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MALDI-TOF Mass Spectrometry Analysis of Amphipol-Trapped Membrane Proteins

Chérine Bechara,[†,§,∥] Gérard Bolbach,[†,§,∥,‡] Paola Bazzaco,[⊥] K. Shivaji Sharma,[#] Grégory Durand,[#,▽] Jean-Luc Popot,[⊥] Francesca Zito,*[,⊥] and Sandrine Sagan*[,#,▽]

[†]Université Pierre et Marie Curie (UPMC Univ Paris 06), Laboratoire des BioMolécules (LBM), and [‡]Plateforme de spectrométrie de masse et protéomique, UPMC, IFR83, F-75005 Paris, France

[§]Centre Nationale de la Recherche Scientifique, Unite Mixte de Recherche (UMR) 7203, LBM, F-75005 Paris, France

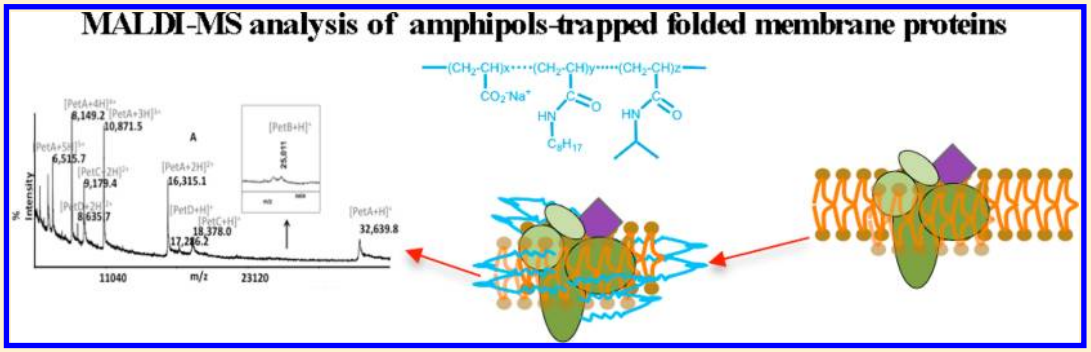
[∥]Ecole Normale Supérieure (ENS), LBM, F-75005 Paris, France

[⊥]Institut de Biologie Physico-Chimique, UMR 7099, CNRS and Université Paris 7, 13 rue Pierre-et-Marie Curie, F-75005 Paris, France

[#]Equipe Chimie Bioorganique et Systèmes Amphiphiles, Université d'Avignon et des Pays de Vaucluse, 33 rue Louis Pasteur, F-84000 Avignon, France

[▽]Faculté de Pharmacie, Institut des Biomolécules Max Mousseron, UMR 5247, CNRS and Universités de Montpellier 1 & 2, 15 avenue Charles Flahault, F-34093 Montpellier Cedex 05, France

Supporting Information



ABSTRACT: Amphipols (APols) are amphipathic polymers with the ability to substitute detergents to keep membrane proteins (MPs) soluble and functional in aqueous solutions. APols also protect MPs against denaturation. Here, we have examined the ability of APol-trapped MPs to be analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). For that purpose, we have used ionic and nonionic APols and as model proteins (i) the transmembrane domain of *Escherichia coli* outer membrane protein A, a β-barrel, eubacterial MP, (ii) *Halobacterium salinarum* bacteriorhodopsin, an α-helical archaebacterial MP with a single cofactor, and (iii, iv) two eukaryotic MP complexes comprising multiple subunits and many cofactors, cytochrome b_6f from the chloroplast of the green alga *Chlamydomonas reinhardtii* and cytochrome bc_1 from beef heart mitochondria. We show that these MP/APol complexes can be readily analyzed by MALDI-TOF-MS; most of the subunits and some lipids and cofactors were identified. APols alone, even ionic ones, had no deleterious effects on MS signals and were not detected in mass spectra. Thus, the combination of MP stabilization by APols and MS analyses provides an interesting new approach to investigating supramolecular interactions in biological membranes.

Membrane proteins (MPs) play a major role in governing exchanges of matter and information across the plasma membrane of cells, as well as between cell compartments. Integral MPs, which are in contact with the hydrophobic phase of membranes, are associated with lipids and, very often, with other MPs and/or cofactors, occasionally forming huge multimolecular aggregates.[1] These assemblies, which may or not be permanent, play an important role in the function and trafficking of MPs, but they are difficult to study. MPs are classically extracted from biological membranes and kept soluble in aqueous solutions using detergents. Detergents are dissociating surfactants. Because they compete with the noncovalent protein/protein and protein/lipid interactions that stabilize MPs and MP complexes, they tend to denature them.[2,3] Many milder alternatives to detergents have been devised to limit this problem,[4] among which are short amphipathic polymers called amphipols (APols).[5,6] After MPs have been solubilized, APols can substitute detergents at their

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hydrophobic surface,[7,8] keeping them water-soluble in the absence of detergent. MPs trapped in APols tend to be more stable than in detergent solution.[4,6] Both direct and indirect evidence suggests that APols favor the retention of lipids by solubilized MPs, which may be one reason why the functional properties of APol-trapped MPs are often closer to those observed when they are membrane-bound rather than in detergent solution.[9−11] Because of their stabilizing effect, APols facilitate the handling and purification of MPs and their complexes and supercomplexes.[8−14] They are therefore useful tools to explore the structure and composition of supramolecular assemblies that do not stand well being exposed to detergents, as illustrated by a recent cryo-electron microscopy study of mitochondrial supercomplexes I, III, and IV.[8]

Mass spectrometry (MS) is an accurate and powerful technique to analyze the sequence of proteins, study their post-translational modifications,[15−17] or determine their stoichiometry in high-molecular-weight complexes.[18−20] In the case of MPs, however, MS studies are far from being routine,[21] because of the difficulty in identifying detergents that make it possible to solubilize and purify them while preserving their native structure and permitting the ionization process.[22,23] MS studies of MPs usually resort to nonionic detergents, such as octyl glucoside or *n*-dodecyl β-D-maltoside (DDM).[24,25] However, the instability of most MPs in detergent solution tends to cause their inactivation, loss of native structure, dissociation, release of cofactors and subunits, and, very often, aggregation. Once aggregated, MPs are difficult to resolubilize, and they do not mix properly with the matrixes used for matrix-assisted laser desorption/ionization (MALDI) MS analyses. Because of their mildness, APols may represent an attractive alternative for MS studies of MPs. Indeed, an early study showed that the mass of an isotopically labeled MP, outer MP X (OmpX) from *Escherichia coli*, could be accurately determined by MS analysis of OmpX/APol complexes.[26]

In the present study, we have resorted to a range of MPs with different origins, functions, and secondary, tertiary, and quaternary structures to examine to which extent MS can be used to identify the proteins, cofactors, and lipids present in APol-trapped preparations, as well as protein post-translational modifications and tryptic fragments. As models, we chose the following MPs: tOmpA, the eight-stranded β-barrel that forms the transmembrane region of outer MP A from *E. coli*,[27] bacteriorhodopsin (BR), a retinal-containing proton pump from the archaebacterium *Halobacterium salinarum*, whose transmembrane domain is folded into seven α-helices,[28] cytochrome b_6f from the green alga *Chlamydomonas reinhardtii*, a superdimer comprising eight different transmembrane subunits and many cofactors and lipids,[29,30] and cytochrome bc_1 from beef heart mitochondria, also a multisubunit superdimer with many cofactors.[31,32] These proteins were chosen because their trapping by APols and the composition, stability, and solution behavior of the complexes thus formed have been extensively characterized before.[5,7,10,11] The two types of APols used in the present study are (i) the first APol to be developed, A8-35[5,33,34] (Figure 1A), an ionic, carboxylate-based polymer with which most of the studies described to-date have been carried out,[6] and (ii) recently developed nonionic, glucose-based APols (NAPols)[35] (Figure 1B), whose electroneutrality gives access to some specific techniques, such as isoelectrofocusing and cell-free synthesis, and which appear advantageous for the handling of particularly fragile MPs.[11] MALDI-MS and MS/MS were used to analyze preparations containing APols alone, MP/APol complexes, or tryptic fragments thereof.

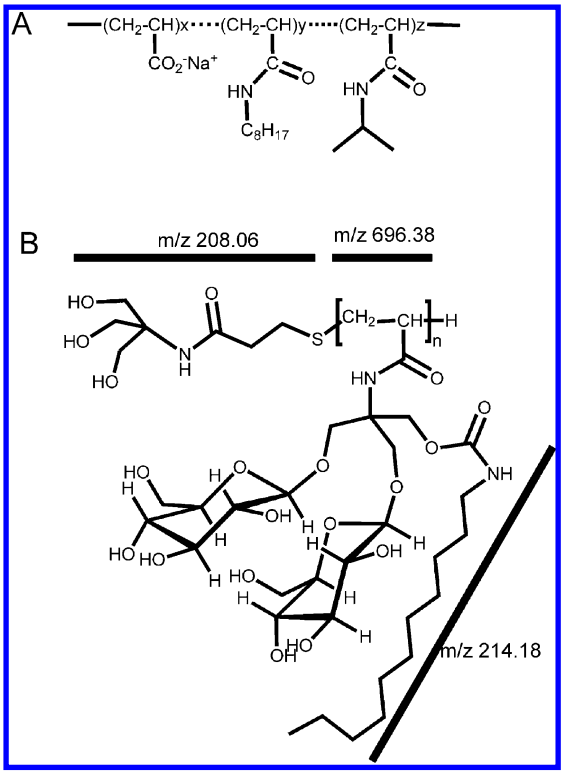

Figure 1. Chemical formulas of the two types of amphipols used in the present study: (A) amphipol A8-35, obtained by grafting a polyacrylate precursor with octylamine and isopropylamine ($x \approx 0.35$, $y \approx 0.40$, $z \approx 0.25$),[5] (B) homopolymeric, glucosylated, nonionic amphipol (NAPol), obtained by telomerization of an amphipathic monomer.[35] The composition of the various batches of APols used in the present study is given in Table S-1 (Supporting Information).

RESULTS AND DISCUSSION

MALDI-MS Spectra of Free Amphipols and Impact of Amphipols on MALDI-MS Analysis of a Test Peptide. MPs are transferred from detergent to APols using an excess of APols over that amount that will actually bind to the protein. As a result, preparations of MP/APol complexes usually contain free APols.[10,36] The behavior of free APols upon MALDI-MS was therefore investigated. The average degree of polymerization, molecular mass, and polydispersity index (PI) of the different APols used are summarized in Table S-1 (Supporting Information). When A8-35 (Figure 1) was mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix and analyzed by MALDI-MS, no signals could be detected whether in positive or negative ion mode, from particles, from intact polymer chains, or from fragments (not shown). The range of concentrations tested in these experiments was similar to that used to trap MPs (1−10 g·L^{-1}), i.e., well above the critical association concentration (~0.002 g·L^{-1}), above which A8-35 molecules assemble into particles.[37] The sample deposits contained typically about 9 μg of matrix and, depending on the concentration of the APol solution, either 4 or 0.4 μg of A8-35. This absence of signal may be related to the molecular polydispersity of the polymer. Indeed, to synthesize standard A8-35, a commercial poly(acrylic acid) (PAA), which features a wide polydispersity in length (PI ≈ 2−2.5) (Table S-1), is randomly derivatized with octylamine and isopropyl-

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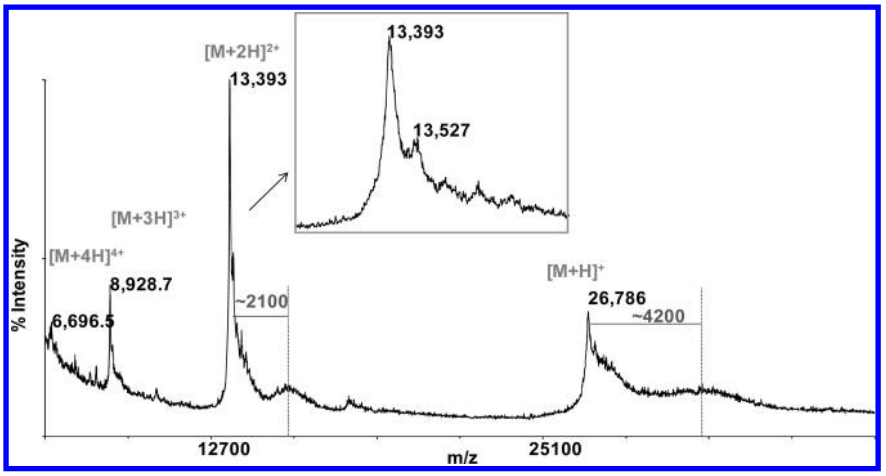

Figure 2. Positive ion MALDI-TOF mass spectrum of BR/NAPol complexes (0.5 μL of BR at 7 μM, trapped with NAPol SS174 at a 1/5 (w/w) BR/NAPol ratio), showing mono- and multicharged ions (+2 to +4). All ions displayed a component at $\Delta m \approx +268$, corresponding to the covalent retinal adduct. Similar spectra were obtained for BR trapped using the other NAPol batches. When BR was solubilized in OTG, the broad peak shifted by ca. +4200 was not detected.

amine.[5,33,34] The combination of these two sources of variability, length dispersity and random grafting, is expected to result in extensive mass polydispersity, which may explain the absence of an MS signal. The particles themselves, while relatively well-defined ($M \approx 40$ kDa), are somewhat polydisperse.[34]

At variance with A8-35, NAPols are homopolymers, the only source of mass polydispersity originating from their variable length (Figure 1B). In aqueous solution, they assemble into ~50 kDa particles.[35] Upon MALDI-MS, full-length NAPols were not observed either, but specific metastable ion species were detected at m/z = 1487, 2184, 2880, and 3576 (Figure S-1, top, Supporting Information). These ions (Figure S-1, top) are not those with the m/z expected from the repeated motif of intact NAPol molecules (Figure 1B). This result likely indicates either prompt fragmentation of NAPols or the presence of small oligomers formed in the course of the synthesis. The mass difference between these ion species is 696 Da, which corresponds to the mass of the repeat motif of NAPols (Figure 1B). In MS/MS, fragmentation of these peaks led to a systematic loss of 215 Da (Figure S-1, bottom), which corresponds to the mass of the undecylcarbamoyl chain (Figure 1), albeit with a difference in mass of −1.

A substance P peptide analogue (M = 1360 Da) was then used as a model peptide to examine the impact of APols on the desorption/ionization of peptides. The peptide (1 pmol, 1.36 ng) was mixed with A8-35 or NAPols at different peptide/APol ratios (1/1, 1/5, and 1/10, w/w), chosen to mimic MP samples. The peptide was easily detected in all cases (data not shown), whatever the nature of the Apol it was mixed with. No signals from APols were detected, except for the lowest peptide/NAPol ratio, for which the species at m/z = 1487 was again detected.

Altogether, these results show that the presence of APols in samples does not seriously hamper MALDI-MS analyses. We followed up with the analysis of various types of MPs that had been first solubilized in detergents and then transferred to either A8-35 or NAPols.

Comparative MALDI-MS Analyses of Intact Membrane Proteins Trapped in Amphipols or Kept in Detergent Solution. The compatibility of APols with MS was examined using four different MPs, two different APols, and, in most cases, detergent-solubilized controls, to form a reasonably general view of the interest of the approach. An exhaustive analysis of each protein in each type of surfactant was, however, beyond the scope of the study.

Bacteriorhodopsin. BR contains a prosthetic group, retinal (267 Da), associated via a Schiff base to Lys216, in the seventh and last transmembrane α-helix. The expected average mass of the mature holoprotein is 26 783 Da, which corresponds to residues 14−261 (SwissProt accession number P02945). Purple membrane was purified from *H. salinarum* and solubilized in *n*-octyl β-D-thioglucopyranoside (OTG) and BR transferred either to A8-35[10] or to NAPols (batches SS174, SS298, and SS325).[11]

Protein ions (+1, +2, +3, and +4) were detected in all NAPol preparations (Figure 2), corresponding to both the full-length holo- and apoproteins (Δm = 267 Da). The mass observed for BR was 26 786 ± 10 Da, which is the calculated average mass from +1 to +3 protein ions (Figure 2). In addition, for each charge state, a broad high-mass shoulder and unresolved ion distribution were present (Figure 2), whose center of mass was shifted from the main peak by ~4200 Da. Because of the relatively high mass of NAPols ($\langle M \rangle \approx 10-39$ kDa; see Table S-1, Supporting Information), it seems unlikely that intact NAPol molecules sticking to BR contribute much to forming the high-mass shoulder, yet we cannot exclude that degradation products of NAPol could be associated with the protein and be responsible for such a signal in MS spectra. More likely, these features might be related to protein-bound lipids. Thin-layer chromatography indicates that the purple membrane lipids are solubilized by OTG along with BR,[10] whereas functional data strongly suggest that, upon transfer to APols, lipids rebind to the protein.[10,38] The broad high-mass shoulder is not seen with OTG-solubilized BR (not shown). This observation supports the view that in the presence of OTG lipids dissociate from BR and that they do rebind upon transfer to NAPols.

Transmembrane Domain of E. coli Outer Membrane Protein A. To examine whether the failure to detect intact BR was related to properties of the BR/A8-35 complexes themselves or to a general effect on the MALDI behavior of samples containing these complexes, A8-35-trapped tOmpA was then analyzed.

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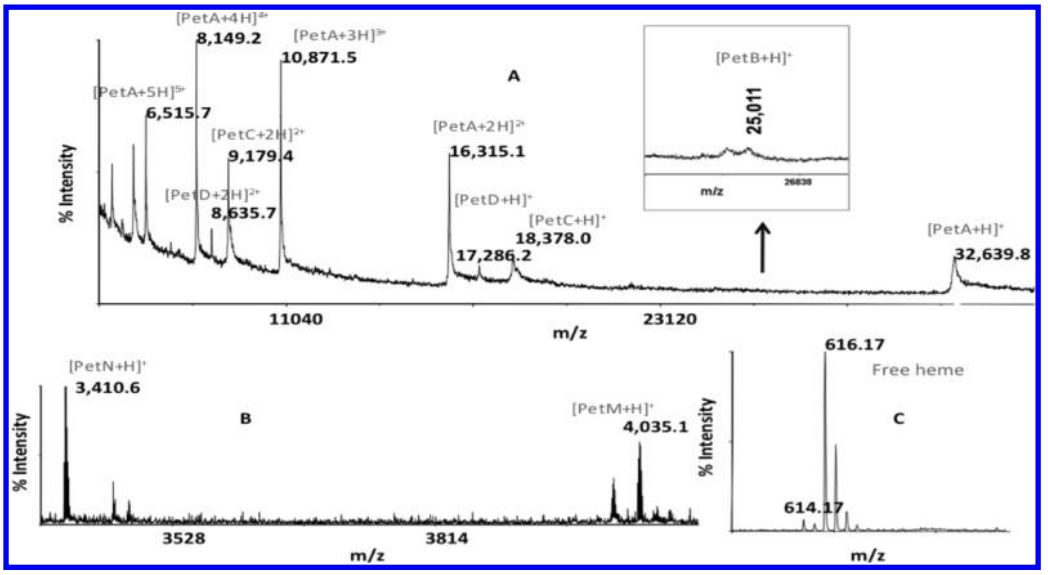

Figure 3. Positive ion MALDI-TOF spectra of b_6f complex (4 μM) trapped with A8-35 (b_6f/A8-35 = 1/5, w/w). The MALDI matrix CHCA was mixed with the complex solution. (A) Linear mode. (B, C) Reflector mode. Combining all the mass spectrometric data (reflector and linear modes), all subunits but PetL could be identified (Table S-2, Supporting Information). (C) Free heme, containing one Fe atom, was detected at low mass with the expected isotopic pattern.

The construct used for the present analysis (tOmpA) is a recombinant form of the transmembrane domain of outer membrane protein A that contains three mutations (F23L, Q34K, and K107Y) and an N-terminal His_8 tag.[7] The protein was expressed in *E. coli* as inclusion bodies, folded in the nonionic detergent *n*-octylpoly(oxyethylene) (C_8-POE), and transferred to C_8E_4 and then to A8-35.[7,39,40] Its expected mass is 20 189.3 Da. tOmpA was easily detected ($(M + H)^+$ at m/z 20 207.4 ± 2.7, average calculated from +1 to +3 charge states of the protein) when trapped in A8-35 (Figure S-2, Supporting Information). The difference between the calculated and measured masses (Δm = +17) likely corresponds to an oxidized form of the protein (the sequence of which contains three methionines). There was no significant difference in the mass analysis whether tOmpA was trapped in A8-35 or kept in C_8-POE solution (not shown), nor after a micropurification step of tOmpA/A8-35 complexes. The detection limit for tOmpA in A8-35 or C_8E_4 was ~200 fmol, i.e., as already noted for other MPs in detergent solutions,[24] ca. 1 order of magnitude above the typical threshold for hydrosoluble proteins of similar mass. The detection limit for BR in NAPols (batch SS174) was estimated in the same range, namely, 300−500 fmol.

A sample containing A8-35-trapped tOmpA, which was readily detected by MALDI-MS, was then mixed with A8-35-trapped BR, which could not be detected. tOmpA, in the presence of BR, was no longer detected (not shown). These observations indicate that the presence of BR/A8-35 complexes somehow negatively influences the protein/matrix mixture and/or the desorption/ionization processes.

Cytochrome b_6f. The photosynthetic cytochrome b_6f complex is a 220 kDa superdimer found in higher plants, in algae, and in cyanobacteria. The complex, along with bound lipids, was purified in DDM from *C. reinhardtii* thylakoids. Each b_6f monomer comprises eight polypeptide subunits, seven prosthetic groups,[29,30,41−44] (Table S-2, Supporting Information), and some lipids,[30] organized as follows: (i) Four large polypeptides (17.5−32 kDa)—cytochromes *f* and b_6, subunit IV, and the Rieske iron−sulfur protein—bind five redox prosthetic groups, namely, two noncovalently bound *b*-type hemes (associated with cytochrome b_6), two covalently bound *c*-type hemes (one bound to cytochrome *f*, the other to cytochrome b_6), and one [2Fe−2S] cluster (covalently bound to the Rieske protein), as well as two noncovalently bound cofactors, a molecule of chlorophyll *a* and one of β-carotene. Chlorophyll *a* interacts with subunit IV, β-carotene primarily with cytochrome b_6. (ii) Four small subunits (3−4 kDa), designated PetG, PetL, PetM, and PetN (Table S-2), form a single transmembrane α-helix each.

Cytochrome b_6 and subunit IV, which contain, respectively, four and three transmembrane helices, form the core of the complex, with which interact the single transmembrane helices of cytochrome *f*, of the Rieske protein, and of the small subunits, which are grouped in two lateral four-helix bundles at the outskirts of the superdimer.[30,41]

The b_6f complex is an interesting model for the present experiments because of its complexity—many subunits comprised of transmembrane α-helices carrying (cytochrome *f* and the Rieske protein) or not (the other subunits) large extramembrane domains, a variety of cofactors, and bound lipids—as well as its fragility: upon exposure to detergents, it very easily inactivates, losing some cofactors and subunits and breaking down into inactive monomers.[29,45] It is thus a good model for fragile noncovalent membrane assemblies. At variance with most MPs, the b_6f complex is not stabilized upon being complexed by A8-35,[5,11] but it is after its transfer to NAPols.[11]

The purified b_6f complex was mixed with the CHCA matrix either as the original preparation in DDM solution or after transfer to either A8-35 or NAPols, and the samples were analyzed by MALDI-MS (Figure 3). The number of subunits detected varied depending on the surfactant (Table S-2, Supporting Information). All subunits were detected in both the DDM-solubilized and A8-35-trapped preparations except for PetL. In general, DDM-solubilized samples gave higher ion signals than A8-35 samples. In NAPol-trapped samples, the subunits PetD, PetG, and PetL yielded no detectable ions.

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Irrespective of the surfactant used, subunits were not all detected with the same efficiency. For instance, cytochromes *f* and b_6 showed strong and weak signals, respectively. Cytochrome b_6 and PetD gave weak signals in all mass spectra. This may be related to the fact that these two subunits are the only ones to feature several transmembrane α-helices, whereas their average hydrophobicity is lower than that of the small subunits PetL and PetN, which were easily detected.

With regard to cytochrome *f*, the discrepancy between the mass expected from the gene sequence (taking into account the reported sequence conflict given in the UniProtKB database) and the measured mass reflects the presence of the covalently bound *c*-type heme (616 Da) (Table S-2, Supporting Information).

For the b_6 subunit, the mass shift varied according to the sample (increase of ∼710, 231, and 145 when using APol, NAPol, and DDM respectively). Excesses of mass compared to the calculated one have also been observed for the b_6 subunit of the *C. reinhardtii* complex studied by MALDI-MS (+165)[46] or for that of the spinach complex by electrospray ionization (ESI) MS (+104),[47] but not for the b_6 subunit from the cyanobacterium *Nostoc* PCC7120.[48] These results show that differences in amino acid sequence and/or in post-translational modifications of the polypeptide exist between complexes from different species. In the case of *C. reinhardtii*, tryptic digestion of the b_6 subunit revealed previously unidentified N-terminal modifications (see below) and thus the presence of different isoforms of this subunit. The fact that we detect an average mass of the various isoforms present can explain the mass shifts observed for subunit b_6, given that APols are clearly *not* associated with these ions (much larger mass increases would be expected; see Table S-1, Supporting Information) and that the mass of b_6f-associated lipids is in the 740−900 Da range.[30,49] The larger mass increase in the case of the A8-35-trapped complex may be due to the retention of one of the two *b*-type hemes noncovalently linked to cytochrome b_6. Free heme ions were detected in the lower mass range (reflector mode), with the isotopic pattern expected from the presence of an iron atom (Figure 3C), characterized by a minor, lighter peak and a major one shifted by two mass units, respectively, due to the presence of ^{54}Fe and ^{56}Fe.

Mass discrepancies were also observed for the other two large subunits, the Rieske protein (PetC) and subunit IV (PetD), for which MS analysis yielded respectively an excess of mass of ∼20 Da and a deficit of ∼150 Da. These mass differences could be due, in the first case, to an oxidation and, in the second, to the loss of the N-terminal Met residue (Table S-2, Supporting Information).

PetG was detected in linear mode but not in reflector mode, indicative of a strong metastable decay. PetM was detected at the expected mass, and the MALDI-TOF-TOF (TOF = time-of-flight) analysis yielded the expected sequence. PetN carried an additional mass (+129 Da), and MS/MS analysis confirmed the expected sequence (Uniprot database), but with an extra glutamyl residue at the N-terminus [EGEPAIVQIGWAATCVMFSFSLSLVVWGRSGL], as already reported.[50] PetL, as mentioned above, was never detected, whereas it has been detected in the *Arabidopsis thaliana* complex analyzed by ESI-MS.[15]

Cytochrome bc_1. Cytochrome bc_1 was analyzed only in A8-35, and 10 out of the 11 subunits were detected in MS spectra[32] (Table S-3, Supporting Information). The undetected subunit is cytochrome *b*, which comprises eight transmembrane helices. As for the b_6f complex, the presence of a *c*-type heme (bound to cytochrome c_1) was detected in MALDI-MS (Figure S-3A, Supporting Information). Interestingly, analysis of these deposits revealed phospholipids at *m/z* 742, 758, 760, 768, and 780 (Figure S-3A). Consistent with this interpretation, the MS/MS analysis of these ions showed a very intense peak at *m/z* 184, the mass of phosphocholine, which is a clear signature of the presence of phospholipids (Figure S-3B).

Tryptic Digestion of Amphipol-Trapped vs Detergent-solubilized Membrane Proteins. To study the susceptibility of APol-trapped MPs to enzymatic treatment, the kinetics of tryptic digestion was analyzed by MALDI-MS. MS data were acquired either directly on the digests or after micropurification and desalting of the samples (C18 reversed phase).

Bacteriorhodopsin. After tryptic digestion of BR kept in OTG solution or transferred to either of the two different types of APols, many ion species were detected in linear mode, while only two ion species were detected in the reflector mode up to *m/z* 4000. The two ion species detected in reflector mode were identified by MS/MS as peptides from the C-terminal extramembrane region of BR. Additional peptides were also identified after tryptic digestion of all BR samples. These peptides originated from contaminant MPs, such as the phosphate ABC transporter (at *m/z* 1645 [^{351}FVTSNDYFALPESR^{364}], 1802 [^{195}EIVDSGVETITIEELR^{210}], and 1897 [^{112}LADYFASEYGYEAGGER^{128}]; peaks at *m/z* 1645 and 1897 are shown in Figure S-4 (Supporting Information) for BR/A8-35 complexes).

Regarding BR-specific signals, an ion species at *m/z* 1452 appeared after 30 min of digestion in samples trapped in A8-35 vs 2 h for BR in NAPols (SS174) or in OTG. The sequence of the peptide, determined by MS/MS, is [^{160}AESMRPEVASTFK^{172}], in the third cytoplasmic loop of the protein, before the sixth transmembrane helix.

A second ion species, at *m/z* 987, was also observed after 30 min in OTG, A8-35, and NAPol SS174 and after 1 h in NAPols SS298 and SS325. The sequence determined by MS/MS (not shown) showed that it corresponds to [^{217}VGFGLILLR^{225}]. Val^{217} lies in the seventh transmembrane helix and immediately follows Lys^{216}, the amino acid that covalently binds retinal. Cleavage at this lysyl residue depends on the Schiff base being hydrolyzed, which occurs spontaneously upon BR denaturation.[51] We therefore measured, in all BR samples, the proportions of bound ($\lambda_{max} \approx 554$ nm) and free ($\lambda_{max} \approx 380$ nm) retinal. The ratio of bound to free retinal in BR was similar in all APol-trapped samples (∼2.5/1), which is typical of freshly prepared samples, while it was much lower for BR in OTG (∼1/1.4), indicative of denaturation (Table S-4, Supporting Information). This observation is consistent with the fact that transfer of BR to either type of APols considerably slows the rate of inactivation as compared to that observed in OTG.[10,11] This assumption was supported by the analysis of BR samples digested for 24 h (Figure S-5, Supporting Information). In linear mode, high-mass ion species were detected at *m/z* ≈ 23 700 for BR trapped in NAPols SS298 and SS325 and to a lesser extent in SS174, while no ion was visible in the samples in OTG or A8-35. For the latter sample, however, the absence of signal could arise from the full digestion of the protein or from an incorrect mixture between the sample and the matrix, similarly to that observed with intact BR. However, the existence of the ion species at *m/z* ≈ 23 700 for BR in NAPol compared to BR in OTG demonstrates that the membrane

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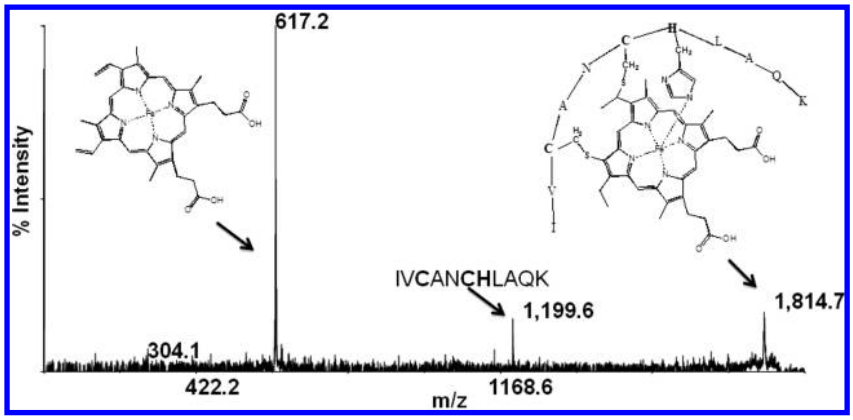

Figure 4. Identification of peptides from trypsin digestion of the A8-35-trapped b_6f complex. MS/MS analysis of the ion at m/z 1814.7 shows the presence of the protonated form of heme (m/z at 617), from cytochrome b_6, and the sequence [^{19}IVCANCHLAQK^{29}], from cytochrome f, with bound heme, at m/z 1815, and without it, at m/z 1199.

protein was less accessible to trypsin when trapped in the nonionic polymer than when solubilized in detergent. The species at m/z 23 700, along with the peptides at m/z 987 and 1452, are similar to the first peptides produced upon tryptic digestion of BR in *Halobacterium* cells or in reconstituted vesicles.[51] Altogether, these results suggest that BR resists trypsin digestion better after trapping with NAPols than in OTG solution, which is consistent with the stabilization afforded by NAPols[11] (see the Conclusion).

Cytochrome b_6f Complex. Limited tryptic digestion of cytochrome b_6f did not show any significant surfactant-dependent differences between samples in terms of the fragments obtained. In terms of kinetics, however, peptide digests appeared more quickly when the b_6f was solubilized in DDM as compared to being trapped in A8-35. As observed with BR, the b_6f complex was most resistant to tryptic digestion when trapped in NAPols, only a few peptides being obtained from such samples.

All tryptic peptides that were identified by MS/MS belonged to the three largest subunits, namely, cytochromes f (PetA) and b_6 (PetB) and, to a lesser extent, the Rieske protein (PetC) (Table S-5, Supporting Information). This is not surprising, given that those are the subunits most exposed to the aqueous solution. Subunit IV (PetD) is mostly buried, while the small subunits are short, mainly transmembrane peptides, with no or few arginyl or lysyl residues. Intact small subunits were occasionally detected in digested samples that had not been micropurified.

Additional, specific peaks were detected when the complex was solubilized in DDM or trapped in A8-35, that is, the two surfactants in which b_6f is least stable.[11] The ion species at m/z 1814.7 did not correspond to any of the possible unmodified tryptic peptide of the b_6f complex. The isotopic pattern of this ion indicated that it contains an iron. Its fragmentation indeed generated two daughter ions, one at m/z 617.2, with side peaks similar to those of free heme,[52] and another at m/z 1199.6, which corresponds to sequence 19−29 of cytochrome f (Figure 4). This peptide includes the sequence CXXCH, which is known to covalently bind heme c through two thioether bonds, the histidine residue being the fifth heme iron ligand.[30,41,53,54]

Trypsin digestion of the b_6 subunit separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out in gel for 24 h. MALDI-TOF mass analysis of the digestion products revealed that cytochrome b_6 is expressed heterogeneously in *C. reinhardtii*. The N-terminal amino acid sequence 1−11 deduced from the genomic one is MSKVYDWFEER (Q00471, UniProtKB). MS/MS sequencing of the tryptic digests led us to identify two unconventional N-terminal sequences, namely, MSKVYDFEER (Δm = −186 Da), in which the tryptophan residue is missing, and (formyl)-PYDWFEER (Δm = −319 Da), in which a formylproline replaces the MSKV N-terminal tetrapeptide. The existence of different molecular isoforms of the b_6 subunit likely results from currently unidentified post-transcriptional modifications. Both of these modifications decrease the mass of the subunit. The limited sequence coverage (~40%) did not permit identification of the origin of the mass excesses observed when analyzing the full-length protein (Table S-2, Supporting Information).

Two samples of the b_6f complex either solubilized in DDM or trapped in NAPols (SS325) were analyzed by blue native−polyacrylamide gel electrophoresis (BN−PAGE).[55,56] Staining of the gel (Figure S-6, Supporting Information) revealed the presence of the dimeric form of the complex in each sample. In-gel trypsin digestion of the monomer and dimer bands yielded the same peptide pattern in MS analysis as that obtained after trypsinolysis of the same samples in solution (not shown). Thus, there is apparently no significant gain in trapping MPs in APols to improve sequence coverage after enzymatic digestion in gel as compared to proteolysis in solution.

CONCLUSION

APols have the potential to preserve in their native state MPs and MP complexes and supercomplexes that do not stand well being exposed to detergents. Part of their stabilizing effect is due to the fact that they do not compete as efficiently as detergents with the noncovalent protein/protein and protein/lipid interactions that determine the structure and function of MPs and MP complexes. As such, APols are highly promising tools to investigate molecular associations that exist in biological membranes but are difficult or impossible to preserve in detergent solutions. Mass spectrometry offers a particularly powerful technology to explore the composition of such complex objects. In the present work, we have examined to what extent APol-trapped MPs and MP complexes are amenable to MALDI-TOF-MS. Two types of APols were tested, the classical, polyacrylate-based ionic APol A8-35[5] and recently developed, glucosylated NAPols.[11,35]

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Our data show that APol-trapped MPs and MP complexes are readily amenable to MALDI-MS analysis, whether of the intact proteins or of fragments generated by proteolysis of APol-trapped proteins, be it in solution or in gels. Lipids were occasionally observed (see below). This is particularly interesting given that one of the mechanisms that underly the stabilizing effect of APols is probably that they preserve MP/lipid interactions better than detergents.[9−11,38] Two monomeric MPs exemplifying different types of secondary structures were examined in the present work, BR and tOmpA, and two MP complexes, cytochrome b_6f (eight types of subunits, five types of cofactors) and cytochrome bc_1 (eleven types of subunits, three types of cofactors). Ten of the eleven subunits of A8-35-trapped cytochrome bc_1 could be identified, as well as lipids and protein-bound and nonbound hemes. All but one of the subunits of cytochrome b_6f could be identified in DDM or A8-35 and all but three in NAPols, as well as protein-bound and nonbound hemes. Hitherto unreported modifications, as compared to the protein sequences expected from those of the genes, could be identified. The relative intensity of the peaks yielded by the various subunits of the b_6f complex was similar for the detergent-solubilized and the APol-trapped complex.

A curious case is that of BR, whose full-length ions could be observed after trapping in NAPols, but not in A8-35, whatever the matrix used, even though tryptic degradation of the same samples yielded peptides that could be readily observed. This behavior may result from some large-scale effect on the sample/matrix mixture. Indeed, tOmpA, tested in APol A8-35, was correctly identified, as had been the structurally comparable MP OmpX in an earlier work.[26] However, tOmpA trapped in A8-35 is not detected anymore if the same sample is mixed with BR/A8-35 complexes. A possibility is that the phenomenon is related to demixing of the samples on the MALDI target, perhaps facilitated by the large amount of lipids retained by BR, but the matter remains undecided.

Lipids were observed either directly or indirectly. BR/NAPol samples give rise to ions corresponding to the naked protein, but also to a collection of heavier ions that are too light to contain the APol and are most likely BR/lipid complexes. Phospholipids were observed upon MALDI-MS analysis of bc_1/A8-35 samples, yielding lipid fragments upon MS/MS, among which is phosphocholine. MP-associated lipids preserved by trapping with APols are therefore accessible to analysis both by thin-layer chromatography after lipid extraction[10] and by MS (this work), which offers interesting opportunities for exploring the lipid environment of MPs. APols themselves are not seen upon MALDI-MS, presumably because of their polydispersity, but fragments of NAPols can be identified. MP/APol ions, if they exist, would probably not be visible either, because of the polydispersity of the polymers.

Trypsinolysis was carried out either in solution or in gels, following BN−PAGE. The peptides observed are similar to those obtained in DDM solution, but they form with slower kinetics in APols, particularly in NAPols. APols have been postulated to damp MP large-scale conformation excursions, which may contribute to their stabilizing character.[6,57] Such a mechanism would provide a reasonable explanation for the higher resistance to trypsin of APol-trapped MPs. With BR, the rate of proteolysis decreased in the order detergent ≥ A8-35 > NAPols. This seems to correlate with the stabilizing potential of the three types of surfactants.[11] These observations suggest that resorting to APols does not improve the sequence coverage of membrane proteins, but may possibly help in sorting between cleavage sites that are always exposed and sites that become so only upon conformational excursions of the protein.

In summary, the present study shows that the compositions of MP complexes trapped and stabilized by APols, as well as the primary structures of the component MPs, are readily amenable to MALDI-MS studies. Given the potential of APols for the stabilization and purification of fragile membrane assemblies, this approach will likely prove helpful in investigating the supramolecular organization of biological membranes.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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Chérine Bechara[1,2,3], Gérard Bolbach[1,2,3,4], Paola Bazzaco[5], K. Shivaji Sharma[6,†], Grégory Durand[6,7], Jean-Luc Popot[5], Francesca Zito[5]*, and Sandrine Sagan[1,2,3]*

[1]UPMC Univ Paris 06, UMR 7203, Laboratoire des BioMolécules, F-75005 Paris, France; [2]CNRS, UMR 7203, LBM, F-75005 Paris, France; [3]ENS, UMR 7203, LBM, F-75005 Paris, France; [4]Plateforme de spectrométrie de masse et protéomique, UPMC, IFR83, F-75005 Paris, France; [5]Unité Mixte de Recherche 7099, Centre National de la Recherche Scientifique and Université Paris-7, Institut de Biologie Physico-Chimique, 13 rue Pierre-et-Marie Curie, F-75005 Paris, France; [6]Université d'Avignon et des Pays de Vaucluse, Equipe Chimie Bioorganique et Systèmes Amphiphiles, 33 rue Louis Pasteur, F-84000 Avignon, France; [7]Unité Mixte de Recherche 5247, Centre National de la Recherche Scientifique and Universités de Montpellier 1 & 2, Faculté de Pharmacie, Institut des Biomolécules Max Mousseron, 15 avenue Charles Flahault, F-34093 Montpellier Cedex 05, France;

MALDI-TOF mass spectrometry analysis of amphipol-trapped membrane proteins

Supporting Information

Supporting Information is given for **Experimental Section**; **Table S-1**. Characteristics of A8-35 and three homopolymeric NAPols used in the present study; **Table S-2.** Expected and observed masses of the b_6f complex subunits from *C. reinhardtii*; **Table S-3.** MALDI MS analysis of A8-35-trapped cytochrome bc_1; **Table S-4.** Preservation of the native structure of BR in surfactant; **Table S-5.** MALDI-TOF-TOF identification of peptides obtained after trypsin digestion of cytochrome b_6f kept in DDM or trapped in either A8-35 or NAPols; **Figure S-1.** MS analysis of amphipols; **Figure S-2.** Positive ion MALDI-TOF mass spectrum of A8-35-trapped tOmpA; **Figure S-3.** MS analysis of A8-35 trapped cytochrome bc_1; **Figure S-4.** MALDI-MS spectrum of BR trapped in A8-35 (1:5 w:w) and digested with trypsin for 120 min; **Figure S-5.** MALDI-MS (linear mode) analysis of BR after 24 h of digestion with trypsin; **Figure S-6.** Blue native gel electrophoresis of cytochrome b_6f; Supplementary references. **Figure S-7.** MALDI-TOF-TOF mass spectrum of the peptide at m/z 3410.7 observed for the b6f complex trapped with A8-35.

EXPERIMENTAL SECTION

1. Materials and Reagents

Matrices (CHCA, SA, 2,5-DHB) and calibrants (peptides and proteins) were purchased from LaserBio Labs (Sophia-Antipolis, France). Trypsin Singles Proteomics Grade was supplied from Sigma (Saint-Quentin Fallavier, France). The substance P analogue, H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Nle-Met-NH2 was synthesized by the tBoc strategy in SPPS.

2. Synthesis of amphipols

A8-35 was synthesized as described in refs [1, 2]. Commercial A8-35 amphipol can be obtained from Affymetrix/Anatrace. The synthesis of the three batches of NAPols used in the present work has been described in ref [3] and their properties are summarized in Table 1.

3. Preparation of membrane proteins and trapping in amphipols

BR, cytochrome b_6f and cytochrome bc_1 were respectively purified from the purple membrane of *H. salinarum*[4], from thylakoid membranes of *C. reinhardtii*[5] and from beef heart mitochondrial membranes[6]. tOmpA was overexpressed as inclusion bodies in *E. coli* and folded as described[7-9]. Before trapping in APols, solutions of MPs were brought to a moderate salt concentration (100 mM NaCl), and a detergent concentration close to its critical micellar concentration (CMC), that is 20 mM C8E4 for tOmpA, 17 mM *n*-octyl-β-D-thioglucopyranoside (OTG) for BR, 0.2 mM DDM for both cytochrome b_6f and bc_1. APols were added at weight ratios of 1:5 PM/APol for tOmpA and BR, 1:3 for cytochrome b_6f and 1:1.5 for cytochrome bc_1. The detergent was removed either by incubating MP/detergent/APol solutions with BioBeads (Bio-Rad) (20 g wet beads per g of detergent) under stirring for 3 h at 4°C (BR, tOmpA), or by incubating several hours with BioBeads, followed by overnight incubation at 4°C as a dilute solution, and reconcentration on an

Amicon filtration device (molecular weight cut-off 50 kDa) (b_6f, bc_1).

4. Sample preparation for MS analysis

The dried-droplet method[10] was found to be the most valuable for the MS studies of MP samples containing OTG, DDM or APols. Typically, 0.5 µL of sample were mixed with a few µL of matrix. Alternatively, a micropurification step using home-made C8 (Poros R2) or ZipTip C18-microcolumns (Millipore) was included. MP elution was performed directly with the matrix and a few µL deposited onto the sample plate.

The matrix giving the best results in terms of sensitivity and resolution was CHCA (α-cyano-4-hydroxycinnamic acid) dissolved at saturation in 0.1% TFA, 50% ACN. Other classical matrices such as sinapinic acid (SA) and 2,5-dihydroxy benzoic acid (DHB) showed very broad peaks with a high-mass tail, likely indicating uncomplete desolvation of matrix-MP complexes (data not shown).

100 $g.L^{-1}$ APol solutions were mixed with 1 or 10 pmol of an analogue of Substance P, at peptide/APol ratios 1/10, 1/5 or 1/1 (w/w). These preparations,as well as APols alone (1, 5 and 10 pmol) and membrane proteins (~ 5-7 pmol) trapped in different APols were then combined with an equal volume of matrix solution (5 $g.L^{-1}$ CHCA in 0.1% TFA, 50% ACN) and 0.5-1.5 µL of this mixture was applied to a stainless steel target plate, and allowed to air-dry at room temperature.

4.2) Limited trypsin digestion of amphipols-trapped membrane proteins

100-150 pmol of the different protein preparations were digested using Trypsin Singles, Proteomics Grade (Sigma), according to the manufacturer's instructions. The samples were incubated at 37°C, for 24 hours. Aliquots from the b_6f and BR digests were taken after 5, 15, 30, 60 and 120 min of trypsinolysis.

After digestion, the samples were acidified with 0.1% TFA, and either mixed with CHCA matrix for direct MS analysis or concentrated on a Zip-Tip C18 column (Millipore) and eluted with the CHCA matrix directly on the MALDI plate.

5. Mass spectrometry analysis

Positive ions MALDI-TOF and MALDI-TOF-TOF mass spectra were performed using the DE-Pro (Applied Biosystems) and 4700 Proteomic Analyzer (Applied Biosytems) in linear mode (proteins) and in reflector mode (low mass units of MP and digests). A high mass detector (CovalX) on the 4700 proteomic Analyzer was also used for the analysis of pure APols and MP complexes. MS/MS studies using N2 as gas for the collision cell at 1keV were only performed on the low mass units of MP and peptides (m/z <4000) after trypsin digestion. MS and MS/MS calibration were obtained using standard proteins and peptides mixtures (LaserBio Labs), in the range of interest.

Table S-1. Characteristics of A8-35 and three homopolymeric NAPols used in the present study.

APol type	DP_n[a]	$\langle M \rangle$[b] (kDa)	PI[c]	$\langle M_{partic.} \rangle$[d] (kDa)
APols				
A8-35	~35[e]	~4.3[e]	2-2.5[e]	~40[f]
NAPols[g]				
SS174	~14	~10	n.d.	n.d.
SS298	~42	~29	n.d.	~51
SS325	~16	~11	n.d.	~51

[a] DPn, degree of polymerization; [b] $\langle M \rangle$, average mass of individual molecules; [c] PI, polydispersity index; [d] $\langle M_{partic.} \rangle$, average mass of free APol particles; [e] F. Giusti and J. Rieger, personal communication; [f] data from ref. [2]; [g] data from ref. [3]; n.d., not determined.

b_6f subunits (UniProtKB)	TM helices	Calculated mass *M*	Measured mass and Δm [a]			Comments
			DDM	A8-35	NAPol	

Table S-2. Expected and observed masses of the b_6f complex subunits from *C. reinhardtii*.

PetA (cytochrome *f*) (+ His_6 tag) (P23577)	1	32,624[b]	(1^+- 6^+) 32,616±20	(1^+- 6^+) 32,615±10	(1^+- 5^+) 32,641± 20	Covalently bound heme *c* (616 Da)
PetB (cytochrome b_6) (Q00471)	4	24,780[b]	(1^+, 2^+) 24,925 Δm=+145	(1^+) 25,490 Δm=+710	(1^+) ~25,011 Δm=+231	Covalently bound heme c_i. Unaccounted-for excess masses of ~145, ~710, and ~231 Da. Retention of a *b*-type heme in the presence of A8-35? Presence of N-terminal modifications (see text).
PetC (Rieske iron-sulfur protein) (P49728)	1	18,332.8	(1^+- 4^+) 18,356±20 Δm=+23±20	(1^+, 2^+) 18,349±20 Δm=+16±20	(1^+- 4^+) 18,358± 20 Δm=+25±20	Oxidation?
PetD (Subunit IV) (P23230)	3	17,441.9	(1^+-2^+) 17,288±30 Δm=-154±30	(1^+, 2^+) 17,274±30 Δm=-168±20	n.d.[c]	Loss of *N*-terminal Met
PetG (Q08362)	1	3,981.2[d]	(1^+) 3,991 Δm=+10	(1^+) 3,991 Δm=+10	n.d.	-
PetL (P50369)	1	4,874.8[d]	n.d	n.d	n.d	-
PetM (Q42496)	1	4,034.19[d]	(1^+) 4,034.11[d] Δm=0	(1^+) 4,034.11[d] Δm=0	(1^+) 4,034.11[d] Δm=0	Sequence checked by MS/MS
PetN(POC1D4)	1	3,281.68[d]	(1^+) 3,410.7 [d]Δm=+129	(1^+) 3,410.65 [d]Δm=+129	(1^+) 3,410.5 [d]Δm=+129	Extra Glu at *N*-terminus

Masses were determined by MALDI-MS for the complex solubilized in DDM or transferred either to A8-35 or to NAPols (batch SS325). [a] Δm: excess or deficit of mass with respect with that deduced from the sequence. [b] calculated mass, taking sequence conflicts and mutations (UniProtKB) into account. [c] n.d.: not detected, [d] m/z of first isotope.

Table S-3. MALDI MS analysis of A8-35-trapped cytochrome bc_1.

Bovine bc_1 subunits	Calculated mass	Measured mass, (Δm) [a]	Comments
Core I (P31800)	49,212	49,112 Δm=-100	weak signal
Core II (P23004)	46,525	46,295 Δm=-230	weak signal
Cyt *b* (Q33995)	42,734	n.d.[b]	-
Cyt c_1 (P00125)	27,288	27,856 Δm=+570	covalently bound heme *c*
Rieske (P13272)	21,610	21,582 Δm=-28	within the uncertainty range
Hinge (P00126)	9,176	9,179 Δm=+3	within the uncertainty range
SU 6 (P00129)	13,345	13,390 Δm=+45	acetylation on Lys or at *N*-terminus ?
QP-C (P13271)	9,532	9,593 Δm=+60	Glycyl lysine[33] isopeptide ?
SU 9[c]	7,998	8,001 Δm=+3	within the uncertainty range
SU 10 (P00130)	7,327	7,327 Δm=0	-
SU 11 (P07552)	6,520	6,698 Δm=+178	?

The uncertainty on mass measurements was estimated to be ± 40 Da in the 30-40 kDa range and ± 5-10 Da in the 10 kDa one. [a] Δm: excess or deficit of mass with respect with that deduced from the sequence. [b] n.d.: not detected. [c] Retained leader sequence of the Rieske protein[11].

Table S-4. Preservation of the native structure of BR in surfactant.

Retinal State	BR absorbance				
	OTG	A8-35	NAPols		
			(SS174)	(SS325)	(SS298)
Bound (A_{554}/A_{280})	0.33	0.45	0.43	0.50	0.46
Free (A_{380}/A_{280})	0.46	0.20	0.18	0.22	0.18
Bound/free (t_0)	0.7	2.2	2.4	2.3	2.5
Bound/free (+ 3 months)	0.5	2.5	2.8	2.8	2.4

As a measure of the degree of preservation of the native structure of BR, the relative concentration of free (λ_{max} = 380 nm) and bound ($\lambda_{max} \approx$ 554 nm) retinal in the samples was determined before tryptic digestion (t_0) and after three months of storage in the dark at -20°C.

Table S-5. MALDI-TOF-TOF identification of peptides obtained after trypsin digestion of cytochrome b_6f kept in DDM or trapped in either A8-35 or NAPols.

Calculated (MH+)	Measured (MH)+	Sequence identified by MS/MS	Subunit	Surfactant (digestion time required for detection)
1,142.8	1,143.51	[4-11] VYDWFEER	PetB (b_6)	DDM (5 min); A8-35 (15 min); NAPols SS174 (60 min), SS298 (15 min) and SS325 (30 min)
1,567.7	1,567.76	[1-13] YPVFAQQNYANPR	PetA (f)	DDM (5 min); A8-35 (5 min)
1,684.7	1,685.81	[97-108] VGNLYYQPYSPEQK	PetA	DDM (>120 min)
1,814.6	1,814.77	heme c + [19-29] IV**C**AN**CH**LAQK	PetA	DDM (120 min); A8-35 (120 min)
2,129.9	2,122.22	[150-170] IVTGVPDAIPGVGGF IVELLR	PetB	DDM and all APols (5 min)
2,480.8	2,480.32	[66-88] KGDLNVGMVLILPE GFELAPPDR	PetA	DDM; A8-35 (>120 min)
2,884.7	2,884.52	[30-55] AVEIEVPQAVLPDTV FEAVIELPYDK	PetA	DDM (>120 min)
3,293.5	3,291.60	[138-168] GPAPLSLALAHCDV AESGLVTFSTWTET DFR	PetC (Rieske)	DDM and all APols (30 min)
3,592.5	3,592.67	[218-251] EGQTVQADQPLTNN PNVGGFGQAETEIVL QNPAR	PetA	DDM; A8-35 (>120 min)

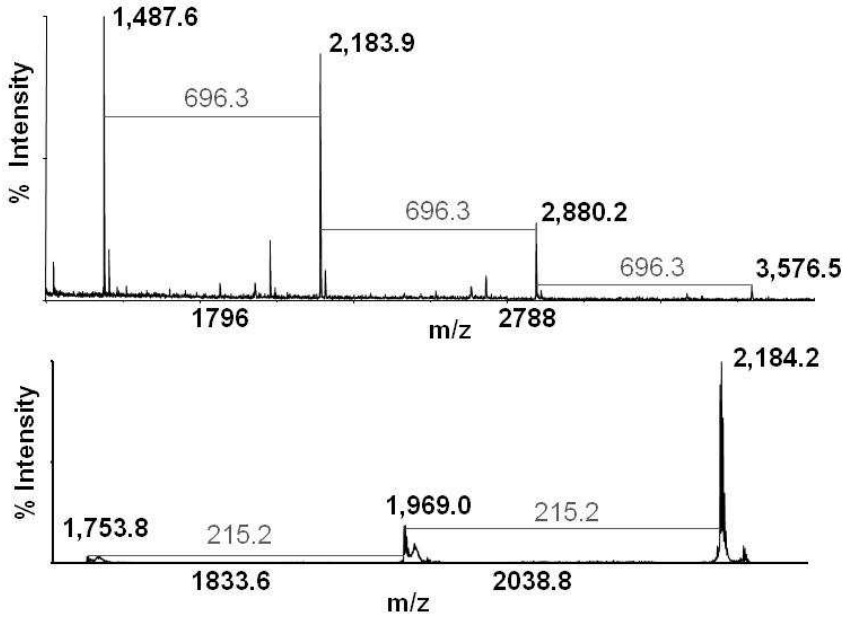

Figure S-1. MS analysis of pure amphipols. *Upper panel*, MALDI mass spectrum in reflector mode of ion species observed with NAPols (batch SS325). These species have a mass difference of 696 Da, which corresponds to the repeat motif of NAPols (Figure 1). *Lower panel*, MS/MS analysis of these ions showed fragments whose mass differed by 215 Da, likely corresponding to the undecylcarbamoyl chain (Figure 1).

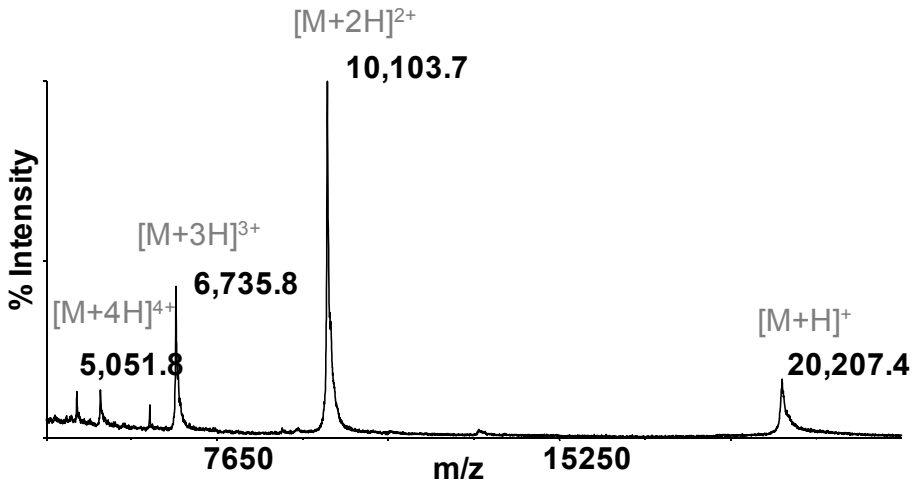

Figure S-2. Positive ion MALDI-TOF mass spectrum of A8-35-trapped tOmpA after micropurification of 5 pmoles. The same result was obtained after 4-fold dilution of the sample. From the different ion charge states, the molecular mass was deduced to be 20,206.4 ± 2.7 Da (expected mass, 20,189.3 Da).

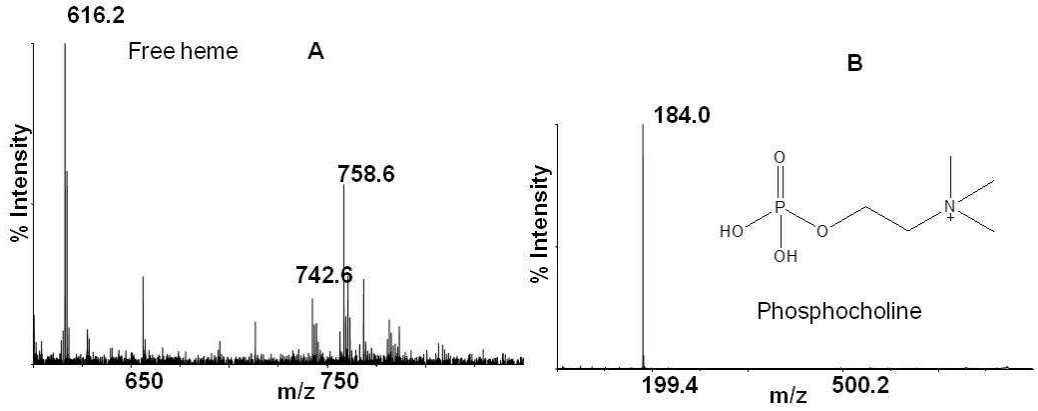

Figure S-3. MS analysis of A8-35-trapped cytochrome bc_1 A. Low-mass region of the MALDI-MS spectrum. The ion at m/z 616.2 corresponds to free heme, those at 742.6 and 758.6 to phospholipids. **B.** MS/MS analysis of the ion at m/z 758, showing the presence of phosphocholine, a phosphatidylcholine-derived fragment, at m/z 184.

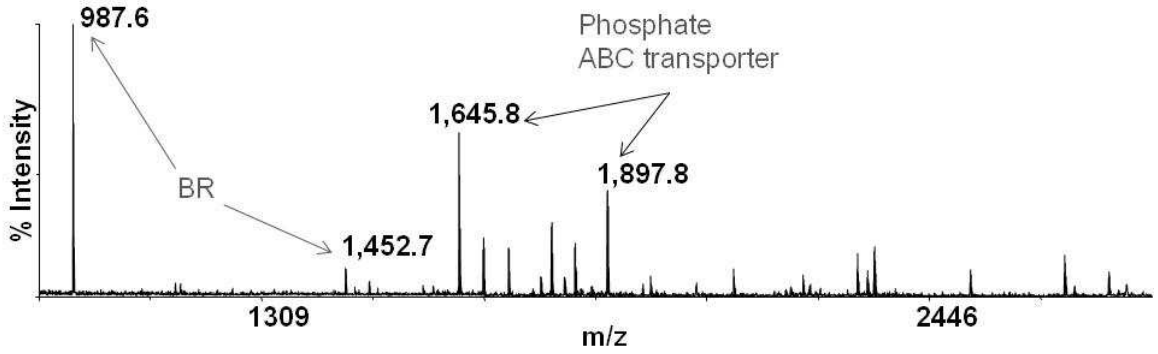

Figure S-4. MALDI-MS spectrum of BR trapped in A8-35 (1:5 w/w) and digested with trypsin for 120 min. The peptide ions observed originated from BR (m/z 987.6 and 1,452.7; sequenced by MALDI-TOF-TOF), but also from other MPs such as the phosphate ABC transporter (ion species at m/z 1,645.8 and 1,897.8 were sequenced by MS/MS: [^{351}FVTSNDYFALPESR^{364}] and [^{112}LADYFASEYGYEAGGER^{128}).

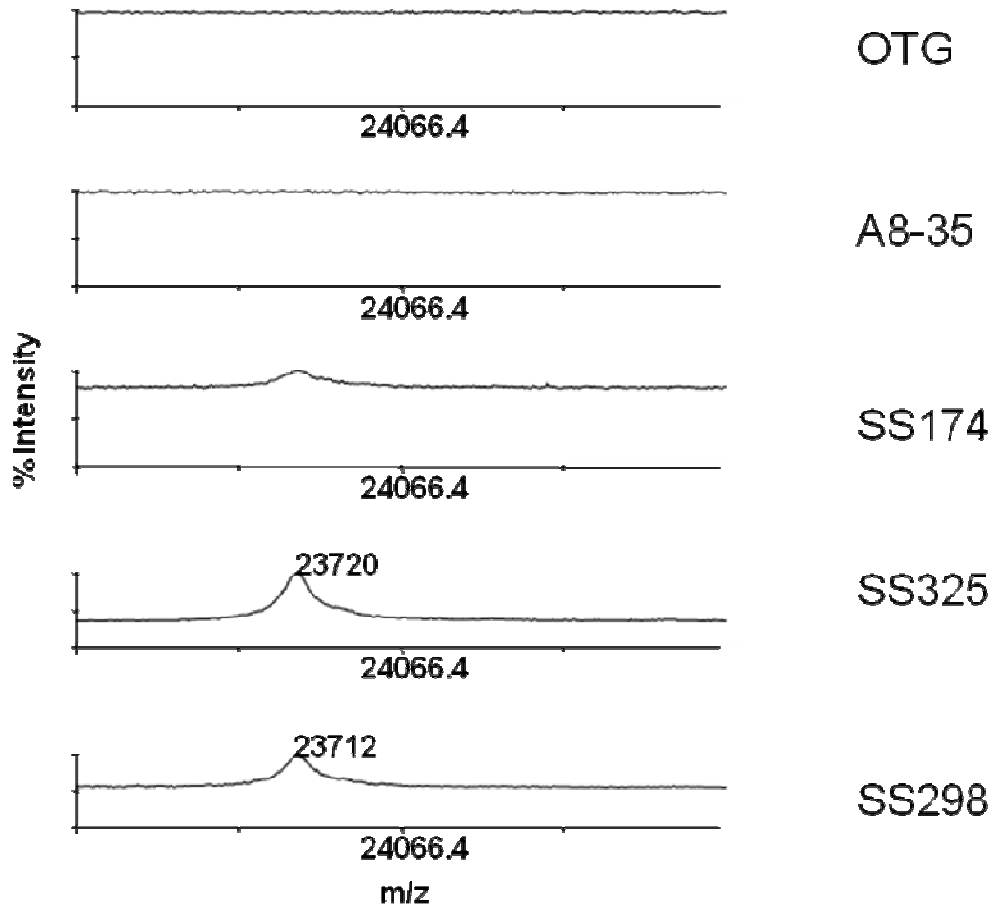

Figure S-5. MALDI-MS (linear mode) analysis of BR after 24 h of digestion with trypsin.

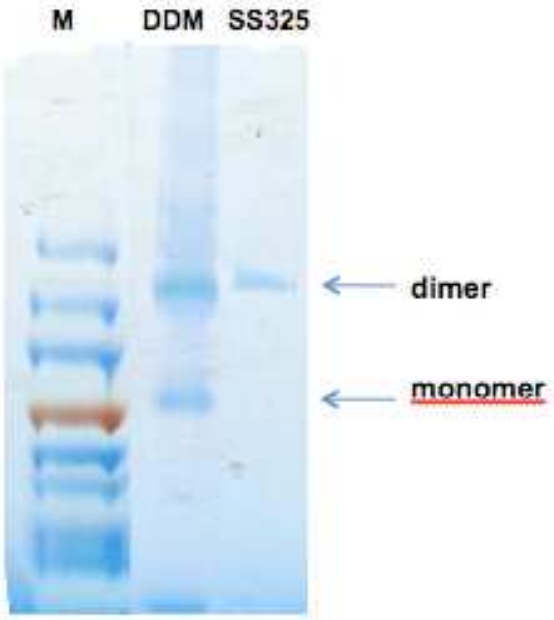

Figure S-6. Blue-native gel electrophoresis of cytochrome b_6f. Samples of b_6f complex either solubilized in DDM or trapped in NAPols (batch SS325) were analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE) on a 4-16% Bis-Tris gel (Invitrogen).

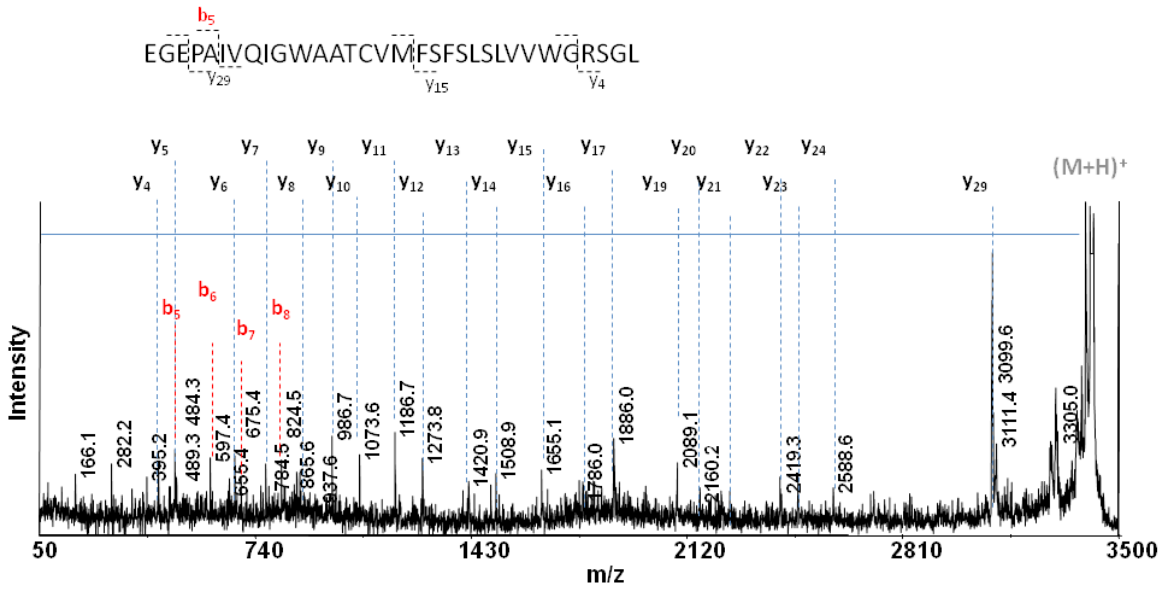

Figure S7. MALDI-TOF-TOF mass spectrum of the peptide at m/z 3410.7 observed for the b_6f complex trapped with A8-35. An almost complete series of fragments (y) found manually shows that the sequence is very similar to that expected for PetN with an extra glutamyl residue at the N-terminus. As shown on the MS/MS spectrum, both calculated series b and y perfectly fit the experimental data.

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