheme I).

According to this kinetic model, the water-soluble enzyme first associates with the interface of the mixed micelle through the interaction with either a substrate or an inhibitor molecule. This depends on the bulk concentration of both the substrate and the inhibitor. In Scheme I, EM represents the enzyme bound to the surface of the micelles; this concentration is the sum of the enzyme species EI and ES. Once on the interface of the micelle, PLA_2 binds either a substrate to form the Michaelis-Menten complex, EMS, or an inhibitor to form the competitive inhibitor complex EMI. This depends on the surface concentration of the substrate or the inhibitor rather than on their bulk concentrations. The kinetic equation for the competitive inhibition of PLA_2 is readily derived, assuming rapid equilibrium kinetics, [20] and is shown in Equation 1.

$$v = \frac{V_{max} X_s}{\frac{K_3}{\frac{[S]}{K_1} + \frac{[I]}{K_2}} + K_3 + X_s\left(1 + \frac{K_3 X_i}{K_4 X_s}\right)} \tag{1}$$

Here K_1, K_2, K_3, and K_4 are the dissociation constants as shown in the kinetic model (Scheme I). V_{max} is equal to $k_5[E]_0$ where $[E]_0$ is the total concentration of enzyme and k_5 is the catalytic rate constant. [S] and [I] are the bulk concentrations of the substrate and the inhibitor. X_s and X_i are the surface concentrations of the substrate and the inhibitor and are expressed in mole fraction units. If both substrate and inhibitor exist predominantly in micelles, X_s and X_i is approximately equal to [S] / ([S] + [I] + [T]) and [I] / ([S] + [I] + [T]), respectively. [T] is the concentration of the Triton X-100 that is actually in micelles and is approximately equal to the total concentration of Triton X-100 minus its cmc.

There are four variables in Equation 1: the bulk concentrations of substrate and inhibitor ([S] and [I]), and the surface concentrations of substrate and inhibitor (X_s and X_i). However, these four variables are not independent of one another. If all of the substrate and inhibitor are incorporated into the micelles, these four concentrations are related by Equation 2.

$$\alpha = \frac{X_i}{X_s} = \frac{[I]}{[S]} \tag{2}$$

Substituting Equation 2 into Equation 1 and rearranging yields Equation 3 which has the form of a simple Michaelis-Menten equation in terms of the surface concentration of the substrate. Thus, a double reciprocal plot of 1/V versus $1/X_s$, where [S], [I], and α are held constant, would yield a straight line with a 1/v intercept of $1/V_{max}^{app}$ (Equation 4) and a slope of K_m^{app}/V_{max}^{app} (Equation 5). X_s is varied in these experiments by changing [T] not [S].

$$v = \frac{V_{max}^{app} X_s}{K_m^{app} + X_s} \tag{3}$$

$$V_{max}^{app} = \frac{V_{max}}{1 + \frac{K_3}{K_4}\alpha} \qquad K_m^{app} = \left\{ \frac{K_3}{[S]\left[\frac{1}{K_1} + \frac{\alpha}{K_2}\right]} + K_3 \right\} \left[1 + \frac{K_3}{K_4}\alpha \right]^{-1}$$

If this experiment is repeated at several different α's, a series of plots are obtained from which the values of the various kinetic constants can be determined. Replotting $1/V_{max}^{app}$ versus α (Equation 4) yields a linear plot with intercepts on the $1/V_{max}^{app}$-axis of $1/V_{max}$ and on the α-axis of K_4/K_3. Note that K_4/K_3 is a measure of how well the inhibitor is bound to the catalytic site relative to how well the substrate is bound. It can be used to compare the potency of a series of inhibitors when the same substrate is employed. K_3 can be determined by replotting the slope (K_m^{app}/V_{max}^{app}) versus 1/[S] of the double reciprocal plots done at the same α but different [S], as shown in Equation 5.

$$\frac{1}{V_{max}^{app}} = \frac{1}{V_{max}} + \frac{K_3}{K_4 V_{max}}\alpha \tag{4}$$

$$\text{slope} = \frac{K_3}{V_{max}\left[\frac{1}{K_1} + \frac{\alpha}{K_2}\right]}\frac{1}{[S]} + \frac{K_3}{V_{max}} \tag{5}$$

This replot also yields a straight line with an intercept on the slope-axis of K_3/V_{max}. Using this data with the V_{max} and K_4 / K_3 obtained from Equation 4, K_3 and K_4 can be calculated. The intercept on the 1/[S]-axis is

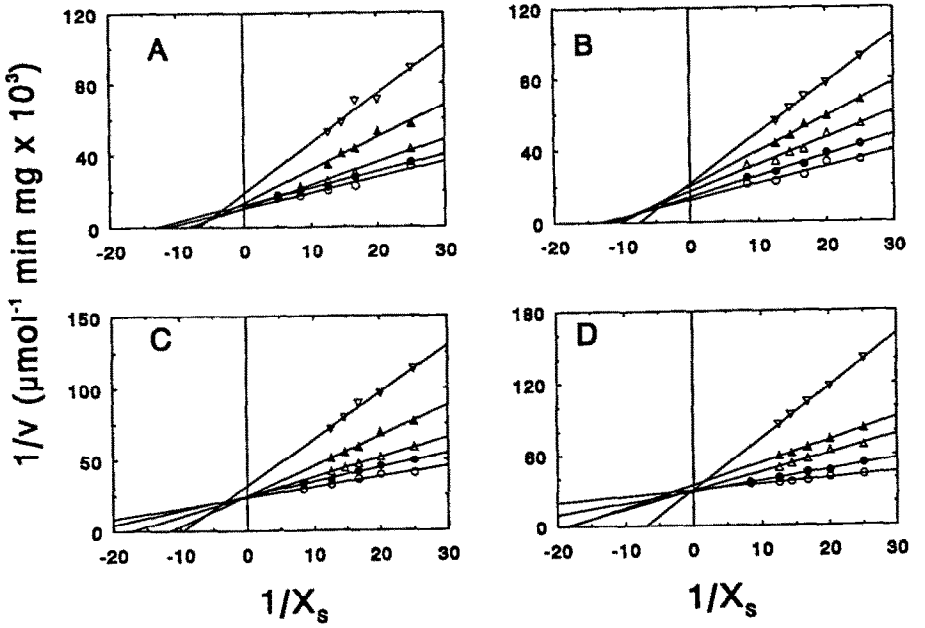

Fig. 1. Activity of PLA_2 toward thio PE in Triton X-100 mixed micelles in the presence of PE amide analogue inhibitor. The enzyme activity was determined using the thio assay at pH 8.5. The ratios of inhibitor to substrate, α, were as follows, in panel A) 0%, B) 0.2%, C) 1.0%, and D) 1.5%. Each line was determined at a fixed bulk concentration of substrate: (○) 0.50 mM, (●) 0.35 mM, (Δ) 0.20 mM, (Δ) 0.10 mM, and (▽) 0.05 mM. The lines drawn are a result of a least-squares analysis of the data.

given by Equation 6. From this, the bulk binding constants K_1 and K_2 can be calculated.

$$-\text{Intercept} = \frac{1}{K_1} + \frac{\alpha}{K_2} \tag{6}$$

The plot of -intercept versus α has a slope of $1/K_2$ and an intercept on the -intercept-axis of $1/K_1$.

In the present study, the inhibition of cobra venom PLA_2 (*Naja naja naja*) by the amide substrate analogue, 1-hexadecylthio-2-hexadecanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphorylethanolamine,[12] was studied. The substrate used was 1,2-bis(decanoylthio)-1,2-didexoy-*sn*-glycero-3-phosphorylethanolamine (thio-PE). PLA_2 activity was measured spectrophotometrically using the thio assay as described previously.[21] The assay was carried out at 30°C in 25 mM Tris buffer (pH 8.5) containing 0.1 M KCl and 10 mM $CaCl_2$. Due to their chain length, both the PE substrate and PE amide analogue should have negligible monomeric concentrations. All of the PE substrate and PE amide analogue are therefore assumed to be in micelles. Thus, Equation 2 holds.

As shown in Figure 1, four sets of experiments were carried out, each at a different value of α (Figure 1.A to 1.D). In each set of experiments, α was held constant and five different values of [S] were employed. In each individual experiment, the surface concentration of the substrate, X_s, was varied by changing the bulk concentration of Triton X-100, [T], while the bulk concentrations of the substrate, [S], and inhibitor, [I], were held constant. The V_{max}^{app} obtained at each α was averaged. Then, the reciprocal of this averaged V_{max}^{app} was plotted as a function of α as shown in Figure 2. V_{max} and the ratio of K_3 to K_4 were determined from the intercept on the $1/V_{max}^{app}$-axis and the intercept on α-axis, respectively. V_{max} is 73 μmol min^{-1} mg^{-1} and K_4/K_3 is 83.

The slopes of the lines in Figure 1 with a given value of α were replotted as a function of 1/[S] (Figure 3). The slopes of these replots are a function of α/K_2, see Equation 5, from which K_2 can be calculated. However, we found that the plots for the four α values that we employed were superimposable, indicating that the slope was not a function of α. This could be due to the fact that the amount of the inhibitor used in these experiments was less than 2% of the substate so that α was always less than 0.02. If, at the same time,

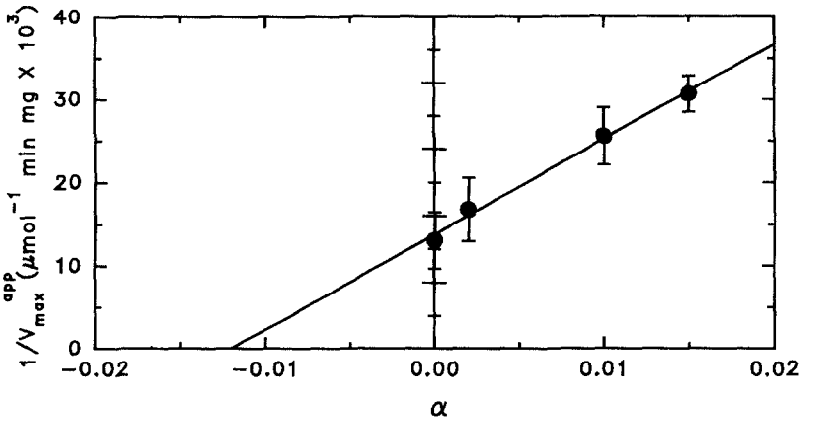

Fig. 2. The reciprocal of V_{max}^{app} as a function of α. The lines drawn are least-squares fits to the data.

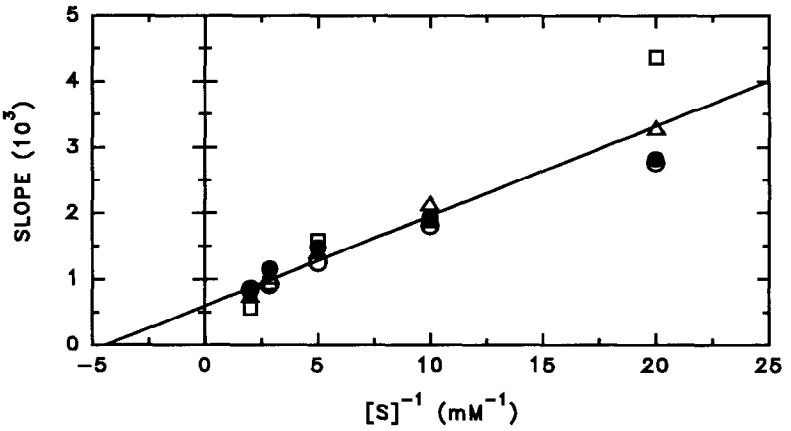

Fig. 3. The replot of the slope from each line in Figure 1 versus the reciprocal of the bulk concentration of substate. α is (○) 0%, (●) 0.2%, (Δ) 1.0%, and (□) 1.5%.

K_1 and K_2 are comparable, the term α/K_2 will be significantly smaller than $1/K_1$, and α will have little effect on the slope. A broader range of α could not be used in this case because this compound was such a potent inhibitor that activities could not be accurately determined at higher inhibitor levels. This precludes one from determining the value of K_2 under these conditions. K_2 could be measured, however, if an inhibitor was employed whose α/K_2 was closer to $1/K_1$.

These replots do, still, allow the calculation of K_1 and K_3. The dissociation constants K_1 and K_3 were determined from the line where α is equal to 0. K_1 is 0.16 mM whereas K_3 is 0.05 mol fraction. Using the ratio of K_4/K_3 obtained above, the dissociation constant of the PE inhibitor, K_4, is 6×10^{-4} mol fraction. The kinetic parameters for the hydrolysis of thio PE obtained in this study are in agreement with the result reported by Hendrickson and Dennis.[16]

One caveat to this discussion must be noted. This kinetic model assumes that the enzyme has equal access to all of the substrate and inhibitor. This can be achieved in two ways. If the enzyme is in a pure hopping mode, as defined by Jain, Gelb, and coworkers (22,23), the enzyme would move rapidly from micelle to micelle and encounter the entire lipid pool. The rapid exchange of lipid between micelles via a collisional process would also insure equal access of the enzyme to the lipid. If these exchange rates are not fast enough to ensure that the enzyme "sees" the entire phospholipid pool, the substrate and inhibitor concentrations used in the equations will not reflect the true concentrations seen by the enzyme and the kinetic model would have to be modified to take this additional process into account. To date, we have no evidence that these exchange rates are affecting catalysis. Neither the hopping rate nor the phospholipid exchange rate have been directly determined experimentally for the cobra PLA_2 Triton X-100 mixed micelle system. The data presented here clearly fits the kinetic model represented in Scheme I and no evidence was found to indicate that a more complicated model should be employed at this time.

In summary, this study presents a new approach to kinetic studies of PLA_2 inhibition in the micellar system. The kinetic results are consistent with the proposed mechanism that the binding of PLA_2 to micellar phospholipid occurs in two steps. In the first step, free water-soluble enzyme binds to the interface of a mi-

celle by binding to phospholipid on the surface. The PE substrate and the PE amide analogue act in the same manner. Both compounds sequester the enzyme to the surface. Thus, the PE amide analogue does not inhibit in this step. The second step is the binding of substrate or inhibitor to the catalytic site of the enzyme in the two-dimensional interface. It is during the second step that the enzyme inhibition occurs. The PE amide analogue binds 80 times tighter than the thio PE substrate in this step. The kinetic considerations presented here represent a useful approach to determine the dissociation constants of PLA_2 inhibitors.

References and Notes

1. Dennis, E. A. in *The Enzymes, Third Edition, Vol. 16*; Boyer, P., Ed.; Academic Press: New York, 1983; pp 307-353.
2. Dennis, E. A., and Plückthun, A. in *Phosphorus-31 NMR: Principles and Applications*; Gorenstein, D., Ed.; Academic Press: New York, 1984; pp 423-446.
3. Plückthun, A., Rohlfs, R., Davidson, F. F., and Dennis, E. A. *Biochemistry* **1985,** *24*, 4201-4208.
4. Reynolds, L. J., Washburn, W. N., Deems, R. A., and Dennis, E. A. in *Methods in Enzymology, 197*; E. A. Dennis, Ed.; Academic Press: Orlando, 1991; pp 3-23.
5. Pieterson, W. A., Vidal, J. C., Volwerk, J. J., and de Haas, G. H. *Biochemistry* **1974,** *13*, 1455-1459.
6. Wells, M. A. *Biochemistry* **1974,** *13*, 2248-2257.
7. Yuan, W., Quinn, D. M., Sigler, P. B., and Gelb, M. H. *Biochemistry* **1990,** *29*, 6082-6094.
8. Lombardo, D., and Dennis, E. A. *J. Biol. Chem.* **1985,** *260*, 7234-7240.
9. Ransac, S., Riviere, C., Soulie, J. M., Verger, R., and de Haas, G.H. *Biochim. Biophys. Acta* **1990,** *1043*, 57-66.
10. Jain, M. K., Yuan, W., and Gelb, M. H. *Biochemistry* **1989,** *28*, 4135-4139.
11. Davidson, F. F., Hajdu, J., and Dennis, E. A. *Biochem. Biophys. Res. Comm.* **1986,** *137*, 587-592.
12. Yu, L., Deems, R. A., Hajdu, J., and Dennis, E. A. *J. Biol. Chem.* **1990,** *265*, 2657-2664.
13. Yu, L., and Dennis, E. A. *Proc. Natl. Acad. Sci. USA* **1991,** *88*, 9325-9329.
14. Dennis, E. A. *Arch. Biochem. Biophys.* **1973,** *158*, 485-493.
15. Deems, R. A., Eaton, B. R., and Dennis, E. A. *J. Biol. Chem.* **1975,** *250*, 9013-9020.
16. Hendrickson, H. S., and Dennis, E. A. *J. Biol. Chem.* **1984,** *259*, 5734-5739.
17. Roberts, M. F., Deems, R. A., and Dennis, E. A. *Proc. Nat'l. Acad. Sci. U.S.A.* **1977,** *74*, 1950-1954.
18. Dennis, E. A. in *Enzymes of Lipid Metabolism*; S. Gatt, L. Freysz, and P. Mandel, Ed.; Plenum Press: New York, 1978; pp 165-175.
19. Hazlett, T. L., Deems, R. A., and Dennis, E. A. in *Biochemistry, Molecular Biology and Physiology of Phospholipase A_2 and Its Regulatory Factors*; A. B. Mukherjee, Ed.; 1990; pp 49-64.
20. Cha, S. *J. Biol. Chem.* **1968,** *243*, 820-825.
21. Yu, L., and Dennis, E. A. *Methods Enzymol.* **1991,** *197*, 65-75.
22. Jain, M. K., and Berg, O. G. *Biochim. Biophys. Acta* **1989,** *1002*, 127-156.
23. Jain, M. K., and Gelb, M. H. *Methods Enzymol.* **1991,** *197*, 65-75.