Regulation of Carboxylester Lipase Adsorption to Surfaces. 2. Physical State Specificity†

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ABSTRACT: The physical specificity of adsorption of porcine pancreatic carboxylester lipase to mixed-lipid surfaces was examined by using films at the argon–buffer interface. They were comprised of 1-palmitoyl-2-oleoylphosphatidylcholine and triolein, 1,3-diolein, methyl oleate, oleonitrile, oleyl alcohol, or 13,16-docosadienoic acid. Under conditions where the surfaces are thermodynamically well-defined, each of these binary systems exhibits the formation of a lipid–lipid complex that is completely miscible with uncomplexed non-phospholipid [Smaby, J. M., & Brockman, H. L. (1985) *Biophys. J. 48*, 701–707]. Initial rates of adsorption of enzyme to the complexes were ≤5% of those measured in the absence of phospholipid and comparable to its rate of adsorption to phospholipid alone. This occurred despite there being up to 46% of the surface area occupied by non-phospholipid in the complexes. Equilibrium binding measurements were made at a composition where phospholipid–fatty acid complex was the predominant species. These showed that the low rates were due to an absence of adsorption sites relative to surfaces of fatty acid alone. With diolein or fatty acid and phospholipid, equilibrium binding was also measured at compositions intermediate between that of the complex and pure non-phospholipid. In both systems surface concentrations of enzyme varied nonideally with respect to either the mole fraction or area fraction of complex and uncomplexed diolein or fatty acid in the film. At area fractions of uncomplexed lipid of 0.35 and 0.67, dissociation constants for enzyme adsorption were increased 10–20-fold relative to pure fatty acid or diolein. Thus, lipid–lipid interactions can be important regulators of lipid–protein interactions. The inhibition of adsorption by complexes is not attributable to the absolute value of the interfacial tension or to the ionization state of the fatty acid but is related to the fraction of the surface area occupied by complex. Qualitatively, such behavior can be understood by consideration of the relative sizes of the enzyme molecule and the lipid domains.

In the preceding paper (Tsujita & Brockman, 1987), the interaction of porcine pancreatic carboxylester lipase with individual lipid–water interfaces was examined. The lipids were in monomolecular films at the point of collapse to a bulk or bilayer phase and had similar aliphatic moieties. These conditions were selected because, on a per chain basis, the areas of the species were identical (Smaby & Brockman, 1985). In addition, by being approximately in equilibrium with bulk or bilayer phases, each lipid should be at a chemical potential closer to what might exist in vivo than if it were at an arbitrarily chosen interfacial tension. The results of this study showed that interaction with the physically similar, but chemically dissimilar, surfaces occurred at the same rate and to the same extent, i.e., was nonspecific. Interpretation of the data was complicated by the occurrence of denaturation that followed adsorption. However, with 13,16-docosadienoic acid (DA)[1] and 1,3-diolein (DO) films, surface denaturation of enzyme was negligible, and saturable monolayer coverage by enzyme was demonstrated. In contrast to these results, adsorption of enzyme to films of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) was minimal.

The difference in binding of enzyme to POPC and DO or DA was of interest because of the interfacial nature of lipolysis [e.g., Brockman (1984)]. Whereas the substrates are often neutral lipids, the preponderance of lipids in naturally occurring surface phases, such as bilayer membranes [e.g., Rouser et al. (1968)] and lipoproteins [e.g., Miller and Small (1983)] is phospholipids. Even in the intestine where carboxylester lipase functions, physical data suggest that phospholipids and other species, not bile salts, are the predominant emulsifiers of neutral lipids like triglycerides (Linthorst et al., 1977). Thus, the regulation of enzyme–surface binding, and hence catalysis, may be regulated by long-chain phospholipids that are nonsubstrates. Within a mixed surface phase of substrate or product and phospholipid, the presence of phospholipid might regulate enzyme–substrate binding directly by competing with substrates or indirectly through lipid–lipid interactions that render substrates unavailable to the enzyme.

At the point of collapse to a bulk or bilayer phase, monomolecular films at the gas–liquid interface are thermodynamically well-defined (Smaby & Brockman, 1984). Moreover, analysis of collapse pressures and areas as a function of lipid composition has revealed the formation of complexes or preferred packing arrays in a number of systems containing phosphatidylcholines and non-phospholipids (Smaby & Brockman, 1985, 1987a,b). The complexation of a non-phospholipid results in a decrease in both its area and hydration, whereas phospholipid behavior is minimally affected. For these systems the complexes are completely miscible with uncomplexed non-phospholipid. To determine how the presence of phospholipids or their interaction with substrates can regulate lipid–protein interaction, we have studied the adsorption of carboxylester lipase to well-characterized, mixed-lipid films containing POPC. The results show that the for-

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DA, 13,16-docosadienoic acid; DO, 1,3-diolein; oleonitrile, 9-octadecenenitrile.

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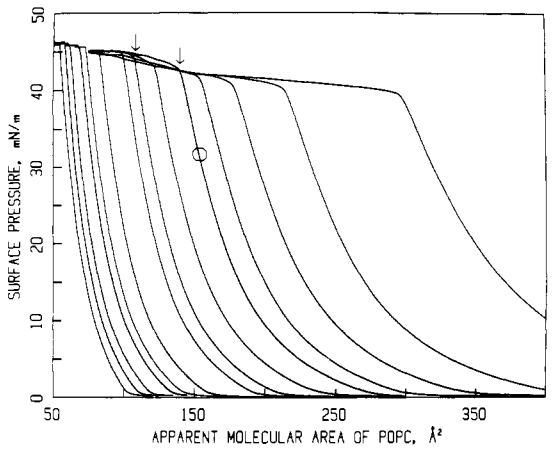

FIGURE 1: Surface pressure–area isotherms for mixtures of DA and POPC. Subphase was 10 mM potassium phosphate, pH 6.6, and 0.10 M NaCl at 24 °C. Mole fractions of DA are, from left to right, 0.0, 0.150, 0.250, 0.349, 0.451, 0.563, 0.659, 0.693, 0.729, 0.769, 0.801, 0.828, 0.861, and 0.901. For the isotherm indicated by the circle the arrows show the phase transitions as described in the text.

mation of lipid–lipid complexes can be an important regulator of the interfacial concentration of the enzyme.

Materials and Methods

Reagents and procedures were as described in the preceding paper (Tsujita & Brockman, 1987).

Results

As discussed in the preceding paper (Tsujita & Brockman, 1987), DA and oleic acid exhibit similar surface behavior on buffers at pH 6.6 containing either 0.1 or 2.0 M NaCl. However, the stability of DA films was superior to that of oleic acid films, making it more useful for studies of fatty acid–protein interactions at interfaces. To determine the usefulness of DA as a substitute for oleic acid with respect to interaction with phospholipids, it was necessary to characterize its behavior in mixed-lipid films at the argon–buffer interface. Sets of surface pressure–area isotherms for DA–POPC mixtures were determined at pH 6.6 on buffers containing either 0.1 or 2.0 M NaCl. Examples from the low-salt set obtained with DA are shown in Figure 1 and resemble those for oleic acid–POPC mixtures under the same conditions (Smaby & Brockman, 1985). At mole fractions of DA up to 0.6–0.7, a single phase transition representing film collapse is observed. At higher DA compositions there are two transitions in each isotherm (e.g., Figure 1, arrows). For the entire set of curves obtained, the phase-transition surface pressures are shown as a function of DA composition in Figure 2a. Figure 2b shows comparable data obtained with 2.00 M NaCl in the aqueous phase. As described earlier (Smaby & Brockman, 1985), this type of behavior indicates formation of a POPC–fatty acid complex, the composition of which is the point of bifurcation of the data in Figure 2a,b. In the compositional region of single transitions, the surfaces at the point of collapse consist of POPC and POPC–DA complex. In the two-transition region at higher DA compositions, the lower transitions define the expulsion of uncomplexed DA from a mixture of uncomplexed DA and POPC–DA complex. The upper transitions show the collapse of complex that occurs upon further reduction in film area. Note that as the composition of the system approaches pure DA, these cannot be detected because the relative mass of complex is so small. Overall, the lower transitions throughout the phase diagram define the phase boundary along which the surface shows regular thermodynamic behavior

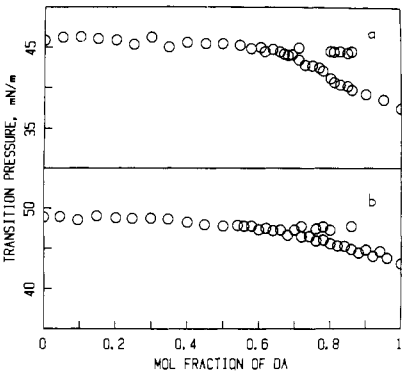

FIGURE 2: Surface pressure–composition phase diagrams for DA–POPC mixtures. Subphase was 10 mM potassium phosphate, pH 6.6, at 24 °C containing (a) 0.10 M NaCl and (b) 2.00 M NaCl.

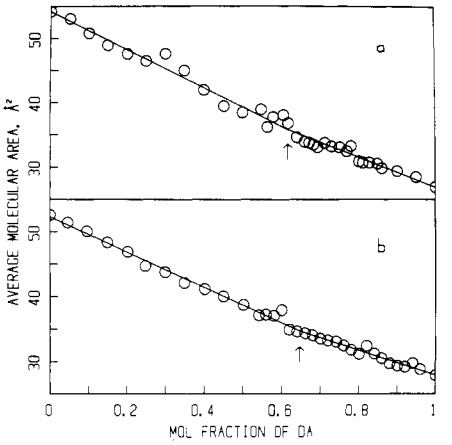

FIGURE 3: Average molecular area–composition isotherms for DA–POPC mixtures at film collapse. Conditions are as in Figure 2: (a) 0.10 M NaCl; (b) 2.00 M NaCl. Arrows indicate the complex composition for each system.

(Smaby & Brockman, 1984, 1985).

From surface pressure–composition data the complex composition can be more precisely defined by a semiempirical method (Smaby & Brockman, 1985). Its application to the data of Figure 2a,b (not shown) gives complex compositions of 0.62 and 0.65 mol fraction of DA on 0.1 and 2.0 M salt. For oleic acid–POPC mixtures on 0.1 M salt, the fatty acid content of the complex was 0.65 (Smaby & Brockman, 1985). At each composition and pressure along the phase boundary there is a corresponding average molecular area. If the system is well-behaved, a plot of area vs composition data should be a biphasic line with a discontinuity at the complex composition. By use of the complex stoichiometries determined from each of the respective phase diagrams, each data set was fitted to a biphasic line by a least-squares procedure. As shown in Figure 3, the data in each segment are linear and, though not required by the model, the slopes are nearly identical. From the slopes and intercepts, the partial molecular areas of uncomplexed POPC, POPC–DA complex, and uncomplexed DA were calculated to be, respectively, 54.2, 93.3, and 26.9 Å^2 on 0.1 M NaCl and 52.3, 99.2, and 27.9 Å^2 on 2.0 M NaCl. These values agree well not only with each other but also with the values of 53.1, 96.2, and 25.8 Å^2 determined earlier from data obtained by using POPC–oleic acid on 0.1 M NaCl (Smaby & Brockman, 1985). Thus, in their miscibility behavior, POPC–DA films are a good model for POPC–oleic acid films.

Their near equivalence in mixtures, together with the superior stability of DA-containing films, makes them useful for studies involving other lipids of which oleic acid is part of a

Table I: Kinetics of Adsorption of Carboxylester Lipase to Mixed Lipid–Buffer Interfaces

non-phospholipid	POPC mole fraction	apparent rate constant[a] × 10^5 (cm/s)
DA	0.39	1.50
oleyl alcohol	0.51	0.19
1,3-diolein	0.76	0.32
methyl oleate	0.81	0.34
oleonitrile	0.80	0.11
triolein	0.96	0.19
none	1.00	0.35[b]

[a] Bulk [enzyme] = 6 nM; subphase was 10 mM potassium phosphate, pH 6.6, 24 °C, 0.10 M NaCl. [b] From Tsujita and Brockman (1987).

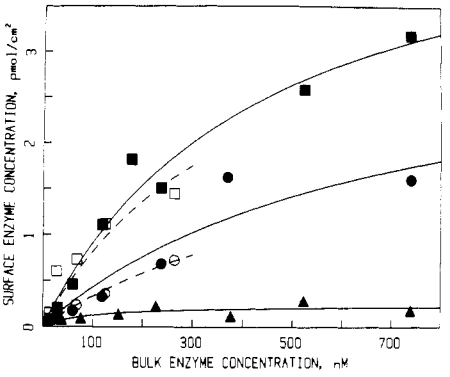

FIGURE 4: Concentration dependence of the adsorption of carboxylester lipase to complex-containing surfaces. Subphase was 10 mM potassium phosphate, pH 6.6, at 24 °C containing either 0.10 M (DO, modified enzyme) or 2.00 M (DA, native enzyme) NaCl. The area fraction of uncomplexed DA was ≤0 (▲), 0.38 (●), or 0.67, (■) and of uncomplexed DO 0.38 (○) or 0.67 (□).

series. One such series includes oleyl alcohol, oleonitrile, methyl oleate, 1,3-diolein, and triolein (Smaby & Brockman, 1985). In mixtures with POPC, each of these lipids exhibits complex formation at POPC compositions approximately 0.95 that shown in Table I. At the compositions given in the table, all non-phospholipid is complexed and complex is the predominant species. The ability of carboxylester lipase to bind to such films at surface pressures near collapse was measured at a subphase enzyme concentration of 6.0 nM. To prevent any possible hydrolysis of substrates, such as diolein and triolein, a catalytically inactive derivative of the enzyme was used. At this level, adsorption of enzyme to pure lipid films is linear with time, and only a few percent of an enzyme monolayer can be adsorbed in 10 min (Tsujita & Brockman, 1987). This allows calculations of pseudo-first-order rate constants for adsorption, and the values obtained are listed in Table I. For all complexes, adsorption is about 0.05 or less of that for the non-phospholipid or DA alone but is comparable to that obtained with POPC alone. During these measurements surface pressures were constant at 40–42 mN/m. Because such high pressures might themselves limit carboxylester lipase adsorption, the measurements with POPC–DA and POPC–DO films were repeated at 35–36 mN/m. The values of 1.50×10^{-5} and 0.61×10^{-5} cm/s obtained indicate that the higher collapse pressures of the complexes relative to pure DA (Figure 2) are not inhibiting adsorption. Another possibility was that derivatization of the active site of the enzyme could impede its binding to complex-covered surfaces. To evaluate this and to better characterize the interaction, the concentration dependence of the adsorption of native enzyme to films with 0.55 mol fraction DA was measured. As shown by the triangles in Figure 4, very little adsorption was observed

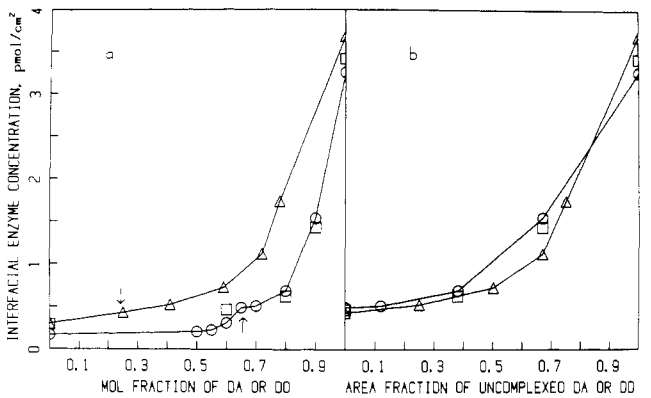

FIGURE 5: Composition dependence of carboxylester lipase adsorption to mixed-lipid surfaces. Subphase was 10 mM potassium phosphate, pH 6.6, at 24 °C containing 0.10 M (DO) or 2.00 M (DA) NaCl. Adsorption of native (○) and [^{3}H]diisopropylphosphoryl (□) enzymes at 220 nM to POPC–DA films; adsorption of diiopropylphosphoryl enzyme (△) at 125 nM to POPC–DO films. The compositions of DA–POPC (↑) and DO–POPC (↓) complexes are indicated by the arrows.

at any concentration. The data were fitted to a Langmuir adsorption isotherm, yielding a dissociation constant of 94 nM and a maximal adsorption of only 0.25 pmol/cm^2. Monolayer coverage would be 4.0 pmol/cm^2 for a protein of this molecular weight.

The enzyme adsorbs readily to uncomplexed DA, DO, and other lipids but not, as shown above, to their complexes with POPC or to POPC itself. It was, therefore, of interest to determine what effect the presence of these complexes would have on enzyme adsorption to surfaces containing uncomplexed lipid. This was examined by using mixed films of POPC and DO or DA with compositions intermediate between the complex composition and pure DA or DO. The latter species were used because they represent a substrate and a product of the reaction catalyzed by the enzyme and because with pure films adsorption of enzyme exhibits saturation behavior at monolayer coverage (Tsujita & Brockman, 1987). For DA-containing films both native and modified enzyme were used at 220 nM, whereas for DO-containing films modified enzyme only was used at 125 nM. On the same subphase these enzyme levels are nearly sufficient to saturate a pure DA or DO surface with enzyme. The composition dependence of adsorption to these films is shown in Figure 5a. For comparison, adsorption to pure POPC and POPC plus complex was also measured at a few compositions. With both DO- and DA-containing films adsorption at compositions near the complex compositions (arrows) were similar to that for POPC alone. Above those values adsorption increased with increasing content of DO or DA and reached near monolayer coverage with the pure lipid. Within error, no differences in adsorption to DA-containing films were observed when modified enzyme was substituted for the native form.

Because enzyme adsorbs to pure DA or DO but not to their complexes with POPC, the amount adsorbed might be expected to vary linearly with the mole fraction of uncomplexed DA or DO relative to uncomplexed lipid plus complex. It is readily shown that the mole fraction of the uncomplexed species varies linearly with the total mole fraction of DA or DO in the system. Hence, Figure 5a shows that composition alone does not regulate enzyme binding. Alternatively, the degree of adsorption at equilibrium might depend on geometric factors. In the simplest case, the level of adsorption could be determined solely by the amount of surface area occupied by uncomplexed DA or DO. If this were so, then the surface concentration of enzyme at equilibrium should vary linearly

Table II: Calculated Parameters for the Adsorption of Carboxylester Lipase to Mixed Lipid–Buffer Interfaces

non-phospholipid				maximal adsorption [b]
species	mole fraction	F_3[a]	K_d[b] (nM)	(pmol/cm^2)
DA	0.55		94 (±40)[c]	0.25 (±0.03)[c]
DA	0.80	0.38	669 (±116)	3.3 (±0.34)
DA	0.90	0.67	447 (±33)	5.0 (±0.19)
DO	0.50	0.38	651 (±23)	2.5 (±0.07)
DO	0.72	0.67	399 (±65)	4.1 (±0.44)

[a] Fraction of area occupied by non-phospholipid as defined in text. [b] Subphase was 10 mM potassium phosphate, pH 6.6, 24 °C, containing 0.10 M (DO) or 2.00 M (DA) NaCl. Adsorption data (Figure 4) were fitted to the Langmiur adsorption isotherm. [c] Standard deviation.

with the fraction of surface area occupied by uncomplexed DA or DO, F_3. At compositions between that of the complex, X_c, and pure DO or DA, the value of F_3 can be calculated at any total mole fraction of DA or DO, X_3, by using the partial molecular area of DO or DA, ω_3, and that of its complex with POPC, ω_c. Specifically

$$F_3 = (X_3 - X_c)\omega_3/[(1 - X_3)(1 - X_c)\omega_c + (X_3 - X_c)\omega_3]$$

As shown in Figure 5b, plotting the adsorption data as a function of F_3 does not linearize it. It does, however, make the data sets nearly superimposable. Given the difference in complex compositions, 0.25 for DO (Smaby & Brockman, 1985) vs 0.65 for DA, this result suggests the importance of geometric factors in regulating enzyme adsorption.

The prediction of simple linearity for Figure 5b was based on the assumption that enzyme could adsorb to all surface area occupied by uncomplexed DO or DA and that the affinity of enzyme for those surfaces was unaltered by the presence of complex. To determine if those criteria were fulfilled, the enzyme concentration dependence of adsorption was measured at area fractions of uncomplexed DA or DO of 0.38 and 0.67. As shown in Figure 4, the curves appear to be approaching saturation. Fitting the data to the Langmuir adsorption isotherm yields the dissociation constants and maximal adsorptions listed in Table II. At both area fractions and with both lipids, the dissociation constants are 10–20-fold higher than those determined with DA or DO alone (Tsujita & Brockman, 1987). Because of these high values, saturation was not experimentally achieved and, therefore, the maximal adsorption values are unreliable. In any case, the data show clearly that the presence of complexes has a pronounced effect on interaction between carboxylester lipase and uncomplexed lipid.

Discussion

In earlier studies of lipid–lipid interactions in surface films, the formation of preferred packing arrays was observed (Smaby & Brockman, 1985, 1987a,b). It appeared that the driving force for complex formation resides primarily in polar group interactions, and this conclusion is supported by comparison of the physical behavior of DA and oleic acid in mixtures with POPC. The two fatty acids differ in chain length, number of double bonds, and position of double bonds. However, comparison of complex compositions and partial molecular areas for the two species shows, within error, no differences. Also, for both DA-containing mixtures at pH 6.6, high salt had minimal effect on physical behavior (Figures 2 and 3). This suggests that charge interactions between fatty acid and the phosphocholine group are not major determinants of complex composition. That they are not is consistent with the values of physical parameters for fatty acids being part of a series with other, uncharged lipids. Thus, overall, the physical studies show the suitability of DA–POPC films as substitutes for the less stable oleic acid–POPC films. As such, they could be used with other oleyl and oleoyl-containing lipids to examine the role of complex formation on the interaction of carboxylester lipase with surfaces.

First to be examined was the effect of complexation alone on enzyme adsorption. This revealed a lack of interaction, both under kinetic and equilibrium conditions. The areas of the complexes, expressed as the apparent areas of POPC, ranged from about 65 to 100 Å^2 (Smaby & Brockman, 1985) compared to 52–54 Å^2 for POPC alone (Figure 3). If the fraction of surface area not occupied by the phosphocholine group were the determining factor, then the rates of binding (Table I) should have been at least 17–46% of that to uncomplexed lipid. Likewise, for the equilibrium binding measurements with DA–POPC complex (Figure 4), maximal adsorption should have been 40–50% of an enzyme monolayer, not 5%. Additionally, control experiments showed that surface pressure itself could not explain the lack of binding. Thus, in complexes POPC does not inhibit binding merely by occupying a fraction of the surface.

The mechanism by which complexation inhibits enzyme adsorption could be approached by measuring adsorption to complexes and to POPC at very large molecular areas. However, the thermodynamic properties of such surfaces are not as well-defined as those for films at collapse, and the high free energy of such surfaces could make denaturation a major experimental problem. An alternative was to address the role of complexes in regulating adsorption of enzyme to uncomplexed lipid. By employing films of uncomplexed DA or DO in mixtures with its POPC-containing complex, it was possible both to have thermodynamically well-defined films and to minimize enzyme denaturation. When enzyme concentrations close to saturating for pure DO or DA films were used, equilibrium measurements of adsorption showed negative deviations from the linearity predicted from compositional considerations (Figure 5a). Nonlinear behavior was also observed when the data were replotted with respect to the fraction of surface area occupied by uncomplexed DA or DO. Surprisingly, this made the data sets nearly superimposable, emphasizing the importance of geometric factors.

As noted in the preceding paper, pH 6.6 is close to the point at which enzyme adsorption is very ionization dependent (Tsujita & Brockman, 1987). Conceivably, as pure DA was progressively diluted by complexes containing POPC, fatty acid ionization could increase (Patil et al., 1975). This could inhibit binding by affecting the dissociation constant for adsorption. The near identity of the normalized data obtained with DA and DO (Figure 5b) shows, however, that composition-dependent changes in ionization, like those in surface tension, are not a major factor in the inhibition. The source of the nonlinearity was indicated by measuring the concentration dependence of adsorption. At 0.38 or 0.67 area fraction of uncomplexed DA or DO, the apparent dissociation constants obtained (Table II) were more than an order of magnitude greater than those obtained with pure DO or DA films. Maximal adsorption values were unreliable because the surfaces could not be saturated; however, the approximate values were much closer to predicted than the dissociation constants. Thus, at intermediate compositions, the presence of complexes decreases primarily the affinity of the interaction between the enzyme and uncomplexed DA or DO.

The mechanism by which this could occur is suggested by the work of Stankowski (1983a,b). It addresses in a generalized way the adsorption of large ligands, like proteins, onto surfaces comprised of an array of much smaller adsorption

or receptor sites. Initially, adsorption proceeds as would be expected from simple mass action models of interaction, such as the Langmuir adsorption isotherm. However, as surface coverage by enzyme increases, adsorption falls short of expected values. This occurs because the presence of a large ligand on the surface effectively blocks the approach of subsequent molecules into an area considerably larger than itself. Because this "excluded area" effect is on the association reaction only and because all unoccupied surface can potentially be occupied, the effect is manifested by changes in the apparent affinity of the enzyme for the surface. Simply, as surface coverage by ligand increases, the dissociation constant for the interaction progressively increases. In the binding of carboxylester lipase to pure DA or DO films apparent agreement with the Langmuir model was observed (Tsujita & Brockman, 1987). That this can occur, even if the above model applies, has been noted for the binding of mellitin to phospholipid vesicles (Standkowski, 1983b). However, the parameters obtained with the simple saturation model underestimate the affinity of the lipid–protein interaction. Thus, the true dissociation constants are probably lower than the reported values.

For mixed surfaces the system is much more complex, but similar concepts may apply. As shown by analysis of surface pressure–composition phase diagrams, complex is completely miscible with uncomplexed DO or DA. Not revealed is the size of complex domains. They may be as small as individual POPC molecules, or they may be comprised of several hundred molecules without behaving as a separate phase [e.g., Mabrey and Sturtevant (1977)]. Within this size range complex domains can be considered analogously to previously adsorbed proteins. If carboxylester lipase cannot adsorb to an area of surface occupied by complex and the excluded area around it, then the adsorption reaction will be slowed. As noted above, this has the result of increasing the apparent dissociation constant. As shown in Table II, the apparent dissociation constants obtained at 0.38 area fraction of uncomplexed lipid are higher than those obtained at 0.67. Moreover, the values of the apparent constants are approximately the same at the same area fraction of coverage by complex. This further emphasizes the geometric nature of the inhibition and suggests similar domain sizes for POPC–DA and POPC–DO complexes. Given the geometric nature of the inhibition, it is possible that lack of binding to complexes and reduced binding to uncomplexed lipid in the presence of complex are reflections of the same phenomenon.

Potentially, the binding data can be analyzed quantitatively to provide information about the sizes of complex domains. Unfortunately, the degree to which adsorption affinity is affected is highly shape, as well as size, dependent (Stankowski, 1983b). Hence, a more detailed analysis of the data must await independent measurement of either parameter or more accurate and extensive data describing the binding of proteins of different sizes. Even if the proposed mechanism is incorrect or insufficient, the data clearly show that binding is inhibited by the presence of complexed ligand. Together with the virtually complete absence of adsorption to complexes alone, the results demonstrate the importance of lipid–lipid interactions within a single phase in regulating the surface concentration of this lipolytic enzyme.

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