Registry No. ATP, 56-65-5; Ca^{2+}, 7440-70-2; ATP synthase, 37205-63-3.

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Regulation of Fatty Acid ^{18}O Exchange Catalyzed by Pancreatic Carboxylester Lipase. 1. Mechanism and Kinetic Properties†

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ABSTRACT: The exchange of ^{18}O between H_2O and long-chain free fatty acids is catalyzed by pancreatic carboxylester lipase (EC 1.1.1.13). For palmitic, oleic, and arachidonic acid in aqueous suspension and for 13,16-*cis*,*cis*-docosadienoic acid (DA) in monomolecular films, carboxyl oxygens were completely exchanged with water oxygens of the bulk aqueous phase. With enzyme at either substrate or catalytic concentrations in the argon-buffer interface, the exchange of DA oxygens obeyed a random sequential mechanism, i.e., $^{18}O,^{18}O$-DA $\rightleftharpoons$ $^{18}O,^{16}O$-DA $\rightleftharpoons$ $^{16}O,^{16}O$-DA. This indicates that the dissociation of the enzyme·DA complex is much faster than the rate-limiting step in the overall exchange reaction. Kinetic analysis of ^{18}O exchange showed a first-order dependence on surface enzyme and DA concentrations, i.e., the reaction was limited by the acylation rate. The values of k_{cat}/K_m, 0.118 cm^2 pmol^{-1} s^{-1}, for the exchange reaction was comparable to that for methyl oleate hydrolysis and 5-fold higher than that for cholesteryl oleate hydrolysis in monolayers [Bhat, S., & Brockman, H. L. (1982) *Biochemistry 21*, 1547]. Thus, fatty acids are good "substrates" for carboxylester lipase. With substrate levels of carboxylester lipase in the interfacial phase, the acylation rate constant k_{cat}/K_m was 200-fold lower than that obtained with catalytic levels of enzyme. This suggests a possible restriction of substrate diffusion in the protein-covered substrate monolayer.

Pancreatic carboxylester lipase (CEL,[1] EC 1.1.1.13) catalyzes the hydrolysis of simple glycerides, lysophospholipids, and vitamin esters in the intestinal lumen (Rudd & Brockman, 1984). Related enzymes are found in the liver (Camulli et al., 1989) and milk of humans and other mammals (Hui & Kissel, 1990). In the digestive process, pancreatic carboxylester lipase functions after the partial digestion of dietary fats by lingual lipase and pancreatic colipase-dependent lipase (Lindstrom et al., 1988; Bernbäck et al., 1990) to effect the complete release of fatty acyl groups from ingested lipids. In addition to this well-recognized role in fat breakdown, carboxylester lipase has been implicated in the catalysis of cholesterol reesterification in the intestinal cell [e.g., Gallo et al. (1984) and Williams et al., (1989)] and, possibly, on the luminal side of the villus membrane (Bhat & Brockman, 1982a). However, these roles for the enzyme are not univ-

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[1] Abbreviations: DA, 13,16-*cis*,*cis*-docosadienoic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CEL, monomeric porcine pancreatic carboxylester lipase; GC-MS, gas chromatography-mass spectrometry.

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ersally accepted [see Williams et al. (1989) and references therein]. Although the importance of the reesterification function under physiological conditions is controversial, the ability of the enzyme to catalyze the acylation of alcohols in vitro is well established (Hyun et al., 1969; Lombardo et al., 1980), and, contrary to these early reports, ester synthesis does not require the presence of bile salts or other detergents (Bhat & Brockman, 1981; Kyger et al., 1990; Bhat & Ansari, 1990).

Mechanistic studies indicate that reactions catalyzed by CEL proceed via an acyl-enzyme intermediate (Lombardo & Guy, 1981; Stout et al., 1985). Early chemical modification studies suggested a serine esterase-like mechanism (Lombardo, 1982), and this was definitively shown by a combination of chemical modification and site-specific mutagenesis (DiPersio et al., 1990). The regulation of the reaction is less well established. Using competition between water and other nucleophiles for the acyl enzyme, both acylation and deacylation were implicated in the limiting step (Lombardo & Guy, 1981). In a similar study, deacylation alone was indicated as the rate-determining step in the hydrolysis of a series of *p*-nitrophenyl esters (Sutton et al., 1990). However, for the closely related milk bile-salt-stimulated lipase (Nilsson et al., 1990), kinetic studies with *p*-nitrophenyl 4-substituted benzoates suggested that neither acylation nor deacylation alone was rate determining (O'Connor & Wallace, 1985). This conclusion is supported by recent measurements of the hydrolysis of fatty acyl anhydrides by the dog pancreatic enzyme (Pieroni & Fourneron, 1990).

A limitation of the above studies and a possible reason for some of the observed differences is their reliance on water-soluble nonphysiological substrates and the presence or absence of detergents. An alternative approach to the study of the regulation of CEL is to use physiological substrates in insoluble monolayers at the gas–liquid interface [e.g., Bhat and Brockman (1982b)]. Using this approach, it has been shown that pancreatic carboxylester lipase binds to surfaces comprised of the substrates or products of the reaction with dissociation constants in the nanomolar range (Tsujita & Brockman, 1987) and that the enzyme retains activity at surface pressures above 20–25 mN/m (Bhat & Brockman, 1982b; Tsujita et al., 1989). This activity does not require the presence of bile salts or other effectors.

In the present study, we use the monolayer approach to investigate the mechanism of CEL-catalyzed exchange of ^{18}O into and from the carboxyl group of free fatty acids. The results show that the enzyme rapidly catalyzes the reaction, consistent with its ability to catalyze net lipid synthesis from free fatty acids and alcohols. Analysis of the product distribution and kinetics in light of earlier studies shows that the rate of dissociation of fatty acid from the enzyme is faster than the rate of deacylation and that the alcohol moiety regulates the rate-determining step for ester hydrolysis in simple one-component lipid monolayers. In the following paper in this issue (Muderhwa & Brockman, 1992), we show that, in the presence of nonsubstrate phospholipid, the mechanism of the reaction is apparently altered by the compositional microheterogeneity of the lipid–water interface.

Experimental Procedures

Lipids. 13,16-Docosadienoic acid (DA), heptadecanoic acid, oleic acid (OA), arachidonic acid, and palmitic acid were purchased from Nu-Chek Prep, Inc. (Elysian, MN), and were shown to be greater than 99% pure by thin-layer chromatography. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Biochemicals (Birmingham, AL). Du Pont–New England Nuclear supplied 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]POPC) (52.6 mCi/mmol). The purity of the phospholipids was also greater than 99% when analyzed by thin-layer chromatography. The phospholipid concentration in stock solutions was determined by assaying aliquots for organic phosphorus (Bartlett, 1959). Lipids were dissolved in hexane containing 5% ethanol (v/v).

Other Reagents. $H_2{}^{18}O$ (>99 atom % ^{18}O) was purchased from Isotec, Inc. (Miamisburg, OH). [^{32}P]Phosphoric acid was obtained from Du Pont–New England Nuclear, and Brij 35 detergent was from Pierce Chemical Company. Calbiochem (San Diego, CA) supplied ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), diisopropylfluorophosphate, and taurodeoxycholic acid, sodium salt. Platinum oxide was obtained from ICN Biochemicals (Irvine, CA), *p*-nitrophenylbutyrate and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) were from Sigma. Ethanol was distilled from KOH and zinc (Perrin et al., 1966). Hexane (non-Spectrograde), acetone, and 2-propanol were from Burdick and Jackson Laboratories, Inc. (Muskegon, MI). Chloroform, diethyl ether, methanol, and benzene were distilled before use. Dimethyl sulfoxide was stored over KOH pellets overnight and then distilled from NaOH pellets at 25 mm Hg (bp 73–75 °C). Petroleum ether (bp 65–66 °C) was purified according to the procedure described earlier (Smaby & Brockman, 1981). Solvents used for monolayer studies were shown to be free of significant levels of surface-active impurities as described elsewhere (Smaby et al., 1983). Hydrophobic paper for surface collection (Whatman type 1PS, 7-cm diameter) was purified for use by solvent washing and water equilibration as previously reported (Tsujita et al., 1989). Water was purified by reverse osmosis, mixed-bed deionization, adsorption on activated charcoal, and filtration through a 0.22-μm Durapore membrane (Millipore Corp., Bedford, MA). Before use, buffers were filtered through a Diaflo hollow fiber with a molecular weight cut-off of 10 000 (Amicon Corp., Danvers, MA), degassed, and stored under argon. All other chemicals were of reagent grade and used without further purification.

Proteins. Colipase was purified from porcine pancreas as reported elsewhere (Cunningham et al., 1989). Purified lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) was obtained by gel filtration on Sephacryl S200 (Pharmacia) of an extract from porcine pancreas as previously described (Cunningham et al., 1989). During purification, lipase and colipase activities were measured by using a modification of a pH-stat assay described by Momsen and Brockman (Cunningham et al., 1989). Protein concentration was determined by the absorbance at 280 nm using an *E*% of 13.3 for lipase (Verger et al., 1971) and 4.0 for colipase (Erlanson & Borgström, 1972). Using these assays for protein and activity, colipase and lipase were found to have specific activities of 28 000 and 7 500 units/mg, respectively, where one unit of activity is 1 μmol of fatty acid released per minute at 25 °C.

The monomeric form of CEL was purified from porcine pancreas using the procedure published elsewhere (Rudd et al., 1987). Its activity was determined from the rate of formation of *p*-nitrophenolate from *p*-nitrophenylbutyrate by a modification of a procedure described by Shirai and Jackson (1982). The assay mixture consisted of 2.94 mL of 50 mM sodium phosphate buffer, pH 7.25, containing 0.1 M NaCl and 0.1% Brij 35, 30 μL of acetonitrile containing 1.5 μmol of *p*-nitrophenylbutyrate, and 30 μL of enzyme solution.

Relative enzyme concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard. To obtain CEL concentrations, the values from the

dye-binding assay were multiplied by 1.75 (Rudd et al., 1987). By using these assays for protein and activity, the enzyme preparation was found to have specific activity between 1050 and 1250 μmol of *p*-nitrophenol released min^{-1}(mg of protein)$^{-1}$.

Incubation of Free Fatty Acids with CEL and $H_2{}^{18}O$. Stock solutions of palmitic acid, oleic acid, and arachidonic acid in ethanol were made separately and standardized by gas chromatography (GC) using heptadecanoic acid as an internal standard. Aliquots (10 μL) of the stock solution (21 μg of palmitic, 26 μg of oleic acid, and 20 μg of arachidonic acid) were introduced into a screw-cap glass tube, and the ethanol was evaporated under a stream of nitrogen. The fatty acids were redissolved in 20 μL of 2-propanol; 100 μL of CEL (410 units) and 25 μL of $H_2{}^{18}O$ were added to provide a final enrichment of 19.8% ^{18}O. In a second incubation, the same amounts of fatty acids were dissolved in 20 μL of dimethyl sulfoxide instead of 2-propanol. Then, 100 μL of buffer (0.2 M NaCl, 0.02% NaN_3, 12 mM taurodeoxycholate, and 20 mM phosphate, pH 6.6) containing 80 units of CEL and 0.5 μmol of diisopropylfluorophosphate was added, followed by 25 μL of $H_2{}^{18}O$. The reaction mixtures were incubated 25 min at 37 °C in a water bath shaker and then the fatty acids were extracted with chloroform/methanol (2:1). The extracts were taken to dryness; the fatty acids were dissolved in diethyl ether/methanol (10:1) and methylated with diazomethane before hydrogenation and GC–mass spectrometry (MS) analysis.

$^{18}O_2$-DA Synthesis and Purification. The synthesis of ^{18}O-labeled DA was achieved enzymatically from DA and $H_2{}^{18}O$ (99.2 atom %) using pancreatic colipase and lipase. Specifically, 50 μL of 2-propanol containing 5 mg of DA was introduced into a 1-mL reacti-vial (Pierce). Following solvent evaporation at 40 °C under a stream of nitrogen, 50 μL of sodium phosphate buffer (0.5 M, pH 7) containing 1 mM taurodeoxycholate and 0.5% Brij 35, and 400 and 780 units of lipase and colipase, respectively, was added. This reaction mixture was then lyophilized for 3 h to remove all solvents. After addition of 5 μL of 2-propanol and 50 μL of $H_2{}^{18}O$ (99.2 atom %), the mixture was stirred magnetically for 2 h at 30 °C. The lyophilization and incubation with $H_2{}^{18}O$ steps were repeated twice, after which the lipid was extracted with three 0.5-mL portions of hexane. The sample was taken to dryness under N_2, redissolved in CH_2Cl_2, and purified by high-performance liquid chromatography on a 5-μm 10 × 250 mm Ultrasphere ODS column (Altex) eluted with methanol/2-propanol/acetic acid (95:5:0.01 v/v/v) at a flow rate of 2.8 mL/min. The eluant stream was monitored at 215 nm. The DA fractions were collected, taken to dryness under a stream of nitrogen at 40 °C, and redissolved in hexane. An aliquot of this sample was taken up in diethyl ether/methanol (10:1) containing 1 mg/mL of heptadecanoic acid, as an internal standard, and treated with sufficient diazomethane, prepared from Diazald according to the procedure of Schlenk and Gellerman (1960), to produce a persistent yellow color. The solvent was removed under a stream of N_2, and the resulting methyl esters were divided into two portions. One portion was hydrogenated in ethyl acetate (1 mL) under 40 psi H_2 with PtO_2 as catalyst for 2.5 h before analysis by GC–MS (Schmid et al., 1988). The second portion was analyzed and quantified by GC on a Packard 428 gas chromatograph equipped with dual flame ionization detectors and a Spectra Physics SP4290 integrator. An aluminum column, 12 ft × 1/8 in. i.d., packed with SP-2330 on 100/120 Chromosorb W/AW (Supelco) was programmed from 185 to 235 °C at 3 °C/min.

^{18}O Exchange and CEL Adsorption Experiments. ^{18}O exchange and enzyme adsorption were measured in separate experiments using similar conditions and procedures. For both types of measurements, surface pressure and lipid molecular area were controlled by a multiprocessor interfacial monitor/controller (Tsujita & Brockman, 1987). For all experiments, DA was spread from hexane/ethanol (95:5) onto an aqueous subphase of 10 mM potassium phosphate buffer, pH 6.6, containing 0.1 M NaCl at 24 °C. Following solvent evaporation, the film was compressed to an area of 26 cm^2, giving a surface pressure of 35–37 mN/m.

The exchange of ^{18}O from DA in lipid films was studied at 24 °C using the incubation procedure for substrate hydrolysis described by Tsujita et al. (1989). Briefly, the aqueous subphase under a DA film (area = 26 cm^2) was stirred at 100 rpm, and an aliquot of enzyme solution was introduced from a microsyringe through a small port in the side of the reaction compartment beneath the film (volume = 27 mL) to achieve the desired subphase enzyme concentration. At the end of the incubation period, during which surface pressure and area were recorded, the film was collected using hydrophobic paper. Adsorbed lipid was eluted by washing with 7 mL (3 + 2 + 2) of chloroform/methanol (2:1) containing 3.7 mM heptadecanoic acid. Results of control experiments showed that no ^{18}O exchange occurred during or after the elution procedure. Following solvent evaporation at 40 °C under a stream of nitrogen, fatty acids were methylated and hydrogenated, according to the procedures described above, before analysis by GC–MS.

CEL adsorption was measured in the same manner except that an aliquot of subphase buffer containing 2.5–4 μCi of [^{32}P]phosphate was added to the reaction compartment and aliquots of the subphase were collected for determination of active enzyme concentration and ^{32}P content (Tsujita & Brockman, 1987; Bhat & Brockman, 1981). For determination of adsorbed CEL, the recovered film was eluted by swirling the hydrophobic paper in a petri dish containing 5.0 mL of 50 mM sodium phosphate buffer, pH 7.25, containing 0.1 M NaCl and 0.2% (w/v) Brij 35. Aliquots of the resulting solution were assayed for active enzyme concentration using the *p*-nitrophenylbutyrate assay as described above (Shirai & Jackson, 1982) and for subphase carry-over by ^{32}P determination using liquid scintillation counting. For CEL adsorption measurement at low subphase enzyme concentration (0.1–5 nM), the 3-mL assay mixture consisted of 0.27–2.47 mL of 50 mM sodium phosphate buffer, pH 7.25 (containing 0.1 M NaCl and 0.1% Brij 35), 30 μL of acetonitrile containing 1.5 μmol of *p*-nitrophenylbutyrate, and 2.7–0.5 mL of enzyme solution. This assay was performed in duplicate and the rate corrected for hydrolysis observed in the absence of enzyme. Typical subphase carry-over was 0.5–2.5 μL/cm^2 of surface. Film recovery was calibrated in separate experiments using [^{14}C]POPC (3.0 mCi/mmol) at surface pressure of 40 mN/m. It was found to be 82 ± 2% of lipid added, and all adsorption measurements reported were corrected to 100% using this value.

Gas Chromatography–Mass Spectrometry. GC–MS was performed according to the procedure published earlier (Schmid et al., 1988; Kuwae et al., 1990) with slight modification. Specifically, a Du Pont DP-102 mass spectrometer equipped with an all-glass separator was used. Hydrogenated and purified methyl esters of DA were separated on a 30 m × 0.25 mm inner diameter fused silica capillary column coated with SP-1000 at a thickness of 0.25 μm (Supelco). The column was operated isothermally at 220 °C with a helium

Table I: ^{18}O Exchange Catalyzed by Carboxylester Lipase in Aqueous Suspension[a]

carboxyl oxygens	experimental (mole fraction) palmitic acid	oleic acid	arachidonic acid	predicted[b]
$^{16}O,^{16}O$	0.709	0.686	0.653	0.643
$^{16}O,^{18}O$	0.259	0.275	0.310	0.318
$^{18}O,^{18}O$	0.032	0.039	0.037	0.039

[a] Incubations were as described under Experimental Procedures. [b] Calculated assuming complete equilibration of fatty acid oxygens with H_2O (19.8% $H_2{}^{18}O$).

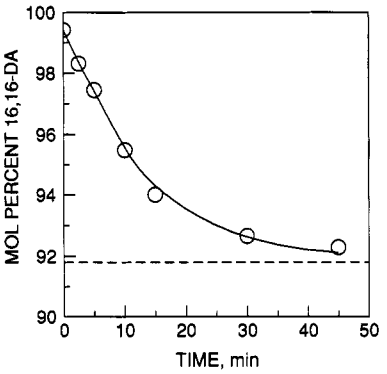


FIGURE 1: Time course of the disappearance of 16,16-DA species at the air–buffer interface. The aqueous subphase (10 mM sodium phosphate buffer, pH 6.6, 24 °C) contained 4.08% $H_2{}^{18}O$, and the bulk CEL concentration was 123 nM. The solid line through the experimental values was calculated using eq 6 as described in the text.

flow of about 1 mL/min. The injector and source temperature were 240 °C. Mass spectra were recorded at an ionization voltage of 70 eV, and the ions from 43 to 100 were scanned at a rate of 90 amu/s. The areas under the peaks of m/z 74, m/z 76, and m/z 78 were integrated and the atom percent of ^{18}O was calculated. Determinations were done in duplicate or triplicate, and reproducibility was generally within 5%.

Results

Specificity of ^{18}O Exchange. The ability of CEL to catalyze the exchange of ^{18}O from $H_2{}^{18}O$ into the carboxyl oxygens of free fatty acids was first addressed by exposing a mixture of palmitic, oleic, and arachidonic acids to the enzyme in an aqueous suspension containing 19.8% $H_2{}^{18}O$. After a 25-min incubation at 37 °C, the free fatty acids were extracted, methylated, and catalytically hydrogenated. The distribution of ^{18}O in the hydrogenated methyl esters of the fatty acids was determined by GC–MS, and the results are shown in Table I. The relative distribution of the species for each fatty acid was similar. Neglecting the natural abundance of ^{18}O in the fatty acid and assuming complete equilibration with ^{18}O in the medium, the mole fraction of fatty acid molecules with 0, 1, or 2 atoms of ^{18}O should be $(1 - F_{18})^2$, $2F_{18}(1 - F_{18})$, and $F_{18}{}^2$, where F_{18} is the atom fraction of ^{18}O in the water. For all three fatty acids, the observed distribution is close to the predicted. This shows that the enzyme is able to exchange completely the carboxyl oxygens of saturated, monosaturated, and polyunsaturated fatty acids. In a parallel experiment, ^{18}O exchange was completely inhibited by 5 mM diisopropylfluorophosphate. Because this inhibitor reacts with a single residue on the pig enzyme (Rudd et al., 1987) which, for the rat enzyme, is the active site serine (DiPersio et al., 1990), the exchange is catalyzed by the normal catalytic pathway.

Mechanism of ^{18}O Exchange. In the absence of added detergents, the free fatty acids are not necessarily monomolecularly dispersed. To achieve a more controlled presentation

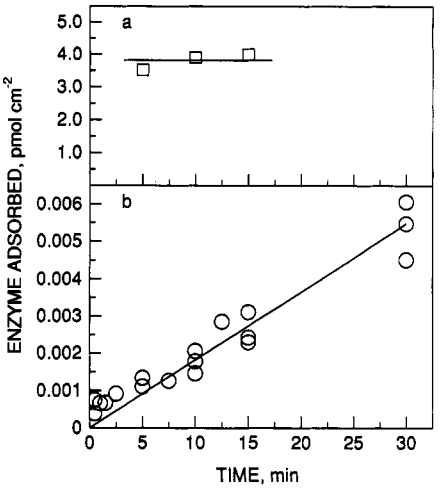


FIGURE 2: Time dependency of CEL adsorption to DA films at the argon–buffer interface. The subphase, 10 mM sodium phosphate buffer, pH 6.6, 24 °C, was stirred at 100 rpm. Bulk phase CEL concentrations were 123 nM (a) and 0.1 nM (b). Adsorption was measured at an initial surface pressure just below film collapse (35–37 mN/m).

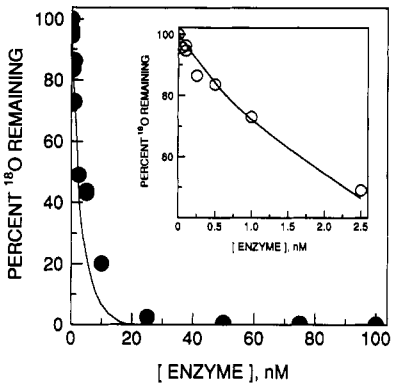


FIGURE 3: Dependence of ^{18}O exchange on CEL concentration. The incubation time was 10 min. (●) All concentrations. (○) 0–2.5 nM.

of fatty acid to the enzyme for mechanistic and kinetic analysis, monomolecular films of 13,16-*cis*,*cis*-docosadienoic acid (DA) at the argon–buffer interface were used. The aqueous subphase contained 4.08% $H_2{}^{18}O$, and the reaction was started by addition of CEL. As shown in Figure 1, analysis of the recovered DA, following methylation and hydrogenation, shows the time-dependent decrease of the $^{16}O,^{16}O$-DA species (16,16-DA) toward a limit of about 92% of all DA present. For several such experiments using different levels of $H_2{}^{18}O$, the atom % of ^{18}O in DA oxygens after a 45-min exposure to CEL equaled that of $H_2{}^{18}O$ in the medium (data not shown). Thus, equilibration is complete, and all molecules of DA in the film are accessible to the enzyme. The concentration of CEL in the subphase, 123 nM, is sufficient to form a monolayer of enzyme beneath the DA monolayer in less than 10 min (Tsujita & Brockman, 1987; Figure 2a). In light of this and the catalytic nature of the enzyme, the rate of equilibration seemed quite slow compared to the rapid and complete hydrolysis of ester substrates which is observed under comparable conditions (Tsujita et al., 1989). While this is in part due to the low ^{18}O content of the medium, it suggests that the reaction could be substrate limited by the high level of enzyme adsorbed to the DA film. To study the reaction with catalytic levels of surface-bound enzyme, but without incurring the expense of using high concentrations of $H_2{}^{18}O$ in the subphase, ^{18}O was incorporated enzymatically to 96.3 atom % in DA and this was used as a substrate for the enzyme. The subphase

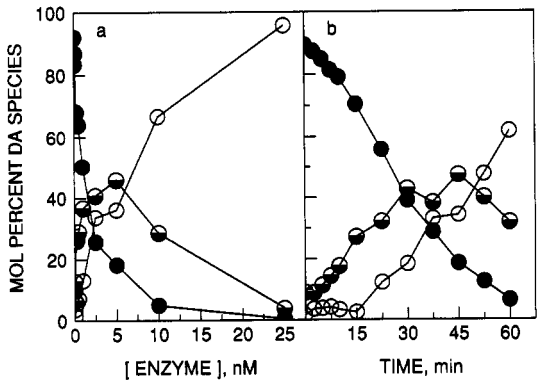
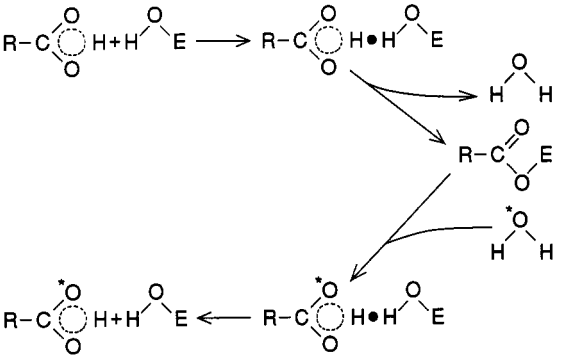


FIGURE 4: DA species distribution as function of CEL concentration (a) and time (b). 18,18-DA (●), 18,16-DA (◒), and 16,16-DA (○).

Scheme I



contained no added $H_2{}^{18}O$. Figure 3 shows that after a 10-min incubation the percent of ^{18}O, relative to that initially present in DA films, decreased to zero as the concentration of carboxylester lipase in the subphase was increased. The inset shows the data for the range of 0–2.5 nM enzyme. The distribution of DA species for the data of Figure 3 up to 25 nM is shown in Figure 4a. Qualitatively, they suggest that the exchange of the carboxyl oxygens is sequential; i.e., 18,18-DA falls continuously toward zero, 18,16-DA increases then falls to zero, and 16,16-DA increases continuously with time toward 100% of DA. If the level of CEL was fixed at 0.02, 0.1, or 0.5 nM and the incubation time varied up to 60 min, a time-dependent decrease in ^{18}O content was observed at each enzyme concentration (data not shown). The time dependence of product distribution obtained in those experiments (e.g., Figure 4b) was similar to that obtained by variation of enzyme concentration (Figure 4a).

Qualitative analysis of the data suggests that accessibility of DA to the enzyme occurs by a random sequential mechanism (Figure 4a,b). Such a mechanism is shown stepwise in Scheme I with the forward and reverse steps of one exchange cycle separated. Oxygen from the medium is shown as *O, CEL as E–O–H, and the dashed circle in the fatty acid carboxyl group signifies the equivalence of the carboxyl oxygens with respect to their exchange. After many exchange cycles, both oxygens should be equilibrated with the medium. However, because the reaction system is heterogeneous and the substrate hydrogen bonds to water, it is possible that product $H_2{}^{18}O$ could remain associated with the lipid monolayer. This was tested using a 1:1 mixed monolayer of DA and oleic acid in which only the DA contained ^{18}O. As shown in Table II, after a 5-min exposure to 10 nM CEL in the subphase, the level of ^{18}O in oleic acid remained at background levels whereas 59.8% of the ^{18}O in DA had been exchanged by the enzyme. In control experiments, oleic acid prelabeled with ^{18}O was shown to be a good substrate for the enzyme

Table II: Equilibration of Liberated $H_2{}^{18}O$ with the Aqueous Subphase[a]

	mol %			
	DA		oleic acid	
carboxyl oxygens	0 min	5 min	0 min	5 min
$^{16}O,^{16}O$	0.40	46.53	99.00	99.02
$^{16}O,^{18}O$	7.40	29.87	1.00	0.98
$^{18}O,^{18}O$	92.20	23.60	0.00	0.00

[a] A mixed monolayer of oleic acid and DA (1:1) in which only the DA contained ^{18}O (96.3 atom %) was used. The subphase enzyme concentration was 10 nM.

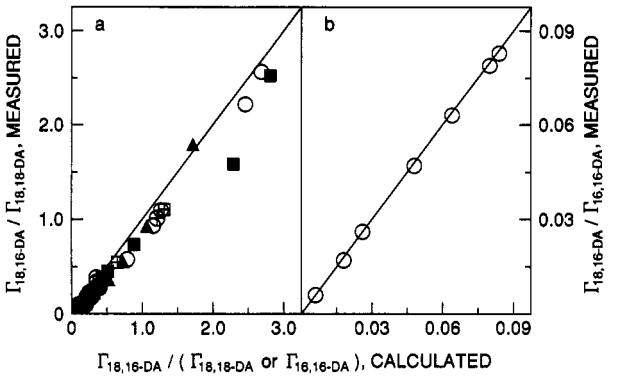


FIGURE 5: Comparison of experimental and calculated DA species distribution. Data in panel a are from experiments described in Figures 3 and 6 obtained at different times using 0.02 (●), 0.1 (○,⊞), and 0.5 (▲) nM CEL or at 10 min with varying CEL concentration (■). Panel b data are from Figure 1. Calculated ratios were determined from total ^{18}O content as described in the text.

under comparable conditions. Thus, the water released during the exchange process rapidly equilibrates with the bulk subphase.

In the experimental system described, the reaction appears to proceed by a random sequential mechanism. If so, and if the ^{18}O-containing DA was randomly labeled, then at any point in the reaction progress curve the surface concentrations of the doubly and singly labeled DA species, $\Gamma_{18,18\text{-DA}}$ and $\Gamma_{18,16\text{-DA}}$, should be given by $\Gamma_{DA}(\%\ ^{18}O)^2/100$ and Γ_{DA}-$(\%^{18}O)(100 - \%^{18}O)/50$, respectively, where $\%^{18}O$ is the atom % of ^{18}O in the DA and Γ_{DA} is the total DA concentration. The mole ratio of the two DA species can be calculated from the value of $\%^{18}O$ determined experimentally, i.e., $(\Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}})_{calc} = 200/\%^{18}O - 2$. This ratio can be compared to the corresponding measured value, $(\Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}})_{exp}$, calculated from the species distribution determined by GC–MS to indicate quantitatively if the irreversible random sequential mechanism is obeyed. Although other forms of comparison could be made, the use of the ratio of the ^{18}O-containing species is particularly advantageous for monolayer experiments where very little DA mass is available for analysis. Specifically, it cancels out distortion caused by the presence of any 22-carbon $^{16}O,^{16}O$-fatty acids present as the most likely contaminants. Figure 5a shows a plot of the experimental ratio vs the calculated ratio for the data of Figure 3 obtained in the range of 0–5 nM CEL, i.e., 0–57% of ^{18}O exchanged. At larger extents of exchange, $\Gamma_{18,18\text{-DA}}$ becomes small, and unrealistic values of the ratio are obtained. Also shown in Figure 5 are the data for the time studies referred to above and exemplified by Figure 4b. All the data lie reasonably close to the theoretical line having a slope of 1.0. This indicates that under all conditions of time and enzyme concentration shown, the reaction proceeds by a random sequential mechanism. For the experiment shown in Figure 1, $\Gamma_{18,18\text{-DA}}$ is too small to allow the test shown in Figure 5a. However, the

comparable ratio, $(\Gamma_{18,16\text{-DA}}/\Gamma_{16,16\text{-DA}})_{calc} = 2(\%^{18}O)/(100 - \%^{18}O)$, can be determined and compared with experimental values. As shown in Figure 5b, even at saturation of the surface with enzyme the mechanism remains random and sequential.

Kinetics of ^{18}O Exchange. From the preceding experiments, the exchange of ^{18}O from water into 16,16-DA can be described as a simple reversible equilibrium:

$$16,16\text{-DA} \underset{k_1\Gamma_E(1-F_{18})/2}{\overset{k_1\Gamma_E F_{18}}{\rightleftharpoons}} 18,16\text{-DA} \underset{k_1\Gamma_E(1-F_{18})}{\overset{k_1\Gamma_E F_{18}/2}{\rightleftharpoons}} 18,18\text{-DA} \quad (1)$$

where k_1 is the rate constant for the exchange reaction, Γ_E is the surface concentration of CEL, and F_{18} is the atom fraction of ^{18}O in the water. With the low values of F_{18} used (Figure 1), the accumulation of 18,18-DA is negligible, allowing the second reaction to be ignored. Integration of the rate expression for the interconversion of 16,16-DA and 18,16-DA with the limits that at $t = 0$ only 16,16-DA is present at a surface concentration, $\Gamma_{16,16\text{-DA},0}$, and at $t = \infty$ equilibration is achieved at a surface concentration, $\Gamma_{16,16\text{-DA},\infty}$, yields the following expression for the surface concentration of 16,16-DA at any time, $\Gamma_{16,16\text{-DA}}$ (Frost & Pearson, 1961):

$$\Gamma_{16,16\text{-DA}} = \Gamma_{16,16\text{-DA},\infty}(\Gamma_{16,16\text{-DA},0} - \Gamma_{16,16\text{-DA},\infty})e^{-k_1\Gamma_E(1+F_{18})t/2} \quad (2)$$

The data of Figure 1 were fitted to this equation, yielding the values of the apparent rate constant, $k_1\Gamma_E(1 + F_{18})/2 = 1.20\ 10^{-3}\ s^{-1}$ and $\Gamma_{16,16\text{-DA},\infty} = 561$ pmol cm^{-2}. Using these and the total surface concentration of DA, $\Gamma_{16,16\text{-DA},0} = 606$ pmol cm^{-2}, the solid line in Figure 1 was generated. From the value of $\Gamma_{16,16\text{-DA},\infty}$, the ^{18}O content of the DA was determined to be 4.10%, which is in good agreement with the value of 4.08% $H_2{}^{18}O$ used in the experiment. Using the apparent rate constant ($1.20 \times 10^{-3}\ s^{-1}$), F_{18} (0.041), and the average values of Γ_E from Figure 2 (3.807 pmol cm^{-2}), k_1 was 6.58×10^{-4} cm^2 s^{-1} pmol^{-1}.

For the irreverisble exchange of ^{18}O from prelabeled DA by bulk phase $H_2{}^{16}O$ (e.g., Figures 3 and 4) the reaction sequence is

$$18,18\text{-DA} \xrightarrow{k_1\Gamma_E} 18,16\text{-DA} \xrightarrow{k_1\Gamma_E/2} 16,16\text{-DA} \quad (3)$$

Under the conditions of the experiments, the value of Γ_E is not constant. However, as shown by Figure 2b and earlier measurements of the concentration dependence of CEL adsorption (Bhat & Brockman, 1982b), the surface concentration of enzyme at any time t following its addition to the aqueous subphase can be reasonably described by

$$\Gamma_E = k_a[E_0]t \quad (4)$$

Integration of the first-order differential equations describing the disappearance of 18,18-DA and 18,16-DA with the condition that at $t = 0$

$$\Gamma_{DA} = \Gamma_{18,18\text{-DA},0} + \Gamma_{18,16\text{-DA},0} + \Gamma_{16,16\text{-DA},0} \quad (5)$$

yields

$$\Gamma_{18,18\text{-DA}} = \Gamma_{18,18\text{-DA},0}e^{-k't^2/2} \quad (6)$$

and

$$\Gamma_{18,16\text{-DA}} = [\Gamma_{18,16\text{-DA},0} + 2\Gamma_{18,18\text{-DA},0}(1 - e^{-k't^2/4})]e^{-k't^2/4} \quad (7)$$

where $k' = k_1k_a[E_0]$. As explained above, the ratio of these concentrations is less contamination prone than the individual values. Dividing eq 7 by eq 6 and taking the log of both sides gives

$$\ln (2 + \Gamma_{18,16\text{-DA}}/\Gamma_{18,18\text{-DA}}) = \ln (2 + \Gamma_{18,16\text{-DA},0}/\Gamma_{18,18\text{-DA},0}) + k't^2/4 \quad (8)$$

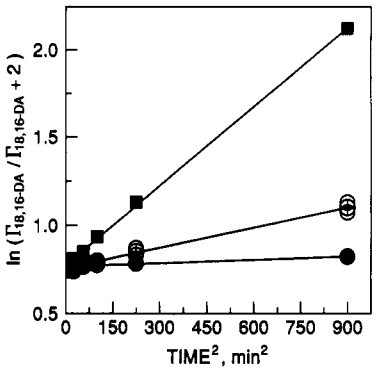


FIGURE 6: Time course for ^{18}O exchange in DA films at the argon–buffer interface. The subphase contained 10 mM sodium phosphate buffer, pH 6.6, 24 °C, with stirring 100 rpm. Bulk phase CEL concentrations were 0.02 nM (●), 0.1 nM (○), 0.1 nM + 5 mM EGTA (⊕), and 0.5 nM (■). Data are plotted according to eq 8, and the lines were determined by linear least-squares fitting of data not involving EGTA.

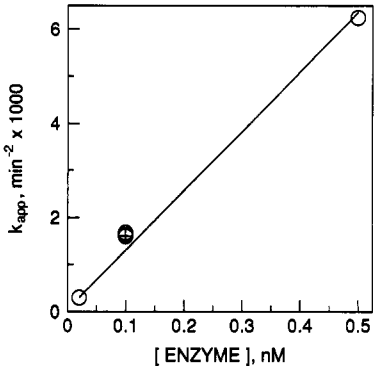


FIGURE 7: Dependence of k' on bulk phase CEL concentration. Values of k' were calculated as 4 times the slopes of the lines shown in Figure 6.

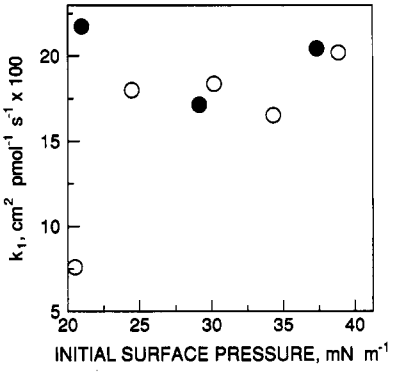


FIGURE 8: Surface pressure dependency of k'. Films were adjusted to the surface pressure shown and exposed to 0.1 nM (●) or 2 nM (○) CEL in the bulk phase for 10 min. k' was calculated from the DA species distribution using eq 8.

The data for the time dependencies obtained at 0.02, 0.1, and 0.5 nM CEL and exemplified by Figure 4b were plotted according to eq 8 as shown in Figure 6. At 0.1 nM, three sets of data are shown. Two (open circles) are duplicates obtained as described above, but the third (crossed circles) was obtained using subphases which contained 5 mM EGTA. The near identity of the results shows that endogeneous levels of divalent metals like Ca^{+2} have no effect on reaction kinetics. The linearity of the data at each of the three enzyme concentrations indicates that the kinetic model is obeyed. The lines share a common intercept which simply reflects the initial isotopic composition of the fatty acid. From the slopes, excluding the data obtained with EGTA, values of $k'/4$ were obtained, and

the k' values are given in Figure 7 as a function of the concentration of CEL present in the subphase. As predicted by eq 4, a straight line through the origin fits the data reasonably well and has a slope, k_1k_a, of 3.56×10^{-6} cm^3 s^{-2} pmol^{-1}. The slope of a line through the origin of Figure 2b gives a slope, k_a, of 30.3×10^{-6} cm s^{-1}. Dividing this into the slope of Figure 7 gives k_1 of 11.8×10^{-2} cm^2 s^{-1} pmol^{-1}.

The preceding kinetic analysis examined the DA species distribution with respect to time and enzyme concentration. Although the isotopic distribution of DA changed with time in the experiments, Γ_{DA} was held constant near 600 pmol cm^{-2}. For reactions occurring at interfaces, factors other than the substrate concentration per se can be important regulators of catalysis. One of these is the stability of the enzyme at lower substrate concentrations, i.e., lower surface pressures, but conformational and other variables may be important. Previous studies have shown that CEL is stable at surface pressures above 25 mN/m (Tsujita & Brockman, 1987) and is reasonably stable from 20 to 25 mN/m (Bhat & Brockman, 1982b; Tsujita et al., 1989). To determine if parameters other than DA concentration were significant regulators of the exchange reaction, the surface pressure dependence of k_1 was estimated from 10-min incubations at either 0.1 or 2.0 nM CEL. At the lower concentration of enzyme, only 10–15% of ^{18}O was lost from DA initially containing 96.3% ^{18}O, whereas at the higher level approximately 75% was lost. At neither concentration, however, was any pressure dependency of k_1 noted (Figure 8), except at the lower limit of surface stability for the enzyme. Thus, factors unique to the interfacial environment are not operative in the range of pressures over which the enzyme is stable. The values of k_1 shown in Figure 8 are somewhat higher than those calculated from the more rigorous analysis presented in Figures 6 and 7. The reasons for this are not clear other than the estimates of Figure 8 being based on single short-time incubations.

Discussion

Exchange of ^{18}O has been used for mechanistic studies of enzyme-catalyzed hydrolyses involving phosphoryl (Van Etten & Risley, 1978; Bock & Cohn, 1978) and acyl (Wells, 1971; van Heusden & van den Bosch, 1979; Lombardo et al., 1986; Fanni et al., 1989; Schmid et al., 1988) groups and for studies of acyl group deacylation–reacylation cycles (Kuwae et al., 1987, 1990; Schmid & Schmid, 1987, Schmid et al., 1991). Previous studies of acyl group hydrolysis involved phospholipids. In all cases they showed that *O*-acyl cleavage was involved and that acylation of the enzyme by free fatty acid did not proceed at measurable rates. Also, the acyl groups liberated by hydrolysis contained only a single atom of ^{18}O, indicating that the rate-determining step in the reaction was not release of the fatty acid by the enzyme. Similar results were obtained for oxygen exchange involving inorganic phosphate, with one exception. When Co^{2+} replaced Zn^{2+}, the mechanism of ^{18}O exchange catalyzed by *E. coli* alkaline phosphatase shifted from sequential to coupled, i.e., the rate of enzyme phosphorylation was faster than the rate of dissociation of the enzyme·phosphate complex (Bock & Cohn, 1978).

In the present study, we have characterized the exchange of ^{18}O to and from long-chain fatty acids of the type encountered in dietary lipids. Qualitatively, CEL catalyzed oxygen exchange involving saturated, monounsaturated, and polyunsaturated fatty acids. The mechanism of that exchange was examined using DA in monomolecular films at the argon–water interface. Exchange proceeded in a time- and enzyme-dependent manner (Figures 1 and 3), almost exclusively by a random sequential mechanism (Figures 4 and 5). It was not perturbed by substrate levels of enzyme in the interface (Figure 2a) or the presence of EGTA in the aqueous medium. The latter indicates a lack of any regulation by calcium ions present in the aqueous phase. Overall, these data indicate that the release of the fatty acid from the enzyme occurs at least 10 times faster than the rate-limiting step in the exchange process. In this regard, oxygen exchange with lipases resembles that seen with phospholipases A_2 (Wells, 1971; Lombardo et al., 1986) and lysophospholipase–transacylase (van Heusden & van den Bosch, 1979).

The kinetics of the exchange were analyzed using rate expressions based on the random sequential mechanism and the limiting conditions. With monolayers of 16,16-DA and substrate levels of enzyme adsorbed to the film, the time dependence for equilibration with low levels of $H_2{}^{18}O$ in the aqueous phase (Figure 3) was described by a reversible first-order process (eq 5) with a rate constant of 6.58×10^{-4} cm^2 s^{-1} pmol^{-1}. Using ^{18}O-labeled DA as a substrate increased the sensitivity and lowered the cost of exchange measurements. The exchange data obtained were well described as sequential irreversible reactions catalyzed by a surface concentration of enzyme increasing linearly with time (eq 8, Figures 6 and 7). Because lipolytic reactions are subject to nontraditional regulation by what has been termed "the quality of the interface" [e.g., Verger and Pieroni (1986)], we also examined the kinetic behavior of the reaction at different surface pressures, i.e., substrate packing densities. No atypical behavior of the kinetics of exchange was observed (Figure 8). The rate constant for exchange determined from time and enzyme dependencies with catalytic levels of adsorbed enzyme was 11.8×10^{-2} cm^2 s^{-1} pmol^{-1}. This value is almost 200-fold higher than that determined with substrate levels of enzyme present. The differences cannot be attributed to a change in the mechanism caused by high enzyme concentration (compare Figure 5a,b). Thus, the lower value of k_1 probably indicates a shift in the rate-determining step due to substrate diffusional restrictions in the protein-covered interface. The trend toward slower rates at higher Γ_E is also seen in Figure 3. The data obtained at low $[E_0]$ are reasonably well described by the solid line (inset) calculated from Figure 7, whereas at high enzyme concentrations the curve falls well below the experimental points.

Comparison of the results of this study with those from an earlier study of substrate hydrolysis rates in monomolecular films (Bhat & Brockman, 1982b) provides insight into the regulation of the hydrolytic reaction. As shown by the first-order dependence of reaction velocity with fatty acid concentration in this and the earlier study, the rate constant k_1 is k_{cat}/K_m. Using an initial rate approach, the hydrolysis of methyl oleate in monolayers gave a rate constant $k_1\Gamma_s$ of 35 s^{-1} where Γ_s was the surface concentration of methyl oleate of 262 pmol cm^{-2} (Bhat & Brockman, 1982b). Division by Γ_s gives $k_1 = 13.4 \times 10^{-2}$ cm^2 s^{-1} pmol^{-1}, a value in good agreement with the k_1 for DA ^{18}O exchange of 11.8×10^{-2} cm^{-2} s^{-1} pmol^{-1} determined in the present study. This agreement shows that CEL interacts similarly with long-chain fatty acids and their methyl esters. However, the rate constant for the hydrolysis of cholesteryl oleate in mixed monolayers with oleic acid is 5-fold lower than that for methyl oleate (Bhat & Brockman, 1982b). Thus, the acylation rate of the enzyme, as reflected by k_1, depends on the alcohol moiety as well as the length of the acyl chain (Sutton et al., 1990).

The similarity among k_{cat}/K_m values for DA, methyl oleate, and cholesteryl oleate points out an important difference between CEL and phospholipase A_2 (Wells, 1971; Lombardo et

al., 1986), namely, the kinetic reversibility of the reaction. CEL does not greatly distinguish fatty acid "hydrolysis" from ester hydrolysis, i.e., both are good substrates. However, there is an important distinction with respect to the interface in which the reaction occurs. Ester hydrolysis generates insoluble products which interact differently with phosphatidylcholine than do substrates (Smaby & Brockman, 1985; Muderhwa & Brockman, 1990). Thus, ester hydrolysis alters the physical milieu in which the reaction occurs. Oxygen exchange between fatty acids and water, however, occurs with no change in the chemical species composition of the interface. Thus, ^{18}O exchange can serve as a nonperturbing probe of the role of physicochemical factors in the regulation of interfacial lipolysis. In the following paper in this issue (Muderhwa & Brockman, 1992), this technique is used to assess the profound effects of a nonsubstrate, phosphatidylcholine, on the availability of DA to CEL.

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Registry No. CEL, 9001-62-1; DA, 17735-98-7; OA, 112-80-1; palmitic acid, 57-10-3; arachidonic acid, 506-32-1.

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