Phosphatidylcholine and yeast mitochondria

On synthesis, transport, distribution, and interaction with proteins

Fosfatidylcholine en gistmitochondriën

Over synthese, transport, distributie en interactie met eiwitten

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Abbreviations

BSA	bovine serum albumin
(bv)PLA$_2$	(bee venom) phospholipase A$_2$
CDP	cytidine diphosphate
CE	cholesteryl oleoyl ether/ester
CL	cardiolipin
CSG	complete synthetic glucose medium
CSL	complete synthetic lactate medium
diSC$_2$(5)	3,3'-diethylthiadicarbocyanine iodide
DMSO	dimethyl sulfoxide
(DO)PC	(dioleolyl)phosphatidylcholine
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-amino-ethyl ether)*N*,*N*,*N*',*N*'-tetraacetic acid
FAD	flavin adenine dinucleotide, oxidized form
FCCP	*p*-trifluoromethoxy carbonylcyanide phenylhydrazine
GUT	glycerol utilization
Hepes	*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid
(HP)TLC	(high performance) thin layer chromatography
IEF	iso-electric focusing
MALDI-TOF	matrix-assisted laser desorption/ionization - time of flight
MAM	mitochondria-associated membranes
MES	2-(*N*-morpholino)ethane-sulfonic acid
MOPS	3-(*N*-morpholino)propane-sulfonic acid
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide
NADH	nicotinamide adenine dinucleotide, reduced form
NEM	*N*-ethylmaleimide
NEPHGE	non-equilibrium pH gradient electrophoresis
OMV	outer membrane vesicles
OG	octylglucoside
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCMBS	para-chloromercuribenzene sulfonic acid
PCR	polymerase chain reaction
PC-TP	PC-specific transfer protein

PDME	phosphatidyldimethylethanolamine, dimethyl PE
PE	phosphatidylethanolamine
PEMT	PE methyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
P_iC	phosphate carrier
PLMT	phospholipid methyltransferase
pmf	proton motive force
PMME	phosphatidylmonomethylethanolamine, monomethyl PE
PMS	phenazine methosulfate
PMSF	phenylmethylsulfonyl fluoride
PNMT	phospholipid-*N*-methyltransferase
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
SAM	*S*-adenosyl-L-methionine
SDS	sodium dodecyl sulfate
SRA	specific radioactivity
SSG	semi-synthetic glucose medium
SSL	semi-synthetic lactate medium
SUV	small unilamellar vesicles
TCA	trichloroacetic acid
TID	3-(trifluoromethyl)-3-phenyldiazirine
[^{125}I]TID-BE	benzoic acid [2-[^{125}I]iodo-4-((trifluoromethyl)-3*H*-diazirin)benzyl] ester
[^{125}I]TID-PC	1-*O*-hexadecanoyl-2-*O*-[9-[[[2-[^{125}I]iodo-4-((trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]oxy]carbonyl]nonanoyl]-*sn*-glycero-3-phosphocholine

Chapter 1

General introduction

Structure, function and biogenesis of mitochondria

Mitochondria are specialized organelles, performing functions central to the life of most eukaryotic cells. The structure and functions of mitochondria from single-celled or complex multicellular organisms are similar. The primary functions are the conservation of oxidatively derived energy and its utilization for ATP synthesis [Tzagoloff, 1982]. Isolated mitochondria are most commonly observed as oval particles, consisting of two distinct membranes (Figure 1A). The major structural elements in mitochondria are proteins and lipids (Figure 1B), mainly phospholipids, among which phosphatidylcholine (PC) is the major phospholipid (Figure 1C). The outer and inner membranes define and enclose two soluble compartments, the matrix and the intermembrane space. The outer membrane is relatively permeable, while the inner membrane is essentially impermeable. The inner membrane is highly invaginated, forming so-called cristae, and is associated with regions of outer membrane at membrane contact sites. In the cell, mitochondria are highly dynamic structures, and it is unknown whether mitochondrial membrane contact sites are fixed structures or form only transiently (for a review, see [Brdiczka, 1991]). The various compartments of mitochondria contain different enzymes, which constitute the metabolic pathways in mitochondria, with the inner membrane containing the enzymes of the respiratory chain [Tzagoloff, 1982].

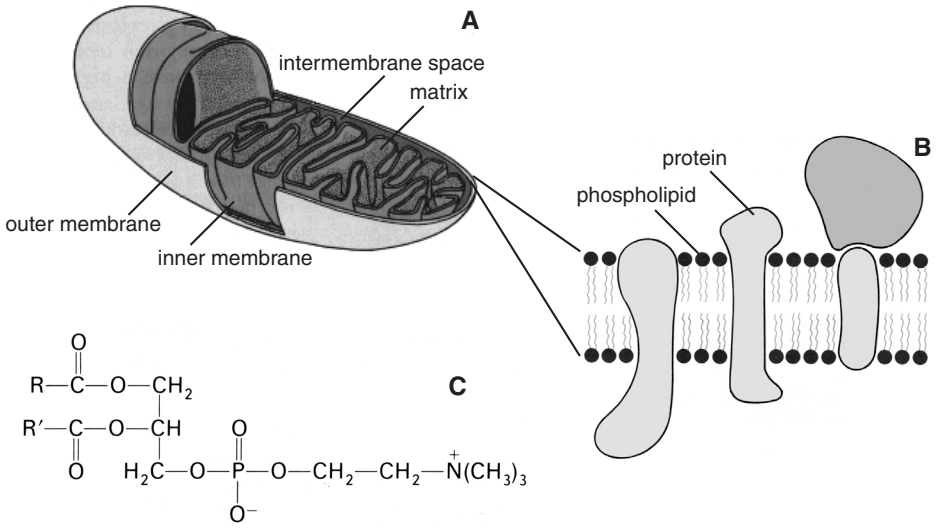

Figure 1. Mitochondria, membranes and phosphatidylcholine. A: schematic representation of the structure of a mitochondrion; B: schematic representation of a membrane; C: chemical structure of phosphatidylcholine. R and R' represent hydrocarbon chains of variable length and degree of unsaturation.

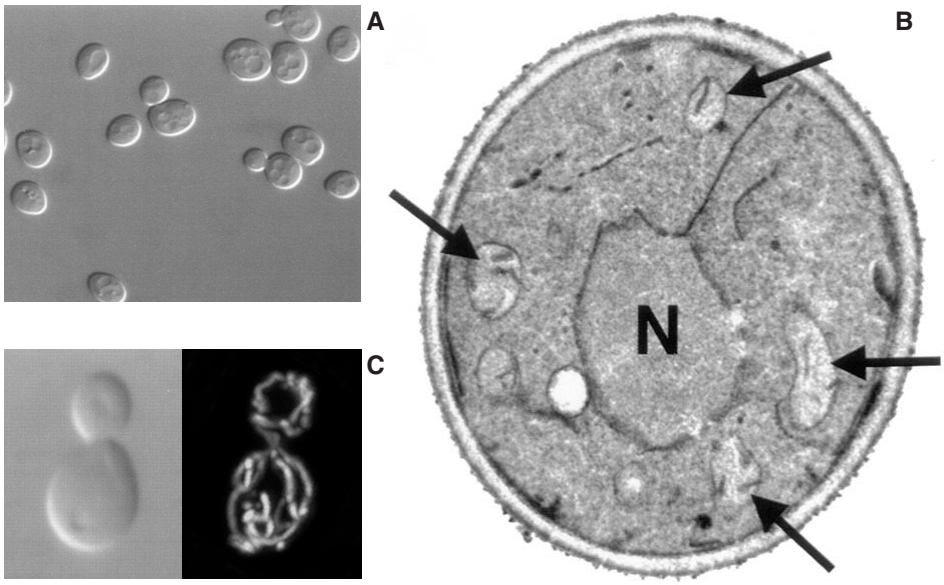

Figure 2. Yeast cells and mitochondria. A: differential interference contrast image of yeast cells [Otsuga *et al.*, 1998]; B: transmission electron microscopy image of a yeast cell. N represents the nucleus, and mitochondria are indicated with arrows [Otsuga *et al.*, 1998]; C: differential interference contrast image of a (budded) yeast cell (left panel) [Otsuga *et al.*, 1998] and confocal microscopy image of a (budded) yeast cell expressing a green fluorescent protein targeted to the mitochondrial matrix (right panel) [Mozdy, McCaffery and Shaw, 2000].

The unicellular eukaryote *Saccharomyces cerevisiae* (yeast) is widely recognized as an ideal eukaryotic microorganism for biochemical and classical genetic studies, and it is amenable to gene cloning and genetic engineering. The cells grow by budding, and Figure 2A shows what they look like under the microscope. Yeast can grow anaerobically as well as aerobically, which makes it a convenient model organism to study mitochondrial biogenesis. Aerobically grown yeast cells contain fully developed mitochondria with a typical respiratory chain, while anaerobically grown cells contain poorly developed structures called promitochondria, which lack a respiratory chain [Schatz and Kovác, 1974; Pon and Schatz, 1991]. Promitochondria develop into respiring yeast mitochondria when the cells shift to aerobic growth. Figure 2B shows the typical mitochondrial ultrastructure in a yeast cell. Yeast mutants defective in oxidative phosphorylation can only grow by fermentation of *e.g.*, glucose, and are unable to grow on non-fermentable carbon sources, such as glycerol or lactate. When grown on glucose-poor media, these cells form smaller colonies than wild-type cells, and are consequently referred to as ‘petites’. Changes in cellular respiration are paralleled by distinctive changes in mitochondrial morphology and cellular volume occupied by mitochondria. Mitochondria are not synthesized *de novo* but develop from existing ones by growth and division. In respiring yeast cells, mitochondria generally form a large

mitochondrial reticulum throughout the cell, as can be seen in Figure 2C, which can undergo fusion and fission [Otsuga *et al.*, 1998; Hermann *et al.*, 1998; Rapaport *et al.*, 1998; Fekkes, Shepard and Yaffe, 2000; Mozdy, McCaffery and Shaw, 2000].

Mitochondria of yeast, like those of other organisms, are only partially autonomous in their biogenesis. Only a small fraction of the constituent protein and lipid molecules is made inside the mitochondrion. The mitochondrial DNA, present in the matrix space, encodes only eight mitochondrial proteins, seven of which are part of the respiratory chain [Pon and Schatz, 1991]. However, most of the mitochondrial proteins are encoded by the nuclear DNA and have to be imported into mitochondria. Studies on mitochondrial biogenesis have benefitted greatly from the awesome power of yeast genetics. Proteins destined for import into mitochondria carry signals both for targeting to the mitochondria and for sorting to the correct intramitochondrial location, *e.g.* most matrix proteins are synthesized as larger precursors containing an N-terminal presequence, which interacts with receptor proteins on the mitochondrial surface. Membrane contact sites have been implicated in the translocation of proteins to the matrix, and the process usually requires a membrane potential and ATP (for a review, see [Voos *et al.*, 1999]). While knowledge of the mechanisms and factors involved in transport and assembly of proteins into different membranes has been rapidly expanding in the past years, knowledge of lipid transport processes is very limited. This thesis focuses on PC, the most abundant phospholipid found in mitochondrial, as well as in other cellular membranes.

Phospholipid composition and biosynthesis

Yeast shares similar membrane phospholipid classes and similar pathways of phospholipid metabolism with higher eukaryotes, with a few exceptions (for reviews see [Carman and Henry, 1989; Paltauf, Kohlwein and Henry, 1992; Carman and Henry, 1999]). The major phospholipids found in yeast membranes are PC, accounting for 40-50% of the total membrane phospholipids [Henry, 1982], phosphatidylethanolamine (PE), phosphatidyl-inositol (PI), and phosphatidylserine (PS). In addition, mitochondrial membranes also contain phosphatidylglycerol (PG) and cardiolipin (CL). Yeast membranes also contain sterols and sphingolipids [Daum *et al.*, 1998]. Contrary to mammalian cells, yeast lipids contain a limited repertoire of acyl chains and lack *e.g.* polyunsaturated species [Schneiter *et al.*, 1999].

The pathways of membrane phospholipid biosynthesis are depicted in Figure 3. Several branches of phospholipid biosynthesis diverge from the precursor cytidine diphosphate (CDP)-diacylglycerol. PI is formed from CDP-diacylglycerol and inositol, and CDP-diacylglycerol is also used in the biosynthesis of PG and CL. The main biosynthetic route leading to PC in yeast starts with the conversion of CDP-diacylglycerol and serine into PS, whereas in higher eukaryotes, PS is synthesized *via* exchange of serine with the

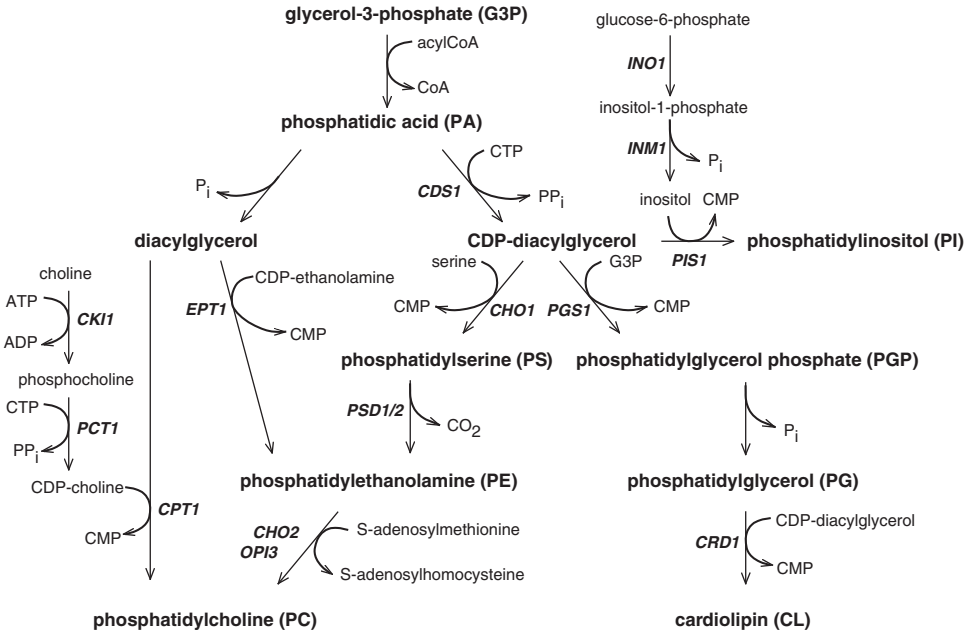

Figure 3. Biosynthesis of membrane phospholipids in yeast. When identified, the genes encoding the enzymes are indicated. The conversion of G3P into PA is catalyzed by glycerophosphate acyltransferase. PA is converted to diacylglycerol by PA phosphatase. CDP-ethanolamine is synthesized from ethanolamine by reactions analogous to the formation of CDP-choline from choline by enzymes encoded by *EKI1* and *ECT1*. PGP is converted into PG by PGP phosphatase. *CDS1*: CDP-diacylglycerol synthase; *CHO1*: PS synthase; *CHO2*: PE methyltransferase; *CKI1*: choline kinase; *CPT1*: choline phosphotransferase; *CRD1*: CL synthase; *ECT1*: phosphoethanolamine cytidylyltransferase; *EKI1*: ethanolamine kinase; *EPT1*: ethanolamine phosphotransferase; *INM1*: inositol monophosphatase; *INO1*: inositol-1-phosphate synthase; *OPI3*: phospholipid methyltransferase; *PCT1*: phosphocholine cytidylyltransferase; *PGS1*: PGP synthase; *PIS1*: PI synthase; *PSD1*: mitochondrial PS decarboxylase; *PSD2*: Golgi/vacuolar PS decarboxylase.

headgroup of PC or PE. PS is decarboxylated to form PE, which is further converted to PC by three sequential methylations. Alternatively, PE and PC can be synthesized from phosphatidic acid (PA) *via* diacylglycerol and CDP-ethanolamine or CDP-choline (Kennedy pathway), respectively. In yeast, the methylation of PE by the phospholipid-*N*-methyl-transferase (PNMT) enzymes is considered the primary pathway of biosynthesis of PC, when cells are grown in the absence of choline.

Many enzymes involved in the biosynthetic routes of phospholipids are coordinately regulated at the transcriptional level in response to phospholipid precursors and growth phase (for recent reviews, see [Carman and Zeimetz, 1996; Henry and Patton-Vogt, 1998; Carman and Henry, 1999]). For example, the *INO1* gene product, which catalyzes the formation of inositol, as well as the enzymes involved in the production of PC *via* CDP-diacylglycerol, are repressed by inositol and choline, and the latter enzymes exhibit maximal

activities in the exponential phase whereas the activities are reduced in the stationary growth phase. In contrast, the expression of PI synthase does not respond to inositol or growth phase, but is regulated by the carbon source [Anderson and Lopes, 1996]. Furthermore, enzymatic activities can be regulated by the membrane lipid composition and phosphorylation (*e.g.* PS synthase [Hromy and Carman, 1986; Kinney and Carman, 1988]).

Cellular roles of phospholipid classes in yeast

From work on yeast mutants with a variety of defects in phospholipid biosynthesis, it has become increasingly clear that yeast cells can tolerate large variations in their phospholipid composition. Furthermore, it is evident that the phospholipid composition of yeast cells and derived subcellular membranes varies with the growth conditions. However, most studies are performed using glucose as the carbon source, and relatively little is known about the consequences of growth on different carbon sources, which is essential knowledge when studying mitochondrial biogenesis. The tolerance of yeast to variations in phospholipid composition complicates the assignment of functional roles to each of the different phospholipid classes.

A specific function in protein import, as well as in other processes, has been attributed to the mitochondrial phospholipid CL, based on a large number of *in vitro* studies, *e.g.* [Eilers, Endo and Schatz, 1989; Endo, Eilers and Schatz, 1989]. Surprisingly, it was recently shown that *crd1* deletion mutants, lacking CL synthase and producing no measurable levels of CL, are still capable of growth on non-fermentable carbon sources [Jiang, Rizavi and Greenberg, 1997; Tuller *et al.*, 1998; Chang *et al.*, 1998]. The lack of CL appears to be compensated by elevated levels of PG.

Another example of extreme tolerance to changes in phospholipid composition are *cho1* mutants, which lack PS synthase and thus are unable to synthesize PS (see Figure 3). The cells have an auxotrophic requirement, which is fulfilled by ethanolamine, monomethylethanolamine, dimethylethanolamine, or choline. These precursors do not restore PS synthesis, but enter the Kennedy pathway, leading to restoration of PC biosynthesis. Null mutants make membranes completely devoid of PS [Atkinson, Fogel and Henry, 1980; Atkinson *et al.*, 1980], but synthesize low levels of PE when grown in the presence of choline but in the absence of ethanolamine, due to turnover of ethanolamine from sphingolipid metabolism. Under these conditions, PI levels are elevated, indicating that PI is able to substitute for PS. The reverse is not true, as mutants in inositol biosynthesis (*ino1*) are strict inositol auxotrophs, and disruption of the gene encoding PI synthase (*PIS1*) is lethal [Nikawa, Kodaki and Yamashita, 1987].

Strains with defects in either one of the methyltransferase enzymes responsible for the conversion of PE to PC (*cho2* and *opi3*, see Figure 3) are not choline auxotrophs [Summers *et al.*, 1988; McGraw and Henry, 1989; Preitschopf *et al.*, 1993]. When grown in

the absence of choline, the *opi3* mutant accumulates phosphatidylmonomethylethanolamine (PMME) and exhibits low or undetectable levels of PC [McGraw and Henry, 1989; Kodaki and Yamashita, 1989], while *cho2* mutants accumulate PE and have low levels of PC [Summers *et al.*, 1988; Kodaki and Yamashita, 1989]. However, double mutants have no phospholipid methylation activity and display a stringent choline auxotrophy [Kodaki and Yamashita, 1987; Summers *et al.*, 1988; Preitschopf *et al.*, 1993], indicating that some level of methylated phospholipid is required. In the absence of exogenous choline, only the methylation pathway provides for the *de novo* synthesis of PC. However, the CDP-choline pathway contributes to PC synthesis by using the choline originating from turnover of PC by phospholipases. There are indications that both pathways of PC biosynthesis serve different functional roles *in vivo*. Mutations in the genes encoding the PNMTs, but not mutations in genes encoding enzymes of the CDP-choline pathway for PC biosynthesis, result in an overproduction of inositol (Opi^-) phenotype, which indicates overexpression of *INO1* [Paltauf, Kohlwein and Henry, 1992]. Furthermore, deacylation of PC occurs only in the presence of an active CDP-choline pathway [Dowd, Bier and Patton-Vogt, 2001]. Moreover, several mutant strains with defects in the routes leading to biosynthesis of PC have the tendency to generate respiratory-deficient petites at high frequency [Griac, Swede and Henry, 1996; Griac and Henry, 1996], suggesting that PC is required for proper mitochondrial function, biogenesis, or maintenance. To date, the only enzyme which is known to be dependent on PC for activity is the mammalian mitochondrial enzyme ß-hydroxybutyrate dehydrogenase [Isaacson *et al.*, 1979], of which no counterpart in yeast has been found.

Recently, it was shown that a minimal level of PE is essential for growth, by analysis of a series of deletion mutants, in which different combinations of the pathways leading to PE were blocked [Birner *et al.*, 2001]. Furthermore, the same authors showed that on non-fermentable carbon sources, a higher level of PE is required for growth than on fermentable carbon sources, and that mutants with low levels of PE exhibit enhanced formation of respiration-deficient cells. In this respect, it is interesting to note that a PE molecule was found embedded in the crystal structure of the mammalian cytochrome *bc1* complex [Iwata *et al.*, 1998; Zhang *et al.*, 1998].

Thus, while the yeast cell is extremely flexible in its phospholipid composition, there seems to be a minimally required level for several phospholipids to maintain viability. Moreover, it should be kept in mind that, although many mutations leading to altered phospholipid composition are not lethal, the cells carrying them are certainly not wild-type in all their properties, with the severity of the phenotype depending on the exact growth conditions.

Import of phospholipids into mitochondria

While the distribution of proteins is usually absolute with respect to specific organelles, the distribution of phospholipids between membranes tends to vary only in the relative amount of each phospholipid. Mitochondrial membranes, like all other cellular membranes, contain the whole set of cellular glycerophospholipids, with PC as the major phospholipid species [Daum, 1985]. Most phospholipids are synthesized in the endoplasmic reticulum and closely related membranes. The contribution of mitochondria to cellular phospholipid biosynthesis is restricted to the formation of PE and CL [Daum and Vance, 1997]. This necessitates efficient import of the phospholipids which are not synthesized in mitochondria to ensure membrane growth, and to maintain membrane lipid composition, integrity, and function, in the growing cell. In addition to transport to the organelle, sorting mechanisms must operate to distribute the phospholipid over the organellar membranes. Furthermore, it has often been shown that phospholipids in biological membranes are asymmetrically distributed over the leaflets of the membranes (for a review, see [Zachowski, 1993]). In yeast mitochondrial outer membranes, PC and PI were found to be equally distributed across both sides of the membrane, while PE preferentially faces the intermembrane space [Sperka-Gottlieb *et al.*, 1988], and CL was found to be enriched in the outer leaflet of the inner mitochondrial membrane [Gallet *et al.*, 1997].

Figure 4 shows the localization of enzymes involved in routes of PC biosynthesis, and the directions in which transport takes place between endoplasmic reticulum and mitochondria. Several mechanisms for the intracellular transport of phospholipids between membranes have been proposed (for reviews, see [Voelker, 1991; Trotter and Voelker, 1994]), including 1) spontaneous or protein-facilitated transport of monomeric phospholipids through the aqueous phase; 2) vesicle-mediated transfer, in which vesicles bud from a donor membrane and fuse with an acceptor membrane; 3) contact between membranes allowing the direct transfer of lipids. For transmembrane transport, the possibilities are limited to spontaneous or protein-mediated flip-flop (for reviews, see [Zachowski, 1993; Menon, 1995]). The spontaneous transport of phospholipids through the aqueous phase is unlikely to be of importance in view of their low solubility in water. Although several soluble proteins have been identified that are capable of transporting lipids between membranes *in vitro*, none of them have been shown to be involved in lipid transport *in vivo* (for a review, see [Wirtz, 1991]). To date, there are also no indications for the occurrence of any form of vesicular transport to the mitochondria. Several experimental observations, in mammalian cells as well as in yeast, are in favor of delivery of lipids to the mitochondria by a mechanism involving contact between membranes (for reviews, see [Voelker, 1991; Daum and Vance, 1997]). A specific subfraction of the endoplasmic reticulum, which is highly enriched in lipid synthesizing activities, *e.g.* PS synthase activity [Vance, 1990; Gaigg *et al.*, 1995], has been implicated in this process. It is referred to as mitochondria-associated membranes (MAM),

since it remains associated with the mitochondria upon isolation. Close proximity of mitochondria and endoplasmic reticulum are often observed by electron microscopy in various cell types (*e.g.* [Bracker and Grove, 1971; Pickett *et al.*, 1980]). Recently, sites of association of endoplasmic reticulum with mitochondria were shown in yeast by a three dimensional reconstruction from serial thin-sections [Achleitner *et al.*, 1999].

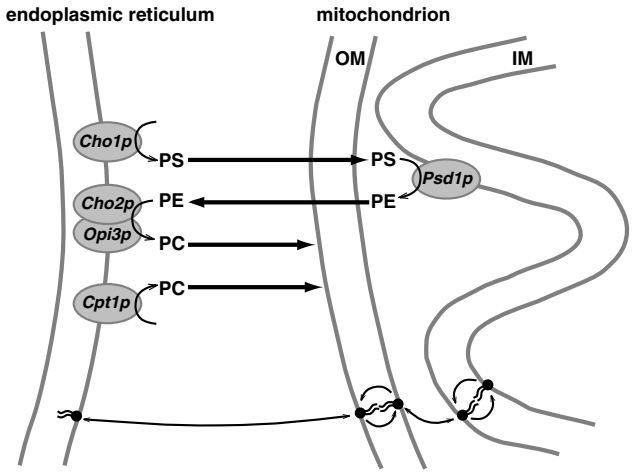

Figure 4. Sites of biosynthesis and main transport routes of PS, PE and PC. PS is synthesized (Cho1p) in the endoplasmic reticulum and in part imported into the mitochondria, where most of it is decarboxylated (Psd1p). PE resulting from PS decarboxylation can be exported to the endoplasmic reticulum. It should be noted that PE synthesized *via* the CDP-ethanolamine pathway or *via* decarboxylation by Psd2p can also be imported into mitochondria, or converted into PC by methylation (Cho2p and Opi3p). PC formed by methylation of PE or by the CDP-choline pathway (Cpt1p) is in part transported to the mitochondria. OM and IM indicate the outer and inner mitochondrial membrane, respectively. The various transport and sorting steps which might be taking place are schematically represented at the bottom of the figure.

The research on phospholipid import into mitochondria has mainly focused on the import of PS. The reason for this is that the metabolic conversion to PE by the PS decarboxylase in the inner membrane can be used to monitor arrival of the phospholipid in the mitochondria. In a similar manner, export of PE from mitochondria to the endoplasmic reticulum can be monitored by its conversion into PC upon arrival in the endoplasmic reticulum. The requirements for the interorganelle transport of PS have been examined, using a radiolabeled serine precursor. In contrast to the results obtained with mammalian cells [Voelker, 1989, 1990 and 1993], no ATP dependence has been observed in yeast for PS import into mitochondria [Achleitner *et al.*, 1995]. Furthermore, it was shown that import of PS in the mitochondria, and export of PE from mitochondria to the endoplasmic reticulum

are processes that do not require a membrane potential [Simbeni, Paltauf and Daum, 1990; Gnamusch *et al.*, 1992], or cytosolic factors [Achleitner *et al.*, 1995]. The import of PS could be reconstituted *in vitro* in a mixture of MAM or microsomes and mitochondria, suggesting that organelle contact is sufficient for this process. Protein factors on the mitochondrial surface appear to be of importance since protease treatment reduced the mitochondrial import of PS [Achleitner *et al.*, 1999]. Contact sites between mitochondrial outer and inner membranes are considered as good candidates for the exchange of lipids between these membranes [Simbeni *et al.*, 1991 and 1993]. However, it remains unclear whether transfer of PS to the inner membrane is an actual prerequisite for conversion to PE [Voelker, 1988; Hovius *et al.*, 1992; Daum and Vance, 1997]. Contact sites were also suggested to be involved in a study on the intramitochondrial transport of PC and PI [Lampl *et al.*, 1994]. In a study using fluorescent PC analogs in rat liver mitochondria, it was found that these were confined to the outer membrane, suggesting that extramitochondrial factors might be necessary to initiate transfer of PC to the inner membrane [Nicolay *et al.*, 1990]. In contrast, Lampl *et al.* found that radiolabeled PC, and also PI, was able to reach the inner membrane when the outer membrane of yeast mitochondria was loaded with these lipids *in vitro*, using a phospholipid transfer protein. These processes required neither an electrochemical gradient across the mitochondrial inner membrane nor ATP. *In vivo* pulse-labeling studies in yeast showed that PC transfer between endoplasmic reticulum and mitochondria continued in the absence of metabolic energy, although at a lower rate as compared to energy-supplemented cells [Daum, Heidorn and Paltauf, 1986]. It has been speculated that synthesis of phospholipids might be the driving force for transport [Simbeni, Paltauf and Daum, 1990]. In a study employing isolated outer membranes from rat liver mitochondria, it was shown that newly introduced PC rapidly equilibrates over both leaflets of the outer membrane [Dolis, De Kroon and De Kruijff, 1996]. Transmembrane movement of PE, PC, and CL in the inner membrane was also found to be rapid, using spin-labeled analogs [Gallet *et al.*, 1999].

The metabolic conversion of PS to PE was used to develop a screen for identifying yeast mutants affected in mitochondrial PS import. In mutants with defects in the *PSD2* gene (which encodes the Golgi/vacuole localized PS decarboxylase), PE can only be synthesized by decarboxylation of PS through the mitochondrial PS decarboxylase (Psd1p), in the absence of ethanolamine. Mutagenization of *psd2* knock-out cells and selection for ethanolamine auxotrophs has yielded a mutant defective in import of PS into the mitochondria in the transport step between the MAM and the mitochondria [Schumacher and Voelker, 2001]. Identification of the responsible gene is underway, and this will enable molecular characterization of the protein involved. A similar strategy, applied to *psd1* knock-out cells, has proven successful for identifying genes involved in transport of PS to PS decarboxylase 2 [Trotter *et al.*, 1998; Wu *et al.*, 2000].

Scope of this thesis

As described in the above, PC is a major phospholipid of all cellular membranes in eukaryotes, and it is also present in large quantities in both mitochondrial membranes. Since the final steps of PC biosynthesis take place in the endoplasmic reticulum (see Figure 4), proper mitochondrial biogenesis necessitates PC transport to the mitochondria and intramitochondrial sorting of PC over both mitochondrial membranes. The mechanisms and regulatory aspects involved in these processes are unknown. The work described in this thesis was primarily aimed at elucidation of the mechanism of PC import and identification of the components involved. The yeast *Saccharomyces cerevisiae* was chosen as the model organism for these investigations, because it shares similar features of cellular organization and phospholipid composition and biosynthesis with higher eukaryotes. Its facultative requirement for mitochondria makes it a convenient model organism to study mitochondrial biogenesis. In addition, it offers the potential for classical genetics and genetic manipulation. This, in combination with its sequenced genome, allows for genetic as well as biochemical studies on candidate genes and gene products involved in PC import.

In chapter 2 of this thesis, the investigation of the transbilayer movement of PC across the outer mitochondrial membrane is described. An *in vitro* approach using isolated mitochondrial outer membrane vesicles (OMV) was applied, and the possible involvement of protein factors in this process was studied. Variations observed in mitochondrial PC and PE content were investigated by addressing the influence of growth phase and carbon source on the phospholipid composition and phospholipid biosynthetic activities, as described in chapter 3. In chapter 4, the possibility was examined, that the methyltransferases, catalyzing the conversion of PE to PC, are able to act upon a substrate, localized in another membrane than the one in which the enzymes reside. Such a mechanism would preclude the need for actual transport of PC between the endoplasmic reticulum and the mitochondria. Subcellular fractions isolated from *opi3* and *cho2* knock-out strains were used. It is important to know that the integrity of mitochondria is preserved in *in vitro* studies, and the buffer requirements for maintaining mitochondrial intactness and membrane potential are reported in chapter 5. The possibility of developing a genetic approach for identifying yeast mutants in PC import depends greatly on knowledge on the role of PC in mitochondria, since such an approach requires an appropriate screening or selection method, preferably making use of PC dependent processes in mitochondria. Chapter 6 explores the interaction of PC with mitochondrial proteins, using a photoactivatable phospholipid analog, since this might shed light on the specific role of PC in mitochondria, or even allow the identification of proteins directly involved in the intramitochondrial transport of PC. Finally, in chapter 7, the results of these studies are summarized and discussed.

Chapter 2

Transbilayer movement of phosphatidylcholine in the mitochondrial outer membrane of *Saccharomyces cerevisiae* is rapid and bidirectional

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Abstract

The process of transmembrane movement of phosphatidylcholine (PC) across the outer membrane of mitochondria was investigated *in vitro* in mitochondrial outer membrane vesicles (OMV) from the yeast *Saccharomyces cerevisiae*. Phosphatidylcholine-transfer protein (PC-TP) was used to extract radiolabeled PC from OMV, with small unilamellar vesicles serving as acceptor system. Endogenously radiolabeled PC synthesized either *via* the CDP-choline pathway or *via* methylation of phosphatidylethanolamine can be extracted completely from the OMV with a $t_{1/2}$ of one min or less at 30°C. The size of the pool of PC in OMV available for exchange by PC-TP is not affected by pretreatment of the OMV with proteinase K or sulfhydryl reagents. In the reverse experiment where radiolabeled PC was introduced into the OMV, similar characteristics for the exchange were found. The accessibility of labeled PC to externally added phospholipase A_2 was used as a measure for its transmembrane distribution. It was found that PC is not exclusively located in the outer leaflet of the OMV. Only 30-35% can be degraded in intact OMV by phospholipase A_2, irrespective of whether the PC is introduced by PC-TP or endogenously synthesized *via* either of the pathways of biosynthesis. The results demonstrate the occurrence of rapid bidirectional transbilayer movement of both endogenous and *in vitro* introduced PC in OMV. Furthermore, there appears to be no preference for mitochondrial import of PC synthesized by either of the pathways *in vivo*.

Introduction

Phosphatidylcholine (PC) is the major phospholipid found in membranes of eukaryotic cells. As in higher eukaryotes, PC is synthesized *via* two distinct pathways in yeast, either *via* the triple methylation of PE or *via* the CDP-choline (Kennedy) pathway (for a recent review, see [Henry and Patton-Vogt, 1998]). In yeast, the methylation of PE is considered to be the primary pathway of biosynthesis of PC when cells are grown in the absence of choline, whereas the CDP-choline pathway is an auxiliary route since it requires exogenous choline for net PC synthesis [McDonough *et al.*, 1995].

PC is also a major constituent phospholipid of both mitochondrial membranes [Zinser *et al.*, 1991]. Several mutant strains with defects in the routes of biosynthesis of PC have the tendency to generate respiratory-deficient petites at high frequency [Griac, Swede and Henry, 1996], suggesting that PC is required for proper mitochondrial function or biogenesis and maintenance. Mitochondria do not contain enzymatic activities capable of PC biosynthesis, the contribution of mitochondria to cellular phospholipid biosynthesis is restricted to the formation of PE and cardiolipin [Daum and Vance, 1997]. The final steps of both routes of PC biosynthesis take place in the endoplasmic reticulum. For the biogenesis of mitochondria, PC must be imported efficiently from its site of synthesis. The mechanisms and regulatory

aspects involved in the mitochondrial import and sorting of PC over both mitochondrial membranes, as well as the relative contributions of the two biosynthetic pathways to mitochondrial PC, are unknown. Several mechanisms for the intracellular transport of phospholipids have been proposed [Trotter and Voelker, 1994]. A combination of intermembrane and transmembrane transport steps is expected to be required for import of PC into mitochondria and the intramitochondrial transport of PC. Previous studies on PC import into yeast mitochondria showed that PC was able to reach the inner membrane when the outer leaflet of the outer membrane was loaded with PC *in vitro*. This intramitochondrial transfer of PC required neither an electrochemical gradient across the mitochondrial inner membrane nor ATP [Lampl *et al.*, 1994]. Furthermore, *in vivo* pulse-labeling studies showed that PC transfer between endoplasmic reticulum and mitochondria continued in the absence of metabolic energy, although at a lower rate as compared to energy-supplemented cells [Daum, Heidorn and Paltauf, 1986].

The mitochondrial outer membrane, which is the site of interaction with the cytosol and other organelles, appears to be the first barrier to be taken by newly synthesized phospholipids on their way into the mitochondrion. In this study the transmembrane movement of PC across the mitochondrial outer membrane was investigated in an *in vitro* approach using isolated mitochondrial outer membrane vesicles (OMV) from the yeast *Saccharomyces cerevisiae*. A phosphatidylcholine specific transfer protein (PC-TP) was used as a tool to extract radiolabeled PC from the OMV or to introduce radiolabeled PC into the OMV, which allowed the determination of the pool size of PC in the OMV which is available for exchange. The accessibility to externally added phospholipase A_2 was assessed to compare the transmembrane distribution of newly introduced and endogenous PC. The results obtained demonstrate the occurrence of rapid transmembrane movement of both endogenous and *in vitro* introduced phosphatidylcholine in isolated mitochondrial outer membrane vesicles with a $t_{1/2}$ of one min or less at 30˚C. The newly introduced radiolabeled PC adopts a transmembrane distribution similar to that of endogenous PC synthesized *via* either of the two biosynthetic pathways. *In vivo* labeling demonstrated no apparent preference of the mitochondria for PC synthesized by one of the routes of biosynthesis.

Materials and methods

Materials

The radiochemicals [1α,2α(n)-^{3}H]cholesteryl oleoyl ether ([^{3}H]CE, 48 Ci/mmol), cholesteryl [1-^{14}C]oleoyl ester ([^{14}C]CE, 56 mCi/mmol), L-[methyl-^{3}H]methionine ([^{3}H]methionine, 79 Ci/mmol), and L-[3-^{3}H]-serine were obtained from Amersham (Amersham, United Kingdom), 1,2-di-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine ([^{14}C]DOPC, 114 Ci/mol) and [methyl-^{14}C]choline chloride ([^{14}C]choline, 54 Ci/mol) were

purchased from DuPont NEN (Brussels, Belgium). DOPC and dioleoylphosphatidic acid (DOPA) were obtained from Avanti Polar Lipids (Birmingham, AL). PC-TP was purified from bovine liver and stored at -20˚C at a concentration of ~0.5 mg/ml in 20 mM Tris-HCl, pH 7.2, 100 mM NaCl, containing 50% (w/v) glycerol [Westerman, Kamp and Wirtz, 1983]. Bee venom phospholipase A_2 (bvPLA_2), trypsin, proteinase K, FITC-dextran (M_r 20,000), octylglucoside, choline oxidase from *Alcaligenes* species, and horseradish peroxidase type II were obtained from Sigma. Enhanced chemiluminescence (ECL) reagents were purchased from DuPont NEN. Zymolyase was obtained from Seikagaku (Japan). All other chemicals were analytical grade.

Isolation of outer membrane vesicles and other subcellular fractions

The wild-type yeast strain *Saccharomyces cerevisiae* D273-10B was grown aerobically to late log (OD600 4-5 (Perkin Elmer Lambda 18 UV/VIS spectrophotometer)) at 30˚C in semi-synthetic lactate medium [Daum, Böhni and Schatz, 1982]. Spheroplasts were prepared using zymolyase as described previously [Daum, Böhni and Schatz, 1982]. The isolation of mitochondria at pH 6.0 and further purification by sucrose gradient centrifugation were based on published procedures [Daum, Böhni and Schatz, 1982; Glick and Pon, 1995; Gaigg *et al.*, 1995]. Microsomes were isolated as the 32,500 x *g* pellet of a 20,200 x *g* post-mitochondrial supernatant. The isolation of mitochondria-associated membranes (MAM) was adapted from a published procedure [Gaigg *et al.*, 1995]. Mitochondrial outer membranes were isolated and purified based on Mayer *et al.* [Mayer, Lill and Neupert, 1993; Mayer *et al.*, 1995]. Full details of all fractionation procedures are presented elsewhere [De Kroon *et al.*, 1999]. The final outer membrane pellet was resuspended in HS buffer (2 mM Hepes, pH 7.4, and 200 mM sucrose). This buffer is used in all experiments unless indicated otherwise. For the preparation of OMV containing radiolabeled phosphatidylcholine synthesized either by the CDP-choline pathway or *via* methylation of PE, mitochondria were isolated from cells grown in the presence of [^{14}C]choline (125 µCi/l) or [^{3}H]methionine (1500 µCi/l), respectively. The radiolabeled mitochondria were mixed with an approximately 24-fold quantity of unlabeled mitochondria for the isolation of the outer membranes. Approximately 98% of the radiolabel in OMV was present in PC when [^{14}C]choline was used and approximately 90% of the lipid-associated radiolabel in OMV was present in PC when [^{3}H]methionine was used.

Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUV) were prepared by sonication of a lipid suspension obtained by hydrating a dry lipid film with HS buffer. The lipid film consisted of DOPC and DOPA at a 95:5 molar ratio and the appropriate amounts of radiochemicals. SUV used as donor vesicles for the introduction of [^{14}C]DOPC into OMV contained 0.5 µCi of

[^{14}C]DOPC and 5 μCi of [^{3}H]CE per μmol of phospholipid, whereas SUV used as acceptor vesicles in the extraction of endogenously synthesized [^{14}C]PC or [^{3}H]PC only contained 0.5 μCi of [^{3}H]CE or 50 nCi of [^{14}C]CE, respectively, per μmol of phospholipid. The phospholipid suspension was subjected to 10 cycles of 30 s ultrasonication at 80 W and 0˚C with 30 s intervals using a Branson B12 sonifier equipped with a microtip. The sonicated phospholipid suspension was centrifuged for 30 min at 390,000 x *g* and 4˚C in a Beckman TL-100 ultracentrifuge. The supernatant containing the SUV was stored at 4˚C and used within two days after preparation.

Exchange of PC by PC-TP

OMV (at a protein concentration of 0.25 mg/ml) were incubated with SUV at the indicated ratios and PC-TP (23 μg/ml) at 30˚C. The ratio of donor and acceptor membranes is always expressed as the molar ratio of PC in SUV over PC in OMV. After the indicated times the OMV and the SUV were separated by centrifuging for 20 min at 230,000 x *g* in a Beckman TL-100 ultracentrifuge at 4˚C. [^{14}C] and [^{3}H] were counted in samples from the incubation mixture and from the supernatant using a Packard 1500 Tricarb Liquid Scintillation analyzer. In the case where OMV containing [^{3}H]PC (labeled *via* the methylation pathway) were used, dried lipid extracts of the samples were counted, because the radiolabel was not exclusively lipid-associated. The extent of the PC exchange was calculated from the enrichment or depletion of the radiolabeled PC in the supernatant containing the SUV. When endogenously radiolabeled OMV were used, a correction was made for the amount of radiolabel associated with lipids other than PC. Furthermore, a correction was made for the contamination of the OMV with co-pelleted SUV using the [^{3}H] or [^{14}C] labeled non-exchangeable marker CE. This contamination typically amounted to 10% of the total amount of SUV added. In the calculation it was assumed that the co-pelleted SUV have specific radioactivities identical to those in the supernatant. In addition to the above-mentioned considerations, the calculation of the pool size of exchangeable PC in the OMV was based on the notion that 65% of the PC in the donor SUV is located in the outer leaflet and thus accessible to PC-TP [Van den Besselaar *et al.*, 1978; Johnson, Hughes and Zilversmit, 1975; Nicolay *et al.*, 1990]. The OMV pellets obtained after introduction of [^{14}C]DOPC by PC-TP for 20 min at 30˚C were resuspended in HS buffer and kept on ice until treatment with phospholipase A_2.

Pretreatment of OMV

Where indicated, OMV (at a concentration of 0.19 mg/ml) were incubated with proteinase K (200 μg/ml) for 10 min at 37˚C, or with *para*-chloromercuribenzene sulfonic acid (PCMBS, 2 mM), or with *N*-ethylmaleimide (NEM, 5 mM) for 10 min at room temperature after a 10 min preincubation at room temperature with dithiothreitol (DTT, 1

mM). After the various incubations, the suspensions were put on ice, diluted two-fold with buffer (to the proteinase K treated sample 1 mM phenylmethylsulfonyl fluoride was also added) and the OMV were reisolated by centrifugation for 20 min at 230,000 x *g* in a Beckman TL-100 ultracentrifuge at 4˚C. The pellets were resuspended in HS buffer and subjected to exchange of PC by PC-TP as above.

Treatment with phospholipase A_2

OMV (at a protein concentration of 0.6 mg/ml) were incubated for 10 min at room temperature with bvPLA$_2$ at the indicated concentrations in the presence of 0.1 mM Ca^{2+}. The specific activity of the phospholipase ($4.2 \cdot 10^3$ U/mg) was determined using egg yolk lipoproteins as substrate [Nieuwenhuizen, Kunze and De Haas, 1974]. Octylglucoside (OG, 40 mM) was used in control experiments to solubilize OMV for maximal degradation of phospholipids by bvPLA$_2$. Phospholipase activity was inhibited by the addition of EGTA to a final concentration of 1 mM and samples were analyzed as described in the following.

Analysis of PC degradation

Phospholipid extracts from samples corresponding to 40-45 µg of protein were analyzed by HP-TLC on silica gel 60, using chloroform/methanol/25% ammonia/water (90:54:5.5:5.5, v/v/v/v) as eluent. The radioactive spots on the TLC plate were quantified by a Berthold Automatic TLC linear analyzer (Wildbad, FRG). The degradation of PC by bvPLA$_2$ was calculated from the amounts of label present in lysoPC and PC. The recovery of radiolabel was not affected by the extent of hydrolysis of PC. No hydrolysis of the newly introduced [^{14}C]DOPC was detected in control experiments without PLA$_2$. The hydrolysis of PC in OMV containing endogenously synthesized radiolabeled PC by bvPLA$_2$ was calculated after correction for the minor amounts of lysoPC present in these vesicles in untreated controls (<4%, expressed as percentage of the sum of radiolabeled lysoPC and PC).

Assessment of the intactness of the OMV

The intactness of the OMV after the phospholipase treatment was assessed by probing the accessibility of Tom40p to trypsin. 2.5 µg aliquots of the OMV suspension were incubated at a protein concentration of 50 µg/ml with trypsin (100 µg/ml) for 20 min on ice. Digitonin (0.2% (w/v)) was added to solubilize the OMV in control experiments. Samples were precipitated with trichloroacetic acid and subjected to SDS-PAGE (10% gel) and Western blotting. To check the intactness during PLA$_2$ treatment, FITC-dextran (M_r 20,000) was introduced in the lumen of the OMV by a freeze-thaw technique essentially as described for isolated mitochondrial outer membrane vesicles from *Neurospora crassa* [Mayer *et al.*, 1995] with the following changes. The inclusion buffer contained 10 mM MOPS, pH 6.5, 0.5

mM EDTA, 0.5 mM EGTA and 0.5% (w/v) BSA. Snap-freezing was performed in the presence of 0.5 mM FITC-dextran at an OMV concentration of 2 mg/ml. The sucrose step gradient to remove non-enclosed FITC-dextran consisted of the sample, mixed with 50% sucrose and 150 mM KCl, overlaid with 45% sucrose and 150 mM KCl, 45% sucrose and 8% sucrose, respectively, all in 10 mM MOPS, pH 7.2, 2.5 mM EDTA (EM buffer). After centrifugation for 30 min at 150,000 x *g* in a Beckman SW60 rotor, the OMV were harvested from the 8-45% sucrose interface, diluted with EM buffer and pelleted by centrifugation. The pellets, containing the OMV with FITC-dextran enclosed in the lumen, were resuspended in HS buffer and used directly for exchange of PC by PC-TP followed by treatment with bvPLA2 as described. After the inactivation of the phospholipase, samples corresponding to 2.5 µg OMV were diluted with buffer and the OMV were pelleted by centrifugation. To determine the leakage of the enclosed fluorophore from OMV during phospholipase treatment, the fluorescence in pellet and supernatant was measured in the presence of 0.1% (v/v) Triton X-100. Fluorescence was measured on an Aminco SLM500C spectrofluorometer using an excitation wavelength of 465 nm and an emission wavelength of 543 nm (bandpass 5 nm).

Other methods

Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin (BSA) as a standard. Phospholipids were extracted according to the method of Bligh and Dyer [Bligh and Dyer, 1959]. The phosphorus content of the organic phase obtained after extraction was determined to yield the phospholipid phosphorus/protein ratio. Phosphate was determined by the method of Fiske and Subbarow [Fiske and Subbarow, 1925]. To determine the PC content of the OMV, phospholipid analysis of lipid extracts from 200 µg OMV was performed by TLC as described [De Kroon *et al.*, 1997]. The choline content of the yeast extract used to prepare the culture medium was measured using an enzymatic assay based on the specific oxidation of choline by choline oxidase [Hise and Mansbach II, 1983]. Samples were vortexed with finely ground charcoal to remove interfering substances and an internal standard was used to correct for any remaining interference. In Western blotting experiments, protein bands of interest were visualized by ECL. When necessary, quantification of protein bands was performed using laser scanning densitometry on an Ultroscan XL (Pharmacia-LKB, Bronna, Sweden). PS synthase activity (expressed as nmol serine metabolized per min per mg protein) in several subcellular fractions was determined in the presence of Triton X-100 as described [Bae-Lee and Carman, 1984], at a concentration of 0.1 mM L-[3-^{3}H]-serine.

Results

Characterization of the OMV

In this study, the transmembrane movement of PC in the mitochondrial outer membrane from yeast was investigated in isolated outer membrane vesicles (OMV). The purity of this membrane preparation was assessed by immunoblotting. Figure 1 shows that compared to the purified mitochondria, the OMV are almost completely depleted of the inner membrane marker P_iC (phosphate carrier) and highly enriched in the outer membrane marker Tom20p. Furthermore, the purified mitochondria are depleted of the endoplasmic reticulum marker Sec61p compared to the homogenate and the crude mitochondria (Figure 1).

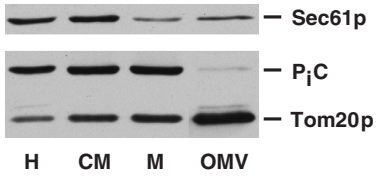

Figure 1. The purity of OMV assessed by immunoblotting. Samples corresponding to 10 μg protein of the homogenate (H), the crude mitochondria (CM), purified mitochondria (M) and OMV were subjected to SDS-PAGE followed by Western blot analysis using antibodies raised against marker proteins for the endoplasmic reticulum (Sec61p) and the mitochondrial inner and outer membranes (P_iC and Tom20p, respectively). The protein bands were visualized by ECL.

It was calculated from quantitative blotting experiments (not shown) that the contamination of the OMV with endoplasmic reticulum is at most 5% based on protein. This corresponds to an even lower contamination on a phospholipid basis, as can be inferred from comparison of the phospholipid/protein ratios of OMV and microsomes [Zinser *et al.*, 1991; Kuchler, Daum and Paltauf, 1986]. Therefore, the contribution of microsomal PC to the total PC pool in OMV can be neglected. The OMV have a phospholipid phosphorus/protein ratio of $(14.2 \pm 2.0)\cdot 10^2$ nmol/mg (n = 7) and a PC content of 35.8 ± 2.5% (n = 4) of total phospholipid. A specific subfraction of the endoplasmic reticulum which remains associated with the mitochondria upon isolation, the so-called MAM, is highly enriched in PS synthase [Gaigg *et al.*, 1995]. Therefore, the activity of this phospholipid biosynthesis enzyme was measured in the OMV and other subcellular fractions. The analysis of PS synthase activity showed that contamination of OMV with the MAM is very low (Table 1) and confirms the minor contamination of OMV with endoplasmic reticulum. The characteristics of the OMV from wild-type yeast strain D273-10B reported here are in agreement with those obtained for strain D273-10B/A1 [De Kroon *et al.*, 1999].

Table 1

The activity of PS synthase in subcellular fractions

fraction	relative specific activity
homogenate	1.0
crude mitochondria	2.6 ± 0.4
purified mitochondria	0.5 ± 0.2
outer membrane vesicles	1.0 ± 0.3
microsomes	16 ± 4
mitochondria associated membranes	35 ± 5

The activity of PS synthase in several subcellular fractions was measured and related to the specific activity of the homogenate.

Extraction of endogenously radiolabeled PC from OMV by PC-TP

Exchange experiments using PC-TP provide information on the occurrence of transmembrane movement of PC. PC-TP catalyzes a one-to-one exchange of PC between membranes, which results in the replacement of the endogenous PC in the acceptor membranes with PC from the donor membranes without changing the PC content of the participating membranes [Helmkamp, 1980]. Therefore, the exchange of PC by PC-TP between OMV and small unilamellar vesicles (SUV) was investigated.

For this purpose, OMV containing endogenous radiolabeled PC synthesized *via* the CDP-choline pathway were isolated from cells grown on medium containing [^{14}C]choline. Because there is no *de novo* biosynthesis of choline in yeast (choline can only be obtained by uptake from the extracellular medium or by turnover of PC [McDonough *et al.*, 1995]), this also allowed for the assessment of the contribution of choline in the medium to the production of PC *via* the CDP-choline pathway. If PC were synthesized exclusively *via* the CDP-choline pathway, the specific radioactivity of cellular PC would be equal to the specific radioactivity of the choline provided in the medium. Therefore, the choline content of the medium was determined, as well as the radioactivity incorporated into the phospholipid. In Table 2 the specific radioactivities of the medium and the whole cell homogenate, mitochondria, and microsomes are compared. The relative contribution of the choline in the medium to cellular PC was found to be approximately 20%. The incorporation of choline in the medium in the PC of whole cells, mitochondria and microsomes is similar.

Table 2

The relative contribution of the CDP-choline pathway

	specific radioactivity of choline [a] or PC [b] (10^3 dpm/nmol)	% PC originating from choline in growth medium
growth medium	39 [a]	-
homogenate	7.4 [b]	19
mitochondria	7.2 [b]	19
microsomes	6.1 [b]	16

The specific radioactivity of PC in several subcellular fractions was measured and related to the specific radioactivity of choline in the growth medium.

To study the kinetics and extent of the PC-TP mediated exchange process, OMV containing endogenous [^{14}C]PC synthesized *via* the CDP-choline pathway were incubated with an excess of SUV, labeled with the non-exchangeable marker [^{3}H]CE (PC^{SUV}/PC^{OMV} molar ratio of 10). The percentage of [^{14}C]PC transferred from the OMV to the SUV after different times of exchange with PC-TP is shown in Figure 2. After approximately 15 min the equilibration of radiolabeled PC over the participating membranes is completed. The halftime of the exchange process is in the order of 1 min. After 20 min of exchange 88 ± 3% (n = 3) of the [^{14}C] labeled PC is transferred to the SUV.

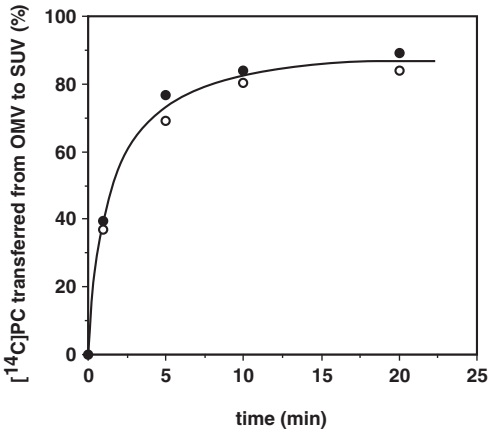

Figure 2. Time course of the extraction of endogenous PC from OMV by PC-TP. The percentage of [^{14}C] label transferred from the OMV to the SUV, corrected for the contamination of the OMV pellet with SUV, was determined in two independent experiments (●,○). OMV containing endogenous [^{14}C]PC synthesized *via* the CDP-choline pathway were incubated at a concentration of 0.25 mg/ml at 30˚C with PC-TP (23 µg/ml) and a tenfold excess of unlabeled SUV (PC^{SUV}/PC^{OMV} molar ratio of 10). At the indicated time points OMV and SUV were separated by centrifugation for analysis as detailed in the 'Materials and methods' section.

From this it was calculated as described under 'Materials and methods' that 99 ± 4% (n = 3) of the PC in the OMV is available for exchange by PC-TP under the conditions used. The exchangeable pool of PC was also determined for OMV isolated from cells grown on medium containing [^{3}H]methionine, in which case the labeling of PC occurs primarily *via* the methylation of PE. As above, the OMV were incubated with an excess of SUV, now labeled with the non-exchangeable marker [^{14}C]CE. Again it was found that all of the PC in the OMV is available for exchange by PC-TP. After 20 min of exchange 89 ± 4% (n = 4) of the [^{3}H]labeled PC is transferred to the SUV, which corresponds to an exchanged pool of 101 ± 5% (n = 4).

Effect of pretreatment of OMV with proteinase K or sulfhydryl reagents on the extent of PC exchange

The effect of proteinase K or sulfhydryl reagents on the size of the exchangeable pool was investigated. This was done by determining the extent of PC exchange in endogenously labeled OMV *via* the CDP-choline pathway as above, after a pretreatment with proteinase K or the sulfhydryl reagents NEM and PCMBS. The size of the PC pool in OMV available for exchange by PC-TP after 20 min was not significantly affected by any of these pretreatments (94.4 ± 1.4% (proteinase K), 96.3 ± 0.6% (NEM) and 96.8 ± 0.9% (PCMBS), n = 3 for all).

Introduction of synthetic radiolabeled PC into OMV by PC-TP and assessment of its accessibility to PLA_2

To determine whether *in vitro* introduced PC equilibrates over both leaflets of the OMV membrane, its transmembrane distribution was investigated. For this purpose, [^{14}C] labeled DOPC was introduced into unlabeled OMV by the action of PC-TP. To minimize the contamination of OMV with SUV, incubations were performed with approximately equal amounts of PC present in the populations of donor and acceptor vesicles (donor/acceptor ratio approximately 1). The depletion of [^{14}C] label from the SUV fraction is used to calculate the size of the exchanged pool in this experimental set-up. This results in lower accuracy of measurement, since the maximally possible relative depletion of label from the donor membranes strongly depends on the donor/acceptor ratio. It was calculated that at these low donor/acceptor ratios up to 80% (n = 2) of the PC present in the OMV had participated in the exchange after 20 min. The kinetics of the exchange were similar to those depicted in Figure 2. The difference in calculated pool size (80 versus 100%) for synthetic and endogenous PC most likely is due to the experimental differences and concomitant technical limitations in the quantification of the exchanged pool size, and not to different behavior of the probes.

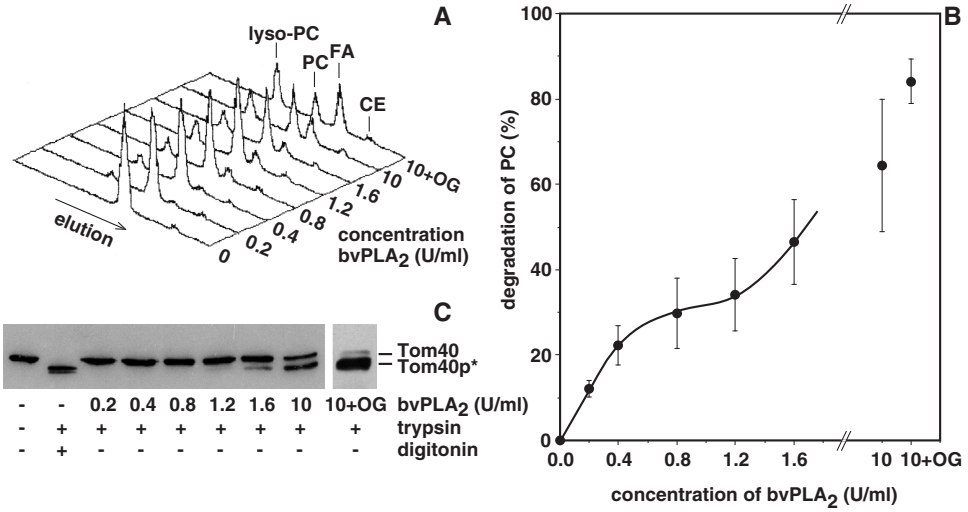

Figure 3. The accessibility of newly introduced [^{14}C]DOPC in OMV to bvPLA$_2$ and the intactness of bvPLA$_2$ treated OMV. [^{14}C]DOPC was introduced into OMV by PC-TP from radiolabeled SUV for 20 min at 30˚C. OMV were reisolated by centrifugation and incubated at a concentration of 0.6 mg/ml for 10 min at room temperature with bvPLA$_2$ at the indicated concentrations in the presence of 0.1 mM Ca^{2+}. Octylglucoside (OG, 40 mM) was added in control experiments to achieve maximal degradation of phospholipids by bvPLA$_2$. Phospholipase activity was inhibited by the addition of EGTA (1 mM). The OMV lipids were extracted and analyzed by HP-TLC. The integrity of the membranes after phospholipase treatment was checked by assessing the accessibility to trypsin of Tom40p. A: radioactivity scan of a TLC plate showing the distribution of the [^{14}C] label over PC, lysoPC, fatty acid (FA) after incubation with different concentrations of phospholipase. The [^{3}H]CE peak originates from the SUV contamination in the OMV pellet; B: quantification of the concentration dependent lipolysis of newly introduced [^{14}C]DOPC by bvPLA$_2$. The data have not been corrected for the [^{14}C] label present in the contaminating SUV, which was estimated to be approximately 10% of the total [^{14}C] label. The error bars represent the standard deviation (n = 4); C: Assessment of the integrity of OMV after phospholipase treatment by analysis of the accessibility of Tom40p to trypsin. A typical Western blot is shown. The first two lanes show the untreated OMV and a control where Tom40p is completely accessible to trypsin after solubilization of the OMV with digitonin (0.2% (w/v)).

To determine whether the newly introduced [^{14}C]DOPC molecules equilibrate over both leaflets, the OMV which were reisolated after an incubation with PC-TP and [^{14}C]DOPC labeled SUV were treated with phospholipase A$_2$ from bee venom (bvPLA$_2$) for 10 min. Figure 3A shows the HP-TLC analysis of OMV phospholipid extracts from a typical experiment. In Figure 3B the degradation of newly introduced [^{14}C]DOPC by increasing concentrations of bvPLA$_2$ is quantitated. Even though the variation between experiments was considerable as can be seen from the error bars representing the standard deviation from four independent experiments, the same type of curve shape was obtained in each case. With increasing concentrations of phospholipase increasing amounts of [^{14}C]DOPC are degraded

and a plateau value of 30 to 35% degradation is reached at 0.8 U PLA_2/ml which is followed by a sudden further increase at 1.6 U/ml. In the presence of an excess of phospholipase and the detergent octylglucoside, 85 ± 5% (n = 4) of the radiolabeled PC is degraded.

The intactness of the OMV after bvPLA_2 treatment was assessed by probing the accessibility of Tom40p to trypsin. Tom40p is not degraded by trypsin when the mitochondrial outer membrane is intact [Hines *et al.*, 1990]. The first two lanes in Figure 3C show the intact Tom40p in untreated OMV and the appearance of the degradation product with a higher electrophoretic mobility upon treating the membrane with trypsin after solubilization with digitonin. Tom40p stays intact when OMV are treated with trypsin after they have been incubated with the phospholipase up to a concentration of 1.2 U/ml, confirming the sealed nature and right-side out orientation of the OMV [De Kroon *et al.*, 1999]. Only after treatment with a bvPLA_2 concentration of 1.6 U/ml and higher, the cleavage site for trypsin becomes exposed and a degradation product of Tom40p appears upon treatment with trypsin. In the presence of an excess of phospholipase and the detergent octylglucoside, Tom40p is virtually completely accessible to trypsin (Figure 3C). To check whether the inaccessibility of Tom40p after phospholipase treatment reflects the closed nature of the membrane during the activity of the phospholipase, *i.e.*, to rule out the possibility that during phospholipase treatment the membrane structure reorganizes itself allowing temporary access of the phospholipase to the lumen of the OMV after which closed structures are formed again, control experiments were performed in which FITC-dextran (M_r 20,000) was enclosed in the OMV lumen. These confirmed that leakage of the fluorophore from the lumen of the OMV only occurred under conditions where Tom40p could be degraded by trypsin (not shown). The sudden increase in the degradation of labeled PC accompanied by the loss of barrier function of the OMV membrane as shown by the accessibility of Tom40p to trypsin at 1.6 U/ml strongly suggest that at this concentration bvPLA_2 gains access to a PC pool located in the inner leaflet of the membrane, which remains protected against degradation while the membrane is still intact. These results imply that a portion of the newly introduced [^{14}C]DOPC has moved to the inner leaflet.

In order to compare the transmembrane distribution of newly introduced PC to the transmembrane distribution of endogenous PC, OMV containing [^{14}C]PC or [^{3}H]PC synthesized *via* the CDP-choline pathway or *via* the methylation of PE, respectively, were also treated with increasing concentrations of bvPLA_2. Figure 4 shows that the accessibility to phospholipase of newly introduced and endogenous PC synthesized *in vivo via* either of the two biosynthetic pathways is very similar. From this it can be concluded that newly introduced PC adopts a transbilayer orientation similar to that of the endogenous PC, and that PC is not exclusively present in the outer leaflet of the OMV. These results and the fact that the PC in OMV is completely available for exchange by PC-TP demonstrate the occurrence of rapid bidirectional transbilayer movement of PC.

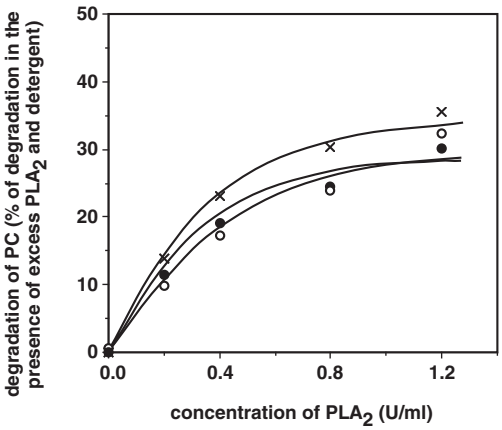

Figure 4. Comparison of the degradation of newly introduced and endogenous PC by bvPLA$_2$. The degradation of radioactive PC, introduced by PC-TP (x) or synthesized *in vivo via* the CDP-choline pathway (●) or *via* methylation of PE (○), upon treating OMV with bvPLA$_2$ at the indicated concentrations is shown. The data is presented as percentage of the maximal degradation of the radiolabeled PC in the presence of excess PLA$_2$ (10 U/ml) and the detergent octylglucoside. [^{14}C]DOPC was introduced into OMV by PC-TP from radiolabeled SUV for 20 min at 30˚C and the OMV were reisolated by centrifugation. OMV containing radiolabeled endogenously synthesized PC *via* either of the two biosynthesis pathways were prepared as described under 'Materials and methods'. OMV were incubated at a concentration of 0.6 mg/ml for 10 min at room temperature with bvPLA$_2$ at the indicated concentrations in the presence of 0.1 mM Ca^{2+}. Phospholipase activity was inhibited by the addition of EGTA (1 mM). The OMV lipids were extracted and analyzed by HP-TLC. The degradation of PC in OMV containing endogenously synthesized radiolabeled PC by bvPLA$_2$ was calculated after correction for the minor amounts of lysoPC present in these vesicles in untreated controls.

Discussion

The present findings demonstrate the occurrence of bidirectional transmembrane movement of both endogenous and *in vitro* introduced PC in isolated mitochondrial outer membrane vesicles from *Saccharomyces cerevisiae*. This process is fast, with a $t_{1/2}$ of one minute or less at 30˚C, and causes newly introduced radiolabeled PC to adopt a transmembrane distribution similar to that of endogenous PC irrespective of its route of biosynthesis, *i.e.*, *via* methylation of PE or *via* the CDP-choline pathway. The extent of the transmembrane movement is not influenced by pretreatment with protein-modifying agents.

In addition, the relative importance of the CDP-choline pathway to net PC biosynthesis was assessed by *in vivo* labeling. It was found that under the culture conditions used approximately 20% of total cellular PC originates from free choline present in the growth medium. However, as pointed out previously by other authors [Trotter and Voelker, 1994], this is probably an underestimate of the total contribution of the CDP-choline pathway

to the production of PC since the choline originating from turnover of PC produced *via* methylation of PE can also be used for production of PC *via* the CDP-choline pathway. Comparison of the amount of choline incorporated into PC and the amount of choline originally present in the growth medium (not shown), suggests that the amount of choline in the medium is limiting its use for PC synthesis under the conditions used. Importantly, no significant differences in the contribution of choline from the medium for production of PC for the whole cell and the mitochondria were found. This result indicates no preference of the mitochondria for importing PC synthesized by either one of the pathways of biosynthesis.

The conclusions regarding the transmembrane movement of PC in the mitochondrial outer membrane are based on several observations. Upon extraction of radiolabeled PC from isolated OMV by PC-TP, 100% of the endogenous PC is available for exchange, with a halftime in the order of 1 min. In the reverse experiment where [^{14}C]DOPC was introduced into OMV, similar characteristics of the exchange process were found, demonstrating that the behavior of the synthetic phospholipid resembles that of the endogenous phosphatidyl-choline.

The complete availability of PC in OMV for exchange by PC-TP could in principle be explained by an exclusive localization of PC in the outer leaflet of the membrane. However, such a complete asymmetry in the mitochondrial outer membrane was considered highly unlikely since it was never found for any other biological phospholipid bilayer and moreover, data from literature argue against it [Sperka-Gottlieb *et al.*, 1988]. In the present study it was demonstrated that 30-35% of the [^{14}C]DOPC introduced in the outer membrane can be degraded by bvPLA$_2$ while the OMV are still intact. The sudden further increase in degradation of labeled PC accompanied by the loss of barrier function strongly suggests that the phospholipase then gains access to a PC pool located in the inner leaflet of the membrane which remains protected against degradation while the membrane is still intact. This is consistent with movement of part of the newly introduced PC to the inner leaflet. The maximal accessibility of PC to bvPLA$_2$ found in intact OMV should only be regarded as a relative measure of the transmembrane distribution and not as an absolute value. Most likely, the intactness of the OMV is affected before all of the phospholipids in the outer leaflet are completely degraded by the phospholipase, due to destabilization of the bilayer structure by the presence of large amounts of lysophospholipids and fatty acids. Nevertheless, the similar accessibility of newly introduced and endogenous PC to bvPLA$_2$ demonstrates that PC introduced by PC-TP assumes a transbilayer orientation similar to that of endogenous PC independent of its route of synthesis. This result in combination with the complete availability of the endogenous PC pool for exchange by PC-TP shows the bidirectional nature of the transmembrane movement and the similar behavior of the synthetic phospholipid and the endogenous PC. The transmembrane movement appears to be at least as fast as the PC-TP

mediated exchange process with a halftime in the order of 1 min, since the data do not allow distinction between the rates of the actual exchange and the PC transmembrane movement.

The fact that the entire pool of PC in OMV is available for exchange by PC-TP whereas only a limited part of the PC is accessible to degradation by PLA_2 in intact OMV may seem paradoxical. The possibility that the presence of Ca^{2+} during phospholipase treatment affects the transmembrane movement was ruled out, since the extent of the exchange as judged from the size of the exchangeable pool after 10 or 20 min was not decreased in the presence of 0.1 mM Ca^{2+} (data not shown). Several other explanations are possible. One is that PC-TP has direct access to the inner leaflet of the OMV but this possibility is considered highly unlikely in view of the molecular size of PC-TP as argued previously [Dolis, De Kroon and De Kruijff, 1996]. Alternatively, PC-TP itself or in combination with OMV membrane components could somehow directly induce the rapid bidirectional transmembrane movement, allowing redistribution of newly introduced and endogenous PC over both leaflets of the OMV. This effect would be rather specific for OMV since there are no indications that PC-TP has this ability in other biomembranes [Rothman *et al.*, 1976; Van Meer *et al.*, 1980] nor does it induce transmembrane movement in protein-free phospholipid membranes [Van den Besselaar *et al.*, 1978; Johnson, Hughes and Zilversmit, 1975]. Another explanation would be that the actual insertion of the phospholipid in the mitochondrial outer membrane rather than the interaction of PC-TP with the membrane somehow triggers flipping of the phospholipid. Alternatively, the redistribution of PC over both leaflets of the OMV could be occurring continuously, regardless of whether PC molecules are inserted or extracted, by a non-stop process which is no longer occurring once the membrane structure is disturbed by hydrolysis of phospholipids or which does not transport the lysoPC molecules produced by the phospholipase.

The results obtained here on the transbilayer orientation of PC are in agreement with the conclusion drawn in a study by Sperka-Gottlieb *et al.* on the lipid topology in OMV from yeast [Sperka-Gottlieb *et al.*, 1988], but at the same time conflict with the results regarding the size of the rapidly exchangeable pool of this phospholipid. They found that only approximately half of the PC in OMV is available for rapid exchange, using a non-PC-specific lipid transfer protein, *i.e.*, the phosphatidylinositol/phosphatidylcholine transfer protein (Sec14p). Extraction of the remaining PC proceeded at a slower rate which was interpreted as slow transbilayer movement of PC located in the inner leaflet ($t_{1/2}$ ~ 50 min). However, the transport protein used by Sperka-Gottlieb *et al.* has a significantly higher relative affinity for phosphatidylinositol than for PC (for a review see [Wirtz, 1991]). This property and the fact that a different donor/acceptor system was used could explain the differences between the two studies.

Mitochondrial contact sites have often been suggested to be zones of intramitochondrial lipid translocation. A study by Lampl *et al.* [Lampl *et al.*, 1994] showed

that, after introduction of radiolabeled PC by a non-PC-specific transfer protein into yeast mitochondria, the radiolabeled PC can be detected almost immediately in the outer membrane as well as in the contact site fraction and the inner membrane at similar specific radioactivities. The present study provides evidence that transport of PC across the outer membrane is not restricted to the contact sites. No conclusions can be drawn as to which step in the overall process of intramitochondrial PC translocation in yeast is rate limiting. Previous work from our laboratory with rat liver OMV and mitochondria strongly suggests that the transmembrane movement across the mitochondrial outer membrane is not rate limiting [Nicolay *et al.*, 1990; Dolis, De Kroon and De Kruijff, 1996]. Considering the short doubling time of the yeast *Saccharomyces cerevisiae*, *in vivo* translocation of phospholipids to and in the mitochondrion has to be very fast to ensure simultaneous development with the rest of the cell. A high rate of PC translocation between endoplasmic reticulum and mitochondria *in vivo* was demonstrated in pulse-chase studies [Daum, Heidorn and Paltauf, 1986]. The short halftime found for transmembrane movement of PC across the mitochondrial outer membrane from yeast *in vitro* is in good agreement with the *in vivo* requirements.

The rate of transmembrane movement of PC in OMV found in the present study would suggest the involvement of proteins in this process, since in protein-free model membranes only very slow transbilayer movement occurs (*e.g.* [Johnson, Hughes and Zilversmit, 1975]). As demonstrated by other authors, several phospholipid transport processes (both intermembrane and transmembrane) in eukaryotic cells require a source of energy and/or are sensitive to protein-modifying agents [Bishop and Bell, 1985; Martin and Pagano, 1987; Auland *et al.*, 1994; Shiao, Balcerzak and Vance, 1998], which is indicative of a protein-mediated process, and in some cases proteins capable of catalyzing such transport processes were also identified [Ruetz and Gros, 1994; Decottignies *et al.*, 1998; Zhou *et al.*, 1997]. However, the extent of the transmembrane movement of PC in the OMV was not influenced by pretreatment with proteinase K, NEM, or PCMBS, which provides no direct clues for the involvement of proteinaceous factors. It is considered unlikely that the process of transmembrane movement demonstrated in the present study has a strict energy requirement since all the experiments described were performed without addition of any possible sources of energy. This is in agreement with observations by other authors that energy depletion did not significantly inhibit the import of phosphatidylcholine into intact mitochondria *in vitro* [Lampl *et al.*, 1994]. Other membranes that were reported to display energy-independent rapid phospholipid transmembrane movement are the endoplasmic reticulum from rat liver [Van den Besselaar *et al.*, 1978], the inner membrane of *Escherichia coli* [Huijbregts, De Kroon and De Kruijff, 1998], and the membrane of *Bacillus megaterium* [Rothman and Kennedy, 1977]. The present study shows for the first time the occurrence of rapid transmembrane movement of a phospholipid in an intracellular membrane (*i.e.* the mitochondrial outer membrane) from the yeast *Saccharomyces cerevisiae*. Rapid PC flip-flop

of *in vitro* introduced synthetic PC was previously shown to occur in isolated mitochondrial outer membranes from rat liver [Dolis, De Kroon and De Kruijff, 1996]. This validates the use of yeast as a model eukaryote for studying the mechanisms of lipid transport across intracellular membranes. Its sequenced genome and potential for classical genetic studies and genetic engineering unequalled in the eukaryotic world render yeast the eukaryote of choice for the eventual elucidation of the putative proteinaceous factors governing these processes. This study provides the basis for future research in that direction.

Acknowledgements

We thank Dr. Roland Lill for the antisera against Tom40p, Tom20p, and P_iC, and Dr. Randy Schekman for the antibodies against Sec61p.

Chapter 3

The phosphatidylcholine to phosphatidylethanolamine ratio of *Saccharomyces cerevisiae* varies with the growth phase

M.J.F.W. Janssen, M.C. Koorengevel, B. de Kruijff, A.I.P.M. de Kroon

Yeast **16** (2000), 641-650.

Abstract

This study compares the effect of the growth phase on the phospholipid composition and the activity of several phospholipid biosynthetic enzymes in a wild-type yeast grown in fermentable (glucose) and non-fermentable (lactate) semi-synthetic and complete synthetic media. Several distinct differences as well as similarities were found. The cellular phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio was found to vary with the growth phase, with increases in PC levels at the expense of PE during the transition to stationary phase. The variation was most pronounced in semi-synthetic lactate medium, which is routinely used for the isolation of mitochondria, where the PC/PE ratio changed from 0.9 to 2.2 during this transition. Similar growth phase dependent changes in PC and PE content were observed in isolated organelles such as mitochondria, mitochondria-associated membranes, and microsomes. Phosphatidylinositol (PI) levels were much higher in cells grown on lactate compared to cells grown on glucose (20% *versus* 5-10%). Irrespective of the medium, PI levels increased upon entering stationary phase. The activities of the phospholipid biosynthetic enzymes phosphatidylserine synthase and the phospholipid-*N*-methyltransferases were found to be maximal at the end of logarithmic growth and to decrease upon entering stationary phase in all media. Cells grown on lactate displayed a significantly higher phospholipid to protein ratio than cells grown on glucose. The results are discussed in terms of regulation of phospholipid biosynthesis and membrane biogenesis in response to growth phase and carbon source.

Introduction

The yeast *Saccharomyces cerevisiae* shares similar patterns of membrane phospholipids and similar pathways of phospholipid metabolism with higher eukaryotes, with a few exceptions (for a review see [Carman and Henry, 1989]). Two branches of phospholipid biosynthesis diverge from the precursor CDP-diacylglycerol. Phosphatidyl-inositol (PI) is formed from CDP-diacylglycerol and inositol, and the main biosynthetic route leading to phosphatidylcholine (PC) in yeast starts with the conversion of CDP-diacylglycerol and serine into phosphatidylserine (PS). PS is decarboxylated to phosphatidyl-ethanolamine (PE) which is converted to PC by three sequential methylations. Alternatively, PE and PC can be synthesized from phosphatidic acid (PA) *via* diacylglycerol and CDP-ethanolamine or CDP-choline (Kennedy pathway), respectively. In yeast, the methylation of PE by the phospholipid-*N*-methyltransferase (PNMT) enzymes is considered the primary pathway of biosynthesis of PC when cells are grown in the absence of choline.

Many enzymes involved in the biosynthetic routes of phospholipids are coordinately regulated at the transcriptional level in response to phospholipid precursors and growth phase (for recent reviews, see [Carman and Zeimetz, 1996; Henry and Patton-Vogt, 1998; Carman

and Henry, 1999]. For example, the *INO1* gene product which catalyzes the formation of inositol, as well as the enzymes involved in the production of PC *via* CDP-diacylglycerol, are repressed by inositol and choline, and the latter enzymes exhibit maximal activities in exponential phase whereas the activities are reduced in stationary growth phase. In contrast, the expression of PI synthase does not respond to inositol or growth phase, but is regulated by the carbon source [Anderson and Lopes, 1996]. Furthermore, enzymatic activities can be regulated by the membrane lipid composition and phosphorylation (*e.g.* PS synthase [Hromy and Carman, 1986; Kinney and Carman, 1988]).

Most phospholipids are synthesized in the endoplasmic reticulum and closely related membranes. The contribution of mitochondria to cellular phospholipid biosynthesis is restricted to the formation of PE and cardiolipin (CL) [Daum and Vance, 1997]. This necessitates efficient import of the phospholipids which are not synthesized in mitochondria to ensure membrane growth and to maintain membrane lipid composition in the growing cell. For studies on mitochondrial biogenesis mitochondria are usually isolated from yeast grown on a non-fermentable carbon source such as lactate. In our research, which focuses on the import of PC into mitochondria, we noticed that the phospholipid composition, more in particular the PC/PE ratio, of these organelles was highly dependent on the moment of harvest in the late log phase. Knowledge on the variation in phospholipid composition and phospholipid biosynthetic enzymes under non-fermenting conditions is limited. It has been reported that regulation of phospholipid biosynthesis and composition depends on the exact growth conditions such as the carbon source [Anderson and Lopes, 1996; Gaynor *et al.*, 1991]. However, most studies on the regulation of phospholipid biosynthesis pertain to yeast cells grown on glucose-based media only. A previous study on cellular phospholipid biosynthesis and composition reported no significant growth phase dependent changes in the PC/PE ratio under the culture conditions used [Homann *et al.*, 1987]. However, in a more recent study the PC and PE content were observed to vary with the growth phase [Jiranek *et al.*, 1998]. This prompted us to carry out a systematic investigation on the influence of both the growth medium and the growth stage on the phospholipid composition of yeast cells and derived subcellular fractions and on the activities of several phospholipid biosynthetic enzymes in a wild-type yeast strain. It is documented for the first time that the PC/PE ratio in yeast membranes varies with the growth phase.

Materials and methods

Materials

S-adenosyl-L-methionine was purchased from Sigma (St. Louis, MO). The radiochemicals L-[3-^{3}H]-serine and *S*-adenosyl-L-[methyl-^{3}H]-methionine were obtained from Amersham (Amersham, United Kingdom). CDP-diacylglycerol was from Doosan

(Korea). Yeast extract and yeast nitrogen base without amino acids were obtained from Sigma and from Difco (Detroit, MI), respectively. Zymolyase was supplied by Seikagaku (Japan). All other chemicals were analytical grade.

Growth conditions

The wild-type yeast strain *Saccharomyces cerevisiae* D273-10B (*MATα*) was grown aerobically at 30˚C. Cells were precultured for 24 h in semi-synthetic lactate medium [Daum *et al.*, 1982]. 5 ml portions of the same preculture were used to inoculate 800 ml batches of the following media: semi-synthetic lactate medium (SSL), semi-synthetic glucose medium (SSG), complete synthetic lactate medium (CSL), and complete synthetic glucose medium (CSG). Semi-synthetic media contained per liter: 3 g yeast extract, 1 g $KH_2PO_4 \cdot 3\ H_2O$, 1 g NH_4Cl, 0.5 g $CaCl_2 \cdot 2\ H_2O$, 1.1 g $MgSO_4 \cdot 7\ H_2O$, 0.5 g NaCl, 0.3 ml of 1% (w/v) $FeCl_3$. Complete synthetic media (based on [Klig *et al.*, 1985]) contained per liter: 6.7 g Difco yeast nitrogen base without amino acids, 20 mg lysine, 10 mg arginine, 10 mg leucine, 30 mg methionine, 10 mg adenine, 10 mg uracil. Lactate media contained per liter: 22 ml 90% lactic acid and 1 g glucose [Daum *et al.*, 1982]. Glucose media contained per liter: 20 g glucose and 1.1 ml lactic acid (to prevent the precipitation of calciumphosphate in SSG during sterilization by autoclaving). The pH of all growth media was adjusted to 5.5 using KOH or HCl before sterilization. Cell growth was monitored by measuring the OD600 (after dilution to an OD600 of approximately 0.3) or by counting cell numbers using a hemocytometer.

Preparation of cell homogenates

Cells were harvested by centrifugation (10 min at 3600 x *g*) at the times indicated in Figures 2-4 and resuspended in ice-cold water at a concentration of 0.1 g/ml (wet weight). Cells were disrupted by vortexing twice for 1 min with glass beads (1.6 g/ml) with a 1 min interval on ice. The broken cell suspension was stored at -80˚C until its use in enzyme assays and phospholipid analysis.

Subcellular fractionation

Cells were harvested at the indicated times from cultures grown on semi-synthetic lactate medium and spheroplasts were prepared using zymolyase as described previously [Daum *et al.*, 1982]. The isolation of mitochondria at pH 6.0 and further purification by sucrose gradient centrifugation were based on a published procedure [Gaigg *et al.*, 1995]. Mitochondrial outer membranes were isolated and purified based on [Mayer *et al.*, 1995]. Microsomes were isolated as the 32,500 x *g* pellet of a 20,200 x *g* post-mitochondrial supernatant. The isolation of mitochondria-associated membranes (MAM) was adapted from a published procedure [Gaigg *et al.*, 1995]. Full details of all fractionation procedures are described elsewhere [De Kroon *et al.*, 1999]. The fractions obtained were stored at -80˚C.

Enzyme assays

PS synthase specific activity (expressed as nmol serine incorporated per min per mg protein) in the cell homogenate was determined in the presence of 4 mM Triton X-100, 0.6 mM $MnCl_2$, 50 mM Tris-HCl, pH 8.0, and 0.2 mM CDP-diacylglycerol as described [Bae-Lee and Carman, 1984], at a concentration of 0.1 mM L-[3-^{3}H]-serine (28,000 dpm/nmol). TLC analysis showed that over 95% of the lipid-incorporated label was present in PS and PE. The combined specific activity of the PNMTs (expressed as nmol *S*-adenosyl-L-methionine metabolized into chloroform soluble material per min per mg protein) was determined by following the methylation of endogenous PE in the cell homogenate in the presence of 0.5 mM *S*-adenosyl-L-[methyl-^{3}H]-methionine (10,000 dpm/nmol) and 50 mM Tris-HCl, pH 8.0 (based on [Gaynor and Carman, 1990]). Over 98% of the lipid-incorporated label was present in monomethyl PE (PMME), dimethyl PE (PDME) and PC as was determined by TLC analysis (not shown). In both enzyme assays cell homogenate samples corresponding to 40 µg of protein were incubated for 10 min at 30°C. Incubations were ended by adding 475 µl of a mixture of chloroform, methanol and 0.5 M HCl (6:12:1, v/v/v) which was followed by lipid extraction [Bligh and Dyer, 1959] and liquid scintillation counting of the dried lipid extracts.

Phospholipid analysis of cell homogenates and subcellular fractions

Phospholipids were extracted according to the method of Bligh and Dyer [Bligh and Dyer, 1959]. The phosphorus content of the organic phase obtained after extraction was determined to yield the phospholipid phosphorus to protein ratio. Phosphorus was determined by the method of Fiske and Subbarow [Fiske and Subbarow, 1925]. Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin (BSA) as a standard. Phospholipid compositions were determined by two-dimensional TLC analysis of lipid extracts containing 200-350 nmol of phospholipid phosphorus as described [De Kroon *et al.*, 1997].

Results

Growth characteristics

This study describes the influence of the growth condition (carbon source and nutritional supplement) and the growth stage on the phospholipid composition and the activities of several phospholipid biosynthetic enzymes in a wild-type yeast strain. Figure 1 shows the growth curves of the wild-type yeast strain D273-10B on both the semi-synthetic and complete synthetic media with fermentable and non-fermentable carbon sources. The semi-synthetic lactate medium was chosen because it is the medium routinely used to culture

semi-synthetic lactate medium was chosen because it is the medium routinely used to culture yeast for the isolation of mitochondria [Daum *et al.*, 1982] and complete synthetic glucose medium was chosen to allow comparison to literature data (*e.g.* [Homann *et al.*, 1987; Jiranek *et al.*, 1998]). In both media the carbon source was varied to distinguish possible effects of carbon source from any effects of the supply of different nutrients.

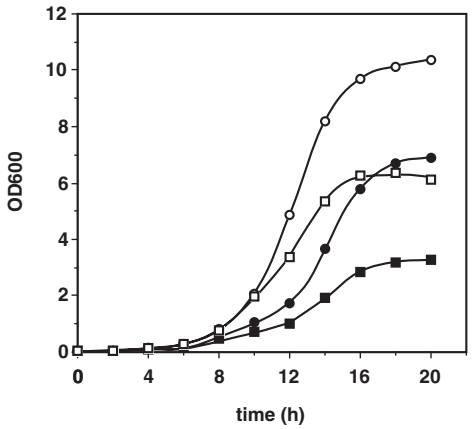

Figure 1. Growth rates of D273-10B in different media. Growth was monitored by measuring at the indicated time points the OD600 of cultures in SSL (●), SSG (○), CSL (■), or CSG (□). One OD unit corresponds to a cell density of approximately $1.5 \cdot 10^7$ cells/ml.

The cultures grow to a lower density and reach the stationary phase earlier in complete synthetic media as compared to semi-synthetic media, which are richer in nutrients (Figure 1). When lactate is used as a carbon source the cells reach the stationary phase later and at a lower cell density than when glucose is used. In all media tested, the doubling time of the cells is a little over 2 h during exponential growth.

Phospholipid content and composition

Figure 2 shows that the carbon source supplied in the medium has a profound influence on the phospholipid to protein ratio of the cells. Cells growing on lactate have at any stage of growth a significantly higher amount of phospholipid per mg of protein. In glucose-based media the phospholipid to protein ratio increases dramatically during exponential growth and levels off in the stationary phase while in lactate-based media it shows a gradual increase going from exponential to stationary phase.

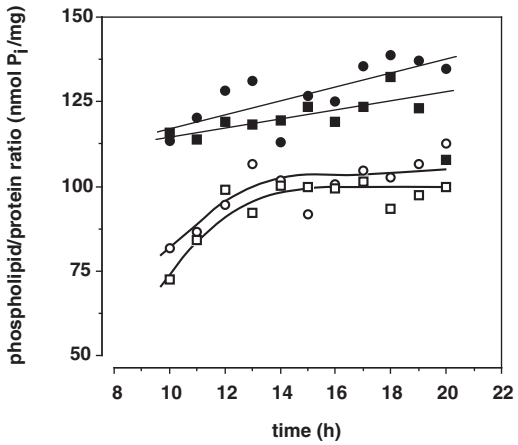

Figure 2. Variation of the phospholipid to protein ratio during growth in different media. Cells grown in SSL (●), SSG (○), CSL (■), or CSG (□) were harvested at the indicated time points and the phospholipid to protein ratio was determined as detailed in the experimental section.

The phospholipid composition of cell homogenates was analyzed at different time points during growth to determine the influence of the different media and the growth stage and is depicted in Figure 3. From this figure it is clear that the phospholipid composition is dynamic throughout the growth stages of yeast. A general feature of the phospholipid composition in all media is the more or less pronounced increase in PC levels at the expense of PE during the transition of the culture from exponential to early stationary phase (*cf.* Figures 1 and 3). In lactate media this is preceded by a gradual decrease in PC and concomitant increase in PE content. During the period of time monitored, the cellular PC/PE ratio varies between 0.9 and 2.2 for SSL, while it varies between 0.9 and 1.8 for SSG, between 1.2 to 2.1 for CSL, and between 1.5 and 2.3 for CSG. For the complete synthetic media the 'turning point', where the PC level starts to increase at the expense of PE, appears to occur earlier than for the semi-synthetic media. Similarly, the 'turning point' seems to occur earlier in glucose media as compared to lactate media.

Remarkably, the PI content of cells grown on the non-fermentable carbon source is much higher (20% *vs.* 5-10%) than that of cells grown on the fermentable carbon source throughout the growth period monitored (Figure 3). Generally, a slight increase in PI content towards the stationary phase is observed. As expected, the cellular level of the mitochondrial phospholipid CL is higher in lactate media than in glucose media and appears to remain fairly constant under the conditions tested, as does the level of PS.

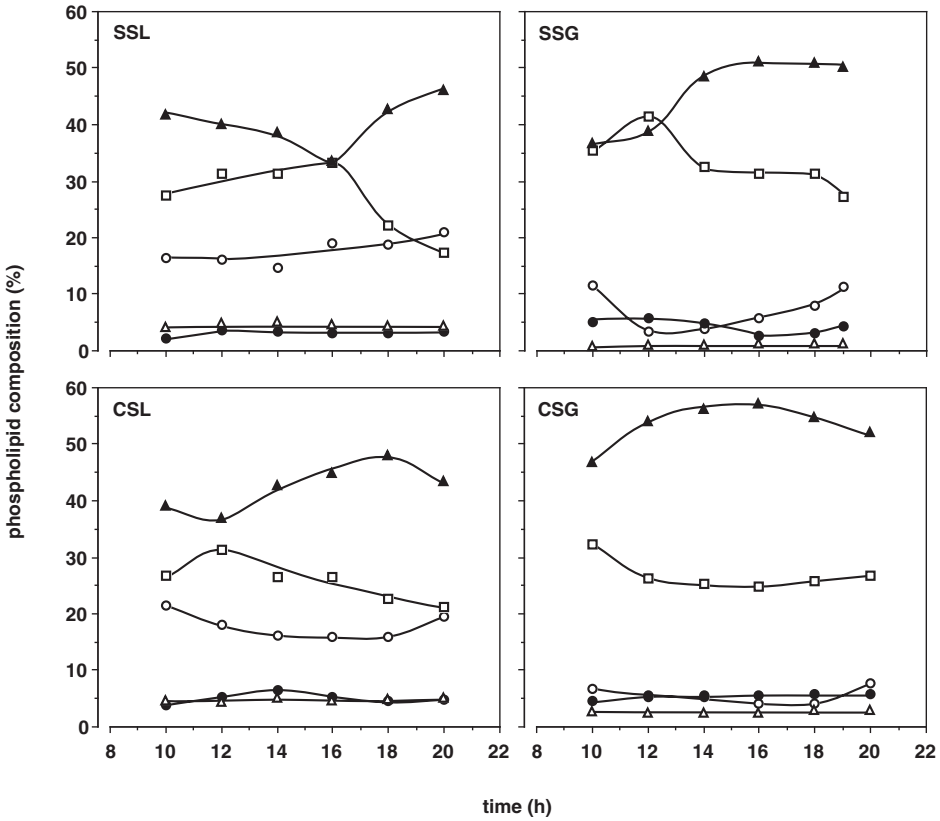

Figure 3. The relative abundance of the five principal phospholipids during growth of D273-10B in different media. Cells grown in SSL, SSG, CSL or CSG were harvested at the indicated time points and the phospholipid composition was determined. For SSL the average of two different growth experiments is shown, in which very similar results were obtained. The contribution of each of the major phospholipids PC (▲), PE (□), PI (○), PS (●) and CL (Δ) is presented as a percentage of total phospholipid. The other phospholipids include lysophospholipids, PA, phosphatidylglycerol (PG), PMME and PDME and together amount to less than 10%.

Phospholipid biosynthetic enzyme activities

The effect of the growth phase on the activity of phospholipid biosynthetic enzymes in different media was examined in parallel. The enzyme PS synthase was chosen as a representative for the coordinately regulated phospholipid biosynthetic enzymes [Carman and Zeimetz, 1996]. The PNMTs were chosen because these effect the conversion of PE into PC. The effect of the growth phase on the activity of PS synthase and the PNMTs in different media is depicted in Figure 4. Maximal specific activities of the enzymes tested were found at the end of the exponential phase or during the shift of the culture to early stationary phase.

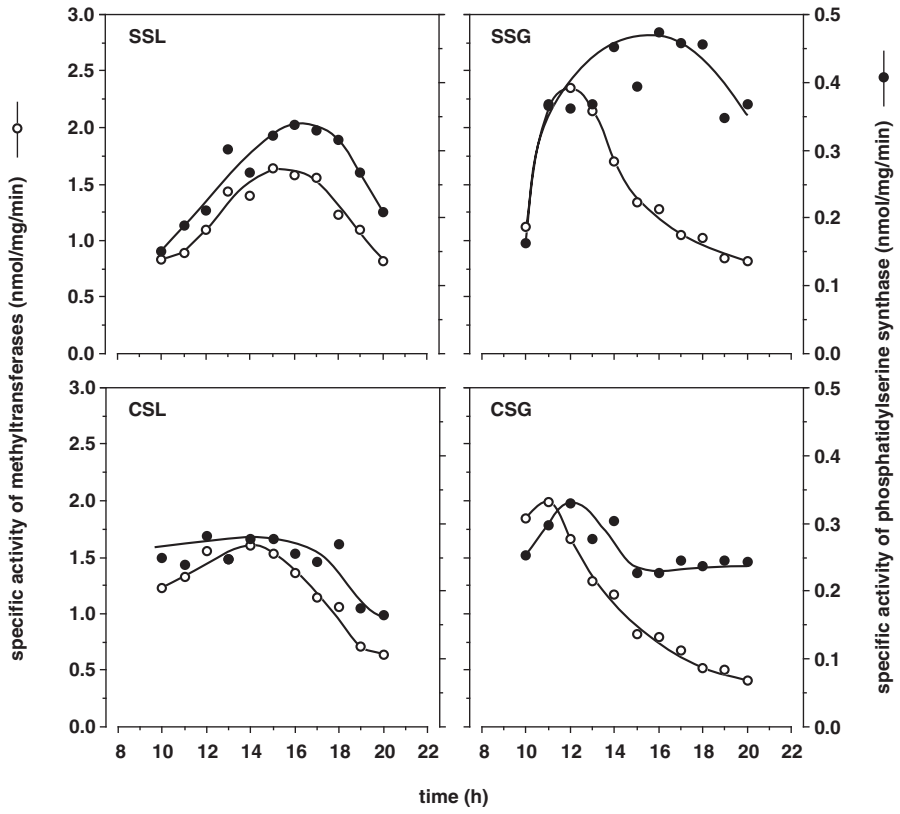

Figure 4. The specific activities of phospholipid biosynthetic enzymes during growth in different media. Cells grown in SSL, SSG, CSL or CSG were harvested at the indicated time points and the specific activities of the phospholipid-*N*-methyltransferases (○) and PS synthase (●) were determined as described in the experimental section.

In the stationary phase the specific activities were found to decrease. In glucose-based media the peak in specific activity of PS synthase seems to lag behind the peak in specific activity of the methyltransferases, whereas the peaks in specific activities coincide in the lactate-based media.

Effect of growth phase on phospholipid composition of subcellular organelles

To get a high yield of the organelle of interest, yeast cells are usually grown to late exponential phase for subcellular fractionation. From the above results it is clear that the phospholipid composition of yeast cells is dependent on the moment of harvest. To investigate to which extent the growth phase related changes in cellular phospholipid composition are reflected in the phospholipid composition of the individual organellar membranes, phospholipid analysis was performed on subcellular fractions from yeast grown

for different times on semi-synthetic lactate medium. Figure 5 compares the levels of the quantitatively most important phospholipids in microsomes, mitochondria-associated membranes (MAM), mitochondria, and mitochondrial outer membrane vesicles (OMV) at two time points at the end of the exponential phase (OD 4 and OD 5.5). The increase in PC content at the expense of PE which is apparent in whole cell homogenates occurs to a similar extent in the subcellular membranes tested. There are no pronounced changes in the concentrations of the other phospholipids.

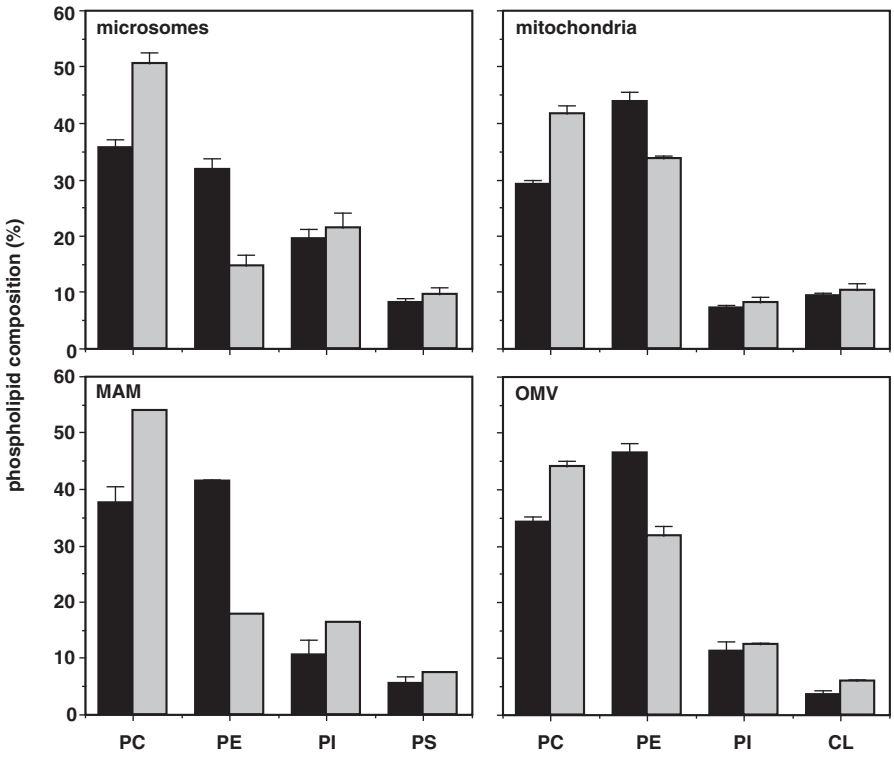

Figure 5. Phospholipid composition of subcellular fractions at the end of logarithmic growth. Cells were cultured in SSL and harvested at OD 4 (black bar, ± standard deviation ($n \geq 3$), or ± variation for MAM ($n = 2$)) or at OD 5.5 (gray bar, ± variation ($n = 2$), except MAM ($n = 1$)). After subcellular fractionation the phospholipid composition of microsomes, mitochondria-associated membranes (MAM), mitochondria and mitochondrial outer membrane vesicles (OMV) was determined. The data for the major phospholipids is presented as a percentage of total phospholipid. Other phospholipids include PS/CL (mitochondria and OMV/microsomes and MAM), lysophospholipids, PA, PG, PDME and PMME and together account for less than 6%.

Discussion

The present study describes the effects of the carbon source and the growth stage on the phospholipid composition and the activities of several phospholipid biosynthetic enzymes in a wild-type yeast strain. It documents for the first time growth phase dependent variations in the PC/PE ratio in yeast cells, of which the extent depends on the carbon source. Typically, the PC content was found to increase at the expense of PE during the transition of the culture to stationary phase. This change in PC to PE ratio is most pronounced when lactate is used as the carbon source. In particular cells grown in SSL medium, the medium routinely used for the isolation of mitochondria, experience a rapid increase in PC/PE ratio as they enter stationary phase. Also, in cells grown on glucose (SSG, CSG), an increase in the PC/PE ratio during the transition from late exponential to early stationary phase was observed, albeit to a lesser extent and much more gradual than in lactate-grown cells. In the time window monitored, the changes occurring in PC and PE content in CSG medium are similar to those observed by Jiranek and co-authors [Jiranek *et al.*, 1998] using a comparable growth medium.

The reason that in previous studies changes in PE/PC ratio went unnoticed [*e.g.* Homann *et al.*, 1987; Gaynor *et al.*, 1991] could be the fact that only two time points were compared, or the use of growth medium in which the effect is less conspicuous, or the use of yeast strains with a different genetic background. Another factor contributing to different results in other studies could be the use of steady-state labeling with $^{32}P_i$ to determine phospholipid compositions, in which the assumption is made that the phospholipid composition no longer changes after growing for five or six generations in the presence of label. However, the present data indicates that this assumption may not always be correct.

Since the CDP-choline route requires exogenous choline for net PC synthesis [Waechter *et al.*, 1969] and as only 20% of the cellular PC is produced *de novo via* this route when yeast is grown on semi-synthetic lactate medium [Janssen *et al.*, 1999], the PNMT enzymes have to be responsible for the observed formation of PC at the expense of PE. In SSL medium the start of the increase in PC/PE ratio appears to concur with the peak in phospholipid biosynthetic activity found at the end of exponential growth. A similar, albeit less pronounced correlation is apparent in the other culture media tested. In general, the order of occurrence of the changes described is CSG < (sooner than) SSG < CSL < SSL.

The phospholipid composition of subcellular fractions from cells isolated at different stages of growth show that the change in PC and PE content which is apparent in whole cell homogenates is also occurring in the subcellular fractions. Within the ranges observed there is no apparent need to ‘protect’ organelles from changes in cellular phospholipid composition. The changes in content are more pronounced in the microsomes and mitochondria-associated membranes than in the mitochondria, as would be expected based on the localization of the PNMT activities in the endoplasmic reticulum [Daum and Vance,

1997] which are responsible for the observed conversion of PE to PC. Interestingly, the changes in lipid composition found for the organelles are even more marked than those found for the whole cell homogenates at the corresponding times. One explanation is that during the pretreatment of the cells to isolate subcellular fractions lipid biosynthesis and transport processes continue such that differences in phospholipid content increase (*cf.* Figure 3A at 14-16 and 16-18 h). An alternative explanation is that the changes in lipid compositions in the organellar membranes subjected to analysis precede those in membranes that remain uninvestigated in this study such as the plasma membrane. The phospholipid composition of yeast mitochondria reported in literature [Zinser and Daum, 1995] is similar to that found in the present study for mitochondria isolated from cultures at OD 5.5 (*cf.* Figure 5) which corresponds to the beginning of the transition of the culture to stationary phase.

One explanation for the increase of PC at the expense of PE could be that during exponential growth and the shift to stationary phase methionine is preferentially used for protein synthesis whereas it may become available in greater quantities for the methylation of PE to form PC once methionine is no longer as urgently needed for protein synthesis. Alternatively, PC may be a low-maintenance phospholipid (*i.e.* an ideal bilayer lipid, with low turnover [Patton-Vogt *et al.*, 1997]) that is preferentially used in cellular membranes in stationary phase, whereas there may be a more pronounced preference for the non-bilayer lipid PE during periods of major physiological activity.

Cells grown in lactate media displayed a significantly higher phospholipid to protein ratio compared to cells grown in glucose media, which implies the need for a higher relative rate of phospholipid biosynthesis on non-fermentable media. Related observations were done in previous studies, more lipid/g dry weight [Jollow *et al.*, 1968] and a higher phospholipid to protein ratio [Jakovcic *et al.*, 1971] were found for aerobically grown cultures (in which 35% of the total cellular phospholipid was recovered in the mitochondrial fraction) than for anaerobically grown cultures. Furthermore, it was found that in synchronized cultures the increase in the amount of phospholipid per generation was higher for cells grown on non-fermentable *versus* fermentable media [Cottrell *et al.*, 1981]. Even though mitochondria do not have an exceptionally high phospholipid to protein ratio compared to other organelles [Zinser and Daum, 1995], it is certainly higher than that of whole cells. Hence, it is likely that the higher ratio of phospholipid to protein is due to the presence of more mitochondrial membranes per cell. Consistent with this explanation is the higher CL content of cells grown on lactate-based medium.

Strikingly, a much higher PI content was found for cells growing on a non-fermentable carbon source as compared to a fermentable carbon source (20% vs 5-10%). This appears to be due to high PI concentrations in membrane systems other than the mitochondria, since the PI content of isolated mitochondrial membranes is well below the cellular content of this phospholipid (compare also Figure 5). The general trend that PI levels increase towards stationary phase which is most pronounced in the glucose-based media, was

also observed in other studies [Homann *et al.*, 1987; Jiranek *et al.*, 1998]. The mechanism that has been proposed to explain this finding is that as a result of the decreased PS synthase activity towards the stationary phase more CDP-diacylglycerol is channeled into the PI synthesis route [Homann *et al.*, 1987]. It should be noted that the growth phase dependent changes in PS synthase and PNMT activity that were observed occurred within a relatively short time span (a few hours, Figure 4). This indicates that mechanisms other than at the transcriptional level contribute to down-regulation of the activities, since growth phase related changes in RNA levels and enzyme subunit levels were observed at a much slower time scale [Lamping *et al.*, 1995; Homann *et al.*, 1987]. An attractive possibility suggested before [*e.g.* Homann *et al.*, 1987] and supported by *in vitro* experiments [Hromy and Carman, 1986] is that an increased PI content decreases the activity of PS synthase which in turn would contribute to a further increase in PI levels. Since no marked differences were found in the average levels of PS synthase activity between glucose- and lactate-based media, the higher PI content on the non-fermentable carbon source cannot be explained by the above mechanism. Neither is it likely to be due to higher levels of PI synthase activity, since *PIS1* gene expression is reported to be lower on the non-fermentable carbon source glycerol than on glucose [Anderson and Lopes, 1996]. It has been reported that the availability of inositol is an important factor determining the levels of PI [Kelley *et al.*, 1988; Jiranek *et al.*, 1998]. Therefore, an alternative explanation for a higher PI content on a non-fermentable carbon source could be increased expression of the *INO1* gene during non-fermentative growth. Support for this latter mechanism comes from a recent study which showed that the expression level of *INO1* increases with a factor 2 or more when glucose is depleted from the medium [DeRisi *et al.*, 1997].

Both the PS synthase and the methyltransferase enzymes show a similar activity profile during growth, with changes correlating to the growth phase in different media and showing a decrease in activity upon shifting to stationary phase as was reported before for growth on complete synthetic media [Homann *et al.*, 1987]. The PNMT activity appears to be a convenient marker for growth phase since in all media tested maximal activities coincide with the end of exponential growth. The decrease in PS synthase activity towards the stationary phase lags somewhat behind that of PNMT when the cells are grown on glucose whereas they parallel each other when the cells are grown on lactate. The higher PI content of the lactate-grown cells may be responsible for the more effective repression of PS synthase activity in the shift to stationary phase.

In conclusion, growth phase and medium have profound effects on the phospholipid composition and content and the activities of several phospholipid biosynthetic enzymes in a wild-type yeast strain. The distinct differences between cells grown on fermentable and non-fermentable media have largely been overlooked so far. This raises an extra interest in studying the biosynthesis and transport of phospholipids under non-fermenting conditions.

Chapter 4

Cooperative activity of phospholipid-*N*-methyltransferases localized in different membranes

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manuscript in preparation

Abstract

The possibility that the phospholipid-*N*-methyltransferase enzymes from yeast are capable of acting upon a phospholipid substrate, localized in a different membrane than in which the enzymes reside ('trans-catalysis' hypothesis), was investigated using subcellular fractions isolated from *cho2* and *opi3* gene disruptant strains, which are defective in phosphatidylethanolamine transferase (PEMT) and phospholipid methyltransferase (PLMT), respectively. When homogenates of an *opi3* and a *cho2* strain are mixed, the combined methyltransferase activity, measured as the incorporation of [^{3}H] label from *S*-adenosyl methionine, exceeds that expected based on the separate activities of PEMT and PLMT. Cooperativity of the enzymes was also found upon mixing *opi3* and *cho2* microsomes, excluding the involvement of soluble factors or membranes other than microsomal membranes. The increased incorporation implies that monomethyl PE generated by PEMT becomes available for PLMT, for which the amount of phospholipid substrate is limiting in the absence of PEMT. Analysis of the products formed provided further support for increased substrate availability. The levels of dimethyl PE and phosphatidylcholine synthesized were increased, but not at the expense of the level of monomethyl PE, indicating that this was also synthesized in increased quantities. Furthermore, the enzymes are capable of methylating [^{14}C]PE presented in small unilamellar vesicles, with an apparent requirement for association between vesicles and microsomes for conversion. Membrane fusion cannot explain the cooperativity between the microsomal membranes, since the possibility that fusion occurs under the experimental conditions employed is remote, as judged from literature data. The kinetics of the cooperativity suggest a collision-based process, enabling either transport of substrate or 'trans-catalysis'.

Introduction

Phosphatidylcholine (PC) is a major phospholipid found in membranes of eukaryotic cells. In yeast it accounts for 40-50% of the total membrane phospholipids [Henry, 1982]. As in higher eukaryotes, PC is synthesized *via* two distinct pathways in yeast, either *via* the triple methylation of phosphatidylethanolamine (PE) or *via* the CDP-choline (Kennedy) pathway (for a recent review, see [Carman and Henry, 1999]). In yeast, the methylation of PE is the primary pathway of biosynthesis of PC when cells are grown in the absence of choline, whereas the CDP-choline pathway is considered to be an auxiliary route since it requires exogenous choline for net PC synthesis [McDonough *et al.*, 1995]. PE undergoes three sequential methylations using *S*-adenosyl methionine (SAM) as the methyl donor, and monomethyl PE (PMME) and dimethyl PE (PDME) are formed as intermediates [Waechter, Steiner and Lester, 1969]. Two enzymes are responsible for the reactions, PE methyltransferase (PEMT) catalyzes the first methylation reaction and phospholipid

methyltransferase (PLMT) catalyzes the second and the third [Kodaki and Yamashita, 1987; Summers *et al.*, 1988; McGraw and Henry, 1989].

The genes encoding PEMT and PLMT are *CHO2* and *OPI3*, respectively, which were cloned by complementation of mutants defective in the corresponding methylation reactions [Kodaki and Yamashita, 1987; Summers *et al.*, 1988; McGraw and Henry, 1989]. The genes are regulated at the level of transcription in response to the soluble phospholipid precursors, inositol and choline, as well as to growth phase (for a recent review, see [Kanipes and Henry, 1997]). The double mutant (*opi3 cho2*) has no phospholipid methylation activity and displays a stringent choline auxotrophy [Summers *et al.*, 1988; Kodaki and Yamashita, 1987; Preitschopf *et al.*, 1993]. The single mutants are not choline auxotrophs [Summers *et al.*, 1988; McGraw and Henry, 1989; Preitschopf *et al.*, 1993]. When grown in the absence of choline, the *opi3* mutant accumulates PMME and exhibits low or undetectable levels of PC [McGraw and Henry, 1989; Kodaki and Yamashita, 1989] while *cho2* mutants accumulate PE and have low levels of PC [Summers *et al.*, 1988; Kodaki and Yamashita, 1989]. The residual synthesis of PC in the *cho2* mutants is due to the ability of the *OPI3* gene product to catalyze, although at a low rate, the first methylation of PE to PMME [Summers *et al.*, 1988; Kodaki and Yamashita, 1989]. This ability is also reflected in the fact that the *OPI3* gene, when present in multiple copies, suppresses the phospholipid methylation defect of a *cho2* mutation [Preitschopf *et al.*, 1993].

The *CHO2* and *OPI3* genes encode proteins with predicted molecular weights of 101 and 25 kDa, respectively, which were predicted to be integral membrane proteins based on hydropathy plots of the amino acid sequences [Kodaki and Yamashita, 1987]. The enzymes have been localized to the endoplasmic reticulum [Kuchler, Daum and Paltauf, 1986; Zinser *et al.*, 1991]. Using membrane extracts from strains carrying *opi3* and *cho2* disruption mutations, the enzymological and kinetic properties of the two individual methyltransferases were analyzed [Gaynor and Carman, 1990]. The pH optima for the reactions catalyzed by PEMT and PLMT were 9.0 and 7.5, respectively. Maximum activity of PEMT occurred in the absence of divalent cations, while the addition of either Mg^{2+} or Ca^{2+} ions to the assay system for PLMT led to a stimulation of activity. The enzymes were inhibited by addition of detergents and by thioreactive compounds, and were unstable above 30˚C. Both enzymes exhibited saturation kinetics when the concentration of the phospholipid substrate was varied while the concentration of SAM was kept constant, and *vice versa*.

In several enzymological studies, the phospholipid substrates were added in the form of sonicated lipid suspensions [Gaynor and Carman, 1990; Kodaki and Yamashita, 1987 and 1989]. However, it remains unclear how the phospholipid substrates subsequently become available to the methyltransferase enzymes localized in the microsomal membranes. There are several possibilities, such as transport of the substrates to the microsomes or fusion of the vesicles with the microsomal membranes. An alternative possibility would be that the

methyltransferase enzymes located in the endoplasmic reticulum can act upon a substrate in another ('trans') membrane. In this respect, an interesting observation was made in our research on the import of PC by mitochondria. It was found in *in vivo* pulse-chase studies where [^{3}H]methionine was provided, that not only [^{3}H] labeled PC but also PMME and PDME appear in the mitochondria. Unexpectedly, the distribution of label over PC, PMME and PDME was similar in microsomes and gradient purified mitochondria [De Kroon *et al.*, submitted for publication]. The so-called 'trans-catalysis' hypothesis would also provide an attractive explanation for this observation.

Subcellular fractions from strains deficient in PEMT or PLMT offer a simple model system that allows us to investigate the possibility of 'trans' methylation. Therefore, subcellular fractions from strains deficient in one or both enzymes required for the methylation of PE were characterized and several aspects of the mechanism of methylation were studied. It was found that upon mixing of membranes from *opi3* and *cho2* strains the incorporation of label increased and more of the methylated PE derivatives were formed than expected based on the activity of each enzyme in the separate fractions. This indicates that PEMT and PLMT localized in different microsomal membranes are capable of cooperating. The findings will be discussed in the light of the 'trans-catalysis' hypothesis.

Materials and methods

Materials

S-Adenosyl-L-methionine (SAM) was purchased from Sigma (St. Louis, MO). The radiochemicals *S*-adenosyl-L-[methyl-^{3}H]-methionine ([^{3}H]SAM, 15 Ci/mmol) and 1,2-dioleoyl-*sn*-glycero-3-phospho-[2-^{14}C]-ethanolamine ([^{14}C]DOPE, 49 mCi/mmol) and [1α,2α(n)-^{3}H]cholesteryl oleoyl ether ([^{3}H]CE, 48 Ci/mmol) were obtained from Amersham (Amersham, United Kingdom). DOPE and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Silicagel 60 HP-TLC plates were from Merck (Darmstadt, FRG). Yeast extract was obtained from Sigma. Zymolyase was supplied by Seikagaku (Japan). Biotinylation reagent (EZ-Link PEO-Maleimide Activated Biotin) and avidin beads (Immobilized NeutrAvidin) were from Pierce. All other chemicals were analytical grade.

Subcellular fractionation

The yeast strains listed in Table 1 were grown aerobically to late log at 30˚C in semi-synthetic lactate medium [Daum, Böhni and Schatz, 1982] supplemented with 1 mM choline and adenine, histidine, leucine, tryptophan, and uracil at a concentration of 20 mg/l each, except for strain D273-10B, which was grown without supplements.

Table 1

Genotypes of strains employed in this study

strain	designated as	genotype
SH921*	(congenic) wild-type	*MATα his3 leu2 ura3 ade2*
SH922*	*opi3 cho2*	*MATα his3 leu2 ura3 ade2 opi3::URA3 cho2::LEU2*
SH414*	*opi3*	*MATa his3 leu2 ura3 ade2 trp1 can1 opi3::URA3*
SH458*	*cho2*	*MATα his3 leu2 ura3 cho2::LEU2*
D273-10B	wild-type	*MATα*

* [Dowd, Bier and Patton-Vogt, 2001]

Spheroplasts were prepared using zymolyase as described previously [Daum, Böhni and Schatz, 1982] and homogenized using a Dounce homogenizer in a buffer containing 10 mM MES, pH 6.0, and 0.6 M sorbitol (D buffer), to which 1 mM PMSF and 0.2% (w/v) BSA were added. Crude mitochondria were isolated using D buffer and were further purified by nycodenz gradient centrifugation as reported [Glick and Pon, 1995], with some minor changes. The nycodenz gradient was made in D buffer and consisted of a layer of 18% (w/v) nycodenz overlaid with a 13.5% (w/v) nycodenz layer. Microsomes were isolated as the 32,500 x *g* pellet of a 20,200 x *g* post-mitochondrial supernatant, as described [De Kroon *et al.*, 1999], with the exception that D buffer was used for all resuspension and wash steps. The subcellular fractions, all in D buffer, were snap-frozen in liquid N_2 and stored at -80˚C.

Measurement of methyltransferase activity and product composition

The activity of the phospholipid-*N*-methyltransferases (PNMTs), expressed as nmol SAM metabolized into chloroform soluble material per min per mg protein, was determined by following the methylation of endogenous PE in the cellular fractions in the presence of 0.5 mM [^{3}H]SAM (10,000 dpm/nmol) and 50 mM Tris-HCl, pH 8.0 (based on [Gaynor and Carman, 1990]). To prevent enhancement of membrane fusion and aggregation by divalent cations, Mg^{2+} was omitted from the incubation mixture, since it was found that in our *in vitro* system, addition of Mg^{2+} did not further increase PNMT enzyme activities (data not shown). Incubations were carried out at 30°C in a final volume of 100 µl and ended by adding 475 µl of a mixture of chloroform, methanol and 0.5 M HCl (6:12:1, v/v/v). This was followed by lipid extraction [Bligh and Dyer, 1959] and liquid scintillation counting of the dried lipid extracts. The incorporation of label was linear with time up to at least 10 min, and with protein concentration at least up to 0.25 mg/ml (determined using microsomes from strain D273-10B, data not shown). To determine product compositions, [^{3}H]SAM was used at 100,000 dpm/nmol to increase the sensitivity of the analysis, and the lipid extracts were analyzed by HP-TLC, using chloroform/methanol/25% ammonia (71:25:4, v/v/v) as eluent. The radioactive spots on the TLC plate were quantified by a Berthold Automatic TLC linear

analyzer (Wildbad, FRG). The product composition is presented as the percentage of [^{3}H] label incorporated into PMME, PDME, and PC, together set at 100%, without correcting for the different number of methyl groups in each methylated PE derivative.

Biotinylation of microsomes

For reduction, microsomes were incubated for 30 min on ice, at a protein concentration of 1 mg/ml in 10 mM Tris-HCl, pH 7.4, containing 155 mM NaCl (TBS), with 5 mM DTT added. After reisolation by centrifugation (10 min at 14,000 rpm (20,700 x *g*) in a microfuge at 4˚C), the microsomes were resuspended in TBS containing 5 mM PEO-maleimide activated biotin, at a protein concentration of 1 mg/ml. After 30 min incubation on ice, the microsomes were reisolated as before and resuspended in TBS at a protein concentration of 2 mg/ml. Avidin beads (125 µl aliquots) were washed three times with 1 ml TBS and incubated for 1 h at 4˚C with 125 µl biotinylated microsomes. After incubation the beads were washed three times with 1 ml TBS and used for further experiments.

Preparation of small unilamellar vesicles

Small unilamellar vesicles (SUV) were prepared by sonication of a lipid suspension obtained by hydrating a dry lipid film with 10 mM Tris-HCl, pH 8.0, to a final lipid concentration of 1 mM. The lipid film consisted of DOPC and DOPE in a 1:1 molar ratio and the appropriate amounts of radiochemicals. SUV contained 7 µCi [^{14}C]DOPE (final specific radioactivity 30,000 dpm/nmol) per µmol of phospholipid and when indicated also 5 µCi of [^{3}H]CE per µmol of phospholipid. The phospholipid suspension was subjected to 10 cycles of 30 s ultrasonication at 80 W and 0˚C with 30 s intervals using a Branson B12 sonifier equipped with a microtip. The sonicated phospholipid suspension was centrifuged for 30 min at 390,000 x *g* and 4˚C in a Beckman TL-100 ultracentrifuge. The supernatant containing the SUV was kept on ice and used immediately after preparation.

Incubations of mixtures of microsomes and SUV

Microsomes and SUV were incubated together for 60 min under the same conditions as used to assay methyltransferase activity. Where indicated, SAM was omitted from the incubation mixture or unlabeled SAM was used. The ratio of microsomal PE to SUV PE was approximately 1:1, corresponding to 50 µg of microsomes per 100 µl incubation and 0.4 mM of total SUV phospholipid. Where indicated, microsomes and SUV were separated by centrifugation for 20 min at 50,000 x *g* in a Beckman TL-100 ultracentrifuge at 4˚C. The distribution of [^{14}C] and [^{3}H] label over pellet and supernatant was determined by liquid scintillation counting of the aqueous samples. After lipid extraction, the formation of labeled phospholipids was determined by liquid scintillation counting of the dried lipid extracts and

by TLC analysis as described. The radioactive spots on TLC were quantified using the linear scanner as described above or by using a Molecular Dynamics phosphorimager.

Other methods

Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin (BSA) as a standard. Phospholipids were extracted according to the method of Bligh and Dyer [Bligh and Dyer, 1959]. Phosphorus was determined by the method of Fiske and Subbarow [Fiske and Subbarow, 1925] after destruction with perchloric acid. Phospholipid compositions were determined by two-dimensional TLC analysis of lipid extracts containing 350 nmol of phospholipid phosphorus as described [De Kroon *et al.*, 1997].

Results

Characterization of methyltransferase activities and product compositions

The specific activity of the methyltransferases in the cellular homogenates of the *opi3* and *cho2* knock-out strains and the congenic wild-type was determined (Figure 1, panel A).

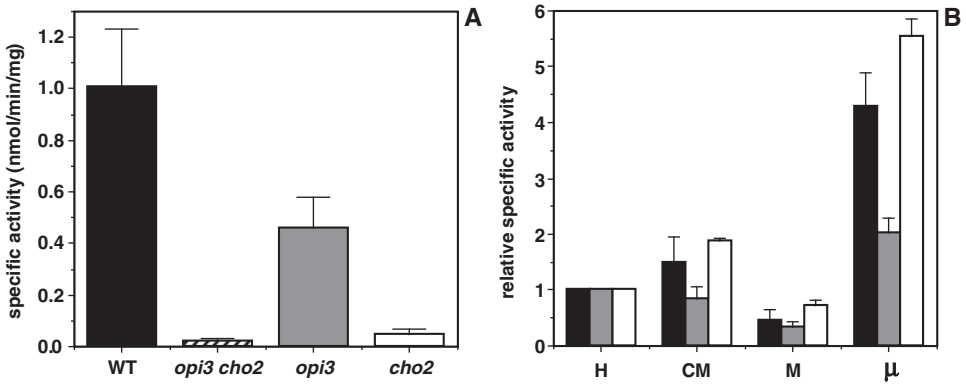

Figure 1. Specific methyltransferase activities in homogenates and distribution over subcellular fractions. The specific activity of the combined methyltransferase enzymes was determined in subcellular fractions from the congenic wild-type (black bars), *opi3 cho2* (hatched bar), *opi3* (gray bars), and *cho2* (white bars) strains. Of the homogenates (H), crude mitochondria (CM) and purified mitochondria (M) 50 μg protein was used and of the microsomal fraction (μ) 10 μg protein was used. The average (± variation) of two independent preparations is shown. A: specific activity of PNMTs in homogenates from the strains indicated; B: the specific activity of subcellular fractions is presented relative to that in the homogenate of the corresponding strain.

A high methyltransferase activity was found in the wild-type strain, whereas no phospholipid methylation activity was found in the double knock-out (*opi3 cho2*) as reported before [Summers *et al.*, 1988; Kodaki and Yamashita, 1987; Preitschopf *et al.*, 1993]. The low residual methylation activity in the double knock-out was attributed to sterol methylation based on TLC analysis (not shown). The methylation activity of the *opi3* and *cho2* single knock-out strains is reduced compared to the wild-type strain (Figure 1A). The specific activity of the strain lacking the PE methyltransferase enzyme (*cho2*) is lower than that of the strain lacking the enzyme catalyzing the second and third methylation (*opi3*). This is explained by differences in substrate availability rather than by differences in the amount of enzyme. The substrate for the first methylation, PE, is expected to be present in ample amounts while a much lower level, or even a complete lack of the preferred substrates is expected for the second and third methylation. To verify this, the phospholipid compositions of the homogenates were determined (Table 2) and found to be very similar for the different strains under the growth conditions employed, *i.e.* in the presence of choline, with PE and PC constituting approximately 30% and 40%, respectively, of the total phospholipids, and PMME and PDME not detected.

Table 2

Phospholipid composition of homogenates

	WT	*opi3 cho2*	*opi3*	*cho2*
PC	44.1 ± 0.9	41.9 ± 0.4	39.7 ± 0.9	43.1 ± 1.6
PE	28.3 ± 1.2	32.7 ± 1.1	30.6 ± 2.9	28.5 ± 1.5
PI	13.3 ± 0.5	12.0 ± 1.8	18.0 ± 4.3	15.7 ± 0.1
PS	7.5 ± 0.6	7.2 ± 0.0	5.1 ± 0.5	6.7 ± 0.2
PA	1.9 ± 0.1	1.1 ± 0.4	1.8 ± 0.1	1.9 ± 0.3
CL	5.0 ± 0.3	5.1 ± 0.1	4.7 ± 0.1	4.0 ± 0.1

The numbers are shown as mole percentage of the phospholipids represented in this table. Other phospholipid classes were not detectable or detected at trace levels. The data shown is the average (± variation) of results from two independent cultures. Strains were grown in the presence of choline.

The similar phospholipid compositions exclude differential effects of phospholipid composition on enzyme activities and indicate that the concentrations of endogenous substrates are similar in all strains. The virtual lack of the preferred substrates for the second methyltransferase implies that this enzyme is to some extent capable of using PE as a substrate, as was found before [Kodaki and Yamashita, 1987; Summers *et al.*, 1988].

Panel B of Figure 1 shows the levels of specific activity relative to the whole cell homogenate, determined for subcellular fractions from the wild-type and the single knock-out strains, respectively. In all three strains, the activity is highly enriched in the microsomal fraction, compared to the homogenate, and is depleted from the mitochondria after gradient purification, in agreement with the ER localization of both methyltransferase enzyme activities [Kuchler, Daum and Paltauf, 1986; Zinser *et al.*, 1991]. However, it should be noted that the enrichment of PEMT in the microsomal fraction is significantly lower than that of PLMT, and that the enrichment of both PNMTs in the microsomal fraction of the wild-type strain is intermediate between the two.

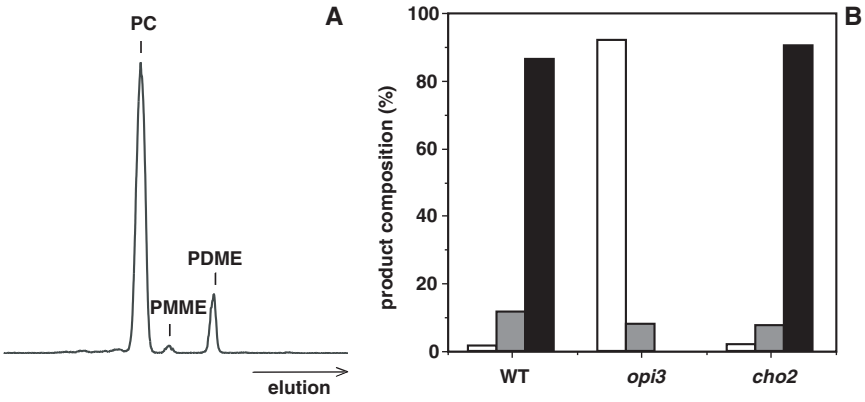

Figure 2. Products formed by the methyltransferase enzymes in microsomes. The products formed by the methylation reactions with [^{3}H]SAM were determined by TLC analysis after a 10 min incubation at 30˚C using 25 µg of microsomal protein. A: TLC analysis of [^{3}H]methyl labeled lipids from wild-type microsomes; B: product composition of the methylation reactions in microsomes of the strains indicated, expressed as the % of [^{3}H] label incorporated into PMME (white bars), PDME (gray bars), and PC (black bars), together set at 100%.

The product compositions of the methylation reactions in microsomes from the wild-type and single disruption strains were analyzed by TLC (Figure 2). The microsomal fractions produce similar relative amounts of methylated PE derivatives as the corresponding homogenates (data not shown). For the wild-type strain, PC is the principal product, with minor amounts of PMME and PDME. The *opi3* strain, which only contains PEMT, produces PMME as major product, as reported before for *opi3* strains [Kodaki and Yamashita, 1987; Summers *et al.*, 1988; Gaynor and Carman, 1990; Preitschopf *et al.*, 1993], although some conversion to PDME was observed. For the *cho2* strain, which only has PLMT, the major product is PC, as reported before for *cho2* strains [Kodaki and Yamashita, 1987; Summers *et al.*, 1988; Preitschopf *et al.*, 1993]. The overall product composition in this strain is very

similar to that in the wild-type, including the presence of some PMME. These results demonstrate that the substrate specificity of both methyltransferase enzymes is not absolute since both are able to utilize the non-preferred substrates, although with a much lower efficiency, in agreement with literature [Kodaki and Yamashita, 1987; Summers *et al.*, 1988].

Cooperativity between PEMT and PLMT activities

To get a first indication whether the methyltransferases are capable of converting substrates provided in another membrane, homogenates from the single knock-out strains were mixed and the incorporation of [^{3}H] label and the composition of the products formed were monitored. Figure 3 shows the time course of the incorporation of [^{3}H]methyl label into a 1:1 mixture of homogenates of both single methyltransferase mutants (100 µg protein each) compared to that in the separate homogenates (100 µg protein each).

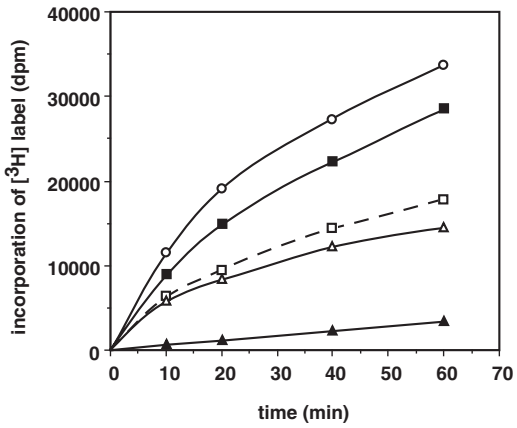

Figure 3. Effect of mixing *opi3* and *cho2* homogenates on incorporation of [^{3}H]methyl label. The incorporation of [^{3}H] label into chloroform soluble material was determined after incubation of homogenate samples (100 µg on protein basis) with [^{3}H]SAM at 30˚C for the times indicated. A typical experiment is shown. The incorporation into wild-type (○), *opi3* (Δ), *cho2* (▲), a 1:1 mixture of *opi3* and *cho2* homogenates (■, 100 µg of each), and the incorporation expected in such a mixture, calculated as the sum of the separate incorporations (□), are depicted.

The combined activity exceeds that expected based upon the separate activities of PEMT and PLMT in 100 µg homogenate of each. At all time points, the amount of radioactivity incorporated in the mixture exceeds the sum of the amounts of radioactivity incorporated in the separate homogenates by some 50%, and approaches the amount of label incorporated into 100 µg of the wild-type homogenate. As a control, the amount of radioactivity incorporated into 200 µg of each homogenate was measured. This was found to be exactly twice the amount of radioactivity incorporated into 100 µg samples (data not shown),

excluding that the difference was due to different extraction efficiencies in samples containing different concentrations of protein and lipid. Furthermore, the increased incorporation was not due to the mere presence of extra microsomal membranes, since the amount of radioactivity, incorporated into a mixture of 100 µg of *cho2* homogenate and 100 µg homogenate of the double knockout (*cho2 opi3*), corresponded exactly to the sum of the amounts of radioactivity, incorporated into the separate samples (data not shown). Thus, it appears that, in a mixture of *opi3* and *cho2* homogenates, the substrate generated by the first methyltransferase enzyme becomes available for the second enzyme, leading to higher incorporation. Figure 4A shows that the composition of the products formed by the mixture of homogenates from the single mutants is shifted towards a higher PC content compared to the product composition calculated based on the incorporation of label and product compositions of the separate homogenates. This is visible even more clearly when the data is presented as the amount of radioactivity in each methylated PE derivative (Figure 4B).

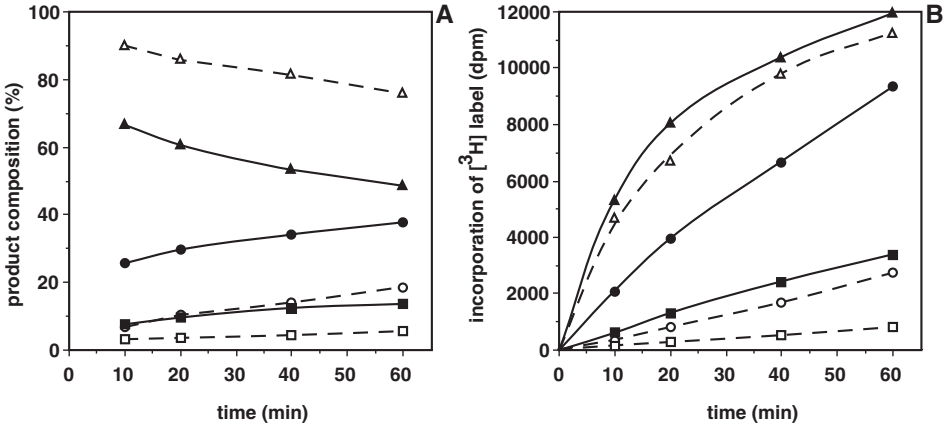

Figure 4. Effect of mixing *opi3* and *cho2* homogenates on the product composition and on the incorporation of [^{3}H] label into the products. The products formed by the methylation reactions with [^{3}H]SAM during incubation at 30˚C for the times indicated were determined by TLC analysis. A typical experiment is shown. The closed symbols and continuous lines represent the values measured in a 1:1 mixture of *opi3* and *cho2* homogenates (100 µg of each, on protein basis) as calculated from the incorporation of [^{3}H] label (see Figure 3) and the corresponding TLC data. The open symbols and dotted lines represent the values expected in such a mixture, as calculated from the incorporation of [^{3}H] label (see Figure 3) and the product composition measured in separate incubations. A: the product composition is expressed as the % of [^{3}H] label incorporated into PMME (▲,Δ), PDME (■,□), and PC (●,○), together set at 100%; B: the data shown in (A) is expressed as the amount of radioactivity incorporated in each of the lipids shown.

While the amount of PMME is hardly affected upon mixing the single mutant homogenates, a large increase is observed in the amounts of PC and PDME produced. This result indicates, indeed, an increased substrate availability for PLMT. Increased synthesis of PDME and PC while the amount of the intermediate PMME is not reduced implies enhanced synthesis of PMME. This suggests that in the absence of the PLMT enzyme there might be some product inhibition of PEMT or that the substrate availability for the first methyltransferase enzyme is also higher in the mixture.

To investigate whether soluble factors or membranes other than microsomal membranes are of importance for the increased substrate availability, the possible cooperativity of the methyltransferases was examined in mixtures of isolated microsomes from the single deletion mutants. Figure 5 shows the incorporation of [^{3}H]SAM into 1:1 mixtures of microsomes of both single methyltransferase mutants and into the separate microsomal fractions at different time points. The cooperative effect observed in the mixture of the microsomal fractions is of similar magnitude as that in the homogenate mixture. Figure 6A shows the product composition of the mixture, compared to what is expected based on the incorporation of label and the product composition of the separate fractions. A decrease in the relative amount of PMME and small increases in the relative amounts of PDME and PC are observed. The change in product formation is more obvious when the data is expressed as the amount of each methylated PE derivative, as shown in Figure 6B.

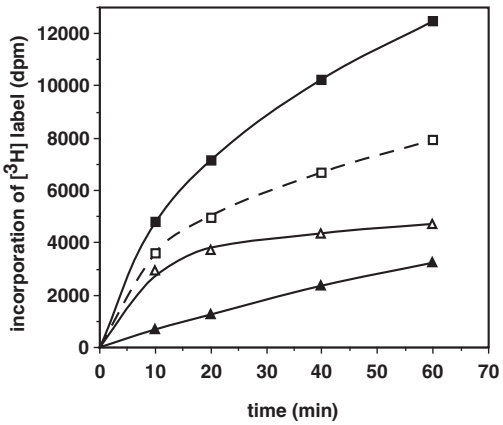

Figure 5. Effect of mixing *opi3* and *cho2* microsomes on the incorporation of [^{3}H]methyl label. The incorporation of [^{3}H] label into chloroform soluble material was determined after incubation of microsomal samples (25 µg on protein basis) with [^{3}H]SAM at 30°C for the times indicated. A typical experiment is shown. The incorporation into *opi3* (Δ), *cho2* (▲), a 1:1 mixture of *opi3* and *cho2* microsomes (■, 25 µg of each), and the incorporation expected in such a mixture, calculated as the sum of the separate incorporations (□), are depicted.

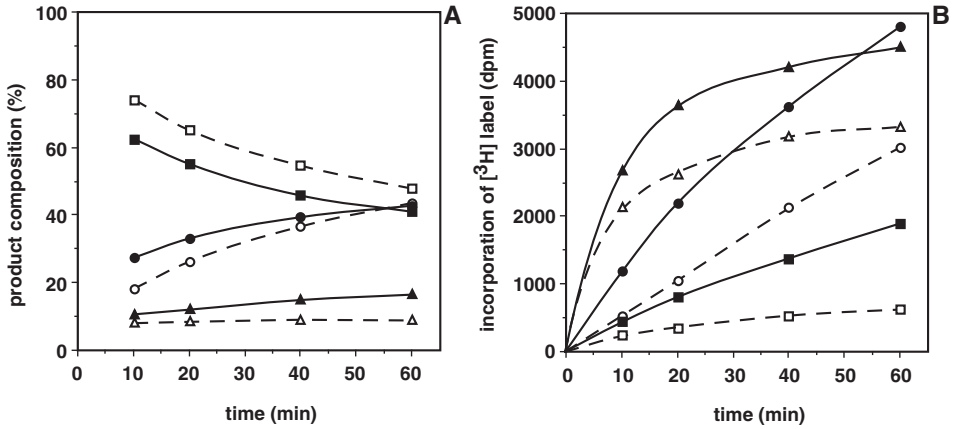

Figure 6. Effect of mixing *cho2* and *opi3* microsomes on the product composition and on the incorporation of [^{3}H] label into the products. The products formed by the methylation reactions with [^{3}H]SAM during incubation at 30˚C for the times indicated were determined by TLC analysis. A typical experiment is shown. The closed symbols and continuous lines represent the values measured in a 1:1 mixture of *opi3* and *cho2* microsomes (25 µg of each, on protein basis) as calculated from the incorporation of [^{3}H] label (see Figure 5) and the corresponding TLC data. The open symbols and dotted lines represent the values expected in such a mixture, as calculated from the incorporation of [^{3}H] label (see Figure 5) and product composition measured in separate incubations. A: the product composition is expressed as the % of [^{3}H] label incorporated into PMME (▲,Δ), PDME (■,□), and PC (●,○), together set at 100%; B: the data shown in (A) is expressed as the amount of radioactivity incorporated in each of the lipids shown.

The amount of PMME product is elevated in the mixture compared to the separate incubations, in accordance with the suggestion of increased substrate availability for PEMT or relief of product inhibition (because of dilution into a larger product matrix). The substrate availability for the second methyltransferase enzyme is increased in the mixtures judged from the fact that the amounts of PDME and PC formed are highly increased, although not to the same extent as in the homogenate mixtures. This could be due to a dependence of the exact product composition on the relative amounts of activity of each enzyme present in the mixture. As mentioned, these differ between microsomes and homogenates, due to different enrichments of PEMT and PLMT enzyme in the microsomal fractions compared to the homogenates (a factor of 2 to 3 in the *opi3* strain *vs.* a factor of 5 to 6 in the *cho2* strain, see Figure 1B). Indeed, as shown in Figure 7, decreasing the relative amount of microsomes containing PLMT results in a higher relative increase in the amount of PC and PDME produced, compared to the separate incubations, than for equal amounts of both types of microsomes. This indicates that the cooperative effect occurring in microsomes is similar to that found in whole cell homogenates.

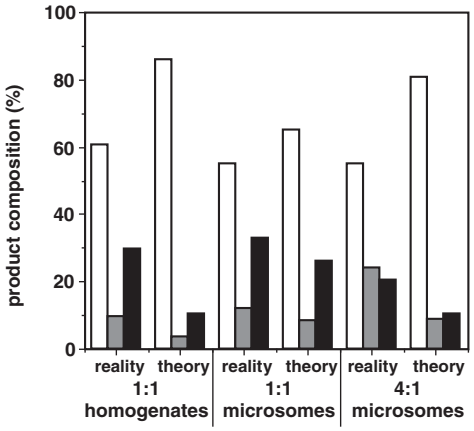

Figure 7. Effect of varying the ratio of PEMT and PLMT on the product composition. The product compositions, measured in mixtures of *opi3* or *cho2* homogenates or microsomes (reality), are compared to the expected product compositions of such mixtures (theory), as calculated from the incorporation of [^{3}H] label and the product composition measured in separate incubations. A 1:1 ratio corresponds to 100 µg samples for homogenates and to 25 µg samples for microsomes of each strain on protein basis. A 4:1 ratio corresponds to 20 and 5 µg microsomal samples on protein basis, of the *opi3* and *cho2* strains, respectively. The product composition is expressed as the % of [^{3}H] label incorporated into PMME (white bars), PDME (gray bars), and PC (black bars), together set at 100%. The products formed by the methylation reactions with [^{3}H]SAM were determined by TLC-analysis after a 20 min incubation at 30˚C.

To get more insight into the mechanism of cooperativity, we wanted to determine whether the substrate for methylation has to be present in the same membrane for the methyltransferase enzymes to act upon it, *i.e.*, does the substrate have to be transported to the membrane containing the enzyme, or can the enzyme convert the substrate present in another membrane? To determine in which microsomal membrane the methylated products reside, one should separate the two populations of microsomes (*opi3* and *cho2*) after the incubation. Therefore, the possibility was explored to separate different populations of microsomes after incubation (data not shown). This might be achieved by immobilizing one population *via* biotinylation and binding to avidin beads so that, after the incubation in the presence of another untreated population, the biotinylated population can be pelleted together with the avidin beads. The biotinylation of microsomes was successful as found in Western blotting experiments using avidin coupled to HRP for detection by ECL. Biotinylation did not affect the methyltransferase activity. However, the biotinylated microsomes could not be pulled down with avidin beads, as judged from Western blotting experiments using avidin-HRP and the lack of association of methyltransferase activity to the avidin beads.

Presentation of phospholipid substrate as SUV

The capability of the methyltransferases in the microsomal membrane to convert substrate from another membrane than microsomes was investigated by presenting substrate in the form of SUV. In such a system it is in principle possible to separate both types of membranes after the incubation by centrifugation. Microsomes from strain D273-10B were used, with a specific activity of the PNMTs of approximately 15 nmol/min/mg. The product composition was similar to that found for the wild-type strain (SH921) used so far in this study (not shown). TLC analysis shows that the microsomes are capable of converting [^{14}C]DOPE presented in SUV to [^{14}C]PC when provided with SAM (Figure 8, *cf.* lanes 2 and 1). Since PC is the major product formed by wild-type microsomes, the conversion of [^{14}C]PE was expressed as the percentage converted into [^{14}C]PC. After 60 min at 30˚C 11 ± 2.7% (n = 8) conversion had occurred (corresponding to approximately 2 nmol of SUV PE used as substrate). The total amount of [^{3}H]SAM incorporated was found to be approximately 12 nmol (not shown) and was similar in the absence or presence of SUV.

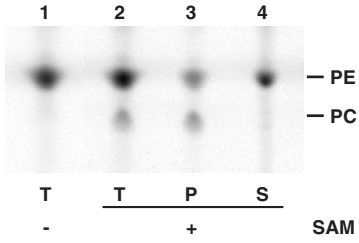

Figure 8. TLC analysis of the conversion of phospholipid substrate presented in the form of SUV by microsomes. Wild-type (D273-10B) microsomes were incubated together with SUV containing [^{14}C]PE for 60 min at 30˚C in the absence (-) or presence (+) of SAM. After the incubation the conversion of [^{14}C]PE to [^{14}C]PC was determined by TLC-analysis, before (T: total sample) or after separating the microsomes from the SUV by centrifugation (P: microsomal pellet; S: supernatant). For more details, see the 'Materials and methods' section.

From these results it can be calculated that about half of the total amount of PE converted to PC in the reaction originates from the SUV, indicating that the PNMTs convert exogenously added phospholipid substrate as readily as endogenous substrate. Upon separation of microsomes and SUV after the incubation, it was found that all of the [^{14}C]PC formed was associated with the microsomal pellet, as can be seen from Figure 8 (lanes 3 and 4). Of the total amount of [^{14}C] label associated with the pellet 33 ± 3% (n = 5) was converted to PC. To see whether the [^{14}C] labeled lipids in the pellet were present in the microsomes or in the SUV, [^{3}H]CE was used as a non-exchangeable marker present in SUV to be able to calculate the amount of contamination of the microsomal pellet with SUV. The amount of [^{3}H]CE

label associated with the pellet was found to be similar to the total amount of [^{14}C]lipid in the pellet (35% and 38%, respectively). These data indicate that the microsomal enzymes are capable of converting phospholipid substrate presented in the form of SUV, and that conversion occurs only in SUV associated with the microsomes.

Discussion

The aim of this study was to investigate the possibility that the phospholipid-*N*-methyltransferases act upon phospholipid substrates localized in a different membrane than the one in which the enzymes reside (the so-called 'trans-catalysis' hypothesis). For this purpose, subcellular fractions were isolated from *opi3* and *cho2* gene disruptant strains. Since choline was provided in the growth medium, the PC levels in the disruptants were similar to that in the wild-type as found on glucose-based media [Kodaki and Yamashita, 1987; McGraw and Henry, 1989]. As was found before for the combined activity of the PNMTs [Kuchler, Daum and Paltauf, 1986; Zinser *et al.*, 1991], both PEMT and PLMT were enriched in the microsomal fraction, although to different extents. The relative enrichment of PEMT in the microsomal fraction is lower than that of PLMT, while the relative enrichment of the combined PNMTs in the microsomal fraction of the wild-type strain is intermediate between the two. This result could indicate a slight difference in localization of both methyltransferase enzymes, as was proposed before based on the appearance of methylation intermediates in the Golgi and the presence of a C-terminal ER retention signal in PLMT but not in PEMT [Schneiter *et al.*, 1999].

Upon mixing of homogenates of the single *cho2* and *opi3* knock-out strains, the combined methyltransferase activity, measured as the incorporation of label from [^{3}H]SAM, exceeds that expected based on the separate activities. A similar cooperativity of the PEMT and PLMT enzymes was found in a mixture of isolated microsomes, excluding the involvement of soluble factors or membranes other than microsomal membranes in the phenomenon. These results indicate that the substrate generated by PEMT becomes available for PLMT, for which enzyme the amount of phospholipid substrate is otherwise limiting the incorporation of label from [^{3}H]SAM. The product composition with increased levels of PC and PDME provided further support for increased substrate availability for PLMT in the mixture. PMME was also synthesized in increased quantities, suggesting either that product inhibition of PEMT occurs in the absence of PLMT activity or that the substrate availability for PEMT is also increased in the mixture.

The exact product composition was found to depend on the relative amount of the activity of each enzyme in the mixture. Decreasing the relative amount of PLMT resulted in a higher relative increase in the amount of PC and PDME produced. Furthermore, it was found that the incorporation of label increased to a similar extent (approximately 50%) relative to what was expected based on the separate levels of incorporation in microsomes for ratios

varying from 1:1 to 1:9 (*opi3*:*cho2*) while keeping the total protein concentration constant (data not shown). These results indicate that the formation of PMME is most likely the rate-limiting step in the cooperative effect.

There are several possibilities to explain the observed increase in substrate availability. It could be due to 'trans' action of the methyltransferase enzymes, or to fusion of membranes or to transport of substrates between membranes. To distinguish between these mechanisms, we wanted to determine whether the substrate for methylation has to be present in the same membrane for the methyltransferase enzymes to act upon it. Therefore, microsomes were mixed with SUV, since this might allow for separation of both types of membranes after the incubation. It was found that wild-type microsomes were capable of converting [^{14}C]PE presented in the form of SUV to [^{14}C]PC and that the exogenous substrate was utilized to a similar extent as the endogenous substrate in the microsomal membranes. However, upon separation by centrifugation all of the product was found associated with the microsomes. Although the data suggest that there is no specific transfer of the [^{14}C] labeled substrate or product from SUV to microsomes, association appears to be a requirement for methylation of PE. Unfortunately, the occurrence of fusion between SUV and microsomes cannot be excluded based on the present data. Previously, studies have appeared where substrates were added in the form of sonicated lipid suspensions to examine the methylation activities of PEMT and PLMT [Gaynor and Carman, 1990; Kodaki and Yamashita, 1987 and 1989]. From the data in those studies, it is clear that the enzymes use the exogenously added substrates and exhibit increased activity when the amount of phospholipid substrate is no longer limiting.

Even though the present results exclude the involvement of soluble cellular factors in the process of cooperativity demonstrated between microsomes, we are unable to pinpoint the effects to any of the possible mechanisms. However, it is very unlikely that fusion of microsomes is involved based on literature data. Fusion of yeast ER membranes *in vitro* is a process that requires ATP and even under optimized conditions it does not exceed 20% after an hour of incubation [Latterich and Schekman, 1994]. In our *in vitro* system, ATP is not present. Furthermore, since after 10 min already a similar relative increase in the incorporation of substrate can be observed as at later times, the cooperativity must be due to a very fast and efficient process. A collision-based process resulting either in transport of substrate or in 'trans-catalysis' thus seems to be more plausible. In favor of the latter explanation, it can be noted that a bacterial phospholipid methyltransferase enzyme has been identified that appears to be a soluble protein [Arondel, Benning and Somerville, 1993].

Subcellular fractions isolated from mutants defective in the PE methylation pathway could also be useful to study the process of transport of PC to mitochondria. In an *in vitro* assay system using isolated organelles, the problem that isolated mitochondria are still contaminated with endoplasmic reticulum, even after purification, can be circumvented by

the use of mutants in PC biosynthesis. When mitochondria from a strain still capable of synthesizing PC are used, one cannot be sure that any PC produced in the assay ending up in the mitochondrial fraction does not originate from adhering endoplasmic reticulum. Use of mitochondria from a strain deficient in the route of PC synthesis employed in the assay would preclude this complication. The present study provides a basis for further investigations in that direction.

Acknowledgements

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Chapter 5

Phosphate is required to maintain the outer membrane integrity and membrane potential of respiring yeast mitochondria

M.J.F.W. Janssen, B. de Kruijff, A.I.P.M. de Kroon

submitted for publication

Abstract

In this study, the buffer requirements to maintain mitochondrial intactness and membrane potential in *in vitro* studies were investigated, using gradient purified yeast mitochondria. It was found that the presence of phosphate is crucial for the generation of a stable membrane potential and for preserving the intactness of the outer membrane, as assessed by probing the accessibility of Tom40p to trypsin and the leakage of cytochrome *b2* from the intermembrane space. Upon addition of respiratory substrate in the absence of phosphate, mitochondria generate a membrane potential that collapses within one minute. Under the same conditions, the mitochondrial outer membrane is disrupted. The presence of phosphate prevents both these phenomena. The ΔpH component of the proton motive force appears to be responsible for the compromised outer membrane integrity. The collapse of the membrane potential is reversible to a limited extent. Only when phosphate is added soon enough after the addition of exogenous respiratory substrate can a stable membrane potential be obtained again. Within a few minutes, this capacity is lost. The presence of Mg^{2+} prevents the rupture of the outer membrane, but does not prevent the rapid dissipation of the membrane potential. Similar results were obtained for mitochondria isolated and stored in the presence of dextran or BSA.

Introduction

For *in vitro* studies using isolated mitochondria, it is important to know how the integrity, bioenergetics, and stability of the organelle are affected by the experimental conditions. In particular when studying the biogenesis of mitochondria, conservation of the membrane barrier properties of the mitochondrial membranes is a prerequisite for interpretation of the data.

Numerous studies have appeared on the bioenergetics of yeast mitochondria and the effects of energization on ultrastructural organization and inner membrane permeability. It has been reported that phosphate protects mitochondrial ultrastructure [Velours, Rigoulet and Guérin, 1977] and counteracts the yeast mitochondria permeability channel by inhibiting changes in inner membrane permeability under conditions of energization (for a review, see [Manon *et al.*, 1998]). A role in the regulation of inner membrane permeability was also attributed to magnesium [Guérin *et al.*, 1994; Jung, Bradshaw and Pfeiffer, 1997]. However, the implications of these findings for *in vitro* studies on mitochondrial biogenesis are not generally appreciated. Although many investigators studying mitochondria *in vitro* routinely add phosphate and magnesium to their buffers [Glick, 1995; Yaffe, 1991; Wagner *et al.*, 1994], it is certainly not common practice (*e.g.* in [Swanson and Roise, 1992; Simbeni *et al.*, 1993; Söllner, Rassow and Pfanner, 1991; Geissler *et al.*, 2000], either both or one of these components were omitted).

There are no reports to date of a systematic analysis of the requirements for conserving outer membrane integrity in combination with a stable membrane potential in yeast mitochondria in response to energization. The aim of the present study is to define the experimental conditions required to maintain mitochondrial intactness and a stable membrane potential upon energization in *in vitro* experiments using isolated yeast mitochondria. The effects of phosphate and magnesium were studied. The integrity of the outer membrane was monitored by assessing the permeability of this membrane to proteins of different sizes, *i.e.* by the accessibility of Tom40p to trypsin (23 kDa) and the leakage of cytochrome *b2* (57 kDa) from the intermembrane space. In parallel, the membrane potential was monitored using the membrane potential sensitive fluorescent probe 3,3'-diethylthiadicarbocyanine iodide ($diSC_2(5)$). It was found that phosphate is required to maintain the outer membrane integrity and membrane potential of respiring yeast mitochondria.

Materials and methods

Materials

ECL reagents were purchased from DuPont NEN. Yeast extract and nycodenz were from Sigma, as was BSA (fatty acid content 0.001%). Zymolyase was obtained from Seikagaku (Japan). Dextran (M_r 40,000) was supplied by Roth (Karlsruhe, FRG). $DiSC_2(5)$ was from Molecular Probes (Leiden, NL). All other chemicals were analytical grade.

Isolation of mitochondria

The wild-type yeast (*Saccharomyces cerevisiae*) strain D273-10B was grown aerobically to late log (OD600 4-5 (Perkin Elmer Lambda 18 UV/VIS spectrophotometer)) at 30˚C in semi-synthetic lactate medium [Daum, Böhni and Schatz, 1982]. Spheroplasts were prepared using zymolyase as described previously [Daum, Böhni and Schatz, 1982]. The isolation and purification of mitochondria were based on published procedures [Daum, Böhni and Schatz, 1982; Gaigg *et al.*, 1995; Glick and Pon, 1995]. The cellular homogenate was prepared using a buffer containing 10 mM MES, pH 6.0, and 0.6 M sorbitol (D buffer), to which 1 mM PMSF was added, and which was supplemented with 0.2% (w/v) BSA or with 0.5% (w/v) dextran instead. Crude mitochondria were isolated using D buffer and were further purified by nycodenz gradient centrifugation as reported [Glick and Pon, 1995], with some minor changes. The nycodenz gradient was made in D buffer and consisted of a layer of 18% nycodenz overlaid with a 13.5% instead of a 14.5% (w/v) nycodenz layer. The final mitochondrial pellet was resuspended in 20 mM Hepes/KOH, pH 7.4 containing 0.6 M sorbitol (H/S buffer) including either 0.5% (w/v) BSA [Kozlowski and Zagorski, 1988] or 5% (w/v) dextran. Aliquots were snap-frozen with liquid nitrogen, stored at -80˚C, and thawed only once before use. The concentration of mitochondria used in experiments is

always expressed as the protein concentration determined by using the BCA method (Pierce) with 0.1% (w/v) SDS added and BSA as a standard. H/S buffer was used in all experiments unless stated otherwise, without further addition of BSA or dextran.

Assessment of effects of incubation conditions on mitochondrial intactness

Mitochondria were thawed on ice before use. All samples were kept on ice unless stated otherwise. To investigate the effect of different conditions on the intactness of the mitochondria, 20 µg protein aliquots of the mitochondrial suspension were incubated for 10 min, on ice or at 20˚C, in a volume of 100 µl H/S buffer, including additives as indicated. NADH and ethanol were used at concentrations of 2 mM or 1% (v/v) (170 mM), respectively. FCCP and valinomycin were added from stock solutions in ethanol to final concentrations of 20 and 5 µM, respectively (final ethanol concentration 1% (v/v)). Potassium phosphate (KH_2PO_4) and $MgCl_2$ were added to concentrations of 2 and 10 mM, respectively.

The intactness of the mitochondrial outer membrane after these treatments was assessed by probing the accessibility of Tom40p to trypsin or, alternatively, by measuring the leakage of cytochrome *b2* from the intermembrane space. In control experiments, digitonin (0.2% (w/v)) was added to destroy the outer membrane, or mitoplasts were prepared by diluting the mitochondria at least a factor of 10 in hypotonic buffer (20 mM Hepes/KOH, pH 7.4), and incubating for 20 min on ice [Jascur, 1991]. Trypsin was added from a 1 mg/ml stock solution in H/S to the samples to a final concentration of 100 µg/ml and after 20 min on ice the action of trypsin was stopped by TCA precipitation, as described below. For measuring leakage of cytochrome *b2*, samples were centrifuged for 12 min at 10,000 rpm (10,600 x *g*) in a microfuge at 4˚C. Pellet and supernatant were separated and the pellet was resuspended in H/S buffer. TCA precipitations were performed by adding one volume of 10% (w/v) TCA and incubating on ice for 20 min. After centrifugation for 15 min at 14,000 rpm, the precipitated protein was washed with 50 µl of ice-cold acetone followed by 20 µl of ice-cold water. Protein pellets were dissolved in SDS-PAGE sample buffer, heated at 95˚C for 5 min and aliquots were subjected to SDS-PAGE (10% gel) and Western blotting. The blots were decorated with antibodies raised against Tom40p and cytochrome *b2*. Protein bands were visualized by ECL according to the manufacturer's instructions.

Measurement of mitochondrial membrane potential and respiration

Mitochondrial membrane potential was monitored by recording the fluorescence of the voltage sensitive dye $diSC_2(5)$ [Sims *et al.*, 1974]. Fluorescence was measured on an Aminco SLM500C spectrofluorometer using excitation and emission wavelengths of 650 nm and 670 nm, respectively (bandpass 5 nm for both). The dye (final concentration 0.5 µM) was added from a 0.5 mM stock solution in DMSO to the mitochondria (final protein

concentration 40 μg/ml) suspended in H/S buffer. Respiratory substrates were added to final concentrations of 0.5 mM and 0.1% (v/v) (17 mM) for NADH and ethanol, respectively. To dissipate the membrane potential, either valinomycin was added from an ethanolic stock to a final concentration of 1 μM, or gramicidin D was added from a 1 mg/ml stock solution in DMSO to a final concentration of 1 μg/ml. Measurements were performed under continuous stirring in a final volume of 2 ml at a temperature of 20˚C.

Respiratory activity of isolated mitochondria was monitored with an Oxygraph respirometer (Anton Paar K.G., Graz, Austria). 160 μg of mitochondria (on protein basis) were used in a volume of 2 ml H/S buffer at a temperature of 20˚C.

Results

Characterization of the mitochondria

In conventional preparations of mitochondria, BSA is often included in the homogenization buffer, and also added to the final preparation for storage in the freezer [Daum, Böhni and Schatz, 1982] and to experimental samples [Glick, 1995] to protect the mitochondria. In our laboratory, we are investigating the import process of phosphatidyl-choline into yeast mitochondria. Since BSA might interfere in *in vitro* studies on phospholipid transport because of its interaction with lipids, it was replaced with dextran in the isolation procedure. It is expected that dextran offers similar protection of mitochondria by maintaining a more physiological colloid osmotic balance across the outer membrane (based on *e.g.* [Wrogemann *et al.*, 1985; Wicker *et al.*, 1993]). Mitochondria isolated and stored in the presence of BSA (BSA mitochondria) or dextran (dextran mitochondria) were found to be similar in every respect in the present study, with the exception of the amount of endogenous respiratory substrate, which was consistently lower in dextran mitochondria than in BSA mitochondria, as will be explained later. All the results shown in this paper were obtained with dextran mitochondria, unless indicated otherwise.

The intactness of the isolated mitochondria was assessed by probing the accessibility of Tom40p to trypsin, and the leakage of cytochrome *b2* from the intermembrane space. Tom40p cannot be degraded by trypsin when the mitochondrial outer membrane is intact [Hines *et al.*, 1990]. Cytochrome *b2*, which is a soluble protein located in the intermembrane space [Daum, Böhni and Schatz, 1982], can only leak out when the outer membrane is disrupted. Figure 1 shows that after incubation of mitochondria with trypsin (lane 4), only a small portion of Tom40p is degraded to a lower molecular weight band (designated Tom40p*). In contrast, in mitoplasts (lane 2) and in mitochondria treated with digitonin (lane 3), trypsin completely converted all of the Tom40p to Tom40p* and also digested all of the cytochrome *b2*. Upon centrifugation of mitochondria, cytochrome *b2* is quantitatively retained in the mitochondrial pellet (*cf.* lanes 7 and 8), while in mitoplasts, cytochrome *b2* is

released into the supernatant (*cf.* lanes 5 and 6). These results indicate that during isolation, a small fraction of the mitochondria is broken, rendering Tom40p accessible to trypsin. However, after storage, no additional membrane rupture occurs, since there is no leakage of intermembrane space proteins into the supernatant. The isolated mitochondria are estimated to be 90-95% intact, similar to what has been reported by others [Kaput *et al.*, 1989].

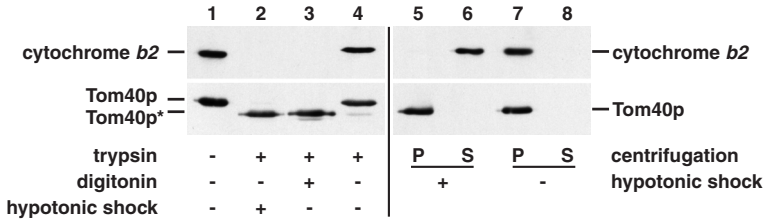

Figure 1. The intactness of isolated mitochondria. The intactness of the mitochondrial outer membrane was probed by subjecting the mitochondria to trypsin treatment. The degradation of Tom40p (to Tom40p*), and of cytochrome *b2* was visualized by Western blotting (left panel). Alternatively, the mitochondria were subjected to centrifugation to examine whether cytochrome *b2* is released from the mitochondria. Pellets (P) and supernatants (S) were subjected to Western blotting (right panel). Controls include disruption of the outer membrane by hypotonic shock, or solubilization of the mitochondria by digitonin, prior to trypsin treatment or centrifugation. For more details, see the 'Materials and methods' section.

Effects of energization

We investigated the effects of energization by addition of a respiratory substrate. Figure 2A shows the oxygraph trace of mitochondria in buffer at 20˚C, displaying a certain low basal level of respiration, depending on the amount of endogenous substrate, and a rapid increase upon addition of exogenous respiratory substrate. Both ethanol and NADH (not shown) result in increased oxygen consumption. These results indicate that the mitochondria are capable of respiration and can metabolize exogenous substrate. Figure 2B depicts the membrane potential measurement performed under similar conditions (20˚C). Addition of the membrane potential sensitive probe to mitochondria leads to a certain fluorescence level. Upon addition of respiratory substrate (ethanol and NADH have a similar effect) the fluorescence decreases, indicating that a membrane potential is generated. However, the fluorescence level returns to the initial value within minutes, demonstrating that the membrane potential is unstable. This was found with ethanol as well as with NADH (not shown). The membrane potential could not be regained by addition of an extra portion of substrate, indicating that depletion of substrate is not the cause of the instability of the membrane potential (not shown).

Figure 2C shows how the mitochondrial intactness is affected by a 10 min incubation under these conditions. The intactness of the outer membrane is no longer maintained, as can be inferred from the complete accessibility of both cytochrome *b2* and Tom40p to trypsin (lane 3), as well as from the appearance of cytochrome *b2* in the supernatant after centrifugation (*cf.* lanes 8 and 9). This was found with ethanol as well as with NADH (not shown). However, the intactness of the mitochondria is maintained when the incubation is performed without addition of exogenous substrate (lanes 1, 4 and 5) or with substrate at 0˚C (lanes 2, 6 and 7). These results indicate that, in a simple buffer containing Hepes and sorbitol, respiration is responsible for rupture of the outer membrane, and that a stable membrane potential is not obtained under these conditions.

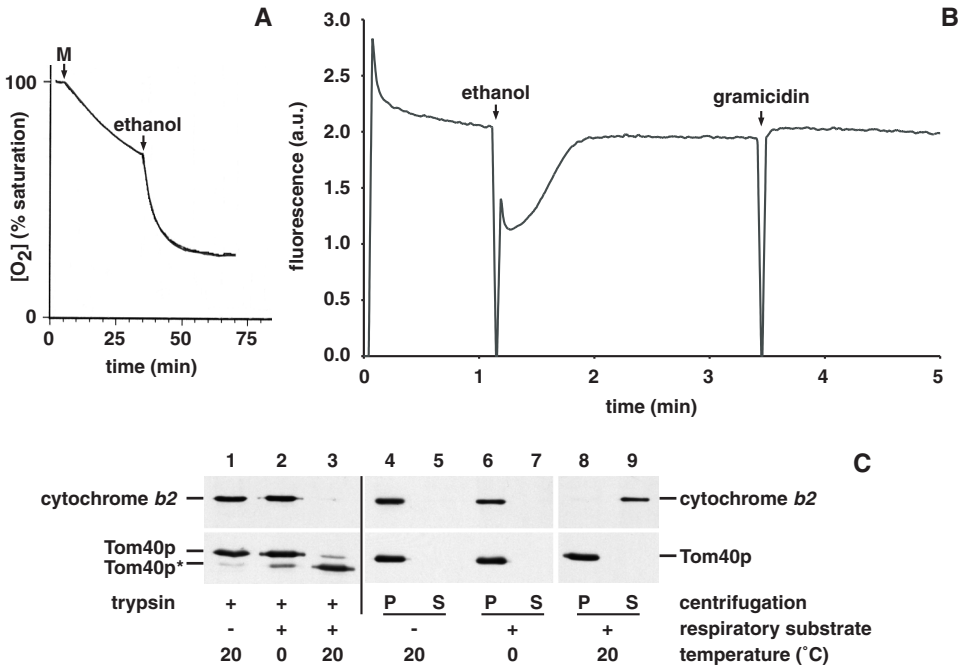

Figure 2. Effects of energization. A: oxygraph trace showing the use of oxygen by mitochondria in buffer. BSA mitochondria were used. The arrows indicate the addition of mitochondria (M) and respiratory substrate. 100% saturation corresponds to approximately 250 µM O_2; B: membrane potential measurement using the membrane potential sensitive fluorescent dye diSC_2(5). At the start of the measurement, diSC_2(5) is added to mitochondria in buffer. The arrows indicate the addition of respiratory substrate, and of gramicidin to dissipate the membrane potential; C: Western blot analysis of the mitochondrial outer membrane intactness. Mitochondria were incubated for 10 min under the conditions indicated. Ethanol was used as a respiratory substrate. Subsequently, the intactness of the outer membrane was probed as described in the legend of Figure 1 by trypsin treatment (left panel), or by centrifugation (right panel, P: pellet; S: supernatant).

To investigate whether the generation of a proton motive force (pmf) induced by respiration in mitochondria is the cause of the rupture of the outer membrane, the uncoupler FCCP was added. Figure 3A shows that dissipation of the pmf by FCCP prevents the rupture of the outer membrane, since Tom40p and cytochrome *b2* are not degraded by trypsin (lane 1), and cytochrome *b2* is retained in the mitochondrial pellet upon centrifugation (*cf.* lanes 3 and 4). This indicates that the (temporary) generation of a pmf leads to the rupture of the outer membrane.

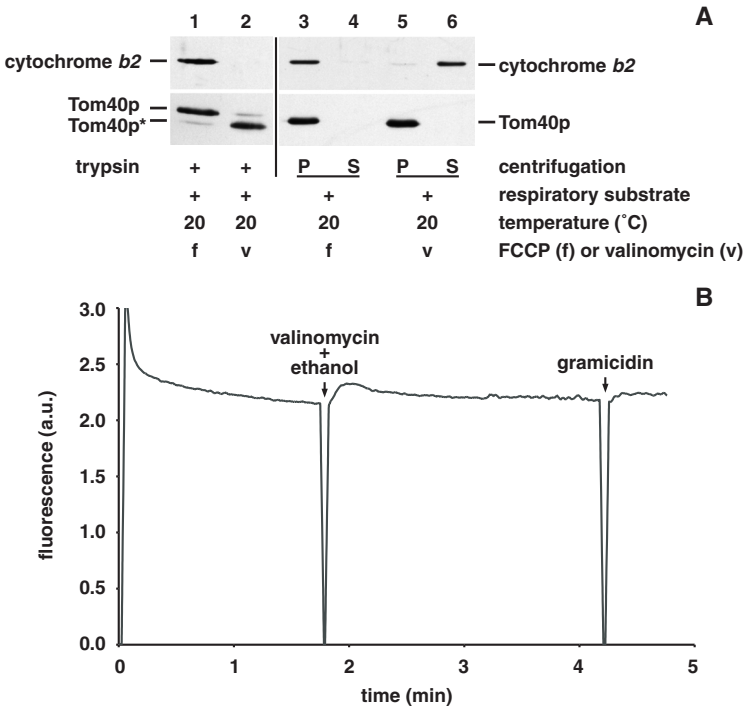

Figure 3. Effects of dissipating the pmf and the membrane potential. A: Western blot analysis of the mitochondrial outer membrane intactness. Mitochondria were incubated for 10 min under the conditions indicated. Ethanol was used as a respiratory substrate. The pmf was dissipated by the addition of FCCP (f), and the membrane potential was dissipated by the addition of valinomycin (v). Subsequently, the intactness of the outer membrane was probed as described in the legend of Figure 1 by trypsin treatment (left panel) or by centrifugation (right panel, P: pellet; S: supernatant); B: membrane potential measurement using the membrane potential sensitive fluorescent dye $diSC_2(5)$. At the start of the measurement, $diSC_2(5)$ is added to mitochondria in buffer. The arrows indicate the addition of valinomycin and respiratory substrate, and of gramicidin to check the dissipation of the membrane potential.

The pmf has two components, a pH gradient (ΔpH) and a membrane potential (ΔΨ) [Rottenberg, 1979]. The membrane potential can be dissipated separately using valinomycin in the presence of K^+ ions [Rottenberg, 1989]. Figure 3B shows that, in the presence of valinomycin and ethanol as a respiratory substrate, the mitochondria are completely unable to form even the temporary membrane potential depicted in Figure 2B, indicating that the potassium concentration in the buffer is sufficient for valinomycin to dissipate the membrane potential. Figure 3A shows that under these conditions the rupture of the mitochondrial membrane is not prevented, as judged from the complete accessibility of Tom40p and cytochrome *b2* to trypsin (lane 2) and the release of cytochrome *b2* into the supernatant after centrifugation (*cf.* lanes 5 and 6). This strongly suggests that the ΔpH component of the pmf is responsible for the damaging effect on the mitochondria.

Buffer requirements for maintenance of mitochondrial integrity and membrane potential

We investigated whether phosphate or magnesium could fulfill the system's requirements for maintaining outer membrane integrity and a stable membrane potential upon respiration. Figure 4A shows that after respiration in the presence of either phosphate or Mg^{2+}, Tom40p is not accessible to trypsin, and that cytochrome *b2* is not degraded by trypsin (lanes 1 and 2) and is retained in the pellet upon centrifugation (*cf.* lanes 3 and 4, and lanes 5 and 6). The membrane potential measurements under both conditions are depicted in Figure 4B. In the presence of phosphate, a stable membrane potential is obtained and maintained for at least an hour if the substrate is replenished (not shown). The membrane potential can be dissipated by addition of either valinomycin or gramicidin. Mg^{2+} (which causes an increase in the initial fluorescence level probably due to decreased association of the probe with the membranes) does not prevent the collapse of the membrane potential shortly after the addition of exogenous respiratory substrate. These data indicate that phosphate and Mg^{2+} are both able to prevent the rupture of the outer membrane, while phosphate, but not Mg^{2+}, is required for maintenance of a stable membrane potential.

Effect of late addition of phosphate

We investigated whether addition of phosphate following the addition of respiratory substrate could result in recovery of the membrane potential. Figure 5 depicts the fluorescence measurements in which phosphate was added at the indicated time points before and after the start of respiration. Addition just prior to the addition of ethanol results in a stable potential (A), as when phosphate is included in the buffer from the start of the experiment (*cf.* Figure 4B). When BSA mitochondria were used, addition of phosphate always resulted in a small decrease in fluorescence (data not shown), most likely due to the presence of some endogenous respiratory substrate which seemed to be absent from dextran

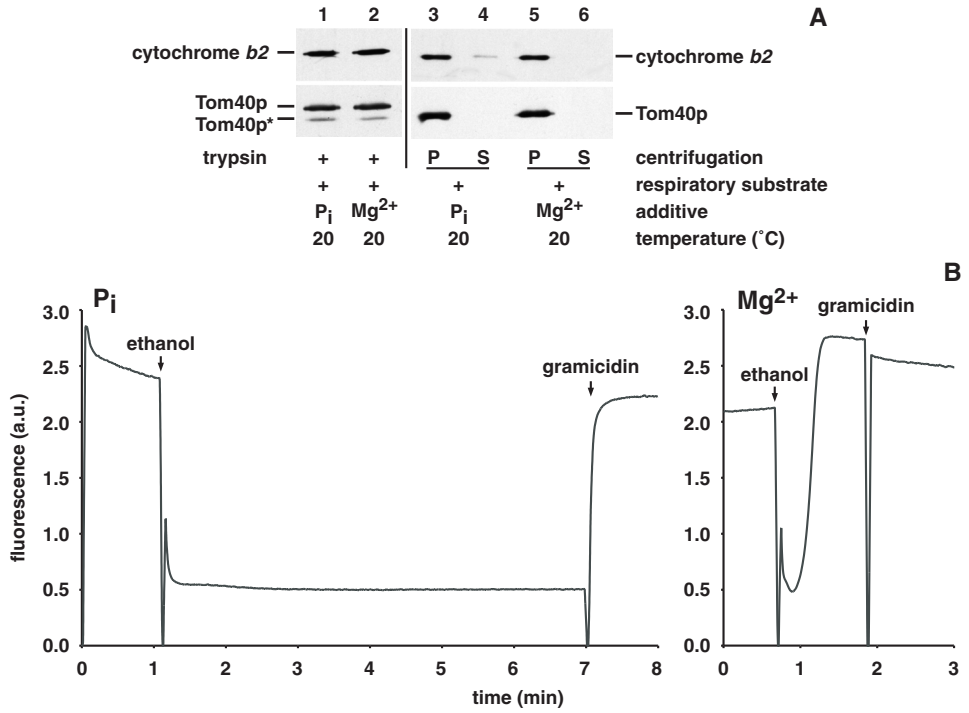

Figure 4. Effects of phosphate and magnesium. A: Western blot analysis of the mitochondrial outer membrane intactness. Mitochondria were incubated for 10 min under the conditions indicated in the presence of phosphate (P_i) or magnesium (Mg^{2+}). Ethanol was used as a respiratory substrate. Subsequently, the intactness of the outer membrane was probed as described in the legend of Figure 1 by trypsin treatment (left panel), or by centrifugation (right panel, P: pellet; S: supernatant); B: membrane potential measurement using the membrane potential sensitive fluorescent dye $diSC_2(5)$. At the start of the measurements, $diSC_2(5)$ is added to mitochondria in buffer in the presence of phosphate (P_i) or magnesium (Mg^{2+}). The arrows indicate the addition of respiratory substrate, and of gramicidin to dissipate the membrane potential. The fluorimeter settings were adjusted for the measurement in the presence of Mg^{2+} to be able to measure the higher level of fluorescence.

mitochondria. When phosphate is added 30 s (B) or 60 s (C) after the addition of respiratory substrate, the fluorescence decreases again to the level obtained when phosphate was present before addition of respiratory substrate (A), indicating that the membrane potential can be recovered. However, when phosphate is added 2 min after the addition of respiratory substrate, only a small decrease in fluorescence is observed (D), and after 5 (E) or 10 min (F) no decrease in the fluorescence is observed, indicating that the membrane potential is no longer recovered. The rate at which the mitochondria undergo the transient formation of the unstable membrane potential and its subsequent dissipation varies slightly from experiment to experiment (as can be seen from Figure 5) and also between different batches of

mitochondria (not shown). Therefore, there is probably also variation in the time allowed for recovery of the membrane potential by addition of phosphate.

Since we found that magnesium protects the mitochondria from outer membrane rupture, we also tested the reversibility of the membrane potential dissipation in the presence of magnesium. The addition of phosphate after 10 min was unable to recover the membrane potential in the presence of Mg^{2+}, while in the presence of Mg^{2+} addition of phosphate 1 min after addition of respiratory substrate resulted in recovery of the membrane potential as it did in the absence of Mg^{2+} (not shown). Therefore, it can be concluded that the addition of phosphate within a short time period after initiation of respiration is important for the recovery of the membrane potential, while Mg^{2+} plays no role in this recovery.

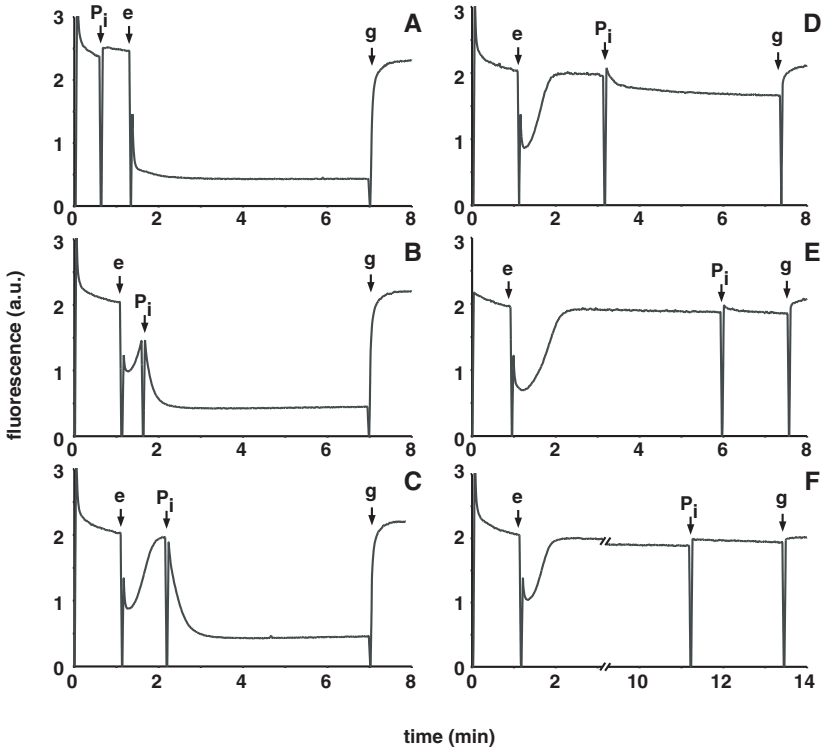

Figure 5. Effect of late addition of phosphate. The membrane potential was measured using the membrane potential sensitive fluorescent dye $diSC_2(5)$. At the start of the measurements, $diSC_2(5)$ is added to mitochondria in buffer. The arrows indicate the addition of phosphate (P_i), ethanol (e) and gramicidin D (g). Phosphate was added before (A), or 30 s (B), 60 s (C), 2 min (D), 5 min (E), or 10 min (F) after the addition of respiratory substrate.

Discussion

The aim of the present study is to define the conditions required to maintain mitochondrial intactness and sustain a stable membrane potential upon energization in *in vitro* experiments using isolated yeast mitochondria. The outer membrane integrity was monitored by assessing the permeability of this membrane to proteins, *i.e.* by the accessibility of Tom40p to trypsin and by the leakage of cytochrome *b2* from the intermembrane space. The membrane potential was monitored using the membrane potential sensitive fluorescent probe $diSC_2(5)$.

It was demonstrated that the presence of phosphate is essential for keeping the mitochondrial outer membrane intact and for the generation of a stable membrane potential upon respiration *in vitro*. In the absence of phosphate only a short-lived membrane potential is generated, and under these conditions, disruption of the mitochondrial outer membrane occurs. The ΔpH component of the pmf appears to be responsible for the compromised integrity of the outer membrane, since uncoupling by FCCP inhibited the rupture of the outer membrane, while dissipation of the ΔΨ component by valinomycin did not. This supports the notion that the inhibitory action of phosphate can be explained by matrix acidification [Velours, Rigoulet and Guérin, 1977; Manon *et al.*, 1998). It has been reported before that extensive swelling of mitochondria occurs upon energization in the absence of phosphate, due to changes in the permeability of the inner membrane [Velours, Rigoulet and Guérin, 1977]. In the present study, it is directly shown that the integrity of the outer membrane is compromised under these conditions in such a way that it no longer functions as a barrier for proteins up to 57 kDa. This suggests strongly that swelling has progressed beyond the physical limit where the outer membrane can still accommodate the matrix volume.

Magnesium alone is also able to prevent the rupture of the outer membrane but does not prevent the collapse of the membrane potential, indicating that the collapse of the membrane potential and the rupture of the outer membrane are related but distinct phenomena. In the absence of phosphate, irreversible changes in the permeability of the inner membrane and/or in the systems generating the membrane potential have occurred after some time. The changes in permeability cause swelling beyond the physical limit inducing rupture of the outer membrane in the absence of magnesium. The presence of magnesium may prevent excessive swelling, as judged from the maintenance of the intactness of the outer membrane under this condition. However, Mg^{2+} is unable to prevent irreversible dissipation of the membrane potential. Alternatively, the presence of Mg^{2+} may delay the mitochondrial swelling and concomitant rupture of the outer membrane in such a way that rupture is not detected after 10 min.

A stable membrane potential could only be regained when phosphate was added shortly after the collapse of the membrane potential. The reason why a time limit to the

recovery went unnoticed in a previous study is probably that in those experiments, phosphate was added within 30 s after the addition of ethanol [Castrejón *et al.*, 1997].

The intent of the present study is to provide clarity regarding the experimental conditions required for *in vitro* experiments with yeast mitochondria. For a more detailed description of the underlying mechanisms of changes in permeability upon respiration, see [Manon *et al.*, 1998]. It can be concluded that phosphate is required to maintain the integrity of the outer membrane of respiring yeast mitochondria, and also for the generation of a stable membrane potential. This knowledge is relevant to a wide variety of *in vitro* studies on mitochondria, *e.g.* related to bioenergetics, the yeast mitochondria permeability channel, and protein or lipid import.

Acknowledgements

We thank Dr. Roland Lill for the antisera against Tom40p and cytochrome *b2*, Frank ter Veld for helping with the oxygraph measurements, and Dr. Miriam Greenberg for reading the manuscript critically.

Chapter 6

Identification of the interaction between phosphatidylcholine and the mitochondrial glycerol-3-phosphate dehydrogenase from yeast (Gut2p) by photolabeling

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manuscript in preparation

Abstract

In search of mitochondrial proteins interacting with phosphatidylcholine (PC), a photolabeling approach was applied, in which photoactivatable probes were added to isolated yeast mitochondria. Using either the PC analog [^{125}I]TID-PC, or the small hydrophobic probe [^{125}I]TID-BE, upon photoactivation, only a limited number of proteins were labeled. The most prominent difference found upon labeling of mitochondria with [^{125}I]TID-PC or [^{125}I]TID-BE was the very specific labeling of a 70 kDa protein by [^{125}I]TID-PC. After separation of mitochondrial proteins by two dimensional electrophoresis, a tryptic digest of the gel spot of interest was analyzed by mass spectrometry. The 70 kDa protein was identified as the *GUT2* gene product, the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase. This was confirmed by the lack of specific labeling in the 70 kDa region in photolabeling experiments on mitochondria from a *gut2* deletion strain. Only under conditions where the inner membrane was accessible to the probe, Gut2p was labeled by [^{125}I]TID-PC, in parallel with increased labeling of the phosphate carrier (P_iC) in the inner membrane. Using a hemagglutinin-tagged version of Gut2p, the protein was shown to be membrane-bound. Carbonate extraction released the protein from the membrane, whereas a high concentration of NaCl did not, demonstrating that Gut2p is a peripheral membrane protein bound to the inner membrane *via* hydrophobic interactions. Interestingly, Gut2p was released from the membrane upon photolabeling with [^{125}I]TID-PC. The significance of the observed interactions between Gut2p and PC is discussed.

Introduction

Phosphatidylcholine (PC) is a major phospholipid found in membranes of eukaryotic cells. As in higher eukaryotes, PC is synthesized *via* two distinct pathways in yeast, either *via* the triple methylation of PE or *via* the CDP-choline (Kennedy) pathway (for reviews see [Carman and Henry, 1989; Henry and Patton-Vogt, 1998]). In yeast, the methylation of PE is the primary pathway of biosynthesis of PC when cells are grown in the absence of choline, whereas the CDP-choline pathway is an auxiliary route requiring exogenous choline for net PC synthesis [McDonough *et al.*, 1995].

PC is also a major constituent phospholipid of both mitochondrial membranes [Zinser *et al.*, 1991]. Several mutant strains with defects in the routes of biosynthesis of PC have the tendency to generate respiratory-deficient petites at high frequency [Griac, Swede and Henry, 1996], suggesting that PC is required for proper mitochondrial function, biogenesis or maintenance. The biogenesis of mitochondria requires efficient import of PC from its site of synthesis, the endoplasmic reticulum. The mechanisms and regulatory aspects involved in the mitochondrial import and sorting of PC over both mitochondrial membranes are unknown. Generally, a major impediment in the study of lipid transport processes is the lack of fast and

reliable methods. With the exception of the decarboxylation of phosphatidylserine (PS) to phosphatidylethanolamine (PE), which is used to monitor the arrival of PS in the mitochondria or the Golgi/vacuole (for a recent review, see [Daum and Vance, 1997]), there is no metabolic conversion that can easily be used for assaying intracellular transport of phospholipids.

To get more insight into the import pathway and also into the role of PC in yeast mitochondria, we chose to apply a photolabeling approach, using a photoactivatable PC analog, in search of proteins interacting with PC. This might allow the identification of proteins directly involved in the intramitochondrial transport of PC, or, alternatively, the identification of proteins with a specific interaction with PC. Knowledge on the specific role of PC in mitochondria might pave the way for development of a genetic screen or selection method to find yeast mutants disturbed in mitochondrial PC import. To date, the only enzyme which is known to be dependent on PC for activity is the mammalian mitochondrial enzyme ß-hydroxybutyrate dehydrogenase [Isaacson *et al.*, 1979].

Photolabeling techniques make use of reagents, which, after targeting to a biological system or component, can be activated with UV light, forming highly reactive intermediates capable of forming covalent bonds with adjacent molecules [Weber and Brunner, 1995]. Generally, hydrophobic photolabeling probes are used to study the exposure of proteins to the apolar core of membranes [Brunner, 1993]. Phospholipid analogs offer an additional advantage since specific interactions of proteins with phospholipids can be monitored. Furthermore, if the localization of proteins is known, photolabeling might also be used to monitor the localization of the phospholipid probe itself.

In the present study, mitochondrial membranes are photolabeled with probes based on radioiodinated 3-trifluoromethyl-3-aryldiazirine ([^{125}I]TID), developed by Brunner and coworkers [Weber and Brunner, 1995; Eichler *et al.*, 1997]. Upon photoactivation of the TID group by irradiation with light of a wavelength around 350 nm, the diazirine is rapidly photolyzed to generate a carbene (Figure 1A) capable of reacting with the full range of functional groups occurring in biomolecules [Weber and Brunner, 1995]. The radioactive iodine enables detection of the crosslink products. Upon comparing protein labeling patterns of mitochondrial membranes photolabeled with the simple hydrophobic probe [^{125}I]TID-BE (Figure 1B) and the PC analog [^{125}I]TID-PC (Figure 1C), several similarities as well as distinct differences were found. The most conspicuous difference was the specific labeling by [^{125}I]TID-PC of a 70 kDa protein, which was identified as the *GUT2* gene product, the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase. The results are discussed in the light of phospholipid-protein interactions.

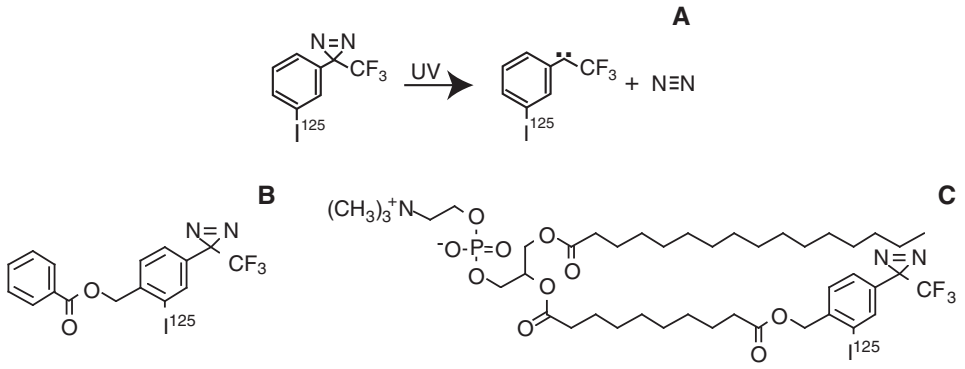


Figure 1. Mechanism of photoactivation and structures of [^{125}I]TID-based photoactivatable reagents. A: photoactivation of the [^{125}I]TID reactive group; B: the structure of [^{125}I]TID-BE; C: the structure of [^{125}I]TID-PC.

Materials and methods

Materials

The radiochemical Na^{125}I (2000 Ci/mmol, 350-600 mCi/ml in NaOH) was obtained from Amersham (Amersham, United Kingdom) or from ICN (Irvine, CA). The tin-based precursors for [^{125}I]TID-BE and [^{125}I]TID-PC were a kind gift from Dr. Josef Brunner (ETH, Zürich). Zymolyase was obtained from Seikagaku (Japan). Peracetic acid was purchased from Sigma. Protein A Sepharose CL-4B beads were from Pharmacia (Sweden). 'Reactivials' were from Pierce (Rockford, IL). PVDF membranes were obtained from Applied Biosystems. Mouse monoclonal antibodies raised against the hemagglutinin epitope were from Boehringer, and the antibiotic G418 was from Calbiochem. All other chemicals were analytical grade.

Isolation of mitochondria and other subcellular fractions

The yeast strains listed in Table 1 were grown aerobically to late log (OD600 4-5 (Perkin Elmer Lambda 18 UV/VIS spectrophotometer)) at 30˚C in semi-synthetic lactate medium [Daum, Böhni and Schatz, 1982]. For the auxotrophic strains, the medium was supplemented with adenine, histidine, lysine, leucine, tryptophan, and uracil at concentrations of 20 mg/l each, as required.

Table 1

Genotypes of strains employed in this study

strain	genotype
D273-10B	*MATα*
W303-1A	*ade 2-1^0 his3-11 trp1-1 ura3-1 can100^0*
W303-1A *gut2Δ*	*ade 2-1^0 his3-11 trp1-1 ura3-1 can100^0 gut2::URA3*
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*

Spheroplasts were prepared using zymolyase as described previously [Daum, Böhni and Schatz, 1982] and homogenized using a Dounce homogenizer in a buffer containing 10 mM MES, pH 6.0, and 0.6 M sorbitol (D buffer), to which 1 mM PMSF and 0.5% (w/v) dextran were added. The isolation and purification of mitochondria were based on published procedures [Daum, Böhni and Schatz, 1982; Gaigg *et al.*, 1995; Glick and Pon, 1995]. Crude mitochondria were isolated using D buffer and were further purified by nycodenz gradient centrifugation as reported [Glick and Pon, 1995], with some minor changes as described [chapter 5]. The final mitochondrial pellet was resuspended in 20 mM Hepes/KOH, pH 7.4 containing 0.6 M sorbitol (H/S buffer) including 5% (w/v) dextran. Mitochondrial outer membranes were isolated as described [De Kroon *et al.*, 1999]. Microsomes were isolated as described [De Kroon *et al.*, 1999], with the exception that H/S buffer was used for all resuspension and wash steps. All subcellular fractions were frozen in liquid N_2 and stored at -80˚C and thawed (on ice) only once before use. All samples were kept on ice unless indicated otherwise.

Preparation of radioiodinated photocrosslinking probes

The hydrophobic photoactivatable probe [^{125}I]TID-BE (Figure 1A) and the photoactivatable phospholipid [^{125}I]TID-PC (Figure 1B) were prepared by radioiodo-destannylation of the tin-based precursors as described ([Eichler *et al.*, 1997] and [Weber and Brunner, 1995], respectively), with minor changes. All steps were performed at room temperature under normal laboratory light. Briefly, 20-50 nmol of the precursors was dried in a 1 ml 'reactivial' under a stream of nitrogen and redissolved in 10 µl of acetic acid. After addition of 2-5 mCi Na^{125}I, the reaction was started by adding 5 µl of peracetic acid. After 2 min the reaction was stopped by addition of 100 µl of 10% (w/v) $Na_2S_2O_5$. The reaction mixture containing [^{125}I]TID-BE was extracted with chloroform and [^{125}I]TID-BE was further purified by HP-TLC using ether/hexane (9:1, v/v) as eluent. The radioactive spot containing [^{125}I]TID-BE was scraped off, and the product was eluted with ethanol and stored at 4˚C. The reaction mixture containing [^{125}I]TID-PC was extracted with chloroform/ methanol (2:1, v/v) and [^{125}I]TID-PC was purified by HP-TLC using chloroform/methanol/

water (65:25:4, v/v/v) as eluent. The radioactive spot containing [^{125}I]TID-PC was scraped off, and the product was eluted with chloroform/methanol (2:1, v/v) and stored at 4˚C.

Labeling of mitochondrial membranes with photoactivatable probes

Solutions of the photoactivatable probes in ethanol, at a concentration of 1 μCi/μl for [^{125}I]TID-BE and of 1-5 μCi/μl for [^{125}I]TID-PC (with 1 μCi corresponding to 0.5 pmol probe), were prepared by drying the probes and redissolving in ethanol. For photolabeling, these solutions were injected into H/S buffer (5-10 μl of ethanolic solution per ml buffer) containing either mitochondria, mitoplasts or mitochondrial outer membranes, under stirring on ice. Aliquots of this suspension were incubated for the indicated times at the indicated temperatures. Thus, different conditions were examined in parallel starting from the same sample. In experiments where the effects of different additives such as FCCP, valinomycin and NADH were investigated, aliquots of the suspension were added to an equal volume of H/S buffer containing the extra ingredients. In all experiments, the final incubation volume of each sample was 100 μl, and a final protein concentration of 100-250 μg/ml was used. NADH and potassium phosphate (KH_2PO_4) were used at a concentration of 2 mM, and FCCP was added from a stock solution in ethanol to a final concentration of 20 μM. The final ethanol concentration never exceeded 1% (v/v). After incubation, the samples (in eppendorf cups) were placed on ice and photolyzed immediately by irradiation with a 15 W UV light source (CAMAC universal lamp) at 366 nm for 2 min at a 1 cm distance. Proteins were precipitated with TCA and analyzed by 12% SDS-PAGE. Gels were dried and autoradiography was performed.

Immunoprecipitation of photolabeled proteins

The protein pellet obtained after TCA precipitation of mitochondrial membranes photolabeled with [^{125}I]TID-BE or [^{125}I]TID-PC (65 μg on protein basis), was dissolved in 25 μl 2% (w/v) SDS by heating for 10 min at 95˚C, and diluted with 625 μl 20 mM Tris-HCl, pH 7.4, containing 310 mM NaCl (2x TBS) and 0.5% (v/v) Triton X-100. After 15 min on ice the solution was precleared by centrifugation for 15 min at 14,000 rpm (20,000 x *g*) in a microfuge at room temperature. A 20 μl aliquot of the supernatant was withdrawn and mixed with 20 μl concentrated SDS-PAGE sample buffer to serve as a 10% standard. Aliquots of 200 μl of the supernatant were subjected to immunoprecipitation, using antibody coated protein A beads (10 mg portions). Incubation and wash steps were done by gently shaking for the indicated times followed by centrifugation for 30 s at 14,000 rpm in a microfuge to pellet the beads. Antibody coated protein A beads were prepared, after 2 wash steps in 1 ml 2x TBS for 10 min at room temperature, by incubating with the appropriate antibody solution diluted to a total volume of 100 μl with 2x TBS, for 1-2 h at room temperature. This was followed by 3 consecutive 10 min wash steps with 1 ml volumes of 2x TBS, 2x TBS containing 0.1%

BSA, and 2x TBS, respectively. The precleared solution of photolabeled mitochondrial proteins was incubated with the beads for 1-2 h at 4˚C. This was followed by three wash steps of 10 min at 4˚C, twice with 1 ml 2x TBS containing 0.1% (v/v) Triton X-100, and once with 1 ml 2x TBS, respectively. 40 µl SDS-PAGE sample buffer was added to the beads. Samples were heated at 95˚C for 5 min and subjected to 12% SDS-PAGE. Gels were dried and autoradiography was performed.

N-terminal sequence analysis

N-terminal sequence analysis was performed (at the Sequence Centre Utrecht) by Edman degradation using an Applied Biosystems Model 47XA protein sequencer on a protein sample bound to PVDF, prepared according to the manufacturer's instructions (estimated amount of protein 30 pmol). Briefly, after a 10 min incubation at 37˚C in SDS-PAGE sample buffer, a mitochondrial sample (300 µg on protein basis) was separated by 7.5% SDS-PAGE and electroblotted onto a PVDF membrane. To prevent N-terminal blocking of proteins during preparation of the sample, 1 mM sodiumthioglycolate was present in the SDS-PAGE running buffer, and the gels were prerun for 1 h and subsequently stored overnight at 4˚C. The PVDF membrane was stained using Coomassie Brilliant Blue R-250 and the protein band of interest was excised from the blot. The correct position of the protein of interest was determined by separating a [^{125}I]TID-PC labeled mitochondrial sample on a 7.5% SDS-PAGE gel and overlaying the autoradiogram and the corresponding coomassie-stained pattern.

Two-dimensional gel electrophoresis and mass spectrometric analysis

Samples destined for unstained gels to be used for mass spectrometric analysis contained 40 µg of [^{125}I]TID-PC labeled mitochondrial protein, while samples destined for gels to be used for silver staining consisted of a mixture of 100 µg of unlabeled mitochondrial protein mixed with 20 µg of [^{125}I]TID-PC labeled mitochondrial protein. The labeled protein sample was prepared by photolabeling mitochondria (190 µg on protein basis) for 10 min at 30˚C with [^{125}I]TID-PC (7.5 µCi) as described. The washed protein pellet obtained after TCA precipitation was stored at -20˚C, and dissolved in 120 µl lysisbuffer [Fey *et al.*, 1997] before use. The unlabeled protein sample was prepared by precipitating protein from unlabeled isolated mitochondria by adding 900 µl ethanol and 900 µl acetone to a 300 µl sample containing 1.2 mg on protein basis, followed by overnight incubation at -20˚C. The pellet obtained after centrifugation for 15 min at 20,000 rpm in a table centrifuge, was washed with 2.1 ml ethanol/acetone/H_2O (2:2:1, v/v/v) and dissolved in 350 µl lysisbuffer. Protein samples were separated in the first dimension on NEPHGE (pH range 7-10) and IEF (pH range 3.5-7) gels and in the second dimension by 12.5% SDS-PAGE, followed by silverstaining, drying, and autoradiography of the gels, as described [Fey *et al.*,

1997]. After autoradiography, the radiolabeled protein spot of interest was cut from the unstained gel and subjected to mass spectrometric analysis, as described [Jensen, Larsen and Roepstorff, 1998]. Briefly, the gel piece was washed, dried in a vacuum centrifuge, and subjected to in-gel protein digestion with trypsin. The tryptic digest was purified by reversed phase chromatography, and mass spectra were recorded using a MALDI-TOF instrument to obtain peptide mass information for database searches.

Measurement of glycerol-3-phosphate dehydrogenase activity

The activity of glycerol-3-phosphate dehydrogenase was measured by monitoring spectrophotometrically the phenazine methosulfate (PMS)-mediated reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) as described [Larsson *et al.*, 1993], with minor changes. The reaction mixture (1 ml) contained 50 mM Hepes, pH 7.5, 10 mM KCN, 0.5 mM MTT, 0.2 mM PMS, 0.05% (v/v) Triton X-100, and 10 mM DL-glycerol-3-phosphate, and in addition, 50 µM FAD. The cofactor FAD stimulates the enzymatic activity, with maximal stimulation at concentrations of 50 µM or higher, as determined using 200 µg purified mitochondria (based on protein) from strain D273-10B. The stimulation by FAD was specific for the Gut2p activity since no activity was found in fractions from the W303-1A *gut2* knock-out strain in the presence or absence of FAD. The increase in extinction at 562 nm was monitored for 10 min after the addition of the protein sample. Activities were calculated from the slope after stabilization of the sample, using an extinction coefficient for reduced MTT of 8.1 $mM^{-1}cm^{-1}$. The numbers were corrected for background activity by repeating each measurement in the absence of glycerol-3-phosphate.

Epitope tagging of the GUT2 *gene*

Gut2p was epitope-tagged at its C-terminus by cloning a hemagglutinin tag at the end of the *GUT2* gene into the yeast genome, *via* homologous recombination with a PCR fragment. The plasmid pU6H3HA (EMBL accession number AJ132966) created by De Antoni and Gallwitz (2000) was used as a template for PCR amplification. The primers used were 5'-AC TTG AAA AAA CTG TGA ACT TCA TCA AGA CGT TTG GTG TCT CC CAC CAC CAT CAT CAT CAC-3', and 5'-T TAT ATT ATG TAT TGG AAA TAG AAT ATA AAC ACT AGG AAG ACT ATA GGG AGA CCG GCA GAT-3'. The PCR product of 1815 bp was transformed into yeast strain BY4742 and positive colonies were selected on G418 containing plates as described [De Antoni and Gallwitz, 2000]. Correct integration of the PCR fragment into the yeast genome was checked by PCR analysis performed on the genomic DNA isolated from transformants. The primers were 5'-GAC GCC AAG GAA GCT TTG AAT G-3' (complementing upstream of the integrated fragment) in combination with 5'-ATT GCG TAT GCA CCA GGA CGT T-3' (complementing downstream of the integrated fragment), resulting in a 2107 bp fragment for positive clones (or a 546 bp

fragment for untransformed cells), or in combination with 5'-GGA TCG CAG TGG TGA GTA ACC-3' (complementing in the integrated fragment), resulting in a 951 bp fragment for positive clones. Expression of the hemagglutinin-tagged Gut2p was verified by SDS-PAGE followed by Western blotting.

Preparation of mitoplasts, carbonate extraction and salt wash

Mitoplasts were prepared by diluting the mitochondria at least a factor of 10 in hypotonic buffer (20 mM Hepes/KOH, pH 7.4), and incubating for 20 min on ice [Jascur, 1991], followed by centrifugation for 12 min at 10,000 rpm (10,600 x *g*) in a microfuge at 4˚C. Pellet and supernatant were separated and the pellet was resuspended in H/S buffer. The mitoplasts were used for photolabeling experiments or subjected to carbonate extraction or salt treatment. These treatments were performed by adding an equal volume of 200 mM Na_2CO_3 (freshly prepared) or of 1 M NaCl and incubating on ice for 5 min followed by centrifugation for 12 min at 10,000 rpm (10,600 x *g*) in a microfuge at 4˚C. Pellet and supernatant were separated and the pellet was resuspended in H/S buffer. Samples were used for measurement of Gut2p activity, or, after TCA precipitation, for analysis by SDS-PAGE followed by Western blotting.

Other methods

Protein concentrations were measured using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin (BSA) as a standard. γ-counting was done with a Packard crystal multidetector RIA system. TCA precipitations were performed by adding one volume of 10% (w/v) TCA and incubating on ice for 20 min. After centrifugation for 15 min at 14,000 rpm, the precipitated protein was washed with 50 µl of ice-cold acetone followed by 20 µl of ice-cold water. Prior to SDS-PAGE, protein samples were heated for 5 min at 95˚C in SDS-PAGE sample buffer, unless indicated otherwise. For autoradiography, a phosphorimager was used. Western blots were decorated with antibodies raised against the indicated proteins and protein bands were visualized by ECL according to the manufacturer's instructions.

Results

Photolabeling of mitochondria and mitochondrial outer membranes with [^{125}I]TID-BE

To explore the feasibility of a photocrosslinking approach to monitor lipid-protein interactions in mitochondrial membranes, mitochondria and isolated mitochondrial outer membranes were labeled at 0˚C with the small hydrophobic photoactivatable probe [^{125}I]TID-BE, added from ethanol.

Figure 2B shows that the labeling is UV dependent (*cf.* lanes with and without UV light) and that the labeling patterns of mitochondria and isolated outer membranes differ from each other, as expected based on their different protein pattern (Figure 2A). Two proteins around 30 kDa are labeled very prominently by [^{125}I]TID-BE in the mitochondria, whereas in the isolated outer membranes there is only one prominent band at 30 kDa. From comparison of the [^{125}I] labeling patterns and the total protein patterns, we hypothesized that the densely labeled band observed both in the outer membrane fraction and the mitochondria was porin, the most prominent protein in the outer membrane, and that the other conspicuous band, absent from the outer membrane, was the abundant phosphate carrier (P_iC) from the inner membrane. The hypotheses were confirmed by immunoprecipitation (Figure 2C). These results show that it is possible to selectively label mitochondrial proteins with a photoactivatable probe.

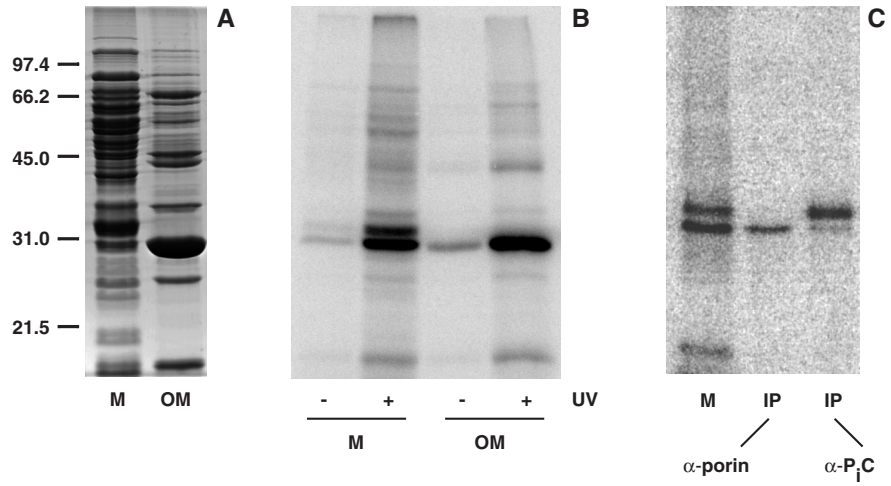


Figure 2. Labeling of mitochondria and mitochondrial outer membranes with [^{125}I]TID-BE. Mitochondria (M) and isolated outer membranes (OM) were incubated for 5 min at 0˚C with [^{125}I]TID-BE, added from ethanol, and were either before (-UV), or after photolysis (+UV), subjected to TCA precipitation followed by 12% SDS-PAGE and autoradiography. A: coomassie stained gel, with the position of molecular weight markers (kDa) indicated; B: autoradiogram; C: identification of the major [^{125}I]TID-BE labeled proteins by immunoprecipitation. Mitochondria were photolabeled for 5 min at 0˚C with [^{125}I]TID-BE, added from ethanol, and immunoprecipitations with antibodies directed against porin and P_iC were performed. A 10% standard of the [^{125}I]TID-BE labeled mitochondria (M), and the immunoprecipitates (IP) were analyzed by 12% SDS-PAGE and autoradiography.

Photolabeling of mitochondria with [^{125}I]TID-PC

When [^{125}I]TID-PC was used for photolabeling mitochondria and outer membranes at 0˚C, a different labeling pattern was obtained than for [^{125}I]TID-BE, but also some similarities were apparent (Figure 3, compare lanes 3 and 5 to lane 1). The outer membrane protein porin is labeled in the mitochondria and outer membranes by [^{125}I]TID-PC, as was also found with [^{125}I]TID-BE. In the mitochondria, P$_i$C is labeled only very faintly by [^{125}I]TID-PC (lane 3), compared to [^{125}I]TID-BE (lane 1), most likely because the phospholipid probe has reduced access to the inner membrane in the intact mitochondria compared to the small hydrophobic probe which appears to partition into all the available membranes. As was found with [^{125}I]TID-BE, P$_i$C labeling by [^{125}I]TID-PC is absent from the isolated outer membranes (Figure 3, lane 5, compare to Figure 2B). However, in the higher molecular weight range, several bands are labeled more strongly at 0˚C by [^{125}I]TID-PC than by [^{125}I]TID-BE, among which Tom70p was identified by immunoprecipitation (data not shown). Tom70p is labeled by [^{125}I]TID-PC both in mitochondria and in isolated outer membranes (lanes 3 and 5). These results suggest that at 0˚C, in intact mitochondria, the phospholipid analog is predominantly confined to the outer membrane. Labeling of proteins with [^{125}I]TID-PC was UV-dependent in a similar manner as with [^{125}I]TID-BE (not shown).

Upon shifting the mitochondria from 0 to 20˚C, they start respiring, using ethanol (from which the probes were added) as respiratory substrate. Upon respiration in the absence of phosphate, the outer membrane barrier is disrupted, while in the presence of phosphate, the mitochondria remain intact [chapter 5]. When mitochondria were incubated with [^{125}I]TID-BE in the absence of phosphate, no changes in labeling pattern or increase of label on P$_i$C are observed, upon raising the temperature (*cf.* lanes 1 and 2), confirming that the probe already has access to both membranes at 0˚C. However, upon photolabeling of mitochondria 20˚C with [^{125}I]TID-PC in the absence of phosphate, conspicuous changes in the labeling pattern occur, such as the appearance of a very prominent band at 70 kDa, and a strong enhancement of P$_i$C labeling (*cf.* lanes 3 and 4). Interestingly, the intensity of labeling of Tom70p is decreased at 20˚C compared to 0˚C, both in mitochondria and in isolated outer membranes (*cf.* lanes 3 and 4, and 5 and 6). The enhancement of P$_i$C labeling is in agreement with the inner membrane becoming accessible to the probe due to rupture of the outer membrane [chapter 5]. The prominent band at 70 kDa is absent from isolated outer membranes (*cf.* lanes 4 and 6), and is also not labeled by [^{125}I]TID-BE (*cf.* lanes 2 and 4). These results suggest that the 70 kDa band corresponds to an inner membrane protein which is very specifically labeled with [^{125}I]TID-PC.

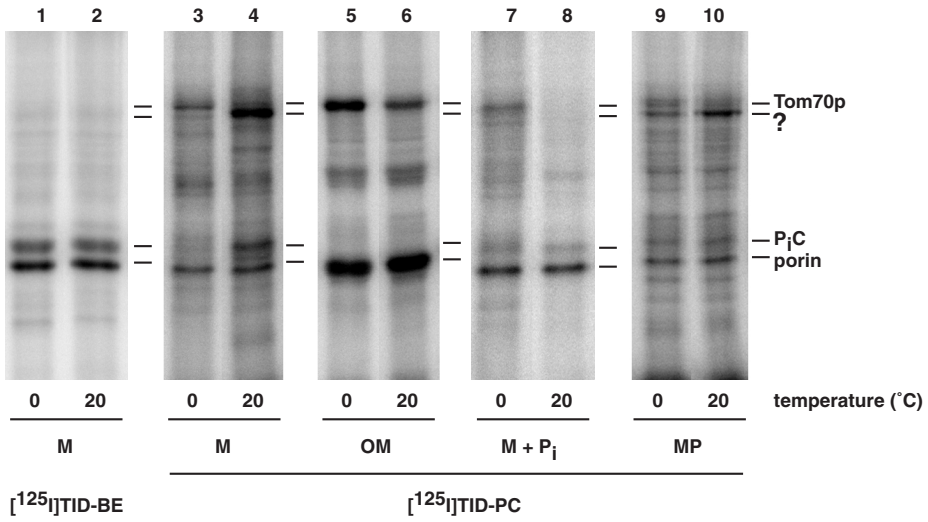


Figure 3. Photolabeling of mitochondrial membranes with [^{125}I]TID-BE and [^{125}I]TID-PC. Mitochondria (M), isolated outer membranes (OM) and mitoplasts (MP) were photolabeled with [^{125}I]TID-BE or [^{125}I]TID-PC for 30 min at 0 or 20˚C, after addition from ethanol. When indicated, the buffer contained 2 mM phosphate (P_i). Samples were analyzed by 12% SDS-PAGE and autoradiography.

In the presence of phosphate, the mitochondrial proteins exhibit similar labeling by [^{125}I]TID-PC at 0 and 20˚C (*cf.* lanes 7 and 8), except for the disappearance of label on Tom70p at 20˚C. The presence of the uncoupler FCCP prevents rupture of the outer membrane upon respiration in the absence of phosphate [chapter 5]. Under these conditions, neither the increase of TID-PC label on P_iC, nor the appearance of the prominent band at 70 kDa was observed (not shown). Addition of [^{125}I]TID-PC from methanol instead of ethanol yielded a similar result as in the presence of phosphate or uncoupler (not shown). These results indicate that also at 20˚C, when the mitochondria remain intact, the probe is confined to the outer membrane. In contrast, when [^{125}I]TID-PC was administered to mitoplasts, in which the outer membrane is disrupted by hypotonic shock, both P_iC and the 70 kDa protein were photolabeled both at 0 and 20˚C (*cf.* lanes 9 and 10). These results confirm that rupture of the outer membrane is a prerequisite for the probe to gain access to the inner membrane.

The labeling patterns obtained at 0˚C did not change with time, while the changes occurring upon shifting to 20˚C were found to be time-dependent, reaching completion after approximately 10-20 min (not shown). The decreased intensity of labeling of Tom70p at 20˚C compared to 0˚C, as observed both in mitochondria and outer membrane vesicles, irrespective of the presence of phosphate, might reflect the flipping of the phospholipid probe to the internal leaflet of the outer mitchondrial membrane. Since phospholipids integrate into membranes in an oriented manner, the probe might in principle be able to label membrane

components selectively from one side of the bilayer [Brunner, 1993]. Taken together, the results indicate that the PC analog is largely confined to the outer membrane when the mitochondria are intact, whereas, upon disruption of the outer membrane, it is also found in the inner membrane, where it labels P_iC and, very prominently, a protein of approximately 70 kDa.

Identification of the 70 kDa protein specifically labeled with [^{125}I]TID-PC

To identify the [^{125}I]TID-PC labeled 70 kDa protein, the corresponding band was excised from a PVDF blot and subjected to N-terminal sequencing by Edman degradation. The obtained sequences, SSHKEL and DPSYMV, originated from the yeast mitochondrial proteins Hsp60p and Gut2p, respectively, as was found by database [EMBL] analysis [Pearson and Lipman, 1988]. The N-terminal sequences, corresponding to the N-termini of the mature proteins, were deposited at the Yeast Protein Database [YPD], to replace the incorrectly predicted mature N-termini.

Since one-dimensional separation by SDS-PAGE was insufficient to obtain a protein sample containing only the protein of interest, mitochondrial protein samples labeled with [^{125}I]TID-PC were subjected to 2D-gel electrophoresis (Figure 4). The heavily [^{125}I] labeled spot at 70 kDa (Figure 4A) does not represent a very abundant protein in the mitochondria, as judged from the intensity of the corresponding spot on the silverstained gel (Figure 4B). The spot was cut from the gel, subjected to tryptic digestion and analyzed by mass spectrometry for peptide mapping. The peptide fingerprint (Figure 5A) matched that of the *GUT2* gene product, the yeast mitochondrial glycerol-3-phosphate dehydrogenase, as was found by database analysis. Figure 5B shows the amino acid sequence of the protein (EMBL accession number P32191) with the matching peptides, which cover 60% of the mature protein sequence. Both the molecular weight and the iso-electric point found on the gel are in agreement with those predicted in the Yeast Protein Database (molecular weight 68.4 kDa and pI 7.1) [YPD].

The identity of the labeled protein was confirmed by performing a photolabeling experiment on mitochondria isolated from a yeast strain (W303-1A) in which the *GUT2* gene was deleted (see Table I). The [^{125}I]TID-PC labeled 70 kDa band is completely absent from the *gut2* knock-out mitochondria whereas it is present in the corresponding wild-type mitochondria (Figure 6). Other features of the labeling pattern, such as the increase of label at the position of P_iC at 20˚C *vs.* 0˚C, are unaffected in the mitochondria from the knock-out. These results demonstrate that the 70 kDa protein which was highly labeled with [^{125}I]TID-PC is indeed Gut2p.

The *GUT2* (Glycerol UTilization) gene is essential for utilization of glycerol as a carbon source [Sprage and Cronan, 1977]. The encoded mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase [Rønnow and Kielland-Brandt, 1993] functions in the

glycerol-3-phosphate shuttle, which delivers reduction equivalents from cytosolic NADH to the mitochondrial respiratory chain, thus contributing to the maintenance of the cytosolic redox balance under aerobic conditions [Larsson *et al.*, 1998; Overkamp *et al.*, 2000]. In this respect, it is interesting to note that when the photolabeling of mitochondria with [^{125}I]TID-PC was performed in the presence of NADH (2 mM), labeling of Gut2p was increased relative to that of P_iC or porin (data not shown).

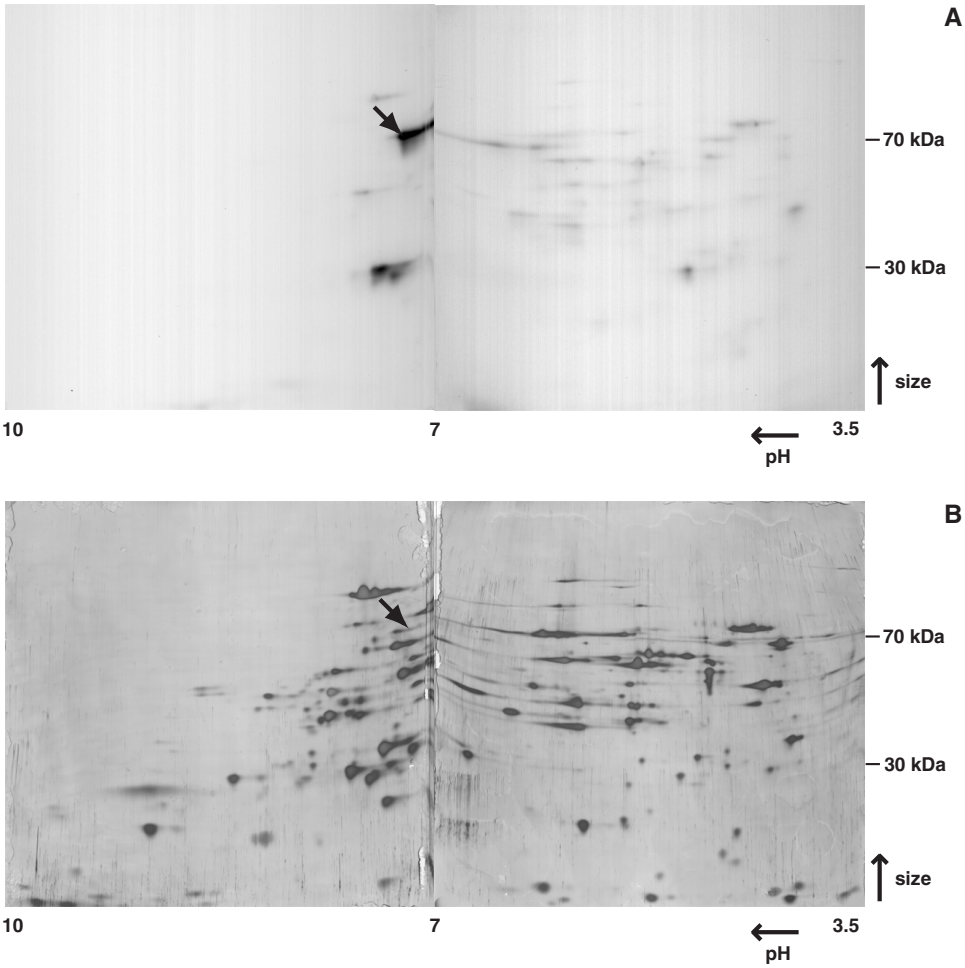
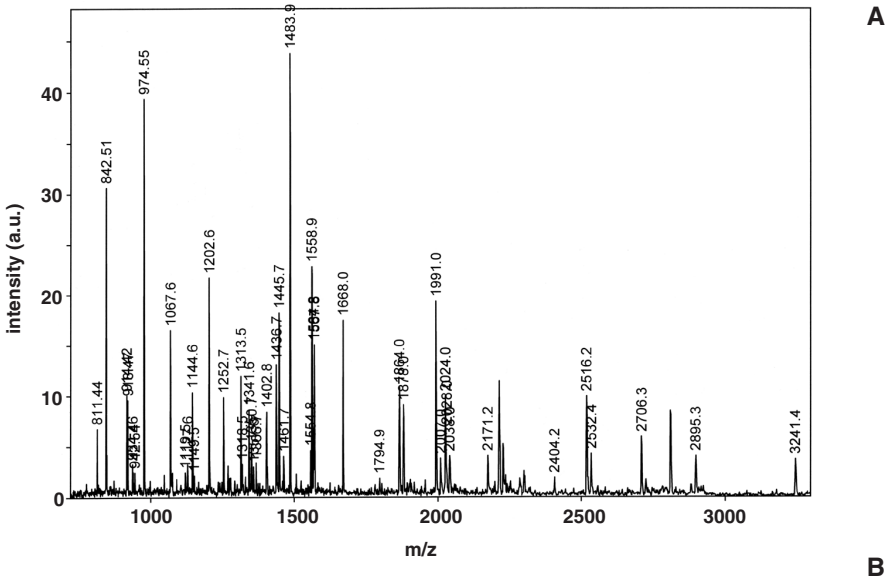


Figure 4. 2D-gel electrophoresis of [^{125}I]TID-PC labeled mitochondria. Mitochondria were photolabeled for 10 min at 30˚C with [^{125}I]TID-PC, added from ethanol. Proteins were separated in the first dimension on NEPHGE (pH range 7-10) and IEF (pH range 3.5-7) gels and in the second dimension by 12.5% SDS-PAGE, followed by silverstaining and autoradiography of the gels. The arrows indicate the position of the spot used for further analysis. A: autoradiogram; B: silver stain pattern.



B

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MFSVTRRRAA GAAAAMATAT GTLYWMTSQG DRPLVHND*PS YMVQFPTAAP PQVSRRDLLD
RLAKTHQFDV LIIGGGATGT GCALDAATRG LNVALVEKGD FASGTSSKST KMIHGGVRYL
EKAFWEFSKA QLDLVIEALN ERKHLINTAP HLCTVLPILI PIYSTWQVPY IYMGCKFYDF
FAGSQNLKKS YLLSKSATVE KAPMLTTDNL KASLVYHDGS FNDSRLNATL AITAVENGAT
VLNYVEVQKL IKDPTSGKVI GAEARDVETN ELVRINAKCV VNATGPYSDA ILQMDRNPSG
LPDSPLNDNS KIKSTFNQIA VMDPKMVIPS IGVHIVLPSF YCPKDMGLLD VRTSDGRVMF
FLPWQGKVLA GTTDIPLKQV PENPMPTEAD IQDILKELQH YIEFPVKRED VLSAWAGVRP
LVRDPRTIPA DGKKGSATQG VVRSHFLFTS DNGLITIAGG KWTTYRQMAE ETVDKVVEVG
GFHNLKPCHT RDIKLAGAEE WTQNYVALLA QNYHLSSKMS NYLVQNYGTR SSIICEFFKE
SMENKLPLSL ADKENNVIYS SEENNLVNFD TFRYPFTIGE LKYSMQYEYC RTPLDFLLRR
TRFAFLDAKE ALNAVHATVK VMGDEFNWSE KKRQWELEKT VNFIKTFGV
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Figure 5. Identification of the [^{125}I]TID-PC labeled 70 kDa protein by peptide mapping. The [^{125}I]TID-PC labeled 70 kDa spot at the position of the arrow in Figure 4A was cut from an unstained 2D-gel and subjected to tryptic digestion. The tryptic digest was subjected to mass spectrometric analysis, yielding a list of 45 peptide masses which was used to search databases. The highest scoring entry was the yeast *GUT2* gene product. A: MALDI-TOF mass spectrum of the tryptic digest; B: the amino acid sequence of the protein encoded by the *GUT2* gene. Matching tryptic peptides are indicated in bold gray and black (the color code was used to distinguish individual peptides). The * indicates the start of the mature protein.

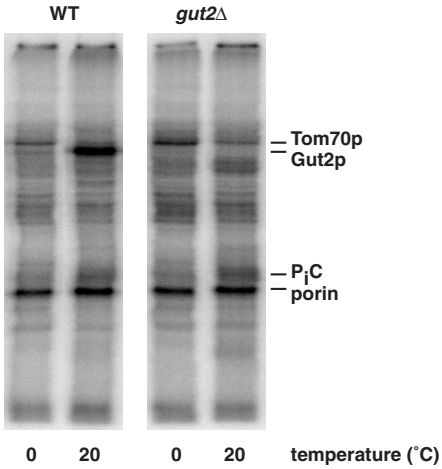

Figure 6. Labeling of wild-type and *gut2*Δ mitochondria with [^{125}I]TID-PC. Mitochondria isolated from a *gut2*Δ strain and the corresponding wild-type (WT) were photolabeled for 30 min at 0 and 20˚C with [^{125}I]TID-PC, added from ethanol. Samples were analyzed by 12% SDS-PAGE and autoradiography.

Localization of Gut2p

To determine the localization of Gut2p, glycerol-3-phosphate dehydrogenase activity was measured, and, in addition, a C-terminal epitope tag (hemagglutinin) was introduced into Gut2p, enabling detection by immunological methods. Figure 7 compares the subcellular distribution of Gut2p specific activity (Figure 7A) and of the hemagglutinin-tagged Gut2p (Figure 7C) to the distribution of the endoplasmic reticulum marker protein Sec61p and the mitochondrial protein P_iC (Figure 7B). Both the distribution of Gut2p activity and of the hemagglutinin-tagged protein is similar to the subcellular distribution of P_iC, in agreement with the mitochondrial localization of the protein [Rønnow and Kielland-Brandt, 1993]. The specific activity of Gut2p in the mitochondrial fraction is significantly higher than the value of 36 nmol/min/mg reported [Rønnow and Kielland-Brandt, 1993], due to the presence of the co-factor FAD in the assay. When mitochondria from strains D273-10B and W303-1A were assayed in the absence of FAD, specific enzyme activities were similar to the literature value.

The effective labeling of Gut2p by [^{125}I]TID-PC indicates that the protein interacts with the membrane. However, upon centrifugation of the [^{125}I]TID-PC labeled mitochondria, the labeled Gut2p is recovered in the supernatant (S), as is the soluble intermembrane space protein cytochrome *b2* [Daum, Böhni and Schatz, 1982] (Figure 8). The [^{125}I]TID-PC labeled porin and the outer membrane protein Tom40p are found in the pellet (P). These results indicate that [^{125}I]TID-PC labeled Gut2p behaves as a soluble protein located in the mitochondrial intermembrane space, that is released upon rupture of the outer membrane under the conditions applied.

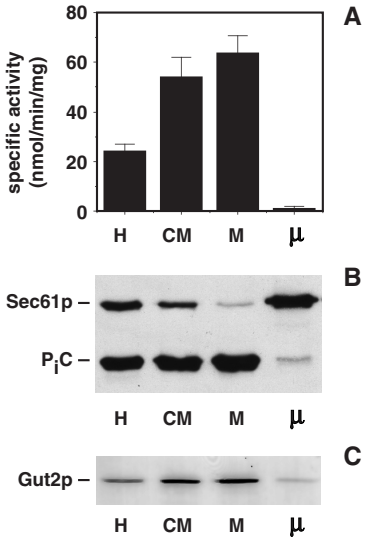

Figure 7. Subcellular distribution of Gut2p activity and epitope-tagged Gut2p. The activity of Gut2p and the presence of hemagglutinin-tagged Gut2p and of endoplasmic reticulum (Sec61p) and mitochondrial (P_iC) markers is shown for homogenates (H), crude mitochondria (CM), purified mitochondria (M) and microsomes (μ). A: subcellular distribution of Gut2p activity. Glycerol-3-phosphate dehydrogenase activity was determined as described in the 'Materials and methods' section using samples from strain D273-10B corresponding to 100 μg on protein basis. The error bars represent the error calculated as the standard deviation from duplicate measurements of both background and activity in a single sample. B: Western blot analysis of the subcellular fractionation. Samples from strain D273-10B corresponding to 10 μg on protein basis were subjected to SDS-PAGE followed by Western blotting using antibodies raised against Sec61p and P_iC. C: Western blot analysis of the subcellular distribution of Gut2p. Samples, corresponding to 5 μg on protein basis, from strain BY4742 expressing hemagglutinin-tagged Gut2p, were subjected to SDS-PAGE followed by Western blotting using antibodies raised against the hemagglutinin epitope.

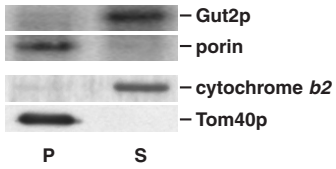

Figure 8. Submitochondrial localization of [^{125}I]TID-PC labeled Gut2p. Mitochondria were incubated for 20 min at 20°C with [^{125}I]TID-PC, added from ethanol. After photolysis, the mitochondria were pelleted by centrifugation. Pellet (P) and supernatant (S) were subjected to TCA precipitation and 12% SDS-PAGE analysis, followed by autoradiography, to visualize [^{125}I]TID-PC labeled Gut2p and porin, or Western blotting using antibodies raised against cytochrome *b2* and Tom40p.

The intramitochondrial localization of the entire Gut2p pool, and not only of the portion labeled with [^{125}I]TID-PC, was studied using the hemagglutinin-tagged version of the protein. Figure 9A shows that upon centrifugation of mitoplasts prepared from mitochondria containing hemagglutinin-tagged Gut2p, the protein is largely found in the pellet together with the membrane protein Tom40p (lane 3) and only a small portion is found in the supernatant, while the release of the intermembrane space protein cytochrome *b2* into the supernatant (lane 4) indicates that the outer membrane is completely disrupted. Activity measurements showed that the activity of Gut2p also remained bound to the membrane fractions (data not shown). When the mitochondrial outer membrane was disrupted due to respiration on ethanol in the absence of phosphate, instead of by hypotonic shock, similar results were obtained, excluding that the observed release of the [^{125}I]TID-PC labeled protein is related to the energization (data not shown). These results demonstrate that Gut2p is a predominantly membrane-bound protein. To investigate whether Gut2p is a peripheral or an integral membrane protein, the mitoplast pellet was subjected to carbonate extraction or salt wash (Figure 9B). Upon treatment with Na_2CO_3 and centrifugation, the protein is completely released from the membrane fraction, while the integral membrane protein Tom40p ends up in the pellet containing the membranes (*cf.* lanes 2 and 3), indicating that Gut2p is peripherally bound to the membrane. Confirmation by enzyme activity measurements was not possible since the carbonate extraction completely abolished the activity of the protein (data not shown). Upon subjecting the membranes to salt wash, Gut2p remains bound to the membrane fraction, since it is found in the pellet together with Tom40p (*cf.* lanes 5 and 6) which was confirmed by the finding that the enzyme activity remained bound to the membrane fraction (not shown), indicating that electrostatic interactions are probably not the most important determinant for interaction with the membrane.

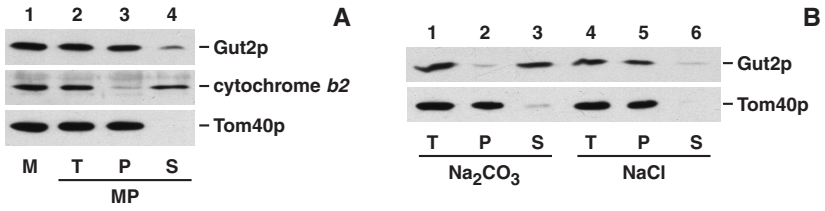

Figure 9. Submitochondrial localization of Gut2p. Mitochondria (M), isolated from strain BY4742 expressing hemagglutinin-tagged Gut2p, were subjected to hypotonic shock to prepare mitoplasts (MP). The mitoplasts were centrifuged and the mitoplast pellet obtained by centrifugation was subjected to carbonate extraction or salt wash, followed by centrifugation. Aliquots (T: complete sample prior to centrifugation; P: pellet; S: supernatant) derived from 5 µg mitochondria on protein basis were analyzed by 12% SDS-PAGE and Western blotting. The distribution of Gut2p was analyzed using antibodies raised against the hemagglutinin epitope. As controls, the distribution of the soluble intermembrane space protein cytochrome *b2* and the outer membrane protein Tom40p was determined using antibodies raised against these proteins. A: Western blot analysis of the conversion to mitoplasts; B: Western blot analysis of mitoplasts after carbonate extraction (Na_2CO_3) or salt wash (NaCl).

Discussion

In search of interactions between phosphatidylcholine and mitochondrial proteins, a photolabeling approach was applied to isolated yeast mitochondria. In comparing the labeling patterns obtained with the small hydrophobic probe [^{125}I]TID-BE and the PC analog [^{125}I]TID-PC, the specific labeling of a 70 kDa protein is the most prominent difference. Only under conditions where the inner membrane was accessible to the probe, the 70 kDa protein was labeled by [^{125}I]TID-PC, in parallel with the inner membrane protein P_iC. In intact mitochondria, the phospholipid probe was largely confined to the outer membrane, and labeled the outer membrane proteins porin and Tom70p.

To identify the [^{125}I]TID-PC labeled 70 kDa protein, a tryptic digest of the spot of interest cut from a 2D-gel, was subjected to mass spectrometry. The peptide fingerprint matches that of Gut2p. The observation that [^{125}I]TID-PC label at the 70 kDa position in mitochondria from a *gut2* deletion strain was completely lacking under conditions where it was heavily labeled in the corresponding wild-type strain, unambiguously identifies the protein as Gut2p. The *GUT2* gene product, also known as the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase, is required for the utilization of glycerol as carbon source [Sprague and Cronan, 1977; Larsson *et al.*, 1993], and it is a component of the glycerol-3-phosphate shuttle, which delivers reduction equivalents from cytosolic NADH to the mitochondrial respiratory chain, thus contributing to the maintenance of the cytosolic redox balance under aerobic conditions [Larsson *et al.*, 1998; Overkamp *et al.*, 2000].

For proteins involved in import of phospholipids into mitochondria, a specific interaction with a phospholipid analog such as [^{125}I]TID-PC might be expected. Photo-activatable lipid analogs have previously been used by others to search for components involved in lipid transport [Schroit and Madsen, 1987; Zachowski *et al.*, 1987; Zegers, Zaal and Hoekstra, 1998]. Although proteins were found that on the basis of labeling by the probes qualified as candidates, so far, no positive identification of the involvement of these proteins in lipid transport has been made. Involvement of Gut2p in PC import into yeast mitochondria was excluded since mitochondria from the *gut2* knock-out strain were found to have a normal PC content, similar to that of the corresponding wild-type strain (data not shown).

From the labeling of Gut2p by [^{125}I]TID-PC an interaction of the protein with the inner membrane can be inferred. Moreover, in view of the physiological function of the protein, which includes delivery of electrons to components of the respiratory chain located in the mitochondrial inner membrane, membrane interactions are expected. However, the Yeast Protein Database states that Gut2p is predicted to be a soluble protein [YPD], while the results of several secondary structure prediction programs available on the internet (mostly based on hydropathy analysis of the amino acid sequence) were variable, with some indicating the possible presence of several transmembrane helices and others predicting a soluble structure, depending on the exact parameters of the programs (data not shown). For

rat mitochondrial glycerol-3-phosphate dehydrogenase, association to the inner mitochondrial membrane has been reported [Klingenberg, 1970]. Gut2p has high similarity to rat and other mammalian mitochondrial glycerol-3-phosphate dehydrogenases, which are generally solubilized from mitochondrial membranes using detergents (*e.g.* [Beleznai, Szalay and Jancsik, 1988; MacDonald and Brown, 1996]), and can be incorporated into phospholipid liposomes (*e.g.* [Beleznai *et al.*, 1990]). Using activity measurements and a hemagglutinin-tagged version of Gut2p, it was shown that the protein exhibits a strong interaction with the mitochondrial (inner) membrane, and was only released upon carbonate extraction, demonstrating that it is a peripheral membrane protein. Hydrophobic rather than electrostatic interactions seem to be of importance for the interaction with the membrane, since the protein could not be released from the membrane using a high salt concentration. Therefore, it was concluded that Gut2p is a peripheral membrane protein, bound to the inner membrane by hydrophobic interactions. Surprisingly, the [^{125}I]TID-PC labeled Gut2p was found in the soluble fraction after pelleting the mitochondria with a disrupted outer membrane. The most likely explanation for finding the [^{125}I]TID-PC labeled protein in the soluble fraction is that only the labeled protein pool is released from the membrane, due to reaction of the phospholipid probe with a hydrophobic part of the protein, thus impairing its association with the inner membrane.

What could be the significance of the observed interaction between PC and Gut2p? An attractive suggestion would be that Gut2p depends on phospholipids for its activity, and that interaction of the protein with the membrane is related to the regulation of the enzymatic activity of the protein. The observation that the labeling of Gut2p is increased in the presence of NADH supports this suggestion. Interestingly, the substrate for Gut2p, glycerol-3-phosphate, is also a precursor for phospholipid biosynthesis. Furthermore, the *GUT1* gene, encoding glycerol kinase, the enzyme catalyzing the formation of glycerol-3-phosphate, was recently shown to have the upstream activating sequence UAS_{INO} in its promotor region [Grauslund, Lopes and Rønnow, 1999]. As a consequence, its expression is co-regulated with many enzymes of phospholipid biosynthesis. This mechanism of regulation might be complemented by down-regulation of Gut2p activity by low phospholipid content, making more glycerol-3-phosphate available for phospholipid biosynthesis. Another interesting observation in this respect, which might point to a specific requirement for PC, is that that yeast strains with defects in the methylation pathway of PC synthesis appear to be more severely affected in growth in the absence of choline on carbon sources where the glycerol-3-phosphate shuttle is normally highly active (*i.e.* under conditions of high cytosolic NADH generation [Larsson *et al.*, 1998] on glycerol and ethanol *vs.* glucose and lactate, unpublished data). Notably, modulation of activity by membrane lipid composition has been shown for mammalian mitochondrial glycerol-3-phosphate dehydrogenases [Nalecz *et al.*, 1980; Amler *et al.*, 1990]. Furthermore, the activity of the aerobic glycerol-3-phosphate dehydrogenase

from *Escherichia coli* (GlpD), which has similarity with Gut2p, was found to be increased in the presence of phospholipids [Schryvers, Lohmeier and Weiner, 1977; Robinson and Weiner, 1980], and involvement of GlpD in regulation of membrane phospholipid biosynthesis was recently suggested [Flower, 2001].

In conclusion, the present study identifies an interaction between the yeast mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) and PC. Although several suggestions were presented, the significance and specificity of this interaction remain to be clarified.

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Chapter 7

Summarizing discussion

This thesis addresses the process of import of phosphatidylcholine (PC) into yeast mitochondria, and describes observations done in studies aimed at elucidation of the mechanism and identification of the factors involved. PC is synthesized in the endoplasmic reticulum *via* two distinct pathways (CDP-choline pathway or methylation of phosphatidyl-ethanolamine (PE)) and has to be imported into mitochondria and sorted over both mitochondrial membranes for proper biogenesis of the organelle.

Intramembrane transport

The import of PC most likely takes place by a combination of intermembrane and transmembrane transport steps. In chapter 2, the process of transmembrane movement across the mitochondrial outer membrane was investigated *in vitro*, using isolated outer membrane vesicles (OMV) from the yeast *Saccharomyces cerevisiae*. It was concluded that rapid bidirectional transmembrane movement of both endogenous and *in vitro* introduced PC occurs in isolated mitochondrial outer membrane vesicles, with a $t_{1/2}$ of one min or less at 30˚C, based on the following observations. The phosphatidylcholine-transfer protein (PC-TP) was used to extract radiolabeled PC from OMV, with small unilamellar vesicles (SUV) serving as acceptor system. Endogenously radiolabeled PC synthesized either *via* the CDP-choline pathway or *via* methylation of PE could be extracted completely from the OMV with a halftime in the order of one min at 30˚C. In the reverse experiment where radiolabeled PC was introduced *in vitro* into the OMV, similar characteristics for the exchange were found, demonstrating that the behavior of the synthetic phospholipid resembles that of the endogenous PC. It was found that PC is not exclusively located in the outer leaflet of the OMV, since only 30-35% can be degraded in intact OMV by phospholipase A_2 (from bee venom, bvPLA$_2$), irrespective of whether the PC is introduced by PC-TP or endogenously synthesized *via* either of the pathways of biosynthesis. A further increase in degradation of labeled PC is accompanied by the loss of barrier function of the OMV. This strongly suggests that the phospholipase then gains access to a PC pool located in the inner leaflet of the membrane which remains protected against degradation while the membrane is still intact, consistent with movement of part of the newly introduced PC to the inner leaflet. Although the maximal accessibility of PC to bvPLA$_2$ found in intact OMV should only be regarded as a relative measure of the transmembrane distribution and not as an absolute value, the similar accessibility of newly introduced and endogenous PC to bvPLA$_2$ demonstrates that PC introduced by PC-TP assumes a transbilayer orientation similar to that of endogenous PC independent of its route of synthesis.

The transmembrane movement appears to be at least as fast as the PC-TP mediated exchange process with a halftime in the order of one min. Considering the short doubling time of the yeast *Saccharomyces cerevisiae*, *in vivo* translocation of phospholipids to and in the mitochondrion has to be very fast to ensure simultaneous development with the rest of

the cell. The short halftime found for transmembrane movement of PC across the mitochondrial outer membrane from yeast *in vitro* is in good agreement with the *in vivo* requirements. The rapid transmembrane movement of PC in OMV would suggest the involvement of proteins in this process, since in protein-free model membranes only very slow transbilayer movement occurs (*e.g.* [Johnson, Hughes and Zilversmit, 1975]). However, the process of transmembrane movement demonstrated has no strict energy requirement, and the extent of the transmembrane movement of PC in the OMV, as judged from the size of the pool of PC in OMV available for exchange by PC-TP, was not influenced by pretreatment with proteinase K or with sulfhydryl reagents, providing no direct clues for the involvement of proteinaceous factors. Similar energy-independent and protease insensitive processes are occurring *e.g.* in bacterial membranes [Huijbregts, De Kroon and De Kruijff, 2000]. In yeast microsomal membranes flipping of aminophospholipids is sensitive to proteases but does not require metabolic energy [Nicolson and Mayinger, 2000]. Since the possibility remained that the kinetics rather than the extent of the exchange might be affected by modification of proteins responsible for the transmembrane movement in OMV, this was investigated in an on-line fluorescence assay with pyrene PC. Using PC-TP, pyrene PC was introduced into OMV from donor SUV, which contained trinitrophenyl-PE to quench pyrene fluorescence. This allowed direct assessment of the exchange process, by measuring the increase of fluorescence upon transfer of pyrene PC to the OMV membrane. However, no effects of pretreatment of OMV with a range of protein modifying agents were detected, other than direct quenching of pyrene fluorescence by a number of these reagents (data not shown).

To identify proteins involved in the process of transmembrane movement across the outer membrane, one might apply a brute force approach, by fractionating and reconstituting outer membrane proteins into model membranes and assaying transmembrane movement of PC. However, the possibility that there is not a dedicated protein mediating the process should also be considered. The mere presence of transmembrane proteins might induce rapid flip-flop by causing local disturbances in the membrane structure, and such a mechanism would explain the insensitivity of the process to protein modifying agents. This hypothesis is currently under investigation in our laboratory. When transmembrane model peptides are incorporated into model membranes transbilayer movement of phospholipids is observed in a peptide and lipid specific manner (Kol *et al.*, 2001).

Variations in phospholipid composition

The relative importance of the CDP-choline pathway to net PC biosynthesis was assessed by *in vivo* labeling (chapter 2). It was found that, under the culture conditions used, approximately 20% of total cellular PC originates from free choline present in the growth medium. This is probably an underestimate of the total contribution of the CDP-choline pathway to the production of PC since the choline originating from turnover of PC produced

via methylation of PE can also be used for production of PC *via* the CDP-choline pathway. The amount of choline in the medium appears to be limiting its use for PC synthesis under the conditions used. Importantly, no significant differences in the contribution of choline from the medium for production of PC for the whole cell and the mitochondria were found. This result indicates no preference of the mitochondria for importing PC synthesized by either one of the pathways of biosynthesis.

During the course of the investigations, it was noticed that the phospholipid composition, more in particular the PC/PE ratio, of the mitochondria was highly dependent on the moment of harvest of the yeast cells in the late log phase. Therefore, a systematic investigation was carried out as described in chapter 3, assessing the influence of both the growth medium and the growth stage on the phospholipid composition of yeast cells, and derived subcellular fractions, and on the activities of several phospholipid biosynthetic enzymes. Fermentable (glucose) and non-fermentable (lactate) semi-synthetic and complete synthetic media were used, and several distinct differences as well as similarities were found. The cellular phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio was found to vary with the growth phase, with increases in PC levels at the expense of PE during the transition to stationary phase. The variation was most pronounced in semi-synthetic lactate medium, which is routinely used for the isolation of mitochondria, where the PC/PE ratio changed from 0.9 to 2.2 during this transition. Since it is known that the CDP-choline route requires exogenous choline for net PC synthesis and as only 20% of the cellular PC is produced de novo *via* this route when yeast is grown on semi-synthetic lactate medium, the PNMT enzymes have to be responsible for the observed formation of PC at the expense of PE. In semi-synthetic lactate medium the start of the increase in PC/PE ratio appears to concur with the peak in phospholipid biosynthetic activity found at the end of logarithmic growth. A similar, albeit less pronounced correlation is apparent in the other culture media tested. The activities of the phospholipid biosynthetic enzymes phosphatidylserine synthase and the phospholipid-N-methyltransferases were found to be maximal at the end of logarithmic growth and to decrease upon entering stationary phase in all media.

Similar growth phase dependent changes in PC and PE content were observed in isolated organelles such as mitochondria, mitochondria-associated membranes, and microsomes. The changes are more pronounced in the microsomes and mitochondria-associated membranes than in the mitochondria, as would be expected based on the localization of the PNMT activities in the endoplasmic reticulum, which are responsible for the observed conversion of PE to PC. The explanation for the increase of PC at the expense of PE could be that during exponential growth and the shift to stationary phase methionine is preferentially used for *e.g.* protein synthesis whereas it may become available in greater quantities for the methylation of PE to form PC once methionine is no longer as urgently needed for protein synthesis. Alternatively, PC may be a ‘low-maintenance’ phospholipid

that is preferentially used in cellular membranes in stationary phase, whereas there may be a more pronounced preference for the non-bilayer lipid PE during periods of major physiological activity.

Strikingly, phosphatidylinositol (PI) levels were much higher in cells grown on lactate compared to cells grown on glucose (20% versus 5-10%). Based on the notion that the availability of inositol is an important factor determining the levels of PI, an attractive explanation is that the higher PI content on non-fermentable carbon source could also be due to increased expression of the *INO1* gene during non-fermentative growth. Cells grown in lactate media displayed a significantly higher phospholipid to protein ratio compared to cells grown in glucose media, which implies the need for a higher relative rate of phospholipid biosynthesis on non-fermentable media. It is likely that the higher ratio of phospholipid to protein is due to the presence of more mitochondrial membranes per cell, consistent with the higher CL content of cells grown on lactate-based medium.

It is established that growth phase and medium have profound effects on the phospholipid composition and content and the activities of several phospholipid biosynthetic enzymes in a wild-type yeast strain. The distinct differences between cells grown on fermentable and non-fermentable media, which have largely been overlooked so far, raise an extra interest in studying the biosynthesis and transport of phospholipids under non-fermenting conditions.

'Trans-catalysis' hypothesis for methyltransferases

In chapter 4, the possibility that the phospholipid-N-methyltransferase enzymes from yeast are capable of acting upon a phospholipid substrate, localized in a different membrane than in which the enzymes reside ('trans-catalysis' hypothesis), was investigated. Such a mechanism would abolish the need for actual transport of PC between endoplasmic reticulum and mitochondria. Subcellular fractions isolated from *opi3* and *cho2* gene disruptant strains were used, which are defective in phosphatidylethanolamine transferase (PEMT), or phospholipid methyltransferase (PLMT), respectively. When homogenates of an *opi3* and a *cho2* strain are mixed, the combined methyltransferase activity, measured as the incorporation of [^{3}H]methyl label from *S*-adenosyl methionine, exceeds that expected based on the separate activities. A similar cooperativity of the PEMT and PLMT enzymes was found upon mixing isolated *opi3* and *cho2* microsomes, excluding the involvement of soluble factors or membranes other than microsomal membranes. The increased incorporation implies that monomethyl PE generated by PEMT becomes available for PLMT, for which enzyme the amount of phospholipid substrate is limiting the incorporation of label from [^{3}H]SAM, in the absence of PEMT. Analysis of the products formed provided further support for increased substrate availability. The levels of dimethyl PE and phosphatidylcholine synthesized were increased but not at the expense of the level of

monomethyl PE, indicating that this was also synthesized in increased quantities. The exact product composition was found to depend on the relative amount of the activity of each enzyme in the mixture.

To distinguish whether the observed increase in substrate availability was due to 'trans' action of the methyltransferase enzymes, or to fusion of membranes or to transport of substrates between membranes, we wanted to determine whether the substrate for methylation has to be present in the same membrane for the methyltransferase enzymes to act upon it. It was found that wild-type microsomes were capable of converting [^{14}C]PE presented in the form of SUV to [^{14}C]PC and that the exogenous substrate was utilized to a similar extent as the endogenous substrate in the microsomal membranes. However, association of SUV to microsomes appears to be a requirement for methylation of PE. Unfortunately, the occurrence of fusion between SUV and microsomes cannot be excluded based on the present data. Nevertheless, membrane fusion cannot explain the cooperativity between the microsomal membranes, since the possibility that fusion occurs under the experimental conditions employed is remote, as judged from literature data. The kinetics of the cooperativity suggest a collision-based process, enabling either transport of substrate or 'trans-catalysis'.

Since we have so far been unable to pinpoint the effects to either of these mechanisms, the need for further research is clearly indicated. The mechanism by which substrate, presented in model membranes, becomes available for the methyltransferase enzymes in microsomes could be investigated in more detail using fluorescence measurements. Fusion or specific transfer of substrate between SUV or, preferably, large unilamellar vesicles, containing pyrene phospholipids (PE or PC or intermediates), and microsomes would manifest itself as relief of (self)quenching of pyrene fluorescence. Furthermore, subcellular fractions isolated from mutants defective in the PE methylation pathway could be useful to study the process of transport of PC to mitochondria. In an *in vitro* assay system using isolated organelles, the problem that isolated mitochondria are still contaminated with endoplasmic reticulum, even after purification, can be circumvented by the use of mutants in PC biosynthesis. When mitochondria from a strain still capable of synthesizing PC are used, one cannot be sure that any PC produced in the assay ending up in the mitochondrial fraction does not originate from adhering endoplasmic reticulum. Use of mitochondria from a strain deficient in the route of PC synthesis employed in the assay would preclude this complication. Chapter 4 also provides a basis for further investigations in that direction.

Protective effect of phosphate on yeast mitochondria

It is important to know that the integrity of mitochondria is preserved in *in vitro* studies. In chapter 5, the buffer requirements to maintain mitochondrial intactness and sustain a stable membrane potential upon energization in *in vitro* studies were investigated, using gradient purified yeast mitochondria. The outer membrane integrity was monitored by assessing the permeability of this membrane to proteins, *i.e.* by the accessibility of Tom40p to trypsin and by the leakage of cytochrome *b2* from the intermembrane space. The membrane potential was monitored using the membrane potential sensitive fluorescent probe $diSC_2(5)$. It was found that the presence of phosphate is crucial for the generation of a stable membrane potential and for preserving the intactness of the outer membrane upon respiration *in vitro*. Upon addition of respiratory substrate in the absence of phosphate, mitochondria generate a membrane potential that collapses within one minute. Under the same conditions, the mitochondrial outer membrane is disrupted. The presence of phosphate prevents both these phenomena. The collapse of the membrane potential is reversible to a limited extent. Only when phosphate is added soon enough after the addition of exogenous respiratory substrate can a stable membrane potential be obtained again. Within a few minutes, this capacity is lost. The presence of Mg^{2+} prevents the rupture of the outer membrane, but does not prevent the rapid dissipation of the membrane potential.

The ΔpH component of the proton motive force (pmf) appears to be responsible for the compromised outer membrane integrity, since uncoupling by FCCP inhibited the rupture of the outer membrane, while dissipation of the $\Delta\Psi$ (membrane potential) component by valinomycin did not. This supports the notion that the inhibitory action of phosphate can be explained by matrix acidification. In chapter 5 it is shown directly that the integrity of the outer membrane is compromised upon respiration in such a way that it no longer functions as a barrier for proteins up to 57 kDa. This suggests strongly that swelling, due to changes in inner membrane permeability, has progressed beyond the physical limit where the outer membrane can still accommodate the matrix volume. This knowledge is relevant to a wide variety of *in vitro* studies on mitochondria, *e.g.* related to bioenergetics, the yeast mitochondria permeability channel, and protein or lipid import. Similar results were obtained for mitochondria isolated and stored in the presence of dextran or BSA. This is important to know since BSA might interfere in *in vitro* studies on phospholipid transport because of its interaction with lipids.

A protective effect of phosphate has been demonstrated before in yeast mitochondria in systematic bioenergetic studies. In 1968 phosphate was already mentioned as a coupling factor in yeast mitochondria [Balcavage and Mattoon, 1968] and in 1977 it was reported that respiration in the presence of K^+ in the absence of phosphate led to irreversible structural damage in yeast mitochondria due to a modified permeability of the inner membrane [Velours, Rigoulet and Guérin, 1977]. It was observed that stimulation of respiration in the

absence of phosphate induced a large swelling as monitored as a decrease in the absorbance at 546 nm, and electron micrographs showed that under these conditions the mitochondria appeared swollen and disintegrated. The structural damage was attributed to a large increase of the internal pH due to entrance of K^+ into the matrix which collapses the membrane potential and increases the respiration rate [Velours, Rigoulet and Guérin, 1977]. It was shown that the effect of potassium on respiration rates was indeed through a dissipation of the membrane potential and that the increase in delta pH consequently stimulates phosphate transport activity [Manon *et al.*, 1995]. It was supposed that the permeability of the inner membrane was altered irreversibly when the proton gradient became too high [Velours, Rigoulet and Guérin, 1977]. In the presence of phosphate, or other permeant ions such as arsenate, acetate, or propionate, the increase in the respiration rate was prevented and a normal morphology was maintained. This observation was recently extended to sulfate [Cortés *et al.*, 2000]. The efficiency of the protecting effect depended on the pK_a of the acid, leading to the conclusion that permeant acids prevent the formation of a too high electrochemical proton gradient [Velours, Rigoulet and Guérin, 1977]. This is in agreement with the observation that the ΔpH which is generated by respiration is high when the phosphate concentration is low and *vice versa*, showing that the ΔpH is 0.3 units when 10 mM phosphate is added to respiring yeast mitochondria instead of 0.8 units at low phosphate concentrations [Beauvoit, Rigoulet, Guérin, 1989].

Similar findings on the protective effects of phosphate were also reported in studies related to the mitochondrial permeability transition pore in yeast which is now often referred to as the yeast mitochondria unselective channel. In a recent review on the similarities and differences between the mammalian permeability transition pore and the yeast mitochondria unselective channel, the observations on large and unselective permeabilities through the inner membrane of yeast mitochondria were discussed, and it was concluded that matrix acidification by phosphate probably inhibits a respiration-induced channel [Manon *et al.*, 1998]. Recently, the same phenomenon was described as a cyclosporin-insensitive permeability transition pore in yeast mitochondria [Jung, Bradshaw and Pfeiffer, 1997]. These authors found that swelling occurs slowly in the absence of phosphate and an exogenous respiratory substrate but accelerates dramatically upon the addition of ethanol or NADH, and is also occurring rapidly in response to ATP even when oligomycin is present. The effects of respiration/energization are strongly antagonized by the presence of phosphate in a concentration dependent manner. The inhibitory action of phosphate on pore opening upon respiration was explained by matrix acidification and this was further supported by experiments in which nigericin, which causes matrix acidification by exchanging internal K^+ for external H^+, or the uncoupler FCCP were able to inhibit respiration-induced swelling in the absence of phosphate. Furthermore, while no explanation was offered, mention was made of the development of a small membrane potential which decays to no measurable value

within 60 s in mitochondria respiring on ethanol, while a large membrane potential is generated and maintained in the presence of a high phosphate concentration, in accordance with the findings in chapter 5.

Comparable observations on the protective effect of phosphate were also done in studies describing a proton permeability pathway in mitochondria as evidenced by swelling induced by ATP [Prieto *et al.*, 1992; Guérin *et al.*, 1994; Prieto, Bouillaud and Rial, 1995 and 1996]. This phenomenon was distinguished from the respiration induced permeability on the basis of the inhibitory phosphate concentration [Roucou *et al.*, 1997] but the possibility remains that both types of permeability are caused by the same system with different modes of regulation [Manon *et al.*, 1998], in accordance with the observations made by Jung and coworkers [Jung, Bradshaw and Pfeiffer, 1997].

In a study examining the function of the phosphate carrier (P_iC) it was briefly mentioned that addition of phosphate to isolated yeast mitochondria (in contrast to mammalian mitochondria) was required for detection of a membrane potential [Zara *et al.*, 1996], with reference to the known coupling activity of phosphate [Balcavage and Mattoon, 1968; Prieto, Bouillaud and Rial, 1995; Velours, Rigoulet and Guérin, 1977]. Furthermore, the same study showed that phosphate import across the inner membrane of yeast mitochondria was required for establishing a membrane potential since mitochondria in which phosphate transport was inactivated by mersalyl, and mitochondria isolated from a mutant lacking the gene encoding for P_iC have a strongly decreased membrane potential.

Furthermore, it was reported that at low concentrations of phosphate, potassium added to yeast mitochondria led to uncoupled respiration and decreased pmf, and that these effects were prevented by increasing concentrations of phosphate [Castrejón *et al.*, 1997]. In the complete absence of potassium and phosphate no membrane potential of significance was detectable. In the presence of a low concentration of phosphate, the addition of increasing concentrations of K^+ resulted in the collapse of membrane potential within 1 min, and it was found that addition of increasing concentrations of phosphate at a later time point resulted in the recovery of the membrane potential. In chapter 5, it was demonstrated that the recovery was only possible when phosphate was added soon enough after the collapse of the membrane potential. The reason why the time limits to the recovery previously went unnoticed is probably because in the experiments of Castrejón *et al.,* phosphate was added within 30 sec after the addition of ethanol. The physiological relevance of the effects of potassium was questioned because the low concentrations of phosphate used in the experiments are probably never reached intracellularly [Castrejón *et al.*, 1997]. As noted by Manon *et al.* [Manon *et al.*, 1998], it remains unclear what the relevance of the yeast mitochondria unselective channel is to yeast bioenergetics since the average phosphate concentration in yeast is 1-2 mM [Beauvoit *et al.*, 1991].

It seems that the observations on the protective action of phosphate made in the field of bioenergetics and permeability transition studies and their implications for *in vitro* studies are not readily picked up on by investigators in these areas, since similar observations are reported from slightly different angles, let alone by investigators not directly involved in these fields of research but who study mitochondria in *in vitro* systems. In the area of protein import phosphate is usually present in the buffer, although also numerous studies (*e.g.* [Martin, Mahlke, Pfanner, 1991; Geissler *et al.*, 2000]) based on the protocol of [Söllner, Rassow and Pfanner, 1991] can be found where phosphate is not added to the buffer while the mitochondrial integrity is maintained upon respiration. This apparent paradox can be explained by the fact that in these studies phosphate is provided in the form of the rabbit reticulocyte lysate [Zara *et al.*, 1996], which is necessary for protein synthesis *in vitro*, and thus satisfies the system's requirement for phosphate. Furthermore, there are several studies in which the assay conditions are clearly not fit to maintain mitochondrial intactness, while this is very important for the interpretation of the data. For example, a study was performed on the binding of a mitochondrial presequence [Swanson and Roise, 1992]. While intending to study binding to unenergized mitochondria, the presequence was added to mitochondria from ethanol, thus energizing the mitochondria. Furthermore, the experiments were performed in the absence of phosphate, which is now shown to lead to compromised mitochondrial intactness, while an important assumption in the interpretation of the data was that the presequence did not diffuse significantly through the pores of the mitochondrial outer membrane. Therefore, the charge density obtained from the binding measurements may not reflect lipids and proteins associated with the outer leaflet of the outer membrane only. Other examples are studies on mitochondrial lipid import in which phosphate was not included while studying the effect of energization/deenergization on arrival of PS in the inner membrane (*e.g.* [Simbeni *et al.*, 1993]).

Fishing for interactions between PC and mitochondrial proteins by photolabeling

In search of mitochondrial proteins interacting with PC, a photolabeling approach was applied in chapter 6, in which photoactivatable probes were added to isolated yeast mitochondria. Using either the PC analog [^{125}I]TID-PC, or the small hydrophobic probe [^{125}I]TID-BE, upon photoactivation, only a limited number of proteins was labeled. When mitochondria were labeled with [^{125}I]TID-BE, both the porin in the outer membrane and P_iC in the inner membrane were labeled intensely, while other proteins were labeled only faintly or were not detected. Since porin is abundantly present in the outer membrane, and P_iC is a prominent protein in the inner membrane, their labeling from the membrane is not surprising. A possible reason for preferential labeling of just these membrane proteins might be a large surface of exposure to the apolar core of the membrane, relative to other membrane proteins. However, the extent of labeling does not necessarily have to be

proportional to the exposed area of the protein to the probe. It could also reflect some specificity in the crosslink reaction, even though TID and other carbene generating probes are in principle able to label any membrane protein regardless of the amino acid composition of its lipid-exposed segment [Brunner, 1993].

The most prominent difference found upon labeling of mitochondria with [^{125}I]TID-PC or [^{125}I]TID-BE was the very specific labeling of a 70 kDa protein by [^{125}I]TID-PC. Only under conditions where the inner membrane was accessible to the probe, the 70 kDa protein was labeled by [^{125}I]TID-PC, in parallel with increased labeling of the P_iC in the inner membrane. After separation of mitochondrial proteins by two dimensional electrophoresis, a tryptic digest of the gel spot of interest was analyzed by mass spectrometry. The 70 kDa protein was identified as the *GUT2* gene product. This was confirmed by the lack of specific labeling in the 70 kDa region in photolabeling experiments on mitochondria from a *gut2* deletion strain. Involvement of Gut2p in PC import into yeast mitochondria was excluded since mitochondria from a *gut2* knock-out strain were found to have a PC content similar to that of the corresponding wild-type strain. The *GUT2* gene product, also known as the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase, is essential for utilization of glycerol as carbon source [Sprague and Cronan, 1977; Larsson *et al.*, 1993], and it is a component of the glycerol-3-phosphate shuttle, which delivers reduction equivalents from cytosolic NADH to the mitochondrial respiratory chain, thus contributing to the maintenance of the cytosolic redox balance under aerobic conditions [Larsson *et al.*, 1998; Overkamp *et al.*, 2000].

Since it can be excluded that differences in labeling by [^{125}I]TID-BE and [^{125}I]TID-PC result from differences in specificity of the reaction, as both probes share the same photoreactive group, the differences might reflect differences in depth of insertion of the photoactivatable group into the membrane for the small hydrophobic probe and the PC analog, resulting in different crosslink products with proteins. The fluidity of membranes and the conformational flexibility of acyl chains allows a photoreactive group attached to such a chain to assume positions within a much wider depth range than would be predicted for an extended chain [Brunner, 1993]. In addition, the degree of access to hydrophobic regions in proteins may be affected by the size of the probe. Alternatively, the differences could reflect a different lateral distribution of the probes in the membrane. Proteins could be preferentially surrounded by a certain type of lipid.

Interestingly, it was observed that during incubation at 20˚C the amount of [^{125}I]TID-PC label on Tom70p decreases, which might reflect the flipping of the phospholipid probe to the internal leaflet of the outer mitchondrial membrane. Since phospholipids integrate into membranes in an oriented manner, the probe might in principle be able to label membrane components selectively from one side of the bilayer [Brunner, 1993]. Under the conditions employed, the photoactivatable PC analog used in chapter 6,

appears to be confined to the outer membrane in intact mitochondria, since no increase of label on inner membrane components, such as P_iC, was observed. Only when the outer membrane is disrupted, a significant amount of label appears on the inner membrane protein P_iC. Nevertheless, the data in chapter 6 might offer a basis for finding conditions where transport of PC to the inner membrane takes place in intact mitochondria. The destination of the [^{125}I]TID-PC introduced in the outer membrane of mitochondria might be studied by examining the amount of radioactivity photocrosslinked to proteins of the outer and inner membrane. Suitable marker proteins for the outer and inner membrane could be porin and P_iC, respectively, or even Gut2p.

From the labeling of Gut2p by [^{125}I]TID-PC an interaction of the protein with the inner membrane can be inferred. Also in view of the physiological function of the protein, and from its similarity with other glycerol-3-phosphate dehydrogenases, membrane interactions are expected. However, secondary structure prediction programs gave no clear-cut answers on the possible membrane association of the protein. Using activity measurements and a hemagglutinin-tagged version of Gut2p, it was shown that the protein exhibits a strong interaction with the mitochondrial (inner) membrane. Since the protein was only released upon carbonate extraction, and not by a high salt concentration, it was concluded that Gut2p is a peripheral membrane protein, bound to the inner membrane by hydrophobic interactions, rather than by electrostatic interactions. Surprisingly, the [^{125}I]TID-PC labeled Gut2p was found in the soluble fraction after pelleting the mitochondria with a disrupted outer membrane. The most likely explanation for finding the [^{125}I]TID-PC labeled protein in the soluble fraction is that only the labeled protein pool is released from the membrane, due to reaction of the phospholipid probe with a hydrophobic part of the protein, thus impairing its association with the inner membrane.

To explain the significance of the observed interaction between Gut2p and PC, an attractive suggestion would be that Gut2p depends on phospholipids for its activity, and that interaction of the protein with the membrane is related to the regulation of the enzymatic activity of the protein. The observation that the labeling of Gut2p is increased in the presence of NADH supports this suggestion. Interestingly, the substrate for Gut2p, glycerol-3-phosphate, is also a precursor for phospholipid biosynthesis. The expression of the *GUT1* gene, encoding the enzyme catalyzing the formation of glycerol-3-phosphate (glycerol kinase), is co-regulated with many enzymes of phospholipid biosynthesis. This mechanism of regulation might be complemented by down-regulation of Gut2p activity by low phospholipid content, making more glycerol-3-phosphate available for phospholipid biosynthesis. Another interesting observation in this respect, which might point to a specific requirement for PC, is that the growth of yeast cells appears to be more dependent on the cellular PC content when the catabolism of the carbon source involves Gut2p. Notably, modulation of activity by membrane lipid composition has been shown for mammalian

mitochondrial glycerol-3-phosphate dehydrogenases [Nalecz *et al.*, 1980; Amler *et al.*, 1990]. Furthermore, the activity of the aerobic glycerol-3-phosphate dehydrogenase from *Escherichia coli* (GlpD), which has similarity with Gut2p, was found to be increased in the presence of phospholipids [Schryvers, Lohmeier and Weiner, 1977; Robinson and Weiner, 1980], and involvement of GlpD in regulation of membrane phospholipid biosynthesis was recently suggested [Flower, 2001].

However, the significance and specificity of the interaction between the yeast mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) and PC remain to be clarified. The hypothesis that Gut2p requires PC for activity can be tested using PC biosynthetic mutants. In these cells the PC content can be manipulated *in vivo*, allowing examination of growth phenotypes under conditions where Gut2p activity is required, and measurement of Gut2p activity in mitochondria with varying PC contents. Furthermore, *in vitro* experiments with purified Gut2p and model membranes of varying phospholipid compositions will permit a systematic evaluation of the requirements for activity and mode of membrane interaction. If the hypothesis turns out to be correct, this might pave the way for development of a genetic screen or selection method to find yeast mutants disturbed in mitochondrial PC import.

Future prospects

From the foregoing, it has become clear that the identification of the factors involved in the import process of PC in yeast mitochondria is not trivial. A step in the right direction is the development of better assay systems to study the transport process itself. The use of mutants defective in PC biosynthesis will certainly contribute, since problems of crosscontamination can then be avoided in reconstituted systems using isolated organelles. However, the major impediment in lipid transport studies is usually the lack of a metabolic conversion of the lipid upon arrival at its destination. Such a conversion would allow rapid assessment of the occurrence of transport, without the need for separation of the participating membranes to see where the lipid is. As discussed, photolabeling might be used to monitor the localization of the phospholipid probe by using suitable marker proteins in each membrane. Another tool which could provide information on the mechanism of PC import into mitochondria is mass spectrometry on phospholipids. The metabolic relationships between PE and PC pools in the endoplasmic reticulum and mitochondrial membrane lipids might follow from the analysis of the molecular species composition found in mitochondrial membranes, MAM and microsomes. This research is currently carried out in our laboratory.

Even if the mechanism of transport is established, identifying the responsible factors is still quite a challenge. Classical genetics could be very useful in this respect, although the applicability is highly dependent on knowledge of the physiological role of the process under study and the correct prediction of related phenotypes. Identification of the interaction

between Gut2p and PC might eventually lead to the development of a genetic screen or selection method to find yeast mutants disturbed in mitochondrial PC import. The photolabeling technique applied to identify this interaction could also be used more generally as a tool to search for specific interactions between proteins and certain lipid classes in a wide range of membranes.

In the pre-genomics era, the most straightforward approach for the identification of protein factors governing lipid transport, would be the reconstitution of candidate proteins and *in vitro* assessment of activities, as proposed in the above for the putative flippase in the mitochondrial outer membrane. However, the technological progress in the fields of genomics and proteomics permits also the use of large scale screening and identification methods. For example, the DNA array chip technique could be used to investigate the involvement of genes in mitochondrial biogenesis by comparison of the right set of conditions. The development of proteomics in the past decade would provide a complementary approach, allowing the direct assessment of the effects on the level of gene products. One of the major challenges for the future will be to integrate all the data obtained by applying the wealth of advanced technologies we have at our disposal. Although each of these techniques and approaches will contribute to solve the puzzle, neither of them is likely to provide all the answers at once.

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Samenvatting

Levende wezens zijn opgebouwd uit cellen, de kleinste eenheden van leven. Een cel wordt omsloten door een soort vliesje, dat membraan genoemd wordt. Dit membraan scheidt binnen van buiten en zorgt er zo voor dat de juiste condities in de cel gehandhaafd kunnen worden en verzorgt ook de communicatie met de buitenwereld. In eenvoudige cellen, zoals bacteriën (prokaryoten), spelen veel biochemische processen zich af in het membraan of in de vloeistof (cytosol), die door dat membraan omvat wordt. In meer complexe cellen, zoals van planten en dieren (eukaryoten), zijn er in de cel ook nog allerlei compartimenten, organellen genoemd, die elk hun eigen functie uitoefenen en door een membraan van de rest van de cel gescheiden zijn. Het mitochondrion is een organel dat als belangrijkste functie heeft het verzorgen van de energiehuishouding van de cel. Het bestaat uit twee membranen, een sterk gevouwen binnenmembraan, omsloten door een buitenmembraan (zie Figuur 1A op pagina 10). In het mitochondrion kunnen zo de optimale omstandigheden in stand gehouden worden om energie te produceren. Deze processen vinden zowel in de membranen als in de vloeistof bevattende ruimtes omsloten door de membranen plaats en worden veelal verzorgd door eiwitten (enzymen).

Biologische membranen zijn voornamelijk opgebouwd uit eiwitten en vetten (lipiden). Zoals iedereen wel weet zijn vetten slecht in water oplosbaar, wat er voor zorgt dat de molekulen in water bij elkaar gaan zitten, denk bijvoorbeeld aan oliedruppels in water. Fosfolipiden zijn een bepaalde klasse vetten, die bestaan uit een deel (kopgroep) dat wel in water op kan lossen (hydrofiel) met daaraan twee staarten die slecht in water oplossen (hydrofoob). Door deze staarten naar elkaar te steken, terwijl de kopgroepen de staarten van het water afschermen, kunnen de lipiden een dubbellaag vormen. Samen met de eiwitten vormen de lipiden zo een biologisch membraan (zie Figuur 1B op pagina 10). De kopgroep van de verschillende fosfolipiden is verschillend en ook de lengte en samenstelling van de staarten kunnen verschillen. Het meest voorkomende fosfolipide in eukaryote cellen is fosfatidylcholine (zie Figuur 1C op pagina 10). De eigenschappen van een membraan worden bepaald door welke lipiden en eiwitten er in zitten en zijn niet hetzelfde voor verschillende membranen. Zo is de samenstelling van het buitenmembraan van het mitochondrion heel anders dan die van het binnenmembraan en ook anders dan die van het buitenste membraan van de cel. Er zijn zeer veel verschillende eiwitten die allemaal hun eigen functie hebben en een bepaald eiwit komt meestal alleen op een zeer specifieke plaats in de cel voor. Voor lipiden is die verdeling wat minder absoluut. Zo bevatten de meeste membranen dezelfde soorten lipiden, maar vaak in verschillende karakteristieke verhoudingen. Wanneer bepaalde eiwitten of lipiden niet gemaakt worden of zich niet op de juiste plek in de cel bevinden leidt dit vaak tot het niet, slecht of anders functioneren van biochemische processen.

Omdat niet alle componenten waaruit het mitochondrion bestaat op de plaats gemaakt worden waar ze uiteindelijk nodig zijn, is er transport nodig. Het mitochondrion is niet volledig zelfstandig in de voorziening van de bouwstenen voor zijn ontwikkeling en moet dus componenten importeren vanuit de rest van de cel. Het grootste deel van de mitochondriële eiwitten wordt geïmporteerd en er is vrij veel bekend over hoe dit werkt. De meeste eiwitten bestemd voor het mitochondrion worden in het cytosol (waarin zich ook de andere organellen bevinden) geproduceerd met een soort postcode eraan vast die door het mitochondrion herkend wordt. Deze postcode wordt er meestal vanaf geknipt zodra het eiwit op zijn bestemming aangekomen is. Ook de meeste fosfolipiden moeten geïmporteerd worden vanuit een ander deel van de cel. Het organel dat de productie van veel lipiden verzorgt heet het endoplasmatisch reticulum. Fosfatidylcholine, het meest voorkomende fosfolipide in de mitochondriële membranen, wordt in het endoplasmatisch reticulum gemaakt via twee verschillende biochemische reactieroutes en moet dus ook geïmporteerd worden in het mitochondrion. In tegenstelling tot eiwitten hebben lipiden echter geen postcode die duidelijk maakt waar ze terecht moeten komen en het transportproces moet dus op een andere manier geregeld zijn. Hier is erg weinig over bekend.

Het in dit proefschrift beschreven onderzoek had als doel om te ontrafelen hoe het proces van import van fosfatidylcholine in het mitochondrion plaatsvindt en welke factoren hierbij van belang zijn. Hiervoor werd de gist *Saccharomyces cerevisiae* gebruikt. Naast het bewezen nut van gist in bijvoorbeeld het bakken van brood of het brouwen van bier, wat niet veel mensen ontgaan zal zijn, is het ook nog eens een buitengewoon handig modelorganisme om onderzoek aan te doen. Niet alleen lijkt de organisatie van gistcellen erg veel op die van bijvoorbeeld de mens en zijn veel processen op dezelfde manier geregeld, ze zijn ook nog eens erg toegankelijk voor genetisch onderzoek en genetische modificatie. Doordat de volledige genetische code van gist bekend is is het mogelijk om verbanden te leggen tussen veranderingen in het genetisch materiaal en biochemische processen in de cel.

In hoofdstuk 2 werd onderzocht hoe fosfatidylcholine zich over het buitenmembraan van het mitochondrion verplaatst. Tijdens import in het mitochondrion moet het waarschijnlijk van de buitenkant van het membraan naar de binnenkant van het membraan getransporteerd worden (transmembraan transport). Er werd gevonden dat het een heel snel proces is wat zowel heen als terug gaat. Binnen slechts enkele minuten kan het merendeel van de molekulen zich van de ene naar de andere kant van het membraan bewegen. Dit is ook nodig gezien het feit dat in de levende cel er snel transport moet plaatsvinden zodat de cel snel genoeg kan groeien. Omdat in een kunstmatig membraan zonder eiwitten slechts langzaam transmembraan transport van lipiden plaatsvindt, werd de eventuele betrokkenheid van eiwitten onderzocht. Hier werden geen directe aanwijzingen voor gevonden. Verder werd gevonden dat er geen voorkeur is van het mitochondrion voor het importeren van fosfatidylcholine geproduceerd op één van de twee verschillende manieren.

In hoofdstuk 3 werd ingegaan op veranderingen in fosfolipidensamenstelling tijdens de groei van gistcellen en hoe deze afhangen van de voedingsstoffen die aanwezig zijn in het groeimedium. Er werd gevonden dat de verhouding van de twee meest voorkomende fosfolipiden, fosfatidylcholine en fosfatidylethanolamine, variëert tijdens de groei, waarbij het fosfatidylcholinegehalte toeneemt ten koste van het fosfatidylethanolaminegehalte wanneer de cellen stoppen met groeien. Deze variatie was het meest uitgesproken in het medium wat meestal wordt gebruikt voor het isoleren van mitochondriën uit gistcellen en werd ook waargenomen in geïsoleerde organellen. Onder condities wanneer de gistcellen erg afhankelijk zijn van hun mitochondriën werd een hoger gehalte van het fosfolipide fosfatidylinositol gevonden en bleken de cellen relatief meer fosfolipiden te bevatten.

In hoofdstuk 4 werd onderzocht of de enzymen verantwoordelijk voor één van de productieroutes van fosfatidylcholine (die zich bevinden in het membraan van het endoplasmatisch reticulum) ook in staat zijn om deze reactie te bewerkstelligen met molekulen gelegen in een ander membraan dan waarin de enzymen zelf zitten ('trans-katalyse'). Als dit namelijk het geval is zou er geen transport tussen endoplasmatisch reticulum en mitochondriën plaats hoeven vinden maar zouden de molekulen in feite op hun bestemming gemaakt kunnen worden. Dit werd onderzocht door twee soorten membranen te mengen die elk een deel van de beoogde reactie kunnen uitvoeren. Samen bleken ze in staat de volledige reactie te volbrengen. Er waren geen extra toevoegingen nodig, wat er op wijst dat alleen membraancomponenten van belang zijn, en het botsen van de membranen met elkaar lijkt een voorwaarde te zijn. Helaas konden geen definitieve conclusies getrokken worden over welk mechanisme er verantwoordelijk is, transport of 'trans-katalyse'.

In hoofdstuk 5 staat beschreven wat de voorwaarden zijn voor het behoud van de intactheid en de functie van mitochondriën bij experimenten met geïsoleerde organellen in de reageerbuis. De aanwezigheid van fosfaat bleek hierbij van essentiëel belang. Deze kennis is van belang voor alle onderzoekers die met mitochondriën werken.

In hoofdstuk 6 werd de interactie van fosfatidylcholine met eiwitten in het mitochondrion bestudeerd om meer informatie te verkrijgen over de functie van het fosfolipide. Hierbij werd gebruik gemaakt van fotoactiveerbare lipiden. Deze molekulen zijn in staat om te reageren met andere molekulen in hun directe omgeving na activering met UV-licht. Verrassend genoeg werd een zeer specifieke interactie gevonden met een mitochondriëel eiwit dat relatief in slechts kleine hoeveelheden aanwezig lijkt te zijn. Dit eiwit werd geïdentificeerd als Gut2p (de mitochondriële glycerol-3-fosfaat dehydrogenase). De interactie heeft eventueel consequenties voor het functioneren van dit eiwit en kan misschien in de toekomst de mogelijkheid bieden om het proces van import van fosfatidylcholine in mitochondriën verder te ontrafelen.

Al met al is dit proefschrift een stap in de richting van een antwoord op de gestelde onderzoeksvraag maar zoals gebruikelijk in wetenschappelijk onderzoek zijn er uiteraard ook tal van nieuwe vragen opgekomen.

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Curriculum vitae

De schrijfster van dit proefschrift werd op 26 februari 1973 geboren in Sevenum. In juni 1991 werd het Gymnasium B diploma behaald aan het Boschveldcollege in Venray. Vervolgens werd in september 1991 aan de studie Scheikunde begonnen aan de Universiteit Utrecht. Het doctoraalexamen werd afgelegd in augustus 1996 (*cum laude*), met als bijvakken Biochemie van Membranen, Bio-Organische Chemie en als hoofdvak Biochemie van Lipiden. Aansluitend volgde een vierjarige aanstelling bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek, via de stichting Chemische Wetenschappen, als Onderzoeker in Opleiding bij de sectie Biochemie van Membranen aan de Universiteit Utrecht. De verkregen onderzoeksresultaten staan beschreven in dit proefschrift.