Technical News Feature

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The Biology, Biochemistry and Technology of Lipases

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Lipid-Lipid Complexes: Properties and Effects on Lipase Binding to Surfaces[1]

Howard L. Brockman, William E. Momsen and **Takahiro Tsujita**
The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912

In the presence of bulk water, the lipase-catalyzed synthesis and hydrolysis of insoluble lipid esters occur at the lipid-water interface. For water-soluble lipases, a necessary step in this process is the partitioning of enzyme from the bulk aqueous phase to the surface phase. In surface phases of phospholipids and the substrates and products of lipolysis, physical studies have demonstrated the formation of preferred packing arrays or lipid-lipid "complexes." Such interactions involve changes in both lipid molecular area and hydration. Binding of pancreatic carboxylester lipase (cholesterol esterase) and colipase to monomolecular films of a phosphatidylcholine and its complexes with fatty acid or diglyceride is negligible. In contrast, saturation of film of pure fatty acid or diglyceride correlates with formation of a protein monolayer. With mixtures of complex and uncomplexed fatty acid or diglyceride, binding to the uncomplexed lipid occurs, but only with colipase can saturation of available sites be achieved. The lower affinity of carboxylester lipase for surfaces containing complexes can be qualitatively explained by differences in the size of lipid and protein molecules. Because it involves no direct interaction between enzyme and complex, such "proxinhibition" of enzyme binding is potentially an important regulation of lipid-protein interactions.

As exemplified by the studies presented in this symposium, lipases are powerful tools for catalyzing not only hydrolysis, but also esterification and transesterification reactions involving water-insoluble esters. To take maximum advantage of these enzymes, as well as to better understand their biological roles, it is important to determine how expression of their activities depends on the surrounding environment. In the case of lipases, this goes far beyond defining experimental conditions such as concentrations of enzymes and substrates, ionic strength, pH, and temperature, because both the reactants and catalyst are distributed non-uniformly within the system. Lipolysis is, in fact, a classic example of heterogeneous biocatalysis (1).

A typical environment in which a lipase might function is shown schematically in Figure 1. It consists of a lipid droplet that is separated from the aqueous milieu by a two-dimensional surface phase. This surface phase is most readily visualized as a monolayer of proteins and amphipathic lipids, like phospholipids, but can be described more precisely by other models (2). Typically, this monolayer phase comprises a negligible fraction of the total mass in the system. However, it is of primary importance with respect to the regulation of lipolysis because it is the site of lipid hydrolysis and esterification (3). Hence, the instantaneous rate and direction of the reaction depend on the concentrations of reactants within the surface phase. These surface concentrations are themselves determined through partitioning reactions between the bulk phases and the surface phase. In this way, the ultimate direction and extent of reaction are controlled by the overall composition of the system. It is important to note in the scheme presented in Figure 1 that the partitioning of the enzyme from the aqueous phase to the surface phase can be, and usually is, distinct from the interac-

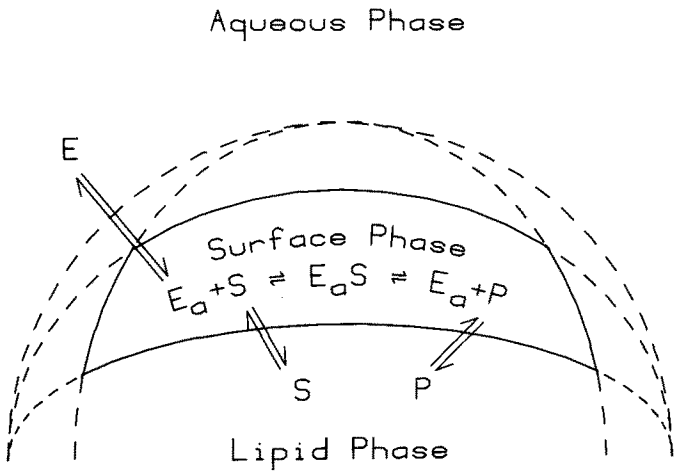

FIG. 1. Schematic model of lipolysis.

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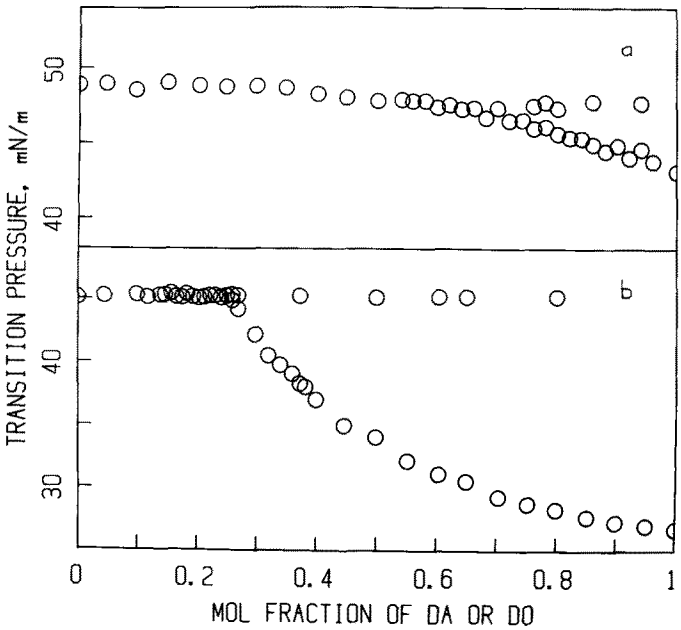

FIG. 2. Surface pressure-composition phase diagrams for (a) 13,16-docosadienoic acid (DA), or (b) 1,3-diolein (DO) with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). Abbreviations are consistent in subsequent figures and tables.

tion of the enzyme with its substrate within the surface phase. For example, under certain conditions the adsorption of pancreatic triglyceride lipase requires a cofactor protein, colipase, that serves to anchor the protein at the interface (4). However, the cofactor has little effect on catalysis by the adsorbed enzyme. Such results imply that the site on the protein for enzyme-surface interaction may be functionally and topographically distinct from the site for enzyme-substrate binding. Hence, an understanding of the regulations of lipolysis requires that the binding and catalytic steps each be investigated with respect to the chemical composition and physical structure of the substrate-containing surface phase. This presentation summarizes results of recent studies of the regulation of the partitioning of proteins between the aqueous and surface phases.

SURFACE STRUCTURE

Even for a relatively simple emulsion or bilayer system consisting of enzyme, substrate, and a surfactant such as a phospholipid, it is difficult to study enzyme partitioning because of the many other reactions and other redistributions of components occurring simultaneously. For example, emulsion and bilayer particles frequently exhibit size heterogeneity and may coalesce with time. For studying lipase adsorption, a useful approach has been to eliminate the bulk lipid phase and to control surface area by using monomolecular films of insoluble lipids at the air-water interface (3,5). Experimentally, lipid composition and packing density in monolayers can be precisely measured and controlled and, near collapse, such films are reasonable models for the surfaces of emulsion and bilayer membranes (6,7). As recently reviewed (3), an important advantage to the use of these lipid films is their amenability to physical characterization under the same conditions in which enzyme-surface interaction is to be studied. Results of such physical studies involving cholesteryl esters mixed with other lipids have shown regularities in lipid interfacial behavior at the point of film collapse to a bilayer of emulsion phase, and more detailed analysis of surface pressure-molecular area-composition behavior supports a model of surface structure based on the hydration of lipids in the film (8). In essence, the surface behaves as if it consists of lipid-water "building blocks," each of which has a characteristic partial molecular area and hydration. These components mix ideally and, hence, the collapse pressure and area of a mixed lipid film are determined simply by the proportion of components.

More recently, studies of other lipid mixtures have extended the applicability of the model and revealed the formation of preferred packing arrays or "complexes" between phospholipids and the substrates and product of lipolysis (9, and work in progress). Within the framework of the model, these complexes behave as pseudo-species, each with a characteristic composition, area and hydration. Examples of surfac phase diagrams showing complex formations are given in Figure 2. Both a substrate, 1,3-diolein (DO), and a

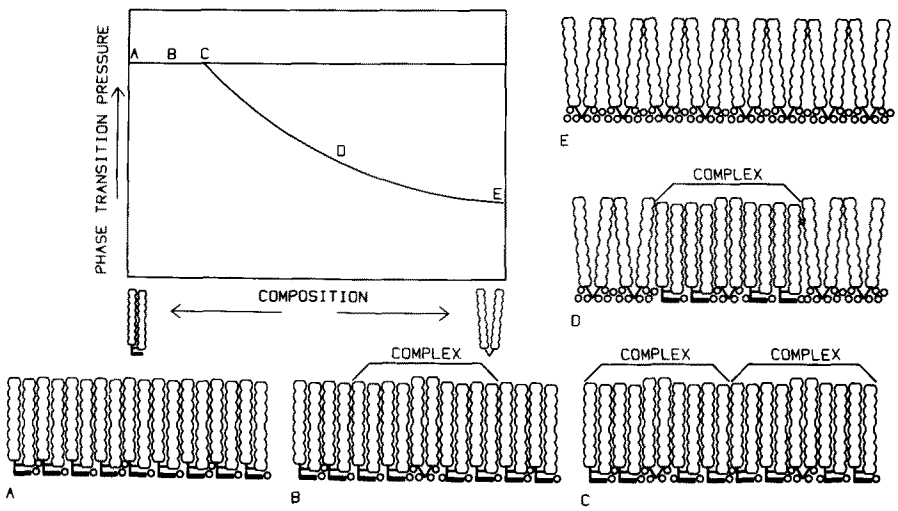

FIG. 3. Schematic phase diagram and models for DO-POPC films.

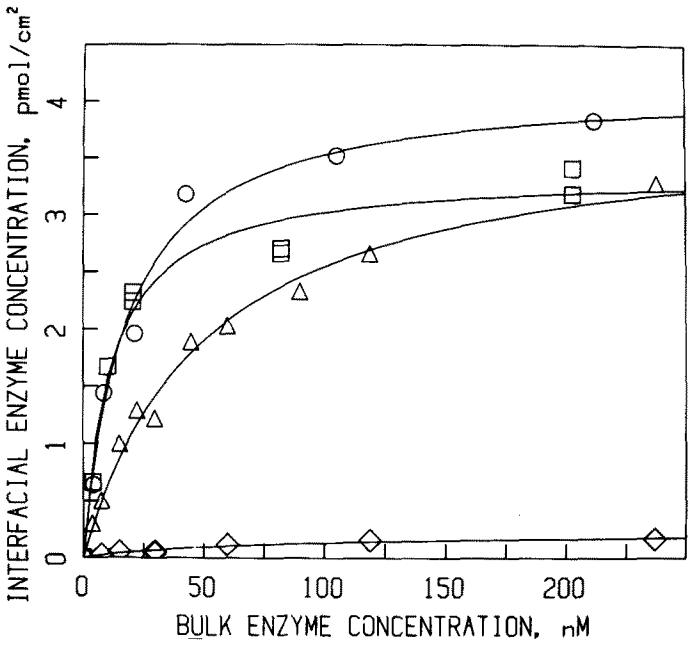

FIG. 4. Enzyme dependence of equilibrium adsorption of carboxylester lipase to lipid films. Native enzyme with DA (△) or POPC (□); diisopropylphosphoryl-enzyme with DA (□) or DO (○).

product 13,16-docosadienoic acid (DA), of lipolysis form complexes with the surfactant, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), at the point of monolayer collapse to a bulk or bilayer phase. The complex compositions are indicated by the discontinuity at 0.20 (DO, Fig. 2A) or 0.67 (DA, Fig. 2B) mol fraction of non-phospholipid.

Comparison of the properties of free and complexed non-phospholipids having exclusively 18:1-chains shows that complexation involves changes in both molecular areaand hydration (9). As shown schematically in Figure 3 for the DO-POPC system, the surface at point A consists of POPC at an area of ~53 Å^2/molecule and at point E of pure DO at a molecular area of ~30 Å^2/acyl chain. Note also that the hydration of the DO is relatively greater per chain than that of POPC. At the composition indicated by point C, the surface consists solely of a complex between POPC and DO. Moreover, on a per-chain basis the area and hydration of complexed DO have decreased by about 7 Å^2 and the equivalent of 0.8 water molecules, respectively. At point B, the surface consists of POPC and complex and these may or may not be miscible in the monolayer phase. At point D, the surface consists of complex and uncomplexed DO, and these components are completely miscible. The behavior of the area of the film as a function of composition (not shown) in the regions denoted by B (if the components are miscible) and D indicates that the mixing is ideal.

LIPID-PROTEIN INTERACTIONS

The observation of such lipid-lipid interactions raised the question of how they might regulate the interactions of lipases and related proteins with interfaces. Using films of the compounds described above, we examined the adsorption to the surface of porcine pancreatic cholesterol esterase (10,11) and, to a lesser extent, porcine pancreatic colipase. This was undertaken in three parts: the adsorption of the proteins to pure lipids, to complexes, and to complexes in the presence of uncomplexed DO or DA. With respect to pure lipids, Figure 4 shows the concentrations of cholesterol esterase at the lipid-water interface as a function of its concentration in the aqueous subphase. For adsorption to DA, the surface becomes saturated with enzyme between 3 and 4 pmol/cm^2, whereas for POPC films, less than 5% of this amount is bound. Because DO is a substrate for pancreatic carboxylester lipase, it was necessary to inactivate the enzymes with diisopropylfluorophosphate (DFP) before performing adsorption studies. This species saturates a DO surface between 3 and 4 pmol/cm^2 and adsorbs to DA films as well. Although not shown, it does not adsorb to POPC films. Based on these similarities, DFP-carboxylester lipase is a reasonable model for the native enzyme. Moreover, the absence of any change in binding specificity following derivatization of the active-site serine indicates that, as noted above, the surface interaction site of this lipase is distinct from its catalytic site.

The lines in Figure 4 were obtained by fitting the data to the Langmiur adsorption isotherm (12). The resulting dissociation constants given in Table 1 show that the affinities of the DFP-enzyme for DA and DO are somewhat greater than the affinity of native enzyme for DA. However, other data (10) show that this is due to differences in ionization state of the enzyme at pH 6.5, and at a pH of 5.6, one unit lower, the

TABLE 1

Equilibrium Binding Parameters for Carboxylester Lipase Adsorption to Surfaces

Lipid	Mol fraction	Enzyme	K_d, μM	Γ_M, pmol/cm^2
DA	1.0	native	52	3.9
DA	1.0	DFP	13	3.5
DO	1.0	DFP	18	4.2
POPC	1.0	native	~72	~0.2
DA/POPC	0.55/0.45	native	~94	~0.25
DA/POPC	0.80/0.20*	native	~669	~3.3
DO/POPC	0.50/0.50*	DFP	~651	~2.5
DA/POPC	0.90/0.10**	native	~447	~5.0
DO/POPC	0.72/0.28**	DFP	~399	~4.1

Area fraction of uncomplexed lipid = 0.38 (*) or 0.67 (**).

TABLE 2

Kinetic Parameters for Carboxylester Lipase Adsorption to Surfaces

Lipid	Mol fraction	Apparent rate constant × 10^5 (cm/s)	
		Native	^{3}H-DFP
DA	1.00	6.30	5.90
DO	1.00	6.70	6.10
POPC	1.00	0.47	0.35
DA/POPC	0.61/0.39	—	1.50
DO/POPC	0.24/0.76	—	0.32

dissociation constant for native enzyme is 12 μM. The maximal adsorption values for all but POPC were about 4 pmol/cm^2. For a spherical protein of 74 kDa mol wt, this is the value expected for formation of an enzyme monolayer adjacent to the lipid film. Thus, all of the DA- and DO-covered surfaces appear to be available for enzyme binding. In contrast, for adsorption to POPC the calculated adsorption limit is near the correction for carryover from the bulk phase and other potential errors. Accordingly, the calculated value of the dissociation constant also has a high uncertainty.

The binding process was further characterized by measuring the initial rate of adsorption of a native and modified enzyme to films of DA, DO or POPC. The results, expressed as apparent rate constants (Table 2) show the same pattern as the equilibrium constants. Enzyme readily adsorbs to DA or DO, but not to POPC. Additional kinetic studies with other lipids (not shown) show that the enzyme binds to many species of non-phospholipids but surface denatures in those cases in which surface pressure is below 25 mN/m. Thus, carboxylester lipase exhibits a lack of specificity for binding to particular lipids but appears to have an inability to bind to POPC. This inhibition also is observed with other phospholipid species such as phosphatidylserine and phosphtidylethanolamine (T. Tsujita and H.L. Brockman, unpublished data).

Given these results, it was of interest to determine if complexation of DA and DO with POPC would affect the ability of the enzyme to bind to them. To test this, surfaces with POPC compositions slightly higher than those of the complex were used. This insured that complex was the predominant species in the surface and that no uncomplexed non-phospholipid was present. As shown in Tables 1 and 2, the lack of binding to complexes was comparable with that observed with POPC alone. Thus, complexation of DA and DO destroys their ability to serve as adsorption sites for the enzyme. The negligible binding to DA-POPC complexes is particularly significant. At the lipid composition used, over half of the lipid is fatty acid, yet maximal adsorption is only near background. The results are equally as dramatic when considered on a molecular area basis.

As noted above, both complexed and uncomplexed DA or DO coexist in a single surface phase in the region corresponding to line CDE on the schematic phase diagram of Figure 3. The marked inhibition of carboxylester lipase adsorption to DA and DO, which accompanies their complexation with POPC, raised an additional question. That is, "can the presence of complex regulate enzyme adsorption to uncomplexed lipid?" To examine this, adsorption of enzyme was measured at various surface pressures and compositions corresponding to points along the line CDE. The bulk enzyme concentration used was five times the respective dissociation constant for adsorption to pure DA or DO, a level that should result in most available binding sites being occupied. If the enzyme adsorbs to all uncomplexed DA or DO, the surface concentration of enzyme should be related linearly to the total mol fraction of DA or DO in the mixture. That this is not the case is shown by comparison of the predicted results with measured surface concentrations (Fig. 5a). Alter-

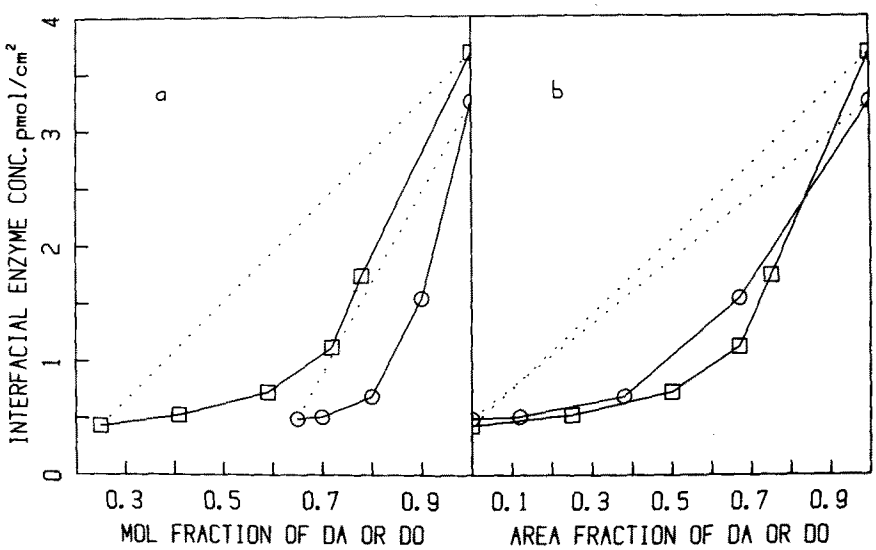

FIG. 5. Composition and area dependence of equilibrium adsorption of carboxylester lipase to mixed lipid films. Predicted values (• • •) for adsorption of native enzyme to POPC-DA (○) and diisopropylphosphoryl-enzyme to POPC-DO (□), assuming that between endpoints , binding is proportional to (a) the mol fraction uncomplexed DO or DA or (b) the fraction of total area it occupies.

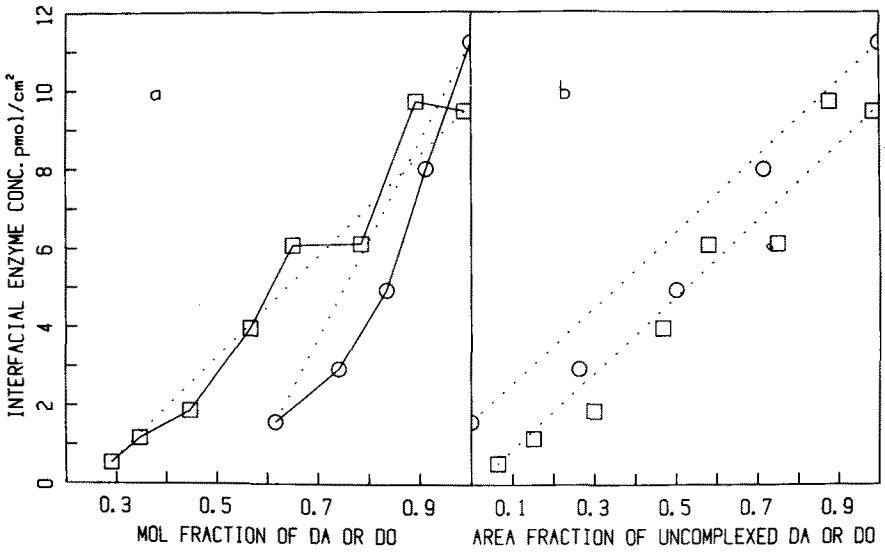

FIG. 6. Composition and area dependence of equilibrium adsorption of colipase to mixed lipid films. Predicted values (• • •) for adsorption of the protein to POPC-DA (○) and POPC-DO (□), assuming that between endpoints binding is proportional to (a) the mol fraction uncomplexed DO or DA or (b) the fraction of total area it occupies.

natively, the level of enzyme binding might depend primarily on the surface area occupied by uncomplexed DA or DO rather than the mol fraction of either species. Using partial molecular areas determined from physical studies of mixed lipid films, the fraction of surface area occupied by uncomplexed DA or DO relative to complex can be calculated at each composition. As shown in Figure 5B, the level of bound enzyme still falls short of the predicted value. Note, however, that replotting the data in this manner makes the two sets superimposable within experimental error. This suggests that geometric factors are an important determinant of enzyme adsorption.

In calculating the theoretical lines in Figures 5a and 5b, it was assumed that the dissociation constant for adsorption to uncomplexed lipid was the same in the presence of complex as in its absence. To test this assumption, dissociation constants were determined for both systems at area fractions of DA or DO of 0.38 and 0.67. Experimentally, saturation could not be achieved and, as shown in Table 1, the apparent dissociation constants are increased by 10–20 fold. Thus, the presence of complexes in mixtures with uncomplexed lipids greatly decreases the affinity of the enzyme for binding to uncomplexed DA or DO. There seems to be a lesser effect on maximal adsorption, but the values obtained are unreliable because saturation could not be achieved experimentally.

In a similar study, the effect of the presence of complex on adsorption of proteins to uncomplexed Da or DO also was investigated using porcine pancreatic colipase A. This small protein serves to anchor pancreatic triglyceride lipase to surfaces, particularly in the presence of bile salts. Its affinity for uncomplexed DA or DO alone is so great that K_d values could not be measured. This experimental limitation exists because at very low concentrations of protein, the binding reaction becomes kinetically limiting relative to film stability. In spite of its higher affinity for uncomplexed lipid, colipase binding to POPC or to its complexes with DA or DO is minimal. However, binding to mixtures of complex and uncomplexed lipid exhibits nearly linear behavior on a composition (Figure 6a) or area fraction basis (Figure 6b), especially with DO-POPC films. In this respect, the interaction of colipase with surfaces is different than that of carboxylester lipase (compare Figures 5a and b with 6a and b).

DISCUSSION

The experimental data presented show clearly that lipid-lipid interactions between phospholipids and substrates and products of lipolysis are important regulators of enzyme-surface interaction. Because this interaction is essential for the activity of water-soluble li-

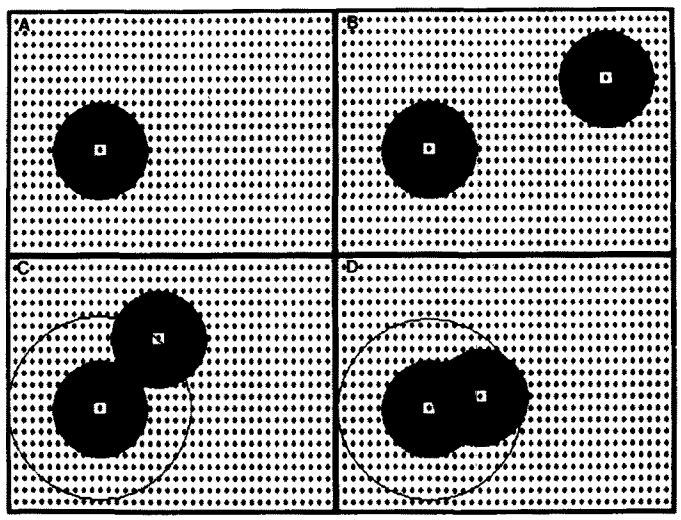

FIG. 7. Schematic model for adsorption of a large protein to a surface occupied by small ligands. Filled circles represent protein with square binding site for small ligand (×). Circle indicates excluded area in which another protein cannot bind, as depicted in lower right panel.

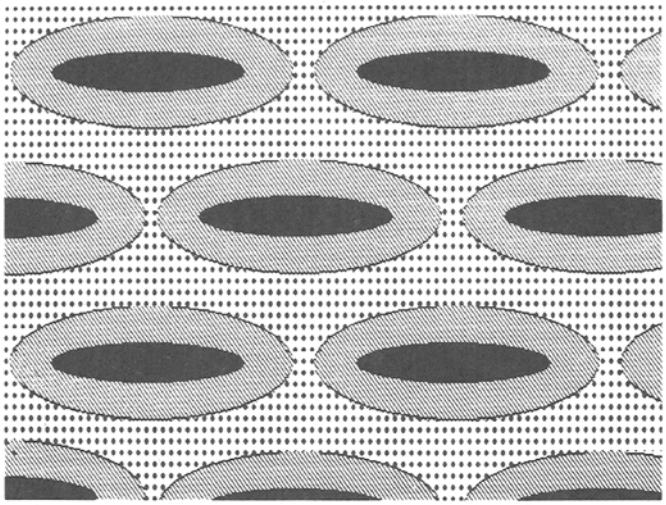

FIG. 8. Schematic model of a lipid surface comprised of both complexed and uncomplexed lipid. Filled ovals indicate complexes and together with shaded areas compose the portion of the surface to which protein, as depicted in Figure 7, cannot bind.

pases to be expressed (Fig. 1), such regulation can be expected to be of importance in controlling the overall catalytic process. A possible mechanism for understanding how POPC and its complexes can inhibit the interaction of lipases with their substrates and products is suggested by the apparent geometric nature of the inhibition (Figs. 5b and 6b). It is based on an extension of concepts presented by Stankowski describing the binding of a large ligand, such as a protein, to a surface covered with relatively small receptors, such as lipid molecules (13,14). The concepts he presented are shown schematically in Figure 7. Panel A shows a protein molecule, represented by the filled circular disk, bound to a ligand, represented by the small dark cross. As long as there are few protein molecules previously bound to the surface, the binding of subsequent molecules of protein from above the plane of the figure is completely unaffected by the presence of first (Panel B). As indicated by the circle in Panel C, the limit of how close protein molecules may bind is twice the radius of the protein. If a molecule approaching the surface from above tries to bind any closer than twice the radius of the enzyme molecule, binding will be prevented, as is depicted in Panel D. This inhibition occurs even though receptors located between the disc and the circle are neither occupied nor covered. Thus, the rate of the binding reaction, which depends upon the number of sites available for binding, is effectively lowered by the presence of previously bound protein molecules. In contrast, the protein, once bound, would have the same dissociation rate in any of Panels A-C, assuming the lack of lateral protein-protein interactions. Thus, the net effect of the presence of previously bound protein is to decrease the net rate of binding and thereby increase the dissociation constant for the enzyme-surface interaction. In practical terms, this means that the apparent dissociation constant will be a function of the fraction of the surface covered with enzyme, increasing with increasing surface coverage. That we may have observed apparent simple saturation of the surface (Fig. 4) is possible (14) but leads to overestimation of the value of the dissociation constant.

Consider next, adsorption of a protein to a mixed surface consisting of domains of POPC or its complex with DA or DO. Such domains are shown schematically as the filled ovals in Figure 8. The size of such domains may be as large as several hundred molecules without any evidence of phase separation (15). Alternatively, they may consist of only one or a few phospholipid molecules. In either case, the inability of protein to bind to such domains may affect its adsorption to uncomplexed lipid in the same manner as previously adsorbed protein molecules do. Thus, their effective area, shown in gray, is much larger than the domains themselves, which are shown in black. Such proximity inhibition or "proxinhibition" is entirely consistent with our observations to date. The apparent absence of such an effect of colipase binding (Fig. 6b) is explained by our inability to measure dissociation constants for the adsorption process. Effectively, the binding of colipase to uncomplexed lipid is so strong that changes in the dissociation constant remain undetected at the bulk concentration of enzyme used in adsorption experiments.

In theory, data of the type presented can be quantitatively analyzed. Unfortunately, however, such analysis requires knowledge of the shape as well as the size of the lipid domains (14). Thus, a detailed understanding of the mechanism behind "proxinhibition" must await further advances in our knowledge of surface structure.

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