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Probing the Membrane Interface-Interacting Proteome Using Photoactivatable Lipid Cross-Linkers

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To analyze proteins interacting at the membrane interface, a phospholipid analogue was used with a photoactivatable headgroup (ASA-DLPE, *N*-(4-azidosalicylamidyl)-1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine) for selective cross-linking. The peripheral membrane protein cytochrome *c* from the inner mitochondrial membrane was rendered carbonate wash-resistant by cross-linking to ASA-DLPE in a model membrane system, validating our approach. Cross-link products of cytochrome *c* and its precursor apocytochrome *c* were demonstrated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and were specifically detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), taking advantage of the intrinsic UV absorbance of the cross-linker. Application of the method to inner mitochondrial membranes from *Saccharomyces cerevisae* revealed cross-link products of both exogenously added apocytochrome *c* and endogenous proteins with molecular weights around 34 and 72 kDa. Liquid chromatograpy (LC)-MS/MS was performed to identify these proteins, resulting in a list of candidate proteins potentially cross-linked at the membrane interface. The approach described here provides methodology for capturing phospholipid−protein interactions in their native environment of the biomembrane using modern proteomics techniques.

Keywords: Photolabeling • ASA-PE • *Saccharomyces cerevisiae* • mitochondria • cytochrome *c* • biomembranes • proteomics • lipid−protein interactions

Introduction

Biological membranes are highly dynamic structures that are not only essential for maintaining the integrity of cells and cell organelles but also play a vital role in numerous cellular processes. While it has been estimated that intrinsic or integral membrane proteins comprise up to 30% of the proteome,[1] emphasizing their importance, they have traditionally been underrepresented in proteomics studies. The main reason for this is their disadvantageous properties such as poor solubility and low abundance, requiring special purification and separation methods.[2,3] Next to intrinsic membrane proteins, many extrinsic or peripheral membrane proteins are known to be associated with the biological membrane. Their function often relies on specific recruitment to the membrane via either protein−protein or protein−lipid interactions. Lipid binding domains, such as C1, C2, PH, FYVE, PX, ENTH, and ANTH,[4] are among the most common types of domains found in the eukaryotic proteome and play an important role in vital processes such as cell signaling and membrane trafficking.[5]

A variety of strategies has been employed that make use of the occurrence of lipid−protein interactions as a tool to study a subset of the membrane protein proteome, such as probing proteome chips with labeled lipids[6] or using lipids bound to resins[7] or a solid support[8] for purifying lipid-binding proteins from cell extracts. Unfortunately, these methods all have the disadvantage that they do not probe the interactions between lipids and proteins in the native environment of the lipid bilayer. An alternative method that can be applied in situ, in a biological membrane, is the use of photoactivatable cross-linkers.[9] These molecules can, once incorporated into a biological system and activated with UV light, form covalent bonds with any adjacent molecule through highly reactive intermediates. For example 3-trifluoromethyl-3-[^{125}I]aryldiazerine-phosphatidylcholine ([^{125}I]-TID-PC) was shown to specifically label the glycerol-3-phosphate dehydrogenase, Gut2p, in mitochondria when this probe was added in vitro.[10] In another approach, photoactivatable [^{3}H]cholesterol, [^{3}H]phosphatidylcholine, and [^{3}H]phosphatidylinositol, containing a photoreactive diazerine moiety, were used to photolabel specific subsets of interacting

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proteins in vivo.[11] Alternatively, cross-linking from proteins or peptides to lipids proved to be a useful tool to study lipid−protein interactions.[12]

Photoreactive labeling has traditionally been applied to the hydrophobic membrane core, mainly by attaching the photoreactive group at the end of a phospholipid acyl chain. Due to the chemical inertness of this environment, the lifetime of the highly reactive intermediates that are generated after UV activation is increased, improving the chance of cross-linking to interacting proteins.[9] Photolabeling at the membrane interface, however, allows for cross-linking to extrinsic membrane proteins and to less hydrophobic regions on target proteins, facilitating subsequent analysis. Successful cross-linking at the membrane interface was reported using the arylazide cross-linker [^{125}I]-ASA-DLPE (*N*-([^{125}I]iodo-4-azidosalicylamidyl)-1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine).[13,14] This probe was used in (proteo)liposomes to cross-link peripheral subunits of the reconstituted bacterial ATP synthase complex,[13] the adhesive protein bindin,[15] and membrane penetrating toxins.[16,17] By incorporating this photoactivatable lipid in biological membranes, specific labeling of proteins was shown.[18,19] For instance, the human erythrocyte band 7.2b was identified as a cross-linked protein, by digestion and Edman sequencing of a radioactively labeled protein band and by immunoprecipitation of the radioactive label with a specific antibody against this protein.[19]

In this study, we used ASA-DLPE in a proteomics approach aimed at selectively cross-linking and identifying proteins interacting with phospholipids at the membrane interface. To be able to use mass spectrometry as a method for detection and identification of cross-link products, we avoided the use of radioactive labels. To test and establish conditions for this approach, the cross-linker was first applied in a model system of large unilamellar vesicles and the well-studied mitochondrial peripheral membrane protein cytochrome *c* and its precursor apocytochrome *c*.[20] Anionic lipids were included in the vesicles to bind (apo-)cytochrome *c* electrostatically. Subsequently the ability of the probe to cross-link (apo-)cytochrome *c* and retain it on the membrane even under stringent wash conditions was tested. On the basis of the results of these experiments, the cross-linker probe was applied to mitochondrial inner membranes of the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), enabling the comparison between the model and biological systems. Using the intrinsic absorbance of the photo-cross-linker for detection, a specific subset of proteins was found to be labeled by the ASA-PE probe.

Materials and Methods

Materials. Cytochrome *c* from horse heart and from yeast was purchased from Sigma (St. Louis, MO). Horse heart apocytochrome *c* was derived from cytochrome *c* by removing the heme group, as described,[21] and stored at 7.2 mg/mL in 10 mM sodium phosphate buffer (pH = 7.3) in small aliquots at −20 °C. 1,2-Dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (DPoPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DOPG) were from Avanti Polar Lipids (Alabaster, AL). *N*-Hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) was obtained from Pierce (Rockford, IL). All other chemicals were at least analytical grade. All handling of photosensitive compounds was performed under red safety light conditions.

Synthesis of Phospholipid Cross-Linkers. The ASA-PE photoactivatable phospholipids were synthesized essentially as described[18] with some modifications:

DLPE, DPPE, DPoPE, or DOPE was mixed with NHS-ASA in $CHCl_3$ to obtain concentrations of 12.5 and 19 mM, respectively. Triethanolamine was added to adjust to a reading of pH 8 on indicator paper, and the reaction was allowed to proceed overnight at 37 °C. Formation of the product was monitored by thin-layer chromatography (TLC) on Silica-HPTLC-60 plates (Merck, Darmstadt, Germany) using 9:2:1.6:1:0.5 $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ (v/v/v/v/v) as eluent. Reaction products were detected by staining with I_2 and by specific staining of phosphate by spraying with a 150 mM molybdate solution in 12.5 N H_2SO_4.

The product was isolated by TLC as above, loading the sample on the whole width of the plate and using a small strip for detection by I_2. Before use, TLC plates were washed with 2:1:0.8 $CHCl_3/CH_3OH/H_2O$ (v/v/v) and dried in the oven for 1 h. The product was scraped off and extracted from the silica in 2 × 3 mL of 2:1 $CHCl_3/CH_3OH$ (v/v). The silica was removed by centrifugation and the solvent evaporated under a N_2 flow. The lipid film was dissolved in 2:1 $CHCl_3/CH_3OH$ (v/v) to a concentration of approximately 5 mM and any remaining silica pelleted in an additional centrifugation step. The product was stored in sealed amber vials at −20 °C.

The identity of the ASA-PEs was established using nano-ESI-MS (MicroMass LCT, Waters, Milford, MA) in the positive-ion mode, and the presence of the photoactivatable moiety was further confirmed by the change in the absorption spectrum in CH_3OH between 250 and 500 nm upon exposure to UV light as described below (data not shown). The typical yield relative to the amount of phospholipid used in the synthesis was 60−70%. The purity of the compounds was routinely tested by TLC as above.

Cross-Linking in Model Membranes. All steps were performed at room temperature. DOPC and DOPG were mixed in a molar ratio of 7:3 in $CHCl_3$ together with 0−4 mol % of photoactivatable phospholipid as indicated. The solvent was evaporated under N_2 and the lipid film dried under vacuum for 2 h. The lipid films were hydrated in H-buffer (50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 7.5) to a lipid concentration of 5 mM and LUVETs (large unilamellar vesicles obtained by extrusion technology) were prepared by eight freeze−thaw cycles and eight passages of the suspension through a 0.2 μm pore size membrane filter (anotop 10, Whatman, Brentford, U.K.).

Cytochrome *c* (from a 1 mg/mL stock solution in H-buffer) or apocytochrome *c* (from a 7.2 mg/mL stock solution as described above) was added to the vesicles to typical final concentrations of approximately 0.24 mg/mL cytochrome *c*, 0.12 mg/mL apocytochrome *c*, and 4−5 mM phospholipid. In the case of cytochrome *c*, these conditions approached saturated binding to the vesicles.[22,23] After 20 min the vesicles were pelleted for 1 h at 200000*g*. The pellets were resuspended in H-buffer to a phospholipid concentration of 4−10 mM and exposed to UV light of 366 nm (Mineralight UVGL-58, UVP Inc., San Gabriel, CA) as described[16] at room temperature for 20 min, unless indicated otherwise. Control samples were left in the dark for corresponding periods of time. After cross-linking, either Na_2CO_3 dissolved at 100 mM in H-buffer was added to a final concentration of 40 mM or H-buffer was added to the

same final volume. After 20 min the vesicles were pelleted at 355000*g* for 30 min.

To quantitate the amount of bound cytochrome *c*, the pellets from the cross-link experiments were dissolved in 200 μL of 5 mM sodium ascorbate, 4% (w/v) sodium cholate, 40 mM NaH_2PO_4, pH 7.3, and the differences in absorbance at 550 and 563 nm were measured in microcuvettes on a double-beam spectrophotometer.[24] To relate the amount of cytochrome *c* to the amount of phospholipid in the pellet, the phospholipid concentration in the supernatants was measured and subtracted from the total amount of phospholipid present. Typically, 80 mol % of the total phospholipid contents was pelleted.

MALDI-TOF MS Analysis of (Apo-)cytochrome *c*. To detect modified proteins, pellets from cross-link experiments were resuspended in 100 μL of H-buffer. The pH of the supernatants containing carbonate was adjusted to pH $\sim$ 7 with 1 M HCl. Proteins from both pellets and supernatants were precipitated in $CHCl_3/CH_3OH$.[25] The resulting protein pellets were air-dried and resuspended in a small volume of 1:1 acetonitrile/H_2O (v/v) containing 0.1% (v/v) trifluoro acetic acid and 0.5% (w/v) sinapic acid. A 1 μL aliquot of each solution was spotted on a stainless steel matrix-assisted laser desorption/ionization (MALDI) plate, and the samples were analyzed on a MALDI-TOF/TOF (TOF = time of flight) instrument (Applied Biosystems 4700, Foster City, CA), equipped with a 200 Hz Nd:YAG laser operating at 355 nm. Experiments were performed in a linear positive ion mode using delayed extraction. Typically, 4000 shots/spectrum were acquired in the MS mode.

In the case of the analysis of digested (apo-)cytochrome *c*, the samples were run on gel as stated below and digested as described under "LC-MS/MS analysis" below. The peptides were purified using ZipTip μC18 tips (Millipore, Billerica, MA) according to the manufacturer's instructions and eluted directly on a MALDI plate using α-cyano-4-hydroxycinnamic acid as matrix. Mass spectrometry (MS) measurements were performed on the instrument described above in a reflectron positive ion mode using delayed extraction.

Analysis by SDS-PAGE. (Apo-)cytochrome *c* from a cross-link experiment was precipitated as above, and protein pellets were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 25 mM dithiotreitol (DTT) and analyzed by SDS-PAGE using 15% (w/v) acrylamide. The gels were fixed 2 × 30 min in 5:1:4 $CH_3OH/CH_3COOH/H_2O$ (v/v/v), washed in H_2O for 5 min, and photographed under UV light (Biorad GelDoc 2000, Hercules, CA), prior to staining with Coomassie brilliant blue.

Isolation of Mitochondrial Inner Membranes. The yeast strain BY4742 (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) was grown aerobically to late log phase at 30 °C in semisynthetic lactate medium[26] supplemented with 20 mg/L histidine, 60 mg/L leucine, 230 mg/L lysine, and 40 mg/L uracil. After washing the cells with 1 mM EDTA, pH 7.4, spheroplasts were prepared using zymolyase as described,[26] and broken in a Dounce homogenizer in buffer containing 0.6 M sorbitol, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.5% (w/v) dextran (Roth), 10 mM EDTA, and 10 mM 4-morpholineethanesulfonic acid (MES), pH 6.0. Crude mitochondria were isolated using differential centrifugation and further purified on a sucrose gradient as described.[27]

Mitochondrial subfractionation was carried out as described.[28] The fraction containing inner membrane vesicles (IMVs) was washed with H/K buffer (10 mM KCl, 2.5 mM EDTA, 5 mM HEPES, pH 7.4). The IMVs were pelleted for 1 h at 200000*g* at 4 °C, resuspended in H/K buffer to a concentration of ~4 mg of protein/mL, frozen in liquid nitrogen, and stored in aliquots at −80 °C.

The purity of the vesicles was assessed by Western blots using antibodies against Sec61p, kindly provided by R. Schekman, against Tom20p and Mir1p, kindly provided by R. Lill, and an antiserum raised against yeast cytochrome *c*[29] as primary antibodies, goat anti-rabbit IgG (H + L)-HPR (Biorad) as a secondary antibody and ECL solutions for visualization.[27]

Cross-Linking in Inner Membrane Vesicles. IMVs corresponding to approximately 200 μg of protein were thawed and H/K buffer containing 1 mM PMSF was added to a total volume of 500 μL. To incorporate the cross-linker, 15 nmol of ASA-DLPE was dried under N_2, dissolved in 10 μL of ethanol, and added to the IMV suspension. The mixtures were incubated at 37 °C for 30 min, immediately chilled on ice, and, to remove non-incorporated ASA-DLPE, layered on top of 500 μL of 0.5 M sucrose in H/K buffer. After centrifugation at 165000*g* for 1 h at 4 °C, the pellets were washed in 200 μL of H/K buffer and centrifuged for 20 min at 200000*g* and 4 °C. The IMV pellet was resuspended in 400 μL of H/K buffer, and, where indicated, 20 μg of apocytochrome *c* from horse heart was added. After 20 min on ice, half of the sample was kept in the dark, and the other half exposed to UV light on ice, as described above. Subsequently, samples were split, and either 60 μL of 100 mM Na_2CO_3 or 60 μL of H/K buffer was added. After 20 min on ice, all four samples were centrifuged for 20 min at 355000*g* and 4 °C.

Pellets, after resuspension in H/K buffer, and supernatants were subjected to protein precipitation as above.[25] The resulting protein pellets were boiled in XT sample buffer (Biorad) supplemented with XT reducing agent (Biorad) and separated on CRITERION XT 4−12% (w/v) Bis-Tris gels (Biorad) in MES running buffer according to the manufacturer's instructions. The gels were fixed 1 × 30 min and 1 × overnight in 5:1:4 $CH_3OH/CH_3COOH/H_2O$ (v/v/v), washed 3 × 15 min in H_2O, and photographed under UV light (Biorad GelDoc 2000), prior to staining with Coomassie brilliant blue.

In a control experiment, 7:3 DOPC/DOPG (mol/mol) LUVETs, corresponding to 120 nmol of phospholipid and prepared as described in "Cross-Linking in Model Membranes", were treated exactly as described above except for the centrifugation step on a sucrose cushion. Instead the vesicles were directly pelleted for 1 h at 200000*g* and 4 °C before resuspension and addition of apocytochrome *c*.

LC-MS/MS Analysis for Protein Identification. Bands of interest were excised and in gel digested with trypsin essentially as described with slight modifications.[30] In brief, the gel pieces were reduced (6.5 mM DTT in 50 mM NH_4HCO_3, pH 8.5) and alkylated (54 mM iodoacetamide in 50 mM NH_4HCO_3, pH 8.5), while washing and dehydrating in between using 50 mM NH_4HCO_3 and acetonitrile, respectively. Proteins were digested overnight at 37 °C by adding 150 ng of trypsin in a final volume of 20 μL of 50 mM NH_4HCO_3. Digestion was stopped by adding an equal volume of 0.6% (v/v) acetic acid.

Nanoscale LC-MS/MS was performed by coupling an Agilent 1100 Series LC system to a LTQ XL quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). Peptide mixtures were concentrated and desalted using an on-line C18 trap column (o.d., 375 μm; i.d., 100 μm packed with 20 mm of 5 μm AQUA C_{18}, RP particles (Phenomenex, Torrance, CA)), and further separation was achieved by gradient elution of peptides onto a C18 reverse-phase column (o.d., 375 μm; i.d., 50 μm packed

with 15 cm of 3 μm C_{18}, Reprosil RP particles (Dr. Maisch, Ammerbuch-Entringen, Germany)). MS detection in the LTQ was achieved by directly spraying the column eluent into the electrospray ionization source of the mass spectrometer via a butt-connected nanoelectrospray ionization emitter (New Objective, Woburn, MA). A linear 30 min gradient (0−50% B) was applied for peptide elution into the MS at a final flow rate of 100 nL/min. The total analysis time was 1 h. Mobile-phase buffers were (A) 0.1 M acetic acid and (B) 80% acetonitrile, 0.1 M acetic acid. The LTQ operated in the positive ion mode, and peptides were fragmented in data-dependent mode. One mass spectrometry survey zoom scan was followed by three data-dependent MS/MS scans.

Database Searching. Tandem mass spectra were extracted and charge state deconvoluted by BioWorks version 3.2. All MS/MS samples were analyzed using Mascot (Matrix Science, London, U.K.; version 2.1.02) and X! Tandem (www.thegpm.org; version 2006.04.01.2). Mascot was set up to search the Yeast 4932 database (6195 entries), and the sample containing horse heart apocytochrome *c* was searched using the UniProt-Swiss-Prot database (version 50.0, 222289 entries). X! Tandem was set up to search the same databases but using a different version of UniProt-Swiss-Prot database (version 48.3, 196277 entries). Both databases were searched with a parent ion tolerance of 0.5 Da and a fragment ion mass tolerance of 0.9 Da. Fixed and variable modifications were the iodoacetamide derivative of cysteine and oxidation of methionine, respectively.

Criteria for Protein Identification. Scaffold (version 01_05_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[31] Protein identifications were accepted if they could be established at greater than 99.0% probability as specified by the Protein Prophet algorithm[32] and contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Other Methods. Phospholipid and phospholipid cross-linker concentrations were measured by phosphorus determination[33] after destruction in perchloric acid at 180 °C using 0−50 nmol of KH_2PO_4 as a standard. In samples derived from yeast mitochondria, the phospholipids were first extracted.[34] Protein concentrations were determined using the BCA method (Pierce) with 0.1% (w/v) SDS added and bovine serum albumin as a standard.

Results

To selectively detect and identify proteins interacting with phospholipids at the membrane interface, we made use of ASA-DLPE as a cross-linker probe. ASA-DLPE (Figure 1) is a phospholipid with a photoactivatable nitrene-generating moiety for cross-linking attached to the headgroup, and with short, C12, acyl chains to allow for incorporation in biological membranes by addition from ethanol solution.[16,19] Once incorporated in a membrane system, the cross-linker should be able to cross-link either peripheral membrane proteins or parts of integral membrane proteins that are exposed to the membrane interface. A subsequent wash using sodium carbonate can be used to wash away non-cross-linked peripheral membrane proteins that primarily have an electrostatic interaction with the membrane.

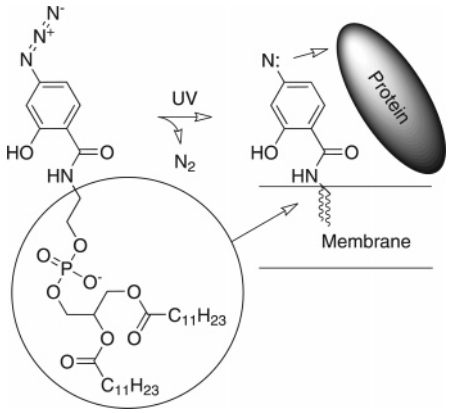

Figure 1. Mechanism of activation of ASA-DLPE in UV light. Upon illumination with UV light, the photoreactive probe ASA-DLPE forms a highly reactive nitrene intermediate that can react with adjacent molecules. Due to the location of the nitrene-generating arylazide on the phospholipid headgroup, molecules interacting at the membrane interface will be preferentially labeled.

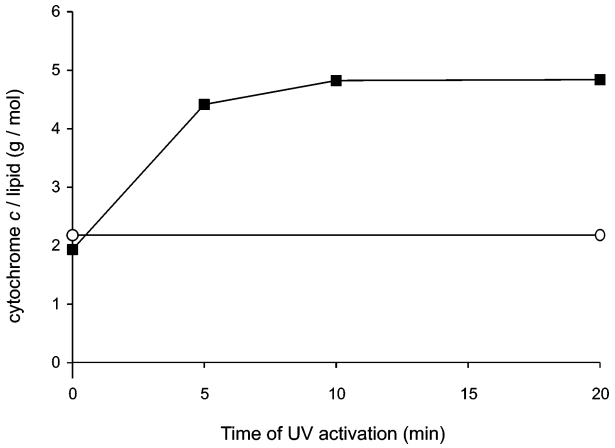

Figure 2. Quantification of the membrane-associated pool of cytochrome *c* that is resistant to carbonate wash after UV activation of ASA-DLPE. LUVETs consisting of DOPC and DOPG (7:3 (mol/mol)), and with (■) or without (○) 4 mol % ASA-DLPE, were incubated at a concentration of 4 mM total phospholipid with 0.24 mg/mL horse heart cytochrome *c*. The vesicles were activated in UV light for the times indicated, washed with carbonate, and pelleted, and the phospholipid and cytochrome *c* contents analyzed by spectrophotometry as described in Materials and Methods. The results of a typical experiment are shown as the amount of cytochrome *c* per mole of lipid in the pellet fraction plotted against the time of UV activation.

UV Activation of LUVETs Containing ASA-PEs in the Presence of Cytochrome *c* Yielding a Carbonate Wash-Resistant Fraction of Cytochrome *c*. To test for cross-linking of the peripheral membrane protein cytochrome *c*, LUVETs were prepared that contained ASA-DLPE. Cytochrome *c* from horse heart was allowed to bind before the vesicles were incubated in UV light or left in the dark, and a wash with sodium carbonate was applied to remove non-cross-linked proteins. The amounts of cytochrome *c* and phospholipid in the vesicles were determined spectrophotometrically and the cytochrome *c*/lipid ratio was calculated. Figure 2 shows the result of a typical experiment. When ASA-DLPE was incorporated in the vesicles, significantly more cytochrome *c* remained bound to the membranes upon treatment with UV light and

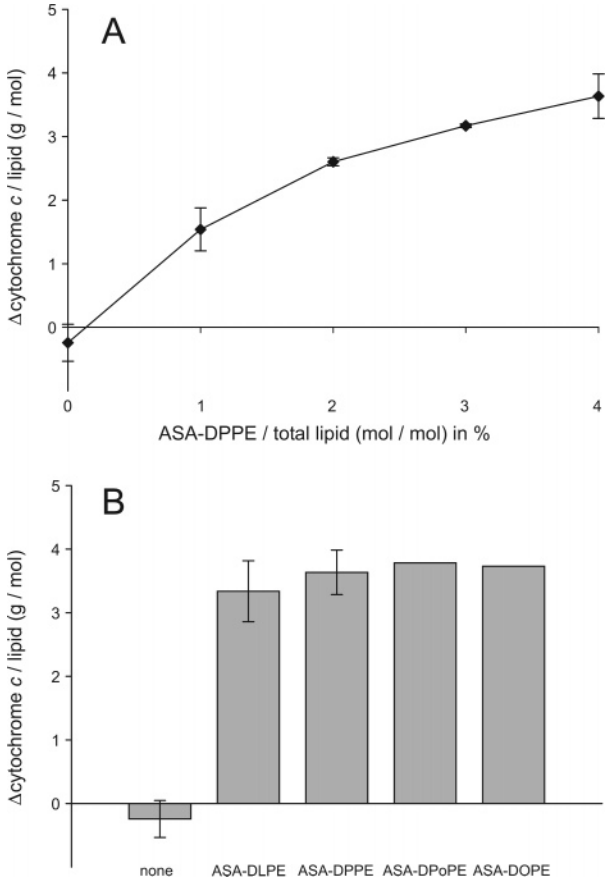

Figure 3. Dependence of the size of the membrane-associated pool of cytochrome *c* resistant to carbonate wash on the concentration and the acyl chain composition of the cross-linker ASA-PE. Cross-link experiments were performed as described in the caption of Figure 2, varying the concentration of ASA-DPPE (A) or the acyl chain composition of the lipid cross-linker (B). The increase in cytochrome *c* associated per total lipid was calculated by subtracting the amount of cytochrome *c* bound per total lipid in the absence of UV treatment from the amount of cytochrome *c* bound per total lipid after UV treatment, both after carbonate wash. The error bars at 1, 2, and 3% ASA-DPPE in panel A represent the maximum variation from the mean ($n = 2$). Error bars at 0 and 4% ASA-DPPE in panel A and error bars in panel B represent the standard deviation ($n \geq 3$).

after carbonate washing than in the absence of ASA-DLPE. The amount of cytochrome *c* bound remained constant after 10 min of activation. When the cross-linker ASA-DLPE was omitted from the system, a constant amount of cytochrome *c* remained bound to the vesicles after the carbonate wash, irrespective of UV activation. In the absence of cytochrome *c*, no signal was observed, indicating that the presence of phospholipids or cross-linker does not interfere with the spectrophotometric detection of cytochrome *c*. Similar results were obtained with yeast cytochrome *c* (data not shown).

Subtraction of the cytochrome *c*/lipid ratios obtained for the samples left in the dark from the values obtained for the samples activated with UV light yielded the cytochrome *c* fraction that became resistant to carbonate wash after activation of ASA-DLPE, and that is presumed to have cross-linked to the phospholipid membranes. Figure 3A shows the dependence of the size of this fraction on the concentration of cross-linker present in the system. The amount of cytochrome *c*

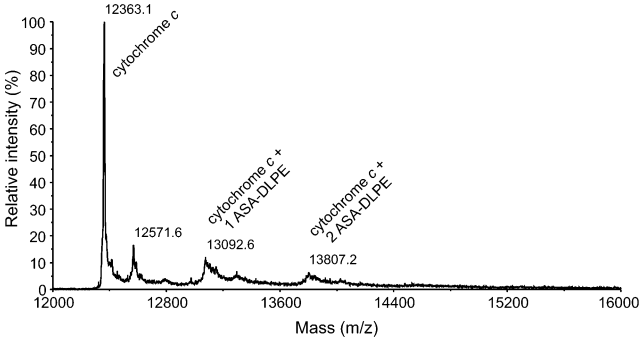

Figure 4. MALDI-TOF MS analysis of cytochrome *c* after cross-linking. After a cross-link experiment as described in the caption of Figure 2, cytochrome *c* was precipitated and analyzed by MALDI-TOF MS in the linear mode. Peaks resulting from horse heart cytochrome *c* (*m*/*z* 12 363.1) and cytochrome *c* with one (*m*/*z* 13 092.6) or two (*m*/*z* 13 807.2) covalently linked lipids were found. The peak at *m*/*z* 12 571.6 most likely represents a matrix adduct.

cross-linked increased with higher ASA-PE concentrations and started to level off around a concentration of 2−3 mol % cross-linker. On the basis of these results it was calculated that when 1% cross-linker was incorporated, approximately 1.2 mol % of the available cross-linker molecules became attached to cytochrome *c*, as compared to 0.7 mol % when 4% cross-linker was used. In these calculations it was assumed that only one cross-linker was bound per protein.

To study the influence of the nature of the acyl chains of the cross-linker on the size of the carbonate wash-resistant fraction of cytochrome *c* after photoactivation, several ASA-PEs, differing in acyl chain composition, were synthesized. Figure 3B compares the results obtained using vesicles containing lipid cross-linkers with saturated acyl chains, ASA-DLPE and ASA-DPPE, two lipid cross-linkers with unsaturated acyl chains, ASA-DPoPE and ASA-DOPE, and vesicles containing no lipid cross-linker. The vesicles containing the different photoactivatable phospholipid species showed a similar increase in the size of the carbonate wash-resistant fraction of cytochrome *c* after UV activation, indicating that upon cross-linking cytochrome *c* was retained on the membrane surface to similar extents, irrespective of the acyl chain composition.

Cross-Linked Cytochrome *c* Demonstrated by Mass Spectrometry. To prove that cytochrome *c* retained on the LUVETs after UV activation and carbonate wash was indeed covalently attached to ASA-PE, the protein was precipitated from the vesicles after a cross-link experiment as described above with 4 mol % ASA-DLPE. The protein precipitate was analyzed directly using MALDI-TOF mass spectrometry (Figure 4). Unmodified cytochrome *c* and cytochrome *c* with one and two lipid cross-linkers attached were detected in the mass spectrum after UV activation. The intensities of the cross-link products were low compared to the signal of the unmodified protein, which is possibly due to the reduced ionization rate of proteins cross-linked to the relatively hydrophobic phospholipids when compared to the free protein. Both cytochrome *c* from horse heart and from yeast showed additional peaks in the spectra after UV incubation with ASA-DLPE or ASA-DPPE, close to the predicted *m*/*z* values of cross-link products of the respective proteins and lipids (Table 1).

Cross-Link Products Visualized Selectively in Gel under UV Illumination. To detect cross-linked proteins in more complex, biological samples, we explored alternative ways to directly

Table 1. MALDI-TOF MS Analysis of Cross-Link Products[a]

		observed (*m/z*)		calculated (*m/z*)
cross-linker	cytochrome *c*	unmodified[b]	cross-linked[c]	cross-linked[d]
ASA-DLPE	horse heart	12 363.1	13 092.6	13 074.9
ASA-DPPE	horse heart	12 363.6	13 162.1	13 187.6
ASA-DLPE	yeast	12 707.6	13 429.2	13 419.4
ASA-DPPE	yeast	12 709.6	13 533.9	13 533.6
ASA-DLPE	horse heart apo	11 741.2	12 463.4	12 453.0
ASA-DPPE	horse heart apo	11 742.3	12 576.9	12 566.3

[a] Cytochrome *c* from horse heart, from yeast, and apocytochrome *c* derived from horse heart cytochrome *c* were used in a cross-link assay with vesicles containing ASA-DLPE or ASA-DPPE as described in the caption of Figure 2. Apocytochrome *c* was used at a concentration of 0.12 mg/mL. After precipitating the proteins from the pellet fraction, the masses of unmodified and cross-linked proteins were measured by MALDI-TOF MS and compared with the calculated values. [b] Mass observed for unmodified protein. [c] Mass observed for protein with one cross-linker attached. [d] Theoretical mass after cross-linking, calculated from the mass observed for the unmodified protein and the mass of the cross-linker used.

visualize cross-link products. Because the ASA-PE probes still absorb UV light after cross-linking,[16] we examined whether this property could be used to detect cross-link products after separation by SDS-PAGE. To avoid interference of the UV absorbing heme with the detection of cross-link products, apocytochrome *c* derived from horse heart cytochrome *c* was used. Apocytochrome *c*, the cytochrome *c* precursor that lacks the heme moiety, is an unfolded protein, exposing hydrophobic residues, and has both electrostatic and hydrophobic interactions with negatively charged membranes.[20] When apocytochrome *c* was added to vesicles containing ASA-DLPE or ASA-DPPE, clear cross-link products were demonstrated after UV activation using MALDI-TOF mass spectrometry (Table 1). SDS-PAGE analysis of the supernatants and pellets from a cross-link experiment (Figure 5A) shows that, after UV activation, there was an increase in carbonate wash-resistance when compared to the un-cross-linked protein (compare lanes 5 and 7 and lanes 6 and 8 and the corresponding supernatants). In addition, a slightly smeared Coomassie stained band appeared above the apocytochrome *c* band in the pellet fractions after cross-linking (lanes 5 and 6) that was hardly present in the supernatants (lanes 1 and 2).

Under UV light a strikingly simple pattern emerged: For apocytochrome *c* only the additional smeared band migrating above apocytochrome *c* after cross-linking was visible (Figure 5B, lanes 5 and 6). Figure 5B also shows a low molecular weight UV absorbing band that appeared after cross-linking below apocytochrome *c* (lanes 5 and 6). Most likely this represents a cross-link product not containing protein, because it is not visible after Coomassie staining (Figure 5A). Experiments with holocytochrome *c* yielded similar results; however, in this case un-cross-linked protein was also visible under UV illumination (data not shown). It should be noted that the carbonate wash was more effective in washing off un-cross-linked holocytochrome *c* from the membrane than apocytochrome *c* due to the differences in interactions of holocytochrome *c* and apocytochrome *c* with phospholipid membranes.[20] However, by using UV illumination we were able to detect cross-linking of both proteins, irrespective of the difference in carbonate wash-resistance.

Apocytochrome *c* Cross-Linked to Mitochondrial Inner Membranes of *S. cerevisiae*. To test whether the approach is applicable to biological membranes, inner mitochondrial membrane vesicles from the yeast *Saccharomyces cerevisiae* were isolated. The purity of the IMVs was assessed by Western blotting using antibodies against markers for ER, mitochondrial outer membranes, and mitochondrial inner membranes, as shown in Figure 6. Similar enrichments were found for the inner membrane protein Mir1p and the peripheral membrane protein cytochrome *c* in the mitochondrial fraction and derived IMVs as compared to the cell homogenate, indicating that peripheral membrane proteins stay attached during the preparation of IMVs. The outer membrane protein Tom20p was enriched in the mitochondrial fraction and largely depleted in the IMVs. The integral membrane protein Sec61p was used as a marker for ER contamination. In agreement with previous studies[27,35] some ER contamination of the mitochondria was found that was slightly enriched in the preparation of IMVs (Figure 6).

The cross-linker with C12 acyl chains, ASA-DLPE, was incorporated into IMVs by addition from solution in ethanol. To enable detection of cross-link products of apocytochrome *c* by UV, this protein was also added. Cross-linking and SDS-PAGE analysis were performed as before, except for the use of gradient gels to obtain better resolution. First, it was verified using LUVETs that incorporation of ASA-DLPE by addition from ethanol and by inclusion of the probe in the lipid film yielded comparable cross-linking results (compare Figure 7A,B to Figure 5A,B). The results obtained were very similar for both methods, although the smear above the protein band after cross-linking was absent in Figure 7A,B. Analysis of these samples on the gels used in Figure 5 resulted in the reappearance of the smeared band (data not shown), indicating that the absence of the smear in Figure 7 was caused by the use of a different gel system.

When cross-linking was performed in IMVs in the presence of horse heart apocytochrome *c*, a similar pattern was obtained for the apocytochrome *c* band by SDS-PAGE as in LUVETs (Figure 7C,D, arrowhead). However, additional UV absorbance was visible in lanes 1, 3, and 8. This indicated the presence of a UV light absorbing protein sensitive to carbonate wash, possibly holocytochrome *c*. In an experiment without cross-linker added to the membranes, this band was also visible, but no change was observed upon UV activation (data not shown). When the experiment was repeated without addition of horse heart apocytochrome *c*, the band was not detected (Figure 7F). These results rule out that the UV absorbance in lanes 1, 3, and 8 is caused by ASA-DLPE or endogenous proteins, leaving the possibility that it originates from the conversion of apocytochrome *c* to cytochrome *c* by heme lyase present in the IMVs.[36]

Identification of Cross-Link Products in IMVs. When cross-linking was performed in IMVs in the absence of apocytochrome *c*, a strikingly simple pattern was observed under UV light (Figure 7F) when compared to the Coomassie stain (Figure 7E). In Coomassie stain, the carbonate wash showed a clear effect in washing specific proteins off the membrane when comparing the buffer washed and carbonate washed pellets in lanes 7 and 8, and the protein contents of the supernatants in lanes 3 and 4. No clear differences were observed when comparing plus and minus cross-linking conditions after Coomassie staining (lanes 5 and 6 vs lanes 7 and 8). In contrast, under UV illumination bands at 34 and 72 kDa showed an increased intensity in the pellet fractions after cross-linking (Figure 7F). The intense band that appeared at 34 kDa could not be washed off by carbonate (lanes 5 and 6), suggesting that at least one abundant membrane protein migrating at this position is cross-linked that is not sensitive to carbonate. At

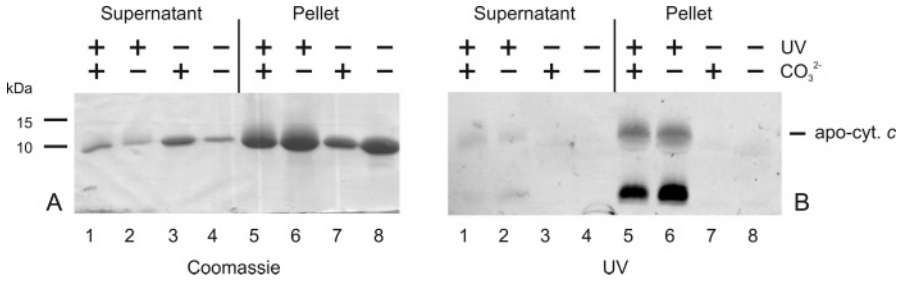

Figure 5. Selective detection of cross-link products in SDS-PAGE gels. A cross-link experiment was performed in LUVETs, as described in the caption of Figure 2, using horse heart apocytochrome *c* (0.12 mg/mL). Samples were either activated in UV light or left in the dark, after which they were washed with or without carbonate, as indicated. Protein precipitates from both pellets and supernatants were boiled in SDS-PAGE sample buffer and separated on 15% polyacrylamide gels. After fixation, the gels were photographed under UV light (B) and subsequently stained with Coomassie (A).

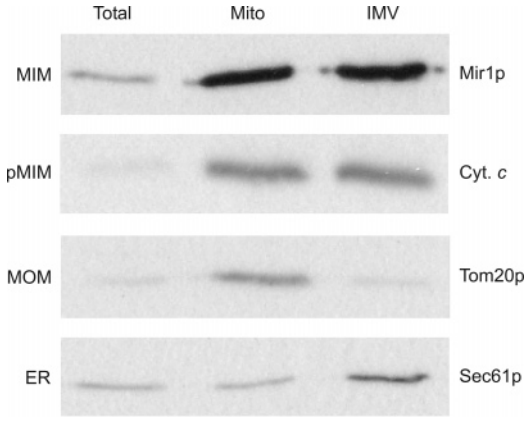

Figure 6. Purity of mitochondrial inner membrane vesicles. IMVs were isolated from yeast mitochondria, and their purity was assessed by immunoblotting. Equal amounts, based on protein content, of total cell lysate (Total), purified mitochondria (Mito), and IMVs were analyzed using antisera against Mir1p, cytochrome *c*, Tom20p, and Sec61p as markers for mitochondrial inner membrane (MIM), peripheral inner membrane proteins (pMIM), mitochondrial outer membrane (MOM), and ER, respectively. For the detection of Mir1p, cytochrome *c*, and Sec61p, 1 μg of protein was loaded per lane and for Tom20p, 2.5 μg of protein.

72 kDa, a UV absorbing band was observed that disappeared after washing the IMVs with carbonate (lanes 3 and 7). However, after cross-linking the signal seemed to increase in the carbonate washed pellet (compare lanes 5 and 7). Possibly, a peripheral membrane protein, containing a UV absorbing group such as heme or flavin, is present in this band that becomes carbonate wash-resistant after cross-linking to ASA-DLPE.

To identify the protein composition of the bands exhibiting increased UV absorbance after UV activation and carbonate wash, the 34 and 72 kDa bands were excised from lanes 5−8 (Figure 7E,F). The proteins were tryptically digested and the resulting peptides subjected to nanoscale LC-MS/MS analysis. To find the most probable cross-link candidates, proteins that were identified in at least two of the lanes and with a predicted mass close (±6 kDa) to the apparent molecular weight of the excised bands are listed in Tables 2 and 3, with the number of unique peptides found for each protein in the carbonate washed pellets indicated. The complete list of identified peptides and proteins is available as Supporting Information. As a control, the apocytochrome *c* band at 12 kDa (Figure 7C,D) was also excised from lane 5, digested, and analyzed. Peptides corresponding to both horse heart and yeast cytochrome *c* were found (see Supporting Information), in agreement with the detection of yeast cytochrome *c* in purified IMVs (Figure 6).

The results in Tables 2 and 3 were analyzed by comparing the number of unique peptides found per protein under the different conditions used. The high number of unique peptides for the mitochondrial ADP/ATP carrier Pet9p in the 34 kDa band (Table 2) suggests a high abundance of this integral membrane protein, consistent with the intense signal in UV illumination after cross-linking. Moreover, as an intrinsic membrane protein, it is expected to be carbonate wash-resistant, a property that is consistent with the lack of changes observed in the signal under UV light, when comparing the buffer and carbonate washed samples (Figure 7F). Therefore this protein is a prime candidate to have been cross-linked to ASA-DLPE. In the 72 kDa band the differences in the number of unique peptides found per protein were less pronounced (Table 3 and Supporting Information), resulting in multiple cross-link candidates.

As judged by the increase in the number of unique peptides identified in the UV-activated sample as compared to the non-UV-activated sample (Tables 2 and 3), a number of proteins in the 34 and 72 kDa bands appears to become more resistant to carbonate washing after UV activation. This behavior that is expected for peripheral membrane proteins that become cross-linked to ASA-PE was found for Lsc1p, Mir1p, Rsm25p, Ilv6p, Mdh1p, Por1p, Mcr1p, Cyt1p, Mrp13p, Qcr2p, Mrps5p, Ssc1p, Ilv2p, Sec63p, and Faa1p (Tables 2 and 3). With the exceptions of the established integral inner membrane proteins Mir1p,[37] and Qcr2p,[38] and the contaminating proteins from the ER (Sec63p) and outer membrane (Por1p and Faa1p), this list indeed only contains mitochondrial peripheral inner membrane or soluble proteins. Strikingly, the list contains multiple components of the small subunit of the mitochondrial ribosome (Rsm25p, Mrp13p, Mrps5p), while the components identified from the large subunit of the mitochondrial ribosome (Mrpl17p, Mrpl22p) do not show this behavior (Table 2). In addition, NADH-cytochrome b_5 reductase, Mcr1p, which is a known component of both mitochondrial outer membranes and the intermembrane space,[39] shows a dramatic rise in the amount of peptides identified after cross-linking and carbonate washing, compared to the non-UV-treated sample (Table 2).

Discussion

In this study we showed that photoactivatable lipids are promising tools for addressing the phospholipid headgroup-interacting proteome. In order to successfully analyze this class

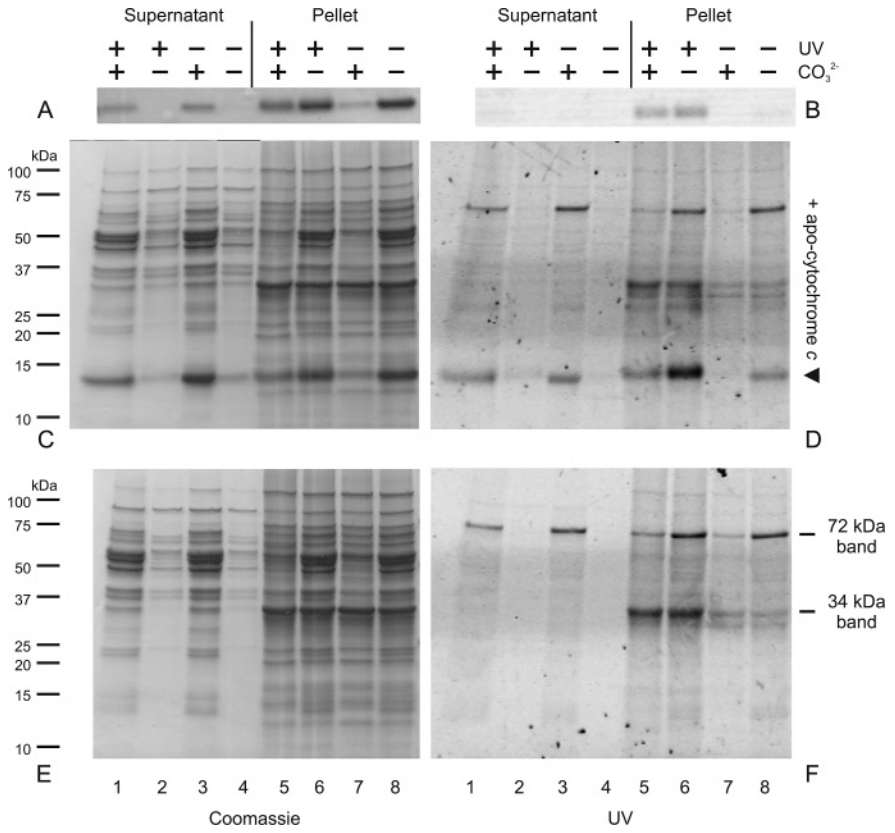

Figure 7. Cross-linking in inner membrane vesicles. Cross-link experiments were performed in LUVETs (A and B) or IMVs (C−F) with ASA-DLPE added from a solution in ethanol and in the presence (A−D) or absence (E and F) of horse heart apocytochrome *c* (arrowhead). Samples were either activated in UV light or left in the dark, and subsequently washed with or without carbonate, as indicated. Protein precipitates from pellets and supernatants were boiled in XT sample buffer (Biorad) and separated on CRITERION XT 4−12% Bis-Tris gels (Biorad) in MES running buffer. After fixation, the gels were photographed under UV light (B, D, and F) and subsequently stained with Coomassie (A, C, and E). For the samples containing LUVETs (A and B) only the apocytochrome *c* region of the gels is shown. Two bands, at approximately 34 and 72 kDa, showed interesting changes under UV illumination after cross-linking and were selected for further analysis.

of proteins, a proteomics approach should be developed that meets a number of requirements. First of all, the method should capture the membrane proteins interacting with phospholipids at the membrane interface, irrespective of whether they are integral or peripheral. In addition, the approach has to include a method to specifically detect these proteins, and, moreover, this method should be compatible with common identification procedures in the field of proteomics, i.e., mass spectrometry. Here, the results will be summarized and discussed in view of the above requirements.

The photoactivatable phospholipid analogue ASA-DLPE was selected, because of the location of the cross-linking moiety on the headgroup of the lipid, enabling cross-linking to peripheral membrane proteins, with the lauroyl acyl chains allowing straightforward incorporation into biological membranes. Addition of the probe to the membrane either via the lipid film or from ethanol solution yielded similar results, indicating that the probe was correctly integrated in the membrane. The cross-linker was first tested in a model membrane system for its ability to cross-link to the peripheral membrane protein cytochrome *c* and was found to be a suitable probe to cross-link and retain this extrinsic membrane protein on the membrane. In this case, carbonate washing could be used to distinguish between cross-linked and non-cross-linked cytochrome *c*. The ability to use stringent wash conditions enables the enrichment of cross-linked and integral membrane proteins, although it should be noted that this method never gives a perfect separation due to the diversity in modes of interaction of proteins with the membrane.[3]

In previous research, ASA-PE has typically been used with a ^{125}I radioactive label for detection.[16] However, because radioactive labeling is generally avoided when performing identification by mass spectrometry, we explored alternative methods for the detection of cross-link products. Identification of cross-linked cytochrome *c* in the model system proved to be possible using mass spectrometry on the undigested protein or using the intrinsic UV absorbance of the cross-linker for detection in SDS-PAGE gels. Although the signal of the cross-link products in MALDI-TOF MS was rather weak when compared to the signal of the unmodified protein, the appearance of a smeared UV light absorbing band in SDS-PAGE just above cytochrome *c* after cross-linking and the results from the quantification assay showed that a considerable amount of protein was modified. Therefore, it seems plausible that the low signal of modified protein in mass spectrometry is due to the hydrophobicity of the cross-linked protein as, for instance, was found for subunits of cytochrome *c* oxidase cross-linked to cardiolipin.[40] We were not successful in finding modified peptides in MALDI-TOF MS after digestion of cross-linked cytochrome *c* (results not shown). Possibly, this is related to the increased hydrophobicity of peptides containing cross-

Table 2. LC-MS/MS Analysis of Potentially Cross-Linked Proteins in IMVs at 34 kDa[a]

			no. of unique peptides			
Swissprot ID	protein	MW (kDa)	+UV[b] + CO_3^{2-}	−UV + CO_3^{2-}	predicted trans-membrane domains[c]	localization[d,e]
P18239	Pet9p	34	34	30	3	MIM
P38077	Atp3p	34	15	16	0	M
P50085	Phb2p	35	19	16	1	MIM
P53252	Pil1p	38	18	15	0	PM/CP/MOM
P40961	Phb1p	31	9	16	0	MIM
Q12230	Lsp1p	38	8	10	0	PM/CP/MOM
Q04013	Yhm2p	34	8	10	0	MIM
Q03028	Odc1p	34	6	8	1	MIM
P53598	Lsc1p	35	7	2	0	M matrix
P23641	Mir1p	33	8	5	0	M
P40496	Rsm25p	30	7	2	0	M
P25605	Ilv6p	34	7	3	0	M matrix
P17505	Mdh1p	36	3	0	0	M matrix
P04840	Por1p	30	6	3	0	MOM
P36060	Mcr1p	34	11	2	1	MOM/MIS
P07143	Cyt1p	34	6	3	0	MIM
P36528	Mrpl17p	32	4	5	0	M matrix
P32331	Ymc1p	33	3	4	0	MIM
P12686	Mrp13p	39	6	0	0	M matrix
P40471	Ayr1p	33	3	4	0	MOM/LP/ER
P33303	Sfc1p	35	4	2	2	MIM
P38988	Ggc1p	33	2	2	0	MIM
Q99297	Odc2p	34	2	3	0	MIM
P28241	Idh2p	40	2	2	0	M matrix
P07257	Qcr2p	40	2	0	0	MIM
P33759	Mrps5p	35	3	0	0	M matrix
P32332	Oac1p	35	3	2	1	MIM
P53881	Mrpl22p	35	0	2	0	M

[a] UV absorbing bands from IMV pellets at 34 kDa, as found by photographing SDS-PAGE gels in UV light (see Figure 7F, lanes 5−8), were identified using LC-MS/MS. Proteins with a predicted mass within 6 kDa of the excised band and present in at least two pellet fractions were selected and sorted according to the average number of unique peptides found in the four lanes. The number of unique peptides for each protein is listed only for the carbonate washed pellets (Figure 7F, lanes 5 and 7). For a complete list of all identified peptides, see the Supporting Information. [b] For experimental conditions see Figure 7 and Materials and Methods. [c] Determined by the TMHMM algorithm as listed in the Saccharomyces Genome Database (SGD). [d] According to SGD, M = mitochondrial, MIM = mitochondrial inner membrane, MOM = mitochondrial outer membrane, MIS = mitochondrial intermembrane space, CP = cytoplasm, ER = endoplasmic reticulum, LP = lipid particle, and PX = peroxisome. [e] Assignment to MIM or MOM implies that the protein is a membrane protein.

Table 3. LC-MS/MS Analysis of Potentially Cross-Linked Proteins in IMVs at 72 kDa[a]

			no. of unique peptides			
Swissprot ID	protein	MW (kDa)	+UV + CO_3^{2-}	−UV + CO_3^{2-}	predicted trans-membrane domains	localization
P32191	Gut2p	72	37	49	0	M
Q00711	Sdh1p	70	29	26	0	MIM
P06208	Leu4p	68	23	29	0	M/CP
P12398	Ssc1p	71	31	22	0	MIM/M matrix
P40053	YER080W	72	21	20	0	M
Q08822	YOR356W	70	7	10	0	M
Q08179	Mdm38p	65	17	21	1	MIM
P36013	Mae1p	74	16	18	0	M
P07342	Ilv2p	75	21	15	0	M
P32796	Cat2p	77	15	12	0	MIM/PX
P32891	Dld1p	65	11	10	0	MIM
P32368	Sac1p	71	5	13	2	ER/Golgi/MOM
P38694	Msc7p	71	3	10	1	ER
P47052	YJL045W	69	0	2	0	M
P46943	Guf1p	73	3	7	0	M
P03874	Cbp2p	74	0	2	0	M
P14906	Sec63p	75	7	3	3	ER
P11484	Ssb1p	66	4	6	0	CP
P38825	Tom71p	72	2	4	0	MOM
P30624	Faa1p	78	3	0	0	MOM/LP

[a] UV absorbing bands from IMV pellets at 72 kDa, as found by photographing SDS-PAGE gels in UV light (see Figure 7F), were excised and analyzed. For details see Table 2.

linked lipid. Alternatively, the protein could be cross-linked at multiple locations, supported by the observation in MALDI-TOF MS of protein with two cross-linkers attached, resulting in the formation of multiple low abundant cross-linker containing peptides after digestion.

Using apocytochrome *c* as a model protein instead of cytochrome *c* enabled the unambiguous detection of cross-link products under UV light in both model membrane systems and IMVs. Apocytochrome *c* showed cross-linking to a similar extent as cytochrome *c* as assessed by SDS-PAGE, but a reduced

sensitivity toward carbonate washing, in agreement with its different mode of interaction with the membrane.[20,41] Despite the presence of other UV absorbing groups, heme in cytochrome *c* and most probably flavin and hemes in IMVs, cross-linked proteins could be clearly detected by comparing cross-linked vs non-cross-linked and carbonate vs buffer washed samples.

Surprisingly, only changes at 34 and 72 kDa were observed under UV illumination in IMVs upon cross-linking, which could be attributed to a preference of ASA-PE for cross-linking to highly abundant proteins or to classes of proteins with similar masses. It should be noted that the signal produced by less abundantly cross-linked proteins, as could be the case for endogenous cytochrome *c*, or cross-linked proteins of low abundance could be too low to detect. Interestingly, exogenously added apocytochrome *c* competed with endogenous proteins for cross-linking, since the differences in UV absorption for other proteins became less pronounced in its presence. To gain insight in which endogenous proteins could have been cross-linked, the proteins in the 34 and 72 kDa bands, which exhibited changes after cross-linking in IMVs when viewed under UV light, were identified yielding a short list of proteins of interest.

As expected, a high number of known membrane proteins was found, confirming that SDS-PAGE combined with LC-MS/MS is a suitable method for the detection of this class of proteins.[42,43] Only 4 (Msc7p, Guf1p, Cbp2p, and Ssb1p) out of the 48 proteins in the list (Tables 2 and 3) were not present in the PROMITO data set obtained after detailed proteomic analysis of highly purified yeast mitochondria.[42,44] Two of these proteins, Ssb1p and Msc7p, are listed by the SGD database as non-mitochondrial. Ssb1p is a cytoplasmic chaperone[45] that was found to bind to lipids in two independent screens.[6,8] Msc7p was localized to the ER in a large-scale GFP-fusion study.[46] In addition, Sec63p, a known ER protein that is listed in the PROMITO data set, was identified. This is consistent with the finding that the IMV preparation contained some contamination with ER. Likewise, the identification of mitochondrial outer membrane proteins is most probably due to contamination.

Analysis of the 34 kDa band (Table 2), which showed a clear cross-link product that was not carbonate wash-sensitive, yielded the ADP/ATP carrier Pet9p/Aac2p, an integral membrane protein, as prime candidate, on the basis of the number of unique peptides identified. Interestingly, the functioning of this abundant inner mitochondrial membrane protein is known to rely heavily on its tight interaction with the anionic phospholipid cardiolipin.[47−49] Moreover, its structure is reported to depend on the interaction with the choline headgroups of zwitterionic lipid phosphatidylcholine[50] and the reconstituted protein also preferentially binds to anionic lipids as rapidly exchangeable annular lipids.[51]

In contrast, we speculate that the γ-chain of ATP synthase (Atp3p), the second protein found in the 34 kDa band, is a less likely cross-link candidate, because in a previous study on the reconstituted bacterial ATP synthase complex [^{125}I]-ASA-DLPE was primarily cross-linked to the α-subunits.[13] The subunits of the prohibitin complex (Phb1p and Phb2p), the next candidates based on the number of unique peptide identified, are required for the stability of mitochondrially encoded proteins and depend on the PE levels in the mitochondrial membranes for proper functioning.[52]

The band analyzed at 72 kDa yielded the glycerol-3-phosphate dehydrogenase Gut2p as prime candidate. Gut2p was previously shown to cross-link to the phospholipid cross-linker [^{125}I]-TID-PC and to be carbonate wash-sensitive,[10,53] consistent with the labeling pattern found in gel. Therefore we propose this protein as main candidate to have been cross-linked.

Interestingly, a number of proteins identified in the 34 and 72 kDa bands from the carbonate washed pellets show an increase in the number of unique peptides identified upon cross-linking, indicating that these proteins are peripheral membrane proteins rendered carbonate wash-resistant by cross-linking. Two known protein complexes residing in the mitochondrial matrix are clear examples of this. First, the three components of the small subunit of mitochondrial ribosomes identified show an increase in the number of peptides identified after cross-linking. This could indicate that the small subunit is in close proximity to the lipids, in agreement with the observation that a large fraction of the ribosomes is associated with the inner membrane in an electrostatic manner in mammals.[54] In contrast, the large subunit was shown to bind to Oxa1p during co-translational protein insertion.[55,56] Possibly, these interactions work together to keep the ribosomes attached to the membrane and functional. The second example is acetolactate synthase, of which the subunits Ilv2p (Table 3) and Ilv6p (Table 2) show similar increases in the amount of peptides identified upon cross-linking. Ilv2p was also found to bind to phospholipids and especially to cardiolipin in a study using lipids immobilized on a solid support.[8]

Another peripheral membrane protein that becomes carbonate wash-resistant, as indicated by the differences of the amount of peptides identified, is the NADH-cytochrome b_5 reductase, Mcr1p. This protein is sorted to both the mitochondrial outer membrane and the intermembrane space via a cleavable anchor sequence.[39] Localized in the latter compartment the protein could be recruited to the inner membrane and therefore be amenable to cross-linking.

In summary, we demonstrated that our method is capable of detecting a phospholipid-interacting proteome and compatible with identification of proteins via mass spectrometry, fulfilling the basic requirements stated above. The present research has opened new avenues to study phospholipid-protein interactions in their native environment using modern proteomics techniques.

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Supporting Information Available: Complete listing of the identified peptides (Supporting Information Table 1) and proteins (Supporting Information Table 2) of the LC-MS/MS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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