Asymmetrical distribution of cardiolipin in yeast inner mitochondrial membrane triggered by carbon catabolite repression

Paul François GALLET*, Jean-Michel PETIT*, Abderrahman MAFTAH*, Alain ZACHOWSKI†‡ and Raymond JULIEN*§

*Institut de Biotechnologie, Université de Limoges, F-87060 Limoges, France and †Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, F-75005 Paris, France

Transmembrane asymmetry of cardiolipin in yeast was monitored during the switch from fermentative to gluconeogenic growth and the reverse. As soon as cells used ethanol as an electron donor to produce ATP by oxidative phosphorylation, rapid and abundant cardiolipin synthesis was observed on the matrix side of the inner mitochondrial membrane followed by a transverse rearrangement between the two leaflets. The cardiolipin distribution changed from about 20:80 (in/out) to 70:30 (in/out), and after translocation towards the outer leaflet it finally became 37:63 (in/out). At the same time, cytochrome *c* oxidase activity remained stable, then increased as a possible result of the topographical rearrangement. During the reverse process from gluconeogenic to fermentative growth, the amount of cardiolipin

rapidly decreased by half, its bilayer distribution apparently changing to a monolayer organization before the 20:80 (in/out) asymmetry of repressed cells was re-established. Experimental impairment of cardiolipin topography by antibiotic inhibition of gene expression or *in situ* dissipation of mitochondrial membrane potential produced data that prove that the amount and transmembrane distribution of the phospholipid are two specific parameters of the mitochondrial inner membrane organization in both fermentative (2.2 fmol/cell and 20:80, in/out) and gluconeogenic (4.2 fmol/cell and 37:63, in/out) growing yeast cells. Finally, the inner mitochondrial membrane topography of cardiolipin appeared to be closely associated with the transmembrane redox potential.

INTRODUCTION

Several lines of evidence have established that cardiolipin may be essential to the functionally active conformation of many mitochondrial inner-membrane proteins such as the ADP/ATP carrier, F_0F_1 -ATPase and cytochrome *c* oxidase [1,2]. However, it is not known how cardiolipin is assembled in the protein complexes and what functions it carries out. Cardiolipin appears to be present predominantly in the inner leaflet of the inner mitochondrial membrane [3], which agrees with topographical evidence that cardiolipin synthase is an integral inner-membrane enzyme with hydrophilic domains exposed to the matrix side and interacts with effectors in the matrix compartment only [4]. It was therefore suggested that cardiolipin, and probably its precursor CDP-diacylglycerol, are synthesized at the inner leaflet of the inner mitochondrial membrane [4]. How newly synthesized cardiolipin becomes accessible to new inner-membrane proteins remains unclear, since most precursors of these proteins are produced outside of the mitochondria. Can cardiolipin diffuse between the two leaflets of the inner membrane as suggested by earlier data [5,6] showing that some cardiolipin is available at the cytoplasmic side?

To understand the assembly of cardiolipin with proteins, one has to know the transverse distribution of cardiolipin *in situ* and the rearrangement that it undergoes when proteins are imported. In the present paper, we provide topographical evidence of the presence of cardiolipin in the two leaflets of yeast inner mitochondrial membrane. It is suggested that, after its synthesis at the inner leaflet, cardiolipin diffuses towards the outer leaflet. This may imply that newly imported proteins become functionally active after this rearrangement and that the topography of

cardiolipin may depend on the redox state of the phosphorylating respiratory chain.

MATERIALS AND METHODS

Materials

Yeast extract, bactopectone and yeast nitrogen base were purchased from Difco Laboratories (Detroit, MI, U.S.A.). Cycloheximide, chloramphenicol, bovine heart cardiolipin and fatty acid-free BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 10-*N*-Nonyl-3,6-bis(dimethylamino)-acridine (NAO) was purchased from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of analytical grade.

Yeast strains and growth conditions

The characteristics of the six *Saccharomyces cerevisiae* strains used in this work are summarized in Table 1. The wild-type strain D 273.10 was grown aerobically on a YM1 medium containing per litre: 5 g of yeast extract, 10 g of bactopectone, 6.7 g of yeast nitrogen base and 10 g of succinic acid. The final pH was adjusted to 5.4 with NaOH. Carbon sources were 40 g/l glucose or 20 g/l lactate.

In order to reduce the repressive effect of glucose in wild-type CW04, respiratory-deficient KL14-4A, W303-1B/50 and 777-3A mutant strains, cells were grown to stationary phase on YM1 medium containing 10 g/l glucose.

TW yeast cell was grown on W3 medium containing per litre: 6.7 g of yeast nitrogen base, 10 ml of glycerol and 20 ml of

Abbreviations used: CL*, spin-labelled cardiolipin; DiOC₆(3), 3,3'-dihexyloxycarbocyanine iodide; NAO, 10-*N*-nonyl-3,6-bis(dimethylamino)acridine; PtdCho, phosphatidylcholine; PtdCho*, 1-palmitoyl-2-(4-doxypentanoylethyl)phosphatidylcholine; RRFI, relative red fluorescence intensity.

‡ Present address: Laboratoire de Physiologie Cellulaire et Moléculaire, URA CNRS 2135, Université Paris VI, case 154, 4 place Jussieu, 75 252 Paris Cedex 05, France.

§ To whom correspondence should be addressed.

Table 1 Characteristics of yeast strains used in this study

Strain	Nuclear genome	Mitochondrial genome
D 273.10	MATa, <i>met1</i>	Wild-type, rho ⁺ mit ⁺
CW04	MATa, <i>ade2, ura3, leu2, trp1, his3</i>	Wild-type from 777-3A, rho ⁺ mit ⁺
W303-1B/50	Same as CW04	rho ^o (complete deletion)
KL14-4A	MATa, <i>his1, trp2</i>	rho ^o (partial deletion)
777-3A	MATa, <i>ade1, op1</i>	Wild-type, rho ⁺ mit ⁺
Strain	Characteristics	
Transformed yeast cell, TW	CW04 strain transformed by pEMBLye30/2-21- <i>urf13TW</i> , a plasmid carrying the complete chimaeric gene consisting of the <i>urf13TW</i> gene fused to the <i>Neurospora crassa</i> ATPase 9 N-terminal peptide sequence used to target the fusion protein to inner mitochondrial membrane [38]	

ethanol. Precultures were made in W3G medium, containing 0.5 g/l glucose. Growth was carried out in an orbital shaker, at 30 °C and 200 rev./min.

Cell preparations

For measurement of cardiolipin asymmetry and content, 1 ml of wild-type (D 273.10, CW04) or respiratory-deficient (KL14-4A, W303-1B/50 and 777-3A) cell suspension at exponential phase of growth was harvested by centrifugation (5 min at 4 °C and 3000 g). The cell pellet was washed twice with 1 ml of 10 mM Tris/HCl buffer, pH 7 (TH buffer), then resuspended in 1 ml of the same buffer. For cell fixation, 2 ml of cold (−20 °C) ethanol was added. The cell suspension was then stored at −20 °C until analysis.

To determine cardiolipin topography during yeast adaptation to oxidation, the wild-type strain D 273.10 was grown for 15 h in YM1 medium containing 40 g/l glucose. From this time, 1 ml of the cell suspension was sampled every 15 min over a period of 12 h. Cells were harvested by centrifugation (5 min at 4 °C and 3000 g); the supernatant was pipetted off and immediately frozen at −20 °C for glucose and ethanol assays. The cell pellet was treated as previously described.

For catabolite-repression induction, cells were first grown to exponential phase in YM1 medium containing 20 g/l lactate. They were harvested by centrifugation (5 min at 4 °C and 3000 g) and then washed twice with autoclaved distilled water. The pellet was resuspended in 5 ml of YM1 containing 40 g/l glucose, and 500 ml of the same medium was inoculated to obtain $A_{600} = 6.5$. Every 10 min over a period of 4 h, a 1 ml aliquot was taken. The cells and corresponding supernatants were treated as described above.

Preparation of mitochondria and mitoplasts

Yeast mitochondria were isolated after cell breakage using 0.45–0.50 mm glass beads [7] by differential centrifugation in a medium containing 0.6 M mannitol, 10 mM Tris/HCl, pH 7.4, and 1 mM PMSF at 4 °C as described by Zinser et al. [8].

Bovine heart mitochondria used for ESR experiments were prepared by the method of Smith [9] by tissue mincing in medium containing 0.27 M sucrose and 10 mM Tris/HCl, pH 7.3. These organelles can be isolated in larger amounts than those of other living tissues and, their inner membranes are much more resistant to freezing and thawing, which makes them useful for preparing mitoplasts. The homogenate was centrifuged for 1 min at 2500 g,

and the pellet was discarded. The mitochondrial pellet obtained after centrifugation (15 min at 4 °C and 10000 g) was washed twice then resuspended in 0.27 M sucrose/10 mM Tris/HCl buffer, pH 7.3.

Bovine heart mitoplasts were prepared from mitochondria by freezing and thawing as described by Brandolin et al. [10]. All mitoplast preparations were controlled by measuring monoamine oxidase [11] and fumarase [12] activities (specific enzymes of the outer membrane and matrix respectively). Membrane polarity was accurately assessed by measuring the specific and irreversible binding of [³H]atractyloside to the outer surface of the inner transmembrane ADP/ATP carrier [13].

Assay of cardiolipin asymmetry

The relative red fluorescence intensity (RRFI) of NAO bound to cardiolipin was measured on a gated cell population [14]. This population was selected according to cytoplasmic granularity (right angle scatter values) and cell size (forward angle scatter values) in order to eliminate cell debris and aggregates (Figure 1).

In order to ensure that the two pools of inner-membrane-associated cardiolipin identified by the titration curves are actually the result of NAO binding to cardiolipin on the inner and outer leaflets, we measured phospholipid distribution in easy-to-prepare bovine heart mitoplasts, by both incorporation of NAO and ESR using spin-labelled cardiolipin (CL*). The cardiolipin analogue is synthesized by reaction of 5-(4,4-dimethyl-3-oxazolidinyloxy)palmitic acid with monolysocardiolipin by a protocol adapted from Fellmann et al. [15]. Mitoplasts (4 mg of protein in 1 ml) were incubated at 30 °C in the presence of 10 nmol of CL* in solution in 10 µl of ethanol, representing 1 % of the total endogenous membrane phospholipids. Aliquots (100 µl) were removed at various times and centrifuged for 2 min at 4 °C and 15000 g. Then mitoplasts were resuspended in 90 µl of cold buffer (0.27 M sucrose, 10 mM Tris/HCl, pH 7.3). The absence of a measurable amount of CL* incorporated into the mitoplast membrane and thus present as pure cardiolipin vesicles was inferred from the absence of a broad ESR spectrum which would result from spin–spin interactions in such a structure. CL* orientation inside the membrane was recorded by measuring reduction of the nitroxide moiety of CL* on the outer leaflet at 4 °C in the presence of 100 mM ascorbate [16]. Two distinct reduction rates were recorded, the faster one corresponding to CL* reduction on the outer leaflet of the membrane and the slower one to reduction of the analogue on the inner leaflet. Extrapolation of the second slope to time zero gives the fraction of CL* on the inner leaflet.

Reorientation of phosphatidylcholine (PtdCho) in artificial membranes

To determine whether NAO affects bilayer integrity, we measured the distribution of a PtdCho analogue in cardiolipin-containing liposomes after the addition of NAO. Large unilamellar vesicles (0.1 µm in diameter) containing 250 nmol of cardiolipin and 5 µmol of PtdCho were prepared by extrusion as described by Hope et al. [17] in 1 ml of 10 mM Hepes, pH 7.3, containing 140 mM NaCl and 0.1 mM EDTA. Spin-labelled 1-palmitoyl-2-(4-doxylopentanoyl)phosphatidylcholine (PtdCho*) was synthesized as described by Fellmann et al. [15]. Some 90 nmol of PtdCho* (2 % of total lipid) in chloroform were dried under reduced pressure, then suspended in 150 µl of buffer and transferred to the tube containing the vesicle suspension. PtdCho* is essentially incorporated into the outer leaflet. If any membrane scrambling occurs as the result of the addition of NAO (0–2 mM), some of the PtdCho* initially present on the outer leaflet of the

Figure 1 *In situ* biparametric analysis of cardiolipin distribution in the inner mitochondrial membrane of yeast cells

Specific light diffusion properties of cells were exploited to draw the biparametric cytogram, dot-plots of forward angle scatter (FAS) on the ordinate against right angle scatter (RAS) on the abscissa from NAO-stained yeast cells (**A**) with the associated monodimensional histograms adjacent to the respective axis (**B** and **C**). Ethanol-fixed yeast cells, washed with TH buffer, adjusted to 1×10^6 /ml, were incubated for 15 min at 20 °C in the presence of increasing concentrations of NAO (0–15 μ M). The red fluorescence of the dye bound to cardiolipin was measured using a flow cytometer ORTHO 50H equipped with a 400 mW argon ion laser for excitation at 488 nm. The red fluorescence was selected by a long-pass filter (615 nm). Mean values of 5×10^3 events (flow rate of 300/s) were calculated using an MCA 3000 computer (Bruker) by reference to 256 fluorescence channels. Cardiolipin distribution in the inner mitochondrial membrane was analysed by following an identical protocol, using amounts corresponding to 20 μ g of mitochondrial protein.

liposomes is relocated to the inner leaflet. After a 2 min incubation in the presence of NAO, 2% (w/v) fatty acid-free BSA (to extract PtdCho* from the outer leaflet) and 60 mM ascorbate (to reduce the extracted PtdCho*) were added, and the ESR spectrum recorded repeatedly. The fraction of the probe in the inner leaflet was then estimated as indicated above.

Kinetics of NAO incorporation in whole cells

Aerobically grown cells (20 g/l lactate as a carbon source) at exponential phase were fixed as previously described, washed with cold TH buffer and counted. Then 5 ml (10^7 cells) of a suspension in TH buffer and 1 μ mol of NAO were mixed at 4 °C and the red fluorescence emission of dye bound to cardiolipin was immediately measured by flow cytometry. Measurements were then repeated every 5 min over a period of 130 min.

Cardiolipin quantification

A calibration curve for cardiolipin quantification was constructed [18] using large unilamellar vesicles prepared as described by Reeves and Dowben [19]. Increasing amounts of vesicles (0–15 μ M cardiolipin) in 0.2 M glucose were mixed with a solution of 45 μ M NAO in 0.2 M glucose, the total volume was adjusted to 1 ml and the mixture incubated for 15 min at 20 °C. Liposomes were pelleted (5 min at 20 °C and 50000 g; Beckman TL-100 ultracentrifuge) then resuspended in 1 ml of 0.2 M glucose. After three washes, the vesicle pellet was resuspended in 3 ml of 0.2 M glucose and the red fluorescence of NAO bound to thin-walled vesicles measured at 640 nm with an LS-5B Perkin-

Elmer spectrofluorimeter (excitation wavelength 450 nm, bandwidths 2.5 nm).

Ethanol-fixed cells were washed twice with 1 ml of TH buffer, sonicated to disperse aggregates and adjusted to 5×10^7 /ml. To increasing amounts of cells (0 – 2.5×10^7), 45 μ M NAO in TH buffer was added and the suspension (final volume 1 ml) was incubated for 15 min at 20 °C before centrifugation (5 min at 20 °C and 3000 g). The supernatant was discarded and the pellet washed three times by centrifugation (5 min at 20 °C and 3000 g). Finally, cells were resuspended in 3 ml of TH buffer. The fluorescence emission of NAO incorporated in the yeast cells was measured at 640 nm as previously described. By comparison with the calibration curve, the red fluorescence value allowed us to determine the cardiolipin content of the sample.

As a control, cardiolipin content of yeast cells was also determined by phosphorus quantification. Ethanol-fixed cells were washed twice with 1 ml of TH buffer, sonicated to disperse aggregates and counted. Total phospholipid was extracted from 10^7 cells and separated as described by Rouser et al. [20]. Cardiolipin phosphorus was then quantified as described by Zhou and Arthur [21].

Measurement of mitochondrial potential of the TW strain

Exponentially growing TW cells were transferred to W3 medium supplemented with 120 mM KCl and 4 mM NaCl in order to abolish the cytoplasmic membrane potential. Then 5×10^7 yeast cells were incubated for 30 min at 30 °C in 5 ml of supplemented medium containing methomyl (150 or 180 μ M final) and stained for 30 min at 30 °C with 0.5 μ M 3,3'-dihexyloxacarbocyanine

iodide [$\text{DiOC}_6(3)$] [22]. Cells were harvested by centrifugation, washed twice and resuspended in the dye-free medium for immediate analysis. The green fluorescence emission of the $\text{DiOC}_6(3)$ incorporated into cells according to the mitochondrial potential was measured at 520 nm using an Ortho 50H flow cytometer.

Assay of cytochrome *c* oxidase activity

Cytochrome *c* oxidase was measured by the method of Warthon and Tzagoloff [23] using reduced cytochrome *c* prepared as described by Carafoli and Pietrobon [24]. One unit of cytochrome *c* oxidase is defined as the amount of enzyme oxidizing 1 $\mu\text{mol}/\text{min}$ reduced cytochrome *c* at 20 °C in 10 mM phosphate buffer, pH 7.2.

Other procedures

Protein concentrations were determined by the Lowry procedure [25] with BSA as a standard. Glucose was assayed as described by Kahle et al. [26], using peroxidase and glucose oxidase, with 2,2'-azinobis-(3-ethylbenzthiazolinesulphonic acid) as chromogenic substrate. Ethanol concentration was determined using the UV method test combination kit from Boehringer (Mannheim, Germany).

RESULTS

Interaction between NAO and mitochondrial membranes

A dimer of NAO, formed by the binding of two dye molecules to the two phosphate groups of the cardiolipin molecule, is characterized by a red fluorescence emission [14]. When dimer formation was followed over time (Figure 2), biphasic kinetics were recorded: a first saturation level was reached in 20 min and this was followed by a slower step which plateaued at 100 min. It is tempting to assume that the rapid step corresponds to NAO

Figure 2 Kinetics of NAO incorporation into yeast

Yeast cells in exponential phase in YM1 plus 20 g/l lactate medium were centrifuged (5 min at 3000 *g*) and adjusted to $2 \times 10^6/\text{ml}$ in TH buffer at 4 °C. Then 5 ml of the suspension was added to an excess of NAO (10 μM final concentration in TH buffer at 4 °C). The RRFI of the dye bound to cardiolipin was selected through a long-pass filter (615 nm) using an Ortho 50H flow cytometer. One measurement lasting 30 s was automatically made every 5 min at a flow rate of 300 cells/s. The mean values of 9×10^3 events were calculated as described in Figure 1.

Figure 3 Cardiolipin distribution as determined by NAO binding to the inner mitochondrial membrane

Flow cytometry measurements of the red fluorescence of NAO bound to cardiolipin were made as described in Figure 1, at a flow rate of 300/s for 5000 events recorded in the experimentally defined gate. Curves obtained with both isolated mitochondria (○) and yeast cells (●) showed two saturation levels. The ratio of RRFI observed at the first level (58 for mitochondria and 63 for whole cells) to those observed at the second (100 for both assays) determined the relative amount of cardiolipin on the outer leaflet. The curves are representative of two independent measurements on isolated mitochondria and more than ten independent measurements on whole cells.

binding to the externally oriented cardiolipin and the second one reflects NAO diffusion through the bilayer and binding to cardiolipin embedded in the inner leaflet. When the fluorescence was measured at a fixed time after the addition of increasing amounts of NAO to yeast cells or isolated mitochondria, RRFI plotted against NAO amount was also biphasic and showed two plateau levels (Figure 3), which could be related to successive saturation of the two cardiolipin pools.

In order to check the validity of these assumptions, cardiolipin distribution was assayed by two independent approaches, NAO binding and distribution of a paramagnetic cardiolipin analogue. Because of the poor sensitivity of ESR spectroscopy, mitochondria were purified from bovine heart which allows large-scale purification protocols. The cardiolipin analogue was inserted into the outer leaflet and its reorientation with time was assayed by reduction of the probe still present in the outer leaflet at different times. Once a steady-state distribution had been reached, the analogue was slightly asymmetrically distributed, $53 \pm 2\%$ of it being present in the inner leaflet (Figure 4A). The NAO saturation curve gave a slightly different, but comparable, result with $58.6 \pm 2.3\%$ of cardiolipin in the outer leaflet (Figure 4B).

We verified that binding of NAO to a cardiolipin-containing membrane was not accompanied by bilayer scrambling, which would modify the endogenous lipid distribution. In artificial membrane (large unilamellar vesicles) made from a mixture of cardiolipin and PtdCho (5:95, mol/mol) and labelled on the outer leaflet with PtdCho*, addition of NAO (up to 2 mol per outward facing cardiolipin molecule) induced a transmembrane reorientation of less than 4% of PtdCho* compared with the incubation in the absence of NAO. Doubling this amount of NAO (i.e. saturating all the membrane cardiolipin) provoked some bilayer destabilization, reflected by the relocation of $13.6 \pm 1.5\%$ of PtdCho in the inner leaflet.

Figure 4 Cardiolipin asymmetry measurements in bovine heart mitochondria

(A) ESR determination of cardiolipin asymmetry. Mitoplasts (4 mg of protein) were incubated at 30 °C with 10 nmol of spin-labelled cardiolipin as described in the Materials and methods section. Aliquots (100 μ l) were taken at different times and centrifuged for 2 min at 4 °C and 15000 g. The CL* signal of the mitoplast pellet resuspended in 90 μ l of cold buffer was recorded over 25 min at 4 °C, in the presence of 100 mM ascorbate. Only the curve at the steady-state distribution of CL* is represented. Reduction kinetics give a biphasic curve, the slow reduction step reflecting the reduction of the CL* present on the inner monolayer. The fraction of CL* in the inner leaflet was estimated by projection of the data curve on the y-axis. (B) Flow cytometric determination of cardiolipin asymmetry. Isolated mitochondria (0.1 mg of protein) were incubated for 15 min at 20 °C in 0.27 M sucrose/10 mM Tris/HCl, pH 7.3, with increasing amounts of NAO (0–250 μ M). The RRFI of the NAO bound to cardiolipin was measured by flow cytometry as described in the Materials and methods section. Analysis was made at a flow rate of 300 events/s for 5000 events recorded in the area of interest. (C) Intensity curves observed with increasing NAO concentrations (a, 10 μ M; b, 30 μ M; c, 80 μ M; d, 140 μ M) during measurement of cardiolipin asymmetry. Curves are representative of two independent experiments.

Cardiolipin quantification

The calibration curve constructed with liposomes added in various amounts displays a linear relationship between the amount of cardiolipin present and the RRFI (Figure 5A). Thus the total red fluorescence emitted by a yeast sample could be converted into the amount of cardiolipin (Figure 5B). As a control, cardiolipin content was also determined by phosphorus quantification after lipid extraction and separation. Although the latter results were less accurate than those obtained by the NAO assay (Table 2), they gave comparable estimated cardiolipin contents.

Change of cardiolipin topography during the diauxic shift from fermentative to gluconeogenic growth and the reverse

Yeast growth with glucose (40 g/l) as a carbon source was divided into three phases [27]. During the first one, characterized by glucose repression of genes coding for mitochondrial proteins [28], only promitochondria were observed [18,29]. Just before glucose depletion (phase II, $\approx$ 17 h), cells became derepressed and acquired a capability of subsequent oxidation of the ethanol accumulated during the previous phase [30]. Mitochondria became fully active and the third phase ended with the complete depletion of the available ethanol (Figure 6A).

Figure 5 *In situ* fluorimetric quantification of cardiolipin

(A) Thin-walled vesicles. A calibration curve was drawn of RRFI of NAO bound against cardiolipin incorporated into thin-walled vesicles. Increasing amounts of liposomes containing cardiolipin were added to 45 μ M NAO (final volume 1 ml). For each of three washes, no phospholipid was detected in the supernatant. The NAO content of the supernatants was zero after three washes. (B) Yeast cells. The cells were incubated for 15 min at 20 °C in the presence of 45 μ M NAO. RRFI of NAO bound to cardiolipin was measured after three washes as previously described (final volume 3 ml). By comparison with the calibration curve, RRFI was used to determine the cardiolipin content of the sample. Curves are representative of three independent experiments.

Table 2 Cardiolipin content of ethanol-fixed yeasts

Ethanol-fixed cells were washed twice with 1 ml of 10 mM Tris/HCl, pH 7, mildly sonicated, then counted. Cardiolipin content was measured in 10^7 cells using the NAO procedure as described in Figure 5 and by phosphorus quantification. Data are expressed as means $\pm$ S.E.M. from three independent experiments.

	Cardiolipin content (nmol/ 10^7 cells)	
	Repressed cells	Derepressed cells
P_i assay	18 $\pm$ 8	62 $\pm$ 17
NAO assay	25 $\pm$ 2	45 $\pm$ 4

As long as glucose concentration was above 17 g/l, cytochrome *c* oxidase activity was low and constant at 100 m-units/mg of protein. Below 17 g/l, glucose repression of gene expression broke down and the enzyme activity increased (Figure 6B).

As soon as cells used ethanol as carbon source (19–20 h), rapid (1 h) and extensive cardiolipin synthesis was detected, increasing the cardiolipin content from 2.2 ± 0.1 to 4.2 ± 0.2 fmol/cell (Figure 6C). It was accompanied by a rearrangement of the transverse phospholipid distribution between the two leaflets of the inner membrane (Figure 6C). Cardiolipin present in the outer leaflet, which was $81.6 \pm 2.5\%$ (mean $\pm$ S.D. from three experiments) during phase I, decreased to $25.0 \pm 7.0\%$ during phase II and finally returned to $62.6 \pm 2.5\%$ during phase III (Figure 6C). At the same time, cytochrome *c* oxidase activity ceased to rise then increased again as a possible result of the topographical rearrangement (Figure 6B).

Figure 6 Yeast adaptation to oxidative phosphorylation

Cell growth in YM1 plus 40 g/l glucose was followed at A_{600} (□). A 1 ml volume of the culture was taken and centrifuged (5 min at 3000 *g*) to pellet the cells. The supernatant was transferred and immediately frozen at -20°C to determine glucose (Δ) and ethanol (▲) concentrations in the medium (A). The pellet was used to determine cytochrome *c* oxidase activity (■; B). Cardiolipin content (●) and asymmetry (○) during the diauxic shift from fermentative to gluconeogenic growth (C) were determined as described in Figures 3 and 4. Curves are representative of three independent experiments.

During the reverse process from gluconeogenic to fermentative growth (Figure 7A), cardiolipin content of exponentially growing yeast cells with lactate as a carbon source rapidly decreased by approx. 40% (from 4.1 ± 0.2 to 2.3 ± 0.1 fmol/cell; Figure 7C). As soon as cells were growing with glucose as carbon source, catabolite repression of gene expression induced a decrease in cytochrome *c* oxidase activity (Figure 7B). At the same time, the bilayer distribution of cardiolipin ($37 \pm 4:63 \pm 4$, in/out) changed transiently to a monolayer organization (100% out) then the characteristic and stable (around 80% out) asymmetry of repressed cells was re-established (Figure 7C).

Cardiolipin topography in antibiotic-treated cells

When the diauxic shift from fermentative to gluconeogenic growth was triggered in the presence of inhibitors of mitochondrial or nucleocytoplasmic protein synthesis, cardiolipin

Figure 7 Yeast adaptation to catabolite repression

Yeast cells at exponential phase of growth in YM1 plus 20 g/l lactate were harvested by centrifugation (5 min at 3000 *g*), washed twice with TH buffer then transferred to YM1 plus 40 g/l glucose medium at a density of $A_{600} = 6.5$. Cell growth in this medium was followed at A_{600} (□). Every 10 min, 1 ml of the culture was taken and centrifuged (5 min, 3000 *g*) to pellet the cells. The supernatant and pellet were immediately frozen at -20°C before the determination glucose concentration (Δ; A) and cytochrome *c* oxidase activity (■; B) respectively. Cardiolipin content (●) and asymmetry (○) in the cells during the diauxic shift from gluconeogenic to fermentative growth (C) were determined as described in Figures 3 and 4. Curves are representative of two independent experiments.

content and asymmetry of the antibiotic-treated cells remained similar to the values observed for the repressed cells (2.5 ± 0.1 fmol/cell and $20.8 \pm 2.1:79.2 \pm 2.1$, in/out) in contrast with derepressed control cells (Table 3).

In another experiment, cardiolipin topography was determined in various mutant strains (Table 4). Cardiolipin content and asymmetry in mitochondrial (ρ^{-} , ρ°) and nuclear (AAC $^{-}$) mutants were again similar to those of the glucose-repressed cells (2.5 fmol/cell and $20:80$ in/out) and different from those of wild-type derepressed cells.

To ascertain the connection between cardiolipin topography and membrane redox potential, we determined the cardiolipin amount and asymmetry in a TW strain carrying the plasmid pEMBLYe30/2-21-*urf13TW* coding for the heterologous T-URF13 protein targeted to inner mitochondria membrane (Table

Table 3 Cardiolipin topography in antibiotic-treated cells

Yeast cells in exponential phase (YM1, 40 g/l glucose medium) were harvested by centrifugation (5 min at 3000 *g*), washed twice with TH buffer, then transferred in YM1 plus 20 g/l lactate. The new medium was free of inhibitor or contained chloramphenicol (CAP) to inhibit mitochondrial protein synthesis or cycloheximide (CHI) to inhibit nucleocytoplasmic protein synthesis. At time zero and after 7 h of culture, samples (1 ml) were ethanol-fixed before cardiolipin quantification and asymmetry determination. Cell viability was controlled by flow cytometry using propidium iodide, a DNA-specific fluorochrome which does not pass through cytoplasmic membranes of viable cells. The cardiolipin content was determined *in situ* by measuring at 640 nm the RRFI of the NAO dimers. Cardiolipin asymmetry was determined *in situ*, by measuring the RRFI of increasing amounts (0–10 μ M) of NAO incubated in the presence of 10^6 cells. For more details see the Materials and methods section. Data are expressed as means $\pm$ S.E.M. from three independent experiments.

	Glucose 40 g/l (<i>t</i> = 0 h)			Lactate 20 g/l (<i>t</i> = 7 h)		
	[Cardiolipin] (fmol/cell)	Cardiolipin in the outer leaflet (%)	Cell viability (%)	[Cardiolipin] (fmol/cell)	Cardiolipin in the outer leaflet (%)	Cell viability (%)
Control	2.5 $\pm$ 0.1	80.2 $\pm$ 2.1	99.0 $\pm$ 0.1	3.6 $\pm$ 0.3	63.0 $\pm$ 4.3	98.0 $\pm$ 0.2
CAP (2 mg/ml)	2.4 $\pm$ 0.1	78.5 $\pm$ 3.5	97.8 $\pm$ 0.1	2.3 $\pm$ 0.1	76.4 $\pm$ 2.5	87.0 $\pm$ 0.3
CHI (50 μ g/ml)	2.4 $\pm$ 0.1	82.5 $\pm$ 0.6	98.0 $\pm$ 0.1	2.3 $\pm$ 0.1	79.4 $\pm$ 3.0	90.0 $\pm$ 0.1

Table 4 Cardiolipin topography in mutant cells

In order to decrease the repressive effect of glucose, respiratory-deficient mutants were grown until stationary phase (24 h) with only 10 g/l glucose in the medium, then cells were collected by centrifugation (5 min at 3000 *g*) and ethanol-fixed. Cell viability and content and asymmetry of cardiolipin were determined as described in Table 3. For more details see the Materials and methods section. Data are expressed as means $\pm$ S.E.M. from three independent experiments.

	Strain			
	Wild-type	AAC [−]	Rho [−]	Rho ^o
Cardiolipin in the outer leaflet (%)	60.2 $\pm$ 2.8	89.6 $\pm$ 0.4	79.4 $\pm$ 0.6	81.7 $\pm$ 5.6
Cardiolipin (fmol/cell)	3.7 $\pm$ 0.2	2.6 $\pm$ 0.2	2.4 $\pm$ 0.1	2.5 $\pm$ 0.2
Cell viability (%)	99.3 $\pm$ 0.1	94.6 $\pm$ 0.3	93.6 $\pm$ 0.6	99.0 $\pm$ 0.5

Table 5 Cardiolipin topography in methomyl-treated TW cells

TW yeast cells (wild-type CW04 strain transformed by pEMBYe30/2-2-*urf13TW* plasmid) grown in W3 medium supplemented with 120 mM KCl and 4 mM NaCl were treated for 30 min with methomyl (0, 150 or 180 μ M). Mitochondrial potential was determined by flow cytometry using 0.1 μ M DiOC₆(3). Cell viability and content and asymmetry of cardiolipin were determined as described in Table 3. For more details see the Materials and methods section. Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP; 5 μ M) stimulated phospholipase activities and prevented determination of cardiolipin amount and asymmetry. N.D., Not determined.

	Methomyl			FCCP
	0	150	180	
DiOC ₆ (3) fluorescence intensity	100	92.3	80	43.5
Cardiolipin in the outer leaflet (%)	59.9	70.4	81.3	N.D.
Cardiolipin (fmol/cell)	3.3	3.1	3.2	N.D.
Cell viability (%)	99.6	96.4	92.7	97.8

1). This 13 kDa maize protein induces an increase in membrane permeability to Ca²⁺ [31], leakage of NAD⁺ [32], changes in the rate of substrate oxidation [33] and dissipation of the transmembrane potential [34] in the presence of fungal pathotoxin HmT. Data presented in Table 5 confirm that, in the presence of the carbamate insecticide methomyl, a functional analogue of the toxin [35], T-URF13 caused the dissipation of mitochondrial redox potential *in situ* as controlled by DiOC₆(3) incorporation [22]. No significant variation in cardiolipin content (3.2 fmol/cell)

was registered. Nevertheless, phospholipid asymmetry in the methomyl-treated cells changed until it reached that of glucose-repressed cells (18.7:81.3, in/out).

DISCUSSION

Evidence that NAO is an effective probe for quantifying cardiolipin in membranes comes from several sources. (i) On hepatocytes [36], L1210 cells [37] and yeast cells [18], no labelling of nuclei or organelles other than mitochondria is observed, showing that phosphate esters such as nucleotides do not bind to the probe. (ii) The dye-binding affinity is high for cardiolipin ($K_a = 2 \times 10^6$ M^{−1}) and 30 times lower ($K_a = 7 \times 10^4$ M^{−1}) for monoacidic phospholipids [36]. (iii) Binding is due to electrostatic interactions between the quaternary ammonium of the probe and the negatively charged phosphate(s) of the phospholipid; stoichiometry was determined as one NAO molecule per monoacidic phospholipid and two NAO molecules per diacidic phospholipid (cardiolipin) [36]. (iv) The fatty acid composition of cardiolipin does not affect the binding affinity of the probe [18]. (v) Dye dimers bound to cardiolipin have a specific red-shifted fluorescence emission, which allows quantification [18]. Monoacidic phospholipids do not significantly affect measurement, allowing cardiolipin quantification in phosphatidylserine- and phosphatidylinositol-rich systems (repressed mitochondria) and in whole cells [18].

When the red fluorescence emission intensity is plotted as a function of the amount of NAO present in the incubation mixture, a biphasic curve is obtained, with two plateaus (see Figure 3). This behaviour has been ascribed to the successive saturation of the outward exposed cardiolipin (first plateau) and the lipid in the inner leaflet [14]. Quantification of total cardiolipin content by either the final level of NAO dimer red fluorescence or phosphate assay after cardiolipin extraction and purification gave comparable results, and one can assume that the dye labelled all the cardiolipin molecules. The validity of this assumption is reinforced by the present data. First, cardiolipin distribution in bovine heart mitochondrial membrane was assayed by two different approaches: NAO-binding assay and determination of the transmembrane distribution of a spin-labelled cardiolipin analogue. Although the two assays did not give strictly identical cardiolipin distributions, the two estimations were very close, differing by less than 10%. As the changes in cardiolipin distribution in the various growth conditions reported in this paper were larger than this range, they can be considered significant. We also verified that NAO binding to the membrane

did not induce bilayer rearrangement along with the change in transmembrane asymmetry. As tested in model systems, saturation of cardiolipin in the outer leaflet by NAO did not perturb the bilayer. Thus the first plateau of the dose-response fluorescence curve represents saturation of the external cardiolipin. Higher amounts of NAO induced limited scrambling, as detected by limited redistribution of the probe originally located in the outer leaflet. Dye-binding kinetics performed at 4 °C show a rapid increase in red fluorescence emission (1.95 RRFI/min) up to the first saturation level, followed by a slower one (0.35 RRFI/min) up to the second (Figure 2). Accordingly, one can attribute the first plateau to association of NAO with cardiolipin located in the outer layer; the second step, leading to the dye complex with the inner cardiolipin, could result from changes in the membrane, either relocation of a fraction of lipids or increased permeability of the bilayer to NAO. Note that the RRFI ratio (0.67) was comparable with the ratio (0.63) observed with increasing concentrations (from 0 to 8 μ M) of dye.

We have used this assay to analyse changes in cardiolipin distribution during the diauxic shift from fermentative to gluconeogenic growth and vice versa. At first, cardiolipin content and asymmetry appeared to be molecular parameters characteristic of both glucose-repressed (2.2 fmol/cell and $81.6 \pm 2.5\%$ out) and derepressed (4.2 fmol/cell and $62.6 \pm 2.5\%$ out) yeast cells. Cardiolipin synthesis on the matrix side of the inner membrane modified the transverse distribution of phospholipid, which changed rapidly ($81.6 \pm 2.5\%$ out to $25.0 \pm 7.0\%$ out), a result that is in good agreement with the topographical evidence that cardiolipin synthase is an integral inner-membrane enzyme [4]. Surprisingly, some of the newly synthesized cardiolipin appeared to move rapidly towards the outer leaflet ($62.6 \pm 2.5\%$), a translocation step that is concomitant with oxidative phosphorylation (ethanol consumption).

Our data on the reverse shift from gluconeogenic to fermentative growth confirm a close connection between cardiolipin content and asymmetry. As soon as cells were transferred to medium containing glucose as a carbon source, the cardiolipin content decreased along with cardiolipin synthase inhibition (probably as the result of cardiolipin synthase gene repression). The bilayer distribution of cardiolipin changed to an apparent monolayer organization (all the cardiolipin present in the outer leaflet) until translocation in the opposite direction towards the inner leaflet produced the characteristic asymmetry of repressed cells (80–85% out). Nevertheless, this reverse translocation took about 30 min and was very rapid compared with induction of cytochrome *c* oxidase activity (see Figure 7). So cardiolipin asymmetry does not appear to be closely connected to mitochondrial protein import. However, cardiolipin topography analysis in antibiotic-treated and mutant cells appeared to indicate that cardiolipin content and asymmetry were linked. This is in contradiction with the analysis of cardiolipin distribution in methomyl-treated cells, which showed that cardiolipin asymmetry was unrelated to cardiolipin content but was closely connected to transmembrane redox potential. Therefore it is not possible at present to establish a clear relationship between the properties of membrane cardiolipin and the physiology of the mitochondria.

How phospholipid molecules move between the two leaflets remains unsolved. We observed only that cardiolipin movement during the switch from fermentative to gluconeogenic growth in yeast and vice versa was rapid (about 30 min), suggesting a

specific mechanism or a sudden change in physicochemical properties of the bilayer allowing such rapid transmembrane diffusion.

We are indebted to Alastair Balloch, Michel Guilloton and Yannis Karamanos for helpful discussion and careful reading of the manuscript. We thank Nathalie Glab for the gift of transformed yeast cells and Paulette Hervé for the synthesis and gift of spin-labelled analogues.

REFERENCES

- Beyer, K. and Klingenberg, M. (1985) *Biochemistry* **24**, 3821–3826
- Eble, K. S., Coleman, W. B., Hantgan, R. R. and Cunningham, C. C. (1990) *J. Biol. Chem.* **265**, 19434–19440
- Krebs, J. J. R., Hauser, H. and Carafoli, E. (1979) *J. Biol. Chem.* **254**, 5308–5316
- Schlame, M. and Holdar, D. (1993) *J. Biol. Chem.* **268**, 74–79
- Cheneval, D., Muller, M., Toni, R., Ruetz, S. T. and Carafoli, E. (1985) *J. Biol. Chem.* **260**, 3839–3843
- Schlame, M. and Augusta, W. (1985) *Biomed. Biochim. Acta* **44**, 1083–1088
- Rickwood, D., Dujon, B. and Darley-Usmar, V. M. (1988) in *Yeast: A Practical Approach*, (Campbell, I. and Duffus, J. H., eds.), pp. 185–254, IRL Press, Oxford and Washington D.C.
- Zinser, E., Sperka-Gottlieb, C. D. M., Fasch, E. V., Kohlwein, S. D., Paltauf, F. and Daum, G. (1991) *J. Bacteriol.* **173**, 2026–2034
- Smith, A. L. (1967) *Methods Enzymol.* **10**, 181–186
- Brandolin, G., Boulay, F., Dalbon, P. and Vignais, P. V. (1989) *Biochemistry* **28**, 1093–1100
- Weissbach, H., Smith, T. E., Daly, J. W., Witkop, B. and Udenfriend, S. (1960) *J. Biol. Chem.* **235**, 1160–1163
- Robinson, J. B., Brent, L. G., Sumegi, B. and Srere, P. A. (1987) in *Mitochondria: A Practical Approach*, (Darley-Usmar, V. M., Rickwood, D. and Wilson, M. T., eds.), pp. 153–170, IRL Press, Oxford and Washington D.C.
- Vignais, P. V., Block, M. R., Boulay, F., Brandolin, G. and Lauquin, G. J. M. (1985) *Structure and Properties of Cell Membranes*, CRC Press, Boca Raton, FL
- Petit, J. M., Huet, O., Gallet, P. F., Maftah, A., Ratinaud, M. H. and Julien, R. (1994) *Eur. J. Biochem.* **220**, 871–879
- Fellmann, P., Zachowski, A. and Devaux, P. F. (1994) *Methods Mol. Biol.* **27**, 161–175
- Kornberg, R. D. and McCornell, H. M. (1971) *Biochemistry* **10**, 1111–1120
- Hope, M. J., Bally, M. B., Webb, G. and Cullis, P. R. (1985) *Biochim. Biophys. Acta* **812**, 55–65
- Gallet, P. F., Maftah, A., Petit, J. M., Denis-Gay, M. and Julien, R. (1995) *Eur. J. Biochem.* **228**, 113–119
- Reeves, J. P. and Dowben, R. M. (1969) *J. Cell. Physiol.* **73**, 49–60
- Rouser, G., Kritchewsky, G., Heller, D. and Lieber, E. (1963) *J. Am. Oil Chem. Soc.* **40**, 425–454
- Zhou, X. and Arthur, G. (1992) *J. Lipid Res.* **33**, 1233–1236
- Glab, N., Petit, P. X. and Slonimski, P. P. (1983) *Mol. Gen. Genet.* **236**, 299–308
- Warthon, D. C. and Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245–250
- Carafoli, E. and Pietrobon, D. (1982) *Membrane Biochemistry*, Springer Verlag, New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Kahle, K., Weiss, L., Klarwein, M. and Wieland, O. (1970) *Z. Anal. Chem.* **252**, 228.
- Käppli, O. (1986) *Adv. Microb. Physiol.* **28**, 181–209
- Perlman, P. S. and Mahler, H. R. (1974) *Arch. Biochem. Biophys.* **162**, 248–271
- Yotsuyanagi, Y. (1962) *J. Ultrastruct. Res.* **7**, 121–140
- Hajek, P. and Bedwell, D. M. (1994) *J. Biol. Chem.* **269**, 7192–7200
- Holden, M. J. and Sze, H. (1984) *Plant Physiol.* **75**, 235–237
- Matthews, D. E., Gregory, P. and Gracen, V. E. (1979) *Plant Physiol.* **63**, 1149–1153
- Miller, R. J. and Koeppe, D. E. (1971) *Science* **173**, 67–69
- Bervillé, A., Ghazi, A., Charbonnier, M. and Bonavent, J. F. (1984) *Plant Physiol.* **76**, 508–517
- Levings, III, C. S. (1990) *Science* **250**, 942–947
- Petit, J. M., Maftah, A., Ratinaud, M. H. and Julien, R. (1992) *Eur. J. Biochem.* **209**, 267–273
- Leprat, P., Ratinaud, M. H., Maftah, A., Petit, J. M. and Julien, R. (1990) *Exp. Cell Res.* **186**, 130–137
- Glab, N., Wise, R. P., Pring, D. R., Jacq, C. and Slonimski, P. (1990) *Mol. Gen. Genet.* **223**, 24–32