Proteome-wide detection of phospholipid–protein interactions in mitochondria by photocrosslinking and click chemistry

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Photoactivatable lipid analogues are uniquely suited for the detection of lipid–protein interactions in biological membranes. Based on photocrosslinking, new methodology has been developed for the proteome-wide detection of lipid–protein interactions. Bifunctional lipid analogues containing a tag for click chemistry in addition to the photoactivatable moiety enable the enrichment of the crosslinked proteins that is required for subsequent identification by mass spectrometry. In principle the phospholipid interaction-based membrane protein proteomics approach is applicable to any biomembrane and any lipid. Here, we review the background and the development of the new methodology. Results obtained with photocrosslinking in purified mitochondrial membranes from the yeast *Saccharomyces cerevisiae* are summarized and future perspectives discussed.

Introduction

Membrane lipids are increasingly being recognized as regulators or modulators of a multitude of cellular processes, in addition to their more passive role as building blocks of biological membranes. The activity of integral membrane proteins is often affected by the surrounding lipid molecules that determine the physical properties of the membrane environment the proteins experience.[1,2] Some lipids bind at specific sites, usually between α-helical transmembrane segments or at protein–protein interfaces of membrane protein complexes, in which case they are regarded as co-factors rather than solvent.[2] The discovery of tightly bound lipids in membrane protein structures obtained by X-ray crystallography has provided new approaches for analyzing the binding specificity and the function of these lipids.[3] The binding of peripheral membrane proteins to lipids constitutes another class of specific lipid–protein interactions that is often accomplished by specialized lipid-binding domains such as PH, FYVE, PX, C2 and ENTH, and involves electrostatic interactions with anionic lipids,[4] and amphipathic secondary structure or hydrophobic domains that can insert in the membrane.[5] This type of interaction is common in cell signaling and membrane trafficking, where lipids and their metabolites are essential.

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How can specific lipid–protein interactions be detected? When the protein of interest has been purified without loss of activity and when a functional assay is available, examination of the dependence of the protein's activity on the presence of various lipids is relatively straightforward. Subsequently, the cell biological relevance of the specific lipid–protein interaction detected should be verified, *e.g.* by manipulating the lipid composition of the biomembrane involved using lipid biosynthesis mutants, or by site-directed mutagenesis of the putative lipid binding site. The detection of lipid–protein interactions in biological membranes from the angle of a lipid of interest requires other approaches. Several proteome-wide methods have been developed to identify candidate proteins that interact with a lipid of interest. For instance, lipids have been covalently bound to resins or other solid supports to find lipid-binding proteins by affinity purification.[6,7] In an inverted setup, immobilized proteins can be probed with labeled lipids on so-called proteome chips.[8] However, in these examples lipid–protein interactions are detected outside the native environment of the lipid bilayer.

In this review, we will describe how our search for mitochondrial proteins interacting with the abundant membrane phospholipid phosphatidylcholine developed into a new proteomics approach for integral and peripheral membrane proteins. Phospholipid interaction-based membrane protein proteomics aims to capture phospholipid–protein interactions in the native environment of the biomembrane. The approach is based on the use of photoactivatable phospholipid analogues that, after incorporation in the biomembrane, are covalently linked to their nearest protein neighbors by UV activation. The crosslink products formed reflect the structural and dynamic organization of the biomembrane and the chemical properties of the reactants.[9] Crosslinked proteins are subsequently identified by mass spectrometry-based proteomics techniques.

Mitochondria from the model eukaryote *Saccharomyces cerevisiae* were used as biomembrane system to set up the approach. The mitochondrial proteome has been studied extensively and encompasses an estimated 700 proteins in yeast.[10–13] Thus, yeast mitochondrial membranes provide an ideal system to develop and benchmark new proteomic techniques for membrane proteins. After a short update on lipid–protein interactions in (yeast) mitochondria and their importance for mitochondrial function, several photoactivatable probes that have been incorporated in membrane lipids for studying lipid–protein interactions will be introduced. Subsequently, photocrosslink studies employing photoactivatable lipids incorporated in mitochondrial membranes will be summarized, and implications and future perspectives will be discussed.

Lipid–protein interactions in mitochondria

The glycerophospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and cardiolipin (CL) are the major lipid constituents of the mitochondrial membranes. PC and PE are zwitterionic phospholipids, whereas PI and CL are anionic, rendering the surface of the mitochondrial membranes negatively charged. PC, the most abundant mitochondrial membrane lipid, and PI are typical bilayer lipids with a cylindrical molecular shape, *i.e.* the cross-sectional areas of the lipid head group and the acyl chains are similar. As a result these lipids self-assemble into bilayers upon hydration. In contrast, in PE the cross-sectional area occupied by the head group is smaller than that occupied by the acyl chains, resulting in a conical molecular shape, and the tendency to adopt non-bilayer structure. CL is a bilayer preferring lipid that acquires non-bilayer propensity upon charge neutralization, *e.g.* in the presence of divalent cations.[14] Non-bilayer lipids confer negative curvature stress to membranes, which is important for processes such as fusion and fission and for proper function of membrane proteins.[1]

Experimental evidence demonstrating the importance of specific glycerophospholipid–protein interactions for the structure and function of mitochondria is rapidly accumulating. In particular, the interactions with proteins of CL, which is synthesized and almost exclusively localized in mitochondria, have been extensively characterized. *In vitro* studies revealed that CL affects the activity of a number of mitochondrial inner membrane proteins, including the respiratory complexes and the ADP/ATP carrier (AAC, reviewed in ref. 15,16). In agreement with these studies, tightly bound CL molecules were identified in the crystal structures of the yeast cytochrome bc_1 and bovine cytochrome *c* oxidase, respiratory complexes III and IV, respectively.[17,18] *In vivo* studies employing a yeast *crd1* mutant lacking cardiolipin synthase, and as a consequence CL, have shown that CL is not essential for growth on non-fermentable carbon sources, which requires fully functional mitochondria. However, the lack of CL did result in sub-optimal ATP production (reviewed in ref. 19). Using a *crd1*Δ strain, CL was shown to be required for supercomplex formation between respiratory complexes III and IV,[20,21] which is thought to facilitate substrate channeling. More recently, CL has been implicated in the interaction of the major ADP/ATP carrier protein Aac2p with other membrane protein complexes in the mitochondrial inner membrane,[22] and in mitochondrial inner membrane fusion through its interaction with the dynamin-related protein Mgm1.[23,24] Moreover, CL has been implicated in the stability of a supercomplex of the prohibitin and the m-AAA protease complexes.[25]

The other major structural phospholipids PC, PE and PI have also been identified in the crystal structures of respiratory complexes III and IV,[17,18] suggesting that they may be important for the structure and activity of these proteins.[26] Other than that, and in contrast to CL, little is known about specific lipid–protein interactions in mitochondria involving these lipids. Insight into their importance for mitochondrial function derives mostly from yeast phospholipid biosynthesis mutants. The growth rate on non-fermentable carbon sources of yeast mutants disturbed in the biosynthesis of PE was found to correlate with the mitochondrial PE content.[27] Furthermore, inactivation of mitochondrial PE synthesis by deletion of the *PSD1* gene was synthetically lethal with deletion of the *CRD1* gene, indicating a mitochondrial requirement for a certain level of phospholipids with non-bilayer propensity.[28] A screen for synthetic lethality with a temperature sensitive allele of *PSD1* revealed functional overlap between the level of mitochondrial PE and the Phb1–Phb2 prohibitin complex.[29]

A synthetic genetic array analysis using *phb1*Δ cells identified 35 so-called GEP (genetic interactors of prohibitins) genes. Lipid analysis of the mitochondria of a majority of the *gep* deletion mutants showed reduced levels of PE and/or CL.[30] These and other findings have led to the proposal that non-bilayer lipids act in concert with the prohibitins as scaffolds required for spatial membrane organization.[31]

Mutant yeast strains with defects in the routes of biosynthesis of PC have the tendency to generate respiratory deficient yeast cells at high frequency,[32] which could be due to impaired PC–protein interactions. Only a few proteins have been identified that have a strong or absolute preference for PC. Delipidated bovine heart *bc*1 complex (retaining some CL) could be reactivated by adding PC or PE.[33] The bovine heart mitochondrial β-hydroxybutyrate dehydrogenase is the only mitochondrial enzyme known to date that shows a strict dependence on PC for activity *in vitro*.[34] To identify yeast mitochondrial proteins that depend on PC for proper function, we chose a photocrosslinking approach using photoactivatable PC analogues.

Photoactivatable lipid crosslinkers: properties and applications

Photoactivatable lipid analogues can be incorporated in biological membranes, and subsequently activated by UV light to crosslink to interacting proteins.[35] A wide variety of lipids containing photoactivatable groups has been synthesized and used to detect interacting proteins, usually by incorporation of a radiolabel in the photoactivatable moiety. For a more detailed treatment of the chemistry of photoactivatable groups and methods for the incorporation of photoactivatable lipids in biomembranes, the reader is referred to a recent review.[36]

The ideal photoactivatable group is small so as not to disturb the system in which it is introduced, stable under non-activating conditions, and highly reactive upon exposure to UV light to avoid long exposures that might damage the biological sample.[35] Photoactivatable groups used in lipid analogues are phenylazides, benzophenones, 3-trifluoromethyl-phenyl diazirines (TPD), and alkyl diazirines.

Upon exposure to UV light, phenylazides (Fig. 1) generate highly reactive nitrenes with a lifetime in the order of 10^{-4} s that subsequently may undergo rapid intramolecular ring expansion. The resulting ketene imine is less reactive, resulting in longer reaction times and a preference to insert into N–H bonds.[35] 4-Azidosalicylate has been attached to the headgroup of the zwitterionic lipid PE *via* an amide bond, and was subsequently radioiodinated. The resulting anionic N-[^{125}I]iodo-4-azidosalicylamidyl-PE ([^{125}I]-ASA-PE) has been used to find new lipid-interacting proteins in *Plasmodium falciparum*[37] and human erythrocytes,[38] and to assay binding of botulinum neurotoxins to liposomes.[39] Picq *et al.*[40] synthesized PA analogues containing an azidotetrafluorobenzylacyl chain at the *sn1*- or *sn2*-position of the glycerol backbone that retained the ability to specifically activate and crosslink a cyclic AMP diesterase isoenzyme. Sedlak *et al.*[41] synthesized CL analogues with a phenylazide group attached to the headgroup or to the acyl chains to probe the CL binding sites in detergent solubilized bovine cytochrome *c* oxidase. Remarkably, the pattern of photolabeling of cytochrome *c* oxidase subunits by the two CL derivatives was qualitatively similar.

Another commonly used photoactivatable moiety is benzophenone (Fig. 1) that upon exposure to UV light forms a triplet diradical, which can efficiently insert in C–H bonds, even in the presence of water and strong nucleophiles (reviewed in ref. 42). Another advantage of this probe is its chemical stability. However, with two phenyl groups, it is more bulky and apolar than the other photoactivatable compounds. Recently, the syntheses of benzophenone-containing PC analogues and of benzophenone-containing cholesterol surrogates have been reported.[43,44] The benzophenone-derivatized sterols were shown to compete with cholesterol for apolipoprotein A-I-induced cellular efflux.[44]

3-Trifluoromethyl-3-phenyl diazirines (TPD) generate carbenes upon UV light exposure that have a life time in the order of nanoseconds.[45] No intramolecular rearrangements are observed and they insert in any type of bond, including non-reactive C–H bonds. Due to the high reactivity, a relatively inert environment such as the core of the lipid bilayer is preferred for efficient photocrosslinking using diazirines.[35] The TPD-based radioiodinated 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl) diazirine ([^{125}I]TID) moiety has been attached to the *sn2*-acyl chain in phospholipids[46] (Fig. 2A) that have been used to study a variety of lipid–protein interactions.[9]

The diazirine group itself is the smallest photoactivatable group. It lacks the stabilizing trifluoromethyl group and is therefore more prone to non-specific labeling *via* the linear diazo-isomers that also form upon UV activation.[35] Diazirines were conveniently incorporated in cholesterol and fatty acids, and have been used for *in vivo* photolabeling of proteins interacting with cholesterol, PC, PI and sphingolipids.[47,48]

These examples show that labeling by photoactivatable lipid analogues is a versatile technique that can be used in many systems under a variety of conditions for detection of lipid-binding proteins. For their identification, mass spectrometry-based proteomics could become a powerful method.

Fishing for interactions between [^{125}I]TID-PC and yeast mitochondrial proteins

To investigate the essential role of PC in mitochondrial function, a search for PC-interacting proteins was performed using 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 ([^{125}I]TID-PC; Fig. 2A).[49] Small quantities of [^{125}I]TID-PC were incorporated in mitochondrial membranes (~0.1 pmol of probe per μg of mitochondrial protein) by addition from a stock solution in ethanol. After incubation under various conditions, samples were irradiated and analyzed by SDS-PAGE and autoradiography. The apolar photoactivatable [^{125}I]TID-benzoic acid ester ([^{125}I]TID-BE; Fig. 2A) that partitions into membranes served as a control for specificity in these experiments. [^{125}I]TID-BE only labeled porin (Por1p) and the phosphate carrier (Mir1p), very abundant integral membrane proteins of the mitochondrial outer and inner membrane, respectively, irrespective of the temperature of incubation (Fig. 2B, lanes 1–2). Photocrosslinking by

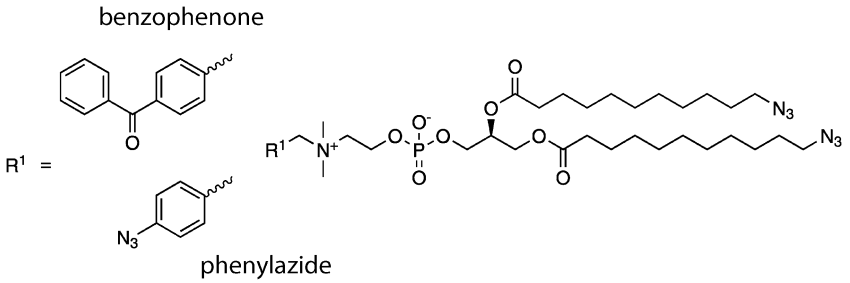

Fig. 1 Structures of phosphatidylcholine analogues containing phenylazide and benzophenone as photoactivatable groups, and containing azide-modified acyl chains.

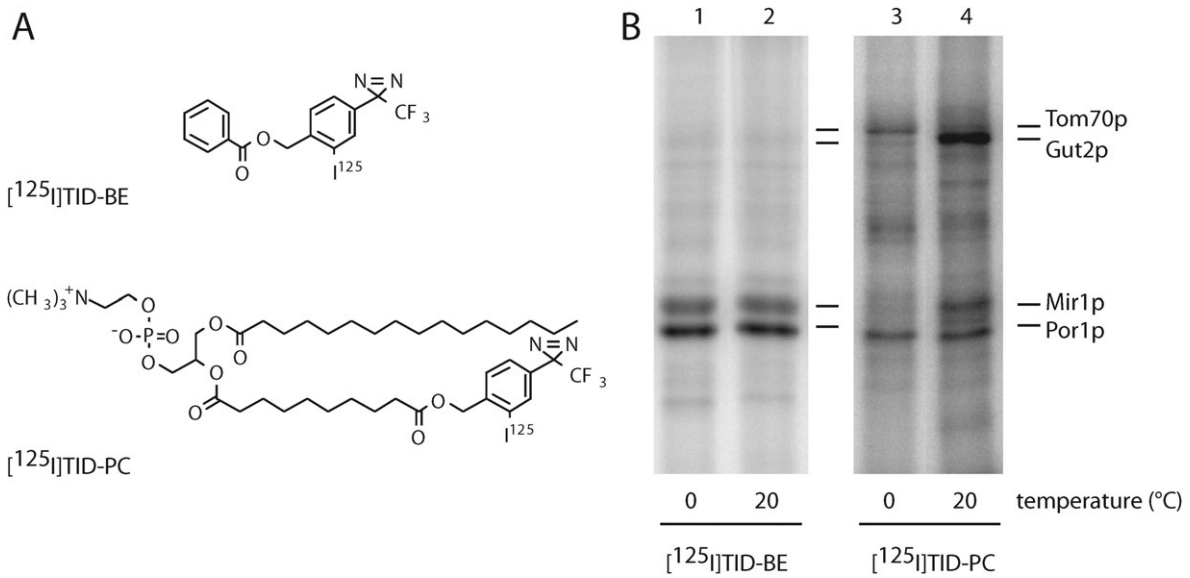

Fig. 2 (A) Structures of [^{125}I]TID-BE and [^{125}I]TID-PC (B) Photolabeling of mitochondria with [^{125}I]TID-BE and [^{125}I]TID-PC. After incubation for 30 min with the probe molecules at the temperature indicated, samples were photoactivated and subsequently analyzed by SDS-PAGE and autoradiography. The identities of Por1p, Mir1p, and Tom70p were established by immune precipitation using specific antibodies, and Gut2p was identified by mass spectrometry and by the absence of labeling in mitochondria from a *gut2*Δ strain. Data reproduced with permission from ref. 49.

[^{125}I]TID-PC revealed only a limited number of proteins, and it showed a remarkable temperature dependence. At 0 °C Por1p and Tom70p of the outer membrane presented the major crosslink products, whereas at 20 °C, the labeling of Tom70p was reduced and prominent labeling of Mir1p and the mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p) appeared (Fig. 2B, lanes 3–4). The temperature dependent labeling of Mir1p and Gut2p labeling was due to rupture of the outer membrane at 20 °C under the experimental conditions used, rendering the inner membrane accessible to the probe. In intact mitochondria the probe was confined to the outer membrane.[49] The reduced labeling of Tom70p at 20 °C was probably due to the phospholipid probe flipping to the inner leaflet of the outer membrane, where the efficiency of crosslinking Tom70p would be less.

Gut2p, which converts glycerol-3-phosphate to dihydroxyacetone phosphate, is an enzyme of the glycerol-3-phosphate shuttle and essential for growth on glycerol. The enzyme is a peripheral membrane protein associated with the mitochondrial inner membrane facing the intermembrane space.[49] The preferential labeling of Gut2p by [^{125}I]TID-PC led us to investigate the role of PC in Gut2p function. PC biosynthesis mutants, in which the PC content was lowered, exhibited reduced growth on glycerol and increased glycerol excretion, indicative for impaired Gut2p activity. However, these effects were most likely indirect since under the conditions tested, PC depletion did not affect the expression level, enzyme activity in isolated mitochondria, or membrane association of Gut2p.[50] With the possible exception of CL, exogenously added phospholipids including PC and PE did not affect the activity of Gut2p in detergent-solubilized mitochondria. In conclusion, no direct evidence for a functional interaction between Gut2p and PC has so far been found. Nevertheless, the relationship between Gut2p and PC discovered by photocrosslinking remains intriguing in view of the influence of membrane lipids on glycerol-3-phosphate dehydrogenases from other species.[51–53] Since Gut2p consumes glycerol-3-phosphate, the precursor of glycerolipid biosynthesis, it is tempting to speculate that the lipid interaction of Gut2p would be involved in the regulation of lipid synthesis. Interestingly, deletion of

the *GUT2* gene in *Y. lipolytica* was recently found to increase the rate of lipid accumulation.[54]

Phospholipid interaction-based membrane protein proteomics

Major challenges in proteomics lie in the area of membrane proteins. In particular, integral membrane proteins have proven difficult to analyze, because of their hydrophobic character and often low expression levels.[55–58] Inspired by the experience with [^{125}I]TID-PC, and the possibilities offered by mass spectrometry-based proteomics techniques, we set up a novel proteomics approach for membrane proteins that is based on lipid–protein interactions. A number of requirements had to be met. As protein analysis by mass spectrometry (MS) with the use of radiolabels is inconvenient, new selective detection methods for crosslink products were required. Most importantly, the photocrosslinked proteins had to be purified from the membrane preparation to allow for detection and quantitation by MS. Because of the relatively small number of proteins crosslinked by [^{125}I]TID-PC, a switch to lipid analogues with the photoactivatable group attached to the lipid headgroup was made, while realizing that the modification of the headgroup was likely to interfere with assigning phospholipid specificity to the interactions detected. The goal of the new proteomics approach was to identify peripheral and integral membrane proteins that interact with lipid headgroups at the membrane interface.

To avoid problems of probe accessibility, the new methodology was tested in purified right side-out yeast mitochondrial inner membranes instead of mitochondria.

Proof of principle of the new approach was obtained employing the well-established ASA-PE probe equipped with C12 acyl chains to enable membrane incorporation by addition from a solution in ethanol.[59] A PC analogue in which the TPD group was attached to the head group did not crosslink proteins, most likely because the short-lived carbene-species generated immediately reacted with water.[60] The ASA-PE probe molecule was validated by demonstrating that UV activation rendered the binding of the positively charged peripheral membrane protein cytochrome *c* to negatively charged liposomes resistant to carbonate wash. This result was recapitulated in mitochondrial inner membrane vesicles (IMV) in which ASA-PE had been incorporated (75 pmol added per μg IMV protein). Taking advantage of the intrinsic UV-absorbance of ASA-PE, crosslinked proteins were visualized by SDS-PAGE, by comparing UV-irradiated, carbonate-washed samples to UV-irradiated, mock-treated controls. Analysis of the protein bands exhibiting increased UV absorbance after photoactivation by liquid chromatography tandem mass spectrometry (LC-MS/MS) and quantifying the number of unique peptides identified yielded a number of candidate integral and peripheral membrane proteins that could have been crosslinked, with the ADP/ATP carrier (Pet9p/Aac2p) and Gut2p as prime candidates.[59] However, due to the background UV-absorbance from flavin- and heme-containing proteins and due to the presence of an excess of uncrosslinked proteins, this first approach fell short of unambiguously identifying crosslinked proteins.

Selective detection and purification of the crosslinked proteome by photoactivatable and clickable phospholipid analogues

To accomplish selective detection and purification (or at least enrichment) of crosslinked proteins, the C11-acyl chains of photoactivatable phospholipid analogues were equipped with azide moieties. The azide moiety serves as a small and relatively apolar chemical handle. Its introduction into lipid acyl chains is not expected to lead to structural perturbation of the membrane. Following photoactivation and solubilisation of the membrane samples, the highly efficient 1,3-dipolar chemoselective copper-catalyzed cycloaddition between azides and alkynes, coined as click chemistry,[61,62] was used for attaching tags to the crosslink products for detection and purification purposes. Contrary to the phenylazide, the alkylazide used for click chemistry is stable upon UV irradiation.[63] Based on the same principle, previously a bifunctional disaccharide probe was synthesized and photocrosslinked to galectin-1 with detection of the product by click chemistry.[64] Click chemistry (recently reviewed by ref. 65) allows the selective labeling of biomolecules with high efficiency and is bioorthogonal since the azide and alkyne do not react with common functional groups present in biological systems.

Two bifunctional phospholipid analogues mimicking PC were synthesized with the photoactivatable phenylazide and benzophenone groups attached to the quaternary ammonium moiety (Fig. 1; ref. 60). Compared to anionic ASA-DLPE,[59] the zwitterionic PC probes were much less effective in crosslinking the positively charged protein cytochrome *c*. The bifunctional PC analogues were validated by demonstrating that membrane-bound apo-cytochrome *c*, the unfolded, more hydrophobic precursor of cytochrome *c*, was rendered carbonate wash resistant after irradiation.[60] Fig. 3 shows the sequence of steps in a crosslink experiment followed by fluorescence detection or purification of the crosslink products. Briefly, phenylazide- or benzophenone-PC was incorporated in IMV by addition from ethanol (75 pmol μg^{-1} IMV protein). Following the removal of non-incorporated probe molecules by centrifugation through a sucrose cushion, samples were UV-irradiated for 20 min on ice, and subjected to carbonate wash as indicated. After solubilising the IMV pellets in SDS, the crosslink products were detected by the click reaction with fluorescent alkyne-conjugated rhodamine.

Photocrosslinking of phenylazide- and benzophenone-PC incorporated in IMV yielded partially overlapping protein profiles (Fig. 4). The differences in labelling were attributed to the different properties of the photoactivatable moieties. Carbonate wash had little effect on fluorescence intensities, indicating that crosslinked peripheral membrane proteins were rendered carbonate resistant by the lipid anchor. Based on the virtual lack of background fluorescence obtained in the absence of UV-activation, phenylazide-PC was considered the more suitable probe. Crosslink products were identified by LC-MS/MS after reaction with a biotin–alkyne conjugate and purification on neutravidin beads (Fig. 3; ref. 60). Out of 37 mitochondrial proteins identified, 20 integral and peripheral membrane proteins were significantly enriched in the carbonate-washed IMV after photoactivation of phenylazide-PC, and

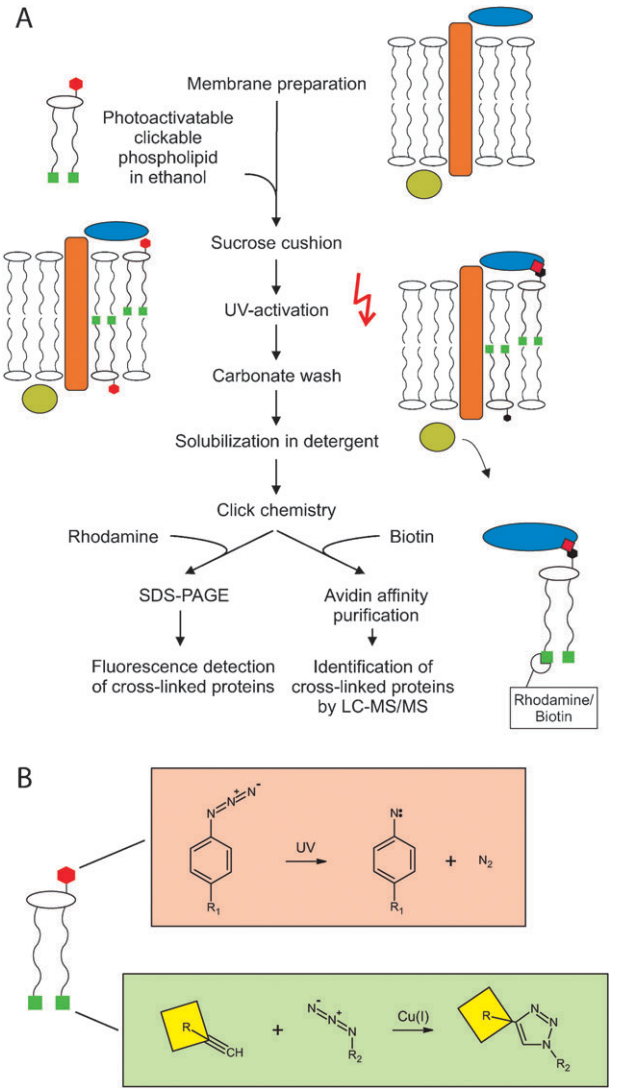

Fig. 3 (A) Flow chart of the sequential steps in phospholipid interaction-based membrane protein proteomics employing photoactivatable and clickable lipid analogues. Proteins are visualized by fluorescence detection and identified by mass spectrometry after separation by SDS-PAGE. For details see text and ref. 60. (B) Bifunctional phenylazide-PC generates a highly reactive nitrene upon photoactivation, and contains azide moieties that enable the 1,3-dipolar chemoselective copper-catalyzed cycloaddition to an alkyne-conjugated reporter. R1 and R2 represent the phosphatidyldimethylethanolamine moiety and the photocrosslink product, respectively.

therefore presumed crosslinked. Most of these were also crosslinked by benzophenone-PC (Table 1).

An important requirement is that the photoactivatable and clickable lipid analogues are correctly incorporated in the IMV. The strongest evidence for proper membrane incorporation of the lipid analogues consists of the carbonate wash-resistance acquired by peripheral membrane proteins after crosslinking. Moreover, peripheral membrane proteins were crosslinked both from the intermembrane space side, such as Gut2p and Nde1p, and from the matrix side of the inner mitochondrial membrane, such as Mrp7p and Ndi1p, indicating that the probes are able to flip and reach both leaflets of the mitochondrial IMV (*cf.* ref. 66).

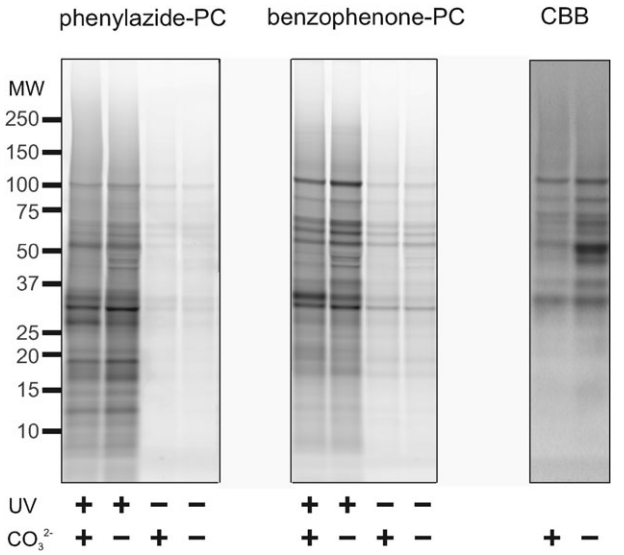

Fig. 4 Fluorescence scans of SDS-PAGE gels showing the crosslink products obtained in mitochondrial IMV after incorporation of phenylazide-PC and benzophenone-PC, and UV irradiation and carbonate wash as indicated. The two lanes shown in the panel on the right hand side represent Coomassie-stained patterns and correspond to the samples in the left two lanes of the phenylazide-panel. Data reproduced with permission from ref. 60.

Implications for lipid–protein interactions in mitochondria

The proteins crosslinked by phenylazide-PC (Table 1) belong to the group of highly expressed proteins, with most proteins present in excess of 5000 copies per cell under the culture conditions used by Ghaemmaghami *et al.*[67] However, proteins were not crosslinked according to abundance, as very abundant inner membrane proteins such as Pet9p/Aac2p and Mir1p did not show significant crosslinking. The lack of low copy number proteins is attributed to limitations of the biotin-purification and the detection by LC-MS/MS.

When it comes to detecting specific lipid–protein interactions, a major concern in using lipid analogues is that the photoactivatable moiety interferes with the interactions. In this respect, it is of interest to compare the results obtained with the anionic ASA-DLPE probe[59] to the results obtained for the zwitterionic PC analogues.[60] The difference in headgroup charge was reflected by the very limited overlap (Gut2p and Cyt1p) between proteins crosslinked by ASA-PE and by phenylazide-PC, both containing a phenylazide moiety.

Based on X-ray analysis of crystallized protein complexes containing glycerophospholipids, the ligands in lipid binding pockets were found to primarily bind to the phosphodiester moiety.[3] PC-binding pockets have less positively charged residues than pockets for anionic lipids, presumably to accommodate the positively charged ammonium group. Considering that the phosphodiester was unaltered in all photoactivatable lipid analogues used, they should be able to bind to these phospholipid binding pockets provided that the more bulky headgroup can be accommodated. The finding that phenylazide-PC crosslinks to the Cor1p and Cyt1p subunits of the cytochrome bc_1 complex directly adjacent to the PC-binding

Table 1 Mitochondrial proteins crosslinked by phenylazide-PC[a]

Protein name	Description	MW/kDa	Localization[b]	X-link to bp-PC[c]	Molecules per cell[d]
Lpd1p	Dihydrolipoamide dehydrogenase component (E3) of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multi-enzyme complexes	54	M	+	2.46E4
Gut2p	Mitochondrial glycerol-3-phosphate dehydrogenase	72	M	+	1.67E3
Ndi1p	NADH:ubiquinone oxidoreductase, transfers electrons from NADH to ubiquinone in the respiratory chain but does not pump protons	57	Matrix	+	5.24E3
Lsc2p	Beta subunit of succinyl-CoA ligase	47	M	+	—
Mrp7p	Mitochondrial ribosomal protein of the large subunit	43	Matrix		2.29E3
Cor1p	Core subunit of the ubiquinol-cytochrome *c* reductase complex (bc1 complex)	50	MIM	+	1.93E4
Dld1p	D-lactate dehydrogenase, oxidizes D-lactate to pyruvate	65	MIM	+	1.08E4
Nde1p	Mitochondrial external NADH dehydrogenase	63	M	+	4.93E3
Cyt1p	Cytochrome *c*1, component of the mitochondrial respiratory chain	34	MIM	+	3.99E4
Atp1p	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase	59	MIM		4.15E4
Cox2p	Subunit II of cytochrome *c* oxidase	29	MIM		—
Cox4p	Subunit IV of cytochrome *c* oxidase	17	MIM		9.41E3
Atp17p	Subunit f of the F0 sector of mitochondrial F1F0 ATP synthase	11	MIM		1.46E4
Ald4p	Mitochondrial aldehyde dehydrogenase	57	M	+	2.22E4
Lat1p	Dihydrolipoamide acetyltransferase component (E2) of pyruvate dehydrogenase complex	52	M	+	5.44E3
Idh2p	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase	40	Matrix	+	4.31E4
Yme1p	Subunit, with Mgr1p, of the mitochondrial inner membrane i-AAA protease complex	82	MIM		2.01E4
Phb2p	Subunit of the prohibitin complex (Phb1p–Phb2p), a 1.2 MDa ring-shaped inner mitochondrial membrane chaperone	34	MIM	+	2.85E3
Atp2p	Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase	55	MIM		1.64E5
Fcj1p	Mitochondrial inner membrane protein involved in formation and molecular structure of crista junctions	61	M		5.73E3

[a] Proteins were considered crosslinked if the ratio of the number of identified spectra with/without photo-activation ≥ 2 in the carbonate washed samples. Data taken from ref. 60. [b] Localization according to SGD (Saccharomyces genome database), M = mitochondrial, MIM = mitochondrial inner membrane, matrix = mitochondrial matrix. [c] Proteins crosslinked by benzophenone-PC[a] are indicated by +. [d] Number of molecules per cell according to ref. 67.

pockets found in its crystal structure,[17,68] indicates that PC is readily substituted for by phenylazide-PC, and supports the notion that the headgroup-modified PC analogues retain the relevant characteristics of PC. Since there is a substantial overlap in the proteins crosslinked by phenylazide-PC and benzophenone-PC it is likely that the nature of the photoactivatable moiety is less important for binding specificity than the charge and structure of the total phospholipid.

The crosslinking of Cox2p and Cox4p by phenylazide PC (Table 1) is consistent with the detection of PC molecules in the crystal structure of the bovine cytochrome *c* oxidase complex.[18] It was gratifying to find Gut2p as a prominent protein crosslinked by both PC probes in view of the highly efficient labeling of Gut2p obtained with [^{125}I]TID-PC at an almost 1000-fold lower concentration compared with its photoactivatable and clickable counterpart.

In addition to established peripheral membrane proteins (*e.g.* Atp1p, Atp2p and Gut2p), some proteins were detected by photocrosslinking that were not previously described as peripheral in yeast mitochondria. Phenylazide-PC was found to crosslink to Lpd1p and Lat1p, subunits of the homologous mitochondrial complexes pyruvate dehydrogenase and/or α-ketogluterate dehydrogenase localized in the matrix. These enzyme complexes were described as membrane-associated in mammals and plants, possibly through complex I.[69,70] Since complex I is not present in yeast mitochondria, association of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase with the membrane could facilitate the transport of electrons through NADH generated by these complexes to Ndi1p and subsequently to ubiquinone. Other proteins identified as peripheral membrane proteins by crosslinking to phenylazide-PC include Ndi1p, Nde1p, Mrp7p and Ald4p (Table 1; ref. 60).

Conclusion and future perspectives

Photocrosslinking is a powerful method to investigate the interactions between biological molecules. Photoactivatable, clickable lipids incorporated in yeast mitochondria *in vitro* have identified a number of proteins interacting with lipid head groups. With respect to the specificity and functional significance of the lipid–protein interactions found, it should be realized that the results of the phospholipid interaction-based proteomics approach can only serve as starting point for in-depth studies addressing the lipid dependence of individual proteins.

In principle, the approach is applicable to any biomembrane and any membrane lipid. Recently, the synthesis of a bifunctional analogue of phosphatidic acid (PA), an important signalling lipid, was reported, containing benzophenone as photoaffinity tag and an azide moiety for purification purposes, both attached to the *sn*-1 carbon of the glycerol backbone.[71] These modifications did not impair the binding of a C2 domain to PA-containing model membranes, suggesting that the probe molecule retains the distinctive features of PA.

Major challenges for future research include improvement of the sensitivity to detect crosslinked membrane proteins with low expression levels, and application of phospholipid interaction-based membrane protein proteomics to detect

lipid–protein interactions in live cells. The demonstration that lipid precursors containing the photoactivatable diazerine moiety,[47,48] ω-alkynyl and ω-azido fatty acids,[72,73] and propargyl-choline[74] are readily biosynthetically incorporated by cells when added to the culture medium, indicates that an *in vivo* approach is feasible. Also here, *Saccharomyces cerevisiae* with its unprecedented tolerance to variation in membrane lipid composition, may serve as test system of choice.

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