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Action of a Microbial Lipase/Acyltransferase on Phospholipid Monolayers†

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ABSTRACT: *Vibrio* species release a lipase which shares many properties with mammalian lecithin–cholesterol acyltransferase. We have studied the action of the enzyme on phospholipid monolayers. At similar surface pressures, reaction velocities were higher with monolayers of dilauroylphosphatidylcholine than with the corresponding phosphatidylglycerol or phosphatidylethanolamine. The dependence of reaction velocity on molecular density was very similar for phosphatidylcholine and phosphatidylethanolamine monolayers. Lag times were shortest with phosphatidylglycerol at low molecular densities, but maximum velocity was reached at considerably lower densities than with the other two lipids. We have found [Hilton, S., McCubbin, N. D., Kay, C., & Buckley, J. T. (1990) *Biochemistry 29*, 9072–9078] that nicking of the enzyme with trypsin or other proteases results in an increase in its activity against lipids in membranes. Here we show that trypsin treatment results in a large change in the surface activity of the lipase, allowing it to penetrate monolayers at pressures higher than 40 $mN \cdot m^{-1}$.

All of the lipases characterized so far appear to be members of a superfamily of esterases which are capable of catalysis at lipid–water interfaces (Kirchgessner et al., 1987, 1989; Komaromy & Schotz, 1987; Wion et al., 1987; Persson et al., 1989). Under the right conditions, lipases will hydrolyze virtually any ester bond, in contrast to the phospholipases, which are often highly specific for the polar head groups of their substrates. In addition, many lipases are active in nonaqueous systems where they may also catalyze stereospecific ester formation (Harwood, 1989). These attributes have generated considerable recent interest as well as proposals for a variety of biotechnological applications. In spite of this, the reaction mechanisms of these enzymes are not well understood. On the basis of active-site sequence homology with the serine proteases (Maraganore & Heinrikson, 1986), the structures of two lipases determined by X-ray crystallography (Winkler et al., 1990; Brady et al., 1990), and some circumstantial evidence obtained by chemical modification (Burstein et al., 1974; Jauhiainen & Dolphin, 1986; De Caro et al., 1989), it is generally assumed that the lipases all have a Ser-His-Asp amino acid triad at the active site. However, there is little direct evidence to support this assumption and no explanation for the differences in specificities and reaction rates which are observed among these enzymes.

The microbial glycerophospholipid–cholesterol acyltransferase (GCAT) and mammalian lecithin–cholesterol acyltransferase (LCAT) both contain short amino acid sequences homologous to the consensus sequence of the lipase superfamily (Maraganore & Heinrikson, 1986; Komaromy & Schotz, 1987; Persson et al., 1989). Like the other lipases, they will hydrolyze ester linkages at lipid–water interfaces, but they are distinguished by their ability to carry out acyl transfer from phospholipids to cholesterol in lipoproteins, bilayers, and inverted micelles (Buckley et al., 1982, 1984; Buckley, 1982, 1983). In addition, there is some evidence that their reaction mechanisms may differ from those of the other lipases. Thus, it has been suggested that at least one cysteine plays a role in LCAT-catalyzed acyl transfer (Jauhiainen & Dolphin 1986; Jauhiainen et al., 1988), and we have shown recently by site-directed mutagenesis that although the serine in the lipase consensus sequence of GCAT is absolutely required for activity, none of the histidines in the enzyme participate in hydrolysis or acyl transfer (Hilton & Buckley, 1990).

We have also shown that, under certain assay conditions, proteolytic nicking results in a profound increase in the activity of GCAT (Hilton et al., 1990). The bacterium releases the

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unprocessed form of the enzyme which is subsequently activated either in the culture supernatant or (presumably) by host proteases during infection. Activation is an important step, not only because it provides a way for the organism to protect itself from its own product but also because it yields an enzyme which is capable of degrading eucaryotic cells. Recently, it has been suggested that the 25-kDa (processed) form of the enzyme may be the most important pathogenic factor in the important fish disease furunculosis caused by *Aeromonas salmonicida* (Lee & Ellis, 1990). The activation process is also interesting from a biochemical point of view as it seems likely that it is due to an increase in the surface activity of the enzyme or to a change in its ability to bind substrate. Surface monolayers of lipids are convenient systems to probe protein–lipid interactions and catalysis at lipid–water interfaces (Verger & de Haas, 1973; Verger & Pattus, 1982), and they have been used to great advantage in studies of a variety of phospholipases and lipases (Verger et al., 1973, 1976; Rietsch et al., 1977; Pattus et al., 1979; Vainio et al., 1983). Here we examine the kinetics of phosphatidylcholine hydrolysis by native and processed GCAT, as well as the surface activity of the two forms of the enzyme, in an attempt to learn more about the mechanism of activation. In addition, we study the ability of native GCAT to hydrolyze monolayers consisting of different phospholipid classes.

Materials and Methods

Lipid Substrates. L-α-Dilauroylphosphatidylcholine (DLPC) was purchased from Sigma. L-α-Dilauroylphosphatidylglycerol (DLPG), L-α-dilauroylphosphatidylethanolamine (DLPE), and sphingomyelin (egg) were obtained from Avanti Polar Lipids. Lipid solutions were prepared in HPLC-grade chloroform.

Enzyme Preparation. GCAT was purified from cell-free culture supernatants of *Aeromonas salmonicida* CB3 pJT2 as described previously (Hilton et al., 1990). Protein concentration was calculated from OD_{280} values using the extinction coefficients we have determined (Hilton et al., 1990). Trypsinolysis was accomplished by incubating 100 μL of GCAT (0.5 mg/mL) with 0.1 μg of TPCK-trypsin (Sigma) at room temperature (22 °C) for 40 min. Nicking was shown to be complete by sodium dodecyl sulfate electrophoresis (Neville, 1971).

Monolayer Experiments. A KSV 2200 surface barostat, fitted with a Teflon trough (total volume 462 mL, surface area 47 020 mm^2, volume of reaction compartment 140 mL) was used to determine the rate of hydrolysis of lipid monolayers at constant surface pressure (Verger & de Haas, 1973; Verger & Pattus, 1982). The aqueous subphase of the reaction compartment was 20 mM Tris-HCl and 150 mM NaCl, pH 7.4, maintained at 25.0 ± 0.3 °C and mixed with two magnetic stirrers (250 rpm). Monolayers were formed by dropwise addition of lipid solutions to the buffer surface. After stabilization (approximately 10 min), 1.5 μg of enzyme was injected into the subphase of the reaction compartment. Surface pressure vs molecular area isotherms were determined independently for each of the phospholipids used. Barrier velocities were taken as the asymptotic slopes of the recorded curves of barrier movement and lag times as the intersections of the slopes with the time axis. Enzyme velocities were calculated by multiplying slopes by the width of the trough and the molecular density of the phospholipid at the specified surface pressures. All velocities and lag times are the means from no fewer than two very similar experiments.

Penetration experiments were carried out in 17-mL Teflon cylindrical troughs (surface area = 12.6 cm^2) with monolayers of sphingomyelin. The enzyme (40 μg) was injected under each stabilized monolayer, and the change in pressure was measured after 1 h.

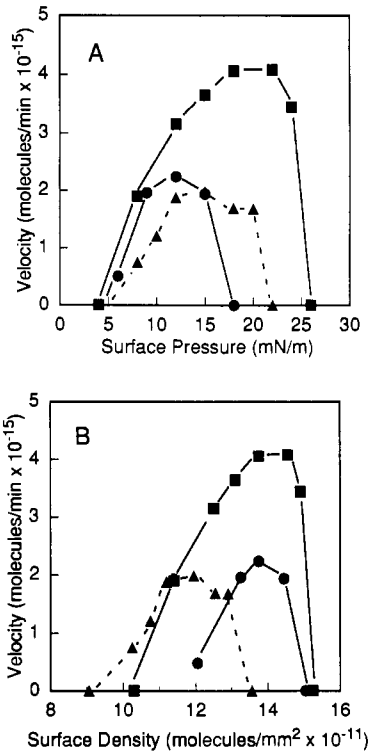

FIGURE 1: Hydrolysis of three phospholipids by native GCAT as a function of surface pressure (A) or surface density (B). Each point represents the mean of at least two separate determinations. (■) DLPC; (●) DLPE; (▲) DLPG.

Results

Hydrolysis of Phospholipid Monolayers by Native GCAT. Three different didodecanoyl phospholipids were used to examine the influence of the phospholipid head group on GCAT-catalyzed hydrolysis. In each case, reaction velocity showed the bell-shaped dependence on surface pressure which is characteristic of lipolytic enzymes (Verger et al., 1976; Vainio et al., 1983). Velocities were highest with DLPC monolayers at all surface pressures. The surface pressure at which maximum velocity was reached was lowest with DLPE (Figure 1A). Since monolayers of phosphatidylcholine are more expanded than monolayers of phosphatidylethanolamine at comparable surface pressures (Phillips & Chapman, 1968), expressing velocity as a function of molecular density led to a somewhat different pattern (Figure 1B). Reaction velocity was always higher with DLPC than with DLPE, as it was in Figure 1A, but the surface density at which maximum velocity was reached was the same with monolayers of both lipids. This suggests that packing of the acyl chains is more important than packing of the polar head groups in limiting penetration of the enzyme into monolayers of these lipids. Pattus et al. (1979) have drawn a similar conclusion for the penetration of phosphatidylcholine monolayers by phospholipase A_2. The dependence of lag time on surface pressure and on surface density is shown in Figure 2. The lag times for monolayers of all three lipids were relatively constant at low surface densities, and increased sharply at surface densities which corresponded to the values at which velocities began to decrease in Figure 1B. At low surface densities, the lag time for GCAT hydrolysis of DLPG monolayers was much lower than the lag times obtained with DLPC and DLPE. This may mean that GCAT

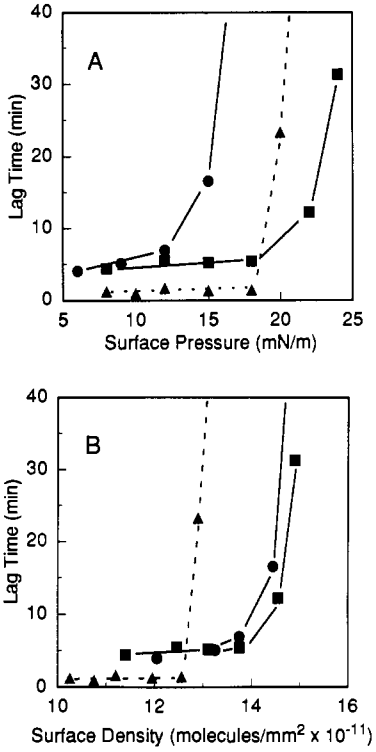

FIGURE 2: Effect of monolayer surface pressure (A) or surface density (B) on the lag time in the hydrolysis by native GCAT. See the legend to Figure 1.

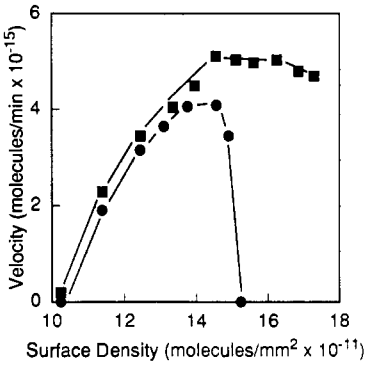

FIGURE 3: Rate of hydrolysis of monolayers of L-α-dilauroyl-PC by native (●) and processed GCAT (■) as a function of surface density. Each point represents the mean of at least two separate determinations.

has a higher affinity for the negatively charged DLPG interface than for zwitterionic interfaces of DLPC and DLPE.

Comparison of Phosphatidylcholine Monolayer Hydrolysis by Native and Trypsinized GCAT. The hydrolysis of DLPC monolayers was measured at different constant surface pressures using native and processed forms of GCAT. The results in Figure 3 show that although the unprocessed enzyme is unable to hydrolyze the monolayers above surface pressures of approximately 26 $mN \cdot m^{-1}$, trypsin-treated GCAT is capable of hydrolysis at pressures up to at least 40 $mN \cdot m^{-1}$, which is near the collapse pressure of the DLPC film. It is interesting to note that below the cutoff pressure for the native enzyme, the rates of hydrolysis are similar for both forms of the enzyme. Lag times for lipolysis by cut GCAT appear to be slightly lower than lag times for the native enzyme at corresponding pressures (Figure 4). This may mean that the processed enzyme can penetrate the lipid film more easily.

Penetration of Sphingomyelin Monolayers by Native and Trypsin-Treated GCAT. The decreased lag times for the action of trypsin-treated GCAT on monolayers of DLPC

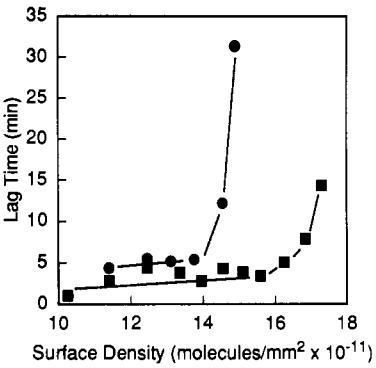

FIGURE 4: Lag time as a function of surface density for the hydrolysis of L-α-dilauroyl-PC by native (●) and trypsin-treated GCAT (■). See the legend to Figure 3.

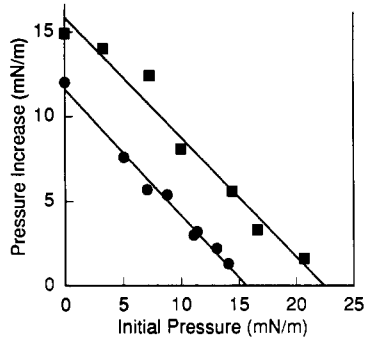

FIGURE 5: Penetration of sphingomyelin monolayers by native (●) and processed GCAT (■). The increase in surface pressure when enzyme (40 μg) was injected under a monolayer of egg sphingomyelin is presented as a function of the initial surface pressure.

(Figure 4) are evidence that processing increases the enzyme's surface activity. Another measure of surface activity or relative penetration capacity is the critical pressure, the initial surface pressure above which a protein cannot penetrate a monolayer (Verger & Pattus, 1982; Vainio et al., 1983; Gargouri et al., 1987, 1989). We measured the critical pressures of native and processed enzyme using sphingomyelin monolayers (sphingomyelin is not a substrate for the enzyme). The results, which are presented in Figure 5, show that the critical pressure was significantly higher for trypsinized GCAT than that for the native enzyme. This is further evidence that there is a large difference in the surface activity or penetration capacity of the two forms of the protein. It should be noted that in general the increase in surface pressure resulting after injection of a protein is not a quantitative measurement of protein penetration (Verger & Pattus, 1982). Therefore, it would be incorrect to conclude from the results in Figure 5 that more molecules of cut GCAT than of native enzyme penetrate the monolayer at a given pressure.

Discussion

We have shown that GCAT from *A. salmonicida* will catalyze acyl transfer to cholesterol from a variety of glycerophospholipids in micelles or liposomes, so it was not surprising to find that the same enzyme from *A. hydrophila* is able to hydrolyze monolayers of several phospholipid classes. Pattus et al. (1979) and later Jain et al. (1986) proposed schemes for interfacial catalysis by phospholipase A_2 in which interaction with the interface is assumed to occur in a single step. However, the differences we observed in the reaction of GCAT with the three dilauroyl phospholipids (Figures 1 and 2) are best explained if the interaction is a two-stage process, such as the one depicted in Figure 6. The first stage

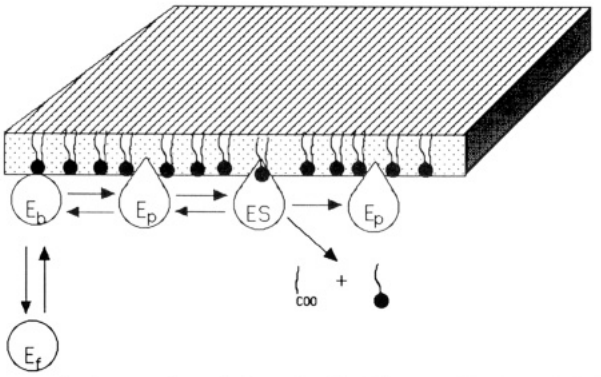

FIGURE 6: Interaction of the microbial lipase with phospholipid monolayers. The enzyme may first bind to the polar head groups at the interface (E_b). This is followed by penetration to form E_p, which is determined at least partly by packing of the acyl chains. The surface charge of the monolayer may also affect penetration. The next step is the formation of the enzyme–substrate complex. Presumably this is influenced by the affinity of the substrate for the active site and the ease of removal of monomers from the monolayer. Binding of substrate is followed by hydrolysis and the loss of the fatty acyl and lysophospholipid products into the aqueous subphase.

might be binding of the lipase to the phospholipid polar head groups. Although this step may not be essential (and our unpublished results show that it certainly is not rate-limiting), it would effectively increase enzyme concentration at the interface and could affect the rate of penetration to the acyl chains in the second step, which determines the lag time. The enzyme may have a higher affinity for the anionic surface of DLPG. At low surface densities, increased binding would lead to a higher penetration rate and correspondingly lower lag time as we saw in Figure 2. As molecular density increases, however, the crowding of negative charges at the interface may inhibit the penetration stage. This would account for the bimodal shape of the velocity curves and for the observation that velocity declined at a lower surface density than was observed for the other two lipids.

The lag times and reaction velocities for hydrolysis of monolayers of DLPE and DLPC show similar dependence on molecular density, indicating that the enzyme's ability to bind to and penetrate both kinds of monolayers is the same. A difference somewhere else in the reaction scheme in Figure 6 must account for the observation that the velocity of DLPC hydrolysis was always about twice the velocity with DLPE. It has been argued that lipolytic enzymes have separate binding sites and active sites (Maraganore & Heinrikson, 1986; De Caro et al., 1989; Hilton & Buckley, 1990), with the former being responsible for the interaction at the oil–water interface and the latter for catalysis. The binding/penetration site of GCAT may not discriminate between the two zwitterionic lipids, whereas the active site may have a higher affinity for DLPC. Other lipases appear to have different specificities. Laboda et al. (1986) have presented evidence that the active site of hepatic lipase has a higher affinity for PE than for PC, whereas lipoprotein lipase appears to hydrolyze PE and PC at similar rates (Jackson & Demel, 1985).

Perhaps the most interesting result of this study is the observation that processed GCAT is able to penetrate and hydrolyze DLPC monolayers at much higher surface pressures than the native enzyme, although the two forms of the enzyme are equally active when the pressure is low. This suggests that it is the binding site of the enzyme and its surface activity which are affected by processing and that the active site itself is not changed. Natural membranes are believed to have surface pressures of 30 $mN \cdot m^{-1}$ (Demel et al., 1975; Blume, 1979) or more, and they would therefore only be vulnerable to the cut form of the enzyme, implying that processing has some physiological importance. Presumably it accounts for the ability of the bacteria to export the protein without self-destruction. Since activation does not occur until the protein is outside, the vulnerable inner and outer membranes are never exposed to a form of the enzyme which is capable of degrading them.

The change in the surface activity with processing also explains the differences we have found previously between the activities of native and cut GCAT when different assay systems are used (Hilton et al., 1990). Thus, processed enzyme is much more active against erythrocyte membranes, which must have high enough surface pressures to limit penetration of the native enzyme. On the other hand, the lower surface pressures of cholesterol/phosphatidylcholine/Triton-X 100 micelles permit both forms of the enzyme to act at similar rates, and there is no difference in the rate of hydrolysis of monodisperse *p*-nitrophenyl esters.

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Registry No. DLPC, 18194-25-7; DLPG, 63644-55-3; DLPE, 59752-57-7; glycerophospholipid–cholesterol acyltransferase, 68009-86-9; trypsin, 9002-07-7.

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Raman Spectroscopic Characterization of Tryptophan Side Chains in Lysozyme Bound to Inhibitors: Role of the Hydrophobic Box in the Enzymatic Function†

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ABSTRACT: The state of H-bonding and the hydrophobic interaction of six tryptophan side chains in lysozyme bound to substrate-analogous inhibitors were investigated by combining H → D exchange labeling and Raman difference spectroscopy. The frequency of the W17 band due to Trp-63 shifts downward upon inhibitor binding, indicating a specific and strong H-bond formation between the N_1 site of the side chain and the inhibitor molecule. On the other hand, the H-bonding state of Trp-62 in the complex is as weak as that in inhibitor-free lysozyme, suggesting no contribution of this residue to the inhibitor binding. Intensity increases of W17 and W18 bands observed upon inhibitor binding are, respectively, ascribed to an increase at Trp-28 and a decrease at Trp-111 in hydrophobic interactions with the environment. The environmental changes are explained consistently by a movement of the Met-105 side chain sandwiched by two indole rings of Trp-28 and 111 in the direction from Trp-111 to Trp-28. The sandwich structure in a core domain, hydrophobic box, and its rearrangement are considered to play an important role in the enzymatic function of lysozyme.

Lysozyme catalyzes the hydrolysis of β-1,4-glycosidic linkages of cell-wall mucopolysaccharides (Jeanloz et al., 1963) and chitin (Berger & Weiser, 1957), a polymer of *N*-acetyl-D-glucosamine. The amino acid composition of hen egg white lysozyme is characterized by high contents of tryptophan (6 Trp at sequence positions 28, 62, 63, 108, 111, and 123 among 129 residues) as well as cystine (4 Cys–Cys) and basic residues (11 Arg + 6 Lys). According to an X-ray diffraction study on a tetragonal crystal of the lysozyme–inhibitor complex (Blake et al. 1967), the active site is composed of six subsites, named A through F, which accommodate six consecutive saccharide units. In subsite C, Trp-62 and -63 are exposed at the edge of active site cleft, and Trp-108 is partially buried underneath the active site. Trp-62 and -63 interact specifically with the inhibitor through hydrogen bonds (H-bonds)[1] from their indole N_1H groups to the oxygen atoms of a saccharide unit that binds to site C. In a recent X-ray study on an orthorhombic form crystallized at 38 °C, however, the H-bond between Trp-62 and the incorporated inhibitor was not found (Bernard et al., 1990). In aqueous solution, on the other hand, the lysozyme–inhibitor interaction has been examined by NMR. The N_1H NMR signals of Trp-63 and -108 shift upon inhibitor binding, but that of Trp-62 remains practically un-

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[1] Abbreviations: GlcNAc, an equilibrium mixture of the α- and β-form of *N*-acetyl-D-glucosamine; $(GlcNAc)_3$, *N*,*N*′,*N*″-triacetylchitotriose; Ac-L-TrpME, *N*-acetyl-L-tryptophan methyl ester; Ac-DL-TrpMA, *N*-acetyl-DL-tryptophan methylamide; L-Trp·HCl, L-tryptophan hydrochloride; H-bond, hydrogen bond; NMR, nuclear magnetic resonance.

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