Synthesis of Cardiolipin Derivatives with Protection of the Free Hydroxyl: Its Application to the Study of Cardiolipin Stimulation of Cytochrome *c* Oxidase†

Marsha P. Dale and Neal C. Robinson*
Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284

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ABSTRACT: Cardiolipin derivatives retaining the free hydroxyl on the polar head group were synthesized. With the use of a tetrahydropyranyl ether to protect this hydroxyl, fatty acyl substitutions were made at both of the 2-positions of cardiolipin (CL). The disubstituted derivatives were obtained in high yields. The stimulation of delipidated cytochrome *c* oxidase activity shows a hyperbolic dependence on the concentration of these CL derivatives. Both activation parameters, the apparent dissociation constant ($K_{d,app}$) and the maximum change in molecular activity (ΔAct_{max}), depend on the chain length of the tails, with less dependence on the degree of saturation. Natural CL (92% $C_{18:2}$, 8% $C_{18:1}$) and CL disubstituted with oleic acid (47% $C_{18:2}$, 52% $C_{18:1}$) were equally effective at stimulating cytochrome *c* oxidase activity, with an apparent dissociation constant of approximately 1 μM when incubated in 0.3% Triton X-100 and assayed in lauryl maltoside. CL disubstituted with hexanoic acid, however, is a poorer activator, with an apparent dissociation constant of 6.8 μM and a ΔAct_{max} that is 50% of that achieved with natural CL. Dilysocardiolipin, with complete removal of two of the fatty acid tails, shows negligible stimulation of cytochrome *c* oxidase activity.

Bovine heart cytochrome *c* oxidase is a multisubunit integral membrane protein complex. Solubilized in detergents, it still catalyzes electron transport. When isolated from beef heart, it contains, in addition to other phospholipids, cardiolipin [1,3-bis(3-*sn*-phosphatidyl)-*sn*-glycerol]. It has been shown (Robinson, 1982) that although most of the phospholipids associated with the enzyme can be readily removed, a small number of cardiolipins, i.e., three to four, remain bound to the oxidase. Most of this tightly bound cardiolipin can be removed by Triton–glycerol gradient centrifugation, leaving delipidated cytochrome *c* oxidase with less than one cardiolipin per oxidase. Delipidated enzyme has a lowered activity which can be stimulated by the addition of cardiolipin. This cardiolipin requirement for maximum activity is specific, as delipidated cytochrome *c* oxidase is only slightly stimulated by high concentrations of other phospholipids. The affinity of cardiolipin for cytochrome *c* oxidase is high, the dissociation constant being on the order of 5 μM in 1% Triton X-100. Approximately two additional cardiolipins can be added back to tight binding sites on the delipidated enzyme (Robinson, 1982).

It is of interest to examine the structural aspects of cardiolipin that are necessary for its specific interaction with cytochrome *c* oxidase. The importance of the fatty acid tails in cardiolipin for stimulation of the activity can be discerned by examining derivatives of cardiolipin with various chain substitutions. Since the effects of acylation of the free hydroxyl have not been fully investigated, one would ideally like cardiolipin derivatives with this hydroxyl unacylated. Cardiolipin derivatives have been previously synthesized by acylating lysocardiolipins; however, the free hydroxyl in cardiolipin is easily acylated, resulting in a low yield of the desired cardiolipin derivative.

We, therefore, have developed a method for the synthesis of cardiolipin derivatives, starting with commercially available cardiolipin, which retain the free hydroxyl group. The reaction involves the introduction of a tetrahydropyranyl protecting group that can be removed by relatively mild conditions. The tetrahydropyranyl ether bond is stable during treatment with phospholipase and under acylation conditions. This scheme was used to synthesize disubstituted cardiolipin derivatives. The effect of the fatty acid substitutions on the stimulation of cytochrome *c* oxidase activity was examined.

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Experimental Procedures

Materials

Cytochrome *c* oxidase was prepared by the method of Fowler et al. (1962) as modified by Capaldi and Hayashi (1972) from Keilin–Hartree heart muscle particles prepared according to Yonetani (1960). The preparation had a molecular activity of 320 μmol of cytochrome *c* oxidized s^{-1} (μmol of cytochrome aa_3)$^{-1}$ when assayed in 0.025 M phosphate buffer, pH 7, with 10 mM lauryl maltoside. The preparation contained 9.1 nmol of heme A/mg of protein. Lipid-depleted cytochrome oxidase was prepared by the Triton X–glycerol procedure of Robinson et al. (1980) at pH 8 and ionic strength of 1. This delipidated enzyme contained 1.8 mol of P/mol of cytochrome aa_3 and had an unstimulated molecular activity of 110 μmol of cytochrome *c* oxidized s^{-1} (μmol of cytochrome aa_3)$^{-1}$.

Type IV cytochrome *c*, *Crotalus atrox* snake venom, fatty acids, and *N*,*N*-dimethylformamide (DMF)[1] were purchased from Sigma Chemical Co. Lauryl maltoside was purchased

[1] Abbreviations: CL, 1,3-bis(3-*sn*-phosphatidyl)-*sn*-glycerol; THP-CL, 1,3-bis(3-*sn*-phosphatidyl)-2-(tetrahydropyranyl)-*sn*-glycerol; DL-THP-CL, 1,3-bis[1-acyl-2-lyso-*sn*-glycero(3)phospho]-2-(tetrahydropyranyl)-*sn*-glycerol; R'_2CL, 1,3-bis[1-acyl-2-R'-*sn*-glycero(3)-phospho]-*sn*-glycerol (where R' is the substituted fatty acyl group); [^{14}C]LACL, 1,3-bis[1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero(3)phospho]-*sn*-glycerol; O_3CL, 1,3-bis[1-acyl-2-oleoyl-*sn*-glycero(3)phospho]-2-oleoyl-*sn*-glycerol; OCL, 1,3-bis(3-*sn*-phosphatidyl)-2-oleoyl-*sn*-glycerol; O_2CL, 1,3-bis[1-acyl-2-oleoyl-*sn*-glycero(3)phospho]-*sn*-glycerol; M_2CL, 1,3-bis[1-acyl-2-myristoyl-*sn*-glycero(3)phospho]-*sn*-glycerol; H_2CL, 1,3-bis[1-acyl-2-hexanoyl-*sn*-glycero(3)phospho]-*sn*-glycerol; MLCL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-lyso-*sn*-glycero(3)phospho]-*sn*-glycerol; DLCL, 1,3-bis[1-acyl-2-lyso-*sn*-glycero(3)phospho]-*sn*-glycerol; DHP, 2,3-dihydro-4*H*-pyran; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; pTSA, *p*-toluenesulfonic acid; DCC, *N*,*N*-dicyclohexylcarbodiimide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $K_{d,app}$, apparent dissociation constant; ΔAct_{max}, maximum stimulation of activity.

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from Calbiochem Co. Bovine heart diphosphatidylglycerol (cardiolipin, CL), monolysocardiolipin (MLCL), and dilysocardiolipin (DLCL) were obtained from Avanti Polar Lipids. Purified Triton X-100 was purchased from Boehringer Mannheim. 2,3-Dihydro-4*H*-pyran (DHP), 4-(*N*,*N*-dimethylamino)pyridine (DMAP), acetyl chloride, hexanoic acid anhydride, *p*-toluenesulfonic acid (pTSA), and *N*,*N*-dicyclohexylcarbodiimide (DCC) were obtained from Aldrich Chemical Co. Grade IV alumina was from Woelm. Gas chromatographic fatty acid methyl ester standards were from Nu-Chek Prep. HPLC-grade acetone, 2-propanol, and cyclohexane were from Fisher. K5 silica gel TLC plates were from Whatman. [^{14}C]Linoleic acid was purchased from New England Nuclear. Other chemicals were of reagent grade and were from Fisher Chemical Co.

Syntheses

Dichloromethane (CH_2Cl_2), diethyl ether, DHP, and DMF were purified and dried by passage over a dry alumina column before use. All CL derivatives that were substituted with two fatty acid tails at the 2-positions were synthesized in a similar manner. Reactions were monitored by spotting on silica gel plates, and the plates were developed with the B solvent system of Touchstone et al. (1980). Spotted plates were developed in acetone to move fatty acids and neutral species to the top of the plate prior to development in solvent B. Spots were identified by comparison with standards, with the phosphate detection spray of Dittmer and Lester (1964) for phospholipids, and/or sulfuric acid charring. All extractions were done by the method of Bligh and Dyer (1959). When possible, manipulations of CL were done under nitrogen or argon.

1,3-Bis(3-sn-phosphatidyl)-2-(tetrahydropyranyl)-sn-glycerol (THP-CL). The blocking of the free hydroxyl of CL by the tetrahydropyranyl (THP) group was done by mixing 30 mg of dry CL with 15.4 mg of pTSA in 2.4 mL of DMF and with 0.6 mL of DHP. This mixture was stirred under nitrogen for 12–16 h. The reaction, as monitored by TLC, goes to completion. Excess pTSA was removed by extraction out of $CHCl_3$ with 0.1 M $NaHCO_3$, followed by a wash of the $CHCl_3$ with 0.1 M NaCl. The $CHCl_3$ layer was dried with sodium sulfate and applied to a 10-mL CC-4 silica column, washed with $CHCl_3$ to remove DHP and DMF and then with methanol to elute the THP-CL. Yields of isolated material were 90–95%, although 1–3% of the THP group came off during silicic acid chromatography (most neutral silicas were found to partially deacylate CL).

1,3-Bis[1-acyl-2-lyso-sn-glycero(3)phospho]-2-(tetrahydropyranyl)-sn-glycerol (DL-THP-CL). Dilyso-THP-CL (DL-THP-CL) was made from THP-CL by using the phospholipase A_2 activity in *C. atrox* snake venom in a buffer system similar to that of van Deenen and de Haas (1963). Dry THP-CL (5 μmol) was dissolved in 2 mL of anhydrous ether, after which 0.3 mL of a 40 mg/mL solution of *C. atrox* venom in 0.15 M sodium borate buffer at pH 7 was added. Then 20 μL of 0.1 M $CaCl_2$ was slowly added with stirring (to avoid CL precipitation). This mixture was incubated at room temperature for approximately 30 h. It was often necessary to add additional 0.2-mL aliquots of venom during the reaction. As judged by TLC, the reaction was complete after 30 h. The reaction was stopped by extraction with 0.1 M EDTA and 0.4 M NaCl, followed by a wash with 0.5 M NaCl. Large losses of the DL-THP-CL are possible at this stage if the ionic strength of the aqueous phase is too low, allowing its extraction into this phase. The $CHCl_3$ layer was dried and applied to a 10-mL silica column and the DL-THP-Cl eluted with methanol. Yields were typically 90%.

1,3-Bis[1-acyl-2-R′-sn-glycero(3)phospho]-sn-glycerol (R′$_2$CL). Although acylation of DL-THP-CL can be achieved with a lower ratio of fatty acid to DL-THP-CL, the following conditions were ideal for the reaction going to completion. DL-THP-CL (2 μmol) and fatty acid (40 μmol) were dried and dissolved in CH_2Cl_2. Solutions of DMAP (112 μmol) and DCC (60 μmol) in CH_2Cl_2 were added with stirring, with a final reaction volume of 0.5 mL. The reaction proceeded with stirring, under nitrogen, for 10–15 h. The resulting R′$_2$-THP-CL (where R′ is the substituted fatty acyl group) was purified on a 1-mL silica column. The THP blocking group was removed by treatment with 0.1 M HCl in 90% methanol at room temperature for 1 h, to produce R′$_2$CL, which is purified on a 1-mL silica column. Alternatively, the acylation mixture can be directly treated with 0.1 M HCl, eliminating the need for the intermediate column. Again, the typical yield was 90–95%. O_3CL[1,3-bis[1-acyl-2-oleoyl-*sn*-glycero(3)-phospho]-2-oleoyl-*sn*-glycerol] was synthesized in a similar manner, starting with DLCL, and OCL [1,3-bis(3-*sn*-phosphatidyl)-2-oleoyl-*sn*-glycerol] was synthesized by starting with natural CL.

The CL derivatives were purified by HPLC on a Waters 5-μm silica Resolve radial-pak column. The HPLC system included a Laboratory Data Control pump and a Gilson detector, monitoring CL elution at 203 nm. The isocratic solvent system of cyclohexane–2-propanol (50:50) of Robinson (unpublished experiments) was used, generally with 3.5% of 50 mM phosphoric acid. This percentage was sometimes varied from 3 to 3.5% to achieve the best possible separation. The purified CL derivatives were extracted from the peak fractions with $CHCl_3$ and 0.1 M NaCl. Recovery ranged from 50 to 90%.

CL was stored in CH_2Cl_2 under nitrogen at −70 °C. Concentrations were determined by phosphate analysis by a modified method of Chen et al. (1956) after digestion in 0.5 mL of perchloric acid according to the procedure of Marinetti (1962). The phosphate concentration of delipidated cytochrome *c* oxidase was determined in the same manner, from a total digest of the protein.

Assays

Cytochrome *c* oxidase assays were done in 0.7 mL of 0.025 M phosphate buffer with 0.5% lauryl maltoside at pH 7 and 25 °C. Cytochrome *c* oxidase, at 2.5×10^{-7} M, was preincubated with CL (0–160 μM) in 0.02 M Tris, 10% glycerol, 0.09 M NaCl, and 0.1 mM EDTA with 0.3% Triton X-100 at pH 8 on ice. CL stock solutions were made up in this buffer, with mild sonication, immediately before use. After incubation for 5 min (longer incubation times did not result in a further increase in activity), the assay was initiated by addition of 5 μL of the cytochrome *c* oxidase–CL solution to 25 μM reduced cytochrome *c* in the assay buffer. The activity was measured spectrophotometrically by following the oxidation of cytochrome *c* with a PM 6K Zeiss spectrophotometer. The decrease in the absorbance at 550 nm was analyzed according to a first-order equation, with a linear regression fit of the slope of the first-order plot. Correction for the concentrations of cytochrome *c* oxidase and cytochrome *c* gave the molecular activity (micromoles of cytochrome *c* oxidized per micromole of cytochrome *c* oxidase per second), as described by Vanneste et al. (1974). The cytochrome *c* oxidase concentration was calculated on the basis of $\epsilon_{422} = 1.54 \times 10^5$ M^{-1} cm^{-1} (van Gelder, 1978).

CL activation data were analyzed in the following manner. The change in molecular activity was found to have a hyperbolic dependence on CL concentration and was analyzed

according to a Michaelis–Menten-type equation with a nonlinear regression fit provided by the ENZFITTER (Elsevier-Biosoft) program. This analysis gave the apparent dissociation constant, $K_{d,app}$, and the maximum stimulation of the activity, ΔAct_{max}. Each concentration point was done at least in duplicate, and the molecular activity was measured at 12 or more different concentrations for each CL derivative.

General

The fatty acid contents of the CL derivatives were analyzed by gas chromatography (GC). CL was hydrolyzed in dry methanol with 10% acetyl chloride at 90 °C for 3 h. The fatty acid methyl esters were extracted with petroleum ether and dried. They were analyzed on an SP2330 column at 150 °C, with the retention times for fatty acids calibrated with a standard mixture. The fatty acid methyl esters were dissolved in carbon disulfide, and 50–100 ng was injected. An internal standard of heptadecanoic acid ($C_{17:0}$) was included in the samples.

CL derivatives were also analyzed for ester content by the method of Snyder and Stephens (1959). A standard solution of methyl stearate was used to calibrate the assay. Approximately 1 μmol of CL derivative was hydrolyzed in alkaline hydroxylamine to calculate the ester content.

All radioactive samples were counted in a Beckman LS230 scintillation counting system.

RESULTS

The scheme for the synthesis of the CL derivatives is shown in Figure 1. The first step involves protection of the free hydroxyl of cardiolipin by the formation of a tetrahydropyranyl (THP) ether bond under acid-catalyzed conditions. Protection of this hydroxyl allows selective acylation of the free hydroxyls that are generated by phospholipase A_2 treatment. This ether bond is stable both to phospholipase A_2 treatment and to the acylation conditions described below.

The next step involves the treatment with phospholipase A_2 to generate the blocked dilyso compound, DL-THP-CL. Although the THP group is not hydrolyzed by phospholipase A_2, it does decrease the rate of CL hydrolysis by this enzyme.

The DL-THP-CL is then acylated with an excess of the desired fatty acid. The reaction proceeds by DMAP-catalyzed acylation of the free hydroxyls by the fatty acid anhydride, and it is more convenient to make the anhydride and do the acylation in one step (Keenan et al., 1982). The reaction essentially goes to completion, and the acylated product, R'_2-THP-CL, is obtained in high yield.

The last step involves the removal of the THP blocking group. This is accomplished by treatment with 0.1 M HCl for 1 h. Incubation of CL in this mild acid does not damage the molecule as judged by its ability to stimulate cytochrome *c* oxidase activity. The final product, R'_2Cl, is purified by HPLC. Three disubstituted derivatives of CL (R'_2CL) were synthesized by the method outlined in Figure 1 and are dioleoyl-CL (O_2CL), dimyristoyl-CL (M_2CL), and dihexanoyl-CL (H_2CL). The syntheses of oleoyl-CL (OCL), from CL, and of trioleoyl-CL (O_3CL), from DLCL, did not utilize the THP blocking reaction but involved the acylation of all free hydroxyls.

Figure 2 shows HPLC traces for some CL derivatives. Under the conditions shown, CL elutes at 5 min, MLCL at 9 min, and DLCL at 16.5 min. Not shown in the figure, fatty acids elute at the solvent front, and THP-CL elutes at 3.5 min, DL-THP-CL at 12 min, and O_3CL slightly before THP-CL. This system is also slightly sensitive to the chain length of the fatty acid tails of CL, since dihexanoyl-CL (H_2CL) elutes at

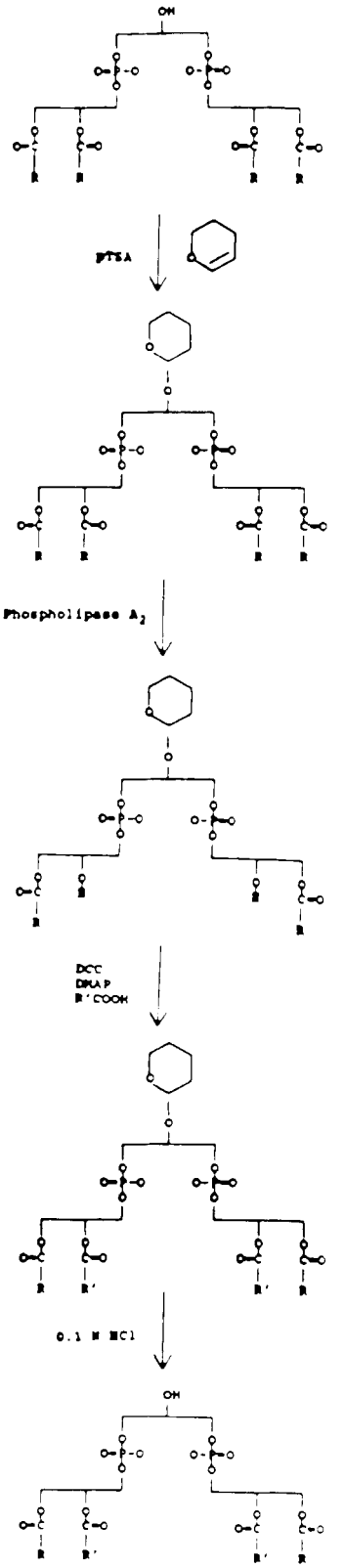

FIGURE 1: Scheme for the synthesis of cardiolipin derivatives. R and R′ represent the fatty acyl tails of natural and synthesized cardiolipin, respectively.

7 min, slightly later than natural CL. It was necessary to modify the system slightly in the purification of O_3CL and THP-CL, with a solvent system containing 3% of 50 mM H_3PO_4, in order to achieve good separation of the desired derivative from minor contaminants, e.g., of CL. To establish that the reaction proceeded as expected, several analytical techniques were applied to some of the derivatives. The

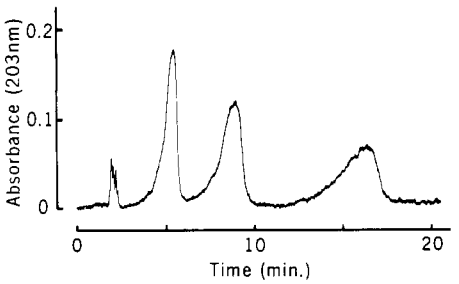

FIGURE 2: HPLC trace of CL, MLCL, and DLCL. HPLC was run with the solvent system as described under Experimental Procedures, containing 3.5% of 50 mM H_3PO_4. A total of 15–20 nmol of each cardiolipin was loaded onto a 0.8 × 10 cm silica radial-pak column and run at 1 mL/min.

Table I: Ester and Gas Chromatography Data

CL derivative	ester/P	theoretical ester/P	% 18:2	% 18:1	theoretical % 18:1
(THP)CL	2.2	2.0	92.6	6.9	
CL	2.1	2.0	92.1	6.5	
MLCL	1.6	1.5			
DLCL	1.1	1.0	97.0	1.4	
OCL			70.5	25.6	25.2
O_2CL	2.3	2.0	47.0	51.7	51.2
O_3CL	2.8	2.5	35.1	63.5	62.6

measurement of the ester content confirms that the proper number of fatty acid tails is present in the synthetic CL molecule. Table I shows the ester content of some of the derivatives, with CL, MLCL, and DLCL included as controls. Within experimental error, all of the ester contents agree with the calculated values.

The CL derivatives also contained the proper proportions of natural and of added fatty acid tails as determined by quantitative GC (Table I). The measured values of the percentage of $C_{18:1}$ in O_3CL, O_2CL, and OCL are close to those calculated on the basis of theoretically containing three, two, and one $C_{18:1}$ tail, respectively. The values for the controls, natural CL and DLCL, and for (THP)CL and DL-(THP)CL, which are from stages in the synthesis of R′CL's, but have had the THP blocking group removed, are also given. It is interesting to note that the percentage of $C_{18:1}$ decreases when two of the tails are clipped off by phospholipase A_2. This decrease was also observed by Okuyama and Nojima (1965).

Once the correct number of ester bonds and correct percentages of fatty acids substituted into CL were confirmed, the positions at which the fatty acids were inserted were determined. Robertson (1962) reported that *C. atrox* snake venom contains only phospholipase A_2, which, by definition, cleaves only at the 2-positions of CL. Therefore, if one of the synthesized CL derivatives is incubated with *C. atrox* snake venom, only the added tails should be removed. This is most easily tested with a CL synthesized with radioactive fatty acid substituted at the 2-positions. If the entire reaction sequence has proceeded as expected, the resulting DLCL should not contain any radioactivity.

CL was prepared containing [^{14}C]linoleic acid at both of the 2-positions, from DL-THP-CL and the radioactive fatty acid. The resulting 1,3-bis[1-acyl-2-[^{14}C]linoleoyl-*sn*-glycero(3)phospho]-*sn*-glycerol ([^{14}C]LACL) was obtained as described for the other derivatives. As in the preparation of DL-THP-CL, 0.017 μmol (80000 cpm) of [^{14}C]LACL was incubated with snake venom for 10 h. The reaction mixture was dried, spotted on a TLC plate, and developed with Touchstone B (Touchstone et al., 1980). Cold CL and DLCL were run along with the reaction mixture. Spots were scraped and counted for ^{14}C. With 97% hydrolysis of [^{14}C]LACL, 94.5% of the total counts were found in the fatty acid spot, with a small amount of radioactivity remaining in DLCL.

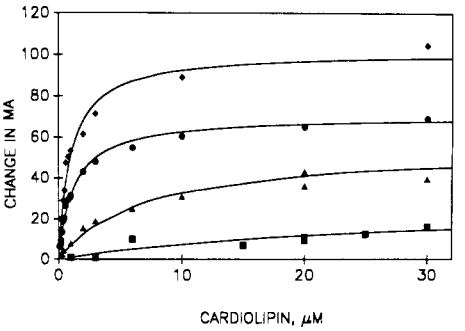

FIGURE 3: Change in the molecular activity of cytochrome *c* oxidase as a function of the cardiolipin derivative concentration. Activities give the stimulation of delipidated cytochrome *c* oxidase. Numbers on the ordinate represent the difference between the molecular activity in the presence of the appropriate CL derivative concentration and the molecular activity in the absence of added CL. Lines illustrate the nonlinear fit to the data. (♦) O_2CL; (●) M_2CL; (▲) H_2CL; (■) DLCL.

The functional effects of replacing two of the linoleoyl chains in natural CL with other fatty acyl groups was tested by the ability of the CL derivative to restore cytochrome *c* oxidase activity. In order to most effectively study the association of cytochrome *c* oxidase with CL, it is necessary to delipidate the enzyme. This delipidated enzyme has a decreased enzymatic activity which can be mostly restored by the addition of CL. This restoration of cytochrome *c* oxidase activity can be used as a guide to the binding of CL to the enzyme. Although it is not possible to completely restore the activity of the delipidated enzyme (CL restores the activity to approximately 70% of the activity before delipidation), ΔAct_{max} for CL is consistent among several highly delipidated enzyme preparations (unpublished results).

The activation of cytochrome *c* oxidase by CL is monitored by the change in the molecular activity. The molecular activity in the presence of CL (or its derivatives) is compared to the activity obtained in the absence of CL. The concentration of the CL derivatives was usually varied from 0 to 60 μM, except with DLCL, where it was necessary to use higher concentrations. Since 0.3% Triton X-100 contains 40 μM micelles, one would expect less than two of the CL derivative per micelle. It is not expected that this has a significant effect on the micelle structure. For all CL derivatives, the change in the molecular activity followed a hyperbolic dependence upon the CL concentration. This change in activity can thus be fitted to a Michaelis–Menten-type equation to give an apparent dissociation constant, $K_{d,app}$, and a maximal increase in activity, ΔAct_{max} (Robinson, 1982).

A plot of the change in molecular activity for O_2CL, M_2CL, H_2CL, and DLCL in Figure 3 gives a general idea of the importance of the fatty acyl tails of CL. Replacement of two $C_{18:2}$ tails with two $C_{18:1}$ tails did not alter either $K_{d,app}$ or ΔAct_{max} significantly, since CL and O_2CL give identical stimulation. For M_2CL, shortening two fatty acyl tails significantly affected only ΔAct_{max}, with ΔAct_{max} 30% lower than it is for CL. When the substituted tails are much shorter, as in H_2CL, the ΔAct_{max} has decreased by 50%, and there is now a significant change in $K_{d,app}$. Upon the removal of both fatty acyl tails, as in the case of DLCL, both parameters are greatly affected. When examined on the same scale as O_2CL, DLCL appears to have no significant effect on cytochrome *c* oxidase activity.

The ΔAct_{max} and $K_{d,app}$ values for the various CL derivatives which were synthesized are given in Table II. O_2CL and CL

Table II: Stimulation of Cytochrome *c* Oxidase Activity[a]

CL derivative	$K_{d,app}$	ΔAct_{max}
(THP)CL	1.3	94
CL	1.3	110
O_2CL	1.0	101
M_2CL	1.2	70
H_2CL	6.8	55
DLCL	28.0	30
O_3CL	1.5	80

[a] The standard errors for $K_{d,app}$ and ΔAct_{max} are as follows: (THP)-Cl, 0.06 and 1.4; CL, 0.12 and 2.0; O_2CL, 0.11 and 3.9; M_2Cl, 0.07 and 1.2; H_2CL, 1.2 and 2.8; DLCL, 5.7 and 2.1; O_3CL, 0.1 and 1.3.

have essentially identical values for ΔAct_{max} and $K_{d,app}$. The greater degree of saturation in O_2CL relative to natural CL (which has 92% linoleic acid) has no significant effect on the activation. Substitution of the slightly shorter $C_{14:0}$ chains, in M_2CL, for the linoleoyl chains has a greater effect on the maximum activation than on the dissociation constant. In this case $K_{d,app}$ is not significantly changed. Substituting with the $C_{6:0}$ chains has a more dramatic effect, with a 50% decrease in the maximum activation and a 6-fold increase in $K_{d,app}$. For DLCL, the maximum possible activity is decreased by 80% compared with CL stimulation, and the apparent binding to the enzyme is decreased by 23-fold. Included as a control is THP-CL which has been acid hydrolyzed to regenerate CL [(THP)CL]. Similar values for the parameters for (THP)CL as for CL demonstrate that the synthetic treatment has not altered the remainder of the CL molecule in a way that affects its binding to cytochrome *c* oxidase. OCL, with a $C_{18:2}$ chain attached to the hydroxyl on the polar head group, exhibits a 30% decrease in ΔAct_{max} compared to that of CL with no change in $K_{d,app}$. No direct equilibrium binding experiments have been done with any of the derivatives.

Discussion

We have described a method for synthesizing derivatives of cardiolipin in which the free hydroxyl remains unsubstituted. The reactions all essentially go to completion, resulting in the desired derivatives obtained in high yield. The synthetic scheme utilizes a facile blocking of the free hydroxyl by the formation of a tetrahydropyranyl ether. This is a fairly common alcohol protecting group [e.g., see Reese (1973)], but one which has not previously been applied to CL synthesis. It is particularly useful for CL synthesis in that only relatively mild conditions are necessary for its removal. Other groups have been used for the protection of the hydroxyl, such as the benzyl (Saunders & Schwarz, 1966) and the *tert*-butyl (van Deenan & de Haas, 1964) groups, but these require considerably harsher conditions for their removal. This is an important consideration in the case of CL, as the molecule is somewhat sensitive to deacylation and oxidation. Additionally, since our reactions start with natural CL, racemized products are not a problem, as in the synthesis by Keana et al. (1986).

Analyses of the synthetic CL derivatives indicate that the synthesis has proceeded as planned. All synthesized CL derivatives contained the correct number of ester bonds, as well as the correct percentages of substituted $C_{18:1}$ fatty acid tails. Phospholipase treatment of the derivative synthesized containing two ^{14}C-labeled fatty acid tails removed nearly all of the radioactivity from the phospholipid, with only a small amount of ^{14}C remaining in the product, DLCL, indicating that the substitution was made almost exclusively at the 2-positions. There has been a report of acyl migration occurring in lysophospholipids (Pluckthun & Dennis, 1982) on silicic acid and under acylation conditions (accelerated by the presence of basic catalysts such as DMAP). The small percentage of acyl exchange we see is of the order of acyl migration observed by these authors, and does not seem to be excessive.

Although we demonstrate this synthesis by making CL derivatives which are disubstituted, the scheme is obviously useful in mono substitutions, or even in the replacement of all four tails of CL. The advantage of starting with natural CL to make a derivative which has homogeneous tails is that the CL will remain optically active.

CL binds to many enzymes in addition to cytochrome *c* oxidase, such as complex I and complex III (Fry & Green, 1981), the mitochondrial phosphate carrier (Mende et al., 1983), and carnitine acylcarnitine translocase (Noel & Pande, 1986). It seems to play a key role in the maintenance of maximum activity, and in cases such as cytochrome *c* oxidase, there is evidence for a specific interaction between CL and the enzyme. It is, therefore, important to study in detail the properties which make CL so ideally suited for the maintenance of activity. As seen in Table II, the acylation with oleic acid at the normally free hydroxyl has a small but significant effect. Since there has not been a comprehensive study of the effect of acylation at this position on CL binding and activation, it was essential to have CL derivatives with this hydroxyl free in order to discern the major structural components of CL binding. This would be particularly important in derivatives containing a single reporter group, such as a spin-label. Our synthesis of derivatives starting with CL allows specific acylation of the lyso molecule in high yield.

Our activity measurements involved incubation of the Triton X-100 solubilized cytochrome *c* oxidase with CL and its derivatives. Although Triton X-100 does inhibit the enzyme, the small amount carried over into the assay is insignificant compared to the large excess of lauryl maltoside, and does not affect the activity. The activation of delipidated cytochrome *c* oxidase by CL also occurs in lauryl maltoside to about the same extent and thus does not seem to represent a specific Triton X-100–CL interaction. However, the study of CL activation in lauryl maltoside requires a detergent exchange out of the Triton X-100 in which the cytochrome *c* oxidase is delipidated, so we chose to study the activation in Triton X-100.

The study of the effect of changing two fatty acid tails in CL upon the activity of cytochrome *c* oxidase provides some interesting results. The hyperbolic dependence of activation on CL concentration allows the determination of parameters of the activation, $K_{d,app}$ and ΔAct_{max}, which give an indication of how well the derivatives stimulate cytochrome *c* oxidase activity. The data clearly show that even the removal of one fatty acid tail affects the amount of stimulation possible. The complete absence of fatty acids at the 2-positions, as in DLCL, greatly decreases the activation, with both $K_{d,app}$ and ΔAct_{max} being affected. In general, cytochrome *c* oxidase is relatively insensitive to the degree of saturation of the fatty acid tails. It is only when the chains are significantly shortened (as in H_2CL) or removed that the differences from CL activation are large. This may in part be due to buffering from the fatty acid tails at the 1-positions, where they still provide a portion of the structure necessary for activation, as long as the 2-positions are acylated. Since there is relatively little difference between the stimulations by CL and O_3CL, the free hydroxyl on the polar head group does not seem to be essential for activation.

It is difficult to make specific conclusions about the requirements for CL activation of cytochrome *c* oxidase from only disubstituted derivatives. Certainly, the hydrophobicity

of the tails plays a key part in the binding. The extent and specific relationship of hydrophobicity to the values of $K_{d,app}$ and ΔAct_{max} can be more easily elucidated by examining tetrasubstituted CL derivatives. These derivatives will provide homogenous CL molecules which will more clearly point out necessary features of CL structure. For example, our data provide no information about the fatty acid tails at the 1-position and how essential they are for binding to cytochrome *c* oxidase. Future experiments attempting to synthesize tetrasubstituted CL derivatives by a similar method are planned.

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