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ARTICLE *in* BIOCHEMISTRY · JUNE 1994

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Characterization of Interfacial Catalysis by *Aeromonas hydrophila* Lipase/Acyltransferase in the Highly Processive Scooting Mode†

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Received August 11, 1993; Revised Manuscript Received December 10, 1993®

ABSTRACT: A glycerophospholipid:cholesterol acyltransferase (GCAT) that also has lipase activity is secreted by the bacterium *Aeromonas hydrophila*. Hydrolysis of the *sn*-2-ester bond of 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol (DMPM) vesicles by this enzyme is shown to occur in a highly processive scooting mode in which the enzyme, substrate, and the products of hydrolysis remain bound to the vesicle interface. This conclusion is based on the following observations. (a) When there is an excess of vesicles over enzyme, the hydrolysis of the *sn*-2-acyl group ceases after only a fraction of the total available substrate is hydrolyzed. Addition of more enzyme, but not of more substrate, leads to a new round of hydrolysis. (b) The extent of hydrolysis of vesicles per enzyme increases with the size of the vesicles, and it corresponds to the total hydrolysis of the outer monolayer of one vesicle by one enzyme. (c) The enzyme bound to vesicles composed of reaction products or of the non-hydrolyzable phospholipid 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol (DTPM) is not able to undergo intervesicle exchange. Instead, intervesicle transfer of the substrate or the bound enzyme due to vesicle fusion promotes hydrolysis of all of the vesicles present in the reaction mixture. (d) Addition of DTPM vesicles to a reaction mixture containing DMPM substrate vesicles and the enzyme has no noticeable effect on the course of hydrolysis. Substrate specificity studies in the scooting mode on DMPM vesicles reveal that GCAT displays essentially no selectivity in the hydrolysis of phospholipids with different polar head groups. Treatment of GCAT with trypsin, which removes a small peptide, results in an enzyme that displays comparable catalytic activity but increased affinity for the interface. Alkyltrifluoromethyl ketones are shown to be tight-binding competitive inhibitors of GCAT. The scooting mode analysis, which has previously been shown to provide a simplified approach for analyzing the steady-state kinetics of interfacial catalysis by secreted phospholipase A_2, is also useful for analyzing the interfacial kinetic behavior of lipases.

The concentration of naturally-occurring phospholipids as solitary monomers in the aqueous phase is very low (Cevc & Marsh, 1987; Jain, 1988) because the hydrophobic effect on such amphiphiles promotes formation of aggregates such as vesicles, micelles, or emulsions. For such reasons, lipolytic enzymes have evolved to carry out interfacial catalysis with great efficiency, whereas solitary substrate molecules in the aqueous phase are relatively poor substrates. As a class, lipases tend to act rather nonspecifically on ester linkages and display a preference for the substrate at the interface (Waite, 1987). Those lipases that have been characterized are serine esterases with no divalent cation requirement. Among lipases, the glycerophospholipid:cholesterol acyltransferase (GCAT)[1] secreted by *Aeromonas hydrophila* is particularly interesting. GCAT transfers the *sn*-2-acyl chain of phospholipids to cholesterol (Buckley et al., 1982; Buckley, 1982, 1983). This transacylation reaction is essentially identical to that of the well-known enzyme lecithin:cholesterol acyl transferase of mammalian plasma. In addition, GCAT hydrolyzes the *sn*-2-ester linkage of phospholipids and other esters in the aqueous phase as well as at the lipid–water interface (Buckley, 1982, 1983; Buckley et al., 1982, 1983; Hilton & Buckley, 1991). The mechanism of interfacial catalysis by such lipases and the factors regulating their partial reactions are not known.

To elaborate a kinetic scheme for interfacial catalysis by GCAT, we have used the protocols that have been successfully developed to study secreted 14-kDa phospholipases A_2 which hydrolyze only the *sn*-2-ester linkages of phospholipids. These enzymes contain calcium as a catalytic cofactor, and lipolysis does not involve acyl enzyme formation (Dennis, 1983; Jain & Berg, 1989; Scott et al., 1990; Thunnissen et al., 1990; Verheij et al., 1981). It is now well established that interfacial catalysis by phospholipase A_2 can be analytically described by Scheme 1. The key elements of this scheme are that the

† Supported by grants from Sterling Inc. (to M.K.J. and M.H.G.), the National Institutes of Health (GM29703 to M.K.J. and Research Development Award GM562 and HL36235 to M.H.G.), and the British Columbia Heart and Stroke Foundation (to J.T.B.). M.H.G. is a Fellow of the Alfred P. Sloan Foundation (1991–1993).

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: $AACOCH_3$ and $AACOCF_3$, analogues of arachidonate in which the carboxyl group is replaced by $COCH_3$ and $COCF_3$; GCAT, lipase/acyltransferase from *Aeromonas hydrophila*; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; DTPC, 1,2-ditetradecyl-*sn*-glycero-3-phosphocholine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; k_{cat}, rate constant for catalytic turnover; k_i, first-order rate constant under substrate-limited conditions for the hydrolysis of all the substrate molecules present in the outer monolayer of the substrate vesicle; K_M^*, interfacial Michaelis–Menten constant for the substrate; K_P^*, interfacial dissociation constant for the products of hydrolysis; N_S, number of substrate molecules in the outer monolayer of the substrate vesicles; T-GCAT, catalytically active trypsin cleavage product of GCAT; v_0, the initial rate of catalytic turnover at substrate mole fraction of 1. All radiolabeled phospholipids contain the designated isotope in the *sn*-2-acyl chain.

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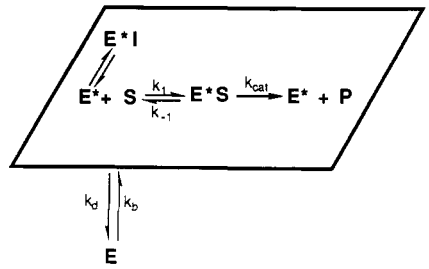
Scheme 1: Minimal Kinetic Scheme for Interfacial Catalysis That Can Account for the Various Features of the Kinetics of Hydrolysis of DMPM Vesicles by GCAT and T-GCAT[a]



[a] Here the enzyme in the aqueous phase (E) binds to the interface (E*), where it can undergo catalytic turnover according to Michaelis–Menten kinetics. The processivity of the enzyme at the interface depends upon the relative rate at which the enzyme leaves the interface during the catalytic turnover time. Thus, for example, in the virtually infinitely processive scooting mode, the bound enzyme does not leave the interface even when all of the substrate has been hydrolyzed. The other extreme is fast hopping, where the enzyme leaves the interface after each catalytic turnover cycle.

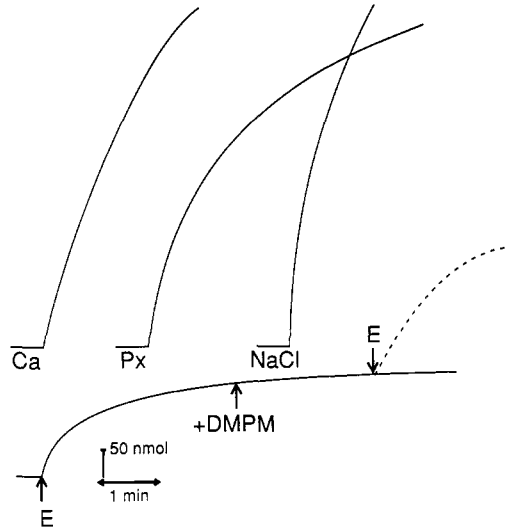


FIGURE 1: (Bottom) Reaction progress curve for the hydrolysis of DMPM vesicles (650 nmol in 4 mL of 1 mM NaCl at pH 8.0) by the addition of 43 pmol of T-GCAT from a stock solution in 2 M KCl and 10 mM Tris at pH 8.0. Addition of more DMPM at any point after the initiation of the reaction did not change the course of the reaction progress. On the other hand, addition of more enzyme led to a new round of hydrolysis, as shown by the dotted curve. (Top) The progress curves in the presence of either 2.5 mM $CaCl_2$, 30 μg of polymyxin B sulfate (Px), or 0.5 M NaCl obtained under the same conditions.

enzyme in the aqueous phase (E) must bind to the interface (E*) for the catalysis to occur [for a review of early literature, seen Jain (1972)] and that the enzyme in the interface must bind a single substrate molecule for the catalytic turnover in the interface (Verger & de Haas, 1976). Many kinetic aspects of lipolysis by secreted phospholipases A_2 have been analytically described by monitoring the reaction kinetics in the highly processive scooting mode, where the enzyme does not desorb from the vesicle interface during several thousand catalytic turnover cycles (Jain et al., 1986a, 1991a, 1993; Berg et al., 1991). This analysis provides a basis for the proper evaluation of competitive inhibitors, to determine the substrate preferences in the interface and to interpret more complex kinetic features arising from the perturbation of the substrate interface. In this paper, we have characterized the *sn*-2-esterolytic activity of GCAT and its trypsin cleavage product (T-GCAT) on vesicles of anionic and zwitterionic phospholipids. Results show that the key features of interfacial catalysis in the scooting mode for the action of GCAT on vesicles can be described within the context of Scheme 1. More complex behavior results under conditions where the enzyme, substrate, or products of hydrolysis can exchange between the vesicles or interfaces.

EXPERIMENTAL PROCEDURES

Materials. The cloned structural gene of GCAT from *A. hydrophila* was overexpressed in *Aeromonas salmonicida* strain CB3 pJT2 and purified from cell culture supernatants as described (Hilton et al., 1990). *DMPM* (Jain & Gelb, 1991), DTPM and DTPC (Jain et al., 1986a,c), 1- and 1,2-thiol analogues of substrates (Jain et al., 1992a), and *N*-dansylhexadecylphosphoethanolamine (Jain & Vaz, 1987) were prepared as described. The arachidonic acid analogues $AACOCF_3$ and $AACOCH_3$ were synthesized as described (Street et al., 1993). The expression system for the Y230F mutant of GCAT will be described elsewhere. All other phospholipids are from Avanti Polar Lipids. Myristoylcholine and polymyxin B sulfate are from Sigma. The sources of the radiolabeled phospholipids are as follows: [^{14}C]dipalmitoylphosphatidylcholine (55 mCi/mmol, Amersham); [^{14}C]-1-stearoyl-2-arachidonoylphosphatidylcholine (52 mCi/mmol, NEN); [^{3}H]dipalmitoylphosphatidylcholine (50 Ci/mmol, NEN); [^{14}C]-1-stearoyl-2-arachidonoylphosphatidylinositol (50 mCi/mmol, NEN); [^{3}H]dipalmitoylphosphatidic acid (5.5 mCi/mmol); and [^{3}H]dipalmitoylphosphatidylethanolamine (12 mCi/mmol) (Ghomashchi et al., 1991). All radiolabeled phospholipids contain the designated isotope in the *sn*-2-acyl chain.

Kinetic Measurements. Preparation and characterization of sonicated vesicles and the kinetic protocols for monitoring the hydrolysis of vesicles with a pH-stat have been previously established for DMPM (Jain et al., 1986a; Jain & Gelb, 1991; Berg et al., 1991) and DMPC (Apitz-Castro et al., 1982) as substrates for phospholipase A_2. These protocols were satifactorily adopted for the hydrolysis by GCAT and T-GCAT without significant changes, and some of the early results also serve as suitable controls for the results reported here. Typically, the reaction mixture at room temperature (23 °C) contained 1 mM NaCl at pH 8.0, and the reaction was initiated by the addition of 0.05–1 μg of enzyme. GCAT and T-GCAT exhibit a tendency to aggregate in buffer of low ionic strength (reveled by analytical ultracentrifugation studies, J. T. Buckley, unpublished results) and to adsorb on glass or plastic surfaces. Therefore, 1–20-μL aliquots of stock solutions of GCAT and TCAT (>0.1 mg/mL) in 1 M NaCl, 0.1 mg/mL γ-globulin (Sigma), and 10 mM Tris-HCl at pH 8.0 were added for initiating the reaction progress. Typically, the time course of hydrolysis by pH-stat titration (Radiometer) was monitored as the volume of 3 mM NaOH required for the neutralization of the protons from released fatty acids. The concentration of the base and the titration efficiency were calibrated by titrating a known amount of myristic acid in the presence of substrate vesicles (Jain et al., 1986a; Jain & Gelb, 1991; Berg et al., 1991).

TLC and NMR analyses of the products formed during the initial course of the reaction showed that only the *sn*-2-acyl chains of the short-chain substrates were hydrolyzed, even in the presence of excess enzyme and after long periods of incubation. Also under the kinetic conditions employed, only the *sn*-2 chain was released from the long-chain phospholipids. Noticeable hydrolysis of the *sn*-1 chain was indicated on a time scale of more than 1 h. Therefore, under the conditions used for monitoring the kinetics (typically <15 min), only hydrolysis of the *sn*-2-acyl chain was occurring.

Preparation of T-GCAT. A solution of 0.3 mg of GCAT in 0.2 mL of 10 mM Tris buffer at pH 7.5 and 23 °C was incubated with 4.0 μg of TPCK-inactivated trypsin (Sigma). Trypsin was devoid of any phospholipase A_2 activity since no product formation was detected when milligram quantities were added to the pH-stat assay with DMPM vesicles. Aliquots from this mixture were tested at different time periods for lipolytic activity using the pH-stat assay with DMPM vesicles as substrate. Results indicated that nicking of GCAT was complete after about 15 min, and further incubation of the mixture at room temperature did not cause any noticeable change in the catalytic properties of the enzyme. This observation is consistent with earlier studies in which it was shown that trypsin cleavage under the same conditions converts the 33-kDa form of GCAT to a single active fragment of 27 kDa and that T-GCAT is quite resistant to further modification by trypsin (Hilton et al., 1990).

Binding of GCAT to Vesicles. Binding studies were carried out by monitoring the fluorescence emission at 335 nm from the tryptophan residues (six present in GCAT) using a SLM 4800S spectrofluorimeter equipped with emission and excitation monochromators. The excitation wavelength was set at 292 nm, and the slit widths for the excitation and emission beams were 4 nm. All fluorescence measurements were carried out in 10 mM Tris buffer at pH 8.0 and 23 °C. Specific conditions and concentrations are given in the figure legends. Protocols for monitoring the binding of the lipases to phospholipid vesicles by the resonance energy-transfer method are the same as developed for phospholipase A_2 (Jain & Vaz, 1987).

Substrate Specificity Studies. Sonicated vesicles of DMPM (0.6 mg in 0.05 mL of water) spiked with ^{14}C- (25–90 nCi) and ^{3}H-labeled (200–500 nCi) substrates were added to 4 mL of 1 mM NaCl in a pH-stat reaction vessel at 21 °C, pH 8.0. The reaction was initiated with 0.9 μg of GCAT. The reaction was stopped after about 1 min by the addition of extraction solvent. Extraction and isolation of the fatty acid product was as described previously (Ghomashchi et al., 1991), except in the case of 1,2-dipalmitoyl-*sn*-glycerol, which required separation of the monoglyceride and the fatty acid by silica gel TLC using petroleum ether:ether:acetic acid (70:30:1). ^{14}C and ^{3}H channel counts were corrected and relative k_{cat}/K_M values calculated from the ratio of counts for the substrates (S_1 and S_2) and for their respective products (P_1 and P_2):

$$(k_{cat}/K_M)_1/(k_{cat}/K_M)_2 = P_1S_2/P_2S_1$$

In all cases, 2–2.5% of the total radiolabeled substrates were hydrolyzed, producing counts per minute of product that were at least 10-fold above background obtained in the absence of enzyme.

RESULTS

Hydrolysis of DMPM Vesicles by GCAT and T-GCAT. The reaction progress curves for the hydrolysis of DMPM vesicles by GCAT and T-GCAT exhibited all of the features characteristic of the hydrolysis occurring in the highly processive scooting mode in which the enzyme remains bound to vesicles (Jain et al., 1986a; Jain & Berg, 1989). As shown in Figure 1 (bottom), hydrolysis of sonicated DMPM vesicles by GCAT starts immediately after the addition of the enzyme. In the presence of vesicles in excess over enzymes, the reaction ceases when only a fraction of the total available substrate is hydrolyzed (Figure 1, bottom). On the other hand, in the presence of enzyme in excess of vesicles, all of the substrate in the outer monolayer of the vesicles (60% of the total DMPM)

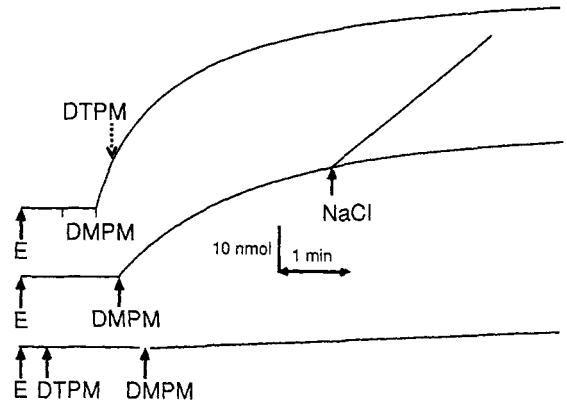


FIGURE 2: Reaction progress curves. (Bottom) T-GCAT (30 pmol in 10 mM KCl) was added to a suspension of DTPM (140 nmol) vesicles, and then 650 nmol of DMPM vesicles was added after 1 min. (Middle) T-GCAT (30 pmol) was added to a suspension of DMPM (650 nmol) vesicles. NaCl (0.1 M final concentration) added near the end of the reaction progress led to a reinitiation of the hydrolysis. (Top) DMPM (650 nmol) was added to T-GCAT (43 pmol) in 1 mM NaCl, and then the reaction was allowed to proceed to completion. The reaction was allowed to proceed uninterrupted, or 140 nmol of DTPM vesicles was added at the arrow. In both cases, the reaction progressed with the same time course.

is hydrolyzed within 20 min, and 100% of the substrate is hydrolyzed after several hours of incubation. These results indicate that all of the vesicles are accessible to the enzyme and that strong product inhibition is not the reason for the reaction cessation. As also shown in the upper part of Figure 1 and elaborated later in this paper, the cessation of the hydrolysis is not due to the inactivation of the enzyme at the interface but due to the depletion of the substrate in the enzyme-containing vesicles. Thus, fusion of vesicles by calcium, or intervesicle transfer of lipids by polymyxin B, or intervesicle exchange of the enzyme promoted by higher salt concentration leads to the hydrolysis of all the substrate present in the reaction mixture (Figure 1, top).

The reaction progress curve for the hydrolysis of a monodisperse population of small sonicated DMPM vesicles by GCAT and T-GCAT is first-order, i.e., the velocity decreases at each point in time (Figure 1, bottom). The first-order exponential constant that describes this curve under the substrate-limiting conditions of small vesicles (Jain et al., 1986a) is designated as k_i. On the other hand, with larger vesicles or with a polydisperse population of vesicles, more complex progress curves were obtained (results not shown). Such progress curves suggest that the enzyme binds essentially irreversibly to DMPM vesicles (Berg et al., 1991). The high-affinity interaction of T-GCAT with DMPM vesicles is underscored by results summarized in Figure 2. T-GCAT added to vesicles of the nonhydrolyzable diether phospholipid analogue, DTPM, is not available for the hydrolysis of DMPM vesicles added subsequently. On the other hand, DMPM vesicles added to T-GCAT are hydrolyzed immediately. As also shown in Figure 2, addition of DTPM vesicles during the course of hydrolysis of DMPM vesicles does not change the time course of hydrolysis. Similarly, additional DMPM vesicles added at the end of the progress curve are not hydrolyzed by the enzyme added initially to DMPM vesicles (not shown). All of these results establish the irreversible nature of the enzyme–vesicle interaction for GCAT and T-GCAT at low salt concentration.

Additional features of the reaction progress under the conditions used for the results obtained in Figures 1 or 2 may be noted. With vesicles in excess over enzymes, adding more enzyme to the reaction mixture at the end of the first round of hydrolysis initiated further hydrolysis (Figure 1, bottom).

Table 1: Kinetic Parameters for the Hydrolysis of DMPM Vesicles by GCAT, Its Y230F Mutant, and T-GCAT

parameter	GCAT	Y230F	T-GCAT
v_0 (s^{-1})	170	38	185
N_S	5950	5570	5400
$N_S k_i$ (s^{-1})	40	14	42
O/S ratio[a]	0.8	0.6	
$X_I(50)$ with			
$AACOCF_3$	0.015	0.023	
$AACOCH_3$	0.14	0.19	
$C_{15}H_{31}COCF_3$	0.08	0.08	
$n_I(50)$ with $AACOCF_3$	0.057		

[a] This is the ratio of v_0 obtained for the hydrolysis of vesicles of DMPM and 1,2-dimyristoyl-*sn*-1,2-mercaptoglycero-3-phosphocholine.

With larger vesicles, the extent of hydrolysis per enzyme under the condition of excess vesicles over enzymes also increased (results not shown). Taken together, these results show that the cessation of the reaction is due to the local depletion of substrate in the enzyme-containing vesicles and that the enzyme does not hop from one vesicle to another to sample the excess vesicles.

Deconvolution of the Reaction Progress Curve. With the kinetic constraints established so far, the time course of hydrolysis in the scooting mode of a monodipserse population of DMPM vesicles can be rigorously analyzed, provided the enzyme-containing vesicles have at most one enzyme per vesicle (Berg et al., 1991). By curve-fitting the reaction progress curves obtained under such constraints to the integrated Michaelis–Menten equation, it is possible to obtain the initial rate of hydrolysis per enzyme, v_0, at the maximum attainable mole fraction of the substrate (mole fraction 1). The parameter v_0 is most reliably obtained from the initial portion of the reaction progress curve for the action of GCAT on large DMPM vesicles. This is because with small sonicated DMPM vesicles, the depletion of substrate occurs relatively quickly in time, and the value of v_0 cannot be obtained as the tangent of the first-order reaction progress curve (Figure 1, bottom) at zero time (Berg et al., 1991). From the progress curve at longer times, the apparent second-order rate constant for the enzymatic turnover ($N_S k_i$) under the substrate-limiting conditions is also obtained. N_S is the number of DMPM molecules in the outer monolayer of the vesicles, and, as described below, in the presence of an excess of vesicles, it is also the extent of the reaction per enzyme. The values of v_0 and $N_S k_i$ for GCAT and T-GCAT are summarized in Table 1. The results show that the catalytic properties of GCAT and T-GCAT are essentially identical.

The steady-state rate parameters for the hydrolysis of vesicles in the scooting mode are related to the primary rate and equilibrium parameters as follows (Berg et al., 1991):

$$v_0 = \frac{k_{cat}}{1 + K_M^*} \quad (1)$$

$$N_S k_i = \frac{k_{cat}}{K_M^*\left(1 + \frac{1}{K_P^*}\right)} \quad (2)$$

Here K_M^*, K_P^*, and k_{cat} have their usual meanings for the catalytic turnover occurring in the interface according to Scheme 1. The asterisks denote that the equilibria are for the species present in the interface.

Experiments indicate that the extent of the reaction per enzyme is essentially identical to the number of DMPM molecules in the outer monolayer of small sonicated vesicles

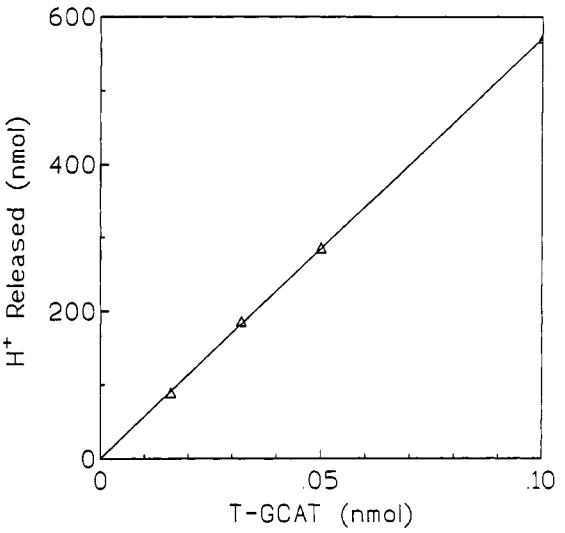


FIGURE 3: Extent of hydrolysis of 1 mM DMPM as a function of the amount of T-GCAT added to the reaction mixture. Other conditions are as given in the legend to Figure 1.

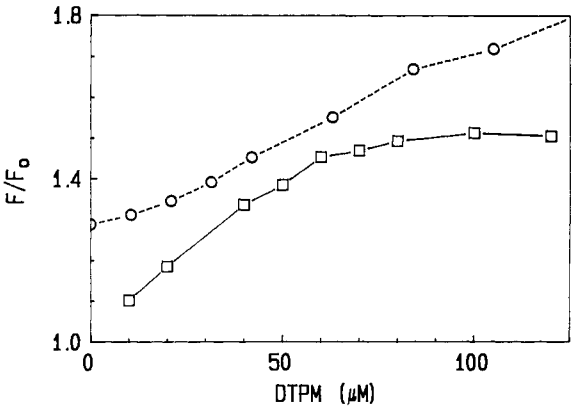


FIGURE 4: Change in fluorescence emission intensity at 335 nm as a function of DTPM concentration added to (□) 0.85 μM T-GCAT or to (○) 0.85 μM GCAT. The fluorescence emission intensities are normalized to the intensity of T-GCAT.

(N_S). As shown in Figure 3, with vesicles in excess over enzymes, the extent of substrate hydrolysis increases linearly with the amount of T-GCAT added initially to the reaction mixture. The slopes of such linear plots gave the extent of hydrolysis per enzyme. As summarized in Table 1, the extent of reaction per enzyme for sonicated DMPM vesicles is between 5400 and 6000 for the progress curves obtained in the presence of low salt (1 mM KCl or NaCl) in the reaction mixture. The extent of reaction per enzyme for the hydrolysis of the same vesicle preparation by the pig pancreatic phospholipase A_2 is 5500. It has been previously shown that the pig pancreatic phospholipase A_2 hydrolyzes essentially all of the DMPM molecules in the outer monolayer of DMPM vesicles and that the enzyme operates as a monomer (Berg et al., 1991; Jain et al., 1991b). Thus, taken together, the data indicate that GCAT or T-GCAT hydrolyze essentially all of the DMPM in the outer monolayer of DMPM vesicles and that the extent of reaction per enzyme (Table 1 and Figure 3) is equal to N_S. The results described thus far unequivocally demonstrate that GCAT and T-GCAT bind essentially irreversibly to vesicles of DTPM, DMPM, or the products of hydrolysis of DMPM at low salt concentrations. These results also show that as a prelude to the high-affinity binding of GCAT and T-GCAT to anionic vesicles, it is not necessary that the enzyme undergo catalytic turnover, nor is it necessary that the interface contain the substrate or the products of hydrolysis.

Binding of GCAT and T-GCAT to the Interface. The binding of GCAT and T-GCAT to DTPM vesicles was compared by spectroscopic methods. As shown in Figure 4, titration of the protein with DTPM vesicles resulted in an increase in the fluorescence emission intensity at 335 nm, presumably due to perturbation of one or more of the six tryptophan residues present in the enzyme molecule. As also shown in this figure, the fluorescence emission intensity of T-GCAT is 30% lower than that of GCAT, possibly because the environment of some of the tryptophan residues is altered after the proteolytic cleavage. The steepness of the initial region of the binding isotherm and the concentration of DTPM required to reach the maximum suggest that the affinity for the binding of GCAT to the interface is noticeably lower than that of T-GCAT. As shown in Figure 4, titration of T-GCAT with DTPM vesicles yields an endpoint in the increase of the fluorescence intensity. From the extrapolation of the initial region to the maximum change in the fluorescence change, it can be calculated that one molecule of T-GCAT binds to approximately 45 molecules of DTPM.

Binding of GCAT and T-GCAT to DTPM vesicles was also measured by energy transfer from the tryptophan residues to the dansyl fluorophore of *N*-dansylhexadecylphosphoethanolamine localized at the interface of the DTPM vesicles. Results confirm that both GCAT and T-GCAT bind to the DTPM interface. The apparent efficiency of energy transfer from GCAT was more pronounced, probably because the emission intensity from the donor tryptophans in GCAT is higher than it is in T-GCAT (Figure 4). Binding of GCAT to DTPC vesicles was also examined by monitoring the change in the intrinsic fluorescence of GCAT or by monitoring resonance energy transfer from the fluorescence emission of GCAT to the dansyl acceptor localized at the interface. No binding of GCAT to DTPC was detected with lipid concentrations up to 0.5 mM, which indicates that the K_d is significantly larger than 0.5 mM. On the other hand, in the presence of 15 mol % products of hydrolysis of DMPC (lysophosphatidylcholine and fatty acid in 1:1 mole ratio), significant binding of the enzyme to the DTPC interface was observed, indicating that GCAT and T-GCAT bind with a higher affinity to anionic interfaces.

Kinetics of Hydrolysis by the Y230F Mutant of GCAT. All the features of interfacial catalysis in the scooting mode observed with wild-type GCAT were also observed with the Y230F mutant. As summarized in Table 1, N_s for the mutant is the same as that for the wild-type enzyme, whereas the values of the rate parameters, $N_S k_i$ and v_0, for the mutant are lower. These results suggest that the side chain of tyrosine-230 is involved in the events of the catalytic turnover cycle rather than the interfacial binding. Independent studies (Robinson et al., in preparation) also support this conclusion.

Both GCAT and the Y230F mutant hydrolyze the *sn*-2-oxy and *sn*-2-thiol ester analogues of DMPM and DMPC. In both cases, the relative rate of hydrolysis of the *sn*-1,2-dithiol analogue is less than 2-fold higher than that for the dioxy analogues (O/S ratio in Table 1).

Inhibition of GCAT by Alkyltrifluoromethyl Ketones. Like other lipases, GCAT is believed to be a serine hydrolase (Hilton et al., 1990). Previous studies have shown that trifluoromethyl ketones can form stable, transition state-like hemiketal adducts with the catalytic serine residue of acetylcholinesterase, serine proteases, and lecithin:cholesterol acyl transferase (Gelb et al., 1985; Brady et al., 1990; Abdel-Aal & Hammock, 1986; Jauhiainen et al., 1989). As depicted in Figure 5 and summarized in Table 1, GCAT is inhibited by the trifluoromethyl ketones, $C_{15}H_{31}COCF_3$ and $AACOCF_3$, whereas

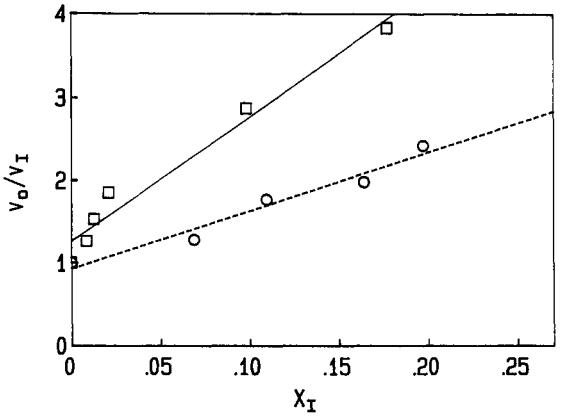


FIGURE 5: Dependence of the relative initial rate of hydrolysis of DMPM vesicles in the presence of varying mole fractions of (□) $AACOCF_3$ or (○) *n*-$C_{15}H_{31}COCF_3$. The reaction mixture (4 mL) contained 1 mM NaCl and 4 μg/mL polymyxin B. The inhibitor and substrate were preincubated for 5 min before the reaction was initiated with GCAT.

the analogous methylketone ($AACOCH_3$) is 10-fold less potent. These studies were carried out by adding inhibitor to DMPM vesicles before adding the enzyme, and the initial velocity v_I was observed within 30 s. Preincubation of the inhibitor with the enzyme for 5 min produces 30% more inhibition. The mole fraction of each inhibitor required to decrease v_0 by 50% is designated as $X_I(50)$, and values of this parameter are given in Table 1. A variety of observations suggest that the inhibition was specific. As stated above, preincubation of the inhibitor with the enzyme in aqueous solution was necessary for maximal inhibition. Since the fluoroketones inhibit GCAT in the scooting mode without changing the extent of hydrolysis (see below), they must bind directly to the enzyme. In addition, several specific competitive inhibitors of secreted phospholipases A_2 with a tetrahedral phosphate or an amide function at the *sn*-2 position [e.g., see Jain et al. (1989a, 1991a,d, 1992b)] do not exhibit any noticeable inhibition on the GCAT-catalyzed hydrolysis of DMPM vesicles when present in vesicles at 0.1 mole fraction.

Inhibition by the fluoroketones is readily reversible because the rate of hydrolysis increases immediately after the addition of excess DMPM vesicles to the reaction mixture containing the inhibitor, substrate, and enzyme (results not shown). Under these conditions, only the inhibitor exchanges between the substrate vesicles, and the mole fraction of the inhibitor at the substrate interface decreases. The $X_I(50)$ values measured with the Y230F mutant are also summarized in Table 1, and the results show that the inhibitor binds to the active site with slightly lower affinity.

The competitive nature of the inhibition by the trifluoromethyl ketone $AACOCF_3$ was demonstrated by kinetic studies designed on the basis of the following obligatory relationship for the effect of a competitive inhibitor on interfacial catalysis (Berg et al., 1991):

$$\frac{v_0}{N_S k_i} = \frac{\left(\frac{1}{X_I(50)} - 1\right)}{\left(\frac{1}{n_I(50)} - 1\right)} = \frac{\left(1 + \frac{1}{K_P^*}\right)}{\left(1 + \frac{1}{K_M^*}\right)} \quad (3)$$

In this equation, $n_I(50)$ designates the mole fraction of inhibitor required for a 50% decrease in the value of $N_S k_i$, and the value for $AACOCF_3$ is listed in Table 1. This value was obtained from the first-order region of the reaction progress curve for the hydrolysis of DMPM vesicles containing the inhibitor. The middle term in eq 3, calculated from the experimentally

Table 2: Relative Substrate Specificities for GCAT on DMPM Vesicles

substrate[a]	relative k_{cat}/K_m^* (SD)[b]
[^{3}H]DPPC vs [^{14}C]DPPC[c]	0.87 (0.04)
[^{3}H]-1-stearoyl-2-arachidonoyl-phosphatidylcholine vs [^{14}C]DPPC	1.8 (0.1)
[^{3}H]dipalmitoylphosphatidyl-ethanolamine vs [^{14}C]DPPC	0.91 (0.04)
[^{14}C]-1-stearoyl-2-arachidonoyl-phosphatidylinositol vs [^{3}H]DPPC	1.23 (0.06)
[^{3}H]-1,2-dipalmitoylglycerol vs [^{14}C]DPPC	1.2 (0.6)
[^{3}H]-1,2-dipalmitoylphosphatidic acid vs [^{14}C]DPPC	0.44 (0.03)

[a] All experiments were carried out with covesicles of DMPM containing either [^{14}C]- or [^{3}H]DPPC (1,2-dipalmitoylphosphatidylcholine) and the indicated additional substrate. [b] The relative k_{cat}/K_M^* value is given as the ratio with respect to DPPC. The numbers in parentheses represent the standard deviation from 3–5 independent trials. [c] This is the control reaction in which the two labeled forms of the same substrate are used.

measured values of $X_I(50)$ and $n_I(50)$, was found to be the same as the ratio $v_0/N_S k_i$ obtained from the reaction progress curve in the absence of an inhibitor, i.e., about 4. This establishes that $AACOCF_3$ is a competitive inhibitor of GCAT (see Berg et al. (1991) and Jain et al. (1991a,d) for detailed discussions).

Intrinsic Substrate Specificities. Intrinsic substrate specificities for interfacial catalysis by GCAT were obtained from the kinetic measurements in a competitive fashion in the scooting mode (Ghomashchi et al., 1991). An analysis of these results gives the ratio of k_{cat}/K_M^* values for the two competing substrates dispersed in DMPM vesicles. Substrate specificity studies employing vesicles composed of phospholipids of one type have little meaning since the differences in reaction velocities will be a reflection of the different fractions of enzyme bound to the different vesicles (Ghomashchi et al., 1991). The results summarized in Table 2 show that GCAT does not significantly discriminate between phospholipids with different polar head groups or with fatty acyl chains of differing chain length and degree of unsaturation. In addition, GCAT does not discriminate between phospholipids and 1,2-dipalmitoyl-*sn*-glycerol. In these experiments, the mole fractions of the competing radiolabeled substrates in DMPM vesicles are less than 0.05, and thus the interface is composed mainly of DMPM in all cases. Although DMPM is being hydrolyzed in these experiments, its reaction products are not detected by the radiometric analysis.

Consequences of Intervesicle Exchange of Enzyme. The experiments described thus far were designed to elaborate the intrinsic catalytic properties of GCAT and T-GCAT which can be rigorously described by the steps of the catalytic turnover cycle at the interface. Under such steady-state conditions for the kinetics in the scooting mode, the binding to and desorption of the enzyme from the interface do not contribute to the observed catalytic turnover. Although these observations unequivocally demonstrate that the catalysis by GCAT and T-GCAT occurs at the interface, the experiments described below show that Scheme 1 can also be used to at least qualitatively interpret the results under conditions in which the enzyme is not irreversibly bound to the interface, and the observed kinetic results are considerably more complex because of the kinetic contribution of parallel exchange processes.

Results in Figures 1 and 6 show that intervesicle exchange of GCAT and T-GCAT is promoted by salt. The shape of the progress curve changes appreciably with the addition of NaCl in a concentration-dependent manner. Noticeable

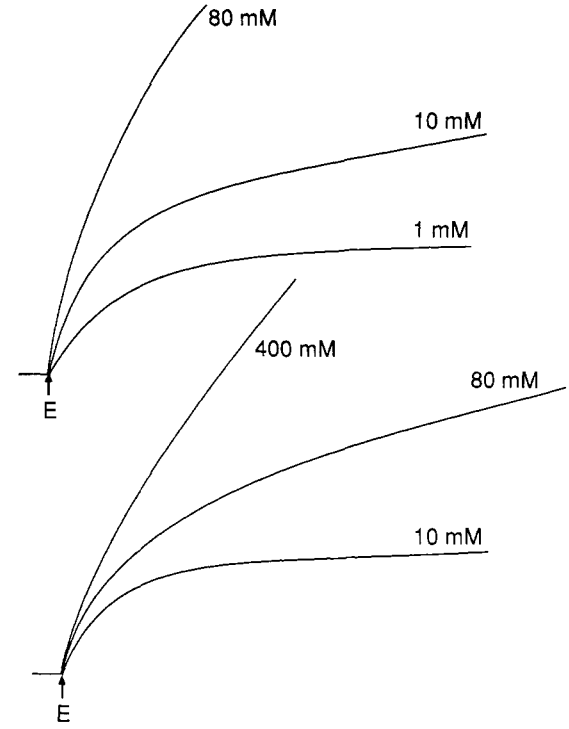


FIGURE 6: Reaction progress curves for the hydrolysis of DMPM vesicles by (bottom) T-GCAT and (top) GCAT in the presence of the indicated concentrations of NaCl. Other conditions are as given in the legend to Figure 1.

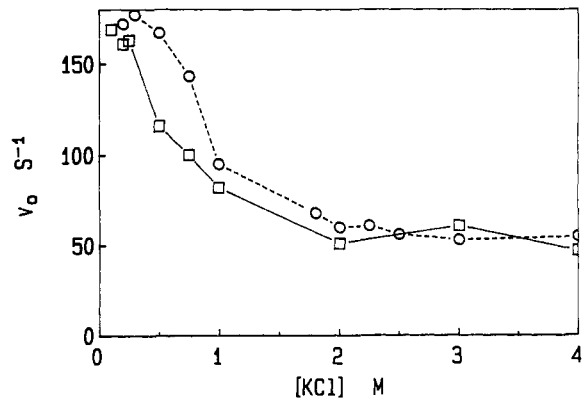


FIGURE 7: Initial rate of hydrolysis of small DMPM (650 nmol/4 mL at pH 8) vesicles by (□) GCAT or (○) T-CGAT as a function of KCl concentration. Other conditions are as given in the legend to Figure 1. These experiments are done with small sonicated vesicles to maximize the effect of the intervesicle exchange of the enzyme [see Jain et al. (1991c) for a detailed discussion of this phenomenon].

increases in the reaction rate at long periods along the progress curve were observed when as little as 10 mM NaCl was added, i.e., the progress curve is no longer first-order and the initial rate (v_0) could be measured reliably only at higher (>0.1 M) salt concentrations [see also Jain et al. (1991c)]. The concentration dependence of the effect of several salts was investigated. As shown in Figure 7, v_0 decreases with increasing concentrations of KCl beyond 0.1 M. This is most likely due to a decrease in the fraction of the enzyme bound to vesicles at higher salt concentrations. From plots of the type shown in Figure 7, the salt concentrations required for a 50% decrease in v_0 were obtained, and these values are summarized in Table 3. These results suggest that the binding of GCAT to the interface is at least partly dependent on ionic interactions of certain positively charged residues on the enzyme with the negatively charged DMPM molecules. As summarized in Table 3, the salt concentration required for desorption of T-GCAT is somewhat higher than that required

Table 3: Salt Concentrations Required for a 50% Decrease in the Maximum Rate of Hydrolysis of DMPM Vesicles by GCAT and T-GCAT[a]

	GCAT		T-GCAT	
salt	[salt] (M)	rate (s^{-1})	[salt] (M)	rate (s^{-1})
KCNS	1.4	187	1.8	210
KCl	0.5	160	0.8	170
K_2SO_4	0.8	174	0.8	133

[a] These results were obtained from plots of the type shown in Figures 6 and 7. The uncertainty in these values is less than 30%. The steady-state rate of hydrolysis was obtained at optimum salt concentration (about 0.2 M).

for GCAT, suggesting that trypsin treatment leads to a change in the number and distribution of enzymic ionic groups involved in the binding of the enzyme to the interface. Such a change could also account for the difference in the tryptophan fluorescence emission from GCAT and T-GCAT (Figure 4).

Consequences of Intervesicle Exchange of Substrate. Calcium concentrations above 1.5 mM promote intervesicle mixing of DMPM by promoting vesicle fusion (Jain et al., 1986a). The polycationic cyclic peptide polymyxin B at concentrations greater than 1 μg/mL also promotes intervesicle exchange of anionic phospholipids by a yet-to-be established mechanism (Jain et al., 1991c). As shown in Figure 1 (top), addition of calcium or polymyxin B also causes an apparent increase in the rate of GCAT-catalyzed hydrolysis of DMPM vesicles, and this increase is seen with concentrations of calcium or polymyxin B that produce apparent activation of many different phospholipases A_2 (Jain et al., 1991c). Since independent experiments (not shown) also show that none of these treatments had any effect on the rate of GCAT-catalyzed hydrolysis of myristoylcholine or short-chain phospholipids, it is concluded that the origin of such effects must lie in the changes in the properties of the substrate interface rather than a direct effect on the intrinsic catalytic properties of the enzyme. The primary kinetic effect of the treatments shown in Figure 1 (top) is that the extent of hydrolysis increases, whereas the initial rate of hydrolysis (v_0 = 150–180 s^{-1}) is virtually the same as that measured in the absence of additives (170 s^{-1}, Table 1). These results are in complete accord with the integrated Michaelis–Menten expression for the reaction progress curve in the scooting mode, which also forms the basis for eqs 1 and 2 (Berg et al., 1991). In the absence of intervesicle exchange of enzyme and substrate, as the hydrolysis of small vesicles progresses, the rate of hydrolysis becomes limited by the availability of the substrate on the enzyme-containing vesicles and not by the bulk substrate concentration. Based on this premise and the arguments developed earlier in this paper for GCAT and elsewhere for phospholipase A_2 (Jain et al., 1986a–d, 1991a–c; Berg et al., 1991), the most likely explanation for the observed increase in the extent of hydrolysis by polymyxin B or calcium is that such treatments allow the excess substrate vesicles to become accessible to the bound enzymes.

The possibility that increasing calcium concentration causes intervesicle exchange of the enzyme and thus the reinitiation of the reaction can be ruled out for the following reasons. Firstly, reinitiation of the reaction occurs in the presence of exactly the same concentration of calcium that causes fusion of DMPM vesicles (Jain et al., 1986a). Secondly, a calcium concentration that causes fusion (2.5 mM) is much lower than concentrations of calcium that would be predicted to cause intervesicle exchange of enzyme; as shown in Figure 6, >400 mM NaCl is required to produce an apparent activation in the reaction seen with 2.5 mM $CaCl_2$. Thirdly, addition of 2.5 mM $CaCl_2$ or 5 μg/mL polymyxin B does not change the fluorescence emission intensity of the type shown in Figure 4 (data not shown), and thus these additives do not cause significant desorption of the enzyme from the DMPM vesicles. Finally, high calcium or polymyxin B concentrations do not change the value of v_0 for the hydrolysis of large DMPM vesicles by GCAT (data not shown); this is because with large vesicles, the initial enzymatic velocity is not limited by substrate depletion.

Table 4: Initial Rates of Hydrolysis (v_0, s^{-1}) and Apparent Dissociation Constants (K_d, mM) for the Enzyme–Interface Complex Measured with Aqueous Dispersions of Phospholipids[a]

	GCAT		Y230F	
substrate	v_0	K_d	v_0	K_d
DMPM	170	<0.1	38	<0.1
DMPC	9		5	
DMPC + products[b]	60		18	
DMPC + deoxycholate	160		30	
myristoylcholine	50		15	
1,2-dioctanoylglycerophosphomethanol	430	0.13	65	0.3
1,2-dioctanoylglycerophosphocholine	275	0.07	82	0.25
1,2-diheptanoylglycerophosphocholine	200	0.8	45	2.5
1,2-dihexanoylglycerophosphocholine	120	1.8	30	10

[a] These measurements were carried out in 0.2 M KCl at pH 8.0 in the presence of 0.2 mM substrate unless K_d values are given, in which case the rates were measured at several bulk substrate concentrations (0.4K_d to 3K_d), and the results were fitted to a rectangular hyperbola. [b] 15 mol % each of 1-myristoyl-*sn*-glycero-3-phosphocholine and myristic acid were codispersed with DMPC vesicles.

Hydrolysis of Micellar Substrates. The kinetics of action of GCAT on aqueous dispersions of several micelle-forming phospholipids was monitored. A comparison of the apparent initial rates of hydrolysis, summarized in Table 4, indicates that: (a) the substrate specificity of GCAT is relatively poor because it hydrolyzes myristoylcholine micelles only 3-fold slower than DMPM vesicles; (b) the overall discrimination between different short-chain phospholipids is relatively minor (less than a factor of 5); and (c) the Y230F mutant is catalytically less efficient to the same degree with virtually all of the substrates. These studies were carried out with sufficiently large substrate concentrations to ensure that all of the enzyme is bound to the substrate aggregates or saturated with monomers if present. These and earlier observations made with substrates at other interfaces (Buckley et al., 1982; Buckley, 1982, 1985; Hilton & Buckley, 1991; Robertson et al., 1992) are consistent with the conclusions of the competitive substrate specificity studies at the interface (Table 2) that GCAT displays broad substrate specificity and that the intrinsic catalytic activity of GCAT does not change appreciably with the factors that control the organization of the interface.

The kinetics of the GCAT-catalyzed hydrolysis of short-chain phospholipids dispersed in the reaction mixture at concentrations below their critical micelle concentrations were studied. The reaction starts immediately after the addition of the enzyme, and all of the substrate is hydrolyzed before the reaction ceases. The initial reaction velocities show a hyperbolic dependence on the substrate concentration, and an anomalous change in the rate is not observed at the critical micelle concentration of the substrate. The apparent maximum velocities V_M and apparent K_M values are listed in Table 4. The apparent K_M values are designated as K_d since they primarily reflect the equilibrium constant for the dissociation of GCAT from the aggregate interface. The maximum initial rate of hydrolysis decreases only slightly with increasing chain length of phosphatidylcholines; however, the K_d values decrease significantly. These values are represented as v_0 because they

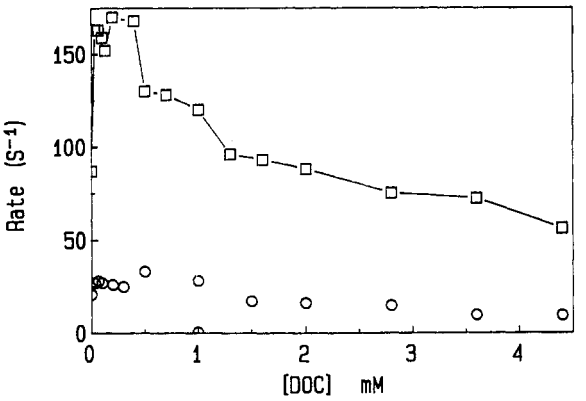


FIGURE 8: Initial rate of hydrolysis of DMPC (1 mM) vesicles by (□) GCAT and (○) Y230F as a function of the deoxycholate concentration in the reaction mixture. Other conditions are as given in the legend to Figure 1.

are the rates at the maximum mole fraction (=1) of the substrate when all the enzyme is bound to the interface. As discussed elsewhere (Rogers et al., 1992), it is difficult to establish the mechanistic significance of these trends, but the results suggest that either GCAT can effectively hydrolyze monomeric substrates or it can form protein–substrate microaggregates in which the reaction proceeds [see Rogers et al. (1992) for a further elaboration of this possibility for phospholipase A_2].

Hydrolysis of DMPC Dispersed in Deoxycholate. One of the criteria for interfacial catalysis is that the apparent reaction rate is modulated by the mole fraction of detergent codispersed with the substrate (Dennis, 1983). As summarized in Figure 8, the initial rate of hydrolysis of DMPC vesicles showed a biphasic dependence on the concentration of deoxycholate in the reaction mixture. At low concentrations of deoxycholate (below 0.2 mM), the apparent initial rate of hydrolysis increases by about 6-fold. On the other hand, the rate of hydrolysis decreases with increasing concentration of the detergent. Similar results were obtained with the Y230F mutant (Figure 8) and with T-GCAT (data not shown). The results at low concentration of deoxycholate are consistent with the interpretation that the introduction of anionic detergent increases the binding of the enzyme to the zwitterionic DMPC interface. At higher concentrations of deoxycholate (>0.5 mM), the rate of the GCAT-catalyzed reaction decreases, and a sharp decrease is seen when the deoxycholate concentration approaches its intermicellar concentration (Figure 8). The decrease in rate at higher concentrations of the detergent is most probably due to the disruption of the bilayer vesicles to form mixed micelles with the detergent. Formation of smaller particles causes a relatively rapid depletion of the substrate on the smaller enzyme-containing particles, which leads to a decrease in the effective rate (Jain et al., 1991a,c, 1992b). The possibility that the decrease in the rate with the increasing concentrations of deoxycholate is due to a decrease in the surface concentration of substrate (surface dilution) can be ruled out on the basis of two key arguments. Firstly, based on eq 1, the surface dilution would cause a decrease in the observed rate at high mole fraction of deoxycholate, and a concave pattern would be seen in the type of plot shown in Figure 8 rather than the observed convex pattern (Jain et al., 1993). Secondly, as shown in Figure 8, compared to the that of the wild-type, the rate of hydrolysis by the Y230F mutant is influenced only slightly by the addition of deoxycholate. This is expected if the detergent-induced decrease is due to substrate depletion because such an effect would be less pronounced with an enzyme that has a lower intrinsic catalytic turnover rate.

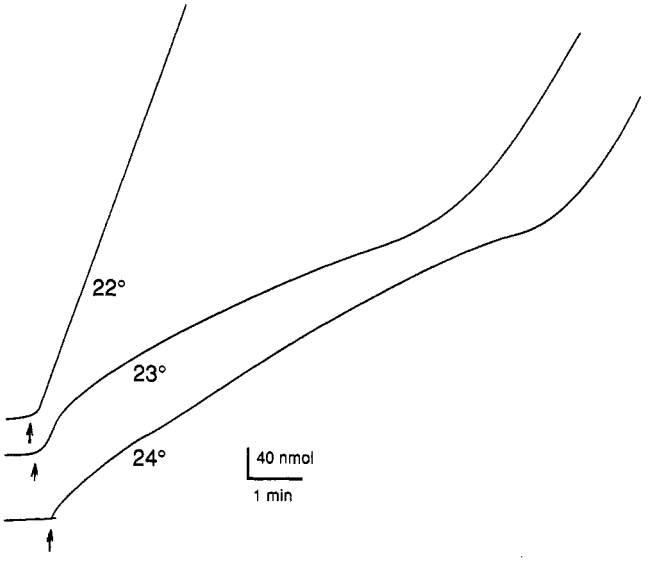


FIGURE 9: Reaction progress curves for the hydrolysis of sonicated DMPC vesicles (0.15 mM) by GCAT (0.19 μg) in 4 mL of reaction mixtures at pH 8.0 containing 0.2 M KCl at 22, 23, and 24 °C. The salt in the reaction mixture was used to emphasize the effects due to the intervesicle exchange of the enzyme. The time of addition of enzyme is indicated by the arrow.

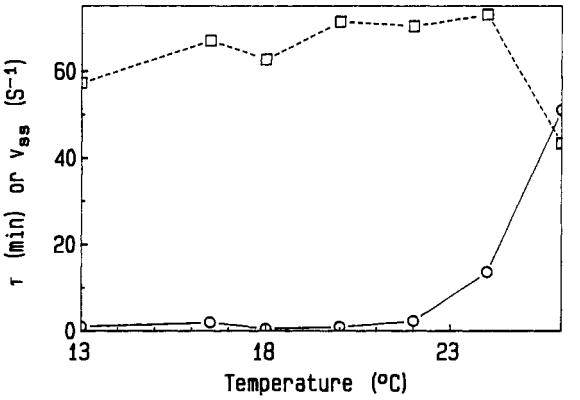


FIGURE 10: Dependence of the (○) latency period (τ) and (□) steady-state rate of hydrolysis (v_{ss}) obtained from the progress curves for the hydrolysis of DMPC vesicles of the type shown in Figure 9 under the same conditions.

The above interpretation is also supported by results with DMPM vesicles. Low concentrations of deoxycholate have no effect on the rate of hydrolysis, presumably because even in the absence of detergent, the enzyme is fully bound to the DMPM vesicles. At higher concentrations of deoxycholate, the turnover number decreases to less than 50 per second (not shown). These results show that while the anionic surface charge on the substrate or the detergent promotes binding of GCAT to the interface, disruption of vesicles at higher detergent concentrations approaching the intermicellar concentration (about 1 mM in this case) leads to a reduction in the rate of hydrolysis. A complete analytical description of this phenomenon is not possible at present; however, such behavior is expected if catalysis occurs at the interface in a highly processive mode (Jain & Berg, 1989; Berg et al., 1991) and if substrate replenishment is rate-limiting for the hydrolysis of DMPM in deoxycholate micelles (Jain et al. 1993).

Hydrolysis of Zwitterionic Vesicles. The progress curves for the hydrolysis of DMPC vesicles by GCAT in the absence of additives are complex, as they show considerable dependence on the phase properties of the bilayer. For example, as shown in Figure 9, the shape of the progress curve changes significantly with the temperature. In such progress curves, the initial slow phase of hydrolysis is followed by an abrupt

increase in the apparent steady-state rate of hydrolysis (v_{ss}) after a lag period, τ. As summarized in Figure 10, τ is less than 1 min when the temperature is near the gel–fluid transition (22 °C for sonicated DMPC vesicles), and it increases sharply above this temperature. On the other hand, v_{ss} remains constant below and at the phase transition temperature but decreases significantly at temperatures above the phase transition temperature. At 26 °C, the latency period increases with increasing substrate concentration and decreases with increasing enzyme concentration (data not shown). The value of v_{ss} at the end of the lag period (about 65 s^{-1}) is the same as that observed at the comparable concentration of DMPC vesicles containing 15 mol % products of its hydrolysis. No lag was observed with these product-containing vesicles.

Generally speaking, the behavior of GCAT on DMPC vesicles is similar to the behavior of pig pancreatic phospholipase A_2 (Apitz-Castro et al., 1982) as well as other phospholipases A_2 (Jain & Berg, 1989) acting on phosphatidylcholine vesicles. Based on this similarity and the evidence already described showing that GCAT binds relatively weakly to DTPC vesicles (compared to the covesicles of DTPC containing the products of hydrolysis of DMPC), it may be concluded that the higher rate of hydrolysis at the end of the lag period is due to the product-assisted shift in the E-to-E* equilibrium. Thus, the lag period is most likely due to the fact that at early times, much of the enzyme is in the aqueous phase. As the lipolysis proceeds, the reaction accelerates as more and more of the enzyme in the aqueous phase is partitioned onto the vesicles. However, a significant difference between the behavior of GCAT and that of phospholipases A_2 may be noted. At 26 °C, the initial rate of hydrolysis immediately after the addition of GCAT to DMPC vesicles is about 20% of that measured after τ. For pig pancreatic phospholipase A_2, the corresponding rate is about 3%.

These kinetic results suggest that the affinity of GCAT for DMPC vesicles is such that about 10% (measured by the experiments of type shown in Figure 4) of the enzyme is at the DTPC interface and about 30% of the enzyme is at the interface of DTPC vesicles containing 15 mol % products of hydrolysis. This is consistent with the kinetic results summarized in Table 4, where the relative rates of hydrolysis of DMPC under the three sets of conditions correlate well with these direct binding results. Direct binding studies also show that the binding of GCAT and T-GCAT to DTPC vesicles is rapid, with a half-time of <3 s. Therefore, the intrinsic rate of binding of the enzyme to the interface cannot be the basis for the lag period.

DISCUSSION

Previous results with secreted phospholipases A_2 (Jain et al., 1986a–d; Jain & Berg, 1989; Berg et al., 1991; Jain et al., 1991a–d) and cytoplasmic phospholipases A_2 (Ghomashchi et al., 1992) led to the design of experiments described in this paper to characterize the kinetics of interfacial catalysis by GCAT and T-GCAT and to interpret the results in terms of Scheme 1. Results show that GCAT- and T-GCAT-catalyzed hydrolysis of DMPM vesicles in the steady sate occurs in the scooting mode. To achieve highly processive catalysis, GCAT and T-GCAT must be studied in solutions of low ionic strength since physiological salt concentrations lead to intervesicle exchange of the enzyme (Figures 1, 2, and 6). However, the turnover numbers of GCAT and T-GCAT are essentially independent of salt concentration in the range 1–500 mM, whereas the initial reaction velocity decreases 3–4-fold in the presence of higher salt concentration, presumably due to a decrease in the fraction of enzyme bound to the vesicles (Figure 7). By studying GCAT and T-GCAT in the scooting mode, the steady-state rate parameters can be obtained. On the other hand, under conditions in which the enzyme, substrate, and products exchange between vesicles, it is difficult to deconvolute the interfacial catalytic parameters from the kinetic contributions of these parallel exchange events (Berg et al., 1991). In short, the catalytic behavior of the vesicle-bound GCAT is consistent with the Michaelis–Menten formalism adapted for catalytic turnover at the interface. The kinetic prediction from this analysis is that tyrosine-230 is involved in the catalytic cycle rather than in interfacial recognition.

The role of the interfacial exchange of the enzyme and substrate on the observed rate was also demonstrated under the conditions that putatively alter the organization and dynamics ("quality") of the interface. Such changes are manifest primarily in the E-to-E* step (Scheme 1), which is a prelude to steady-state catalytic turnover at the vesicle interface. The overall influence of the E-to-E* binding step on the observed rate comes from the fact that it controls not only the fraction of the enzyme at the interface but also the rate of intervesicle or intermicelle exchange of the bound enzyme between the catalytic turnover cycles. Both of these processes therefore determine the number of catalytic turnovers during each visit of the enzyme to the interface, i.e., processivity. Thus, physical factors such as temperature, salt, and detergent concentration can anomalously modulate the observed rate of hydrolysis by modulating the E-to-E* equilibrium even if such factors do not significantly affect the primary rate parameters of the turnover cycle. The contribution of the organization and the dynamic properties of the interface to the observed steady-state rate is minimized by choosing a set of experimental conditions in which all of the enzyme is tightly bound to substrate vesicles. For example, the turnover number for the action of GCAT on DMPM vesicles shows no anomalous behavior at temperatures in the vicinity of the phase transition of DMPM (28 °C); the value of v_0 for this reaction increases 2-fold when the temperature is increased from 23 °C to 34 °C (data not shown).

The hydrolysis of phospholipid vesicles or micelles by GCAT under most conditions that were examined occurs without a detectable latency period, i.e., hydrolysis is initiated within 3 s after the addition of enzyme. The fact that GCAT binds essentially irreversibly to DTPM vesicles rules out the possibility that acylation of the enzyme is required for the high-affinity binding of GCAT to the substrate interface. Along the same lines, dimerization of GCAT is not needed for its action on DMPM vesicles, as is the case for phospholipase A_2 from different sources (Jain et al., 1991b). If GCAT was functional as a dimer, the N_s value obtained in the scooting mode would be 2-fold smaller than the value observed in this study.

Complex progress curves are observed for the hydrolysis of DMPC vesicles at temperatures near the gel–fluid phase transition (Figure 9). By analogy with the behavior of phospholipase A_2 and the phase properties of the product containing DMPC vesicles (Jain & Berg, 1989), it appears that the binding of GCAT to the interface may require lateral "segregation" of anionic charges (Jain et al., 1989b). It is pertinent to recall that during the GCAT-catalyzed hydrolysis of phosphatidylcholine monolayers at the air–water interface, a latency period of several minutes was observed, and the steady-state rate was less than 2 s^{-1} if it was assumed that all the enzyme was in the interface (Hilton & Buckley, 1991). Based on the weight of the evidence developed in this paper for GCAT, the origin of the latency observed with monolayers

must reside in the factors that are characteristic of the monolayer interface rather than in the kinetics of the changes in the presteady-state behavior of the enzyme. As discussed elsewhere (Jain & Berg, 1989), the origin of the latency period in monolayers could be due to one of three possibilities, none of which invokes a slow change in the catalytic state of the enzyme on the binding to the interface. (a) The geometrical constraints of the monolayer trough and the diffusion across the unstirred layer could produce a lag period. (b) Due to a low affinity of GCAT for the zwitterionic interface, accumulation of the products of hydrolysis would shift the E-to-E* equilibrium and increase the effective rate of hydrolysis due to an increased fraction of the enzyme in the interface. (c) A significant portion of the enzyme in the aqueous phase could be adsorbed onto the (Teflon) trough, which is then partitioned into the zwitterionic monolayer as the mole fraction of the products increases. At present it is not possible to dissect the contribution of such factors to the behavior of GCAT on monolayers; however, it appears that in this case the anionic charge of the products of hydrolysis shift the equilibrium between the enzyme adsorbed on the surface of the trough and the enzyme bound to the phospholipid monolayers.

There are several reports of changes in the kinetic properties of lipases on proteolytic treatment [see Hilton et al. (1990) for a minireview]. The results described here show that T-GCAT retains the ability to carry out interfacial catalysis and appears to have a somewhat higher affinity for the interface than does GCAT. This mechanism of "activation" is reminiscent of the conversion of the zymogen form of pancreatic phospholipase A_2 to the active enzyme (Verheij et al., 1981). In this latter case, the zymogen and processed enzymes are catalytically active, but only the processed enzyme is able to bind with high affinity to anionic interfaces and to exhibit catalysis in the highly processive scooting mode. Compared to the pig pancreatic phospholipase A_2 (Jain et al., 1989), the difference in interfacial binding affinities for anionic vesicles measured with GCAT and T-GCAT is relatively small.

Several questions are raised by the demonstration that GCAT is an interfacial enzyme and acts on anionic interfaces with virtually infinite processivity. GCAT can also function as an *sn*-1- and *sn*-2-phospholipase, a lysophospholipase, a diglyceride lipase, and a cholesterol, glycerophospholipid acyltransferase. Could it be that intervesicle hopping promotes one type of activity, whereas scooting leads to another? With the understanding of the interfacial kinetic and binding behaviors of GCAT, such questions can now be addressed by using radiolabeled substrates and appropriate phospholipid matrices, which should permit a study of the effect of surface properties on the partial reactions of this versatile enzyme.

REFERENCES

Abdel-Aal, Y. A. I., & Hammock, B. D. (1986) *Science 233*, 1073–1076.

Apitz-Castro, R. J., Jain, M. K., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 349–356.

Berg, O. G, Yu, B.-Z., Rogers, J., & Jain, M. K. (1991) *Biochemistry 30*, 7283–7297.

Brady, K., Wei, A., Ringe, D., & Abeles, R. H. (1990) *Biochemistry 29*, 7600–7607.

Buckley, J. T. (1982) *Biochemistry 21*, 6699–6703.

Buckley, J. T. (1983) *Biochemistry 22*, 5490–5493.

Buckley, J. T. (1985) *Can. J. Biochem. Cell. Biol. 63*, 263–267.

Buckley, J. T., Halsa, L. N., & MacIntyre, S. (1982) *J. Biol. Chem. 257*, 3320–3325.

Cevc, G., & Marsh, D. (1987) in *Phospholipid Bilayers*, Wiley, New York.

Dennis, E. A. (1983) *The Enzymes 16*, 307–353.

Gelb, M. H., Svarer, J. P., & Abeles, R. H. (1985) *Biochemistry 24*, 1813–1817.

Ghomashchi, F., Yu, B.-Z., Berg, O. G., Jain, M. K., & Gelb, M. G. (1991) *Biochemistry 30*, 7318–7329.

Ghomashchi, F., Schuttel, S., Jain, M. K., & Gelb, M. H. (1992) *Biochemistry 31*, 3814–3824.

Hilton, S., & Buckley, J. T. (1991) *Biochemistry 30*, 6070–6074.

Hilton, S., McCubbin, W. D., Kay, C. M., & Buckley, J. T. (1990) *Biochemistry 29*, 9072–9078.

Jain, M. K. (1972) *Curr. Top. Membr. Transp. 4*, 175–254.

Jain, M. K. (1988) in *Introduction to Biomembranes*, p 423, John Wiley, New York.

Jain, M. K., & Vaz, W. L. C. (1987) *Biochim. Biophys. Acta 905*, 1–8.

Jain, M. K., & Berg, O. (1989) *Biochim. Biophys. Acta 1002*, 127–156.

Jain, M. K., & Gelb, M. H. (1991) *Methods Enzymol. 197*, 112–125.

Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., & Ramirez, F. (1986a) *Biochim. Biophys. Acta 860*, 435–447.

Jain, M. K., Maliwal, B. P., de Haas, G. H., & Slotboom, A. J. (1986b) *Biochim. Biophys. Acta 860*, 448–461.

Jain, M. K., Rogers, J., Marecek, J. F., Ramirez, F., & Eibl, H. (1986c) *Biochim. Biophys. Acta 860*, 462–474.

Jain, M. K., de Haas, G. H., Marecek, J. F., & Ramirez, F. (1986d) *Biochim. Biophys. Acta 860*, 475–483.

Jain, M. K., Rogers, J., & de Haas, G. H. (1988) *Biochim. Biophys. Acta 940*, 51–62.

Jain, M. K., Yuan, W., & Gelb, M. H. (1988a) *Biochemistry 28*, 4135–4139.

Jain, M. K., Yu, B.-Z., & Kozubek, A. (1989b) *Biochim. Biophys. Acta 980*, 23–32.

Jain, M. K., Yu, B.-Z., Rogers, J., Ranadive, G. N., & Berg, O. G. (1991a) *Biochemistry 30*, 7306–7317.

Jain, M. K., Ranadive, G. N., Yu, B.-Z., & Verheij, H. M. (1991b) *Biochemistry 30*, 7330–7340.

Jain, M. K., Rogers, J., Berg, O. G., & Gelb, M. H. (1991c) *Biochemistry 30*, 7340–7348.

Jain, M. K., Tao, W., Rogers, J., Arenson, C., Eibl, H., & Yu, B.-Z. (1991d) *Biochemistry 30*, 10256–10268.

Jain, M. K., Yu, B.-Z., Rogers, J., Gelb, M. H., Tsai, M. D., Hendrickson, E. K., Hendrickson, H. S. (1992a) *Biochemistry 31*, 7841–7847.

Jain, M. K., Yu, B.-Z., Gelb, M. H., & Berg, O. G. (1992b) *Mediators Inflammation 1*, 85–100.

Jain, M. K., Rogers, J., Hendrickson, H. S., Berg, O. G. (1993) *Biochemistry* (in press).

Jauhiainen, M., Yuan, W., Gelb, M. H., & Dolphin, P. J. (1989) *J. Biol. Chem. 264*, 1963–1967.

Robertson, D. L., Hilton, S., & Buckley, J. T. (1992) *Biochemistry 31*, 4974–4980.

Rogers, J., Yu, B.-Z., & Jain, M. K. (1992) *Biochemistry 31*, 6056–6062.

Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., & Sigler, P. B. (1990) *Science 250*, 1541–1546.

Street, I. P., Liu, H.-K., Laliberte, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N. M., Huang, Z., Weech, P. K., & Gelb, M. H. (1993) *Biochemistry 32*, 5935–5940.

Thornton, J., Howard, S. P., & Buckley, J. T. (1988) *Biochim. Biophys. Acta 959*, 153–159.

Thunnissen, M. M. G. M., Eiso, A. B., Kalk, K. H., Drenth, J., Dijkstra, B. W., Kuipers, O. P., Dijkman, R., de Haas, G. H., & Verheij, H. M. (1990) *Nature 347*, 689–691.

Verger, R., & de Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.

Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol. 91*, 91–203.

Waite, M. (1987) *The Phospholipase*, Plenum, New York.