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Regulation of Carboxylester Lipase Adsorption to Surfaces. 1. Chemical Specificity†

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ABSTRACT: The chemical specificity of the adsorption of porcine pancreatic carboxyl ester lipase to pure lipid surfaces was examined. Adsorption of native and catalytically inactivated enzyme was measured at the argon–buffer interface by using lipid films near the point of collapse. Protein adsorbed readily to films of triolein, 1,3-diolein, methyl oleate, oleonitrile, oleyl alcohol, and 13,16-docosadienoic acid. However, recovery of enzyme activity was variable. These differences and the changes in surface pressure accompanying adsorption indicated the occurrence of enzyme denaturation at the interface. Denaturation was controlled largely by surface free energy but showed some chemical specificity at high surface pressures. Adsorption of protein to the lipids was comparable when measured under either equilibrium or initial rate conditions. Together with surface pressure changes that accompany adsorption, the data indicate a relative lack of specificity for the enzyme–surface interaction. Adsorption to 13,16-docosadienoic acid and 1,3-diolein obeyed the Langmuir adsorption isotherm. Dissociation constants ranged from 10 to 50 nM, depending on enzyme form, ionic strength, and pH. With both lipids, a monolayer of enzyme was adsorbed at saturation. In contrast to these results, adsorption of enzyme activity and protein to films of 1-palmitoyl-2-oleoylphosphatidylcholine was ≤5% of that observed with the other lipids under all conditions. Comparison of rate constants for adsorption to 13,16-docosadienoic and 1,3-diolein as a function of subphase pH indicated a marked dependence on the ionization state of the fatty acid. Overall, the data suggest that the presence of zwitterionic and anionic lipids may regulate the interaction of the enzyme with substrate-containing surfaces in vivo.

Lipolytic enzymes are a class of esterases that exhibit a high activity toward water-insoluble lipids. In contrast to their substrates, many lipases and phospholipases are well-behaved, water-soluble proteins. Thus, for catalysis to occur it is necessary that enzyme and substrate partition to a common phase. As recently reviewed (Brockman, 1984), the site of lipolysis is the interphase that exists between bulk lipid and water phases. Previous studies with lipases and other proteins have shown that both the rate and extent of protein adsorption from aqueous phases to interphases, as well as denaturation at interfaces, are in some way regulated by the physical properties of the surface phase. Taken together, those physical properties of interphases such as charge, lipid composition, and lipid-packing density have been termed the "quality" of the interface (Verger, 1980).

Although it is reasonable to postulate that changes in surface quality accompany changes in lipid packing or composition, it has been difficult to quantitatively apply the concept to understanding the regulation of protein adsorption to lipid–water interfaces. In large part, this difficulty has arisen from lack of an adequate model to describe the structure of such interfaces. Recently, however, it has been shown that monomolecular lipid films at the point of collapse to a bulk or bilayer phase display regular behavior with respect to the area of each lipid species as well as interfacial tension (Smaby & Brockman, 1984). Analysis of this behavior has yielded an equation of state that describes the surface in terms of lipid–water "building blocks". In one of these studies, the properties of a series of lipids containing exclusively oleoyl or oleyl

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moieties in mixtures with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)[1] were examined (Smaby & Brockman, 1985). Comparison of the area and hydration parameters for the pure, non-POPC lipids revealed the dominance of the aliphatic chain in regulating their physical behavior.

The thermodynamic regularity of the behavior of the non-POPC lipids at film collapse provides an ideal basis for studying the specificity of interaction of water-soluble proteins and other surfactants with interfaces. Using such surfaces, it should be possible to discern to what extent differences in adsorption behavior are related to the chemical nature of the lipid polar groups, the interfacial tension, and the hydration of the species. In this study, the adsorption of porcine pancreatic carboxylester lipase to such surfaces and to POPC was characterized. In contrast to previous work, the results for non-POPC lipids indicate a relative lack of head-group specificity. They also reveal denaturation of the protein that is regulated primarily by interfacial tension.

Materials and Methods

Reagents. [1,3-^{3}H]Diisopropyl fluorophosphate, [*oleoyl*-1-^{14}C]-1-palmitoyl-2-oleoylphosphatidylcholine, and [^{32}P]-phosphoric acid were obtained from New England Nuclear (Boston, MA). Triolein, 1,3-diolein, oleic acid, 13,16-docosadienoic acid (DA), oleonitrile, oleyl alcohol, and methyl oleate were from NuChek Prep (Elysian, MN) and were shown to be ≥99% pure by thin-layer chromatography. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) from Avanti Biochemicals (Birmingham, AL) was also ≥99% pure. Diisopropyl fluorophosphate, *p*-nitrophenyl butyrate, and *p*-nitrophenol were from Sigma (St. Louis, MO), and Brij-35 detergent was from Pierce Chemical Co. (Rockford, IL). Hydrophobic paper for surface collection (Type 1PS) was from Whatman, Inc. (Clifton, NJ) and was cut into 6 cm diameter circles that were washed with chloroform/methanol (2:1 v/v), methanol, and petroleum ether. After air-drying the circles were dried at 110 °C for 30 min. Chloroform and methanol were redistilled before use, and petroleum ether (bp 65–66 °C) was purified (Smaby & Brockman, 1981) and shown to be free of surface-active impurities (Smaby et al., 1983) as previously described. Water for studies involving lipid films was purified by reverse osmosis, ion exchange, charcoal adsorption, and filtration at 0.2 μm. The monomeric form of pancreatic carboxylester lipase (cholesterol esterase EC 3.1.1.13, M_r 74000) was purified to homogeneity from porcine pancreas (Rudd et al., 1987). All other chemicals were of reagent grade and used without further purification.

Methods. The concentration of active carboxylester lipase was determined from the rate of release of *p*-nitrophenol from *p*-nitrophenyl butyrate by a modification of the procedure described by Shirai and Jackson (1982). The assay mixture consisted of 2.94 mL of 50 mM sodium phosphate buffer, pH 7.25, containing 0.1 M NaCl and 0.1% Brij-35, 30 μL of acetonitrile containing 1.5 μmol of *p*-nitrophenyl butyrate, and 30 μL of enzyme solution. Relative protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA), using bovine serum albumin as a standard. Protein concentrations were converted to a dry weight of carboxylester lipase by multiplying by 1.75 (Rudd et al., 1987). By use of these assays for protein and activity, the enzyme preparations were found to have specific activities between 900 and 1100 μmol of *p*-nitrophenol released min^{-1} (mg of protein)$^{-1}$. The amount of native enzyme protein recovered with lipid films was in some instances determined fluorometrically by the method of Benson and Hare (1975) modified as previously described (Bhat & Brockman, 1981).

Radiolabeled, catalytically inactive carboxylester lipase was prepared by treating native enzyme with [^{3}H]diisopropyl fluorophosphate. To purified enzyme (4.1 mg in 2.4 mL of 50 mM piperizine–acetate buffer, pH 6.6) was added 120 μL of isopropyl alcohol containing 114 nmol of [^{3}H]diisopropyl fluorophosphate at a specific activity of 220 Ci/mol. After a 1-h incubation at 25 °C, an additional aliquot of unlabeled reagent was added to remove traces of activity remaining. After an additional hour, unreacted reagent was removed by chromatography on a 1.5 × 22 cm diameter column of Sephadex G-25 equilibrated with 10 mM potassium phosphate buffer, pH 6.6, containing 0.1 M NaCl. The specific catalytic activity of the labeled protein was $\leq 3 \times 10^{-5}$ that of native enzyme. Incorporation of diisopropyl fluorophosphate was 0.76 mol/74000 g of protein, consistent with previous chemical titration data (Rudd et al., 1987).

Surface pressure–area isotherms were determined by using a fully automated Langmuir film balance (Brockman et al., 1980, 1984). All films were spread from 51.67 μL of petroleum ether onto a subphase of 10 mM potassium phosphate buffer, pH 6.6, containing 0.1 or 2.0 M NaCl as required. The atmosphere was humidified argon. After standing at a large molecular area for 4 min, the films were compressed at ≤5 Å^2 min^{-1} molecule^{-1}. Phase-transition areas and surface pressures were identified by using second and third derivatives, as previously described (Brockman et al., 1980).

For studies of protein adsorption, surface tension and area were controlled by a multiprocessor, interfacial monitor/controller. The surface tension of lipid films was measured by the Wilhelmy method using a Cahn Model 27 microbalance from which was suspended 24 gauge diameter nichrome wire. The wire was calibrated by using solvents of known surface tension, and measurements were accurate to within ±0.1 mN/m. The aqueous subphase was contained in a brass–Teflon laminated trough with one circular (diameter = 5.6 cm) and one rectangular (1.78 cm × 12.5 cm) compartment connected by a shallow channel. Temperature control was achieved by mounting the trough on a thermostated base plate. Surface area was controlled by a movable Teflon barrier connected to a linear transport driven by a microprocessor-controlled stepper motor. Positioning of the barrier was controllable within 12.5 μm, and movement was by microsteps of 0.2 μm to minimize surface vibrations. Positioning of the microbalance and Wilhelmy wire was similarly controlled. Stirring of the circular aqueous compartment was achieved with a magnetic bar driven by a magnet connected to a computer-controlled stepper motor. In this manner, stirring speed was precisely controllable between 0.01 and 60.00 rpm with ramping of speed to avoid surface disruption. Monitoring and control of these components were through a distribution processor connected to a user interface. Overall, the instrument provided real-time component control as well as data acquisition, manipulation, display, and storage.

All lipid dissolution and protein adsorption measurements in this study were made at 24 °C in an atmosphere of humidified argon. The argon–buffer interface was cleaned by compression and aspiration, reducing the volume of liquid in the circular compartment to 27 mL. To form lipid films of known surface area and pressure, an aliquot of lipid in petroleum ether was spread over the cleaned aqueous surface in both compartments, the solvent was allowed to evaporate

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

for 3–4 min, and the barrier was moved slowly toward the center of the channel. If necessary, small amounts of lipid were removed by aspiration so that the barrier was centered in the channel when the desired pressure was achieved. Unless otherwise noted, each film was formed at a surface pressure 1–4 mN/m below its dynamically measured collapse pressure. This was necessitated by the instability of the films at the collapse pressure itself.

For measuring film dissolution, the stirring speed was ramped linearly to 60 rpm over 1 min after which surface tension was monitored as a function of time. Surface pressures were empirically converted to surface concentrations by using the appropriate surface pressure–area isotherms, and concentration vs time data were plotted semilogarithmically. The maximal rate constant for dissolution was defined as the slope near zero time. For measuring enzyme–surface interactions, stirring of the aqueous subphase under the lipid film was ramped to 60 rpm and 10–20 μL of subphase buffer containing 3–4 μCi of [^{32}P]phosphate was introduced from a microsyringe through a small port in the side of the circular compartment. One minute later an aliquot of enzyme solution was added in the same manner. After the desired time of interaction with the surface, during which surface tension was recorded, the Wilhelmy wire was removed and the film collected using hydrophobic paper as previously described (Bhat & Brockman, 1981). Aliquots of the subphase were also collected for determination of active enzyme or tritiated enzyme and for ^{32}P determination. It was empirically determined that 10 min was sufficient for equilibration of binding to DA films at enzyme concentrations ≥25 nM, and 30 min was sufficient below this level.

Active enzyme adsorbed to the recovered film was eluted by swirling the hydrophobic paper gently in a Petri dish containing 5.0 mL of 10 mM potassium phosphate buffer, pH 6.6, containing 0.15% Brij-35. As noted earlier (Bhat & Brockman, 1981), this achieves quantitative removal of the enzyme from the paper, which is then removed from the dish. Aliquots of the resulting solution were assayed for active enzyme concentration or protein concentration (fluorometric assay) and subphase carry-over (see below). To measure adsorption of [^{3}H]diisopropylphosphoryl enzyme following film recovery, the paper was not eluted but cut into pieces and radioactivity determined by liquid scintillation counting. By use of the measured concentrations of ^{32}P and active or tritiated enzyme in the subphase, enzyme and protein adsorption data were corrected for carry-over of aqueous phase on the hydrophobic paper. Typical subphase carry-over was 1–3 μL/cm^2 of surface. Film recovery was calibrated in separate experiments using [*oleoyl*-1-^{14}C]POPC at surface pressures from 30 to 45 mN/m. It was found to be 0.92 ± 0.03 of lipid added, and all adsorption measurements reported were corrected to 100% recovery by using this value.

Results

Earlier studies of the interaction of pancreatic carboxylester lipase with lipid–water interfaces showed an apparent affinity of the enzyme for surfaces containing oleic acid. For the most part, these studies were conducted at surface pressures far below the collapse pressure of the lipid films. This was necessitated by the instability of oleic acid films at higher pressures. For example, at a surface pressure of 37 mN/m the initial rate of decay of surface pressure is 2.3 mN m^{-1} min^{-1} on a subphase of 10 mM potassium phosphate at pH 6.6 containing 0.1 M NaCl. This instability at high surface pressures and near neutral pH is a result of film dissolution into the aqueous phase (Patil et al., 1973). In order to correlate

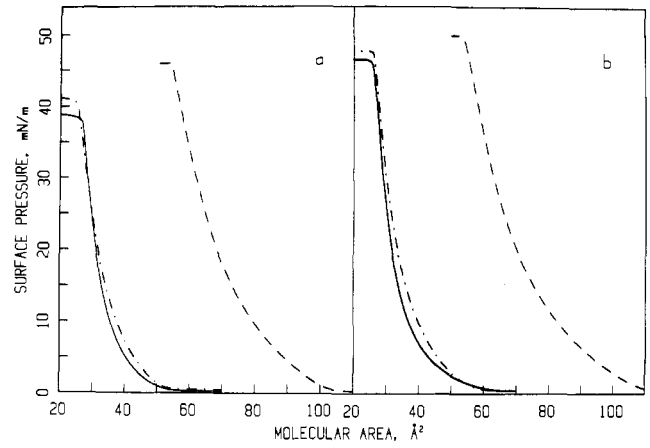

FIGURE 1: Surface pressure–area isotherms for 13,16-docosadienoic acid (—), oleic acid (–·–), and 1-palmitoyl-2-oleoylphosphatidylcholine (– – –). The subphase was 10 mM potassium phosphate buffer, pH 6.6, 24 °C, containing (a) 0.10 M NaCl and (b) 2.00 M NaCl.

enzyme adsorption with surface structure, however, measurements must be made at or near the collapse pressures of the films. Because dissolution involves hydration of the hydrocarbon moiety, its rate is sensitive to the nature and concentration of ionic species present (Patil et al., 1973). For example, at surface pressures near collapse, increasing the sodium chloride concentration in the stirred aqueous subphase from 0.1 to 2.0 M reduced the dissolution rate constant for oleic acid from 4×10^{-4} to 7×10^{-5} s^{-1}. Although film stability was improved by high salt concentration, the surface pressure still declined at an initial rate of about 0.5 mN m^{-1} min^{-1}. In an effort to achieve greater film stability, the dissolution rates of several free fatty acids with longer aliphatic chains were measured. The most satisfactory was DA, which at pressures near collapse had dissolution rate constants of 2×10^{-5} and 7×10^{-6} s^{-1} on subphases of 10 mM potassium phosphate at pH 6.6 containing 0.1 and 2.0 M sodium chloride. The latter rate constant corresponds to an initial rate of surface pressure decrease of less than 0.1 mN m^{-1} min^{-1}. As shown by the surface pressure–area isotherms in Figure 1 the surface pressure–area characteristics of DA and oleic acid are similar on both 0.10 and 2.00 M salt. For both fatty acids the main effect of increasing salt is to increase the collapse pressure by 7–8 mN/m (compare parts a and b of Figure 1). Thus, the physical properties of DA and oleic acid are qualitatively the same and quantitatively similar at both high and low ionic strength. These similarities suggest that DA should be a reasonable model for oleic acid but form more stable films at pressures near its collapse.

The ability of pancreatic carboxylester lipase to interact with films of DA on 0.1 M NaCl was determined by measuring its surface concentration as a function of its concentration in the aqueous phase. As shown in Figure 2, the surface becomes saturated with enzyme. Fitting the data to the Langmuir adsorption isotherm (Adamson, 1967) gives a dissociation constant of 13 nM and a maximal surface concentration of 4.2 pmol/cm^2 (Table I). With oleic acid at a much lower surface pressure, a maximal adsorption of 3.5 pmol/cm^2 was previously observed (Bhat & Brockman, 1981). As noted, this level of adsorption corresponds, within error, to the formation of a monolayer of native enzyme at the interface. Thus, in spite of the substitution of DA for oleic acid and the large increase in initial surface pressure compared to earlier experiments, the interaction of the enzyme with the surface was minimally altered. An adsorption isotherm was also measured on a subphase containing 2.0 M NaCl (Figure 2). Saturation

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

lipid	initial surface pressure (mN/m)	subphase pH	subphase [NaCl]	enzyme	K_d (nM)	maximal absorption (pmol/cm^2)
DA	36 (±0.50)[a]	6.6	0.10	native	13 (±1.8)[a]	4.2 (±0.17)[a]
DA	41 (±0.37)	6.6	2.00	native	52 (±2.5)	3.9 (±0.08)
DA	38 (±0.21)	5.6	2.00	native	12 (±1.7)	4.1 (±0.15)
DA	40 (±0.33)	6.6	2.00	^{3}H	13 (±1.4)	3.5 (±0.08)
1,3-diolein	27 (±0.35)	6.6	0.10	^{3}H	18 (±2.6)	4.2 (±0.27)
POPC	44 (±0.21)	6.6	2.00	native	72 (±8.7)	0.2 (±0.01)

[a] Standard deviation.

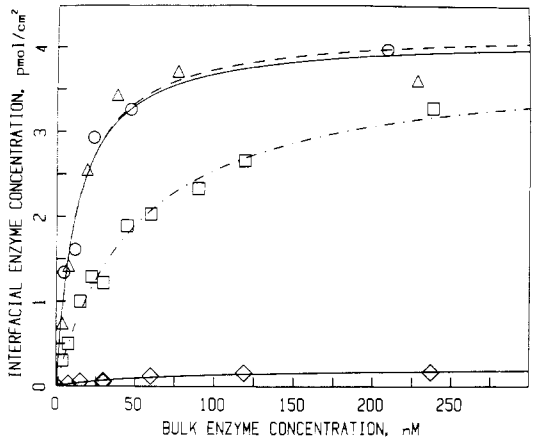

FIGURE 2: Concentration dependence of carboxylester lipase adsorption to lipid films. Subphases contained 10 mM potassium phosphate buffer at 24 °C, and initial surface pressures are given in Table I. (○) DA, subphase pH 6.6 with 0.10 M NaCl; (□) DA, subphase pH 6.6 with 2.00 M NaCl; (△) DA, subphase pH 5.6 with 2.00 M NaCl; (◇) POPC, subphase pH 6.6 with 2.00 M NaCl. Theoretical curves were generated by fitting the data to the Langmuir adsorption isotherm.

was again observed with a higher dissociation constant but a comparable maximal binding (Table I). Thus, the presence of high salt that stabilizes the films affects primarily the dissociation constant for the interaction. One difference noted between these and earlier results is that the surface pressure did not increase upon the addition of enzyme; in fact, it decreased slightly.

As with fatty acid films, the main effect of 2.00 M salt on the surface properties of POPC is to increase its collapse pressure from 45.9 to 49.7 mN/m (Figure 1). In contrast to its behavior toward oleic acid, adsorption to POPC films at low surface pressure and ionic strength was minimal (Bhat & Brockman, 1981). As shown in Figure 2, comparable results are obtained for adsorption to POPC on a subphase containing 2.0 M NaCl. The dissociation constant determined from the data was similar to that for binding to DA on the same medium, but the saturation adsorption was less than 5% of a monolayer (Table I). The change in surface pressure during the adsorption period was negligible.

Because oleic acid (Smaby & Brockman, 1985) and DA (Figure 1 and Tsujita et al., 1987) have been shown to exhibit physical behavior at interfaces similar to that for a number of other lipids containing *cis*-9-octadecenyl aliphatic chains, it was of interest to determine if enzyme would bind to other members of the series. Of those lipids listed in Table II, only DA, oleyl alcohol, oleonitrile, and the lipid-free surface are not potential substrates for the enzyme. At a concentration of 220 nM carboxylester lipase in the aqueous phase, a surface concentration of 3.3 pmol/cm^2 was achieved for adsorption to DA. In contrast, adsorption to oleyl alcohol was only 0.9 pmol/cm^2, and no measurable enzyme activity was associated with either a film of oleonitrile or the argon–buffer interface (Table II). These latter results were surprising because the

Table II: Equilibrium Adsorption of Carboxylester Lipase to Lipid–Buffer Interfaces

lipid	initial surface pressure (mN/m)	native enzyme[a] (pmol/cm^2) activity	native enzyme[a] (pmol/cm^2) protein	[^{3}H]diisopropylphosphoryl enzyme[a] (pmol/cm^2)
DA	38	3.3	3.2	3.4
oleyl alcohol	30	0.9		2.6
1,3-diolein	27			3.1
methyl oleate	15			2.5
oleonitrile	14	0.0	2.4	2.3
triolein	12			2.9
none	0	0.0		2.8

[a] Bulk [enzyme] = 220 nM; the subphase was 10 mM potassium phosphate, pH 6.6, 24 °C, 0.10 M NaCl.

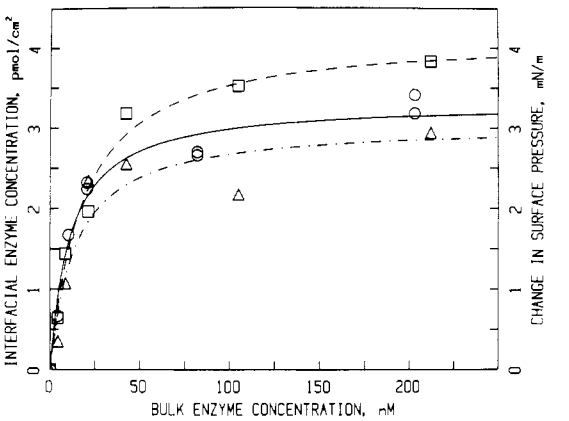

FIGURE 3: Concentration dependence of [^{3}H]diisopropylfluorophosphoryl carboxylester lipase adsorption to lipid films. Subphases contained 10 mM potassium phosphate, pH 6.6, 24 °C, and initial surface pressures are given in Table I. (○) DA on 2.00 M NaCl; (□) diolein on 0.10 M NaCl; (△) surface pressure changes vs enzyme concentration for adsorption to diolein. Theoretical curves were generated by fitting the data to the Langmuir adsorption isotherm.

introduction of enzyme into the subphase under films of oleonitrile and no lipid increased the surface pressure 9.5 and 10.7 mN/m, respectively. This suggested that enzyme adsorption was occurring but was accompanied by rapid enzyme denaturation either at the interface or during the film isolation procedure. To examine this possibility, the experiment was repeated with DA and oleonitrile films, but the protein recovered from the interface was quantitated by using a chemical (fluorometric) assay rather than by measuring its catalytic activity. This revealed (Table II) protein adsorption to DA comparable to that measured by enzyme activity. Adsorption of protein to the oleonitrile film was about two-thirds of that to the DA film, in sharp contrast to the activity measurements. Thus, surface denaturation can occur within the time course of our experiments and isolation procedure.

A limitation to measuring adsorption specificity with native enzyme is the requirement for nonsubstrate lipids. To overcome this, the adsorption of catalytically inactivated enzyme

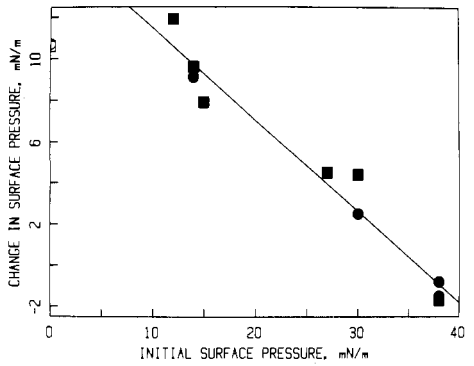

FIGURE 4: Change in surface pressure vs initial surface pressure for adsorption of carboxylester lipase to lipid films. The data were fitted to a straight line by using a least-squares algorithm. Experimental conditions are described in the legend to Table II. (●) Native enzyme; (■) [^{3}H]diisopropylphosphoryl enzyme; open symbols indicate data obtained in the absence of lipid, which were excluded from the least-squares line.

was measured. Inactivation was achieved with [^{3}H]diisopropyl fluorophosphate, which modifies one amino acid residue per enzyme molecule (Rudd et al., 1987). As shown in Figure 3, the modified enzyme readily adsorbs to DA films on 2.0 M NaCl with characteristics comparable to those for the interaction of native enzyme with DA at lower ionic strength (Table I). At 220 nM, a bulk concentration sufficient to nearly saturate a DA film, adsorption of this modified species to POPC on 2.0 M NaCl was only 0.3 pmol/cm^2. Thus, qualitatively, the modified enzyme exhibits the same characteristics as the native enzyme. The adsorption of the inactivated enzyme at 220 nM to both substrate and nonsubstrate lipid films is shown in Table II. With DA, adsorption is comparable to that observed with native enzyme, approaching monolayer coverage of the surface. Adsorption to the non-POPC surfaces was in all cases greater than half monolayer coverage, suggesting a relative lack of specificity for the interaction.

The interaction of any surfactant with a given lipid may alter the surface pressure. If the surfactant concentration is kept constant in the aqueous phase, a plot of the change in surface pressure induced by surfactant versus the initial surface pressure of the lipid film is often linear [e.g., Bougis et al. (1981)]. For all the adsorption experiments indicated in Table II, the surfactant (enzyme) concentration was constant at 220 nM, but the chemical nature of the lipid species and, hence, the initial surface pressure varied (Table II). In spite of the use of different lipids, however, all the surface pressure changes associated with the measurements shown in Table II, with the exception of the no-lipid control, define a reasonably straight line (Figure 4) when plotted as described above. This linearity is consistent with the lack of specificity indicated by direct binding measurements and supports the applicability of the modified enzyme as a model for the native enzyme.

To better understand the relationship between specificity of adsorption of native enzyme and its surface denaturation, we compared surface concentrations of native and modified enzyme under conditions where adsorption is not equilibrated as above but is linear with time. Limiting total adsorption to about 5% of monolayer coverage should emphasize specificity differences in the adsorption step. Also, denaturation should not be impeded by neighboring protein molecules. As shown in Table III for DA and 1,3-diolein films near collapse, measurements of adsorbed activity and protein were comparable. With oleoyl alcohol, recovered activity was about one-third of recovered protein, and in all other cases, except for POPC, activity was only 5–10% of protein adsorbed. For POPC films, adsorption of both active enzyme and protein was

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

lipid	initial surface pressure (mN/m)	apparent rate constant[a] × 10^5 (cm/s) native	apparent rate constant[a] × 10^5 (cm/s) [^{3}H]diisopropylphosphoryl
POPC	42	0.47	0.35
DA	38	6.30	5.90
oleyl alcohol	30	2.00	5.70
1,3-diolein	27	6.70	6.10
methyl oleate	15	0.33	6.40
oleonitrile	14	0.17	6.40
triolein	12	0.47	6.40
none	0	0.17	5.80
POPC	10	0.50	1.90
DA	10	0.44	5.80
1,3-diolein	10	1.60	6.70

[a] Bulk [enzyme] = 6.0 nM; the subphase was 10 mM potassium phosphate, pH 6.6, 24 °C, 0.10 M NaCl.

only 5–10% of protein adsorbed to the non-phospholipids. During these experiments surface pressures changed by ≤2 mN/m. Extending the incubation time from 10 to 60 min did not alter protein adsorption with POPC but did increase it 4–6-fold for the other films (data not shown). Hence, at surface pressures near collapse the enzyme does not adsorb to or denature on POPC films, adsorbs in an undenatured state to DA and possibly 1,3-diolein films, but appears to partially or totally denature upon interaction with other films or a surface containing no lipid.

To help understand the role of surface pressure in regulating denaturation, adsorption to DA, 1,3-diolein, and POPC films was also measured at 10 mN/m, a surface pressure far below collapse for these lipids. As shown in Table II, adsorption to POPC was low, measured either as activity or protein. In contrast, protein adsorption to DA and diolein films was the same as at higher pressures, but recovered activity was comparable to that observed with lipids exhibiting lower collapse pressures. Thus, surface pressure is a major determinant of surface denaturation. Comparison of results for DA and diolein with those for POPC at both high and low surface pressures (Table III) suggests that adsorption of native enzyme to the surface must precede its denaturation.

The data in Tables II and III indicate that both native and modified enzymes adsorb to films of diolein at levels comparable to those with DA films. Figure 3 shows that this adsorption, measured with the modified enzyme, is saturable with a K_d of 18 nM and a maximal adsorption of 4.2 pmol/cm^2. At saturation this adsorption is accompanied by increases in surface pressure of up to about 3 mN/m. Under such conditions, if structural perturbation of the surface is minimal, the change in surface pressure should be proportional to the surface excess of enzyme present. If so, a plot of surface pressure vs bulk enzyme concentration should exhibit saturation behavior from which the enzyme–surface dissociation constant can be calculated (Pethica, 1955). This was the case for the adsorption of carboxylester lipase to diolein, and the data are shown in Figure 3. The calculated dissociation constant was 12 nM.

An earlier study of the interaction of pancreatic carboxylester lipase with oleic acid at low surface pressures suggested the involvement of two or more ionizable groups in the lipid–protein interaction (Bhat & Brockman, 1982). Not addressed by that data was whether one of the groups might be the fatty acid which comprised the lipid film. With the observed similarities of modified and native enzyme, rates of adsorption to films of DA and diolein could be compared as a function

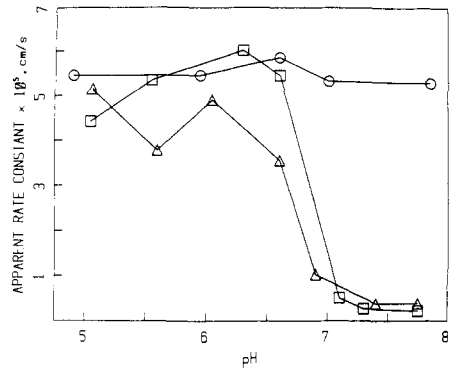

FIGURE 5: Rate of dependence on pH of adsorption of native and modified carboxylester lipase to lipid films. Subphase contained 10 mM potassium phosphate buffer and 0.10 M (diolein) or 2.00 M (DA) NaCl, 24 °C. Adsorption time 10 min for modified enzyme with diolein (○) and DA (□) and for native enzyme with DA (△).

of pH. As shown in Figure 5, the apparent rate constant for adsorption of modified enzyme to DA decreased to near zero between pH 6.5 and pH 7.0. In contrast, the rate of adsorption of the modified enzyme to diolein shows no pH dependency. That the difference was not due to modification of the enzyme is shown by comparable measurements of native enzyme binding to DA films. For the measurements shown in Figure 5, the lipid-packing density of the films was maintained at the value used at pH 6.6. For DA, but not diolein, this caused increases in the surface pressures to greater than 40 mN/m at pH values above 7. Because surface pressure, rather than ionization, could be inhibiting adsorption, binding of both native and modified enzyme to DA films was also measured at pH 7.6 but at 35–36 mN/m. Even though this pressure was slightly below the value at pH 6.6, results were comparable to those for pH 7.6 at higher surface pressure (Figure 5). Thus, it is the ionization state of the fatty acid that plays the determining role in enzyme–surface interaction. The ionization dependence revealed by these data also suggests that the 4-fold higher K_d for binding to DA on 2.0 M salt as compared to 0.1 M salt (Table I) may be a consequence of the increased ionization of the fatty acid expected at higher ionic strengths (Patil et al., 1975). An adsorption isotherm for native enzyme binding to DA on 2.0 M sodium chloride at pH 5.6 supports this hypothesis (Figure 2). The dissociation constant is reduced from 52 to 12 nM enzyme, while the maximal binding is not significantly changed (Table I).

DISCUSSION

To help understand how lipid physical state and chemical composition can regulate lipid–protein binding, the specificity of carboxylester lipase adsorption to thermodynamically well-characterized surfaces was examined. The surface properties of DA were found to be analogous to those of oleic acid, the only major difference being its rate of dissolution. The characterization of DA films was necessary if it were to be used in enzyme adsorption studies with other lipids containing oleyl and oleoyl moieties. Its similarities with oleic acid made it useful not only for those studies but, potentially, for any studies in which the relatively rapid dissolution of oleic acid is a detrimental factor.

The adsorption of carboxylester lipase to DA films at high surface pressures was analogous to earlier results obtained at low pressure (Bhat & Brockman, 1981). Not only was saturation of the surface observed but binding occurred over the same concentration range. The maximal adsorptions approached monolayer coverage (Table I), which, for a globular protein of M_r 74 000, is predicted to be 4.0 pmol/cm^2. Carboxylester lipase is readily inactivated by 1 mol of diisopropyl fluorophosphate (Rudd et al., 1987), suggesting a serine esterase type of mechanism. Because fatty acid is a substrate for the reverse of lipid hydrolysis catalyzed by the enzyme (Bhat & Brockman, 1981), adsorption to fatty acid films could be driven by the formation of an acyl-enzyme intermediate. However, this notion is dispelled by the adsorption characteristics of the diisopropyl fluorophosphate modified enzyme (Figure 3 and Table I). Additionally, the similarities in adsorption between native and modified enzyme support the postulated topological, as well as functional, separation of catalytic and surface binding sites on such enzymes.

The initial rate of binding to DA films was highly pH dependent, decreasing to near zero between pH 6.6 and 7.6 as a result of fatty acid ionization. A pH of 6.6 was used in binding studies because it is the optimum for enzyme activity (Bhat & Brockman, 1981). The difference between the dissociation constants measured at pH 6.6 on 2.0 M NaCl and those measured at 5.6 on 2.0 M NaCl or at 6.6 on 0.1 M NaCl (Table I) probably arise also from a difference in fatty acid ionization. If so, the kinetic and equilibrium measurements taken together would indicate that it is the affinity of the enzyme for the surface which is pH dependent. More extensive physical characterization of the pH dependence of DA films will be required to establish what changes in film structure occur. However, the pH range is that in which the fatty acid should be changing from the stable acid-anion to the fully ionized species (Small, 1986). Because fatty acids in the gut are generated by the action of carboxylester lipase and other lipases, their accumulation at pH values between 6 and 7 could be a regulatory mechanism for controlling enzyme distribution among substrate-containing particles.

Adsorption of the catalytically inactive form of the enzyme to diolein occurs to the same extent and with the same affinity as to DA (Table I). This argues against there being a chemically specific interaction between the enzyme and either lipid. Supporting this conclusion are the equilibrium and kinetic measurements of adsorption to surfaces comprised of other neutral lipids. Comparison of binding rates and extents is complicated by the rapid denaturation that occurs in some cases. However, if we assume that adsorption of native enzyme precedes its denaturation, the data indicate a lack of adsorption specificity. This is further supported by the linearity of the surface pressure changes that accompany binding to different lipids (Figure 4). Even with a single lipid, a change in the mode of adsorption can induce pronounced nonlinearity [e.g., Bougis et al. (1981)].

The pressure change with binding to the argon–buffer interface alone did not fall on the line with the other data (Figure 4), but protein binding was comparable to other surfaces. This may indicate a more extensive denaturation of the proteins or, possibly, some specificity with respect to denaturation. The latter possibility is also suggested by comparison of adsorption rates to oleyl alcohol (Table III) and oleic acid (Bhat & Brockman, 1982). In the earlier study the rate of adsorption of native enzyme to oleic acid films was highly dependent on surface pressure up to 30 mN/m, but between 30 and 40 mN/m, adsorption rates were constant. This suggests that minimal denaturation was occurring at the higher pressures. Under comparable conditions, however, low activity was recovered with oleyl alcohol films at 30 mN/m (Table III). The enzyme was also apparently stable with diolein at 27 mN/m (Table III), but some hydrolysis could have occurred, generating oleic acid. At saturating levels of modified enzyme the maximal adsorption to diolein of 4.2 pmol/cm^2 also sug-

gests enzyme stability. For fully denatured enzyme a maximal concentration of 1.2 pmol/cm^2 would be expected (Sobotka & Trurnit, 1961). Regardless of whether any real lipid specificity exists for denaturation, the data clearly show that surface pressure, i.e., free energy, is the dominant factor.

The instability of the enzyme at high-energy surfaces helps to explain several earlier observations. Indirectly, rapid denaturation might explain the reported lack of activity of the human carboxylester lipase toward emulsified olive oil and triolein in the absence of bile salts (Lombardo et al., 1980). It may also be a factor in the apparent lack of activity of the same enzyme toward medium-chain di- and triglycerides in monomolecular films (Lombardo et al., 1980). More directly, as noted above the apparent rate constant for adsorption of the porcine enzyme to oleic acid–cholesteryl oleate films, measured by recovered activity, undergoes a 30-fold increase between 10 and 30 mN/m (Bhat & Brockman, 1982). This increase is independent of film composition, suggesting that denaturation was responsible for the apparently low adsorption rate for native enzyme at low surface pressures. Also, the proportional dependence of the adsorption rate constant on the oleic acid content of oleic acid–methyl oleate films at 13 mN/m (Bhat & Brockman, 1982) may reflect a slower rate of denaturation on the fraction of surface covered with fatty acid, not specificity of adsorption. This notion is supported by the observation that, under similar conditions where enzyme adsorption is kinetically controlled, hydrolysis of methyl oleate at 15 mN/m by native enzyme is only a few percent in 10 min. In contrast, at higher surface pressure and with 33 mol % POPC present, hydrolysis is complete (T. Tsujita and H. Brockman, unpublished results). The minimal hydrolysis of methyl oleate at low pressure also supports the conclusion that denaturation occurs primarily during the adsorption phase, 600–1800 s, rather than during film collection, $\leq$10 s.

The molecules of DA and the neutral lipids were, by virtue of the chosen experimental conditions, in similar physical states. In contrast, POPC films are at higher surface pressures, exhibit smaller chain areas, and have possibly lower hydration than the non-phospholipids (Smaby & Brockman, 1985). However, these differences are probably not directly responsible for the lack of interaction of the enzyme with POPC. Even when the pressure was decreased to 10 mN/m, concomitantly increasing molecular area by about 50% (Figure 1) and, presumably, increasing the water content of the films (Gaines, 1978), low binding was observed compared to that to diolein or DA (Table III). This argues that the presence of the phosphocholine group blocks enzyme–surface interaction. In this sense the enzyme exhibits a marked specificity against binding to POPC. The accompanying paper (Tsujita et al., 1987) addresses this phenomenon with respect to lipid mixtures containing POPC.

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