Regulation of CTP:Phosphocholine Cytidylyltransferase by Lipids. 2. Surface Curvature, Acyl Chain Length, and Lipid-Phase Dependence for Activation†

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ABSTRACT: We are investigating the mechanism of the activation of phosphocholine cytidylyltransferase by selective lipids. In the previous paper [Cornell, R. B. (1991) Biochemistry (preceding paper in this issue)] we found that the Triton X-100 present in the purified enzyme preparation interfered with activation of the enzyme by neutral lipid activators. The enzyme preparation was also contaminated with micromolar levels of phospholipids and fatty acids. To eliminate these interferences in our analysis, we removed 99.9% of the Triton and 99% of the endogenous lipid by DEAE-Sepharose chromatography. This preparation was inactive but could be reactivated in a lipid-specific manner up to 40-fold when added back to preformed liposomes. Small unilamellar vesicles were more effective than multilamellar vesicles of the same composition. The transferase showed a requirement for membrane acyl chains longer than 12 carbons and was poorly activated by lipids in the gel phase. Using PG–PC liposomes that undergo phase transitions within a useful temperature range (27 and 36 °C), we found an anomalous enhancement of enzyme activity around the phase transition temperature. These results support the idea that cytidylyltransferase activation depends on intercalation into the membrane bilayer.

The synthesis of phosphatidylcholine (PC)[1] in higher eucaryotes is regulated by the lipid-specific membrane interactions of the rate-limiting enzyme in the CDP-choline pathway, phosphocholine cytidylyltransferase (Vance, 1989). Previous work on the lipid specificity for activation of cytidylyltransferase (CT) in vitro has utilized either crude enzyme extracts or purified enzyme preparations contaminated with detergent and traces of lipid. When crude extracts were used, hydrolysis of the added lipids such as lyso-PC and fatty acyl CoA by contaminating lipases led to mistaken identification of these lipids as CT activators (Cornell & Vance, 1987a). Studies using purified CT have not considered the effect of varying ratios of Triton X-100/lipid in the assay. The presence of detergent could influence the activation by added lipid vesicles in light of the ability of trace concentrations of detergent to catalyze insertion of proteins into preformed bilayers (Eytan et al., 1976).

In the preceding paper (Cornell, 1991) we examined the relative effectiveness of anionic and neutral lipids as activators of the purified transferase using a Triton X-100 mixed micelle system in which the lipid concentration was expressed as a mole percent. We found that only the anionic phospholipids could fully activate CT when presented in micellar form. Triton seriously interfered with the activation of CT by fatty acids, fatty alcohol, or diacylglycerol. We have developed a protocol, described herein, using DEAE-Sepharose for preparing detergent-depleted CT. Ninety-nine percent of the endogenous lipids contaminating the enzyme preparation were also removed by the DEAE column. The Triton- and lipid-depleted CT had very low activity without added lipids, but could be reactivated up to 40-fold. This preparation can be used to compare the effectiveness of membranes of various lipid compositions and physical states on the activity and binding of CT, and ultimately to determine the chemical and/or physical basis of the lipid specificity for binding and activation. In this paper we describe the influence of the PC and fatty acid chain length, vesicle curvature, and the role of the lipid phase in the activation of CT.

Materials and Methods

Materials. Phospholipids were from Avanti (Birmingham, AL) except for egg PC, which was from Sigma (St. Louis, MO) or Avanti. The phospholipids were >99‰ pure as assessed by thin-layer chromatography. As little as 3 nmol of a fatty acid standard could be readily detected by H_2SO_4 charring, but no spot corresponding to fatty acid was visible in the lane containing 600 nmol of egg PC. Oleic acid and Triton X-100 were from Sigma. *sn*-1,2-Diacylglycerol was prepared from egg PC by phospholipase C digestion (Myher & Kuksis, 1979). Only minor traces (<5%) of the 1,3 isomer were detectable by TLC. [^{3}H]Phosphocholine was prepared from [^{3}H]choline as previously described (Cornell, 1989). All radioisotopic compounds were from New England Nuclear (Boston, MA): [*phenyl*-^{3}H]Triton X-100, 1.3 mCi/mg; [*methylcholine*-^{14}C]dipalmitoyl-PC, 150 mCi/mmol; [^{3}H]-palmitic acid, 0.5 Ci/mmol; and [*methyl*-^{3}H]choline, 88 Ci/mmol. DEAE-Sepharose CL-6B and Sephadex G-25 were from Pharmacia (Uppsala, Sweden).

Purification and Assay of CT. CT was purified according to Weinhold et al. (1986) with some modifications (Cornell, 1989). The enzyme activity was assayed as described previously (Cornell, 1989, 1991). One unit of enzyme forms 1 nmol of product/min. The protein content of the Weinhold preparation and the Triton-depleted preparation was assayed by the method of Bradford (1976) or a modification of the Lowry method (Peterson, 1977), with ovalbumin as a standard. We were unable to assay the protein concentration of the Tri-

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[1] Abbreviations: CT, CTP:phosphocholine cytidylyltransferase; PC, phosphatidylcholine; PG, phosphatidylglycerol; BSA, bovine serum albumin; DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MLV, multilamellar vesicle; SUV, small unilamellar vesicle.

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ton-depleted CT with each experiment; to do so would have required the entire sample. Therefore, CT activity is expressed as units/mL. One unit is 1 nmol of CDP-choline formed per minute. Standardization of the activities for the multiple CT preparations and column runs was accomplished by assaying the activity in the presence of 100 μM egg PC–oleic acid (1/1) vesicles.

Analysis of Lipids in the Weinhold CT Preparation. Lipids were extracted (Bligh & Dyer, 1959) from 0.3–0.6 mL of purified CT (Weinhold et al., 1986). For phosphorus determination (Bartlett, 1959) the chloroform extract was washed three times with theoretical upper phase to remove inorganic phosphate, which was present in the buffer. Triplicate buffer blanks were analyzed simultaneously. Fatty acids and phospholipids were separated by TLC in hexane/diethyl ether/acetic acid (60/40/1). After chromatography, an exact amount of an internal standard, heptadecanoic acid, was applied onto the fatty acid and phospholipid spots. The silica was scraped and transferred to a Pasteur pipet column with a glass wool plug, and the lipids were eluted with successive 2.5-mL washes of $CHCl_3$/MeOH (1/2, 1/1, and 2/1). Methyl esters were generated by using BF_3 as a catalyst (Cornell & Vance, 1987b) and were analyzed on a DB-1 capillary column in a Hewlett Packard 5890A gas chromatograph. Only major peaks corresponding to 16:0, 17:0, 18:0, 18:1, 18:2, 20:4, and 22:6 fatty acids were used in the calculation.

Removal of Triton and Lipids by DEAE-Sepharose Chromatography. DEAE-Sepharose CL-6B was suspended in 1 M NaCl and 5 mM Triton X-100 and packed in a siliconized glass column, 0.7 cm × 0.7 cm (0.25 mL bed volume). The packing solution eliminated sticking of DEAE to the sides of the column. The DEAE was overlaid with 1–2 mm of swollen Sephadex G-25 to protect the ion-exchange resin surface. After extensive washing with H_2O (20 mL), the column was equilibrated with ~10 mL of buffer D (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 2 mM DTT). Thirty to sixty units (3–6 μg) of purified CT (Weinhold et al., 1986) was diluted 6-fold in buffer D and applied to the column. The column was washed with 7 mL of buffer D at a rate of 0.6 mL/min. CT was eluted with 2.5 mL of high-salt buffer: 50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.2 M K_2HPO_4, 1 mM EDTA, and 2 mM DTT. Fractions (0.1–0.3 mL) were collected. Approximately 35% of the applied activity and ~21% of the applied protein were recovered in this peak. A second peak of activity was eluted with 2.5 mL of the above buffer containing 0.5 mM Triton X-100. Roughly equal amounts of CT activity were recovered in the first and second peak. The first peak contained the Triton-depleted CT. The column was recycled ~6 times. It was reconditioned by washing with 1 M NaCl and 5 mM Triton X-100, and then with excess H_2O. In some experiments radiolabeled Triton or lipid trackers were added to the sample prior to column application. The radiolabeled compounds in $CHCl_3$ were dried under nitrogen and solubilized in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 2 mM DTT, 0.025% sodium azide, 0.2 M K_2HPO_4, and 0.5 mM Triton. An aliquot was added to the purified CT which was in the same buffered solution.

Preparation of Liposomes. The concentrations of lipid stocks were checked by phosphorus assay (Bartlett, 1959). Sonicated vesicles were prepared as follows: Lipids (1–5 μmol) were mixed in chloroform in a 5-mL round-bottom flask. The solvent was removed by rotary evaporation. The dried film was resuspended by vortexing in 1 mL of 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA. The suspension was sonicated under a stream of nitrogen by using a Heat Systems 375-W sonicator with a fine titanium probe at 22% output until the turbid suspension had clarified. This required only a few minutes in the case of mixtures containing acidic lipids and >10 min for PC. The temperatures for the rehydration and sonication steps were at least 5 °C above the T_c of the lipid mixture. The sonicated vesicles were centrifuged for 3 min at 10000*g* to pellet any multilamellar lipid and titanium debris. In some cases the size of the vesicles was determined by light scattering using a Nicomp Model 270 particle sizer. Gaussian distribution profiles of vesicle diameters were recorded at 37 °C, with vesicles suspended at concentrations of 2–5 mM in CT assay buffer (Cornell, 1989). Egg PC–fatty acid and PC–PG unilamellar vesicles were physically stable for several days when stored under N_2 at 4 °C (no sedimentation). PC vesicles and saturated PC–fatty acid mixtures tended to aggregate and were used within a few hours after preparation.

Multilamellar liposomes were prepared for DSC and CT activation as follows: Lipids were mixed in $CHCl_3$ or $CHCL_3$/MeOH (1/1), the solvent was evaporated under N_2, and the samples were lyophilized overnight. The dried film was resuspended in 2 mL of 40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$, and 0.1 M NaCl. The lipids were hydrated at 10 °C above the T_c for >30 min, were cooled slowly in a water bath, and were centrifuged at 100000*g* for 1 h at 10 °C. The supernatants were free of turbidity. The wet pellets were transfered to pans for DSC or were resuspended in buffer D by gentle vortexing for CT activity measurements. By use of this protocol the liposomes for DSC were prepared in a buffer containing the same salt and Mg^{2+} concentration as was present in the CT assay. The samples used for DSC contained no CT. Inclusion of the enzyme at the concentration used in the activity assay would not have affected the phase transition since the phospholipid/CT weight ratio was 10^4–10^5.

Differential scanning calorimetry was performed on a Du Pont Model 910 scanning calorimeter. The instrument was calibrated with H_2O and gallium (T_m = 29.8 °C). The lipids were prepared as described above. Samples contained ~3 μmol of phospholipid and were ~25% lipid by weight. Samples were scanned at a rate of 5 °C/min. T_c values were measured at the intersection of the tangent to the rising portion of the peak with the base line.

Gel Electrophoresis. Protein was concentrated by precipitation with 10% TCA at 0 °C for 1 h. Precipitates were washed with 0.1% TCA. The protein was dissolved in sample buffer and electrophoresed according to Laemmli (1970), using 12% acrylamide minigels. The gel was stained with silver (de Moreno et al., 1985).

Results

Endogenous Lipid in the Purified Preparation. The specific activity of the CT preparation purified by the method of Weinhold et al. (1986) in the absence of exogenous lipids was typically 1000–1500 nmol/(min·mg of protein) and was activated at most 10-fold by PC–oleic acid (1/1) vesicles. The high CT activity measured in the absence of exogenous lipid could be explained by contamination of the preparation with endogenous lipid. This CT preparation contained 2.5 ± 1.2 μM phospholipid (N = 4) as determined by lipid phosphorus analysis. Fatty acid and phospholipid content was also analyzed by thin-layer and gas chromatography. By this analysis the phospholipid concentration was 4.5 μM (N = 1) and the fatty acid content was 36 ± 1 μM (N = 2). The origins of the lipid were the egg PC–oleic acid vesicles added at the first step of the purification (Weinhold et al., 1986) and the lipid

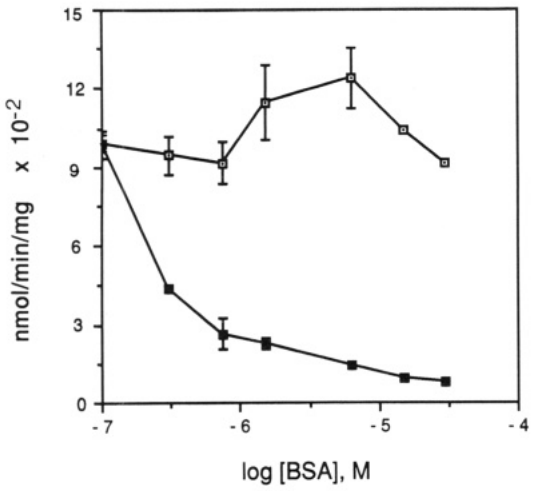

FIGURE 1: Effect of BSA on CT activity. CT activity was assayed in the absence of lipid vesicles. (⊡) Oleate-reconstituted BSA; (■) fatty acid free BSA. Data are duplicates of a single experiment.

present in the liver cytosol. Oleic acid was the major free fatty acid, but at least four other fatty acids appeared in the chromatograms. Other classes of lipids besides phospholipid and fatty acid were not examined. The activity was reduced to near-background levels by addition of high concentrations of fatty acid free serum albumin (Figure 1). By contrast, albumin reconstituted with fatty acid did not appreciably alter the activity (Figure 1). These results indicate that endogenous fatty acids accounted for the basal activity of the purified transferase. The activity of *cytosolic* CT can also be reduced by albumin treatment (Sleight & Dao, 1990).

Removal of Triton and Endogenous Lipid by DEAE-Sepharose. We had to solve the problems of Triton inhibition of activity (Cornell, 1991) and contamination of the enzyme preparation with endogenous lipids prior to undertaking a comparison of the effectiveness of a series of lipids as modulators of CT activity. We attempted to remove the Triton from the enzyme while leaving it in a form that could be reactivated by lipids. Of all the methods we tried, including Bio-Beads, ultracentifugation, hydroxylapatite, and exchange to a dialyzable detergent, only DEAE-Sepharose chromatography gave nearly complete removal of the detergent with reasonable recovery of activity (~35%) (Figure 2). A total of 99.9% of the Triton but none of the CT activity was eluted with a low ionic strength wash. A peak of enzyme activity was eluted with high salt concentration (peak A, Figure 2). Another peak was eluted with high salt concentration plus 0.5 mM Triton (peak B). The gel profiles of the applied, peak A, and peak B samples were virtually identical (Figure 2, inset).

Although removal of Triton was our primary intention with the DEAE column, we found that it was also effective in reducing the endogenous lipid from the enzyme preparation (Figure 2). Removal of Triton and lipid was monitored by spiking the samples with radiolabeled Triton, PC, or fatty acid prior to chromatography. This method is commonly emplowed for monitoring detergent removal in reconstitution studies (Cornelius & Skou, 1984; Kramer & Klingenberg, 1979; Konigsberg, 1982; Anderson et al., 1983). The small peaks of Triton and lipid that coelute with the peaks of CT activity do not represent Triton or lipid tightly bound to the enzyme. Peaks of the same magnitude appeared at precisely these positions when the samples applied lacked CT (buffer blanks; data not shown). Thus these peaks represent Triton and lipid that bind to and elute from the column at the same ionic strength as CT. After DEAE-Sepharose the enzyme contained only 0.1% of the original Triton, 1–2% of the original phospholipid, and 0.2–0.3% of the original fatty acid. The eluant was diluted 10-fold to assay; thus the Triton concentration in the activity assay was just 50 nM and the concentrations of endogenous phospholipid and fatty acid in the enzyme assay were ~5 and 10 nM, respectively, and this concentration was insufficient to activate the enzyme.

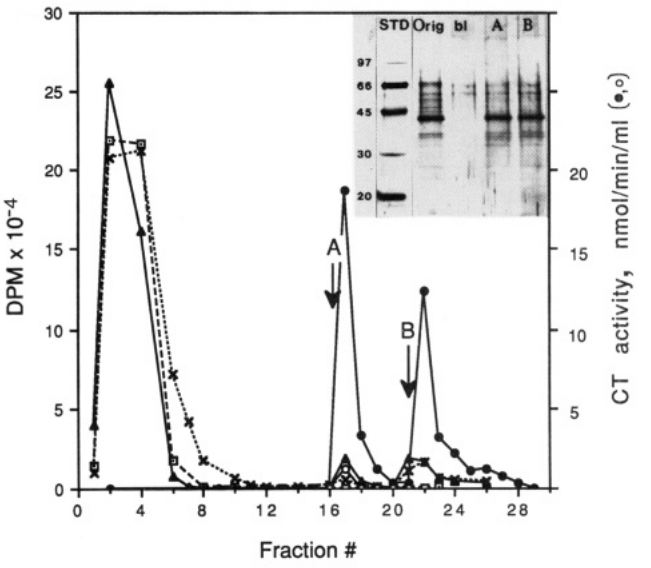

FIGURE 2: DEAE-Sepharose chromatography. Prior to application onto the column the sample (63 units) was mixed with 1 μCi of [^{3}H]palmitic acid, [^{14}C]DPPC, or [^{3}H]Triton and incubated for 5 min at 22 °C. (A) High ionic strength buffer was added; (B) high ionic strength buffer with 0.5 mM Triton was added. See Materials and Methods for details. (⊡) [^{3}H]Triton profile; (×) [^{3}H]palmitic acid profile; (▲) [^{14}C]DPPC profile; (●, ○) CT activity assayed in the presence (closed circles) or absence (open circles) of 0.2 mM PC–oleic acid (1/1) vesicles. Inset: 12% polyacrylamide–SDS gel of CT applied and recovered from the DEAE-Sepharose column. (Orig) 100 μL of the original CT applied to the column; 0.7 mL was applied. (Bl) Buffer blank. (A) Pooled peak A. (B) Pooled peak B.

The CT eluted from the DEAE column was diluted by a factor of ~5 compared to the applied enzyme as estimated by micro Lowry, by densitometry of silver-stained gels, and by activity. The detergent-depleted enzyme was inactivated but could be reactivated up to 40-fold by 100 μM PC–oleic acid vesicles. The specific activity of the CT in peak A was equal to or greater than that of the applied CT [~10 μmol/(min·mg)] when assayed in the presence of 0.2 mM egg Pc–oleic acid (1/1) vesicles. The Triton concentration in the assay was <50 nM. This method was particularly useful because the Triton could be removed prior to the addition of lipid vesicles, rather than a simultaneous exchange of Triton for lipid. Thus it was possible to test a variety of liposomes over a concentration range with a single preparation of the detergent-depleted CT. This preparation (peak A from the DEAE column) had limited stability. The $t_{1/2}$ for decay of activity was 3.5 h at 4 °C. Reactivation by lipids was tested within 30 min after elution from the column.

Lipid Selectivity of the Reconstitution of Activity. In the absence of added lipid, CT activity was near the limit of detection in our assay (Figures 2, 3, and 7). Palmitic or oleic acid dispersions had no effect on enzyme activity (Figure 3), in agreement with a previous study (Weinhold et al., 1986). Myristic and stearic acids were also inactive (data not shown). Diacylglycerol dispersions also were without effect. PC liposomes containing equimolar palmitic or oleic acid or 25 mol % diacylglycerol activated CT nearly 40-fold at 10 μM lipid (Figure 3). This was the maximum stimulation observed.

Egg PC liposomes were moderately stimulatory, showing

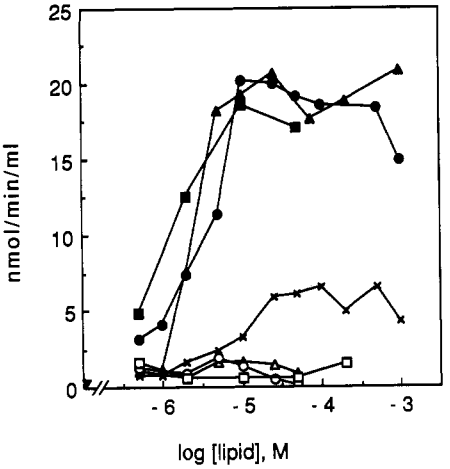

FIGURE 3: Reactivation of Triton-depleted CT by lipids. Sonicated lipid suspensions: (●) egg PC–oleic acid (1/1); (▲) egg PC–palmitic acid (1/1); (■) egg PC–diacylglycerol (3/1); (×) egg PC; (○) oleic acid; (△) palmitic acid; (□) diacylglycerol; (▼) no lipid added. The total lipid concentration is plotted. Data are averages of 2–10 separate experiments.

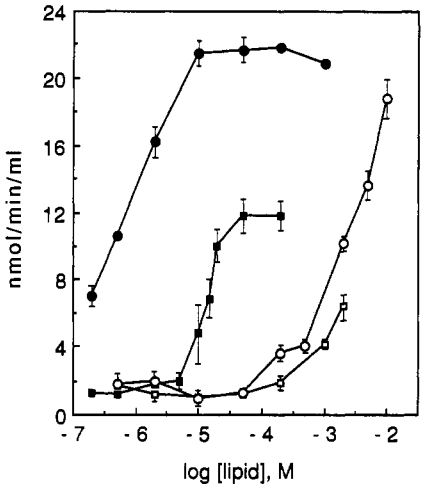

FIGURE 4: Activity dependence on MLVs vs SUVs. Triton-depleted CT was assayed in the presence of (○, ●) egg PC–egg PC (1/3) or (□, ■) egg PC. Open symbols, MLVs; closed symbols, SUVs. Data are duplicates of a single experiment which was repeated with similar results.

an activation of ~10-fold above 25 μM (Figures 3 and 4). Egg PC from two different commercial sources (Avanti and Sigma) gave similar activation. Modest stimulation by PC was reported previously in the presence of 50 μM Triton (Feldman & Weinhold, 1987). Since two other studies suggested that crude cytosolic CT does not bind to pure PC vesicles (Feldman et al., 1985; Cornell & Vance, 1987b), we considered whether this activation could be due to residual fatty acid in the enzyme preparation. Residual fatty acid in combination with the added PC would make a strongly activating membrane. If the stimulation was due entirely to traces of fatty acid, the stimulation would diminish as we increased the PC concentration due to a dilution effect. The opposite was observed; there was no stimulation below 2 μM PC and a plateau above 25 μM (Figures 3 and 4). The purified, amphiphile-free CT may be less selective in its binding to added vesicles than crude cytosolic CT.

Effect of Vesicle Size/Curvature. The effectiveness of sonicated vesicles (SUVs) vs multilamellar suspensions (MLVs) was investigated. Egg PC and DOPG/egg PC (1/3) suspensions were centrifuged to obtain multilamellar vesicles. SUVs were prepared by sonication and sized by light scattering. The mean diameter of the sonicated vesicles was 27 nm for the PG–PC vesicles and 42 nm for the egg PC vesicles. Vesicles of the size of the PG–PC SUVs are highly curved and asymmetric with respect to the distribution of phospholipids on the outer vs inner monolayer (Huang & Mason, 1978; Wetterau & Jonas, 1982). The 42-nm PC vesicles would be less strained and asymmetric, but would differ in lipid packing from planar bilayers (Wetterau & Jonas, 1982). The concentration curves for activation by SUVs vs MLVs differed by a factor of $\sim 10^3$ for the PG–PC vesicles and $> 10^2$ for the PC vesicles (Figure 4). The differences between SUVs and MLVs cannot be accounted for on the basis of available lipid surface area alone. The method used to prepare MLVs normally yields at least 5% of the lipid on the external lamella (Schwartz & McConnell, 1978). These data suggest a correlation between vesicle size and stimulation of CT. Small highly curved vesicles are more effective than planar bilayers of the same lipid composition.

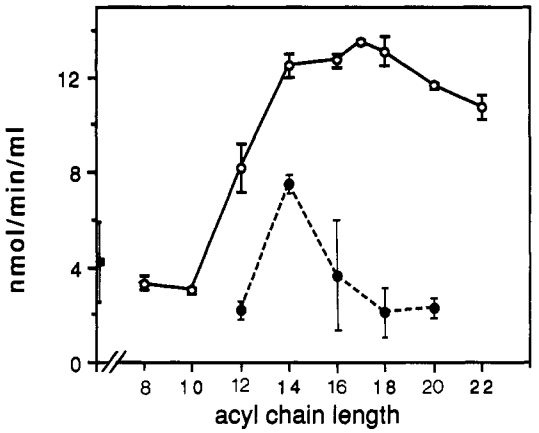

FIGURE 5: Acyl chain length dependence of lipid stimulation. Sonicated vesicles consisted of PC and 33 mol % of the saturated fatty acid. The concentration of PC in the assay was 50 μM. (○) Egg PC plus the indicated saturated fatty acid; (●) disaturated PC and saturated fatty acid of the indicated length; (■) egg PC alone. Data are averages of 2-4 separate determinations.

Acyl Chain Selectivity. The acyl chain requirement for CT activation was investigated with egg PC liposomes containing a series of saturated fatty acids at a 2/1 molar ratio of PC/fatty acid (Figure 5, open-circle curve). Fatty acids shorter than 12 carbons did not activate CT. Saturated fatty acids 14–22 carbons in length activated CT to about the same extent as oleic acid (*cis*-18:1). The unsaturated acids *cis*-18:2, -18:3, and -20:4 were also as potent as oleic acid (data not shown). The activation by egg PC–dodecanoic acid was intermediate between that by egg PC alone and the vesicles containing the longer chain fatty acids. The acyl chain composition of PC from egg is heterogeneous, but contains on average one saturated and one monounsaturated chain per molecule. The lipids were liquid crystalline at the assay temperature, 37 °C.

When saturated fatty acids from 12 to 20 carbons were tested with PCs containing the same saturated chains instead of egg PC, the activity was much reduced (Figure 5, closed-circle curve). Vesicles composed of 12:0 chains, although liquid crystalline, were poor activators, and this may be due to the shorter chain length. Perhaps for the same reason vesicles composed of 14:0 chains were less active than the egg PC–14:0 mixture. The other lipids had sufficiently long chains but had phase transitions well above the assay temperature. Vesicles composed of entirely 18:0 or 20:0 chains did not activate the enzyme. The degree of activation by DPPC–palmitic acid was variable but was always reduced relative to the activation by

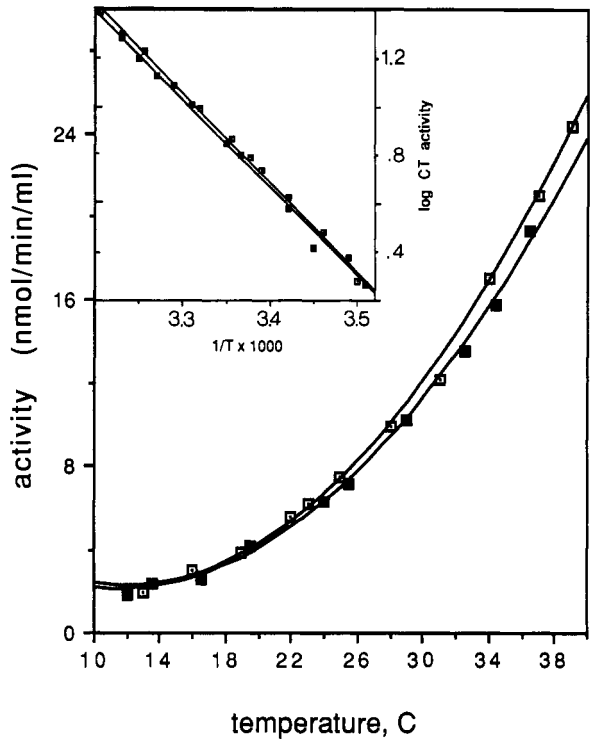

FIGURE 6: Temperature–activity profiles with liquid-crystalline membranes. Triton-depleted CT was assayed in the presence of 6 mM egg PG–egg PC (1/3) MLVs (⊡) or 0.2 mM egg PC–oleic acid (1/1) SUVs (■). Inset: Arrhenius plots. Lines were computed by linear regression. Correlation coefficients were >0.996. Data are averages of two independent experiments.

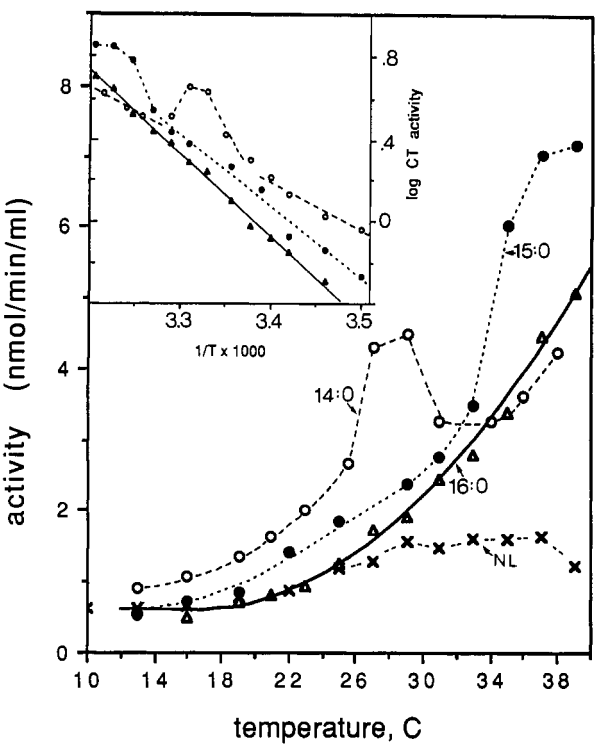

FIGURE 7: Temperature–activity profiles. Triton-depleted CT was assayed in the presence of MLVs (3–6 mM lipid) of the following compositions: (○) di-14:0-PG–di-14:0-PC (1/3); (●) di-15:0-PG–di 15:0 PC (1/3); (△) di-16:0-PG–di-16:0-PC (1/3). (×) No lipid added. Each of these experiments was repeated 1–9 times. Inset: Arrhenius plots. Correlation coefficients for the linear portions were >0.996, except for (●) di-15:0-PG–PC, which was 0.992.

egg PC–palmitic acid (Figure 5).

Lipid-Phase Dependence. These results suggested a preference for a membrane in the liquid-crystalline state. To further study this preference, we examined the temperature dependence of the activity of CT in the presence of several membranes with well-defined phase transitions. We chose PG–PC (1/3) mixtures of multilamellar liposomes for several reasons. (1) Membranes with this composition are potent activators. (2) These two lipids exhibit ideal mixing and have nearly identical phase transition temperatures for identical acyl chains (Findlay & Barton, 1978). (3) A ratio of 1/3 was chosen because the enzyme assay contained 10 mM $MgCl_2$. Ca^{2+} and Mg^{2+} dramatically raise the transition temperature of pure PG liposomes (Jacobson & Papahadjopoulos, 1975) but have only a slight effect when the PG is mixed with PC at ratios <1/1 (Findlay & Barton, 1978). (4) Multilamellar membranes were chosen because their transitions are better defined than SUVs. As shown in Figure 4, PG–PC MLVs were capable of activating CT to the same degree as SUVs of the same composition provided the concentration was sufficiently high.

In our protocol the enzyme was mixed with liposomes 15 min prior to initiation of the reaction. Since the added CT was in the form of an aggregate (Cornell, 1989), and since the association of aggregated proteins with membranes may be a slow process (Jain & Zakim, 1987; Epand & Surwicz, 1984), we tested the effect of preincubation time on the activity measurements obtained in the presence of dipentadecanoyl-PG–PC and egg PC–oleic acid mixtures at three different temperature (22, 30, and 37 °C). There was no effect between 10 and 40 min (not shown), suggesting that the binding reaction had reached equilibrium within 10 min.

Temperature–activity profiles for CT in the presence of liquid-crystalline membranes are shown in Figure 6. The profile for egg PG–egg PC fitted a smooth parabola (Figure 6) and gave a straight line in an Arrhenius plot (Figure 6, inset), the slope of which gave an E_a of 3.72 kcal/mol. The curve dropped off above 39 °C due to heat inactivation of the enzyme. A similar curve was obtained for egg PC–oleic acid (1/1) vesicles, and the E_a derived from the Arrhenius plot was 3.65 kcal/mol. Neither of these membranes undergoes a phase transition over the temperature range tested.

The temperature–activity profile for dipalmitoyl-PG–PC (1/3) also gave a relatively smooth parabola, and although the rates (activities) at each temperature were much lower than those obtained in the presence of the fluid membranes, there was a similar temperature dependence (Figure 7). An E_a of 4.0 was calculated from the Arrhenius plot (Figure 7). The phase transition of these membranes was determined simultaneously by DSC. A value of 46 °C was obtained for the main transition. The profile for dipentadecanoyl-PG–PC (1/3) was entirely different (Figure 7). The temperature of the main transition was 36 °C. The temperature dependence below 33 °C resembled that obtained for dipalmitoyl-PG–PC, and an E_a of 3.65 was obtained. However, between 33 and 35 °C the activity rose sharply. The E_a estimated from this portion of the Arrhenius plot was 10 kcal/mol, significantly higher than the value obtained for the fluid-phase (e.g., egg) membranes. The profile for dimyristoyl-PG–PC (1/3) indicated a relatively poorly activating membrane except for a striking activation between 26 and 30 °C. The T_c for these membranes was 27 °C. The calculated E_a value for this curve, ignoring the anomaly between 23 and 31 °C, was 2.34 kcal/mol. The E_a for the region between 25 and 27 °C was estimated at 10 kcal/mol.

DSC scans of the three phospholipid mixtures showed single sharp transitions (width at half peak height <3 °C) with pretransitions beginning 4–7 °C below the T_c. No other transitions were observed up to 75 °C. Literature values for

di-14:0-, di-15:0-, and di-16:0-PG and -PC are 3–5 °C lower than those we obtained (Lentz et al., 1980; Findlay & Barton, 1978; Jacobson & Papahadjopoulos, 1975). On the basis of the studies of Findlay and Barton (1978), we suspected that the presence of 10 mM Mg^{2+} in our buffer would account for the slightly higher transition temperatures. This was confirmed when we prepared di 15:0-PG–PC (1/3) mixtures in the presence or absence of $MgCl_2$. The T_c values differed by 3 °C (without Mg^{2+}, 33 °C; +10 mM $MgCl_2$, 36 °C).

Discussion

Suitability of the Triton- and Lipid-Depleted CT for Examining Lipid Structure–Activity Relationships. To study the lipid interactions of CT without the interference of Triton, we first removed the detergent from the enzyme and then added preformed lipid vesicles, rather than substituting lipid for detergent by simultaneous exchange as is often done in membrane reconstitution. Thus, problems related to asymmetric disposition of enzyme were avoided. We have previously shown that the Triton-depleted form is a self-aggregate (Cornell, 1989). The aggregation is probably initiated by hydrophobic interactions of the lipid-binding domain. The CT aggregate can be dissociated into dimers by addition of Triton or PC–oleic acid vesicles (Cornell, 1989). The Triton-depleted preparation is suitable for investigating the activity dependence on membrane physical properties such as the lipid packing density and lipid-phase preference. In the temperature–activity studies the mole percent of Triton in the system was 0.001. The mole percent of endogenous lipid was even lower. The removal of endogenous lipid from CT resulted in a degree of activation by added lipid much higher than previously observed (40-fold). The lipid class selectivity for activation of the amphiphile-free form of CT is similar to that reported for less pure preparations (Feldman & Weinhold, 1987). We found that the detergent- and lipid-depleted CT was moderately activated by egg PC, as previously noted (Feldman & Weinhold, 1987), and this appeared not to be due to residual fatty acid. In addition, we showed that diacylglycerol is as effective as fatty acid in the activation of CT when provided as a component of a PC vesicle. The direct activation of CT by membrane-bound diacylglycerol could explain the previous observations that exogenous addition of short-chain diacylglycerols to cells stimulated PC synthesis (Kolesnick & Paley, 1987; Liscovitsch et al., 1987; Kolesnick & Hemer, 1990).

CT Requires Long Fatty Acyl Chains for Activation. While there have been several previous reports on the fatty acid chain specificity for stimulation of PC synthesis and CT translocation by addition of fatty acids to whole cells (Pelech et al., 1983, 1984; Mock et al., 1986; Aeberhard et al., 1986; Burkhardt et al., 1988), a systematic examination of the activation of the transferase by fatty acids in vitro has never been presented. Our results, suggesting that chain lengths shorter than 12 carbons are ineffective as activators of purified CT, agrees with the in vivo requirement for stimulation of PC synthesis (Pelech et al., 1983, 1984). A few studies reported lower stimulation by saturated fatty acids compared to unsaturated fatty acids (Anderson et al., 1985; Aeberhard et al., 1986; Pelech et al., 1984). The apparent differences may have been due to differential solubility or differential effects of Ca^{2+} in the culture medium toward the fatty acids (Cornell & Vance, 1987a).

Membranes containing short chains in both the PC and fatty acid were poor activators of the purified transferase. The optimum chain length was >14 carbons. In addition, the combination of long-chain (egg) PC and fatty acids shorter than 12 carbons did not support activity. Such membranes may also have had a shorter bilayer width due to the tendency of the alkyl chains to pack so as to leave no gaps. This requirement for long chains differs from the chain length dependence for activation of protein kinase C in which lipids with six-carbon chains (which form micelles) are sufficiently long (Ebeling et al., 1985) and suggests that bilayer width may play a role in stabilizing the interaction between CT and membrane. Transmembrane proteins, for example, the sarcoplasmic reticulum Ca^{2+} ATPase (Johannsson et al., 1981) and bacteriorhodopsin (Lewis & Engelman, 1983), show minimum bilayer width requirements related to the length of the hydrophobic helices spanning the bilayer. The amino acid sequence of CT however does not contain any uninterrupted stretches of nonpolar amino acids longer than five (Kalmar et al., 1990). Therefore, this surprising dependence on long-chain lipids remains to be explained.

Preference for Highly Curved Surfaces. The greater effectiveness of SUVs vs MLVs in the activation of CT may be related to the increased curvature and accompanying looser packing in the head group region. This would facilitate intercalation of portions of the protein between the lipids. Feldman et al. (1978) have previously noted that cytosolic CT from rat lung can be activated more effectively by SUVs than MLVs of PG. Mellitin (Kuchinka & Seelig, 1989), cytochrome b_5 (Greenhut et al., 1986), apolipoprotein A-1 (Wetterau & Jonas, 1982), and a PC exchange protein (Berkhout et al., 1984) also show preferences for highly curved SUV vs planar bilayers.

Anomalous Activation at the Lipid-Phase Transition. The dependence on the lipid phase for CT activation was suggested by the marked drop in activity at 37 °C when the acyl chains of both the PC and fatty acid were longer than 14 carbons. However, more thorough temperature studies of PG–PC mixtures (Figures 6 and 7) showed that gel-phase lipids can activate CT but are not as effective as fluid-phase lipids and that neither of these phases was as effective as the transition between them. At the phase transition the temperature dependence of the activity rose sharply and the energy of activation for the CT reaction tripled. When CT was assayed in the presence of fluid-phase membranes, the activity was significantly higher than when assayed in the presence of gel-phase membranes. However, for lipids with acyl chain lengths longer than 14 carbons the temperature dependence and energies of activation for the two types of membranes were similar. One interpretation is that the lipid phase is critical for the membrane binding of CT but has little influence on the enzyme function once bound. Hence the activity would be higher in the presence of the fluid membranes because more of the CT would be membrane-bound.

Other membrane–protein interactions that are enhanced in the vicinity of the phase transition include Apo A-1 (Pownall et al., 1981; Weinstein et al., 1981), phospholipase A_2 (Op de Kamp et al., 1975; Gabriel et al., 1987), phospholipase C (Gabriel et al., 1987), tubulin (Klausner et al., 1981), glucagon (Epand & Epand, 1980), and M_{13} coat protein (Wickner, 1977). The basis for the effect has been attributed to the disruptions in lipid packing that occur as solid domains interface with fluid domains during the transition (Epand & Surewicz, 1984; Jain & Zakim, 1987). The disruptions would lower the membrane barrier against adsorption and intercalcation of peptides. This barrier includes the hydration layer and hydrogen-bonding and ionic interactions between neighboring phospholipid head groups, as well as the van der Waals interactions of the hydrocarbon chains. The enhanced permeability (Klausner et al., 1981) and fusion (Myers et al., 1987) that can occur at the phase transition are also indicative

of the disruptions in the lipid packing.

CT Activation Depends on Membrane Intercalation. We have recently obtained the complete amino acid sequence of rat liver CT by cDNA cloning (Kalmar et al., 1990). We have proposed that the membrane-binding domain consists of an extensive amphipathic helix located toward the C terminus and that its interaction with membranes would involve a surface interaction and intercalation of its hydrophobic face into the lipid bilayer (Kalmar et al., 1990). One segment of this helix is highly enriched with basic amino acids. In the preceding paper we characterized an electrostatic component of the CT–membrane association, which could serve to concentrate the protein at the membrane surface. In the present work we have identified membrane features which activate CT that could serve to facilitate the intercalation of an amphipathic peptide into the hydrophobic domain of the lipid bilayer by weakening lipid–lipid interactions. These features are (1) a high degree of vesicle curvature and (2) induction of the gel–liquid-crystalline phase transition. The activation associated with lipids that have small polar head groups (e.g., diacylglycerol) could also be attributed to facilitation of peptide intercalation by the generation of packing defects.

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Registry No. CTP:phosphocholine cytidylyltransferase, 9026-34-0; Mg, 7439-95-4; (*Z*)-$HO_2C(CH_2)_7CH{=}CH(CH_2)_7CH_3$, 112-80-1; $HO_2C(CH_2)_{14}CH_3$, 57-10-3; dimyristoylphosphatidylglycerol, 61361-72-6; dimyristoylphosphatidylcholine, 18194-24-6; dipalmitoylphosphatidylcholine, 63-89-8; dipalmitoylphosphatidylglycerol, 4537-77-3; di-15:0-phosphatidylcholine, 3355-27-9; Triton X-100, 9002-93-1.

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Cholesterol Esterase Catalyzed Hydrolysis of Mixed Micellar Thiophosphatidylcholines: A Possible Charge-Relay Mechanism†

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ABSTRACT: Mechanistic features of cholesterol esterase catalyzed hydrolysis of two thiophospholipids, *rac*-1-(hexanoylthio)-2-hexanoyl-3-glycerophosphorylcholine (6TPC) and *rac*-1-(decanoylthio)-2-decanoyl-3-glycerophosphorylcholine (10TPC), have been characterized. The hydrolysis of 10TPC that is contained in mixed micelles with Triton X-100 occurs strictly at the micellar interface, since the reaction rate is independent of the micelle concentration but depends hyperbolically on the mole fraction of the substrate in the micelles. This latter observation allows one to calculate the interfacial kinetic parameters V^*_{max} and K^*_m. The hydrolyses of 10TPC and *p*-nitrophenyl butyrate are similarly inhibited by the transition state analogue inhibitor phenyl-*n*-butylborinic acid, and therefore, physiological and nonphysiological substrates are processed at the same active site. The similarity of k^*_{cat} values for the acyl-similar substrates 10TPC and *p*-nitrophenyl decanoate indicates that the phospholipase A_1 activity of cholesterol esterase is partially rate limited by turnover of a decanoyl-enzyme intermediate. Solvent isotope effects on V^*_{max} and V^*_{max}/K^*_m (which monitors acylation only) are ~2-3 and are consistent with transition states that are stabilized by general acid-base proton transfers. Proton inventories of V^*_{max}/K^*_m indicate that simultaneous proton transfers stabilize the acylation transition state, which requires a multifunctional acid-base machinery (perhaps a charge-relay system) in the cholesterol esterase active site. Similar results are obtained for the 6TPC reaction, both in the presence and absence of Triton X-100 micelles.

Pancreatic cholesterol esterase (CEase)[1] is a digestive lipolytic enzyme that is secreted into the duodenum in response to a dietary fat load. CEase is a rather promiscuous catalyst. In the intestinal lumen the enzyme catalyzes the hydrolysis of such diverse esters as triacylglycerols, cholesteryl esters, and phospholipids (Brockerhoff & Jensen, 1974; Kritchevsky & Kothari, 1978; Rudd & Brockman, 1984), with consequent release of amphipathic molecules that can be taken up by the cells of the intestinal mucosa. Accordingly, CEase activity is necessary for the full absorption of dietary fats (Bhat & Brockman, 1982; Gallo et al., 1984). In vitro CEase activity is increased by interaction with bile salts, which are physiological activators of the enzyme (Brockerhoff & Jensen, 1974; Kritchevsky & Kothari, 1978; Rudd & Brockman, 1984).

CEase catalyzes ester turnover via an acylenzyme mechanism (Stout et al., 1985; Lombardo & Guy, 1981) that is reminiscent of the mechanism of serine protease catalysis (Stroud, 1974; Blow, 1976; Kraut, 1977; Polgar, 1987). The best defined CEase reactions, in a mechanistic sense, are those for turnover of lipid *p*-nitrophenyl esters (Sutton et al., 1990; Sutton & Quinn, 1990). Proton inventory experiments (Schowen, 1978; Schowen & Schowen, 1982) with these substrates invariably indicate that chemical transition states are stabilized by simple general acid-base (i.e., one-proton) catalysis. However, recent sequence comparisons among CEase (Kissel et al., 1989), AChE (Schumacher et al., 1986), and BuChE (Lockridge et al., 1987a) suggest that the three enzymes may contain active site catalytic triads like those of the serine proteases. Scheme I compares three subsequences of CEase and the cholinesterases that contain (in the CEase sequence) S194, H435, and D79. Since the corresponding serine residues of the cholinesterases are the active site nucleophiles, the inescapable conclusion is that S194 plays this

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[1] Abbreviations: 6TPC, *rac*-1-(hexanoylthio)-2-hexanoyl-3-glycerophosphorylcholine; 10TPC, *rac*-1-(decanoylthio)-2-decanoyl-3-glycerophosphorylcholine; CEase, cholesterol esterase; BuChE, butyrylcholinesterase; AChE, acetylcholinesterase; V^*_{max}, interfacial $V_{max} = k^*_{cat}[E]_T$; K^*_m, interfacial K_m; TX100, Triton X-100; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $^{D_2O}V^*_{max} = (V^*_{max})^{H_2O}/(V^*_{max})^{D_2O}$, solvent isotope effect on V^*_{max}; $^{D_2O}V^*_{max}/K^*_m$, corresponding solvent isotope effect on V^*_{max}/K^*_m; V_i, initial velocity; X_S, mole fraction of substrate in mixed micelles; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PBBA, phenyl-*n*-butylborinic acid; MeCN, acetonitrile; NaTC, sodium taurocholate. Single-letter amino acid codes used are C, cysteine; D, aspartate; H, histidine; and S, serine.

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