Regulation of Fatty Acid ^{18}O Exchange Catalyzed by Pancreatic Carboxylester Lipase. 2. Effects of Lateral Lipid Distribution in Mixtures with Phosphatidylcholine†

Jean M. Muderhwa and Howard L. Brockman*

The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

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ABSTRACT: The lipase-catalyzed exchange of the carboxyl oxygens of 13,16-*cis*,*cis*-docosadienoic acid (DA) was studied in the presence of a nonsubstrate matrix lipid, 1-palmitoyl-2-oleoylphosphatidylcholine. For mixed lipid films at the argon–water interface exposed to pancreatic carboxylester lipase (EC 1.1.1.13), the extent of oxygen exchange showed an abrupt increase as the abundance of DA in the interface was increased from 0.5 to 0.6 mole fraction. This compositional range was independent of the level of enzyme used and of the surface pressure, i.e., lipid packing density, of the film. Concomitant with the transition was a change in the apparent mechanism of exchange from coupled to random sequential. Like the extent of oxygen exchanged, the shift in mechanism was independent of all variables except the lipid composition of the interface. The absence of any chemical or physical change accompanying the exchange reaction precludes mechanistic explanations based on the generation of reaction products by the enzyme. Instead, the results suggest that the lateral distribution of DA in phosphatidylcholine–DA interfaces regulates the expression of carboxylester lipase activity and its apparent mechanism. Preliminary measurements give an average cluster size of 1825 molecules of DA when its mole fraction is 0.35. As the DA content of the interface reaches 0.5–0.6, there appears to be a lipid head-group based percolative transition in which DA becomes the continuum. Because this transition involves the lateral organization of the lipids themselves, other interfacially active enzymes may be regulated similarly.

Enzymatic reactions occurring in solution normally depend on the concentration of substrate in a manner described by Michaelis–Menten kinetics or one of the more complex variants thereof. Typically, substrate and enzyme concentrations are low compared to the concentration of water. In such a dilute system, the enzyme and substrate are brought into juxtaposition by specific noncovalent interactions which reduce a bimolecular reaction to a unimolecular one and thereby increase the efficiency of catalysis [e.g., Segel (1975)]. Biological processes like lipolysis may involve a water-insoluble substrate and a water-soluble enzyme. For such reactions, enzyme adsorption to the substrate-containing surface, but not necessarily to the substrate itself, can increase catalytic efficiency by orienting and confining the reactants to the small volume of the essentially two-dimensional interphase (Brockman, 1984). In this surface phase, the "solvent" for the reaction is not only interfacial water but also the lipid matrix in which the substrate and enzyme reside. A consequence for an enzyme which functions in an interfacial environment is that its partitioning to the surface and its access to substrate may be regulated in ways not simply related to the two-dimensional substrate concentration.

Surface-related regulation of catalysis has been observed for several (phospho)lipases acting on their respective substrates in bilayer and monolayer membranes (Pieroni & Verger, 1979, 1983; Alsina et al., 1989; Cunningham et al., 1989; Bhat & Brockman, 1981, 1982). It is manifested as unusual dependencies of enzyme binding and activity on the surface pressure, i.e., lipid packing density, and lipid composition. Additional factors now recognized to have contributed to these effects in early studies are the surface instability of the enzymes at low surface pressures in monolayer films and the transient accumulation of "soluble" reaction products in the interface when medium-chain substrates are used [e.g., Brockman (1984) and Verger and Pieroni (1986)]. However, even if these are eliminated by using monolayers at higher surface pressures with long-chain substrates, nontraditional regulatory effects persist. For example, pancreatic carboxylester lipase (CEL)[1] activity toward a variety of ester substrates is inhibited in monomolecular films rich in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) (Tsujita et al., 1989). POPC reduces enzyme adsorption to these films, but the size of this effect is insufficient to explain the limited extent of substrate hydrolysis (Tsujita et al., 1987; Muderhwa & Brockman, 1990). CEL adsorption to all substrate-containing phosphatidylcholine films was similar on a lipid area adjusted basis but was distinct from adsorption to films of POPC and 13,16-*cis*,*cis*-docosadienoic acid (DA). This difference suggested that the lateral organization of the lipids in the interface is an important regulator of enzyme adsorption. In an extension of this argument (Brockman & Muderhwa, 1991), it was proposed that the abrupt change in ester substrate accessibility by surface-bound enzyme at a critical lipid composition (Bhat & Brockman, 1981; Tsujita et al., 1989) reflects a percolation-based transition in lipid head-group organization.

In all studies involving ester substrates noted above some hydrolysis products were formed. These may alter the lateral distribution of lipids in the interface and thereby complicate the interpretation of results. This occurs because the physical

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* Address correspondence to this author.

[1] Abbreviations: DA, 13,16-*cis*,*cis*-docosadienoic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; CEL, monomeric porcine pancreatic carboxylester lipase.

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as well as chemical properties of the surface are changing as lipid hydrolysis proceeds. In the preceding paper in this issue, it was shown that the exchange of ^{18}O between free fatty acids in monomolecular films and medium water was catalyzed at rates comparable to those for the hydrolysis of natural ester substrates in monolayers and of *p*-nitrophenyl esters in micelles (Muderhwa et al., 1992). Thus, in its interactions with CEL, free fatty acid is a good "substrate" for the enzyme. Compared to other substrates, however, the "hydrolysis" of fatty acid is unique because the lipid species composition of the interface remains unaltered by the isotope exchange. This property of the exchange reaction suggested that ^{18}O exchange could be used to probe the role of matrix lipids like POPC in regulating the activity of CEL at interfaces. The results reported herein show that the extent of ^{18}O exchange in mixed films exhibits a composition-dependent critical transition like those observed with ester substrates. Associated with the critical transition is an apparent shift in the mechanism of the oxygen exchange reaction which is attributable to the lateral distribution of lipids in the interface.

Experimental Procedures

Most reagents and procedures for CEL adsorption and ^{18}O exchange experiments were as described in the preceding paper (Muderhwa et al., 1992). All experiments were performed at pH 6.6 on a subphase containing 0.01 M potassium phosphate buffer and 0.1 M NaCl at 24 °C. Unless otherwise indicated, in all experiments the subphase was stirred at 100 rpm. For the determination of the ^{18}O content of DA in samples containing POPC, thin-layer chromatography was used in addition to the procedures described earlier. Specifically, following methylation and hydrogenation of free fatty acid, the lipids were dissolved in 200 μL of chloroform, containing 0.1 mg/mL methyl heptadecanoate as an internal standard, and applied on layers of silica gel H. Hydrogenated methyl esters and POPC were separated by development with hexane/diethyl ether/acetone (90:5:5 v/v/v). Purified methyl esters scraped from the plates were eluted with 6 mL (3 + 3) of diethyl ether, taken to dryness under a stream of nitrogen, and redissolved in 10–20 μL of heptane for analysis by gas chromatography–mass spectrometry.

Surface pressure–area isotherms for POPC/DA mixtures were determined using an automated Langmuir film balance described earlier (Brockman et al., 1980, 1984). The aqueous subphase was identical to that described above for kinetic experiments. Procedures for reagent preparation and quality assessment have been recently described (Smaby & Brockman, 1990, 1991). For each isotherm, the surface pressure at the onset of the liquid-expanded to liquid-condensed phase transition was determined by analysis of second and third derivatives of surface pressure with respect to area (Brockman et al., 1980).

Results

Composition Dependence of ^{18}O Exchange. Earlier studies had shown that ester substrate/POPC monolayers were miscible in all proportions (Smaby & Brockman, 1985). However, when such films were exposed to a relatively high concentration of CEL (123 nM), a composition-dependent extent of substrate hydrolysis was observed (Bhat & Brockman, 1981; Tsujita et al., 1989). Specifically, in POPC-rich films the extent of hydrolysis was low, generally less than 10% of substrate present, whereas in substrate-rich films hydrolysis was complete. The transition between these regions occurred over a narrow range of compositions, suggesting the existence of a critical composition. Using the exchange of the carboxyl oxygens of 13,16-*cis*,*cis*-docosadienoic acid (DA) in monolayers, the accessibility of the fatty acid by the enzyme can be monitored (Muderhwa et al., 1992). With monolayers of pure DA and a CEL concentration of 30 nM, exchange is essentially complete in 10 min. Using this concentration, the POPC composition dependence of ^{18}O exchange from prelabeled DA was measured. As shown in Figure 1, the extent of ^{18}O exchange in mixed films shows the same type of lipid concentration dependence observed earlier with ester substrate hydrolysis (Tsujita et al., 1989). Specifically, there is an apparent critical concentration at about 0.5–0.6 mole fraction of DA. Below this DA composition, about 10% of carboxyl oxygens were exchanged for ^{16}O from the medium, whereas above it exchange was essentially complete. At a more catalytic level of enzyme in the aqueous phase, 1.0 nM, the results are qualitatively similar but quantitatively different (Figure 1). The extent of exchange is the same in the POPC-rich region but is limited to about 50% between 0.75 and 1.0 mole fraction of DA. The similarities at low mole fractions of DA suggest exchange limited by substrate availability, whereas the differences at high mole fractions suggest exchange limited by catalytic turnover.

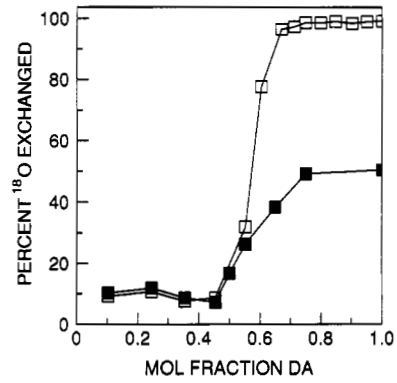

FIGURE 1: Lipid composition dependence of DA ^{18}O exchange in mixed films with POPC catalyzed by CEL. Bulk phase CEL concentrations were 1 (■) and 30 (□) nM. ^{18}O exchange was measured at initial surface pressures just below film collapse [44–35 mN/m, (Tsujita et al. 1987)].

Kinetics of ^{18}O Exchange. In monolayers of DA alone, ^{18}O exchange catalyzed by CEL is first order in time, interfacial enzyme concentration (Γ_E), and DA concentration (Muderhwa et al., 1991). Combined with the first-order dependence of Γ_E on time and bulk enzyme concentration [E_o], the progress of the reaction at low (<10 nM) enzyme concentrations is described by

$$\ln (2 + \Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}}) = \ln (2 + \Gamma_{18,16\text{-DA},0}/\Gamma_{18,18\text{-DA},0}) + k't^2/4 \quad (1)$$

where $\Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}}$ is the molar ratio of the singly and doubly ^{18}O-labeled DA species at any time t, and the subscript 0 refers to their value at $t = 0$. The apparent rate constant, k', is

$$k' = k_1 k_a [E_0] \quad (2)$$

where k_1 ($= k_{cat}/K_m$) is the rate constant for the reaction, k_a is the rate constant for adsorption of CEL to the interface, and [E_0] is the bulk enzyme concentration. At a bulk enzyme concentration of 2 nM and a DA mole fraction of 0.45, about 59% of the ^{18}O initially present in DA is exchanged in 60 min. For incubations terminated during that period, exchange data are shown in Figure 2, plotted according to eq 1. In contrast to results of similar experiments at a DA mole fraction of 1.0 (Muderhwa et al., 1992), the plot is nonlinear, precluding

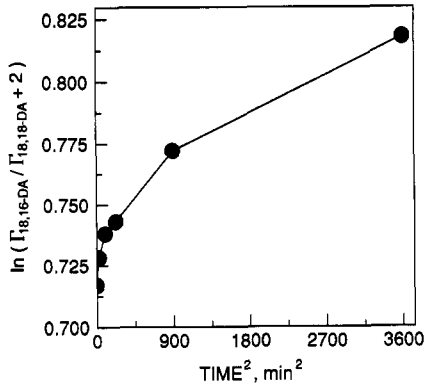

FIGURE 2: Time course for DA ^{18}O exchange in mixed films with POPC. The initial film composition was 0.45 mole fraction of DA, the bulk CEL concentration was 2 nM, and the initial surface pressure was 41 mN/m. Data are plotted according to eq 1.

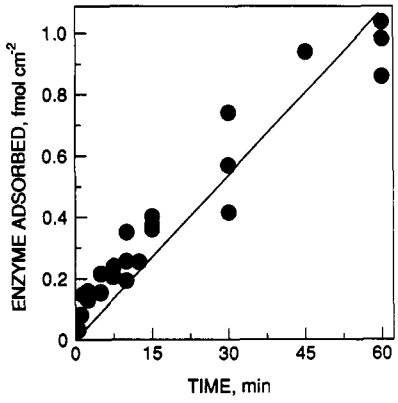

FIGURE 3: Time dependency of CEL adsorption to DA/POPC films at the argon–buffer interface. CEL added was 2 nM, the initial film composition was 0.45 mole fraction of DA, and the initial surface pressure was 41 mN/m.

determination of k'. One reason for this behavior could be a lack of proportionality between Γ_E and time at a constant $[E_0]$. This possibility was tested using films containing 0.45 mole fraction of DA at $[E_0] = 2.0$ nM, and the results are shown in Figure 3. When fitted to a line forced through the origin, reasonable proportionality is obtained and the slope yields a rate constant of 0.15×10^{-6} s^{-1} cm. The linearity of Figure 3 is comparable to that obtained in similar experiments with films of DA alone, but the value of k_a is 200-fold lower (Muderhwa et al., 1992). Thus, alteration of enzyme binding kinetics at 0.45 mole fraction of DA can explain the decrease in the extent of the reaction relative to pure DA films (Figure 1) but not the marked nonlinearity of Figure 2. In control experiments, results comparable to those shown in Figure 2 were obtained if 5 mM EGTA was present in the aqueous subphase (data not shown). This indicates that interaction of DA with calcium ions is not responsible for the nonlinearity of the data in Figure 2.

Mechanism of ^{18}O Exchange. To better understand the failure of the kinetic model to describe data obtained below the critical composition, DA species distributed was examined. For the experiment shown in Figure 2 the time course of the relative concentrations of the three isotopic DA species is given in Figure 4a. While it shows the expected decline in 18,18-DA (filled circles) and increase in 16,16-DA (open circles) with time, the relative abundance of 18,16-DA (half-filled circles) is atypical. If the reaction proceeded by a random sequential mechanism, as it does in pure DA films (Muderhwa et al., 1992), 18,16-DA should transiently accumulate prior to the buildup of 16,16-DA. Yet, even at the 5-min time point when

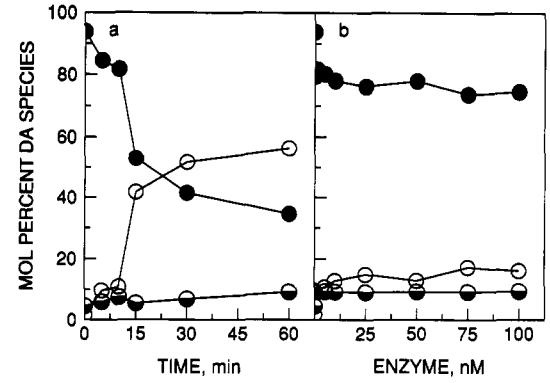

FIGURE 4: DA species distribution as function of (a) time and (b) CEL concentration. The initial film composition was 0.45 mole fraction of DA in POPC, the bulk CEL concentration was 2 nM, and the initial surface pressure was 41 mN/m. The relative percent of 18,18-DA (●), 16,18-DA (◒), and 16,16-DA (○) is shown.

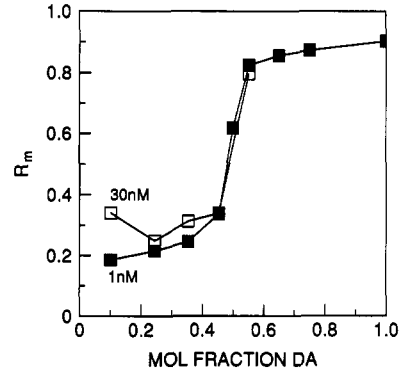

FIGURE 5: Lipid composition dependence of the mechanism parameter, R_m, in DA/POPC monolayers. R_m values were calculated using data from the experiments shown in Figure 1 using eq 3.

little ^{18}O has been exchanged overall, 16,16-DA is more abundant. This pattern of product accumulation suggests a shift in the reaction mechanism from random sequential to either concerted or coupled, i.e., both carboxyl oxygens are exchanged simultaneously or sequentially before the fatty acid is released into the global fatty acid pool. Another anomaly in ^{18}O exchange at this lipid composition is revealed by measurement of product distribution after 10 min as a function of the bulk enzyme concentration (Figure 4b). It again shows unexpectedly low levels of the singly ^{18}O-labeled DA species, and, regardless of how much enzyme is present, the extent of the reaction is approximately constant. Together with Figure 4a, this suggests that not only is the mechanism of ^{18}O exchange altered in POPC-rich surfaces but also that the DA exists in at least two pools with respect to the rate of its accessibility by the enzyme.

In the preceding paper in this issue, the mechanism for oxygen exchange occurring in pure DA films was characterized by plotting the ratio of the surface concentrations of the singly and doubly ^{18}O-labeled species determined experimentally, $\Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}}$, against the corresponding ratio calculated for a randomly labeled sample with the same total content of ^{18}O (Muderhwa et al., 1992). To examine the dependence of the mechanism on other parameters like lipid composition, we define the mechanism parameter

$$R_m = (\Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}})_{exp}/(\Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}})_{calc} \quad (3)$$

For any experimental point, this parameter will have a value of 1.0 if the exchange reaction proceeds by a random sequential mechanism and a value of zero if the mechanism is entirely coupled or concerted, i.e., no 18,16-DA accumulates. For the data shown in Figure 1, realistic values of R_m can be calculated

Table I: Dependence of the Mechanism Parameter R_m on Experimental Parameters

mole fract DA	carboxylester lipase (nM)	no. of det	%^{18}O exchd	init surf press (mN/m)	R_m
0.35	0.1–100	12	2–19	42.51 ± 0.72	0.24 ± 0.05
0.45	0.1–100[a]	14	5–18	40.88 ± 1.09	0.31 ± 0.07
0.55	0.25–7.5	6	3–54	40.40 ± 1.90	0.70 ± 0.08
0.65	0.05–7.5	5	7–66	38.10 ± 1.06	0.81 ± 0.04
1.00	0.05–7.5	8	6–57	35.63 ± 0.25	0.84 ± 0.06

[a] All incubations were for 10 min at 24 °C, except at 0.45 mole fraction DA, for which incubation times were 2.5–30 min.

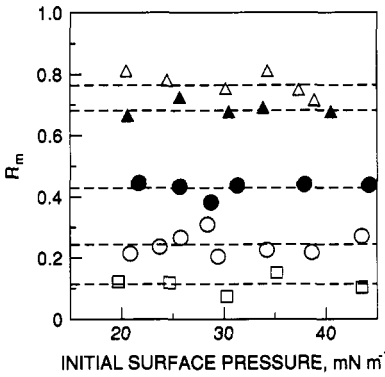

FIGURE 6: Initial surface pressure dependency of the mechanism parameter, R_m, in DA/POPC monolayers. The bulk phase CEL concentration was 2 nM, and initial mole fractions of DA were 0.25 (□), 0.45 (○), 0.50 (●), 0.65 (▲), and 1.00 (△).

for all points where the extent of ^{18}O exchanged is ≤80%. At higher extents of exchange, the level of the doubly ^{18}O-labeled species approaches the limit of detection of our GC–MS apparatus for the small amounts of lipid available for analysis from a 26 cm^2 monolayer. In Figure 5, R_m is shown as a function of lipid composition for the data of Figure 1. The values range from a low of 0.2 to a high of 0.9 for DA alone, and the composition dependence of R_m reflects the critical composition indicated by the percent exchange data of Figure 1. In separate experiments at selected lipid compositions, the dependence of R_m on [E_0], and hence the extent of ^{18}O exchange, was tested, and, at 0.45 mole fraction of DA, incubation time was also varied. The results in Table I show that R_m is independent of those experimental variables over the ranges measured. In another set of experiments at selected lipid compositions, the lipid packing density, i.e., initial surface pressure, was varied over the range in which the enzyme is stable with respect to surface denaturation. As shown in Figure 6, the value of R_m at each lipid composition is essentially constant, and the increase in R_m with increasing DA mole fraction parallels the trend seen in Figure 5 and Table I. Together, these data clearly show that the shift in mechanism indicated by R_m is determined largely, if not solely, by the lipid composition of the interface.

Lipid Distribution in the Interface. As noted above, comparison of the time and enzyme dependencies of DA species distribution during exposure to CEL (Figure 4a,b) suggests pooling of the DA in films at a DA mole fraction of 0.45. One reason for such behavior could be the proximity of this composition to the critical composition if substrate exists as slowly and rapidly accessible pools. Another could be that a pool of DA is initially accessible to the enzyme and that the mechanical stirring used to bring the enzyme to the interface is slowly generating another pool. To determine if such factors could be regulating DA accessibility, parallel sets of ^{18}O exchange and enzyme adsorption measurements were made as a function of time at 0.35 mole fraction of DA. Because the rate constant determined from Figure 3 was 200-fold lower

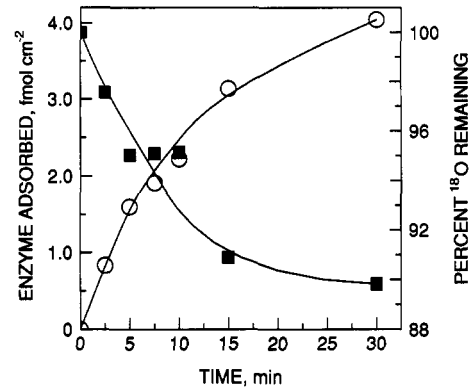

FIGURE 7: Time course for CEL adsorption and DA ^{18}O exchange in DA/POPC films. The initial film composition was 0.35 mole fraction of DA, the bulk CEL concentration was 5 nM, and the initial surface pressure was 42.5 mN/m. (○) Surface concentration of enzyme; (■) percentage of ^{18}O remaining in DA.

than that obtained under the same conditions but with monolayers of DA alone, adsorption of carboxylester lipase is apparently not limited by its rate of arrival at the lipid–water interface. Accordingly, after 1 min of mixing at 100 rpm to disperse the enzyme, stirring was halted. The results (Figure 7) show that under these conditions ^{18}O exchange seems to approach a limit after 10–15% of the ^{18}O initially present has been exchanged. Concomitantly, the surface concentration of enzyme appears to be approaching a limiting value of 4–6 fmol cm^{-2}. These data support the notion that away from the critical composition and with mechanical perturbations minimized, DA exists in accessible (10–15%) and inaccessible (85–90%) pools in the interface.

Discussion

The use of ^{18}O exchange to probe the role of lipid–lipid interactions in the regulation of lipolysis is based on the observation that fatty acids are good "substrates" for CEL. Chemical, and hence physical, alterations of the surface accompany the hydrolysis of ester substrates. Because these perturbations do not occur with ^{18}O exchange, the existence of the critical composition (Figure 1) cannot be ascribed to product generation or to hydrolysis-dependent kinetic lags observed with substrate-containing interfaces in the presence of catalytic levels of (phospho)lipases (Verger & Pieroni, 1986). Also, it is independent of the concentration of enzyme in the aqueous subphase (Figure 1 and Table I), and, at the initial surface pressures and enzyme concentrations used in most experiments, the enzyme does not significantly perturb the interface (Muderhwa & Brockman, 1990). Thus, the critical composition appears to reflect interaction between the enzyme and preexisting lipid organization.

The importance of lipid organization as a regulator of substrate availability to lipases has been inferred from earlier studies of the hydrolysis of ester substrates in mixed films with POPC (Tsujita et al., 1989; Brockman & Muderhwa, 1991) and in bilayer membranes of 1-stearoyl-2-oleoyl-phosphatidylcholine and 1,3-dioleoylglycerol (Cunningham et al., 1989). It was also indicated from comparison of CEL adsorption to ester substrate/POPC and DA/POPC monolayers (Muderhwa & Brockman, 1990). In previous studies of ester substrate hydrolysis, the critical composition was observed in a compositional and thermal range over which the components were completely miscible and their acyl chains were melted, i.e., monolayers were liquid expanded and bilayers were liquid crystalline. Thus, those critical transitions were not directly associated with lateral phase separation [e.g., Kanehisa and

Tsong (1978)], which is known to regulate the activity of phospholipases A_2 [e.g., Jain and Jahagirdar (1985), Kensil and Dennis (1985), Lichtenberg et al. (1986), and Grainger et al. (1990)]. For monolayers of DA and POPC, miscibility in the critical transition range for ^{18}O exchange is suggested by physical data (Tsujita et al., 1987) but cannot be unequivocally determined. The same is true for data obtained with mixed monolayers of similar fatty acids and phosphatidylcholine (Torosian & Lemberger, 1968; Lundberg & Ekman, 1979). However, mixtures of DA with disaturated phosphatidylcholines are miscible over the compositional range of the critical transition (J. M. Smaby and H. L. Brockman, unpublished). Thus, the existence of a critical composition for ^{18}O exchange does not appear to reflect macroscopic lateral phase separation of DA and POPC.

From studies of ester substrate hydrolysis, we have suggested that the critical transition of substrate utilization in monolayers reflects a lipid organizational transition from a phosphatidylcholine-dominated to a substrate-dominated surface (Muderhwa & Brockman, 1990) in a manner consistent with percolation theory (Brockman & Muderhwa, 1991). The data of Figure 5 show that throughout the compositional range of the critical transition for DA oxygen exchange, the mechanism parameter, R_m, is constant at each lipid composition. If the area created by lowering the lipid packing density was equivalent to adding more nonphospholipid, i.e., DA, to the surface, R_m should have been lipid packing dependent. Specifically, at some composition having a value of R_m between its upper and lower limits, decreasing the surface pressure should have caused R_m to increase. The absence of such a dependence suggests that the controlling percolation process is matrix based rather than continuum based (Gawlinski & Stanley, 1981). If the underlying matrix is determined by the melted hydrocarbon chains of the lipids, it is most likely trigonal, i.e., hexagonal, packing.

In the context of percolation theory, at DA mole fractions above the critical composition, the surface should consist of essentially a single continuous domain of DA in which microdomains of POPC are present. This interpretation is qualitatively consistent with the extent of ^{18}O exchange being essentially constant from ~0.7 mole fraction of DA to 1.0, even at low bulk enzyme concentration (Figure 1). At lower mole fractions of DA, where the curves in Figure 1 are nearly identical, DA should be clustered in POPC. That fatty acids may be clustered in predominantly phosphatidylcholine surfaces has been inferred earlier from electrophoretic mobility and Ca^{2+} binding data (Hauser et al., 1979). Because the adsorption of CEL is severely inhibited by POPC at DA mole fractions up to 0.5, the small extent of exchange over that range reflects in part a reduction of catalyst in the surface [Figure 3 and Muderhwa and Brockman (1990)]. At 0.45 mole fraction of DA, the rate constant for enzyme adsorption is reduced by 200-fold, an amount far in excess of the reduction in fraction of surface area occupied by DA in the mixed-lipid films compared to DA alone. This is consistent with percolation theory if DA domains comprised of many DA molecules are required for enzyme adsorption. Except for compositions near the percolation threshold, i.e., the critical transition, clusters of DA should remain on average small [e.g., Hoshen and Kopelman (1976)]. In addition to such purely statistical considerations, lipid–lipid interactions between POPC and DA should also regulate domain size (Kertesz et al., 1983). That interaction-based regulation occurs with either ester substrates or DA and POPC is suggested by marked differences in CEL adsorption to mixed films (Muderhwa & Brockman, 1990).

A totally unexpected result was the shift in mechanism from random sequential to largely coupled or concerted which occurs in the transition region. Such a shift could reflect a specific interaction between the enzyme and the POPC, but this seems unlikely for two reasons. First, enzyme adsorption data do not suggest any such interaction; indeed, POPC inhibits adsorption of the enzyme by what appears to be steric exclusion (Muderhwa & Brockman, 1990). Secondly, the mechanism shifts abruptly with DA composition (Figure 5). This could indicate a high-order dependence of lipid–protein interaction on the surface concentration of phospholipid. However, for different substrates in films with POPC the critical composition occurs at different mole fractions of substrate and is independent of lipid packing density (Tsujita et al., 1989). Similar independence of the critical composition on lipid concentration for DA/POPC films is indicated by Figure 6. A less direct role for POPC could be its regulation of the orientation or conformation of the enzyme, i.e., interfacial activation, through the size of clusters of DA. This could explain both the observed transitions in activity (Figure 1) and mechanism (Figure 5). Specifically, if the organization of POPC-rich surfaces prevented the opening of a flap protecting a buried active site (Winkler et al., 1990; Brzozowski et al., 1991), the rate of entrance of DA to the active site could be diminished. Moreover, once in the active site, DA egress could be slowed, allowing multiple cycles of ^{18}O exchange to occur with each DA molecule in the active site. At high mole fractions of DA the flap may open, increasing the rates of DA binding and dissociation.

Alternatively, if direct perturbation of the enzyme does not occur, can the mechanism appear to be coupled or concerted as a consequence of the proposed lateral organization of the lipids described above? Statistically, in the phospholipid-rich surface the DA should exist as individual molecules or clusters in a continuum of POPC. Because CEL adsorption is severely inhibited in such surfaces, there are presumably only a limited number of DA clusters of sufficient size for it to adsorb. Once adsorbed to a cluster, the enzyme molecule will be presented with the fatty acid molecules in that cluster. If those fatty acid molecules do not rapidly equilibrate with DA in other clusters, relative to the rate of ^{18}O exchange catalyzed by the enzyme, then each will rapidly undergo several cycles of exchange. In essence, the exchange mechanism may remain sequential, but not random. This is formally equivalent to a coupled exchange mechanism except that the DA remains in the proximity of the enzyme not as a consequence of enzyme–fatty acid binding in the classical sense, but by virtue of the fatty acid being confined to the enzyme-associated DA cluster. Within the cluster, translational and rotational diffusion of DA may still be rapid. The net result would be that those DA molecules in the enzyme-associated domain will rapidly become 16,16-DA, while those in other DA clusters will remain 18,18-DA. On a global level, the mechanism of ^{18}O exchange reaction would appear to be coupled.

For the above mechanism to operate, the enzyme must remain associated with the DA cluster long enough to catalyze the exchange of all ^{18}O in the fatty acid. The dissociation constant for CEL bound to films of DA only under the conditions used herein is 13 nM (Tsujita & Brockman, 1987). Affinity decreases if phospholipid is present for reasons relating to phospholipid interference with the adsorption rate (Tsujita et al., 1987; Figure 3). The dissociation rate has not been measured directly, but its highest likely value can be estimated (Brockman et al., 1973). At saturation with CEL, the surface concentration of enzyme is 4.2 pmol cm^{-2} (Tsujita & Brock-

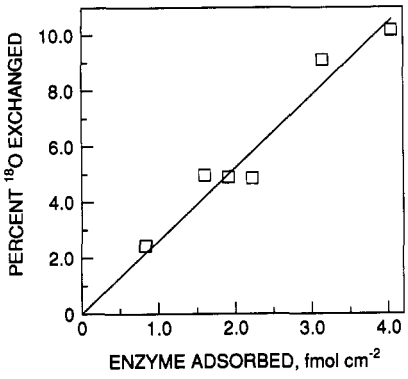

FIGURE 8: Determination of average DA cluster size. The change in the percentage of ^{18}O remaining is plotted versus the level of CEL adsorbed for each of the time points shown in Figure 7.

man, 1987), corresponding to a molar area of 2.4×10^{-11} cm^2 mol^{-1}. If adsorption is diffusion controlled with a diffusion constant of $\sim 7.0 \times 10^{-7}$ cm^2 s^{-1} and if the smallest unstirred layer possible is 10^{-4} cm (Drost-Hansen, 1971), then the maximum rate constant for dissociation is 2.2×10^{-3} s^{-1}. Thus, the half-life of the adsorbed protein should be ≥ 5 min. The same calculation performed using the rate constant for adsorption to DA of 3×10^{-5} cm s^{-1} determined in the preceding paper in this issue (Muderhwa et al., 1992) gives a half-life of about 20 h. However, this is an upper limit because adsorption is not stirring independent under the conditions used (H. L. Brockman and W. E. Momsen, unpublished). Although these half-lives are determined using data obtained in the absence of phospholipid, they indicate that the assumption of a long residence time for enzyme bound to a DA cluster is not unreasonable.

If CEL adsorbs to a cluster of DA molecules, exchanges ^{18}O, and remains bound, then the extent of ^{18}O exchanged and the quantity of enzyme adsorbed should be proportional. Plotting the changes in the data of Figure 7 against each other shows such a proportionality exists (Figure 8). From the slope and the total surface concentration of DA, the average cluster size is 1825 molecules. This corresponds to a diameter of 26 nm if the cluster is circular. For comparison, the diameter of the enzyme calculated from its molecular weight is about 7 nm. Although the prospect of measuring the average size of fatty acid "nanodomains" to which the enzyme binds is exciting, this preliminary result must be treated cautiously. More experiments will be needed to determine if the enzyme binds uniformly throughout the monolayer or only at its periphery and if the proportion of bindable clusters is constant with age of the film. Regardless of the outcome, the critical transition is clearly a global phenomenon of great importance to the regulation of expression of CEL activity. Whether or not it involves interfacial activation of CEL, it appears to be based on the lateral organization of the lipids which comprise the interface. Thus, other lipases may also respond similarly. The exact form this will take may vary, however, due to differences in the adsorption and catalytic characteristics of the specific enzymes.

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Serine Hydroxymethyltransferase: Origin of Substrate Specificity†

Sebastiana Angelaccio,‡ Stefano Pascarella,‡ Elena Fattori,‡ Francesco Bossa,‡ William Strong,§ and Verne Schirch*,§

Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298, and Dipartimento di Scienze Biochimiche and Centro di Biologia Molecolare del Consiglio Nazionale delle Ricerche, Università La Sapienza, 00185 Roma, Italy

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ABSTRACT: All forms of serine hydroxymethyltransferase, for which a primary structure is known, have five threonine residues near the active-site lysyl residue (K229) that forms the internal aldimine with pyridoxal phosphate. For *Escherichia coli* serine hydroxymethyltransferase each of these threonine residues has been changed to an alanine residue. The resulting five mutant enzymes were purified and characterized with respect to kinetic and spectral properties. The mutant enzymes T224A and T227A showed no significant changes in kinetic and spectral properties compared to the wild-type enzyme. The T225A and T230A enzymes exhibited differences in K_m and k_{cat} values but exhibited the same spectral properties as the wild-type enzyme. The four threonine residues at positions 224, 225, 227, and 230 do not play a critical role in the mechanism of the enzyme. The T226A enzyme had nearly normal affinity for substrates and coenzymes but had only 3% of the catalytic activity of the wild-type enzyme. The spectrum of the T226A enzyme in the presence of amino acid substrates showed a large absorption maximum at 343 nm with only a small absorption band at 425 nm, unlike the wild-type enzyme whose enzyme–substrate complexes absorb at 425 nm. Rapid reaction studies showed that when amino acid substrates and substrate analogues were added to the T226A enzyme, the internal aldimine absorbing at 422 nm was rapidly converted to a complex absorbing at 343 nm in a second-order process. This was followed by a very slow first-order formation of a complex absorbing at 425 nm. Variation of the initial rapid second-order process as a function of pH suggested that the anionic form of the amino acid forms the first complex with the enzyme. The results are interpreted as being due to the rapid formation of a *gem*-diamine complex between amino acids and T226A enzyme with a rate-determining formation of the external aldimine. This suggests that Thr-226 plays an important role in converting the *gem*-diamine complex to the external aldimine complex. Variation of the kinetic constants with amino acid structure suggests that the T226A enzyme distinguishes between substrates and substrate analogues in the formation of the *gem*-diamine complex.

Serine hydroxymethyltransferase (SHMT)[1] catalyzes the conversion of serine and tetrahydrofolate (H_4folate) to glycine and 5,10-methyleneH_4folate. This reaction is present in a wide variety of cells and is the major source of one-carbon groups required in the biosynthesis of methionine, choline, thymidylate, and purines (Schirch, 1982). We have previously purified and determined the primary structure of cytosolic and mitochondrial isoenzymes from rabbit liver (Martini et al., 1987, 1989). SHMT has also been purified and characterized from expression of the *Escherichia coli* cloned *glyA* gene (Plamann et al., 1983; Schirch et al., 1985). SHMT activity is dependent on the two coenzymes pyridoxal-P and H_4folate. Pyridoxal-P is covalently attached at the active site and serves as a spectrophotometric probe in determining the structure of enzyme–substrate intermediates in the reaction pathway (Schirch, 1982). As with all pyridoxal-P enzymes, the site of covalent attachment is an internal aldimine between the 4′-aldehyde group on the coenzyme and an ϵ-amino group of a lysyl residue (Davis and Metzler, 1972). Reduction of this external aldimine converts it to a stable secondary amine, which permits isolation of a peptide from proteolytic digests containing the bound pyridoxal-P (Bossa, et al., 1976). These active site peptides have been isolated and sequenced from numerous pyridoxal-P containing enzymes (Vaaler and Snell, 1989; Tanizawa, et al., 1989). The three forms of SHMT which we have studied all contain the nine-residue conserved sequence V-V-T-T-T-T-H-K(Pyr)-T (Martini et al., 1989).

The active-site nonapeptide from SHMT is unusual in that five Thr residues have been conserved in the *E. coli* enzyme and the mammalian isoenzymes in rabbit liver. This suggests that these Thr residues have some functional role in this en-

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* To whom correspondence should be addressed.
‡ Università La Sapienza.
§ Virginia Commonwealth University.

[1] Abbreviations: SHMT, serine hydroxymethyltransferase; H_4folate, tetrahydrofolate; pyridoxal-P, pyridoxal 5′-phosphate.

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