Kinetic model for surface-active enzymes based on the Langmuir adsorption isotherm: Phospholipase C (Bacillus cereus) activity toward dimyristoyl phosphatidylcholine/detergent micelles

(ABSTRACT) A simple kinetic model for the enzymatic activity of surface-active proteins against mixed micelles has been developed. This model uses the Langmuir adsorption isotherm, the classic equation for the binding of gas molecules to metal surfaces, to characterize enzyme adsorption to micelles. The number of available enzyme binding sites is equated with the number of substrate and inhibitor molecules attached to micelles; enzyme molecules are attracted to the micelle due to the affinity of the enzyme active site for the molecules in the micelle. Phospholipase C (Bacillus cereus) kinetics in a wide variety of dimyristoyl phosphatidylcholine/detergent micelles are readily explained by this model and the assumption of competitive binding of the detergent at the enzyme active site. Binding of phospholipase C to pure detergent micelles is demonstrated by gel filtration chromatography. The experimentally determined enzyme–detergent micelle binding constants are used directly in the rate equation. The Langmuir adsorption model predicts a variety of the characteristics observed for phospholipase kinetics, such as differential inhibition by various charged, uncharged, and zwitterionic detergents and surface dilution inhibition. The essential idea of this model, that proteins can be attracted and bound to bilayers or micelles by possessing a binding site for the molecules composing the surface, may have wider application in the study of water-soluble (extrinsic) protein–membrane interactions.

The interaction of water-soluble proteins with biomembrane surfaces plays an important role in fat digestion, cell–cell communication, and numerous other cellular functions. Characterization of these phenomena is difficult because of the complexity of biomembranes and the need for sensitive assays. The interaction of water-soluble phospholipases with micellar structures offers a useful model system for extrinsic protein–membrane interactions. Many phospholipases have been purified to homogeneity and are available in relatively large quantities (1–3). Micelles form optically clear solutions and can be studied by a variety of conventional physical techniques (4–7). If one of the micellar components is a substrate for the phospholipase, then the observed activity serves as a direct test for theories of enzyme–micelle interactions.

Phospholipase action toward phospholipid molecules in a surface is much greater than that toward monomeric substrates ("interfacial activation") (1). Enzyme-specific activity also depends on the matrix used to form the surface—i.e., detergent mixed micelles (8), short-chain lecithin micelles (9, 10), bilayers (11), monolayers (12). A variety of kinetic models have been applied to these phenomena. The simplest model, applied to snake venom phospholipase A2 action toward short-chain lecithins, proposes normal Michaelis–Menten kinetics and different Vₘ and Kₘ values for monomeric and micellar lipid with the monomer as a competitive inhibitor of micellar lecithin (13). Another model, proposed for pancreatic phospholipase A₂, accounts for interfacial activation by proposing a second site on the enzyme that "anchors" or "recognizes" surfaces (14). Different surface-active molecules can interact differentially with the two sites and hence modulate the activity. These models have not been extended in a systematic fashion to binary or more complex surfaces except in cases in which the added surface molecule is a substrate analogue. The only detailed binary component kinetic model is that of Dennis and co-workers (15). This "surface as cofactor" model was developed for phospholipase A₂ and phospholipase C kinetics using Triton X-100/lecithin micelles as substrates. The model is quite complex, requiring estimation of the surface area/head-group ratio and several assumptions (16) to fit observed activities. It is based on surface association of the enzyme followed by substrate binding in the active site to form the Michaelis complex; i.e., two distinct binding steps are involved.

To generalize a kinetic model for surface-active enzymes such as the phospholipases, we have examined the action of phospholipase C (Bacillus cereus) toward dimyristoyl phosphatidylcholine (Pl₄PtdCho) in mixed micelles with four different detergents: Triton X-100 (nonionic), Zwittergent-3-14 (zwitterionic), deoxycholate (anionic), and trimethylated ammonium bromide (Me₃CetNBr; cationic). The data are interpreted by using a simple model based on the Langmuir adsorption isotherm and competitive inhibition of detergent. Detergent binding is estimated independently by gel filtration. This model, which postulates a single binding site on phospholipase C that has different affinities for amphiphilic molecules, yields unique kinetic constants for processing Pl₄PtdCho and predicts the surface saturation and surface-dilution inhibition kinetics experimentally observed in each detergent system.

MATERIALS AND METHODS

Materials. Myr₂PtdCho was obtained from Calbiochem. Phospholipid purity was monitored by TLC in CHCl₃/CH₃OH/H₂O (65:24:4). Triton X-100 (Amersham), Zwittergent 3-14 (Calbiochem), and Me₃CetNBr and sodium deoxycholate (Sigma) were used without further purification.

Enzymatic Assays. Phospholipase C (B. cereus) was purified as described (17). Enzymatic hydrolysis of Myr₂PtdCho was measured by pH-stat (pH 8 end point) (8) at 30°C. Assay mixtures contained 0.1–20 mM Myr₂PtdCho and 0.5–100 mM detergent.

Abbreviations: Myr₂PtdCho, dimyristoyl phosphatidylcholine; Me₃CetNBr, trimethylammonium bromide; cmc, critical micelle concentration.

* To whom reprint requests should be addressed.
**RESULTS AND DISCUSSION**

**Derivation of Kinetic Model.** We propose that the observed initial velocity, $V_i$, for a surface-active enzyme (Fig. 1) is

$$ V_i = k_{cm}(ES_m) + k_{c}[ES]. $$

The different catalytic rate constants for monomeric and micellar substrate are not important in this study; the aqueous solubility of Myr2PtdCho is very low, and kinetic studies of phospholipase C (9) and phospholipase A2 (13) with pure short-chain lecithin monomers and micelles suggest that $k_c \ll k_{cm}$. The monomer term is retained in the derivation for generality.

We propose normal Michaelis–Menten kinetics for monomers; the steady-state approximation ($d(ES)/dt = 0$) yields

$$ ES = E S_m K_{cm}, $$

where $K_c = k_{cm}^{-1} + k_d^{-1}/k_d$. For micellar substrate, the Langmuir adsorption isotherm is proposed as the appropriate relationship for the steady-state approximation ($d(ES_m)/dt = 0$). In the Langmuir equation, formation and breakdown of the enzyme–micellar substrate complex are as follows:

$$ E + S \xrightarrow{k_{c+1}} ES \xrightarrow{k_{c-1}} E + P $$

$$ E + D_m \xrightarrow{k_f} ED_m $$

**Fig. 1.** Parameters for kinetic model. All concentrations are bulk average solution concentrations. $E$, enzyme concentration; $S$, monomeric substrate concentration; $P$, monomeric product concentration; $S_m$, micellar substrate concentration; $D_m$, micellar detergent concentration; $P_m$, monomeric and micellar product concentrations, respectively. Complexes are indicated by combining the appropriate symbols.

$$ $${}

**Association** = rate constant × concentration of free enzyme in solution above the surface × fraction of binding sites unoccupied

$$ = k_d(E)\left(1 - \frac{ES_m}{S_m + ES_m}\right) \equiv k_d(E)\left(1 - \frac{ES_m}{S_m}\right) $$

This approximation is valid because 10 nM enzyme is typically used in an assay, while micellar substrate is in the millimolar concentration range, and

**Dissociation** = rate constant × fraction of sites occupied

$$ = \frac{k_d(ES)}{S_m}. $$

This treatment circumvents the surface dimensionality problem (16). The full steady-state approximation for micellar substrate is therefore

$$ \frac{d[ES_m]}{dt} = 0 = k_d(E)\left(1 - \frac{ES_m}{S_m}\right) - k_d(ES_m) - k_{cm}(ES_m). $$

Solving this equation and assuming that

$$ \frac{k_d + k_{cm}S_m}{k_d} \gg (E) $$

we find that

$$ \frac{ES_m}{E} = \frac{S_mk_d}{k_d + k_{cm}S_m}. $$

Defining

$$ \frac{k_d + k_{cm}S_m}{k_d} = K_{cm} \quad [= f(S_m)] $$

then gives

$$ \frac{ES_m}{E} = \frac{S_m}{K_{cm}}. $$

This term is of the same form as the corresponding term for monomers, but $K_{cm}$ is a function of $S_m$, the micellar substrate concentration. The approximation shown above, that ($k_d +
$k_{\text{on}} S_m / k_{\text{off}} \gg E$ is now $K_{\text{on}} \gg E$. $K_{\text{on}}$ has a binding term ($k_d / k_a$) and a kinetic component. Other studies have estimated lecithin or analogue binding constants to phospholipases ranging from 0.1 to 5 mM. This term alone is greater than $E$ (the total enzyme concentration is typically 10 nM); the additional kinetic term will only increase the difference. $S_m$ in these assays is 0.1–20 mM. As shown below with the kinetic constants determined for this system, $K_{\text{on}}$ is 4–8 mM, while total enzyme concentration is typically 10 nM and free enzyme concentration is even less, verifying the approximation.

**Langmuir Binding Term for Inhibitors.** Steady-state approximation for this complex yields
\[
\frac{d[ED_m]}{dt} = 0 = k_d (E) \left( 1 - \frac{E D_m}{D_m} \right) - k_{-1} \left( \frac{E D_m}{D_m} \right).
\]
giving
\[
\frac{E}{ED_m} = \frac{k_{-1}}{k_1} \left( \frac{1}{D_m} \right) + \frac{E}{D_m}.
\]
Defining $k_{-1} / k_1 = K_{\text{Dm}}$ and assuming again that $K_{\text{Dm}} \gg E$ gives
\[
\frac{E}{ED_m} = K_{\text{Dm}} = \frac{D_m}{D_m} \frac{K_{\text{Dm}}}{K_{\text{Dm}}}
\]
or
\[
\frac{ED_m}{E} = \frac{D_m}{K_{\text{Dm}}}.
\]
As long as $K_{\text{Dm}} \gg E$, the binding of enzyme to detergent micelles occurs in a form similar to that in bulk (isotropic) solution. If the affinity of the protein for the binding site is stronger—the $K_{\text{Dm}} \leq E$, then the appropriate binding term would be $(ED_m / E) = (K_{\text{Dm}} + E) / D_m$, which can be solved iteratively.

**Derivation of Kinetic Equation.** Returning to the original rate equation, we have
\[
V_i = k_{\text{on}} [ES_m] + k_s [E]
\]
\[
= (E) \left[ \frac{k_{\text{on}} S_m}{K_{\text{on}} S_m} + \frac{k_s S_{m}}{K_{s}} \right]
\]
and
\[
E_T = E + ES + ES_m + ED + E_{\text{Dm}}
\]
\[
= E \left( 1 + \frac{S_m}{K_s} + \frac{S_m}{K_{\text{on}} S_m} + \frac{D_m}{K_D} + \frac{D_m}{K_{\text{Dm}}} \right).
\]
In this study where relative inhibition of monomeric versus micellar detergent was not studied carefully, we will assume that
\[
K_D = K_{\text{on}}.
\]
Since, under our assay conditions, the concentrations of monomeric detergents are much less (1–10%) than those of the micellar species, $K_D$ would have to be 10 to 100 times $K_{\text{on}}$ to affect the kinetics. The final rate equation is
\[
E_T \left[ \frac{k_{\text{on}} S_m}{K_{\text{on}} S_m} + \frac{k_s S_{m}}{K_{s}} \right]
\]
\[
V_i = \frac{S}{1 + \frac{S_m}{K_s} + \frac{D_m}{K_D} + \frac{D_m}{K_{\text{Dm}}} + \frac{K_{\text{Dm}} D_m}{E_T}}.
\]
where $D_T$ is the total detergent concentration.

**Results of the Kinetic Model.** The kinetic parameters determined for Myr₂PtdCho/Triton X-100, Zwittergent 3-14, deoxycholate, and Me₃CetNBr micelles are given in Table 1. The values of the free parameters $k_d$, $k_i$, and $K_{\text{on}}$ were derived by minimizing the sum
\[
\sum_{i=1}^{49} | V_{\text{Calc}} - V_{\text{Obs}} |
\]
for forty-nine assays (done in duplicate) distributed among the four mixed micellar systems. The best-fit values of $K_{\text{Dm}}$ for each detergent (for concentrations around the experimentally estimated $K_{\text{Dm}}$) were also determined. Good agreement between best-fit and experimental $K_{\text{Dm}}$ values (which have a fairly large error) is observed. Binding of phospholipase C to Triton X-100 is difficult to estimate. In the presence of 1 mM Zn²⁺, the enzyme shows very weak affinity for Triton. When excess Zn²⁺ is removed from the system, the enzyme shows enhanced Triton binding. Exact evaluation is complicated by a general "stickiness" that phospholipase C develops in solution lacking excess Zn²⁺ and in low ionic strength buffers. Assays are done under conditions in which there is no excess Zn²⁺. Therefore, the "experimental" $K_{\text{Dm}}$ is probably somewhere between these two values.

The $K_{\text{Dm}}$ value for Me₃CetNBr is less than the cme for this detergent. Although direct comparison of these $K_{\text{Dm}}$ values with true solution concentrations cannot be proven through this model, the direct correspondence of the gel chromatographic $K_D$ values and the best-fit $K_{\text{Dm}}$ values supports this conclusion. This in turn suggests tight binding of monomeric Me₃CetNBr to phospholipase C. Preliminary UV difference spectra of the enzyme (0.7 mg/ml) without and with Me₃CetNBr (<0.6 mM) show that a strong interaction does occur: the enzyme–detergent complex first precipitates and then is resolubilized as larger amounts of detergent are added.

The value of $K_D$ is irrelevant in these assays because the cme of Myr₂PtdCho is so low (0.1 μM) (21) that monomer hydrolysis does not significantly contribute to the rate. The Myr₂PtdCho cme would need to be wrong by several orders of magnitude for monomer hydrolysis to be kinetically important. For comparison, Littie (9) has found $K_m$ for hydrolysis of monomeric dibutyrylphosphatidylcholine to be 37 mM. The average error per assay for the optimized model is approximately three times

**Table 1. Kinetic constants for the Langmuir adsorption model of phospholipase C activity toward Myr₂PtdCho/detergent micelles**

<table>
<thead>
<tr>
<th>Constant</th>
<th>Calculated</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_d$, mM⁻¹</td>
<td>20,000</td>
<td>—</td>
</tr>
<tr>
<td>$k_i$, s⁻¹</td>
<td>5,000</td>
<td>—</td>
</tr>
<tr>
<td>$K_{\text{on}}$, s⁻¹</td>
<td>1,000</td>
<td>—</td>
</tr>
<tr>
<td>$K_{\text{Dm}}$, mM</td>
<td>Triton X-100</td>
<td>40 ±60;</td>
</tr>
<tr>
<td>Zwittergent</td>
<td>3</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>12</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Me₃CetNBr</td>
<td>0.2</td>
<td>2.4 ± 0.2;</td>
</tr>
<tr>
<td>Average specific activity per assay, nmol/min μg⁻¹</td>
<td>370</td>
<td>—</td>
</tr>
<tr>
<td>Average error per assay, nmol/min μg⁻¹</td>
<td>97</td>
<td>30</td>
</tr>
</tbody>
</table>

*Unless otherwise noted, $K_{\text{Dm}}$ values were measured by gel filtration in the presence of 1 mM Zn²⁺.

² Estimated by UV difference spectra suggesting that phospholipase C binds to Me₃CetNBr monomers with a $K_D$ of ~0.5 mM.

³ Estimated by gel filtration in the absence of Zn²⁺ ions.

* Estimated by UV difference spectra suggesting that phospholipase C binds to Me₃CetNBr monomers with a $K_D$ of ~0.5 mM.

* Experimental SD per assay.
the average experimental SD per assay. However, we believe that the SD of two assays done the same day does not accurately reflect the error for a large assay set done over a 4-month period with phospholipase C obtained from three separate purifications. The ability of this model to predict observed specific activities in mixed micellar systems with four structurally dissimilar detergents is a significant improvement over previous models of phospholipase kinetics.

As shown in the derivation of this model,

$$K_{sm} = \frac{k_d + k_{cm}S_m}{k_a} = \frac{k_d}{k_a} + \frac{k_{cm}S_m}{k_a}.$$  

If we compare this with the derivation of the equation for detergent binding and equate $k_d/k_a$ to the binding constant (analogous to $K_{DM}$) for Myr₂PtdCho, we obtain

$$K_{sm} = \text{binding constant} + \text{kinetic term.}$$

Substituting the appropriate kinetic constants gives

$$K_{sm} = 4.0 \text{ mM} + 0.2 \text{ S}_m,$$

where $S_m$ is millimolar. The binding constant for Myr₂PtdCho is similar to the value of $K_{DM}$ for Zwittergent and larger than that for Me₃CetNBr. All these molecules contain a quaternary nitrogen and linear aliphatic chains. For the Me₃CetNBr system, the effective inhibition of phospholipase activity is not caused by bromide ion; added NaBr has no effect on other assays (data not shown). Phospholipase C shows quite low substrate specificity, with binding energy relationships probably dominated by hydrophobic interactions. Structural analyses of this kind can readily be extended to other substrates and detergents to determine the binding specificity of phospholipases.

The maximal specific activity for phospholipase C can be calculated by examining the term $S_m/K_{sm}$ as $S_m$ goes to infinity:

$$S_m = \frac{S_m k_a}{k_d + k_{cm}S_m} = \frac{S_m k_a}{k_a} = k_a.$$  

This result and the assumption that monomer kinetic contributions and detergent inhibition are negligible yield

$$V = \frac{k_a k_{cm}}{k_d + k_{cm}} = 2,100 \text{ µmol.min}^{-1}\text{mg}^{-1}.$$  

This value is similar to the maximum velocity for phospholipase C activity extrapolated for the Triton X-100/egg lecithin system using a surface-as-cofactor model (16).

**Surface Dilution** Kinetics. At a fixed mole fraction of lecithin, phospholipase C activity depends on the total concentration of surfactant (lecithin plus detergent). For the Triton X-100 and deoxycholate-containing micelles, curves somewhat reminiscent of substrate saturation kinetics are observed (Fig. 2A and C). For the Zwittergent and Me₃CetNBr micellar systems, the activity is constant and markedly inhibited (Fig. 2B and D). In each graph, the line connects the theoretical values; the calculated activities are found at the phospholipid concentrations corresponding to the experimental points. It is not easy to see how direct surface binding can be obtained from these curves, as suggested by Dennis (22).

If, rather than holding the mole fraction of lecithin constant, we maintain a fixed lecithin concentration and vary the detergent concentration, distinct inhibition is observed (Fig. 3). This type of phenomenon, termed surface dilution, has been explained by Dennis and co-workers in terms of a complex kinetic model involving a nonspecific surface binding site and a specific catalytic site on the enzyme. The experimental data points for phospholipase C in the four detergent systems are quite well fit by our Langmuir adsorption model (solid lines), in which only a single enzyme site is postulated.

A powerful technique for understanding the kinetics of surface-active enzymes is a three-dimensional plot in which total substrate, total detergent, and observed activity form the $x$, $y$, and $z$ axes. Two-dimensional slices of such plots are shown in

**FIG. 2.** Experimental and theoretical enzymatic activities at a fixed Myr₂PtdCho mole fraction ($f_I$). Results are experimental values ± SD (absence of error bars reflect SD values smaller than the point size). — Activity determined by hydrophobic specificities, not detergent (data shown). guitar, activities calculated by the kinetic model; the calculated activity is found at the same total Myr₂PtdCho concentration as the corresponding experimental point. (A) Myr₂PtdCho/Triton X-100 micelles: $f_I = 0.19 ± 0.01$. (B) Myr₂PtdCho/Zwittergent micelles: $f_I = 0.19 ± 0.01$. (C) Myr₂PtdCho/deoxycholate micelles: $f_I = 0.20 ± 0.02$. (D) Myr₂PtdCho/Me₃CetNBr micelles: $f_I = 0.16 ± 0.03$.

**FIG. 3.** Surface-dilution experiments for Myr₂PtdCho/detergent micelles. Observed phospholipase C specific activity at roughly constant Myr₂PtdCho concentrations is plotted as a function of total detergent concentration. Results are expressed as in Fig. 2. (A) Myr₂PtdCho/Triton X-100 micelles; average [Myr₂PtdCho] = 0.4 ± 0.1 mM. (B) Myr₂PtdCho/Zwittergent micelles; average [Myr₂PtdCho] = 4.9 ± 0.1 mM. (C) Myr₂PtdCho/deoxycholate micelles; average [Myr₂PtdCho] = 2.1 ± 0.1 mM. (D) Myr₂PtdCho/Me₃CetNBr micelles; average [Myr₂PtdCho] = 2.4 ± 0.3 mM. The apparent peak in the theoretical curve in A is caused by variations in [Myr₂PtdCho].
Fig. 4. The assay series for total surface concentration at a fixed mole fraction of lecithin is represented on this plot by a straight line that intersects the origin. For example, the data from Fig. 2A for Myr₂PtdCho/Triton X-100 micelles are shown in Fig. 4B as points connected by the line constant Myr₂PtdCho/detergent = 4. At low substrate concentrations, this surface concentration activity line cuts across some of the specific activity contour lines, showing some change in activity. At higher concentrations (i.e., the upper two-thirds of the line), this line runs parallel to the activity isobars, so no change in activity with increasing concentration is indicated. The surface-dilution series is seen on this graph as a line parallel to the abscissa. It can intersect a large number of activity isobars indicating inhibition. An optimized set of experiments is given by the crossline in Fig. 4A, which represents fixed total surfactant (phospholipid and detergent) but various mole fractions of phospholipid.

Inspection of the Myr₂PtdCho concentration axes in Fig. 4A and B indicates different activities in the absence of detergent. The failure of this model to converge to a common activity in the absence of detergent cannot be examined experimentally for this system because mixed micelles that have high proportions of Myr₂PtdCho are not stable soluble micelles. However, mixed micellar systems with short-chain lecithins and detergents are soluble in all proportions, making the entire line shown in Fig. 4A accessible. The cme of several of the short-chain lecithins are in experimentally convenient concentration ranges, so a full kinetic analysis for monomeric and micellar substrate and monomeric and micellar detergent is possible.

**Extrapolation to Other Substrate Aggregates.** The basic idea of the Langmuir adsorption kinetic model is that an enzyme is attracted to a surface via a binding site for individual molecules composing the surface. This allows us to reinterpret the activity of phospholipases in other mixed systems and to predict enzyme affinities for different surface components. For example, phospholipase C (B. cereus) activity is sensitive to the presence of cholesterol (23) but not to that of triglyceride (24). Rather than strictly relating "surface" concentrations of these components, this means that phospholipase C must have a strong affinity for cholesterol but little for triglyceride compared with lecithin. The report of Sundler et al. (25) that a phosphatidylinositol-specific phospholipase C displayed surface dilution inhibition in Triton X-100/phosphatidylinositol micelles but not with lecithin/phosphatidylinositol sonicated vesicles can be explained by a stronger affinity of that enzyme for Triton than for lecithin. This suggests that the detergent hydroxy group or oxygen-rich oxethylene units mimic inositol binding to that enzyme.

We thank Dr. William Gilbert, Massachusetts Institute of Technology, for assistance in computer programming and Prof. Gregory Petsko, Massachusetts Institute of Technology, for access to his PDP 1100. This work was supported by Grant GM 26762 from the National Institutes of Health. R.A.B. is a Whitaker College (Massachusetts Institute of Technology) predoctoral fellow.