# Acylation of monolysocardiolipin in rat heart

**Brian J. Ma,* William A. Taylor,* Vernon W. Dolinsky,[1,*] and Grant M. Hatch[2,*,†]**

Departments of Pharmacology and Therapeutics,* Internal Medicine and Biochemistry and Molecular Biology,† Center On Aging, University of Manitoba, Winnipeg, Manitoba, Canada, R3E 0W3

**Abstract** **Cardiolipin is a major mitochondrial membrane glycerophospholipid in the mammalian heart. In this study, the ability of the isolated intact rat heart to remodel cardiolipin and the mitochondrial enzyme activities that reacylate monolysocardiolipin to cardiolipin in vitro were characterized. Adult rat heart cardiolipin was found to contain primarily linoleic and oleic acids. Perfusion of the isolated intact rat heart in the Langendorff mode with various radioactive fatty acids, followed by analysis of radioactivity incorporated into cardiolipin and its immediate precursor phosphatidylglycerol, indicated that unsaturated fatty acids entered into cardiolipin mainly by deacylation followed by reacylation. The in vitro mitochondrial acylation of monolysocardiolipin to cardiolipin was coenzyme A-dependent with a pH optimum in the alkaline range. Significant activity was also present at physiological pH. With oleoyl-coenzyme A as substrate, the apparent $K_m$ for oleoyl-coenzyme A and monolysocardiolipin were 12.5 μM and 138.9 μM, respectively. With linoleoyl-coenzyme A as substrate, the apparent $K_m$ for linoleoyl-coenzyme A and monolysocardiolipin were 6.7 μM and 59.9 μM, respectively. Pre-incubation at 50°C resulted in different profiles of enzyme inactivation for the two activities. Both activities were affected similarly by phospholipids, triacsin C, and various lipid binding proteins but were affected differently by various detergents and myristoyl-coenzyme A. [$^3$H]cardiolipin was not formed from monolyso[$^3$H]cardiolipin in the absence of acyl-coenzyme A. Monolysocardiolipin acyltransferase activities were observed in mitochondria prepared from various other rat tissues. In summary, the data suggest that the isolated intact rat heart has the ability to rapidly remodel cardiolipin and that rat heart mitochondria contain coenzyme A-dependent acyltransferase(s) for the acylation of monolysocardiolipin to cardiolipin. A simple and reproducible in vitro assay for the determination of acyl-coenzyme A-dependent monolysocardiolipin acyltransferase activity in mammalian tissues with exogenous monolysocardiolipin substrate is also presented.**—Ma, B. J., W. A. Taylor, V. W. Dolinsky, and G. M. Hatch. **Acylation of monolysocardiolipin in rat heart.** ***J. Lipid Res.*** **1999.** 40: **1837–1845.**

**Supplementary key words** cardiolipin • heart • monolysocardiolipin • acyltransferase

Cardiolipin (CL), the first polyglycerophospholipid ever discovered, was initially isolated from beef heart by Mary Pangborn in 1942 (1). CL is a major membrane glycerophospholipid of mammalian mitochondria and, in the rat heart, CL comprises approximately 15% of the entire cardiac glycerophospholipid mass (for reviews see 2–5). CL is characteristically associated with the inner mitochondrial membrane where it may constitute as much as 21% of the total membrane glycerophospholipid mass of that organelle (6). In addition to its inner membrane localization some CL has been identified in the mitochondrial outer membrane (7). CL was shown to be required for the reconstituted activity of a number of key mammalian mitochondrial enzymes involved in cellular energy metabolism including cytochrome c oxidase (8), carnitine palmitoyltransferase (9), creatine phosphokinase (10), pyruvate translocator (11), tricarboxylate carrier (12), mitochondrial glycerol-3-phosphate dehydrogenase (13), phosphate transporter (14), ADP/ATP carrier (15), and the ATP synthase (16). CL interaction with the above proteins appeared to be specific as substitution with other mitochondrial phospholipids (for example, phosphatidylcholine and phosphatidylethanolamine) did not fully reconstitute activity (for review see 5). Under experimental conditions in which CL was removed, denaturation and complete loss in activity of many of these proteins was observed. Thus, the appropriate content of CL may be an important requirement for activation of enzymes involved in mitochondrial respiration.

CL was shown to be synthesized via the cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) pathway in the isolated rat heart (17). In this pathway, phosphatidic acid (PA) was converted to CDP-DG. CDP-DG was then condensed with *sn*-glycerol-3-phosphate to form phosphatidylglycerol (PG) phosphate which was rapidly converted to PG. Finally, PG condensed with another CDP-DG molecule to form CL. CL contains four fatty acid side chains and

Abbreviations: AT, acyltransferase; MLCL AT, monolysocardiolipin acyltransferase; CL, cardiolipin; MLCL, monolysocardiolipin; PG, phosphatidylglycerol; CDP-DG, cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol; PA, phosphatidic acid; FABP, fatty acid binding protein; LBP, lipid binding protein; ACBP, acyl-coenzyme A binding protein.

[1] Present address: Lipid and Lipoprotein Research Group, 328 Heritage Medical Research Center, University of Alberta, Edmonton, Alberta, Canada, T6G 2S2.

[2] To whom correspondence should be addressed.

the molecular composition of CL appeared to be in part dependent upon the type of fatty acids provided in the diet (2, 18). The proportion of CL symmetrical molecular species was determined to be 50–65% and the four acyl positions were shown to be occupied by monounsaturated and diunsaturated chains of 16–18 carbons in length (19). The hydrophobic double unsaturated linoleic diacylglycerol species appeared to be an important structural requirement for the high protein binding affinity of CL (20). Dietary modification of the molecular species composition of CL was shown to alter the oxygen consumption in cardiac mitochondria (21, 22). In addition, the activity of delipidated rat liver cytochrome c oxidase was reconstituted by the addition of CL. The specific activity of the reconstituted cytochrome c oxidase varied markedly and significantly with different molecular species of CL. Thus, in addition to CL content, the appropriate molecular composition of CL may be critical for optimum mitochondrial respiratory performance.

The deacylation–reacylation cycle for the molecular remodeling of glycerophospholipids was first described by Lands (23). The acyltransferase (AT) activities for mammalian phosphatidylcholine remodeling have been extensively investigated (for review see 24). However, limited information was available on the ATs that are involved in the molecular remodeling of CL. An acyl-coenzyme A-dependent reacylation of dilysocardiolipin in rat liver microsomes was reported (25). This process was not specific for linoleoyl-coenzyme A and was inactive in rat liver mitochondria. More recently, a deacylation–reacylation cycle for the molecular remodeling of endogenous CL in rat liver mitochondria was proposed (26). Endogenous CL was deacylated to monolysocardiolipin (MLCL) and then reacylated with linoleoyl-coenzyme A, derived from phosphatidylcholine, to form CL. Such a deacylation followed by reacylation scheme for CL seems logical as mitochondrial phospholipase $A_2$ was shown to readily hydrolyze endogenous and exogenous CL (27–30). However, AT(s) activities involved in the reacylation of MLCL to CL had never been identified or characterized in any mammalian tissue. In this study, we show that the isolated intact rat heart has the ability to rapidly and readily remodel CL with unsaturated fatty acids. In addition, we have characterized the enzyme activities that acylate MLCL to CL in rat heart mitochondrial fractions. To our knowledge this study represents the first detailed characterization of mitochondrial acyl-coenzyme A-dependent MLCL AT activity assayed with exogenous MLCL. We present a simple and reproducible in vitro assay for the determination of this activity. A preliminary report of this work was published in abstract form (31).

## MATERIALS AND METHODS

### Materials

Adult male Sprague-Dawley rats (150–300 g body weight) were maintained on Purina rat chow and tap water ad libitum, in a light- and temperature-controlled room. Treatment of animals conformed to the Guidelines of the Canadian Council on Animal Care. Rat heart fatty acid binding protein (FABP) and mouse adipocyte FABP were obtained from Dr. Judith Storch (Rutgers University). Adipocyte and keratinocyte lipid binding protein (LBP) were obtained from Dr. David Bernlohr (University of Minnesota). Rat liver acyl CoA binding protein (ACBP) was obtained from Dr. Jens Knudsen (Odense University, Denmark). H9c2 cells were obtained from the American Type Culture Collection. [1-$^{14}$C]oleic acid, [1-$^{14}$C]linoleic acid, [1-$^{14}$C]palmitic acid, [1-$^{14}$C]oleoyl-coenzyme A, and [1,3-$^{3}$H]glycerol were obtained from Mandel Scientific (Missassauga, Ontario, Canada). [1-$^{14}$C]linoleoyl-coenzyme A and [1-$^{14}$C]palmitoyl-coenzyme A were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Oleic acid, linoleic acid, palmitic acid and acyl-coenzyme As were obtained from Serdary Research Laboratories (Englewood Cliffs, NJ). MLCL (a mixture of 1′(1-acyl-*sn*-glycerol-3-phosphoryl)-3′(1′′,2′′:-diacyl-*sn*-glycerol-3-phosphoryl)glycerol and 1′ (1,2-diacyl-*sn*-glycerol-3-phosphoryl)-3′(1′′-acyl-*sn*-glycerol-3-phosphoryl)glycerol, produced by phospholipase $A_2$ hydrolysis of bovine heart CL, was obtained from Avanti Polar Lipids (Alabaster, AL). Purity of the MLCL substrate was checked by two-dimensional thin-layer chromatography as described (17). The fatty acyl molecular species composition of the MLCL substrate was examined as described (32) and was comprised mainly of linoleic (90.3%) and oleic (8.6%) acids. Dilysocardiolipin was synthesized from CL as described (28). Ecolite scintillation cocktail was obtained from ICN Biochemicals (Costa Mesa, CA) and thin-layer plates (silica gel 60, 0.25 mm thickness) were obtained from Fisher Scientific (Winnipeg, Manitoba, Canada). *N. mocambique mocambique* phospholipase $A_2$ and all other biochemicals were of analytical grade and obtained from either Fisher Scientific (Edmonton, Alberta, Canada) or Sigma Chemical Company (St. Louis, MO).

### Heart perfusion studies

For fatty acid perfusion experiments, the animal was killed by decapitation and the heart was quickly removed and cannulated via the aorta using a modified syringe needle (18 gauge). The remaining blood in the coronary circulation was removed by injecting the heart with freshly prepared Krebs-Henseleit buffer (33) using a 10 cm$^3$ syringe. The heart was placed on a perfusion apparatus and perfused for 5 min or until electrical stabilization was achieved. The viability of the heart throughout the perfusion experiment was monitored via electrocardiac analysis. Subsequent to stabilization, the heart was perfused for 30 min in the Langendorff mode (34) with 12.5 ml of Krebs-Henseleit buffer containing 0.1 mm [1-$^{14}$C]oleic acid (0.4 μCi/ml) bound to bovine serum albumin in a 1:1 molar ratio (35). In some experiments, the heart was perfused with 0.1 mm [1-$^{14}$C]linoleic acid (0.4 μCi/ml) or 0.1 mm [1-$^{14}$C]palmitic acid (0.4 μCi/ml) bound 1:1 to bovine serum albumin as above. The lipids were extracted and radioactivity incorporated into PG and CL was determined exactly as described [17].

### Assay of MLCL AT activity

For assay of MLCL AT activities, a 10% (w/v) rat heart mitochondrial fraction (or mitochondria from other rat tissues) was prepared by homogenizing the heart (Polytron 20 sec burst) in 0.25 m sucrose, 10 mm Tris-HCL, pH 7.4, 2 mm EDTA followed by differential centrifugation. The homogenate was centrifuged at 1,000 *g* for 5 min and the resulting supernatant was centrifuged at 12,000 *g* for 10 min. The pellet obtained from this centrifugation was washed once in homogenizing buffer and then resuspended in 1–2 ml of homogenizing buffer using a tight-fitting Dounce A homogenizer and designated the mitochondrial fraction. Rat heart mitochondrial fractions (50 μg protein) were incubated for 30 min at 25°C in 50 mm Tris-HCl, pH 9.0, 93 μm [1-$^{14}$C]oleoyl-coenzyme A (6,700 dpm/nmol), or 33 μm [1-$^{14}$C]linoleoyl-coenzyme A (68,700 dpm/nmol) at pH 8.0, or 33 μm [1-$^{14}$C]palmitoyl-coenzyme

A (80,200 dpm/nmol) at pH 8.0, 300 μm MLCL in a final volume of 0.35 ml. Under these optimum assay conditions the reactions were linear up to 150 μg protein and at least 40 min. The MLCL substrate in chloroform was dried under nitrogen and resuspended in double distilled water via sonication in a bath sonicator for 45 min prior to addition to the assay mixture. The temperature of the bath sonicator was maintained at 4°C by ice. The reaction was initiated by the addition of the radioactive acyl-coenzyme A substrate and terminated by the addition of 3 ml of chloroform–methanol 2:1 (v/v). To facilitate phase separation, 0.8 ml of 0.9% KCl was added. The aqueous phase was removed and the organic phase was washed with 2 ml of chloroform–methanol–0.9% NaCl 3:48:47 (v/v/v). The resulting organic fraction was dried under nitrogen and resuspended in 25 μl of chloroform–methanol 2:1 (v/v). A 20-μl aliquot of the resuspended organic phase was placed on a thin-layer plate and CL was separated from other phospholipids in a solvent system containing chloroform–hexane–methanol–acetic acid 50:30:10:5 (v/v/v/v). [$^3$H]CL was prepared as described below and after addition of 0.1 μm [$^3$H]CL (1,000 dpm/nmol) to the incubation mixture the recovery of labeled CL was 93% (average of two determinations) indicating little loss of the final product during the incubation. In some experiments, separation of CL from other lipids was confirmed using a two-dimensional thin-layer chromatography system described previously [17]. The silica gel corresponding to CL was removed and placed in a plastic scintillation vial and 5 ml of scintillant added. Radioactivity incorporated into CL was examined approximately 24 h later using a liquid scintillation counter. MLCL AT activity was taken as radioactivity incorporated into CL in the presence of the MLCL substrate minus radioactivity incorporated into CL in the absence of the MLCL substrate. In some experiments, 0.5 mm phospholipid, or 0.05% detergent was included in the assay mixture. In other experiments, mitochondrial fractions were preincubated for 5 min with 14 μm triacsin C prior to assay. In other experiments, 16.5 or 33 μm rat heart FABP, mouse adipocyte FABP, keratinocyte LBP, adipocyte LBP, rat liver ACBP, or albumin were included in the incubation mixture. In other experiments, 10 mm ATP, 1.0 mm coenzyme A, and 33 μm [1-$^{14}$C]oleic acid (11,944 dpm/nmol) were added in place of [1-$^{14}$C]oleoyl-coenzyme A (or 33 μm [1-$^{14}$C]linoleic acid (13,907 dpm/nmol) added in place of [1-$^{14}$C]linoleoyl-coenzyme A). In acyl-coenzyme A competition experiments, 33 μm of the competing acyl-coenzyme A was added at a 1:1 molar ratio with the appropriate radioactive acyl-coenzyme A substrate.

### Other analyses

H9c2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated newborn calf serum, 100 U/ml penicillin G, 10 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cell cultures were maintained at 37°C saturated with humidified air/5% carbon dioxide. H9c2 cells were incubated for 24 h with [1,3-$^3$H]glycerol and the radioactive CL was isolated by thin-layer chromatography as described [17]. The radioactive CL was removed from the silica gel [36] and treated with 0.5 units of phospholipase $A_2$ for 30 min in 0.5 ml buffer containing 50 mm Tris-HCl, pH 8.0, 3 mm $CaCl_2$ at 37°C. The [$^3$H]MLCL formed was isolated by thin-layer chromatography as described [17] and removed from the silica gel as above. The [$^3$H]MLCL preparation was added to assay incubation mixtures (20,000 dpm/tube) and the formation of [$^3$H]CL was monitored in the standard assay system described above minus acyl-coenzyme A. For fatty acid analysis, PG and CL were extracted from the silica gel as described [36] and fatty acid methyl esters were prepared and analyzed as described [32]. Protein was determined as described [37].

TABLE 1. Molecular composition of rat heart cardiolipin and phosphatidylglycerol

| | Relative Percentage | |
|---|---|---|
| Fatty Acid | Cardiolipin | Phosphatidylglycerol |
| Palmitic acid (16:0) | 1.1 | 36.8 |
| Stearic acid (18:0) | <1.0 | 15.5 |
| Oleic acid (18:1) | 7.7 | 35.9 |
| Linoleic acid (18:2) | 88.2 | 10.1 |
| All others combined | 2.0 | 2.7 |

CL and PG were isolated from rat heart and the fatty acid composition was determined as described in Materials and Methods. Values are means of four hearts and are expressed as a relative percentage of total fatty acid.

## RESULTS

To examine whether the isolated intact rat heart could reacylate MLCL to CL, we initially examined the molecular composition of CL and its immediate precursor PG in rat heart. As seen in **Table 1**, CL was comprised of mainly linoleate (18:2) and oleate (18:1) molecular species. In contrast, PG was comprised of mainly palmitic and oleic acids with significant amounts of stearic and linoleic acids. Thus, the molecular composition of rat heart CL differed markedly from its immediate phospholipid precursor PG. We then perfused isolated rat hearts in the Langendorff mode for 30 min with oleic, linoleic, and palmitic acids (0.1 mm) bound to albumin in a 1:1 molar ratio. As seen in **Table 2** perfusion of the isolated rat heart with labeled fatty acids resulted in considerable incorporation of these fatty acids into CL and PG. The incorporation of radioactive unsaturated fatty acids (oleate, 18:1; linoleate, 18:2) into CL was approximately 3- to 4-times higher than the saturated fatty acid (palmitate, 16:0). In addition, incorporation of radioactive unsaturated fatty acids into CL was higher than PG. In contrast, incorporation of radioactive palmitic acid into PG was much greater than into CL. Because saturated fatty acids such as palmitic acid enter into glycerophospholipids mainly by glycerophospholipid de novo biosynthetic pathways (38), the data suggest that cardiac CL is remodeled to obtain its appropriate molecular composition in vivo.

MLCL AT activity had not been demonstrated or characterized in the heart. We thus examined the ability of rat

TABLE 2. Incorporation of [1-$^{14}$C]palmitate, [1-$^{14}$C]oleate, and [1-$^{14}$C]linoleate into rat heart cardiolipin and phosphatidylglycerol

| | Radioactivity incorporated into Cardiolipin and Phosphatidylglycerol | |
|---|---|---|
| Fatty acid | Cardiolipin | Phosphatidylglycerol |
| | *dpm × 10$^4$/g freeze-dried heart* | |
| Palmitic acid (16:0) | 0.4 ± 0.1 | 1.5 ± 0.2 |
| Oleic acid (18:1) | 1.4 ± 0.4 | 1.0 ± 0.3 |
| Linoleic acid (18:2) | 1.7 ± 0.3 | 0.7 ± 0.2 |

Isolated hearts were perfused for 30 min in the Langendorff mode with Krebs-Henseleit buffer containing [1-$^{14}$C]palmitic or [1-$^{14}$C]oleic or [1-$^{14}$C]linoleic acids and the radioactivity incorporated into CL and PG was determined as described in Materials and Methods. Values are means ± standard deviation of three hearts.

heart mitochondria to reacylate MLCL to CL in vitro. MLCL AT activities were determined in freshly prepared isolated rat heart mitochondrial fractions. Oleoyl-coenzyme A and linoleoyl-coenzyme A were used as the acyl-coenzyme A substrates in this study as these were the major molecular species found in rat heart CL. The acylation of MLCL to CL was found to have a pH optimum in the alkaline range using oleoyl-coenzyme A and linoleoyl-coenzyme A as substrates (**Fig. 1**). When oleoyl-coenzyme A was used as substrate, the pH optimum range for the reaction was 8.0–9.0. With linoleoyl-coenzyme A as substrate, the pH optimum range for the reaction was 7.5–8.0. A gradual loss of activity with both substrates was observed when the pH was decreased (data not shown). However, with both substrates significant MLCL AT activity was observed at physiological pH. When dilysocardiolipin was added to these incubations, in place of MLCL, [$^{14}$C]CL formation was not observed. In the presence of [1-$^{14}$C]oleic acid, ATP and coenzyme A, MLCL and mitochondrial protein, some formation of [$^{14}$C]CL was observed (9 pmol/min·mg protein). In addition, in the presence of [1-$^{14}$C]linoleic acid, ATP and coenzyme A, MLCL and mitochondrial protein, some formation of [$^{14}$C]CL was observed (4 pmol/min·mg protein). However, [$^{14}$C]CL was not formed when [1-$^{14}$C]oleic acid or [1-$^{14}$C]linoleic acid was added alone, indicating the reaction was coenzyme A-dependent. When [1-$^{14}$C]palmitoyl-coenzyme A was used as substrate, the formation of [$^{14}$C]CL was 13 ± pmol/min·mg protein (average of three determinations) and was approximately 5% of the activity observed with [1-$^{14}$C]oleoyl-coenzyme A as substrate. We examined whether MLCL could be converted to CL by transacylase activity. [$^{3}$H]MLCL was synthesized by phospholipase $A_2$ hydrolysis of [$^{3}$H]CL isolated from

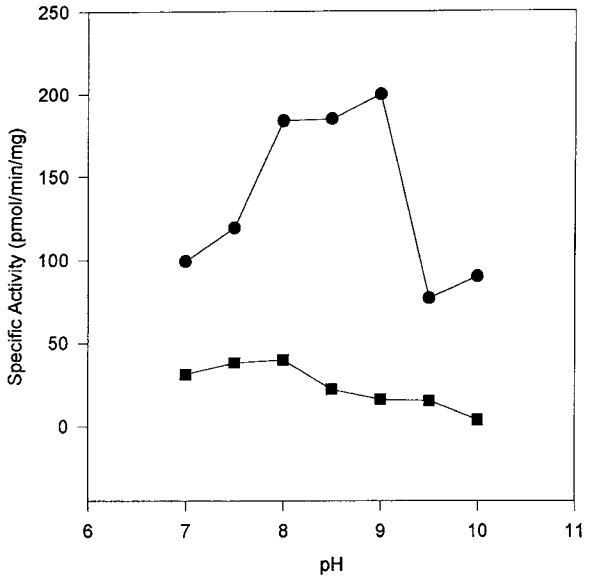


**Fig. 1.** pH profile of MLCL AT activities in rat heart mitochondria. MLCL AT activities were determined in the presence of [1-$^{14}$C]oleoyl-coenzyme A (circles) or [1-$^{14}$C]linoleoyl-coenzyme A (squares) at various pH. Values represent the mean of two separate experiments performed in duplicate. The results between samples differed by less than 15%.

TABLE 3. Effect of albumin, FABP, and ACBP on MLCL AT activity

| | Relative MLCL AT Activities | |
|---|---|---|
| | With [1-$^{14}$C]Oleoyl-Coenzyme A | With [1-$^{14}$C]Linoleoyl-Coenzyme A |
| Protein added | | |
| None | 100 | 100 |
| Albumin | 85 (87) | 74 (68) |
| Rat heart FABP | 91 (117) | 68 (75) |
| Mouse adipocyte FABP | 98 (97) | 67 (68) |
| Rat liver ACBP | 119 (76) | 66 (49) |

Rat heart mitochondrial fractions were incubated in the absence (none) or presence of 16.5 μm FABP or ACBP or albumin and MLCL AT activities were determined with either [1-$^{14}$C]oleoyl-coenzyme A or [1-$^{14}$C]linoleoyl-coenzyme A as substrates. Results represent the mean of two experiments and are expressed as a percent of control. With [1-$^{14}$C]oleoyl-coenzyme A as substrate, control = 237 pmol/min·mg protein. With [1-$^{14}$C]linoleoyl-coenzyme A as substrate, control = 45 pmol/min·mg protein. The results between samples differed by less than 15%. Results in brackets represent incubation with 33 μm FABP, ACBP, or albumin.

H9c2 cells and added directly to assay incubation mixtures in the absence of acyl-coenzyme A. No significant formation of [$^{3}$H]CL was observed, suggesting that transacylase activity does not contribute significantly to the acylation of MLCL in the heart. A previous study had shown that fatty acid binding proteins increased the incorporation of oleoyl-coenzyme A into phosphatidic acid (39). The presence of albumin, rat liver ACBP, mouse adipocyte FABP, or rat heart FABP did not stimulate the formation of [1-$^{14}$C]CL from [1-$^{14}$C]oleoyl-coenzyme A or from [1-$^{14}$C]linoleoyl-coenzyme A (**Table 3**). Similarly, the presence of adipocyte LBP or keratinocyte LBP did not affect the formation of [1-$^{14}$C]CL from [1-$^{14}$C]oleoyl-coenzyme A or from [1-$^{14}$C]linoleoyl-coenzyme A (data not shown).

The apparent affinities for oleoyl-coenzyme A, linoleoyl-coenzyme A, and MLCL substrates were then determined. The activities of the mitochondrial MLCL AT at different oleoyl-coenzyme A concentrations in the presence of a fixed amount of MLCL were determined and the results are depicted in a Lineweaver-Burk plot. From this plot the apparent $K_m$ of oleoyl-coenzyme A was determined to be 12.5 μm (**Fig. 2A**). The activities of the mitochondrial MLCL AT at different MLCL concentrations in the presence of a fixed amount of oleoyl-coenzyme A were then determined and the results are depicted in a Lineweaver-Burk plot. From this plot the apparent $K_m$ of MLCL was determined to be 138.9 μm (Fig. 2B). The activities of the mitochondrial MLCL AT at different linoleoyl-coenzyme A concentrations in the presence of a fixed amount of MLCL were determined and the results are depicted in a Lineweaver-Burk plot. From this plot the apparent $K_m$ of linoleoyl-coenzyme A was determined to be 6.7 μm (**Fig. 3A**). The activities of the mitochondrial MLCL AT at different MLCL concentrations in the presence of a fixed amount of linoleoyl-coenzyme A were then determined and the results are depicted in a Lineweaver-Burk plot. From this plot the apparent $K_m$ of MLCL was determined to be 59.9 μm (Fig. 3B).

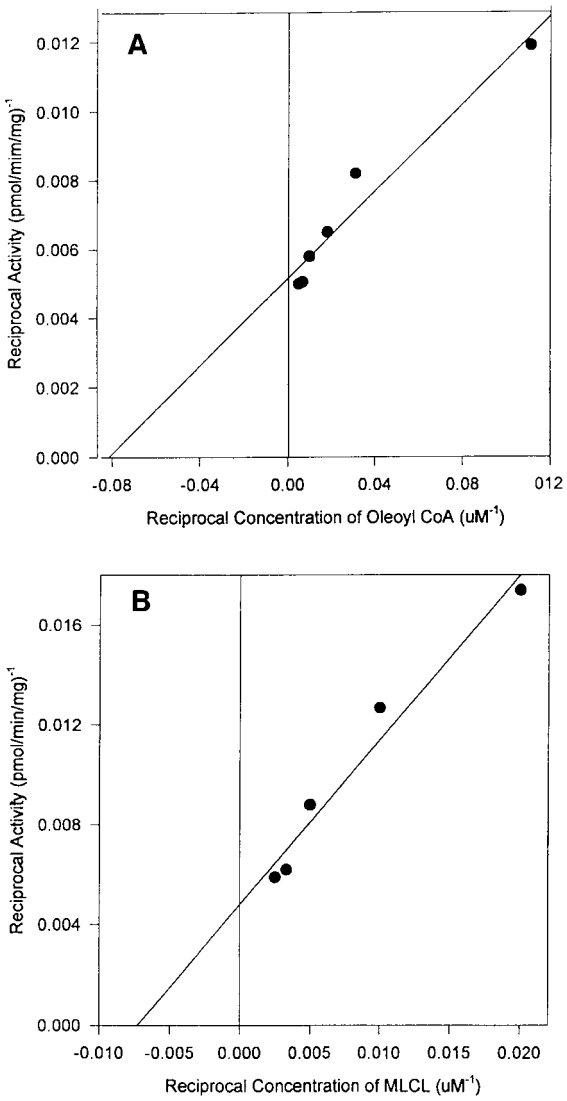


**Fig. 2.** MLCL AT activities in rat heart mitochondria in the presence of various concentrations of oleoyl-coenzyme A and MLCL. A: MLCL AT activities were determined in rat heart mitochondrial fractions in the presence of 0.3 mm MLCL and various concentrations of [1-$^{14}$C]oleoyl-coenzyme A and the results are depicted in a Lineweaver-Burk plot ($r^2 = 0.95$). B: MLCL AT activities were determined in rat heart mitochondrial fractions in the presence of 93 μm [1-$^{14}$C]oleoyl-coenzyme A and various concentrations of MLCL and the results are depicted in a Lineweaver-Burk plot ($r^2 = 0.95$). Results represent the mean of two separate experiments performed in duplicate. The results between samples differed by less than 15%.

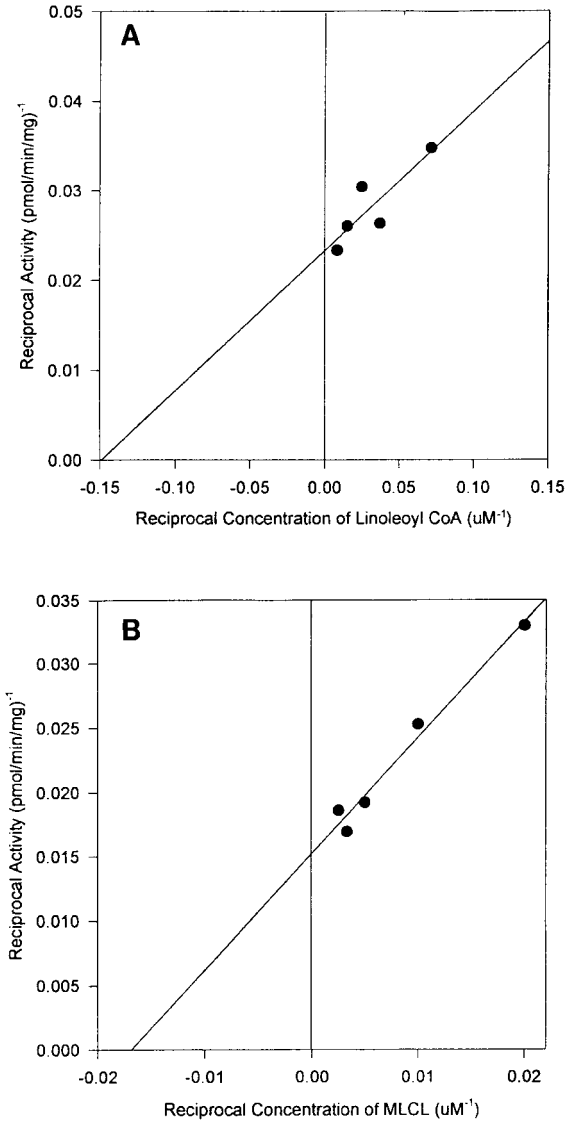


**Fig. 3.** MLCL AT activities in rat heart mitochondria in the presence of various concentrations of linoleoyl-coenzyme A and MLCL. A: MLCL AT activities were determined in rat heart mitochondrial fractions in the presence of 0.3 mm MLCL and various concentrations of [1-$^{14}$C]linoleoyl-coenzyme A and the results are depicted in a Lineweaver-Burk plot ($r^2 = 0.95$). B: MLCL AT activities were determined in rat heart mitochondrial fractions in the presence of 33 μm [1-$^{14}$C]linoleoyl-coenzyme A and various concentrations of MLCL and the results are depicted in a Lineweaver-Burk plot ($r^2 = 0.95$). Results represent the mean of two separate experiments performed in duplicate. The results between samples differed by less than 15%.

We next examined the heat inactivation profile of the MLCL AT activities. Rat heart mitochondrial fractions were pre-incubated at 55°C for up to 10 min and MLCL AT activity was subsequently determined. Enzyme activity was completely lost by pre-incubation at 55°C (data not shown). Thus, the cardiac MLCL AT activities were heat labile. Milder pre-incubation conditions (40–45°C) resulted in little inactivation. However, pre-incubation at 50°C resulted in a different rate of inactivation of the MLCL AT activities between oleoyl-coenzyme A and linoleoyl-coenzyme A substrates (**Fig. 4**). With linoleoyl-coenzyme A as substrate, pre-incubation for 2 min resulted in a 33% reduction in enzyme activity. In contrast, with oleoyl-coenzyme A as substrate, pre-incubation for 2 min resulted in a 70% reduction in enzyme activity.

The above difference in heat-dependent enzyme inactivation suggested the possible presence of separate AT activities for the acylation of MLCL to CL in rat heart mitochondria. We examined the ability of various acyl-coenzyme As to compete with [$^{14}$C]CL formation from [1-$^{14}$C]oleoyl-coenzyme A or [1-$^{14}$C]linoleoyl-coenzyme A. The pres-

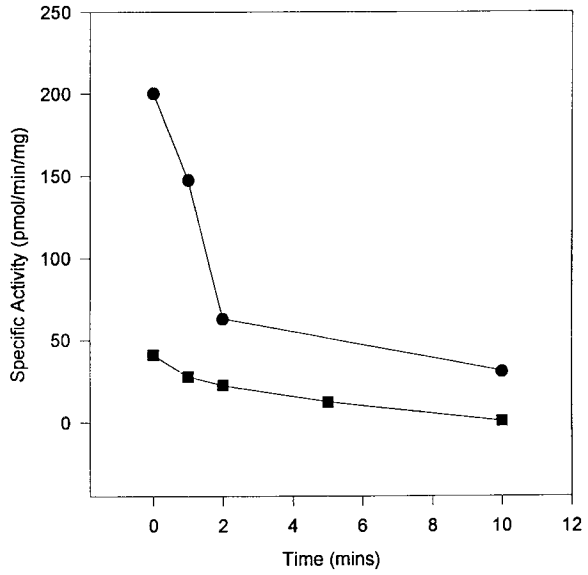


**Fig. 4.** Heat inactivation profile of MLCL AT. Rat heart mitochondrial fractions were pre-incubated at 50°C for up to 10 min and MLCL AT activities were determined with either [1-$^{14}$C]oleoyl-coenzyme A (circles) or [1-$^{14}$C]linoleoyl-coenzyme A (squares) in the presence of MLCL at their respective pH optimums. Results represent the mean of two separate experiments performed in duplicate. The results between samples differed by less than 15%.

ence of a 1:1 molar ratio of most acyl-coenzyme As with [$^{14}$C]oleoyl-coenzyme A or [1-$^{14}$C]linoleoyl-coenzyme A in the assay incubation mixture reduced the formation of [1-$^{14}$C]CL (**Table 4**). However, the presence of myristoyl-coenzyme A inhibited formation of [1-$^{14}$C]CL from [1-$^{14}$C] oleoyl-coenzyme A but not from [1-$^{14}$C]linoleoyl-coenzyme A. We next examined whether MLCL AT activity could be affected by various detergents or phospholipids or the acyl-coenzyme A synthetase inhibitor, triacsin C. **Table 5** shows that compared to the acylation of MLCL with

TABLE 4. Effect of competitory acyl-coenzyme A substrates on MLCL AT activity

| | Relative MLCL AT Activities | |
|---|---|---|
| | With [1-$^{14}$C]Oleoyl-Coenzyme A | With [1-$^{14}$C]Linoleoyl-Coenzyme A |
| Competitor | | |
| None | 100 | 100 |
| Myristoyl-CoA | 53 | 106 |
| Palmitoyl-CoA | 37 | 50 |
| Palmitoleoyl-CoA | 49 | 69 |
| Stearoyl-CoA | 48 | 52 |
| Linoleoyl-CoA | 50 | ND |
| Oleoyl-CoA | ND | 66 |

Rat heart mitochondrial fractions were incubated in the absence (none) or presence of various acyl-coenzyme As and [1-$^{14}$C]oleoyl-coenzyme A or [1-$^{14}$C]linoleoyl-coenzyme A (1:1 molar ratio with the acyl-coenzyme A competitor) and MLCL AT activities were determined. Results represent the mean of two experiments and are expressed as a percent of control. With [1-$^{14}$C]oleoyl-coenzyme A as substrate, control = 237 pmol/min·mg protein. With [1-$^{14}$C]linoleoyl-coenzyme A as substrate control = 45 pmol/min·mg protein. The results between samples differed by less than 15%; ND, not determined.

TABLE 5. Effect of various detergents and phospholipids on MLCL AT activities

| | Relative MLCL AT Activities | |
|---|---|---|
| | With [1-$^{14}$C]Oleoyl-Coenzyme A | With [1-$^{14}$C]Linoleoyl-Coenzyme A |
| Control | 100 | 100 |
| Detergent | | |
| Triton X-100 | 26 | 105 |
| Miranol H2M | 225 | 15 |
| Tween 20 | 120 | 26 |
| Tyloxapol | 98 | 65 |
| CHAPS | 45 | 48 |
| Phospholipid | | |
| Phosphatidylcholine | 83 | 73 |
| Phosphatidylethanolamine | 76 | 67 |
| Lysophosphatidylcholine | 46 | 62 |
| Lysophosphatidylethanolamine | 82 | 98 |

Rat heart mitochondrial fractions were incubated in the absence (control) or presence of various detergents (0.05% final concentration) or various phospholipids (0.5 mm final concentration) and MLCL AT activities were determined. Results represent the mean of two experiments and are expressed as a percent of control. The results between samples differed by less than 15%. With [1-$^{14}$C]oleoyl-coenzyme A as substrate, control = 250 pmol/min·mg protein. With [1-$^{14}$C]linoleoyl-coenzyme A as substrate, control = 40 pmol/min·mg protein.

[1-$^{14}$C]linoleoyl-coenzyme A, the acylation of MLCL with [1-$^{14}$C]oleoyl-coenzyme A was affected differentially in the presence of various detergents (added at a final concentration of 0.05%). Both MLCL AT activities were affected similarly by the addition of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and lysophosphatidylethanolamine (added at a final concentration of 0.5 mm). In the presence of 14 μm triacsin C, both activities were unaltered (data not shown). We next examined MLCL AT activities in various rat tissues. Significant MLCL AT activity was observed in the mitochondrial fractions prepared from all tissues examined (**Table 6**). The highest activities observed were for those of the liver and lung with brain having the lowest MLCL AT activity. Taken together, the results suggest that the isolated intact rat

TABLE 6. MLCL AT activities in various tissues

| | Relative MLCL AT Activities | |
|---|---|---|
| Tissue | With [1-$^{14}$C]Oleoyl-Coenzyme A | With [1-$^{14}$C]Linoleoyl-Coenzyme A |
| Heart | 100 | 100 |
| Liver | 992 | 1030 |
| Lung | 345 | 495 |
| Spleen | 340 | 278 |
| Kidney | 698 | 427 |
| Brain | 29 | 47 |

Mitochondrial fractions were prepared from various tissues and MLCL AT activities were assayed as described in Materials and Methods. Results represent the mean of two experiments and are expressed as a percent of heart activity. The results between samples differed by less than 15%. With [1-$^{14}$C]oleoyl-coenzyme A and [1-$^{14}$C]linoleoyl-coenzyme A, heart activities were 272 pmol/min·mg protein and 42 pmol/min·mg protein, respectively.

heart may rapidly and readily remodel CL and this involves the participation of a MLCL coenzyme A-dependent AT(s).

## DISCUSSION

The objectives of this study were to determine whether the isolated intact rat heart had the ability to remodel CL, to develop a simple and reproducible in vitro assay for the measurement of MLCL AT activity with exogenous MLCL substrate, and to characterize the enzyme activities that acylate MLCL to CL with physiological substrates in rat heart mitochondrial fractions. Two clear pieces of evidence indicated that MLCL could be acylated to CL in the heart. First, perfusion of the isolated intact rat heart in the Langendorff mode with various fatty acids indicated that the incorporation of unsaturated fatty acids (linoleic acid and oleic acid) into CL was greatly enhanced compared to that of a saturated fatty acid (palmitic acid) which would enter CL primarily via de novo biosynthesis. Secondly, in vitro acyl-coenzyme A-dependent MLCL AT activity was observed, even at physiological pH, in isolated rat heart mitochondrial fractions using either linoleoyl-coenzyme A or oleoyl-coenzyme A as substrates and these fatty acids represent the major molecular species found in adult rat heart CL.

It was initially thought that the rat liver enzymes that use CDP-DG for the de novo biosynthesis of CL exhibited some fatty acyl substrate selectivity (for review see 2). However, the species patterns of PA, CDP-DG, and PG were similar enough to imply that the rat liver enzymes of the CL biosynthetic pathway were not molecular species-selective (40). The rat liver CL synthase was shown to have no selective preference for various molecular species of CDP-DG (41). The fatty acid composition of the various phospholipid species in the heart was shown to be dependent upon a variety of factors including composition of diet (for review see 42). A recent study in rats indicated that dietary elaidic acid (*trans*-9 18:1) was desaturated to *cis*-5, *trans*-9 18:2, and incorporated into most phospholipids of several tissues, including heart (43). However, *cis*-5, *trans*-9 18:2 was not incorporated into CL, which implied specificity of CL molecular remodeling in the rat heart. Such a selectivity in CL molecular remodeling might implicate the existence of ATs involved in the molecular remodeling of CL that are distinct from those that remodel other phospholipids.

In the present study, we characterized the coenzyme-A dependent enzyme activities that reacylate MLCL to CL in rat heart mitochondria. The apparent $K_m$ values observed for the oleoyl-coenzyme A (12.5 μm) and linoleoyl-coenzyme A (6.7 μm) substrates might initially suggest that these enzymes have a high affinity for addition of these molecular species onto MLCL. Such a high affinity could explain why there is an enrichment of adult rat heart mitochondrial CL with linoleate and oleate molecular species. However, the estimated in vivo levels of acyl-coenzyme As are approximately 15–35 μm (42). The apparent $K_m$ (59.9 μm) for MLCL with linoleoyl-coenzyme A as substrate was approximately half the apparent $K_m$ (138.9 μm) for MLCL with oleoyl-coenzyme A as substrate. As the estimated in vivo concentration of MLCL is approximately 34 μm (44, 45), it is likely that the level of MLCL is rate-limiting for the MLCL AT. This observation might explain the enrichment of linoleic over oleic molecular species in CL. Although these are apparent $K_m$ values, the complex natural mitochondrial membrane used in this study may provide a more accurate estimate of the natural activities of these enzymes than compared to a purified enzyme reconstituted in artificially defined substrates and detergents. Such a situation has been observed in studies of phospholipase $A_2$ hydrolysis of U987 monocyte membranes (46). However, as MLCL is found in only trace amounts in mitochondria (44, 45), low concentrations of lyso derivatives of CL might result in a selectivity of acyl donors in vivo (25). Therefore, it must be stressed that the observed differences in apparent $K_m$s of the substrates may simply be due to differences in the solubility of these substrates within the mitochondrial membrane preparation.

Previous studies have indicated that FABPs and ACBP may interact with and affect the utilization of acyl-coenzyme As (39, and for review see 47). For example, rat liver FABP, but not intestinal FABP, increased the incorporation of oleoyl-coenzyme A into phosphatidic acid (39). In our study, addition of albumin or lipid binding proteins (obtained from three independent laboratories) did not elevate enzyme activity. These data indicated that lipid binding proteins may not mediate specific targeting of the acyl-coenzyme A substrate to the MLCL AT. Interestingly, when linoleoyl-coenzyme A was used as substrate, these lipid binding proteins appeared to reduce enzyme activity. It is possible that lipid binding proteins such as ACBP bind acyl-coenzyme A and prevent it from entering into the mitochondria. Studies with various phospholipid additions and triacsin C addition indicated that MLCL AT activities, when assayed with either oleoyl-coenzyme A or linoleoyl-coenzyme A substrates, were affected similarly. In contrast, the activities were markedly different in the presence of various detergents, when assayed with either oleoyl-coenzyme A or linoleoyl-coenzyme A substrates, indicating that the acylation of MLCL to CL may be catalyzed by different enzymes. In addition, some differences were noted when various acyl-coenzyme As were added in the competition experiments. Specifically, the presence of myristoyl-coenzyme A inhibited MLCL AT activity when oleoyl-coenzyme A was used as substrate but not when linoleoyl-coenzyme A was used as substrate. Acyl-coenzyme As themselves may act as detergents. Thus, caution must be used in interpretation of this data as surface dilution effects occur when membrane-bound enzymes are assayed in the presence of detergents, and detergent addition will likely alter the concentration of the membrane MLCL and acyl-coenzyme A substrates. Structural stability is an intrinsic property of every protein and this is influenced by temperature (48). The difference in heat inactivation profiles between the two activities also argues for the possibility of distinct enzymes or active sites involved in the acylation of MLCL to CL in rat heart mitochondria.

When dilysocardiolipin was used as substrate, radioactive CL production was not observed. This was in agreement with a previous study performed in isolated rat liver mitochondria (26). Biosynthesis of the polyglycerophospholipid bis(monoacylglycero)phosphate from PG occurs by phospholipase A followed by transacylase activity (49). The lack of [$^3$H]MLCL acylation to [$^3$H]CL in the absence of acyl-coenzyme A indicated that MLCL acylation by transacylase activity does not contribute to CL remodeling in rat heart mitochondria. In rat liver, transacylase activity for the remodeling of CL was not observed (26). As mitochondria contain significant phospholipase $A_2$ activity toward CL (27, 29, 30) and MLCL inhibits this activity (26), it is unlikely that the [$^{14}$C]CL formed in our assay incubation mixtures was significantly hydrolyzed by phospholipase $A_2$ due to the presence of MLCL. The molecular remodeling of CL in rat heart mitochondria likely follows the CL deacylation/MLCL reacylation scheme outlined in **Fig. 5** as first hypothesized by Schlame and Rustow (26) for endogenous rat liver mitochondrial CL.

A previous study indicated that lysophosphatidylglycerol could be reacylated to PG via a coenzyme A-dependent pathway in rat heart mitochondria (50). It might be argued that the radioactive CL formed from the oleoyl-coenzyme A or linoleoyl-coenzyme A substrates in the presence of the lysophospholipid MLCL might have been due to conversion of the radioactive PG to CL via CL synthase. This was unlikely as lysophospholipids were shown to be potent inhibitors of the partially purified rat liver CL synthase (19). In addition, in the absence of exogenous lysophosphatidylglycerol, limited radioactive PG is formed in the presence of [$^{14}$C]acyl-coenzyme A (50). Further evidence against this supposition was provided by the isolated perfused rat heart studies. Incorporation of [1-$^{14}$C]oleate and [1-$^{14}$C]linoleate into CL in perfused hearts exceeded the amount of incorporation into PG. In contrast, incorporation of the PG and CL de novo precursors [1,3-$^3$H]glycerol or [$^{32}$P]Pi into PG was much greater than the incorporation of these into CL in perfused hearts (17). This finding was not surprising as unsaturated fatty acids enter into glycerophospholipids mainly by deacylation followed by reacylation (38). The MLCL used in the current study was a mixture of 1′(1-acyl-*sn*-glycerol-3-phosphoryl)-3′(1′′,2′′-diacyl-*sn*-glycerol-3-phosphoryl)glycerol and 1′(1,2-diacyl-*sn*-glycerol-3-phosphoryl)-3′(1′′-acyl-*sn*-glycerol-3-phosphoryl)glycerol). As a result, the positional specificity of the added oleate or linoleate molecular species is to either *sn*-2 ester position. The current assay system does not distinguish between these two positions.

The acylation of MLCL to CL was observed in all rat tissues examined. MLCL AT in rat liver, lung, spleen, and kidney exceeded that in both the heart and brain. Interestingly, the lowest activity was observed in the two tissues heart and brain, which are electrically active and do not secrete phospholipids. CL remodeling has been examined in H9c2 cardiac myoblast cells (51). Incubation of these cells with [1-$^{14}$C]linoleic acid resulted in rapid incorporation of the labeled precursor into CL independent of alterations in de novo CL biosynthesis. In CCL16-B2 cells, a Chinese hamster ovary cell line deficient in oxidative energy consumption, the incorporation of [1-$^{14}$C]oleic acid into CL was reduced when de novo CL biosynthesis was elevated (52). Incorporation of [1-$^{14}$C]oleic acid into CL was dramatically elevated in *C. trachomatis*-infected HeLa cells (53). The above studies and the current study indicate that the in vivo molecular remodeling of mitochondrial CL is a complex process and is likely regulated under several levels of control including substrates, remodeling enzymes, cell or tissue type and the pathological state of the cell.

We thank Dr. Judith Storch for the generous gift of rat heart FABP and mouse adipocyte FABP, Dr. David Bernlohr for the generous gift of adipocyte LBP and keratinocyte LBP, Dr. Jens Knudsen for the generous gift of rat liver ACBP and Andrew Deonarine for technical assistance. This work was supported by a grant from the Medical Research Council of Canada to G. M. H. (MT-14261). B.J.M. is the recipient of a National Sciences and Engineering Research Council Studentship. V. W. D. was the recipient of a Manitoba Health Research Council Traineeship. G.M.H. is a Heart and Stroke Foundation of Canada Scholar.

*Manuscript received 15 December 1998 and in revised form 10 June 1999.*

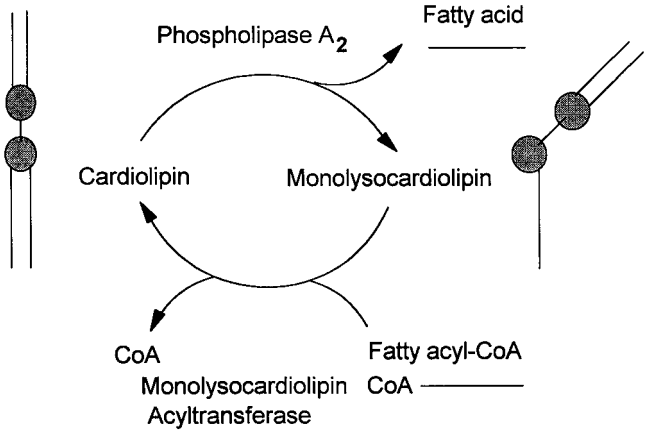


**Fig. 5.** Proposed model of cardiolipin molecular remodeling in rat heart mitochondria.

## REFERENCES

1. Pangborn, M. 1942. Isolation and purification of a serologically active phospholipid from beef heart. *J. Biol. Chem.* **143:** 247–256.
2. Hostetler, K. Y. 1982. Polyglycerophospholipids: phosphatidylglycerol, diphosphatidylglycerol and bis(monoacylglycerol)phosphate. *In* Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Press, Amsterdam. 215–261.
3. Daum, G. 1985. Lipids of mitochondria. *Biochim. Biophys. Acta.* **822:** 1–42.
4. Hatch, G. M. 1998. Cardiolipin: biosynthesis, remodeling and trafficking in the heart and mammalian tissues. *Int. J. Mol. Med.* **1:** 33–41.
5. Hoch, F. L. 1992. Cardiolipins and biomembrane function. *Biochim. Biophys. Acta.* **1113:** 71–133.
6. Stoffel, W., and H-G. Schiefer. 1968. Biosynthesis and composition of phosphatides in outer and inner mitochondrial membranes. *Hoppe-Seyler's Z. Physiol. Chem.* **349:** 1017–1026.
7. Hovius, R., H. Lambrechts, K. Nicolay, and B. de Kruijff. 1990. Improved methods to isolate and subfractionate rat liver mitochon-

dria. Lipid composition of the inner and outer membrane. *Biochim. Biophys. Acta.* **1021:** 217–226.
8. Vik, S. B., G. Georgevich, and R. A. Capaldi. 1981. Diphosphatidylglycerol is required for optimal activity of beef heart cytochrome c oxidase. *Proc. Natl. Acad. Sci. USA.* **78:** 1456–1460.
9. Fiol, C. J., and L. L. Bieber. 1984. Sigmoidal kinetics of purified beef heart mitochondrial carnitine palmitoyltransferase. *J. Biol. Chem.* **259:** 13084–13088.
10. Muller, M., R. Moser, D. Cheneval, and E. Carafoli. 1985. Cardiolipin is the membrane receptor for creatine phosphokinase. *J. Biol. Chem.* **260:** 3839–3843.
11. Hutson, S. M., S. Roten, and R. S. Kaplan. 1990. Solubilization and reconstitution of the branched-chain-alpha-keto acid transporter from rat heart mitochondria. *Proc. Natl. Acad. Sci. USA.* **87:** 1028–1031.
12. Kaplan, R. S., J. A. Mayor, N. Johnston, and D. L. Oliveira. 1990. Purification and characterization of the reconstitutively active tricarboxylate transporter from rat liver mitochondria. *J. Biol. Chem.* **265:** 13379–13385.
13. Belezani, Z. S., and V. Janesik. 1989. Role of cardiolipin in the functioning of the L-glycerol-3-phosphate dehydrogenase. *Biochem. Biophys. Res. Commun.* **159:** 132–139.
14. Kadenbach, B., P. Mende, H. V. Kolbe, I. Stipani, and F. Palmieri. 1982. The mitochondrial phosphate carrier has an essential requirement for cardiolipin. *FEBS Lett.* **139:** 109–112.
15. Hoffman, B., A. Stockl, M. Schlame, K. Beyer, and M. Klingenberg. 1994. The reconstituted ADP/ATP carrier activity has an absolute requirement for cardiolipin as shown in cysteine mutants. *J. Biol. Chem.* **269:** 1940–1944.
16. Eble, K. S., W. B. Coleman, R. R. Hantgan, and C. C. Cunningham. 1990. Tightly associated cardiolipin in bovine heart mitochondrial ATP synthase as analyzed by $^{31}$P nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* **265:** 19434–19440.
17. Hatch, G. M. 1994. Cardiolipin biosynthesis in the isolated heart. *Biochem. J.* **297:** 201–208.
18. Berger, A., and J. B. German. 1990. Phospholipid fatty acid composition of various mouse tissues after feeding α-linoleate or eicosatrienoate. *Lipids.* **25:** 473–480.
19. Schlame, M., S. Brody, and K. Y. Hostetler. 1993. Mitochondrial cardiolipin in diverse eukaryotes: comparison of biosynthetic reactions and molecular acyl species. *Eur. J. Biochem.* **212:** 727–735.
20. Schlame, M., L. Hovath, and L. Vigh. 1990. Relationship between lipid saturation and lipid-protein interaction in liver mitochondria modified by catalytic hydrogenation with reference to cardiolipin molecular species. *Biochem. J.* **265:** 79–85.
21. Yamaoka, S., R. Urade, and M. Kido. 1990. Cardiolipin molecular species in rat heart mitochondria are sensitive to essential fatty acid-deficient dietary lipids. *J. Nutr.* **120:** 415–421.
22. Yamaoka-Koseki, S., R. Urade, and M. Kito. 1991. Cardiolipins from rats fed different diets affect bovine heart cytochrome c oxidase activity. *J. Nutr.* **121:** 956–958.
23. Lands, W. E. M. 1960. Metabolism of glycerides II. The acylation of lysolecithin. *J. Biol. Chem.* **253:** 2233–2237.
24. Choy, P. C., M. Skrzypczak, D. Lee, and F. T. Jay. 1997. Acyl-GPC and alkenyl/alkyl-GPC:acyl-CoA acyltransferases. *Biochim. Biophys. Acta.* **1348:** 124–133.
25. Eichberg, J. 1974. The reacylation of deacylated derivatives of diphosphatidylglycerol by microsomes and mitochondria from rat liver. *J. Biol. Chem.* **249:** 3423–3429.
26. Schlame, M., and B. Rustow. 1990. Lysocardiolipin formation and regulation in isolated rat liver mitochondria. *Biochem. J.* **272:** 589–595.
27. De Winter, J. M., H. B. M. Lenting, F. W. Neys, and H. Van Den Bosch. 1987. Hydrolysis of membrane-associated phosphoglycerides by mitochondrial phospholipase $A_2$. *Biochim. Biophys. Acta.* **917:** 169–177.
28. Buckland, A. G., A. R. Kinkaid, and D. C. Wilton. 1998. Cardiolipin hydrolysis by human phospholipases $A_2$: The multiple enzymatic activities of human cytosolic phospholipase $A_2$. *Biochim. Biophys. Acta.* **1390:** 65–72.
29. Waite, M., and P. Sisson. 1971. Partial purification and characterization of the phospholipase $A_2$ from rat liver mitochondria. *Biochemistry.* **10:** 2377–2383.
30. Hostetler, K. Y., B. D. Zenner, and H. P. Morris. 1978. Altered subcellular and submitochondrial localization of CTP:phosphatidate cytidylyltransferase in the Morris 7777 hepatoma. *J. Lipid Res.* **19:** 553–560.
31. Taylor, W. A., V. W. Dolinsky, and G. M. Hatch. 1998. Physiological regulation of cardiolipin remodeling in rat heart. *Can. Fed. Biol. Soc.* **41**: P003.
32. Tardi, P. G., J. J. Mukherjee, and P. C. Choy. 1992. The quantitation of long chain acyl-CoA in mammalian tissues. *Lipids.* **27:** 65–67.
33. Krebs, H. A., and K. Henseleit. 1932. Untersuchungen uber die Harstoffbildung in Tierkorper. *Hoppe Seyler's Z. Physiol. Chem.* **210:** 33–36.
34. Langendorff, O. 1896. Untersuchungen am uberlebenden saugetierberzen. *Pfluegers Arch.* **61:** 291–332.
35. Cao, S. G., and G. M. Hatch. 1995. Oleate stimulation of incorporation of exogenous glycerol into cardiolipin does not involve direct activation of the CDP-DG pathway. *Biochem. Cell Biol.* **73:** 299–305.
36. Arvidson, G. A. E. 1968. Structural and metabolic heterogeneity of rat liver glycerophospholipids. *Eur. J. Biochem.* **4:** 478–486.
37. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **72:** 248–254.
38. Akesson, B. 1970. Initial esterification and conversion of intraportally injected [1-$^{14}$C]linoleic acid in rat liver. *Biochim. Biophys. Acta.* **218:** 57–70.
39. Hubbell, T., W. D. Behnke, J. K. Woodford, and F. Schroeder. 1994. Recombinant liver fatty acid binding protein interacts with fatty acyl-Coenzyme A. *Biochemistry USA.* **33:** 3327–3334.
40. Rustow, B., M. Schlame, H. Rabe, G. Reichman, and D. Kunze. 1989. Species pattern of phosphatidic acid, diacylglycerol, CDP-diacylglycerol and phosphatidylglycerol synthesized *de novo* in rat liver mitochondria. *Biochim. Biophys. Acta.* **1002:** 261–263.
41. Hostetler, K. Y., J. M. Galesloot, P. Boer, and H. van den Bosch. 1975. Further studies on the formation of cardiolipin and phosphatidylglycerol in rat liver mitochondria: effect of divalent cations and the fatty acid composition of CDP-diglyceride. *Biochim. Biophys. Acta.* **380:** 382–389.
42. Van der Vusse, G. J., J. F. C. Glatz, H. C. G. Stam, and R. S. Reneman. 1992. Fatty acid homeostasis in the normoxic and ischemic heart. *Physiol. Rev.* **72:** 881–940.
43. Wolff, R. L. 1995. Structural importance of the *cis*-5 ethylenic bond in the endogenous desaturation product of dietary elaidic acid, *cis*-5, *trans*-9 18:2 acid, for the acylation of rat liver mitochondrial phosphatidylinositol. *Lipids.* **30:** 893–898.
44. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
45. Hof, H., and G. Simon. 1970. Phospholipid content of human and guinea pig muscle: post-mortem changes and variations with muscle composition. *Lipids.* **5:** 485–487.
46. Diez, E., F. H. Chilton, G. Stroup, R. J. Mayer, J. D. Winkler, and A. N. Foneth. 1994. Fatty acid and phospholipid selectivity of different phospholipase $A_2$ enzymes studied by using a mammalian membrane as substrate. *Biochem. J.* **301:** 721–726.
47. Faergeman, N. J., and Knudsen, J. 1997. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and cell signaling. *Biochem. J.* **323:** 1–12.
48. Cowan, D. 1995. Protein stability at high temperatures. *Essays Biochem.* **29:** 193–207.
49. Amidon, B., A. Brown, and M. Waite. 1996. Transacylase and phospholipases in the synthesis of bis(monoacylglycero)phosphate. *Biochemistry.* **35:** 13995–14002.
50. Cheng, P., V. Dolinsky, and G. M. Hatch. 1996. The acylation of lysophosphatidylglycerol in rat heart: Evidence for both *in vitro* and *in vivo* activities. *Biochim. Biophys. Acta.* **1302:** 61–68.
51. Hatch, G. M. and G. McClarty. 1996. Regulation of cardiolipin biosynthesis in H9c2 cardiac myoblasts by cytidine-5′-triphosphate. *J. Biol. Chem.* **271:** 25810–25816.
52. Rusnak, A., R. Mangat, F. Xu, G. McClarty, and G. M. Hatch. 1997. Cardiolipin remodeling in a Chinese hamster lung fibroblast cell line deficient in oxidative energy production. *J. Bioenerg. Biomembr.* **29:** 291–298.
53. Hatch, G. M., and G. McClarty. 1998. Cardiolipin remodeling in eukaryotic cells infected with *Chlamydia trachomatis* is linked to elevated mitochondrial metabolism. *Biochem. Biophys. Res. Commun.* **243:** 356–360.