Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 32, Issue of August 11, pp. 18711–18714, 1995
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Printed in U.S.A.

Lipid Signaling Enzymes and Surface Dilution Kinetics*

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In recent years, considerable effort has been devoted to defining lipid signaling pathways, purification of the involved enzymes, and cloning of the genes encoding for these enzymes. However, much less is known about the mechanism, specificity of reaction, and the regulation of the catalytic activity of lipid-dependent enzymes and enzymes involved in generating lipid second messengers. The enzymes in lipid signaling pathways include lipid biosynthetic enzymes, lipid degrading enzymes, and enzymes that utilize lipid activators (cofactors). These enzymes have hydrophobic or amphipathic lipid substrates, activators, inhibitors, and products that exist as large aggregated structures in water. Many of these enzymes are themselves water-insoluble integral membrane proteins. Thus, lipid-dependent enzymes act in an environment in which both three-dimensional bulk interactions occur in solution and two-dimensional surface interactions occur, both of which must be taken into account by any kinetic model that describes the action of these enzymes. A kinetic model, termed "surface dilution kinetics," that takes into account both types of interaction has been developed (1, 2). This work introduced the important concept that both the surface concentration of lipid and the bulk concentration play critical roles in defining the kinetic parameters of lipid-dependent enzymes.

This review focuses on both theoretical and experimental considerations of surface dilution kinetics and its application to defining the kinetic parameters of a large variety of lipid-dependent enzymes. While surface dilution kinetics applies to all lipid interfaces, be they micelles, vesicles, liposomes, or membranes, the detergent mixed micelle system offers several advantages for carrying out *in vitro* surface dilution studies. Since the Triton X-100 mixed micelle system has been successfully employed with numerous lipid-dependent enzymes, we have focused our discussion on examples that employ this detergent.

Surface Dilution Kinetics

Dennis and co-workers (1–4) developed surface dilution kinetics to explain the action of cobra venom phospholipase A_2 on Triton X-100/phosphatidylcholine mixed micelles. This system will be used to describe the principles of surface dilution kinetics that are presented in Equation 1.

$$\begin{array}{cc} \text{Bulk step} & \text{Surface step} \\ \overset{k_1}{\underset{k_{-1}}{\rightleftarrows}} & \overset{k_2}{\underset{k_{-2}}{\rightleftarrows}} \quad \overset{k_3}{\underset{k_{-3}}{\rightleftarrows}} \\ E + \text{A} \rightleftarrows E\text{A} & E\text{A} + \text{B} \rightleftarrows E\text{AB} \rightleftarrows E\text{A} + \text{Q} \end{array} \qquad \text{(Eq. 1)}$$

In this scheme, a soluble enzyme (E) first associates with the Triton X-100/lipid mixed micelle (A) to form an enzyme-mixed micelle complex (EA). This association is a function of the bulk concentrations of both E and A. If the enzyme nonspecifically binds to the micelle surface, the term A is defined as the sum of the molar concentrations of Triton X-100 and lipid substrate. This model is illustrated in Fig. 1 as the "surface binding model." If, on the other hand, the enzyme specifically binds to a substrate molecule in the micelle surface during this first binding step, the term A is defined as the molar concentration of lipid substrate. This model is illustrated in Fig. 1 as the "phospholipid binding model."

Once the enzyme has associated with the Triton X-100/lipid mixed micelle, it then binds an individual lipid molecule (B) in the catalytic site forming the EAB complex. Since both components of this association are sequestered at the surface, the association is a function of the surface concentrations of both EA and B. Thus, the concentration of lipids in the mixed micelle, term B, is expressed in surface concentration terms such as mole fraction, [lipid]/([lipid] + [detergent]) or mol % (mole fraction × 100). As catalysis occurs, the products (Q) are formed and EA is regenerated.

The illustrations shown in Fig. 1 are representative of water-soluble enzymes (*e.g.* cobra venom phospholipase A_2 (1–4)). For a water-insoluble integral membrane enzyme (*e.g.* phosphatidylserine decarboxylase (5) or phosphatidylinositol 4-kinase (6, 7)), the protein is delivered to the assay as a detergent/protein mixed micelle. In this case, the enzyme concentration, E, actually represents the concentration of the enzyme-detergent complex. This differs from the phospholipase A_2 model where a soluble enzyme interacts with a surface. With integral membrane enzymes, two surfaces are interacting, one containing enzyme and the other containing lipid. The enzyme/micelle can maintain its integrity and bounce from micelle to micelle or it can fuse with lipid/micelles. In either case, this confers the bulk concentration dependence to the kinetics (5, 6, 8, 9).

The kinetic equation for the surface dilution scheme has been derived (2, 4) and is given in Equation 2,

$$V = \frac{V_{max}(\text{A})(\text{B})}{K_s^{\text{A}}K_m^{\text{B}} + K_m^{\text{B}}(\text{A}) + (\text{A})(\text{B})} \qquad \text{(Eq. 2)}$$

where $K_s^{\text{A}} = k_{-1}/k_1$ and $K_m^{\text{B}} = (k_{-2} + k_3)/k_2$. The V_{max} is the true V_{max} at an infinite mole fraction and an infinite bulk concentration of phospholipid substrate, K_m^{B} is the interfacial Michaelis constant (expressed in surface concentration units), and K_s^{A} is the dissociation constant (expressed in bulk concentration terms) for the mixed micelle binding site. The following assumptions are made with this scheme (2). The size and aggregation number of mixed micelles remain constant over the concentration range employed as the bulk concentration or surface concentration of substrate is varied; the average surface area per molecule of Triton X-100 is the same as the lipid substrate; both remain constant as the bulk concentration or the surface concentration of substrate is varied; the micellar surface area per enzyme binding site remains constant; and the enzyme has access to the entire lipid pool.

Mixed Micelles

To carry out a surface dilution kinetic analysis of a lipid-dependent enzyme, both the substrate's bulk concentration, expressed in terms of molarity, and its surface concentration, expressed in terms of mole fraction, must be varied. This produces a matrix of kinetic data points that is then analyzed to determine the various kinetic parameters. While varying the bulk substrate concentration is trivial, varying its surface concentration is not. To accomplish the latter, the mole fraction of substrate in the surface is varied by adding or removing other components of the surface. Ideally, these other surface components should be neutral dilutors, that is they should not interact directly with the catalytic or modulator sites of the enzyme. They can, however, interact in nonspecific ways with the enzyme as Triton X-100 does when it is used to solubilize integral membrane proteins. In addition, changing the surface concentration of the substrate with a neutral dilutor should not

* This minireview will be reprinted in the 1995 Minireview Compendium, which will be available in December, 1995. This work was supported by United States Public Health Service Grants GM-28140 and GM-35655 (to G. M. C.) and GM-20501, GM-51606, and HD-26171 (to E. A. D.) from the National Institutes of Health.
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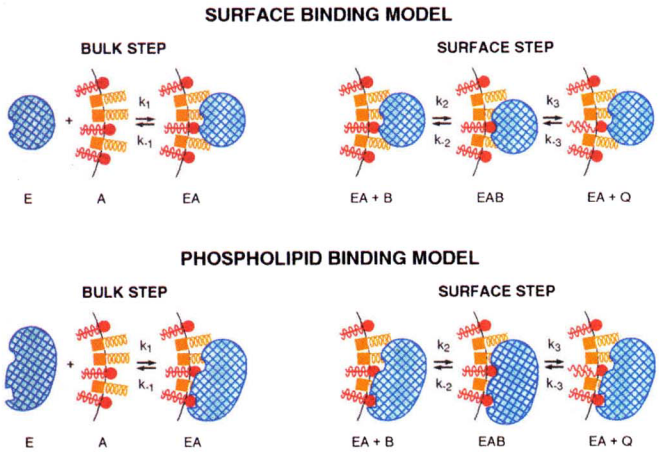

FIG. 1. **Illustration of the application of "surface dilution kinetics" to the phospholipase A_2-catalyzed hydrolysis of phospholipids contained in mixed micelles with nonionic surfactants such as Triton X-100.** Two possibilities are shown: *top*, the "surface binding model" whereby the enzyme first associates nonspecifically with the micelle surface; and *bottom*, the "phospholipid binding model" whereby the enzyme first associates specifically with phospholipid in the micelle surface. In both cases, in a subsequent step, the enzyme associated with the micelle binds a phospholipid substrate molecule in the micelle in its catalytic site and carries out hydrolysis producing as products a lysophospholipid and a fatty acid, which may be released to solution or be retained in the micelle surface. Phospholipid molecules are depicted in *red*, detergent molecules in *gold*, and the enzyme in *blue*. Adapted with permission from Refs. 2 and 3.

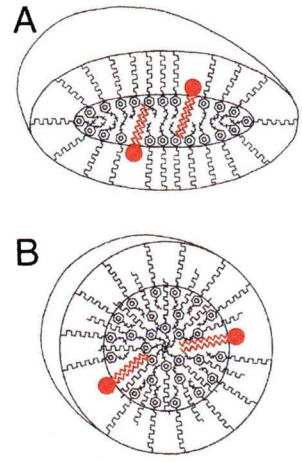

FIG. 2. **Schematic view of the "classical" oblate ellipsoid model and the "non-classical" spherical model for a mixed micelle containing the nonionic surfactant Triton X-100 and a low molecular fraction of phospholipid (<0.1).** The micelle model shapes were calculated (14) based on a Stokes radius of 44 Å at 40 °C and a hydration (taken from the value for the Triton X-100 micelle at 25 °C) of 1.3 g of water/g of Triton X-100. Using volume/density calculations for the hydrophobic core, *A* is a classical micelle with the shape of an oblate ellipsoid with an approximately 2:1 axial ratio. For the spherical micelle model *B*, the octylphenyl groups cannot pack in a spherical core to form a classical micelle. Therefore, in this model some oxyethylene units must be included in the hydrophobic core. It is not possible to precisely calculate the arrangement of groups in this non-classical model because one does not have the limits imposed by a distinct hydrophobic/hydrophilic boundary, but all of the octylphenyl groups are shown in the core plus the relevant portion of the oxyethylene chains that are attached to the octylphenyl groups. It is assumed that the hydrophilic region extends one oxyethylene chain length (16 Å) beyond the hydrophobic core making the radius of the whole micelle about 44 Å. Reproduced with permission from Ref. 16.

dramatically change the physical characteristics of the surface, *e.g.* size, area per molecule, or surface charge. Such changes can dramatically affect the activities of some enzymes and would complicate any kinetic analysis.

We have found that nonionic detergents come close to meeting these requirements. Nonionic detergents form micelles of relatively uniform size at concentrations above their critical micelle concentration (reviewed in Refs. 10–13). The addition of low concentrations of lipids into these detergent micelles appears to have little effect on micelle structure and results in the solubilization of these molecules into a homogeneous micelle phase. One of the most frequently employed detergents is Triton X-100. Triton X-100 mixed micelles, illustrated in Fig. 2, containing up to 15 mol % lipids, are similar to the structure of pure Triton X-100 micelles (14) but are slightly larger in size (13, 15, 16). Triton X-100 has been shown to form uniform mixed micelles with a variety of lipid molecules including phosphatidylcholine (13, 15, 17), phosphatidylethanolamine (16), phosphatidylinositol (6), phosphatidate (9), phosphatidylserine (5), CDP-diacylglycerol (8), diacylglycerol (18), palmitic acid (16), and sphingoid bases (16, 19, 20).

These nonionic detergents are also often required to solubilize integral membrane proteins (11–13). In such cases, their inclusion into assays is often unavoidable. If the assay system is using vesicles, liposomes, or membranes, the introduction of these detergents can alter the physical state of the substrate and in turn enzymatic activity. Using a detergent mixed micelle assay system, containing the same detergent used in the solubilization and purification of the enzyme, can greatly simplify the kinetic analysis and helps to ensure that the substrate will be maintained in the same physical state.

Experimental Considerations, Phosphatidylinositol 4-Kinase as an Example

To examine an enzyme according to surface dilution kinetics, activity is measured as a function of the bulk concentration of lipid substrate (A of Equation 2) at a series of set surface concentrations of lipid substrate (B of Equation 2). An example of a surface dilution kinetic analysis is shown in Figs. 3 and 4. In this example, yeast phosphatidylinositol 4-kinase activity is plotted as a function of the substrate's bulk concentration at set surface concentrations of phosphatidylinositol. These data are plotted in two ways. In Fig. 3*A* the surface binding model is employed, and the enzyme activity is plotted *versus* the sum of the molar concentrations of detergent and substrate, *i.e.* the enzyme binds nonspecifically to the entire surface. In Fig. 4*A* the phospholipid binding model is employed,

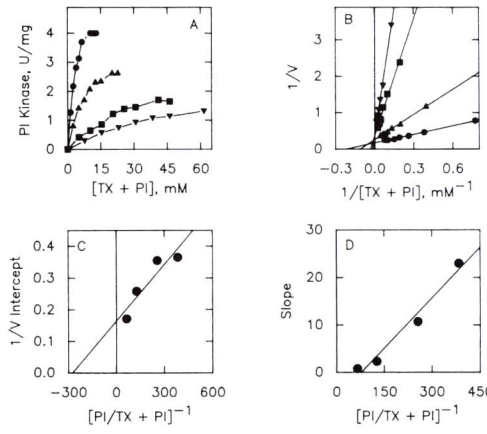

FIG. 3. **Activity of phosphatidylinositol 4-kinase toward phosphatidylinositol in mixed micelles with Triton X-100 according to the "surface binding model."** *Panel A*, phosphatidylinositol 4-kinase activity (μmol/min/mg) was measured as a function of the sum of the molar concentrations of Triton X-100 (*TX*) plus phosphatidylinositol (*PI*) at set mole fractions of phosphatidylinositol: ●, 0.0157; ▲, 0.0079; ■, 0.0039; ▼, 0.0026. *Panel B*, reciprocal plot of the data in *panel A*. *Panel C*, replot of 1/*V* intercepts obtained in *panel B versus* the reciprocal of the mole fraction of phosphatidylinositol. *Panel D*, replot of slopes obtained in *B versus* the reciprocal of the mole fraction of phosphatidylinositol. The *lines* drawn are a result of a least-squares analysis of the data. Reproduced with permission from Ref. 6.

and the same data used in Fig. 3 are now plotted as activity *versus* the concentration of substrate alone, *i.e.* the enzyme binds specific phospholipids in the surface and does not bind to the other components that make up the surface.

As predicted by the models, phosphatidylinositol 4-kinase activity exhibits typical saturation kinetics with respect to the bulk concentration of substrate, whether the latter is represented as Triton plus phospholipid or as phospholipid alone. Also, as the surface concentration of phosphatidylinositol in the mixed micelle is diluted, there is a decrease in the apparent V_{max} (Figs. 3*A* and

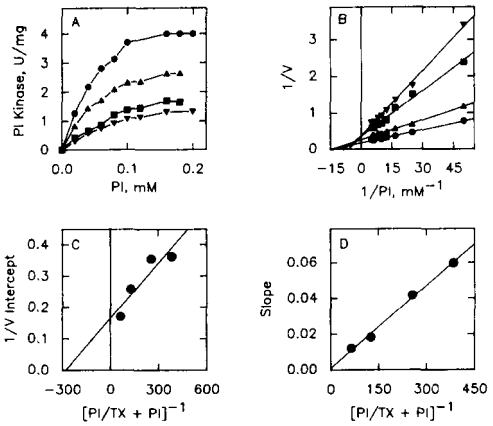

FIG. 4. **Activity of phosphatidylinositol 4-kinase toward phosphatidylinositol in mixed micelles with Triton X-100 according to the "phospholipid binding model."** The same data as plotted in Fig. 3 are replotted as follows. *Panel A*, phosphatidylinositol 4-kinase activity (μmol/min/mg) was measured as a function of the molar concentration of phosphatidylinositol (*PI*) at set mole fractions of phosphatidylinositol: ●, 0.0157; ▲, 0.0079; ■, 0.0039; ▼, 0.0026. *Panel B*, reciprocal plot of the data in *panel A*. *Panel C*, replot of 1/*V* intercepts obtained in *panel B versus* the reciprocal of the mole fraction of phosphatidylinositol. *Panel D*, replot of slopes obtained in *B versus* the reciprocal of the mole fraction of phosphatidylinositol. The *lines* drawn are a result of a least-squares analysis of the data. *TX*, Triton X-100. Reproduced with permission from Ref. 6.

4*A*). Hence, the reason for describing this phenomenon as "surface dilution kinetics."

Double-reciprocal plots confirm that the kinetic data exhibit saturation kinetics when the bulk concentration of phosphatidylinositol is varied at each fixed surface concentration of phosphatidylinositol (Figs. 3*B* and 4*B*). The pattern of lines shown in Figs. 3*B* and 4*B* is consistent with an equilibrium-ordered reaction as predicted by Equation 2. Equation 2 predicts that a replot of the 1/*V* intercepts from Figs. 3*B* and 4*B versus* 1/*B* should be linear, that the intercept of the 1/*V* intercept axis of the replot is $1/V_{max}$, and that the intercept of the 1/*B* axis is $-1/K_m^B$. Replots of the data are linear (Figs. 3*C* and 4*C*) and yield the same values for V_{max} and K_m^B (6.1 μmol/min/mg and 0.0036 mole fraction, respectively).

Equation 2 also predicts that a replot of the slopes from Figs. 3*B* and 4*B versus* 1/*B* should be linear and pass through the origin. The slope of the replot is equal to $K_s^A K_m^B/V_{max}$. From this, K_s^A can be calculated since K_m^B and V_{max} are determined above. The replot of slopes *versus* 1/mole fraction of phosphatidylinositol from Fig. 4*B versus* 1/*B*, shown in Fig. 4*D*, is linear and passes through the origin as predicted by the phospholipid binding model. Although the analogous plot in Fig. 3*D* is also linear, the line does not pass through the origin as predicted by the surface binding model. The dissociation constants (K_s^A) calculated for the mixed micelle binding site from the data in Figs. 3*D* and 4*D* are 118 and 0.26 mM, respectively. While this analysis indicates that the phospholipid binding model fits better, one should not conclude that the enzyme follows this model unless supported by other independent data. The slope replot is two replots away from the primary data and thus is subject to a fair amount of error and should not be used as a rigorous proof of which model applies.

The example of phosphatidylinositol 4-kinase demonstrates the utility of surface dilution kinetics. It should be noted that this linearized, graphical method is a traditional tool used by biochemists to analyze kinetic data. This method is helpful in visualizing the data and how well the data fit the models. Modern non-linear regression computer algorithms are also important tools to be used in analyzing surface dilution kinetics.

It should also be noted that a common practice is to hold one of the terms, either A or B, constant, often at saturating levels, while varying the other, thus creating a single kinetic line. The kinetic constants obtained from this single line are only apparent kinetic constants. These constants contain both the true kinetic constant and terms due to the variable that was held constant. In order to separate these terms and obtain the true constants both variables must be varied. In addition, to get good kinetic data, all terms must be varied over a concentration range that extends well above and below the various binding constants. This can often be a problem with lipid-dependent enzymes because many substrates are soluble over narrow ranges or can be solubilized in micelles only over a small surface concentration range.

Examples of Enzymes That Follow the Surface Dilution Kinetic Model

Surface dilution kinetics has been useful for the kinetic analysis of several lipid-dependent enzymes. These enzymes include phospholipid biosynthetic enzymes (phosphatidylinositol 4-kinase (6, 7), phosphatidylserine synthase (8), phosphatidylserine decarboxylase (5), phosphatidate phosphatase (9), and phosphatidate kinase (21)) as well as phospholipid degrading enzymes (phospholipase A_1 (22), phospholipase A_2 (1, 2, 23), phosphatidylcholine-phospholipase C (24), and phosphatidylinositol-phospholipase C (25)). Whereas some enzymes are most consistent with the surface binding model (*e.g.* phosphatidylserine synthase (8), phosphatidylserine decarboxylase (5), phosphatidate phosphatase (9), and phosphatidate kinase (21)), other enzymes are most consistent with the phospholipid binding model (*e.g.* phosphatidylinositol 4-kinase (6, 7), phospholipase A_1 (22)).

Substrate Specificity Studies

Substrate specificity with respect to acyl chain length and degree of fatty acyl unsaturation is of great interest in lipid enzymology. The study of lipid substrate specificities of phospholipid biosynthetic enzymes is required to explain the mechanism by which these enzymes contribute to the molecular species distribution of the membrane phospholipid bilayer (26). Phospholipid acyl group composition governs membrane structure and function and regulates lipid signaling pathways. Studies that attempt to determine the specificity of lipid-dependent enzymes for different phospholipids are plagued by changes in the phospholipid physical state. Changing the head group or fatty acid chain composition of a phospholipid can produce significant changes in the characteristics of the interfaces they form. These changes have been shown to dramatically affect the activity of these enzymes. Therefore, an observed difference in activity of an enzyme hydrolyzing two different phospholipids could be due to either a true specificity difference or to changes in the physical characteristics of the surface. The nonionic detergent mixed micelle systems offer a distinct advantage in regard to this problem. These mixed micelles present the phospholipid in a uniform matrix of detergent. Incorporation of different phospholipids into these structures cause much less distortion.

The utilization of surface dilution kinetics has resulted in useful information on acyl group specificity for substrates of several lipid-dependent enzymes including phosphatidate phosphatase (9), phosphatidylserine synthase (8), phosphatidylethanolamine *N*-methyltransferase (27), diacylglycerol kinase (28, 29), phospholipase A_1 (22, 30), and phosphatidylinositol-phospholipase C (31).

Expanding the Surface Dilution Model

The surface dilution kinetic scheme was developed for nonspecific (surface binding model) and specific (phospholipid binding model) binding of cobra venom phospholipase A_2 to Triton X-100/phospholipid mixed micelles (1–4). This enzyme catalyzes the hydrolysis of a single substrate molecule. However, many phospholipid biosynthetic enzymes carry out more complicated reactions. For example, yeast phosphatidylinositol 4-kinase catalyzes a BiBi reaction involving phosphatidylinositol and ATP. The surface dilution model can be expanded to include these more complex enzymatic reactions. In the case of phosphatidylinositol 4-kinase, Equation 1 is modified to include the reaction of ATP. A rate equation can then be derived that is comparable with Equation 2 by using either the rapid equilibrium or the steady state assumptions and standard kinetic derivations (32). Care must be taken to distinguish between bulk events and surface events and to ensure that the appropriate concentration terms are used. For phosphatidylinositol 4-kinase, the binding of the enzyme to the surface is in bulk terms, the binding of the substrate to the catalytic site of the enzyme is in surface terms, and the binding of ATP to the enzyme is in bulk terms because ATP does not intercalate into the surface. A detailed kinetic analysis, similar to that described above, can then be carried out and analyzed either graphically or via non-

linear regression to determine if the model fits the data and if it does to determine the kinetic parameters. Carman and co-workers (6, 7) have used surface dilution kinetics to determine kinetic constants for phosphatidylinositol and ATP.

Activation, Inhibition, and Surface Dilution Kinetics

One of the current challenges of lipid enzymology is developing pharmacologically active modulators of enzymes involved in signal transduction pathways. In addition, the activity of many membrane-associated enzymes is dependent on or regulated by membrane lipids (33). Most of the activators and inhibitors found to date are themselves surface-active compounds that readily incorporate into lipid surfaces. This fact makes the use of surface dilution kinetics imperative in analyzing inhibition and activation data. For example, numerous phospholipid analogs have been found that will inhibit the various intracellular and extracellular phospholipase A_2s. As one increases the concentration of such an inhibitor in the assay, two things occur. The inhibitor interacts with the enzyme directly to produce inhibition, *e.g.* competitive inhibition. At the same time, the inhibitor increases the surface area of the interface because it is a surface-active compound and incorporates into the surface. This effectively decreases the surface concentration of the substrate, even though its bulk concentration remains constant, which can then decrease the enzymatic rate. This would in itself appear as competitive inhibition even if the compound did not interact with the enzyme directly. These two effects must be separated to accurately determine the nature and extent of the true inhibition. Yu and Dennis (34) have derived the surface dilution kinetic equations for competitive inhibition and applied them to the evaluation of thioether amide analogs of phospholipids with phospholipase A_2 (35). Similar treatments are also possible for other forms of inhibition.

The use of surface dilution kinetics has facilitated well defined kinetic analyses of lipid activator requirements and lipid inhibitors of several lipid-dependent enzymes (for review, see Ref. 26). These include phospholipid biosynthetic enzymes (*e.g.* phosphatidate phosphatase (36), phosphatidylserine synthase (37), phosphatidylinositol synthase (37), diacylglycerol cholinephosphotransferase (38), diacylglycerol ethanolaminephosphotransferase (38), phosphocholine cytidylyltransferase (39), phosphatidylinositol 4-kinase (40), and phosphatidylinositol 4-phosphate kinase (41)), enzymes of neutral lipid metabolism (*e.g.* monoacylglycerol acyltransferase (42) and diacylglycerol kinase (28, 43–45)), lipid degrading enzymes (phospholipase A_2 (23) and phosphatidylinositol-phospholipase C (46)), and lipid signal transduction enzymes (*e.g.* protein kinase C (18, 20, 47–51); for review, see Ref. 52). The analyses of these enzymes using surface dilution kinetics have resulted in: (*a*) the identification of lipid activators and inhibitors; (*b*) information on the structural requirements for activation and inhibition; (*c*) determination of the mechanism of activation and inhibition; (*d*) information on lipid-protein interactions; and (*e*) information on the regulatory roles of lipid activators and inhibitors of enzymes *in vivo*.

Concluding Comments

In this review, we have discussed the theory, experimental considerations, and applications of surface dilution kinetics. This approach provides a useful framework for the kinetic analysis of lipid-dependent enzymes involved in biosynthesis, degradation, and lipid signaling pathways. With this basis, we should be able to advance our fundamental understanding of the mechanism of action, activation, and inhibition of enzymes that act in or on membranes and other lipid-water interfaces. This approach should allow investigators to better study and characterize important biological processes and phenomena that reside in membranes.

Acknowledgments—We are grateful to the numerous undergraduate, graduate, and postdoctoral students and colleagues who have contributed to the "surface dilution" studies in our laboratories.

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