Photolabeling Identifies an Interaction between Phosphatidylcholine and Glycerol-3-phosphate Dehydrogenase (Gut2p) in Yeast Mitochondria[†]

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ABSTRACT: In search of mitochondrial proteins interacting with phosphatidylcholine (PC), a photolabeling approach was applied, in which photoactivatable probes were incorporated into isolated yeast mitochondria. Only a limited number of proteins were labeled upon photoactivation, using either the PC analogue [^{125}I]TID-PC or the small hydrophobic probe [^{125}I]TID-BE. The most prominent difference was the very specific labeling of a 70 kDa protein by [^{125}I]TID-PC. Mass spectrometric analysis of a tryptic digest of the corresponding 2D-gel spot identified the protein as the *GUT2* gene product, the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase. This was confirmed by the lack of specific labeling in 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. A hemagglutinin-tagged version of Gut2p 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. The significance of the observed interactions between Gut2p and PC is discussed.

Phosphatidylcholine (PC)[1] 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 phosphatidylethanolamine (PE) or via the CDP-choline (Kennedy) pathway [for reviews, see (*1*, *2*)]. 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 (*3*).

PC is also a major constituent phospholipid of both mitochondrial membranes, representing 40−50% of the total mitochondrial phospholipid content (*4*). The biogenesis of mitochondria requires efficient import of PC from its site of synthesis, the endoplasmic reticulum. Several mutant strains with defects in the routes of biosynthesis of PC have the tendency to generate respiratory-deficient petites at high frequency (*5*), suggesting that PC is required for proper mitochondrial function, biogenesis, or maintenance. The basis for such a requirement is unknown. One possibility is that PC is required to maintain the barrier properties of the mitochondrial membranes, but it is more likely that specific PC−protein interactions are involved. The molecular interactions of exposure of membrane proteins to the surrounding lipids may have functional implications which are currently unknown.

Little, if anything, is known about interactions between PC and proteins in yeast mitochondria. Using the classical approach to gain information on lipid−protein interactions, i.e., by studying purified proteins in reconstituted systems, it has been shown that the mammalian mitochondrial enzyme β-hydroxybutyrate dehydrogenase is dependent on PC for activity in vitro (*6*). In a quest for yeast mitochondrial proteins interacting with PC, a photolabeling strategy is used in situ in the present study. This might allow the identification of proteins with a specific interaction with PC and generate knowledge on the specific role of PC in yeast mitochondria.

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[1] Abbreviations: FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazine; GUT, glycerol utilization; IEF, isoelectric focusing; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NEPHGE, nonequilibrium pH gradient electrophoresis; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PCR, polymerase chain reaction; P_iC, phosphate carrier; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate; TID, 3-(trifluoromethyl)-3-phenyldiazirine; TCA, trichloroacetic acid; [^{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.

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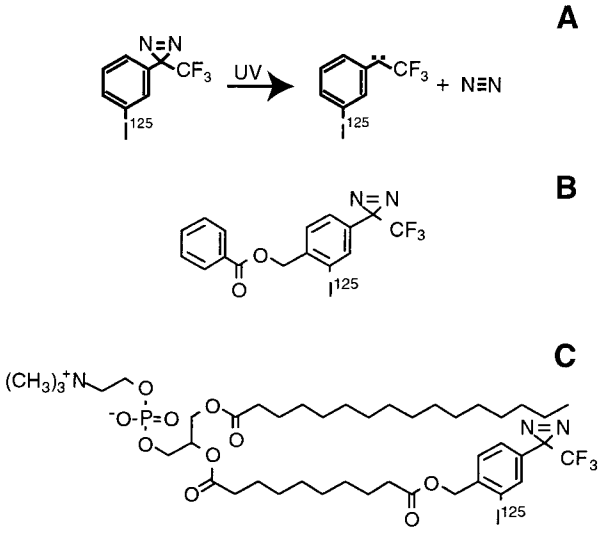

FIGURE 1: Mechanism of photoactivation and structures of [^{125}I]TID-based photoactivatable reagents. (A) Photoactivation of the [^{125}I]TID reactive group; (B) the structure of [^{125}I]TID-BE; (C) the structure of [^{125}I]TID-PC.

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 (*7*). Generally, hydrophobic photolabeling probes are used to study the exposure of proteins to the apolar core of membranes (*8*). Phospholipid analogues offer an additional advantage since specific interactions of proteins with phospholipids can be monitored and identified. Recently, such probes were successfully applied to investigate the exposure of SecA to phospholipid acyl chains in (proteo)liposomes (*9*).

In the present study, yeast mitochondrial membranes are photolabeled with probes based on radioiodinated 3-trifluoromethyl-3-aryldiazirine ([^{125}I]TID) which forms a reactive carbene upon photolysis (Figure 1A), and which was developed by Brunner and co-workers (*7*, *10*). Upon comparing protein labeling patterns of mitochondrial membranes photolabeled with the simple hydrophobic probe [^{125}I]TID-BE (Figure 1B) and the PC analogue [^{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 submitochondrial localization of Gut2p was determined. The significance of the interaction between Gut2p and PC will be discussed.

EXPERIMENTAL PROCEDURES

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). 'Reactivials' were from Pierce (Rockford, IL). Mouse monoclonal antibodies raised against the hemagglutinin epitope were from Boehringer, and the antibiotic G418 was from Calbiochem. All other chemicals were analytical grade.

Table 1: Genotypes of Strains Employed in This Study

strain	genotype
D273-10B	*MAT*α
W303-1A	*ade 2-1^0 his3-11 trp1-1 ura3-1 can100^0*
W303-1A *gut2*Δ	*ade 2-1^0 his3-11 trp1-1 ura3-1 can100^0 gut2::URA3*
BY4742	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ

Isolation of Mitochondria and Other Subcellular Fractions. The yeast strains listed in Table 1 were grown aerobically to late-log phase [OD600 4-5 (Perkin-Elmer Lambda 18 UV/VIS spectrophotometer)] at 30 °C in semi-synthetic lactate medium (*11*). 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. Spheroplasts were prepared using zymolyase as described previously (*11*) 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 phenylmethylsulfonyl fluoride and 0.5% (w/v) dextran (M_r 40 000) were added (*12*). The isolation and purification of mitochondria using Nycodenz gradient centrifugation were based on published procedures (*11*, *13*, *14*) as described (*12*). 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 and microsomes were isolated as described (*15*). 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 Photo-Cross-Linking Probes. The hydrophobic photoactivatable probe [^{125}I]TID-BE (Figure 1B) and the photoactivatable phospholipid [^{125}I]TID-PC (Figure 1C) were prepared by radioiododestannylation of the tin-based precursors as described [(*10*) and (*7*), 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 of Na^{125}I, the reaction was started by adding 5 μL of peracetic acid (Sigma). 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 of 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/mL of buffer) containing either mitochon-

dria, mitoplasts, or mitochondrial outer membranes, under stirring on ice. Aliquots of this suspension were incubated for the indicated times at the indicated temperatures. Alternatively, aliquots of the suspension were immediately mixed (on ice) with an equal volume of H/S buffer containing extra ingredients such as FCCP or NADH, prior to incubation, to investigate the effect of these additives. Thus, different conditions were examined in parallel starting from the same sample. 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 final concentrations 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 Photo-Cross-Linked Proteins. A 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 of 2% (w/v) SDS by heating for 10 min at 95 °C, and diluted with 625 μL of 20 mM Tris/HCl (pH 7.4) containing 310 mM NaCl and 0.5% (v/v) Triton X-100. After 15 min on ice, the solution was precleared by centrifugation. A 20 μL aliquot of the supernatant was withdrawn and mixed with 20 μL of concentrated SDS−PAGE buffer to serve as a 10% standard, and 200 μL aliquots of the supernatant were subjected to immunoprecipitation, using antibody-coated protein A beads (Pharmacia, Sweden). After immunoprecipitation, 40 μL of 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.

Two-Dimensional Gel Electrophoresis and Mass Spectrometric Analysis. Mitochondrial proteins were labeled for 10 min at 30 °C with [^{125}I]TID-PC. After precipitation, proteins were dissolved in lysis buffer and separated in the first dimension on NEPHGE (pH range 6−10) and IEF (pH range 3.5−7) gels and in the second dimension by 12.5% SDS−PAGE, followed by silver staining, drying, and autoradiography of the gels, as described (*16*). After autoradiography, the radiolabeled protein spot of interest was cut from an unstained gel to increase recovery, digested with trypsin, and subjected to mass spectrometric analysis, as described (*17*).

Measurement of Glycerol-3-phosphate Dehydrogenase Activity. The activity of glycerol-3-phosphate dehydrogenase was measured by monitoring spectophotometrically the phenazine methosulfate (PMS)-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described (*18*), 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 50 μM or above, as determined using 200 μg of purified mitochondria (protein based) 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, 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 (*19*). 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 was used to transform yeast strain BY4742, and positive colonies were selected on G418-containing plates as described (*19*). Correct integration of the PCR fragment into the yeast genome was checked by PCR analysis performed on the genomic DNA isolated from transformants. 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 (*20*), followed by centrifugation for 12 min at 10 000 rpm (10600*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 (10600*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 as a standard. γ-Counting was done with a Packard crystal multidetector RIA system. TCA precipitations were performed by adding 1 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, X-ray film or a phosphorimager (Molecular Dynamics) was used. Western blots were decorated with antibodies raised against the indicated proteins, and protein bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (DuPont NEN).

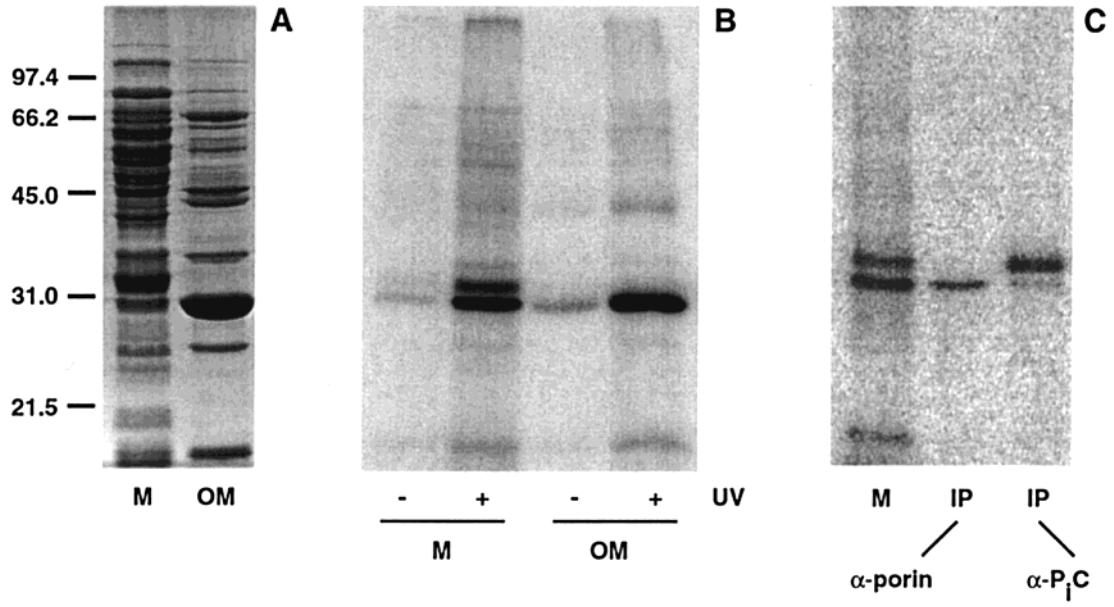

FIGURE 2: Labeling of mitochondria and mitochondrial outer membranes with [^{125}I]TID-BE. Mitochondria (M) and isolated outer membranes (OM) were incubated for 5 min at 0 °C with [^{125}I]TID-BE, added from ethanol, and were, either before (−UV) or after photolysis (+UV), subjected to TCA precipitation followed by 12% SDS−PAGE and autoradiography. (A) Coomassie-stained gel, with the position of molecular mass markers (kDa) indicated; (B) autoradiogram; (C) identification of the major [^{125}I]TID-BE-labeled proteins by immunoprecipitation. Photolysis was performed on mitochondria incubated 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.

RESULTS

Photolabeling of Mitochondria and Mitochondrial Outer Membranes with [^{125}I]TID-BE. To explore the feasibility of a photo-cross-linking 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 dependent on irradiation with UV light and that a relatively simple labeling pattern is obtained both for mitochondria and for isolated outer membranes, as compared to their total 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 in 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 label mitochondrial membrane proteins with a hydrophobic photoactivatable probe.

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 found with [^{125}I]TID-BE. In the mitochondria, P_iC 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. The labeling of both porin and P_iC was confirmed by immunoprecipitation (data not shown). As expected, P_iC 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 analogue 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 (*12*). 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_iC 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 at 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_iC labeling (cf. lanes 3 and 4). Interestingly, the intensity of labeling of Tom70p is decreased at 20 °C compared to 0

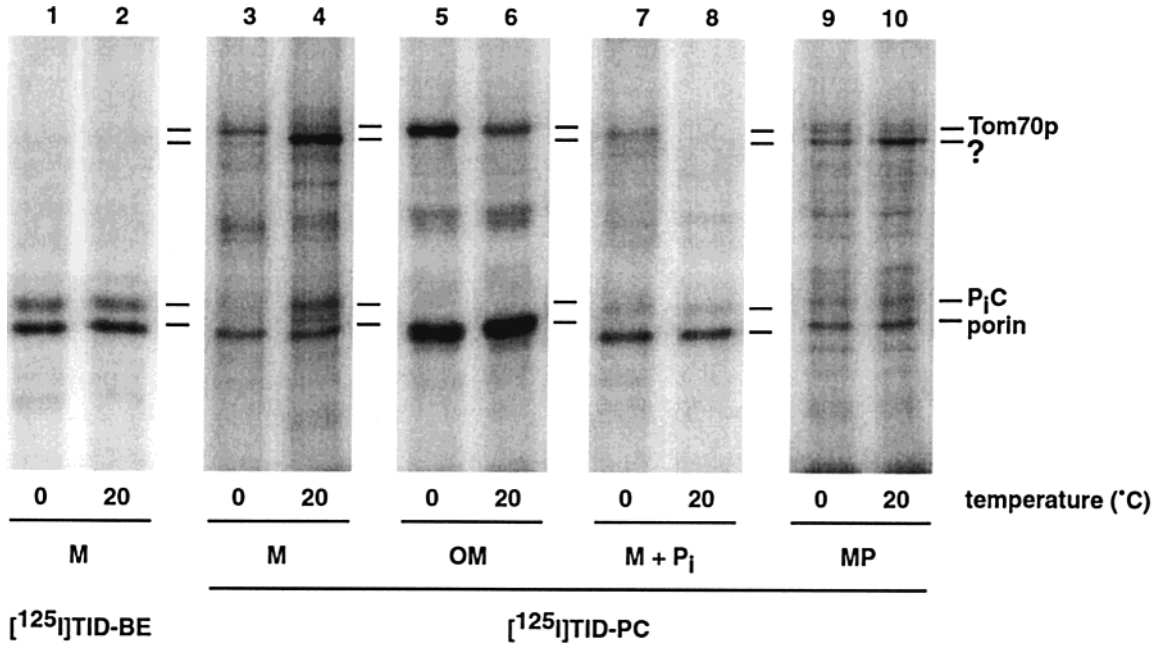

FIGURE 3: Photolabeling of mitochondrial membranes with [^{125}I]TID-BE and [^{125}I]TID-PC. Samples containing mitochondria (M), isolated outer membranes (OM), and mitoplasts (MP) were photolyzed after incubation for 30 min at 0 or 20 °C with [^{125}I]TID-BE or [^{125}I]TID-PC, added from ethanol. When indicated, the buffer contained 2 mM phosphate (P_i). Samples were analyzed by 12% SDS−PAGE and autoradiography. The positions of Tom70p, P_iC, porin, and the heavily labeled 70 kDa protein (?) are indicated.

°C, both in mitochondria and in isolated outer membranes (cf. lanes 3 and 4, and 5 and 6). The enhancement of P_iC labeling is in agreement with the inner membrane becoming accessible to the probe due to rupture of the outer membrane (*12*). 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.

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. Neither the increase of TID-PC label on P_iC nor the appearance of the prominent band at 70 kDa was observed (not shown). These results indicate that also at 20 °C, when the mitochondria remain intact, the probe is confined to the outer membrane. Rupture of the outer membrane in the absence of phosphate can be prevented by the presence of the uncoupler FCCP during respiration (*12*), or by adding [^{125}I]TID-PC from methanol instead of ethanol, in which case respiration is not induced. Under these conditions, a similar result as in the presence of phosphate was yielded (not shown). 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 at 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 and to label the 70 kDa protein.

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). Taken together, the results indicate that the PC analogue 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. Since one-dimensional separation by SDS−PAGE was insufficient to obtain a protein sample containing only the protein of interest (data not shown), 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 silver-stained gel (Figure 4B). The spot was cut from an unstained 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. The tryptic peptides covered 60% of the mature protein sequence (Figure 5B). Both the molecular mass and the isoelectric point found on the gel are in agreement with those predicted in the Yeast Protein Database (molecular mass 68.4 kDa and p*I* 7.1) (*21*). The start of the mature protein was determined to be DPSYMV by N-terminal sequencing (Edman degradation) and was deposited at the Yeast Protein Database, to replace the incorrectly predicted mature N-terminus (*21*).

The identity of the labeled protein was confirmed by performing a photolabeling experiment on mitochondria isolated from a yeast strain in which the *GUT2* gene was deleted (see Table 1). 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 vs 0 °C, are unaffected in the mitochondria from the knock-out. These results demonstrate that the 70 kDa protein which

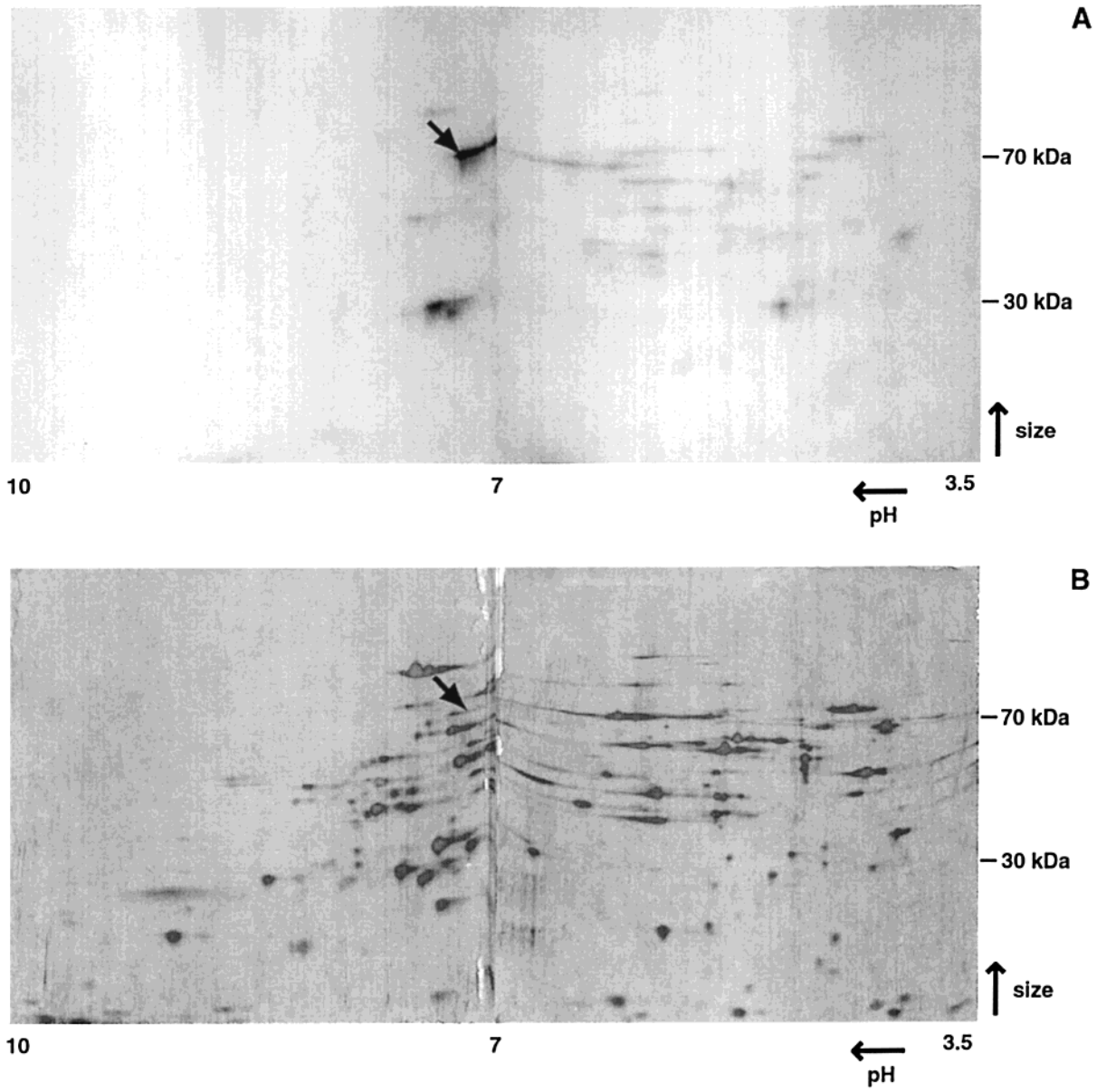

FIGURE 4: 2D-gel electrophoresis of [^{125}I]TID-PC-labeled mitochondria. Photolysis was performed on mitochondria after a 10 min incubation at 30 °C with [^{125}I]TID-PC, added from ethanol. Proteins were separated in the first dimension on NEPHGE (pH range 6−10) and IEF (pH range 3.5−7) gels and in the second dimension by 12.5% SDS−PAGE, followed by silver staining and autoradiography of the gels. The arrows indicate the position of the spot used for further analysis. (A) Autoradiogram; (B) silver stain pattern.

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 (*22*), and the encoded mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (*23*) functions in the glycerol 3-phosphate shuttle (*24*, *25*).

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 shows that both the distributions of Gut2p activity (Figure 7A) and the hemagglutinin-tagged protein (Figure 7C) are similar to the subcellular distribution of P_iC (Figure 7B), in agreement with the mitochondrial localization of the protein (*23*).

The effective labeling of Gut2p by [^{125}I]TID-PC indicates that the protein interacts with the membrane. The intramitochondrial localization of Gut2p was studied using the hemagglutinin-tagged version of the protein. Figure 8A 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 b_2 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 (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

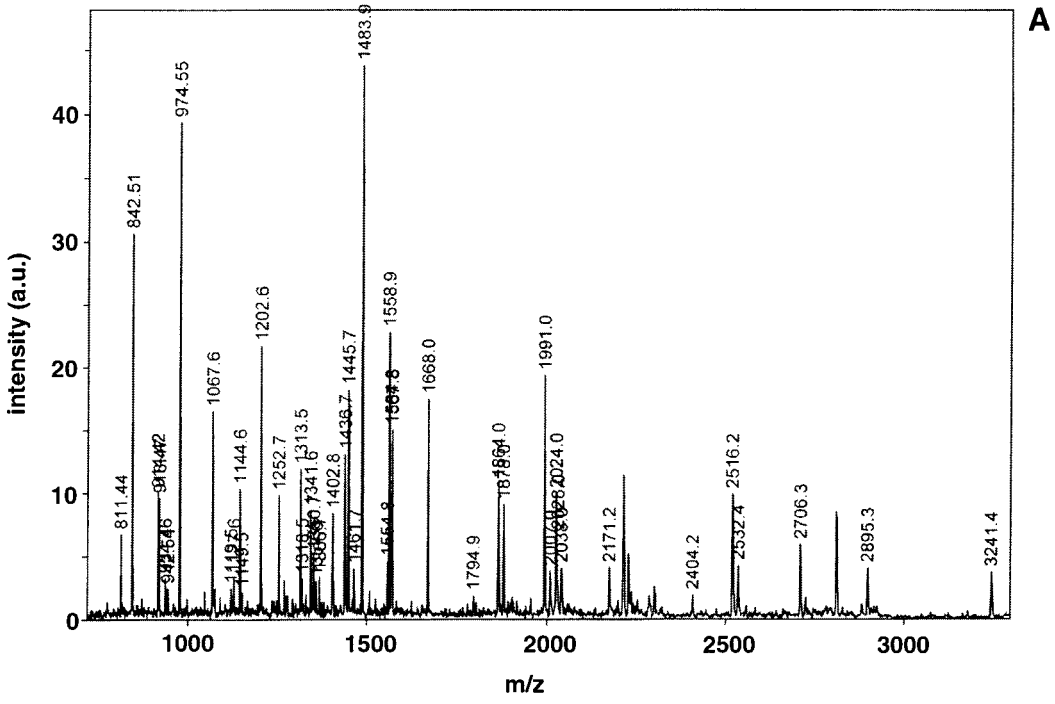

FIGURE 5: Identification of the [^{125}I]TID-PC-labeled 70 kDa protein by peptide mapping. The [^{125}I]TID-PC-labeled 70 kDa spot at the position of the arrow in Figure 4A was cut from an unstained 2D-gel and subjected to tryptic digestion. The tryptic digest was subjected to mass spectrometric analysis, 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 distinghuish individual peptides). The asterisk indicates the start of the mature protein. As examples, the masses of four major peaks in (A) are indicated above the corresponding peptide sequences in (B).

carbonate extraction or salt wash (Figure 8B). 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.

DISCUSSION

In search of interactions between phosphatidylcholine and mitochondrial proteins, a photolabeling approach was applied to isolated yeast mitochondria. Using either the PC analogue [^{125}I]TID-PC or the small hydrophobic probe [^{125}I]TID-BE, only a limited number of proteins were 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

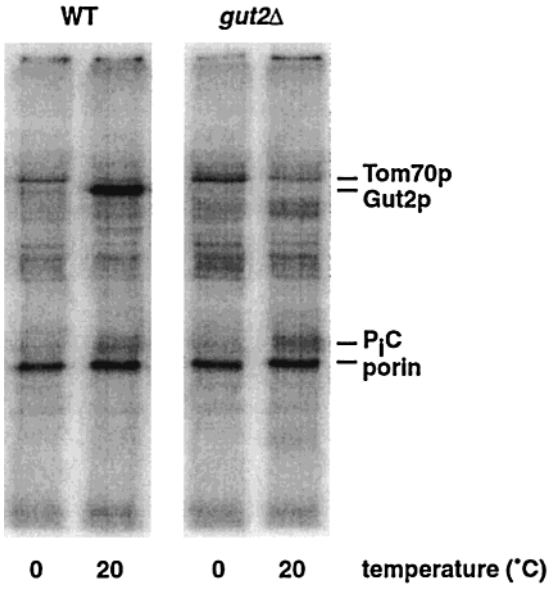

FIGURE 6: Labeling of wild-type and *gut2*Δ mitochondria with [^{125}I]TID-PC. Photolysis was performed on samples containing mitochondria isolated from a *gut2*Δ strain and the corresponding wild-type (WT) after a 30 min incubation at 0 or 20 °C with [^{125}I]TID-PC, added from ethanol. Samples were analyzed by 12% SDS−PAGE and autoradiography.

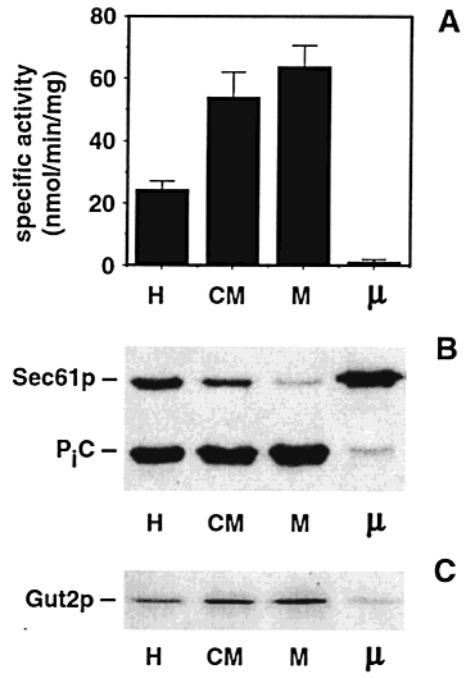

FIGURE 7: Subcellular distribution of Gut2p activity and epitope-tagged Gut2p. The activity of Gut2p and the presence of hemagglutinin-tagged Gut2p and of endoplasmic reticulum (Sec61p) and mitochondrial (P_iC) markers are shown for homogenates (H), crude mitochondria (CM), purified mitochondria (M), and microsomes (μ). (A) Subcellular distribution of Gut2p activity. Glycerol-3-phosphate dehydrogenase activity was determined as described under Experimental Procedures using samples from strain D273-10B corresponding to 100 μg on protein basis. The error bars represent the deviation from the average calculated 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 (endoplasmic reticulum marker) and P_iC (mitochondrial marker). (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.

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. Furthermore, the results demonstrate that both β-barrel proteins and helical bundle proteins, exemplified by porin and P_iC, respectively, are susceptible to photolabeling. In addition to their relative abundance, 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, or some specificity in the cross-link reaction.

In comparing the labeling patterns obtained with [^{125}I]TID-BE and [^{125}I]TID-PC, a number of interesting observations were made. In intact mitochondria, the phospholipid probe was largely confined to the outer membrane, and labeled the outer membrane proteins porin and Tom70p. A decreased intensity of labeling of Tom70p at 20 °C compared to 0 °C was observed both in mitochondria and in outer membrane vesicles, irrespective of the presence of phosphate. This might reflect the flipping of the phospholipid probe to the internal leaflet of the outer mitchondrial membrane. Tom70p has a large cytosolic domain (*26*, *27*) and might thus be more accessible for labeling from the outer leaflet of the membrane. Since phospholipids integrate into membranes in an oriented manner, it might in principle be possible to label membrane components selectively from one side of the bilayer (*8*).

The most conspicuous difference between the labeling patterns obtained with [^{125}I]TID-BE and [^{125}I]TID-PC was the 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 the inner membrane protein P_iC. 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, and the observation that [^{125}I]TID-PC label at the 70 kDa position is lacking from a *gut2* deletion strain, 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 (*18*, *22*), 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 (*24*, *25*).

From the labeling of Gut2p by [^{125}I]TID-PC, it can be concluded that a direct interaction of the protein with the inner membrane occurs in the biological system. The active site of the rat mitochondrial glycerol-3-phosphate dehydrogenase was reported to be located in the intermembrane space (*28*). Interaction of the protein with the inner membrane most likely is required for its physiological function, since this includes delivery of electrons to components of the respiratory chain located in the mitochondrial inner membrane. However, the Yeast Protein Database states that Gut2p is

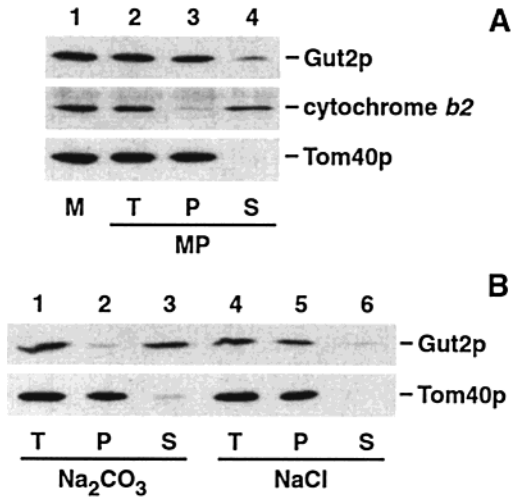

FIGURE 8: Submitochondrial localization of Gut2p. Mitochondria (M), isolated from strain BY4742 expressing hemagglutinin-tagged Gut2p, were subjected to hypotonic shock to prepare mitoplasts (MP). The mitoplasts were centrifuged, and the mitoplast pellet obtained by centrifugation was subjected to carbonate extraction or salt wash, followed by centrifugation. Aliquots (T, complete sample prior to centrifugation; P, pellet; S, supernatant) derived from 5 μg of 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 b_2 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).

predicted to be a soluble protein (*21*), 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. 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 appear 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. Based on these data, it is unlikely that transmembrane helices are involved in the mode of membrane interaction. A more plausible explanation is the presence of a hydrophobic region that ensures a peripheral localization perhaps by partial insertion. A similar mechanism could apply to rat and other mammalian mitochondrial glycerol-3-phosphate dehydrogenases, which have high similarity to Gut2p, and this would explain the need for detergents to solubilize these enzymes from the mitochondrial membranes [e.g., (*29*, *30*)]. Recently, it was found that Gut2p was present in a supramolecular complex containing yeast mitochondrial dehydrogenases (*31*). This indicates that besides protein−lipid also protein−protein interactions may be of importance for membrane association.

At this point, we can only speculate about the significance of the observed interaction between Gut2p and PC. One explanation could be a direct involvement of Gut2p in the import of PC into yeast mitochondria. However, we have no direct evidence for this 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). Another attractive suggestion to explain the significance of the observed interaction between PC and Gut2p 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. Such a mechanism could involve increased binding to the membrane when high activity is required and dissociation from the membrane for down-regulation of activity. Support for this suggestion comes from the observation 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). 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 (*32*). 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, which might point to a specific requirement for PC, is 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 (*24*) 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 (*33*, *34*). 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 (*35*, *36*), and involvement of GlpD in regulation of membrane phospholipid biosynthesis was recently suggested (*37*).

By demonstrating a direct interaction between PC and the mitochondrial glycerol-3-phosphate dehydrogenase in the biological system, the applied photolabeling strategy may serve as a paradigm for identifying (specific) interactions between lipids and proteins in a wide range of biological systems.

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