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Lipid Hydrolyses Catalyzed by Pancreatic Cholesterol Esterase. Regulation by Substrate and Product Phase Distribution and Packing Density†

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ABSTRACT: The role of oleic acid in the regulation of the hydrolysis of cholesteryl oleate in lipid films at the air–buffer interface was investigated by using initial rate techniques. A small quantity of enzyme is rapidly adsorbed to substrate-containing films; however, a much greater, although slower, adsorption occurs if oleic acid is present. The rate constant for the slow adsorption is independent of the phase distribution of cholesteryl oleate but is markedly dependent upon both the concentration of oleic acid head groups and the acyl chain packing density in the film. Adsorption is controlled by two ionizable groups, one of which may be the carboxyl group of oleic acid. In contrast to adsorption, catalysis by the surface excess of enzyme is pH independent between 5.5 and 7.5 and is relatively specific for substrate in the monolayer phase. The second-order rate constants for the hydrolysis of cholesteryl oleate in the monolayer phase and the interfacial layer of the double-layer phase are 27 and 2 $cm^2\ s^{-1}\ fmol^{-1}$. These results indicate that adsorption and catalysis occur at functionally, if not physically, distinct sites on the protein. The adsorption of enzyme to a hydrolysis product, oleic acid, constitutes a form of product activation which presumably helps keep it at the interface during intraluminal fat digestion. The catalytic properties of the adsorbed enzyme suggest that substrate specificities determined for cholesterol esterase in complex reaction systems may largely reflect the availability of substrate in the appropriate physical state at the lipid–water interface.

Cholesteryl esters in tissues and serum reside primarily in bulk lipid deposits such as arterial lipid droplets [e.g., see Smith et al. (1967)] and lipoproteins [e.g., see Keim (1979)]. Based on physical studies in model systems (Janiak et al., 1974; Smaby & Brockman, 1981b), it is likely that a small fraction of cholesteryl esters are present in one of two surface phases at the lipid–water interface (Smaby & Brockman, 1981a). The hydrolysis of these esters is a prerequisite for cholesterol utilization in tissues [e.g., see Kritchevsky (1972)] as well as for its absorption in the intestine (Treadwell & Vahouny, 1968). Little is known, however, about the regulation of this reaction, particularly with regard to the interfacial structure.

In a recent study of the properties of the hydrolysis reaction, equilibrium could be achieved over only a relatively narrow range of pH (Bhat & Brockman, 1981). This kinetic deficiency reflects the properties of the water-soluble catalyst employed, porcine pancreatic cholesterol esterase (EC 3.1.1.13). However, it does not reveal if in this two-phase reaction system the lack of catalysis results from a separation of enzyme and substrate or catalytic inactivity of the protein. At the optimum pH, adsorption of enzyme to films consisting of products and reactants does occur and was shown to be a time-dependent, saturable process. In contrast to these results, the human pancreatic cholesterol esterase in the absence of bile salts exhibited negligible activity toward cholesteryl esters and other substrates in films at the air–water interface (Lombardo et al., 1980).

Aside from species differences, the data suggested that this apparent discrepancy in results may be related to the presence of oleic acid at the interface. For porcine pancreatic lipase–colipase acting on phospholipid–triglyceride emulsions, oleic acid decreases the acceleration period for catalysis (Borgström, 1980). Mechanistic studies have shown that this involves the binding of fatty acid to colipase, enhancing its affinity for lipase 100-fold (Larsson & Erlanson-Albertsson, 1981). The potential regulatory significance of these product activation effects prompted us to investigate the role of oleic acid in cholesterol esterase adsorption to interfaces and the subsequent catalytic reaction. Their relationships to lipid packing density and interfacial composition were of particular interest because these parameters determine the amount and phase distribution of cholesteryl esters at the lipid–water interface (Smaby & Brockman, 1981a,b). For simplification of the interpretation of the results, all experiments were, unless noted, confined to the monolayer and monolayer–double-layer coexistence regions of the cholesteryl oleate–oleic acid phase diagrams where no bulk lipid phases are present. The results of these studies using initial rate techniques applied to surface films indicate that adsorption and catalysis are independently regulated and that the rate constants for these processes may be useful as probes

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of the structure of the lipid–water interface in more complex systems.

Materials and Methods

Reagents. [^{14}C]Cholesteryl oleate was obtained from New England Nuclear, Boston, MA, and had a radiopurity ≥99%. Cholesteryl oleate, oleic acid (9-*cis*-octadecenoic acid), methyl oleate, and erucic acid (13-*cis*-docosenoic acid) were obtained from Nu-Chek Prep, Elysian, MN, and were shown to be ≥99% pure by thin-layer chromatography. *p*-Nitrophenyl acetate was from Sigma, St. Louis, MO, and sodium taurocholate was from Calbiochem-Behring Corp., La Jolla, CA. Hydrophobic paper (phase separators, IPS grade) was obtained from Whatman Inc., Clifton, NJ, and plastic sheets (polyester transparencies, SP-500) were from J. H. Sherburn & Sons, Inc., Fort Worth, TX. [1-^{14}C]Methyl oleate was synthesized by methylating the carboxyl group of [1-^{14}C]oleic acid (specific activity 48.62 mCi/mmol) with diazomethane (Schlenk & Gellerman, 1960) and purified by silicic acid chromatography to a radiopurity ≥99%. All other chemicals were reagent grade and used without further purification. Water and petroleum ether (bp 66–67 °C) for interfacial experiments were purified as previously described (Smaby & Brockman, 1981a).

Methods. Cholesterol esterase from porcine pancreas was purified and assayed as previously described (Momsen & Brockman, 1977). The enzyme used had a specific activity ≥700 units/mg of protein where a unit of activity is 1 μmol of *p*-nitrophenyl acetate released/h. Protein concentration was estimated at 280 and 260 nm by using a nomograph based on protein and nucleic acid extinction coefficients given by Warburg & Christian (1942).

Before use, small circles of hydrophobic paper were exposed to water vapor in a closed container for 1 day. Plastic sheets were washed with chloroform–methanol (2:1) and air-dried. Immediately before use, static electricity was removed with a ^{210}Po source.

For a typical kinetic measurement, 13 mL of 0.25 M potassium phosphate buffer was added to a 4-cm diameter Teflon trough. The surface was cleaned by compression with a Teflon bar followed by aspiration of the surface. In this manner, the volume was reduced to 10 mL. An aliquot (usually about 5 μL) of petroleum ether containing the desired lipids was slowly added to give the desired surface pressure as measured with the du Nöuy ring technique (Brockman et al., 1975). After 5–10 min was allowed for complete evaporation of solvent, enzyme (usually 0.5–1.0 mg/mL in 0.1 M KPO_4, pH 6.5) was injected into the subphase which was stirred at 80 rpm. At the desired time, the monolayer was collected for analysis of reaction products of adsorbed enzyme activity by using a novel adsorption technique (Bhat & Brockman, 1981). Briefly, for analysis of reaction products in the lipid film containing radioactively labeled substrate, a plastic disk with dimensions about 1 cm larger than those of the film was placed on the surface. After 1 min, the sheet was removed by slowly pulling it parallel to the surface. This process was repeated with the other side of the sheet, after which it was cut into pieces and adsorbed lipid was eluted by washing with 5 mL (3 + 1 + 1) of chloroform–methanol (2:1) containing unlabeled substrate and products. Following solvent evaporation, the sample was dissolved in a small volume of elution solvent, and the products and reactants were separated by thin-layer chromatography. Products were quantitated by liquid scintillation counting. For measurement of active enzyme adsorbed to lipid films, the surface was collected on one side of a hydrophobic filter paper disk as described above. The adsorbed enzyme was quantitatively eluted by placing the paper, monolayer face down, in a petri dish containing 1.5 mL of 0.25 M potassium phosphate buffer, pH 6.5, and 5 mM sodium taurocholate and gently swirling the solution. The paper was then removed, and the enzyme activity in the eluate was measured by following the release of *p*-nitrophenol from *p*-nitrophenyl acetate at 400 nm.

In cases where the quantity of adsorbed enzyme was very low, the sensitivity of the assay was increased by assaying the desorbed enzyme with a radiolabeled substrate. Into 0.5 mL of eluate at 37 °C was injected 68 000 dpm of [^{14}C]methyl oleate (specific activity 48.62 mCi/mmol) in 5 μL of ethanol, and the sample was incubated at 37 °C for 10 min. The reaction was stopped by freezing the reaction mixture in 2-propanol–dry ice. For quantitation of the oleic acid formed, the method of Warner et al. (1979) was used. The sample was rapidly thawed, and oleic acid was extracted with 1.0 mL of carbon tetrachloride–hexane (2:1 v/v), 1 mM Triton X-100, 0.1 mM oleic acid, 1.5 mL of ethanol–water (3:1 v/v), and 70 mM NaOH. After the extraction was vigorously mixed for 30 s, the resulting emulsion was cleared into two phases by centrifugation (1000*g*, 8 min, 24 °C). The amount of oleic acid in a 1.0-mL aliquot of the upper phase was determined by scintillation counting. Background substrate hydrolysis was about 0.6% of that added and did not increase with time. Product formation was linear with time up to 10 min provided no more than 10% of the substrate was consumed and was linear with protein added up to 38 ng.

Surface pressure and surface potential isotherms were measured at 24 °C with a microprocessor-controlled Langmuir film balance. From the pressure–area isotherms, the coordinates of discontinuities which are indicative of phase transitions were determined by the use of second and third derivatives of surface pressure with respect to molecular area (Brockman et al., 1980). Surface dipole moments were calculated from surface potential measurements as described by Gaines (1966). The subphase for all experiments was 0.25 M potassium phosphate buffer, pH 6.5, unless otherwise indicated.

Results

If pancreatic cholesterol esterase is present at about 100 nM in the aqueous phase under a monomolecular film of oleic acid, it rapidly forms an adsorbed monolayer of enzyme at the lipid–water interface (Bhat & Brockman, 1981). At bulk concentrations lower than 10 nM, we observed that time-dependent adsorption of enzyme can still be conveniently measured even though the quantity of enzyme adsorbed in 15 min is less than one-tenth the saturation value. Under these conditions, adsorption produces a negligible change in the surface pressure. Representative examples of the time dependencies for the adsorption of enzyme to mixed films of oleic acid and cholesteryl oleate are shown in Figure 1a. In these and other experiments, such linearity was routinely observed for periods up to 30 min, substrate hydrolysis was normally less than 10%, and the quantities of enzyme adsorbed were 5% or less of that added to the bulk phase. Cholesteryl oleate was not required for adsorption to occur. In subsequent studies, unless noted, the rate of cholesterol esterase adsorption per square centimeter of surface, $d\Gamma_e/dt$, was calculated from a single adsorption measurement obtained 15 min following addition of enzyme to the subphase.

The bulk phase enzyme concentration dependence of the rate of adsorption to oleic acid films is shown in Figure 1b. Together with Figure 1a, the observed linearity is consistent with the lack of a significant desorption rate. Thus, the rate of adsorption can be described by the simple relationship

$$d\Gamma_e/dt = k_a[E_0] \quad (1)$$

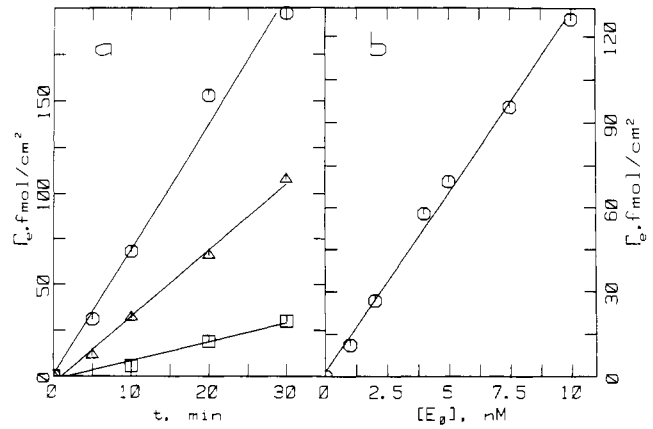

FIGURE 1: Representative time and bulk phase enzyme concentration dependencies for cholesterol esterase adsorption to oleic acid–cholesteryl oleate films. (a) $[E_0]$ = 4.9 nM, 24 °C; mole fraction of cholesteryl oleate 0.05 at 7 dyn/cm (□), 0.50 at 25 dyn/cm (○), and 0.80 at 15 dyn/cm (△). (b) Oleic acid film at 15 dyn/cm, 24 °C, 15-min adsorption time.

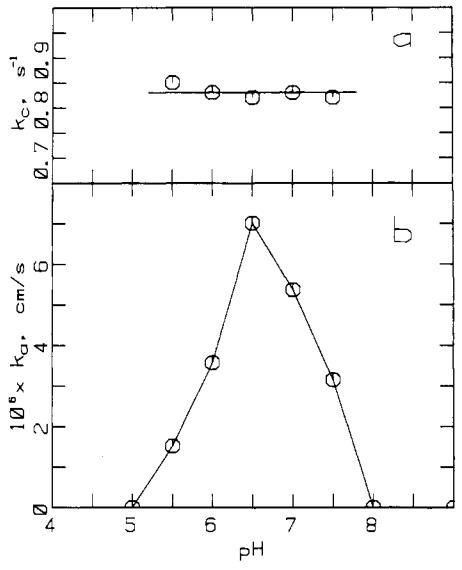

FIGURE 2: pH dependence of k_a and k_c. Subphase 0.25 M potassium phosphate, 24 °C. (a) $[E_0]$ = 2.5 nM; k_c's were calculated from products formed in 15 min as described in the text. (b) $[E_0]$ = 7.5 nM; k_a's were calculated from enzyme adsorbed in 15 min by using eq 1.

where Γ_e is measured in moles per square centimeter and $[E_0]$ is the subphase enzyme concentration in moles per cubic centimeter. The apparent rate constant for adsorption, k_a, provides a convenient parameter for characterizing the adsorption reaction. As shown in Figure 2b, k_a exhibits a relatively narrow pH optimum at 6.5, apparently governed by two ionizable groups with pK_as between 6.0 and 7.5. Accordingly, all subsequent experiments were performed at a pH of 6.5.

k_a was measured as a function of the packing of oleic acid molecules in pure and mixed lipid films to better understand the surface structural requirements for adsorption. Figure 3 shows that adsorption to oleic acid is relatively inefficient until the surface pressure of the film reaches 10–12 dyn/cm, after which it abruptly increases. The plateau reached at 25 dyn/cm is possibly artifactual because at pressures above 20 dyn/cm oleic acid containing films are highly unstable, even with 0.25 M phosphate buffer in the aqueous subphase. In a related experiment, k_a's for adsorption to erucic acid monolayers (data not shown) exhibited similar behavior up to the phase transition which such films undergo at 28 dyn/cm.

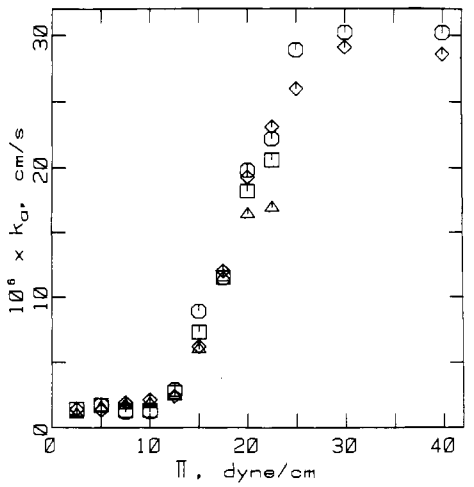

FIGURE 3: Variation of k_a with surface pressure in oleic acid–cholesteryl oleate films. $[E_0]$ = 2.5 nM, 24 °C; k_a's were calculated from enzyme adsorbed in 15 min by using eq 1. Mole fraction of cholesteryl oleate: 0.0 (○), 0.05 (◇), 0.50 (□), and 0.80 (△).

The abrupt increase in adsorption rate described above suggested a possible reorientation of the lipid molecules at that pressure. Accordingly, we calculated the surface pressure dependence of the surface dipole moment and the second derivative of surface pressure with respect to molecular area (Brockman et al., 1980) from the surface pressure and potential measurements for oleic acid. The absence of any discontinuity or inflection in these parameters at 10–12 dyn/cm (data not shown) is not consistent with an abrupt change in film structure.

Interestingly, the k_a data shown in Figure 3 were obtained at mole fractions of cholesteryl oleate from 0.0 to 0.8; yet they are superimposable. Over this range, the film changes from pure monolayer to essentially pure double layer with a bulk phase present (Smaby & Brockman, 1981b). Thus, the phase distribution of the film constituents has negligible bearing on the adsorption process. It should be noted that with changing film composition the concentration of oleic acid should not remain constant at a given surface pressure. Accordingly, the data were replotted with oleic acid concentration as the abscissa (not shown). Qualitatively, each curve was similar in shape to its counterpart in Figure 3, but the various data sets were no longer superimposable. However, the displacements were sufficiently small to preclude an analysis of the data to separate effects of surface pressure from oleic acid concentration. This occurs because the area of the interfacial film occupied by cholesteryl oleate is relatively small, between 10 and 20 dyn/cm, the range over which the largest changes in k_a occur.

Methyl oleate was employed as a surface diluent to help separate the roles of lipid packing and oleic acid concentration on adsorption. The oleic acid–methyl oleate phase diagram shown in Figure 4b indicates the complete miscibility of these compounds up to 15 dyn/cm. More importantly, the average molecular area–composition data at 13 dyn/cm shown in Figure 4a demonstrate the near-ideal miscibility of these compounds in two dimensions. Thus, at constant surface pressure, a change in film composition changes primarily the head-group composition while the molecular packing density remains essentially constant.

The k_a's for cholesterol esterase adsorption to such films were determined at 13 dyn/cm. Obtaining meaningful data from these experiments was complicated by the relatively high rate at which methyl oleate was hydrolyzed by the enzyme. This necessitated the use of enzyme concentrations less than

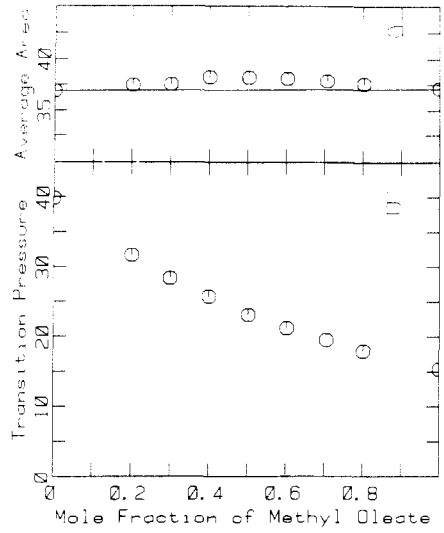

FIGURE 4: Physical characterization of oleic acid–methyl oleate mixtures at the air–buffer interface. (a) Average molecular area. Solid line shows expected values for ideal miscibility. (b) Phase transition pressures determined as described under Materials and Methods, 24 °C.

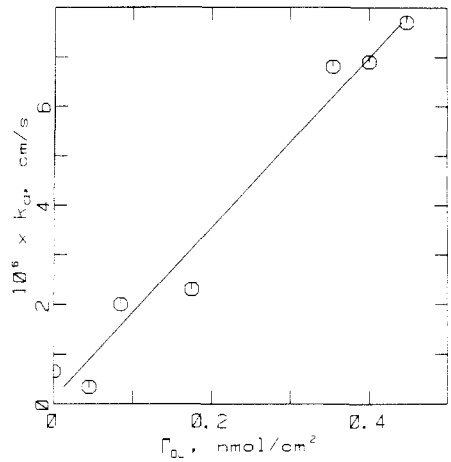

FIGURE 5: Dependence of k_a on oleic acid concentration, Γ_{OL}, at constant acyl chain packing for oleic acid–methyl oleate films. $[E_0]$ = 0.95 nM, 24 °C, surface pressure 13 dyn/cm; k_a's were calculated from enzyme adsorbed at 5, 10, and 15 min as described in the text.

1.0 nM, below which bulk enzyme concentration was not proportional to enzyme added. Also, we observed a small, rapid adsorption of enzyme to the monolayer which increased with the mole fraction of methyl oleate. Thus, it was necessary to perform these enzyme adsorption measurements at a constant bulk enzyme concentration and for each k_a determination to calculate $d\Gamma_e/dt$ from the slope of a plot of Γ_e vs. t with values measured at 5, 10, and 15 min. The values of k_a obtained from these slopes are shown in Figure 5. In contrast to those values obtained at variable surface pressure with oleic acid alone or in mixtures with cholesteryl oleate, these k_a's show a linear dependence on oleic acid concentration. The range of concentrations covered duplicates that of Figure 3. Thus, a part of the complex behavior shown in Figure 3 is a simple dependence of k_a on the concentration of oleic acid head groups.

In the preceding experiments, substrate hydrolysis did occur as the enzyme was adsorbed to the lipid–water interface. However, conditions were chosen such that the products, particularly oleic acid, did not change the surface pressure and did not significantly alter the oleic acid concentration. Under conditions where hydrolysis is restricted to less than 10% of substrate present, it appeared possible to obtain kinetic in-

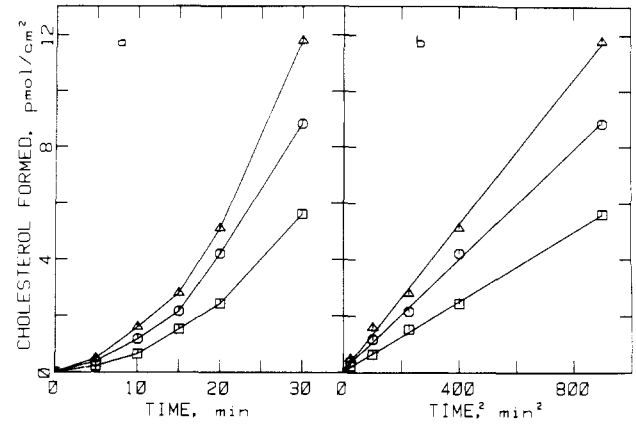

FIGURE 6: (a) Time course for hydrolysis of cholesteryl oleate in mixed films with oleic acid. $[E_0]$ = 2.5 nM, 24 °C, initial surface pressure of 15 dyn/cm; mole fraction of cholesteryl oleate, 0.05 (□), 0.2 (○), and 0.5 (△). (b) Data of panel a plotted according to eq 3.

formation reflecting the catalytic properties of the adsorbed enzyme. Representative time courses for the hydrolysis of cholesteryl oleate in mixed films with oleic acid are shown in Figure 6a. Note the increasing slope of each data set with time. This is expected if the reaction is catalyzed by the surface excess of enzyme, not that in the bulk aqueous phase. In the simplest case at constant substrate concentration

$$d\Gamma_p/dt = k_c\Gamma_e \quad (2)$$

where Γ_p is the surface concentration of free cholesterol or fatty acid released from cholesteryl ester. Because the enzyme adsorption rate is constant under these conditions, eq 1 can be integrated, $\Gamma_e = k_a[E_0]$, and substituted into eq 2. Subsequent integration of this equation with the conditions that Γ_e and Γ_p are zero at zero time yields

$$\Gamma_p = k_a k_c[E_0]t^2/2 \quad (3)$$

Figure 6b shows that if the data of 6a are plotted according to eq 3 the predicted linearity is observed up to 30 min.

The examples shown cover regions of both the monolayer phase and the coexistence of monolayer and double-layer phases, suggesting that eq 3 is valid over a wide range of experimental conditions. At any surface pressure, k_a is constant and known from Figure 3. Thus, by use of that parameter and $[E_0]$, k_0 values can be readily calculated from the slopes of lines of the type shown in Figure 6b or, as was normally done, from the product formed in 15 min. The pH dependency of k_c determined in this manner is shown in Figure 2a. Its constancy over the range in which k_a undergoes large changes shows clearly our ability to separate the processes of adsorption and catalysis in this complex reaction system.

Because at any surface pressure k_a is a constant (Figure 3), it is apparent from the slopes in Figure 6b that k_c is lipid composition dependent. To allow a more detailed analysis of this dependence, we determined k_c at 15 dyn/cm from 0.0 to 0.5 mole fraction of cholesteryl oleate. On the phase diagram for this system, this corresponds to nearly the entire range over which monolayer and double-layer phases exist in the absence of a bulk cholesteryl ester phase (Smaby & Brockman, 1981b). As shown in Figure 7, k_c increases throughout the region of existence of the monolayer phase and then decreases as the monolayer phase is replaced by the double-layer phase. This suggested that the enzyme preferentially hydrolyzes substrate in the monolayer phase. If so, and if the rate of hydrolysis in each phase is proportional to the amount of the substrate per unit area in that phase at the lipid–water interface, then

$$k_c = k_1\Gamma_1 + k_2\Gamma_2 \quad (4)$$

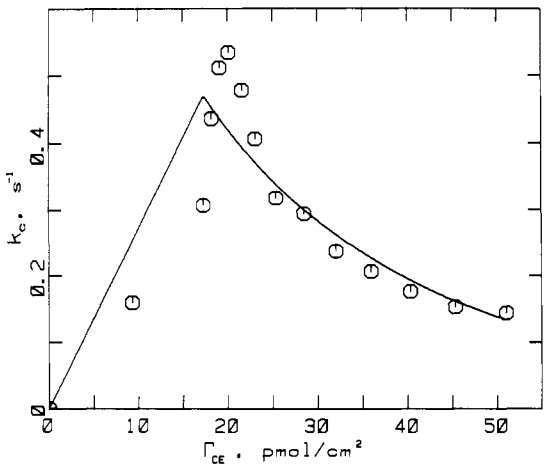

FIGURE 7: Variation of the rate constant for cholesteryl ester hydrolysis, k_c, with cholesteryl oleate concentration in mixed films with oleic acid. $[E_0]$ = 4.95 nM, 24 °C, initial surface pressure 15 dyn/cm. Values of k_c were calculated from products formed in 15 min as described in the text, using $k_a = 7.1 \times 10^{-6}$ cm/s. The theoretical curve was calculated as described in the text.

where Γ_1 is the apparent concentration of monolayer cholesteryl ester and Γ_2 is that for the interfacial layer of the double-layer phase. These concentrations were calculated at each composition by applying the model for these phases (Smaby & Brockman, 1981a,b) to average molecular composition data for these particular components at 15 dyn/cm. The data of Figure 7 were fit to eq 4 by using a modified Marquardt strategy, yielding $k_1 = 27 \pm 2$ and $k_2 = 2 \pm 0.4$ cm^2 s^{-1} fmol^{-1} with a root mean square deviation for k_c of 0.071/s. The 15-fold difference in these parameters indicates that pancreatic cholesterol esterase is relatively specific for substrate in the monolayer phase. The solid line in Figure 7 is drawn by using k_1 and the appropriate Γ_1 for each total cholesteryl oleate concentration to calculate k_c. Although not studied in detail, the hydrolysis of methyl oleate at 13 dyn/cm proceeded with a k_c of approximately 35/s at a substrate concentration of 262 pmol/cm^2, yielding a k_1 of 130 cm^2 s^{-1} fmol^{-1}.

Discussion

The hydrolysis of water-insoluble lipids by water-soluble lipases occurs in two distinct steps, adsorption of enzyme to the interface (not necessarily the substrate) followed by substrate hydrolysis within the interfacial plane [for a review, see Verger & de Hass (1976)]. In this work, we have used an initial rate approach to study the regulation of both the adsorption and the catalytic steps of the reaction with films containing oleic acid, methyl oleate, and cholesteryl oleate, all of which are substrates.

With methyl oleate, where significant substrate was present at the interface in the monolayer phase, we observed a small rapid association of enzyme with the substrate film. This can explain how the reaction is initiated with a pure cholesteryl oleate film (Bhat & Brockman, 1981). The small burst of adsorption was followed by a slower phase of binding which at constant acyl chain packing correlates directly to the concentration of fatty acid in the film. Esterified substrate is not required for this latter reaction which constitutes a form of product activation. Similar effects of fatty acids have been observed for colipase adsorption to a triglyceride–phospholipid emulsion (Borgström, 1980).

The apparent rate constant for enzyme adsorption, k_a, exhibits a narrow pH optimum, whereas catalysis is pH independent over this range. Thus, catalysis at constant substrate concentration is controlled primarily by the availability of enzyme at the interface. In contrast, the overall catalytic activity of this enzyme toward the short-chain substrate, *p*-nitrophenyl acetate (Momsen & Brockman, 1977), and of the human enzyme toward tributyrin (Lombardo & Guy, 1981) shows dependence on only a single ionizable group with a pK_a of 5.8 with constant activity above pH 6.5. This difference suggests that in the monolayer system the decrease in k_a above pH 6.5 may be related to pH-dependent changes in the structure of the interface, not ionization of the enzyme.

If k_a was measured at variable surface pressure, an apparent cooperativity with respect to oleic acid concentration was observed. This must be considered apparent because the lack of adsorption at low surface pressure might, in part, reflect rapid surface denaturation of the adsorbed enzyme at the relatively empty lipid–water interface. On the other hand, if acyl chain packing and pressure were held constant, a linear dependence was observed. This suggests two components may regulate adsorption rate, the concentration of oleic acid head groups and the concentration or a particular arrangement of acyl chains. From data for the adsorption of $^{45}Ca^{2+}$ to fatty acid containing films and electrophoretic mobilities of liposomes, clustering of oleic acid molecules has been postulated (Hauser et al., 1979). Recently, however, this conclusion has been questioned (von Tscharner & Radda, 1981). If the observed cooperativity of adsorption at variable acyl chain packing reflects clustering, the observed linear dependence at constant packing necessitates that either all or only a small fraction of the oleic acid is clustered in the constant packing experiment.

Because k_a had dimensions of centimeters per second, it is not directly comparable to rate constants reported for the adsorption of enzymes such as triglyceride lipase to lipid–water interfaces. It is reasonable to assume that, as for lipase, the adsorption of active enzyme is reversible and follows the Langmuir adsorption isotherm. Under our initial rate conditions, it can be readily shown that $k_a = k_f/a_0$, where k_f is the second-order rate constant for adsorption and a_0 is the area of an enzyme adsorption site at the lipid–water interface (Brockman et al., 1973). Saturation of the interface with enzyme has been attained at 3.5 pmol/cm^2 which corresponds to formation of a hexagonally close-packed monolayer of native enzyme molecules (Bhat & Brockman, 1981) with an a_0 of 2.9×10^{11} cm^2/mol. By use of this value and the highest k_a determined, about 30×10^{-6} cm/s, k_f is 8.6×10^6 cm^3 mol^{-1} s^{-1}. This value is about 100-fold lower than that measured for lipase (M_r 50 000) which is believed to adsorb via a diffusion-controlled mechanism (Momsen & Brockman, 1976). Thus, either the adsorption of cholesterol esterase (M_r 80 000) is not diffusion controlled or the concentration of clusters or adsorption sites is small. This latter possibility would not preclude saturation of the interface with enzyme since the cross-sectional area of an enzyme molecule is more than 100-fold greater than that of an oleic acid molecule.

Clearly, adsorption is a complex process which depends on packing, concentration, and possibly ionization of oleic acid. A more complete understanding of this process will require more extensive kinetic and physical studies. Ultimately, however, it should be possible to define the minimum requirements to create an adsorption site and the relation of site formation, if any, to fatty acid clustering at the lipid–water interface.

Adsorption of cholesterol esterase to oleic acid–substrate films leads to a parabolic increase in the formation of reaction products with time. This is consistent with product being generated by the surface excess of enzyme formed by oleic acid

dependent enzyme adsorption. By using the integrated form of the rate expression and knowledge of k_a and $[E_0]$, we calculated the apparent rate constant for catalysis, k_c, under conditions where substrate hydrolysis was less than 10%. The presence of relatively high concentrations of oleic acid in the film makes it necessary to consider if the rate of synthesis of cholesteryl oleate interferes with the determination of k_c. Calculations of the ratio of synthetic and hydrolytic rates using 1.4×10^{-8} mol/cm^2 as the equilibrium constant (Bhat & Brockman, 1981) show, however, that the rates of synthesis are negligible under all conditions studied.

Because k_c should reflect the substrate concentration dependence of the reaction, it was determined as a function of cholesteryl oleate concentration throughout the region of monolayer and double-layer phase coexistence. Given the 15-fold difference between the second-order rate constants for hydrolysis between k_1 and k_2, the maximum in the data of Figure 7 should indicate the monolayer/double-layer phase boundary at 0.04 mol fraction of cholesteryl oleate (Smaby & Brockman, 1981b). That it is at a concentration corresponding to approximately 0.1 mol fraction suggests a small, quantitative difference in the physical properties of a static film compared to those measured dynamically. The latter were used to calculate Γ_1 and Γ_2. This difference is likely responsible for the poorer fit of the data to eq 4 at low cholesteryl ester concentrations. An alternative treatment of the data is to use just the data from the monolayer region to calculate k_1. This can then be used in eq 4 with the data of Figure 7 above 25 pmol/cm^2 to calculate k_2. The values obtained in this manner for k_1 and k_2, 18 and 3 cm^2 s^{-1} fmol^{-1}, show reasonable agreement with those calculated by using the entire data set. By either method of analysis, our results show that the enzyme is relatively specific for substrate in the monomolecular phase. The mathematical model describing the double-layer phase was based on assumed similarities between cholesteryl ester in the monolayer phase and the interfacial layer of the double-layer phase (Smaby & Brockman, 1981a). Apparently, however, the similarities extend only to the cross-sectional area of the substrate molecules, not their state within the phase. That the packing or orientation of substrate molecules is different in the interfacial layer of the double-layer phase is also indicated by the higher solubility of cholesteryl oleate in the interfacial layer of the double layer at any surface pressure compared to the monomolecular phase (Smaby & Brockman, 1981b).

The observed phase specificity of the adsorbed enzyme strongly suggests that it functions only at the lipid–water interface and does not, as commonly assumed (Khoo et al., 1979; Lombardo & Guy, 1981), penetrate the surface monolayer to hydrolyze bulk cholesteryl ester. The significance of this observation is that in binary systems the concentration of a particular cholesteryl ester in the monolayer phase is markedly dependent on the structure of its acyl moiety (Smaby & Brockman, 1981b). Thus, the hydrolysis of that cholesteryl ester in the intestine and its hydrolysis or transport in other tissues may well exhibit an apparent specificity based on substrate availability in the proper phase at the lipid–water interface. If so, then measurements of k_c in more complex systems, such as arterial lipid inclusions, should be useful in determining the phase distribution of cholesteryl ester species at the lipid–water interface.

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