Cardiolipin Synthase Is Associated with a Large Complex in Yeast Mitochondria*

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The phospholipid cardiolipin (CL) is ubiquitous in eucaryotes and is unique in structure, subcellular localization, and potential function. Previous studies have shown that CL is associated with major respiratory complexes in the mitochondrial membrane. To determine whether CL biosynthesis requires the presence of intact respiratory complexes, we measured activity of CL synthase, which catalyzes the synthesis of CL from cytidine diphosphate diacylglycerol and phosphatidylglycerol, in Saccharomyces cerevisiae strains with genetic defects in the oxidative phosphorylation system. Assembly mutants of cytochrome oxidase had significantly reduced CL synthase activity, while assembly mutants of respiratory complex III and the F$_0$F$_1$-ATPase were less inhibited. To obtain further information on the activity of CL synthase, we purified the enzyme and compared the size of the catalytic protein with the functional molecular mass. The enzyme was solubilized by Triton X-100 from KSCN-extracted mitochondrial membranes of S. cerevisiae. The functional molecular mass of Triton-solubilized CL synthase, determined by radiation inactivation, was 150–240 kDa, indicating that the functional enzyme was a large complex. After partial purification, the enzyme eluted from a Superose 12 gel filtration column with an apparent molecular mass of 70 kDa. CL synthase was further purified by hydroxyapatite and cytidine diphosphate diacylglycerol affinity chromatographies, Mono Q anion exchange FPLC, and preparative gel electrophoresis. These steps led to identification of a 28-kDa protein, which had catalytic activity when eluted from an SDS-polyacrylamide gel. This 28-kDa protein also reacted with an antiserum that inactivated the enzyme. We conclude that yeast CL synthase is a 28-kDa protein, which forms an oligomeric complex whose biogenesis and/or activity is influenced by the assembly of cytochrome oxidase.

Cardiolipin (1,3-diphosphatidyl-sn-glycerol; CL) is an anionic phospholipid found in procaryotic cells (1) and in the inner mitochondrial membrane of all eucaryotic cells (2, 3). It is a unique phospholipid with a dimeric structure in which two phosphatidyl moieties are linked by a glycerol bridge. The physiological role of CL has remained obscure, but it has been shown to interact strongly with a large variety of apparently unrelated proteins. In mitochondria, CL displays characteristic combinations of acyl groups (4), distinguishing it from other phospholipids and implying some specific functional involvement. Mitochondrial CL is associated with the major proteins of oxidative phosphorylation, including the respiratory complexes I (NADH-ubiquinone reductase) (5), III (ubiquinol-cytochrome c reductase) (6) and IV (cytochrome oxidase) (7), the F$_0$F$_1$-ATPase (complex V) (8), as well as the carrier proteins for phosphate (9) and adenine nucleotides (10).

There is a common eucaryotic reaction for CL formation involving phosphatidyl transfer from CDP-diacylglycerol to phosphatidylglycerol (4), in contrast to the bacterial reaction in which CL is formed by the condensation of two molecules of phosphatidylglycerol (11). Most of what is known about CL biosynthesis has originated from studies in rat liver mitochondria. In this tissue, the eucaryotic pathway was discovered (12) and its absolute dependence on divalent cations was established (13). Rat liver CL synthase was purified as an acidic 50-kDa enzyme whose activity was optimal in the presence of Co$^{2+}$, phosphatidylylethanolamine, and alkaline pH (14). This enzyme is located in the mitochondrial inner membrane (15, 16), with the catalytic site facing the matrix (16), suggesting that CL is formed in the inner leaflet of the inner membrane. There is also evidence that the de novo formation of CL is succeeded by a deacylation-reacylation reaction via the intermediate monolysocardiolipin to generate the specific acyl pattern of CL (17).

The yeast Saccharomyces cerevisiae has proven to be an excellent model to study the biogenesis of mitochondria by combined biochemical and genetic approaches. Therefore, we chose this organism for experiments addressing the role of CL in eucaryotic cell function. Since CL interacts with several protein complexes of the mitochondrial inner membrane, it would be particularly interesting to determine how CL biosynthesis is coordinated with import and assembly of mitochondrial proteins. Therefore, it is desirable to identify the molecular components involved in CL formation as well as their supramolecular organization. In earlier studies, we solubilized yeast CL synthase by Triton X-100, characterized some of its properties, and investigated the mechanism of CL synthesis in a mixed micellar system containing Triton and the partially purified enzyme (18). There are striking similarities between CL synthases from yeast and mammals, such as the high pH optimum, the absolute requirement for divalent cations, strong inhibition by lysophosphatidylglycerol, and insensitivity to the SH-reactant N-ethylmaleimide. However, in contrast to the mammalian enzyme, the yeast enzyme prefers Mg$^{2+}$ over Co$^{2+}$ for activation. The kinetic data were compatible with a model,
Yeast Cardiolipin Synthase

Yeast cells were grown to mid-log phase in agitated Erlenmeyer flasks. The growth medium contained 2% (w/v) galactose, 2% (w/v) peptone, and 1% (w/v) yeast extract. When indicated by an asterisk, galactose was replaced by glucose. Mitochondria were prepared exactly as described by Daum et al. (34). Cardiolipin synthase activity was assayed in the presence of 305 mg of mitochondrial protein. The incubation time was 20 min, a period for which the activity was found to be linear. The activity of the mutant mitochondria is expressed as percent of the activity in the parent strain mitochondria. In the parent strains, the following cardiolipin formation was measured: strain W303-1A, 12.3 ± 2.6 pmol (n = 7, carbon source: galactose); strain YPH500, 13.5 ± 2.5 pmol (n = 2, carbon source: galactose); strain DL-1a, 8.1 ± 0.3 pmol (n = 4, carbon source: glucose); strain JHRY1–2C, 6.9 ± 0.4 pmol (n = 4, carbon source: glucose) and 24 ± 2 (n = 2, carbon source: galactose). N, number of experiments; QCR, ubiquinol-cytochrome c reductase; COX, cytochrome oxidase; mt, mitochondrial.

| Affected complex | Strain | Parent strain | Reference | Deletion | Defective function | Cardiolipin synthase |
|------------------|--------|---------------|-----------|----------|--------------------|---------------------|
| III (QCR)        | aW303ACOR1 | W303–1A        | (35)      | QCR-subunit 1 | Assembly of QCR    | 77 ± 16 6          |
| III (QCR)        | aW303ACOR2 | W303–1A        | *         | QCR-subunit 2 | Assembly of QCR    | 62 ± 8 4           |
| JP1              | W303–1A    | (36)           | QCR-subunit 5 | Fe-S Protein | 107 ± 31 4        |
| MMY18            | YPH500    | b               | QCR-subunit 6 | Activity of QCR | 71 ± 2 2          |
| IV (COX)         | WDI*      | W303–1A        | (37)      | QCR-subunit 9 | Assembly of QCR    | 100 ± 16 2         |
| IV (COX)         | WDI*      | W303–1A        | *         | Assembly of COX | 48 ± 6 4          |
| IV (COX)         | WDI*      | W303–1A        | *         | Assembly of COX | 54 ± 14 2         |
| IV (COX)         | J3α*      | JHRY1–2Ca      | (24)      | COX-subunit 6a | Activity of COX    | 83 ± 14 2          |
| IV (COX)         | J3α*      | JHRY1–2Ca      | *         | COX-subunit 7 | 56 ± 10 4         |
| V (F,F,-ATPase)  | aW303ATP10 | W303–1A        | (40)      | ATP10    | Assembly of F,D     | 87 ± 19 4          |
| V (F,F,-ATPase)  | aW303ATP11 | W303–1A        | (41)      | ATP11    | Assembly of F,D     | 77 ± 13 4          |
| V (F,F,-ATPase)  | aW303ATP12 | W303–1A        | (42)      | ATP12    | Assembly of F,D     | 65 ± 5 2           |
| III, IV, V       | aW303Spb  | W303–1A        | *         | mt DNA   | Assembly of QCR, COX, F,F | 42 ± 12 2         |

* A. Tzagoloff, personal communication.

b L. B. Trumpower, personal communication.

in which CL synthase forms a ternary complex with both phospholipid substrates and exercises catalysis at the surface of mixed phospholipid/detergent micelles. Since Mg^{2+} increased the maximal velocity but not the substrate affinity, we suggested that it binds to the enzyme rather than the substrates.

In the work described here, we report the purification of CL synthase from S. cerevisiae to apparent homogeneity, and show that activity is dependent on the assembly of specific respiratory complexes in the mitochondrial membrane.

EXPERIMENTAL PROCEDURES

Materials—Phenylmethanesulfonyl fluoride, leupeptin, and aprotinin were obtained from Boehringer (Mannheim, Germany). N-Ethylmaleimide, pepstatin A, 1-3-(aminophosphatidyl-1)-glycerol (derived from egg yolk phosphatidylcholine), CDP-diacylglycerol-1,2-diacylglycerol (derived from egg lecithin), and Coomassie Brilliant Blue R-250 were from Sigma. Triton X-100 and Silica Gel 60 H were purchased from EM Science. Florisil was from J.T. Baker. SDS (electrophoresis grade) was from Fisher. Peptone and yeast extract were obtained from Difco. Hydroxyapatite (Bio-Gel HT), and ampholites (pH 3–10) were from Bio-Rad. The ultrafiltration equipment, including YM 10 ultrafiltration membrane, the pressure dialysis device, and Centricon 10 microconcentrators were from Amicon. Florisil was from J.T. Baker. SDS (electrophoresis grade) was from Fisher. Peptone and yeast extract were obtained from Difco. Hydroxyapatite (Bio-Gel HT), and ampholites (pH 3–10) were from Bio-Rad. The ultrafiltration equipment, including YM 10 ultrafiltration membranes, the pressure dialysis device, and Centricon 10 microconcentrator tubes were made by Amicon (Beverly, MA). Agarose adipic acid hydrazide ([4% beaded agarose]-NHNNH-CO-(CH_2)_3-CO-NHNNH_2) and the Mono Q HR 5/5 anion exchange column were from Pharmacia (Uppsala, Sweden). Protein electrophoresis was performed with the Mini-PROTEAN II electrophoresis system from Bio-Rad.

Preparation of Substrate CDP-diacylglycerol—1,2-Dimyristoyl-sn-[U-14C]glycerol-3-phospho-4'-cytidine (14C]CDP-diacylglycerol), specific activity 88 dpm/pmol, and 1,2-dimyristoyl-sn-[2-14H]glycerol-3-phospho-4'-cytidine (14H]CDP-diacylglycerol), specific activity 1923 dpm/pmol, were synthesized as described (18, 19).

Growth of Cells—The wild-type S. cerevisiae strain 2737–10B (met6 MATa) was grown to mid-logarithmic phase at 30 °C in a medium containing 1% yeast extract, 2% peptone, and 2% glucose in a 100-liter fermentor (New Brunswick Scientific Co.). Air at a rate of 15 liters/min was passed through the culture, which was stirred at 200 rpm. The culture was grown to an A_600 of 1.5. Cells were harvested in a Sharples AS-16 Super-centrifuge at 13,000 × g at a flow rate of 4 liters/min. The cell paste was stored at −80 °C. All subsequent procedures were performed at 4 °C.

Preparation of Mitochondria, and Chatoptic Extraction and Solubilization of Mitochondrial Membranes—Frozen cells (100 g) were thawed, washed with water, and suspended in 400 ml of medium containing 50 mM Tris-HCl (pH 7.5), 20% (w/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 4 mM NaF. Mitochondria were prepared as described (20, 21). Extraction of membranes with 1 m KSCN and solubilization in 2% Triton X-100 have been described in detail previously (18). During solubilization and all chromatographic procedures, the following mixture of protease inhibitors was present: 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 2 mM aprotinin.

Hydroxylapatite (HA) Chromatography—The Triton extract (14 ml) was loaded on a freshly prepared HA column (1.6 × 15 cm) which was equilibrated in a buffer containing 10% (v/v) glycerol, 0.08% (w/v) Triton X-100, 0.05 m KCl, and 10 mM potassium phosphate (pH 7.2). A 140-ml gradient was run from 0 to 500 mM phosphate in the equilibration buffer, and 5-ml fractions were collected (flow rate, 1 ml/min). Thirty-ml aliquots of each fraction were assayed for CL synthase activity. The peak fractions were pooled, and their total volume was reduced to 5 ml by ultrafiltration through an Amicon YM 10 membrane. The sample was diluted 2-fold with Medium A and then rechromatographed. Three dilution-reconcentration cycles were performed to reduce the salt concentration about 8-fold.

CDP-diacylglycerol Affinity Chromatography—The concentrated and desalted enzyme fraction from HA chromatography was incubated with CDP-diacylglycerol affinity resin, which was prepared as described (22) and equilibrated with medium A containing 0.5% Triton X-100. After a 2-h incubation, the mixture was packed into a column (1 × 5 cm) and incubated for an additional 1 h. CL synthase was eluted with 15 ml of 1 m KCl in medium A containing 0.1% Triton X-100. Fractions of 1 ml were collected, and 30-μl aliquots were assayed for CL synthase activity. For this and subsequent steps, an overnight incubation time was necessary due to low enzyme activity. Three peak fractions containing CL synthase were pooled, concentrated, and desalted 8-fold using Centricon 10 concentrators.

Mono Q Anion Exchange FPLC—The concentrated and desalted peak fractions from either the HA or CDP-DG affinity column were loaded on a Mono Q column (HR 5/5), which was equilibrated with medium A containing 0.1% Triton X-100 and run at a flow rate of 0.25 ml/min using a 15-ml gradient from 0 to 1 m KCl. Fractions of 1 ml were collected, and 30-μl aliquots were assayed. CL synthase eluted at about 0.4 M. Three peak fractions were pooled, concentrated, and desalted 8-fold using Centricon 10 concentrators.

Preparative Gel Electrophoresis—The concentrated and desalted enzyme fraction from the Mono Q column was further purified by preparative gel electrophoresis in a Bio-Rad Prep Cell apparatus, following the directions of the manufacturer, and using the discontinuous Laemmli buffer system for SDS-PAGE (23). One mg of protein in 1 ml was applied to a 12.5% polyacrylamide separating gel poured to 6.0 cm (height) with a 1-cm 4% polyacrylamide stacking gel. The lower chamber buffer consisted of the cooling core of the Prep Cell at 100 ml/min. The enzyme sample was electrophoresed at a constant current
were assayed. The enzyme assay mixture was incubated for 3 days.

Bradley with medium A containing 0.2% (w/v) Triton X-100 and 1M KCl were applied to a Superose 12 HR 10/30 gel filtration column equilibrated with 20 mM Tris (0.5 M, pH 6.8), 20 μl of SDS (10%) and 1 μl of 2-mercaptoethanol and then incubated at 4 °C for 1 h. The sample was electrophoresed on 10×7-cm slab minigels with a thickness of 0.75 mm. The Laemmli system (23) was used at a polyacrylamide concentration of 12.5% (w/v). The gels were stained by Silver Stain Plus (Bio-Rad).

Protein Assay — Protein concentrations of organelle and membrane fractions were determined by the procedure of Lowry et al. (25). In partially purified fractions, protein was assayed by the method of Bradford (26) using a Bio-Rad assay kit. Bovine serum albumin (Sigma) was used as a standard, and blanks contained the same buffers used for the protein samples.

RESULTS

Relation between CL Synthase and the Oligomeric Complexes of Oxidative Phosphorylation

We studied CL synthase activity in yeast mutants that had defined genetic defects in the oxidative phosphorylation system (Table I). Specific assembly defects of complex III (ubiquinol:cytochrome c reductase) and complex V (F1F0-ATPase) resulted in only small reductions in CL synthase activity. More strikingly, full activity was lost in the FA-6 strain, which lacks mitochondrial complex I.

FPLC Gel Filtration — The peak fractions from the Mono Q column were applied to a Superose 12 HR 10/30 gel filtration column equilibrated with medium A containing 0.2% (w/v) Triton X-100 and 1M KCl (flow rate 0.25 ml/min). Fractions of 0.8 ml were collected and assayed for activity. CL synthase was eluted after a volume of 12 ml.

Assay of CL Synthase — The Triton-solubilized enzyme was treated with γ rays for various lengths of time after which CL synthase activity was measured. The activities are given relative to the control (no radiation). Squares and triangles represent two different experiments. The $D_{37}$ value (dose at which the activity drops to 37% of the control) was obtained by nonlinear regression analysis. The result of the fit was $D_{37} = 2.6 \pm 0.2$ Mrad, corresponding to a molecular mass of 240 ± 20 kDa (26). The inset shows a semilogarithmic plot of the data demonstrating their exponential character.

Preparation of Antibody against CL Synthase — Four mice were injected with the partially purified (HA, Mono Q, and Prep Cell) CL synthase as antigen. Western blotting was carried out using HA peak fractions as antigens. Several days after the fourth injection, serum was determined by enzyme-linked immunosorbent assays using HA-purified enzyme as antigen. Western blotting was carried out using standard procedures.

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changes were caused by an impaired assembly of complex IV (cytochrome oxidase), which led to the loss of about half of CL synthase activity. The extent of reduction of CL synthase in complex IV mutants is the same as that observed in mutants lacking mitochondrial DNA (ρ0), in which none of the complexes of oxidative phosphorylation can assemble due to the lack of mitochondrially encoded subunits. Only those defects of cytochrome oxidase that affected the assembly of the complex (deletion of subunit 4, 6, or 7) decreased CL synthase activity significantly, while the deletion of subunit 6a, a modulator of complex IV activity (27), had little effect on CL synthase.

The fact that CL synthase was still detectable in a ρ0 mutant clearly demonstrated the nuclear location of its gene.

**Physical Characterization of CL Synthase**

**Solubilization of CL Synthase**—CL synthase was solubilized from mitochondrial membranes by Triton X-100 or Triton X-114. When mitochondrial membranes were solubilized by Triton X-114, the total CL synthase activity dropped to 26 ± 5% (n = 3). Most of this activity (93 ± 19%, n = 3) partitioned into the detergent phase after Triton X-114 phase separation, suggesting that CL synthase is a very hydrophobic membrane protein.

For the purpose of enzyme purification, Triton X-100 was chosen because of its compatibility with enzyme activity and its suitability for various chromatographic procedures. Mitochondria were isolated and separated into a membrane fraction and a soluble fraction. The membranes were treated with the chaotropic salt KSCN to remove loosely attached proteins. This procedure yielded a highly hydrophobic membrane residue, which could be partially solubilized by Triton X-100.

**Radiation Inactivation Analysis of the Triton-solubilized Enzyme**—CL synthase was solubilized with Triton X-100 and radiation inactivation was used to determine its functional size. The radiation inactivation curve of the Triton-solubilized enzyme revealed a target size of 240 ± 20 kDa (Fig. 1). Given that Triton X-100 may artificially increase the target size by up to 55% (28), the functional molecular weight of CL synthase is at least 150 kDa.

**Gel Filtration Analysis of the Partially Purified Protein**—Following purification, the enzyme lost activity dramatically. Radiation inactivation could not, therefore, be used to assess the functional size. Therefore, we analyzed the functional molecular weight of partially purified CL synthase by gel filtration.

![Fig. 3. A. CDP-diacylglycerol affinity chromatography. The concentrated and desalted enzyme fraction from the hydroxylapatite column was incubated with CDP-diacylglycerol affinity resin, which was prepared as described (24). The resin was equilibrated with medium A containing 0.5% Triton X-100. After a 2-h incubation, the mixture was packed into a column (1 × 5 cm²) and incubated for an additional 1 h. The column was eluted with 15 ml of 1 M KCl in medium A containing 0.1% Triton X-100. Fractions of 2 ml were collected, and 30-μl aliquots were assayed for CL synthase activity. B. preparative gel electrophoresis. Peak fractions from the Mono Q column were pooled, concentrated, and then subjected to preparative gel electrophoresis on the Bio-Rad Prep Cell. The enzyme sample was electrophoresed in the cold for 10 h at a flow rate of 0.75 ml/min, using a 12.5% polyacrylamide Laemmli system (25). Fractions of 4 ml were collected, and 30-μl aliquots were assayed for CL synthase activity.

![Fig. 4. SDS-PAGE analysis of purified CL synthase. Peak fractions of CL synthase from the Prep Cell were concentrated using Centricon-10 concentrators (Mr, 10,000 cut-off), and analyzed by SDS-PAGE. The gel, which had been loaded with 30 μl of concentrated peak fraction (10 μg of protein), was stained by Silver Stain Plus (Bio-Rad). Lane 1, Rainbow molecular weight markers (14.3–200 kDa); Lane 2, Prep Cell peak fraction.](image-url)
tion. After solubilization from the membranes, the enzyme eluted in the void volume of a Bio-Gel A-1.5m column (exclusion limit about 1,500 kDa) (data not shown). However, after chromatographic purification, the enzyme eluted with an apparent molecular mass of 70 kDa from a Superose 12 column (Fig. 2). Depending on the number of Triton molecules bound to the enzyme at this point, the data are consistent with an active enzyme consisting of a dimer or trimer.

**Purification of CL Synthase**

CL synthase was solubilized in Triton X-100 as described above and purified from the Triton extract by successive chromatography steps, as follows.

**HA Chromatography**—HA chromatography was performed as described. A280 during the elution and protein assay of each fraction indicated that a major protein peak was eluted in the wash. CL synthase eluted at about 0.2 M phosphate. Nearly 100% of applied CL synthase was recovered in peak fractions containing only 8% of the applied protein. The increase in purification is thus 12-fold for this step. CL synthase was very stable to freeze-thaw cycles at this point and could be stored at −80 °C for up to 6 months.

**CDP-DG Affinity Chromatography**—The concentrated and desalted peak enzyme fraction from hydroxylapatite chromatography was incubated with CDP-DG affinity resin, which had been equilibrated with medium A containing 0.5% Triton X-100, the optimal Triton concentration for enzyme binding. The batch method allowed maximal binding of CL synthase to the resin. After a 2-h incubation, the mixture was packed into a column and precipitated for another hour. The column was eluted with 15 ml of 1 M KCl in medium A containing 0.1% Triton X-100, the optimal concentration for elution.

The majority of protein came off the CDP-DG affinity column in the flow-through (Fig. 3A), resulting in substantial purification. However, over 90% of CL synthase activity was lost in this purification step. An attempt was made to recover enzyme activity in the peak fractions by adding a phospholipid mixture containing lipid in the ratio CL/phosphatidylethanolamine/phosphatidylcholine/phosphatidylinositol/phosphatidylserine (19:29:36:8:2) found in the inner membrane of yeast mitochondria (2). However, this was not successful.

CL synthase after CDP-DG affinity chromatography was very unstable to freeze-thaw cycles and could only be stored at −80 °C for a few days.

**Mono Q Anion Exchange FPLC**—The concentrated and desalted enzyme fraction from the CDP-DG affinity column was loaded onto a 1-ml Mono Q FPLC column (HR 5/5). A 15-ml gradient was run from 0 to 1 M KCl in equilibration buffer, and CL synthase was eluted with 0.4 M KCl. The A280 profile showed that a major protein peak eluted at 0.8 M KCl. CL synthase was very unstable to freeze-thaw cycles at this point. The sample could be stored at −80 °C for only a few days.

**Preparative Gel Electrophoresis**—The pooled, concentrated, and desalted peak fractions from the Mono Q column were further purified by preparative gel electrophoresis using the Bio-Rad Prep Cell. Preparative SDS-PAGE was run according to the discontinuous Laemmli buffer system (23). A total volume of 1 ml (1 mg of protein) was applied to the Prep Cell. The enzyme sample was electrophoresed at a constant current of 40 mA in the cold for 10 h. Starting at the dye front, 80 3-ml fractions were collected at an elution rate of 0.75 ml/min.

To assay enzyme activity in each fraction, a 3-day incubation was necessary, since CL synthase activity was extremely low at this point. The peak fractions of CL synthase were eluted after 270 ml. The elution profile from preparative gel electrophoresis is shown in Fig. 3B. CL synthase was very unstable to freeze-thaw cycles at this point. The sample could be stored at −80 °C for only a few days.

In a typical purification protocol, 100 g wet weight of yeast cell paste gave about 100 μg of CL synthase.

**FIG. 5.** Reconstitution of CL synthase activity from SDS-PAGE gel slices. Fifty μl of partially purified CL synthase were incubated with 1% SDS, Tris (pH 6.8), and 2-mercaptoethanol and then electrophoresed. Gel slices of 5 mm in length were cut out and assayed for CL synthase activity.
CL Synthase Catalytic Activity Is Associated with a 28-kDa Protein

**SDS-PAGE Analysis of Purified CL Synthase**—Fig. 4 shows the SDS-PAGE analysis of the purified CL synthase. A single protein is present in the purified sample, indicating that the catalytic CL synthase is a 28-kDa protein.

**Determination of CL Synthase Activity in Polyacrylamide Gel Slices**—Because active enzyme was compatible with a low concentration of SDS, we identified the enzyme following elution from an SDS-PAGE gel. CL synthase was purified by hydroxylapatite and Mono Q chromatographies and then analyzed by SDS-PAGE as described, except that the sample was not boiled. Individual gel slices were assayed for CL synthase activity, and activity was recovered in the 25–29-kDa region (Fig. 5).

**CL Synthase Is Inactivated by Antibody That Reacts with a 28-kDa Protein**—Mice were immunized with partially purified enzyme following hydroxylapatite, Mono Q, and Prep Cell steps (without affinity purification). SDS-PAGE analysis of the enzyme preparation showed three or four major protein bands, in the range of 26–29 kDa (data not shown). Antiserum from a mouse immunized with partially purified CL synthase strongly inhibited enzyme activity (Fig. 6A). Western blotting indicated that the antibodies in this serum react with a 28-kDa protein (Fig. 6B).

**DISCUSSION**

In this paper, we report that CL synthase activity is dependent on the assembly of respiratory complex IV in the mitochondrial membrane. Furthermore, we purified the enzyme to homogeneity and show that, while catalytic activity is associated with a 28-kDa protein, the functional protein is much larger.

The data in this paper demonstrate that the catalytic activity for CL synthase resides in a 28-kDa integral membrane protein that is encoded by nuclear DNA. Identification of the enzyme was based on the reconstitution of activity in an electrophoretically isolated band (Fig. 5), as well as on chromatographic copurification of this protein with CL synthase activity (Fig. 4). In addition, mouse polyclonal antibodies that inhibited CL synthase activity reacted with a 28-kDa protein (Fig. 6). The hydrophobic nature of CL synthase was demonstrated by Triton X-114 phase partition and by the inability of a chaotropic salt to solubilize it. The presence of CL synthase activity in a ρ0 mutant (Table I) was evidence for the nuclear location of its gene. We have recently identified the likely structural gene coding for CL synthase, CLS1 (29). A CLS1 null mutant has no CL synthase activity and no CL in its membranes. The CLS1 ORF predicts a protein of 32 kDa, which is consistent with the size of the catalytic protein reported in this paper and allows for the possibility of proteolytic cleavage of the protein upon import into the mitochondria.

There are two problems with using CL synthase activity as an indicator of enzyme purity. 1) Changes of salt and Triton concentration may affect the enzyme conformation, and 2) in this study, Triton induces a surface dilution effect that decreases CL synthase activity even if the enzyme conformation remains unharmed (18). Since we cannot control the Triton concentration at each step of the purification protocol, it is virtually impossible to estimate the amount of enzyme from CL synthase activity assays.

Radiation inactivation is a well-established method to determine the molecular size of proteins, and many examples demonstrate the validity of this procedure (24), including membrane proteins (28) and the Triton-solubilized CDP-DG synthase from yeast (30). The target size of CL synthase, as determined by radiation inactivation, translated into a molecular mass of approximately 200 kDa (Fig. 1). How can this be reconciled with the data showing that the catalytic activity was associated with a 28-kDa polypeptide, which was purified by gel filtration as a 70-kDa micelle? It is not clear whether the 70-kDa species represented a homopolymer of the 28-kDa protein, or whether it was an assembly of the 28-kDa protein with other proteins. The binding of detergent may also have contributed significantly to the molecular mass of the 70-kDa form. In any case, the high target size suggested that the functional CL synthase was a complex much larger than 70 kDa. The fact that CL synthase activity was detectable in the isolated 28-kDa protein does not preclude a requirement for other protein components, because only rudimentary activity was recovered after SDS-PAGE. Thus, while the 28-kDa protein is clearly the catalytic peptide, other proteins and/or lipids may be involved in stimulating its activity.

Ohtsuka et al. (31) described a temperature-dependent mutation of a CHO cell line in which the content of CL was reduced due to a defect in phosphatidylglycerophosphate synthase. They further studied the effect of this mutation on individual respiratory enzymes (32). Reduced levels of CL in the CHO cells led to altered mitochondrial morphology and to reduced oxygen consumption, but they were accompanied by little changes in the activities of individual respiratory enzymes, except for a reduction in complex I activity in the absence of exogenous electron acceptors. In the present work, we took a complementary approach by studying the impact on CL synthase of certain defects in the complexes of oxidative phosphorylation (Table I). Most mutants had a small reduction of their CL synthase activity, but this could most likely be ascribed to an unspecified pleiotropic effect. However, assembly defects of complex IV had a more severe impact, reducing CL synthase activity by about 50% (which is the extent to which activity is reduced in the ρ0 mutant). It was specifically the assembly of complex IV rather than its redox activity that affected CL synthase. Interestingly, Ohtsuka et al. (32) found an almost 2-fold increase in complex IV activity in the CHO mutant at the permissive temperature, whereas other respiratory enzymes remained unaffected. While no explanation is readily available, both the data of Ohtsuka et al. (32) and our data suggest that there might be a regulatory relation between cytochrome oxidase assembly and the CL pathway. There is evidence that respiratory complexes participate in functions other than energy transduction, such as the transfer of acyl groups (33, 34) or the proteolytic processing of mitochondrial precursor proteins (35, 36). The common aspect between those functions and CL synthase might be their involvement in mitochondrial membrane biogenesis.

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