Published in final edited form as:

Mol Membr Biol. 2011 April ; 28(3): . doi:10.3109/09687688.2011.552440.

A class of mild surfactants that keep integral membrane proteins water-soluble for functional studies and crystallization

Jens Hovers^a, Meike Potschies^a, Ange Polidori^b, Bernard Pucci^b, Simon Raynal^b, Françoise Bonneté^c, Maria J. Serrano-Vega^d, Christopher G. Tate^d, Daniel Picot^e, Yves Pierre^e, Jean-Luc Popot^e, Rony Nehmé^f, Michel Bidet^f, Isabelle Mus-Veteau^f, Holger Bußkamp^g, Karl-Heinz Jung^g, Andreas Marx^g, Peter A. Timmins^h, and Wolfram Welte^{a,*}

^aFachbereich Biologie, Universität Konstanz, Universitätsstr. 10, 78457 Konstanz, Germany

^bLaboratoire de Chimie Bioorganique et des Systèmes Moléculaires Vectoriels, 33 rue L. Pasteur, Université d'Avignon, 84000 Avignon, France.

^cCINAM, CNRS-UPR 3118, Université de Luminy, 13288 Marseille Cedex 9, France

^dMRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK

^eLaboratoire de Physico-Chimie Moléculaire des Protéines Membranaires, UMR 7099, CNRS/ Université Paris-7, Institut de Biologie Physico-Chimique, 13, rue Pierre-et-Marie-Curie, F-75005 Paris, France.

^fUniversité de Nice-Sophia Antipolis, Institute of Developmental Biology and Cancer UMR-CNRS 6543, Parc Valrose, 06108 Nice Cedex 02, France

^gFachbereich Chemie, Universität Konstanz, Universitätsstr. 10, 78457 Konstanz, Germany

^hInstitut Laue-Langevin, BP156, 38042 Grenoble Cedex 9, France

Abstract

Mixed protein-surfactant micelles are used for *in vitro* studies and 3D crystallization when solutions of pure, monodisperse integral membrane proteins are required. However, many membrane proteins undergo inactivation when transferred from the biomembrane into micelles of conventional surfactants with alkyl chains as hydrophobic moieties.

Here we describe the development of surfactants with rigid, saturated or aromatic hydrocarbon groups as hydrophobic parts. Their stabilizing properties are demonstrated with three different integral membrane proteins. The temperature at which 50% of the binding sites for specific ligands are lost is used as a measure of stability and dodecyl- β -D-maltoside ("C12-b-M") as a reference for conventional surfactants. One surfactant increased the stability of two different G protein-coupled receptors by approximately 10°C compared to C12-b-M. Another surfactant yielded a stabilization of the human Patched protein receptor by 13°C.

In addition, one of the surfactants was successfully used to stabilize and crystallize the cytochrome *b₆f* complex from *Chlamydomonas reinhardtii*. The structure was solved to the same resolution as previously reported in C12-b-M.

Keywords

membrane protein solubilisation; surfactant; membrane protein crystallization

*Corresponding author: Tel: +49 7531 88 2206, Fax.: +49 7531 88 3183, wolfram.welte@uni-konstanz.de.

Declaration of Interest statement The authors report no declarations of interest.

Mixed protein-surfactant micelles are often used for *in vitro* studies when solutions of pure, monodisperse integral membrane proteins are needed (Garavito and Ferguson-Miller, 2001). Because integral membrane proteins evolved in a lipid bilayer environment, the exchange of lipid for surfactant frequently causes a destabilization of the protein. This can result in increased activity followed by increased rate of denaturation, increased susceptibility to protease attack, accelerated heat denaturation, oxidative damage, loss of activity regulation and lack of crystallization (Tanford and Reynolds, 1976; Tate, 2010).

At the beginning of membrane protein research only surfactants developed as detergents for consumer or industrial use were available many of which were inhomogenous. In-vitro studies of solubilized membrane proteins and structural work (Rosenbusch et al., 1981; Michel and Oesterhelt, 1980) led to an increasing demand in tailored surfactants. In the micellar solution the protein should be functional and stable for extended periods of time and the micellar belt attached to the protein should be small in order to leave much of the polar protein surfaces uncovered for crystal lattice contacts (Michel, 1983).

Studies of the micellar mass revealed the relation between the chemical structure of a surfactant and the size of its micelle. It was found to depend upon the ratio of the polar cross-section and the length of the extended hydrophobic chain (Israelachvili et al., 1976). In case of spherical micelles the latter matches the radius of the hydrophobic core. With alkyl chains as hydrophobic moieties of surfactants proteins require a length between an extended octyl and a dodecyl chain (Iwata, 2003).

Small micelles are formed by β -octylglucoside and lauryldimethylamineoxide (LDAO) with masses of 26 kDa (He et al., 2000) and 16 kDa (Timmins et al., 1988), respectively. Medium sized micelles are formed by dodecyl- β -maltoside with a mass of 66 kDa (Timmins et al., 1988).

Attention has recently turned to the eukaryotic membrane proteins that are often far less stable than their prokaryotic counterparts. Some of these had been studied very early because of important function and sufficient abundance in natural sources. One example is bovine rhodopsin, a G protein-coupled receptor (GPCR). The MetaI-MetaII equilibrium is a sensitive monitor for a native-like occupation of conformations. In rod disk membranes the equilibrium of dark-adapted bovine rhodopsin was on the Meta I side while in most surfactants it was shifted towards MetaII (Schleicher et al., 1987). Only one surfactant, the steroid-based digitonin, retained the native equilibrium (Szundi et al., 2005). Baldwin and Hubbell, 1985, suggested that shifts in the MetaI-MetaII equilibrium of rhodopsin in artificial lipid bilayers result from changes in the interfacial tension or excess surface free energy (ESFE). This suggested that surfactant micelles that maintain native-like conditions (henceforward referred to as “mild conditions”) require a certain surface tension at the interface between the hydrophobic moiety of the surfactant and the transmembrane surface of the protein. The interactions between the hydrophobic surface of the membrane protein and the hydrocarbon groups of lipids or surfactants will be dominated by van der Waals contacts. Consequently it was assumed (Schleicher et al., 1987) that the ratio of the densities of van der Waals contacts which the hydrophobic moiety of a lipid or surfactant experiences in the bulk of the micelle and at the surface with the protein, respectively, determines its ESFE. For example ratios near one lead to a low value and to a destabilizing surfactant while higher ratios lead to a high value and a mild surfactant.

It was further assumed that this ratio differs for a protein in a lipid bilayer and in a micelle assuming that both contain alkyl chains as hydrophobic moieties. In a bilayer in liquid crystalline phase, the alkyl chains assemble by more-or-less parallel packing with a restricted set of conformations distributed around the all-trans conformation (Senak et al.,

1991), allowing for a high number of van der Waals contacts in the bulk of the bilayer. Alkyl chains adjacent to the irregularly shaped membrane embedded protein surface will thus feature an ESFE because they experience a smaller density of van der Waals contacts than chains in the bulk and/or because they adopt a specific subset of conformations complementary to the protein surface which raises their entropy. According to Marsh and Horvath, 1998, the second possibility is unlikely.

In a micelle, however, the alkyl chains must adopt a broad distribution of conformations to pack into a spheroidal or ellipsoidal aggregate (Szeleifer and Ben-Shaul, 1985; Holler and Callis, 1989). The number of van der Waals contacts in the bulk thus will be smaller and the difference in density of van der Waals contacts of bulk alkyl chains compared to interface alkyl chains will also be smaller. The surface tension experienced by the protein thus will be lower than in the bilayer. A way to increase the ESFE in a micelle therefore should be to use surfactants with less flexible hydrophobic moieties, so that their ability to form van der Waals contacts in the bulk is increased and with the protein surface decreased. This could explain the “mildness” of digitonin. To test this concept, a compound was synthesized, which contained a hydrophobic allyloxybiphenyl group esterified with an octaoxyethylene headgroup (Schleicher et al., 1987). It was expected that the rigid phenyl ring systems should increase the ESFE but still possess sufficient flexibility to form smaller micelles than digitonin. The results supported the hypothesis.

However, the solubilizing efficiency of the biphenyl compound was low and did not retain the functionality of all proteins tested. The electrostatic field of the π -electron system in the hydrophobic part of the membrane may act to destabilize the arrangement of the adjacent helical bundle of the protein. Interactions with protein aromatic networks (Haeger et al., 2010) or edge-to-face interactions (Burley and Petsko, 1986) between surfactant molecules may be the cause. Therefore, in the work described here we synthesized a series of surfactants by exchanging one or both aromatics by cyclohexyl groups.

The allyloxybiphenyl group was changed to an alkoxyphenylcyclohexyl group or an alkylbicyclohexyl group. The alkyl was either a butyl or a propyl group. The polar headgroup was changed to a maltosyl because of stabilising effects (Xie and Timasheff, 1997) and because of its compactness compared to ethyleneoxide groups.

These maltoside surfactants were made in purein both anomeric forms because of known differences in solubility and surfactant action between the anomers (Brown et al., 1970; Focher et al., 1989; Hunte et al., 2005). Finally, to exclude oxygen from the hydrophobic moiety which was suspected to render the hydrophobic part more aggressive due to its electronegativity, we synthesized propylphenylcyclohexylmaltoside and its butyl variant.

Methods

The Supplementary information includes detailed descriptions of the following:

1. Synthesis of the novel surfactants PoPC-a-M, BoPC-a-M, PPC-b-M, BPC-b-M, PCC-a-M, BCC-a-M
2. Determination of micellar mass by SANS and SAXS.
3. Temperature stability of turkey β_1 -adrenergic receptor β_1AR_{34-324} .
4. Expression, purification and temperature stability of hSmo and hPtc.
5. Purification and structure analysis of cytochrome b_6f .
6. Figures S1 – S3.

Thermostability of β_1 AR₃₄₋₃₂₄

In brief, the truncated wild-type turkey β_1 receptor (β_1 AR₃₄₋₃₂₄) was functionally expressed in *E. coli* with a maltose binding protein fused to the N-terminus and a His-tag at the C-terminus. β_1 AR₃₄₋₃₂₄ was solubilised and purified from membranes with C12-b-M. Aliquots were used to exchange the detergent for PCC-a-M, BPC-b-M, PPC-b-M and C12-b-M. The solubilised protein was incubated at the specified temperature for 30 min, quenching the reaction on ice, and 3 H-dihydroalprenolol (3 H-DHA) added to determine the amount of binding by the receptor. Receptor-bound and free radioligand were separated by gel filtration. The temperature was determined at which half of the binding was lost (“T₅₀”).

Thermostability of hSmo and hPtc

In brief, hSmo and hPtc carrying a multitag affinity purification sequence (MAP) at their C-terminal part were solubilized from membrane fractions expressing hSmo-MAP or hPtc-MAP with C12-b-M and loaded on a calmodulin column. Surfactant exchange was achieved by washing with buffers supplemented with either PCC-a-M, PPC-b-M, BPC-b-M or C12-b-M and elution. SPR sensorgrams were measured using a sensor chip with ligand-coated flow cell, CPN for Smo or N-SHh for Ptc, and control, unliganded flow cell as described (Nehmé et al., 2010; Joubert et al., 2009). Prior to measurement, receptors were incubated for 30 minutes at temperatures ranging from 4 to 70°C. The percentage of remaining activity with respect to its own unheated control was determined. The inflection point of the sensorgram signals, which was assumed to be equivalent to T₅₀, was determined by fitting with a Boltzmann sigmoidal equation.

Crystal structure of cytochrome *b₆ f* complex

In brief, cytochrome *b₆ f* was solubilised and purified in C12-b-M. The surfactant was exchanged for 0.2 mM PCC-a-M by Ni-NTA chromatography. All subsequent steps including crystallization were done in 0.2 mM PCC-a-M. Crystals isomorphic to those in C12-b-M were obtained. Diffraction data to 2.8 Å were collected at the synchrotron SOLEIL (St Aubain, France). A model containing 8 PCC-a-M molecules was built and refined. Plastoquinol plasto cyanine oxidoreductase activity was measured as described (Pierre et al., 1995).

Results

Structures of the surfactants and their physicochemical data

Members of three surfactant families were synthesized :

1. 4-(4'-alkoxyphenyl)-*trans*-cyclohexyl- α -D-maltoside family : Propoxy (“PoPC-a-M”) and Butoxy (“BoPC-a-M”).
2. *trans*-4-(4'-alkylphenyl)cyclohexyl- β -D-maltoside family: Propyl (“PPC-b-M”) and Butyl (“BPC-b-M”).
3. *trans*-4-(*trans*-4'-alkylcyclohexyl)cyclohexyl- α -D-maltoside family: Propyl (“PCC-a-M”) and Butyl (“BCC-a-M”).

For details see Supplementary information. Structures are shown in Fig. 1. Critical micelle concentrations are given in Tab. 1.

Physicochemical data—Assuming a fully extended all-*trans* conformation, the radius of the hydrophobic core of micelles from the widely-used n-dodecyl- β -D-maltoside (C12-b-M) would be 13.7 Å (1.25 Å per C-C bond). For PCC-a-M, using 7.46 Å for the length of an all-*trans* bicyclohexyl group (Walz et al., 1989) and 3.76 Å for an all-*trans* propyl moiety, a

maximal radius of 11.2 Å results, which resembles the length of an extended decyl chain. With regard to the critical micelle concentration (cmc), C12-b-M (0.17 mM) is more polar than PCC-a-M (0.036 mM) as expected from the larger number of hydrocarbon groups in the hydrophobic moiety of the latter.

The exchange of an aromatic group for a cyclohexyl group in PoPC-a-M and PPC-b-M increases the cmc to 0.41 mM and 1.11 mM, respectively, indicating the relative polarity of an unsaturated as compared to a saturated 6-membered hydrocarbon ring. The cmc-s decrease slightly between room temperature and 50°C (see Table 1).

The micellar mass was determined by Small angle neutron scattering (SANS) for PoPC-a-M and BCC-a-M and by Small angle X ray scattering (SAXS) for PCC-a-M. The micellar radius was determined by SANS for PoPC-a-M and BCC-a-M and by dynamic light scattering for PPC-b-M and BPC-b-M (Table 1 and Supplementary information). The surfactants C12-b-M and PCC-a-M form micelles of similar mass (66 kDa (Timmins et al., 1988) and 63 kDa, respectively). BCC-a-M forms larger micelles (85 kDa), while those of PoPC-a-M are smaller (36 kDa). The cone-shaped phenyl-cyclohexyl group of PoPC-a-M likely facilitates packing into a smaller micelle whereas the brick-shaped bicyclohexyl group promotes a micelle of smaller curvature, similar to the micelle of C12-b-M.

Thermostability of a β_1 -adrenergic receptor in the surfactants—The surfactants were tested on a truncated version of the turkey β_1 adrenergic receptor (β_1 AR₃₄₋₃₂₄) a GPCR, which also contains a C-terminal His₆ tag (Serrano-Vega et al., 2008; Warne et al., 2009; Warne et al., 2003). Functional β_1 AR₃₄₋₃₂₄ was expressed in *E. coli*. The receptors were solubilised with C12-b-M, bound to NiNTA beads and a chromatographic procedure was performed to exchange C12-b-M for PCC-a-M, BPC-b-M, PPC-b-M as described in Supplementary information. An T₅₀ of 40°C for β_1 AR₃₄₋₃₂₄ in PCC-a-M indicated that the surfactant stabilized the receptor compared to C12-b-M, for which a T₅₀ of 30°C was observed (Fig. 2 and Table 2). In contrast, β_1 AR₃₄₋₃₂₄ in either PPC-b-M or BPC-b-M was less stable than in C12-b-M, with apparent T₅₀-s of 21°C and 25°C, respectively. Thus PCC-a-M significantly stabilizes β_1 AR₃₄₋₃₂₄ in comparison to C12-b-M.

Thermostability of Patched and Smoothened solubilized in the novel surfactants—The Sonic Hedgehog (SHh) signalling pathway involves two membrane receptors Patched (Ptc), a twelve transmembrane domain protein receptor of the SHh protein, and Smoothened (Smo), a GPCR responsible for the transduction of the signal (Varjosalo and Taipale, 2008). For *in vitro* studies, human Ptc and human Smo fused with a multi-affinity tag (MAP) at their C-terminus have been expressed at the plasma membrane of the yeast *Saccharomyces cerevisiae* and purified in C12-b-M in a native and stable conformation, as assessed by ligand binding using Surface Plasmon Resonance (SPR) studies (Nehmé et al., 2010; Joubert et al., 2009) (Supplementary information).

C12-b-M was exchanged for each of the surfactants (Supplementary information) and as a control for C12-b-M, i.e. 2 mM each of C12-b-M, PCC-a-M, PPC-b-M and BPC-b-M. Western blots of 8% SDS PAGE gels loaded with hPtc and hSmo purified in the four surfactants and probed with anti-hemagglutinin (HA) antibodies directed against the HA tag of the MAP at their C-terminus showed bands at apparent molecular weights of approximately 180 kDa and 90 kDa corresponding to hPtc and hSmo, respectively, (Fig. S1), suggesting that the surfactants maintained both receptors in a soluble state similar as in C12-b-M.

The ability of purified hSmo and hPtc to bind their ligands was tested using SPR. Purified hSmo and hPtc were injected in both the reference flow cell (Fc1) and the CPN-(for hSmo)

or Sonic Hedgehog-(for hPtc) coupled flow cell (Fc2). See Supplementary information for details. The difference between the sensorgrams recorded on Fc2 and Fc1 gave signals varying between 150 RU and 700 RU for the two proteins in each of the four surfactants, indicating that hSmo and hPtc were able to interact with their respective ligand after purification in each surfactant. The observed variations in the SPR response could be due to variations in the protein amount injected but also due to intrinsic properties like the variations in the refractive index of the surfactants.

For thermostability experiments, hSmo samples and hPtc samples were incubated for 30 minutes at increasing temperatures prior to SPR analysis as described above and under Supplementary information. The percentage of remaining native hSmo was determined by comparing the amount of binding (in RU) after heating with its unheated control. T_{50} was calculated and reported (see Materials and Methods and Supplementary information). hSmo (Figure 3a) exhibited comparable thermostability in BPC-b-M and in C12-b-M ($T_{50} = 39.8 \pm 3$ °C, 39.4 ± 0.9 °C respectively, Table 2), and lower thermostability in PPC-b-M ($T_{50} = 31 \pm 2.7$ °C, Table 2), whereas hSmo thermostability was enhanced by about 12 °C in PCC-a-M ($T_{50} = 52.1 \pm 0.7$ °C, Table 2). These results indicated that PCC-a-M strongly stabilized the GPCR hSmo in solution.

hPtc (Figure 3b) showed an apparent T_{50} in C12-b-M of about 23 °C (Table 2). It was enhanced by about 3 °C, 9 °C and 13 °C in BPC-b-M ($T_{50m} = 26.4 \pm 0.1$ °C), PCC-a-M ($T_{50} = 32 \pm 1.5$ °C) and PPC-b-M ($T_{50} = 36.4 \pm 1.7$ °C) respectively (Table 2). These results indicated that hPtc was more stable in these surfactants than in C12-b-M, PPC-b-M being the best surfactant for hPtc.

Enzymatic activity and structural analysis of cytochrome b_6f complex from *Chlamydomonas reinhardtii*—The cytochrome b_6f complex was purified with C12-b-M, as described by Stroebel et al., 2003, except that the detergent was exchanged for PCC-a-M in the last chromatographic step on NiNTA (Supplementary information). This exchange of 0.2 mM C12-b-M for 0.2 mM PCC-a-M during the purification of the protein did not affect the biochemical properties of the cytochrome b_6f complex. The oxidoreductase activity of the complex (Pierre et al., 1995) and its stability over extended storage at 4 °C were similar to those observed in 0.2 mM C12-b-M (Fig 4) i.e. at a concentration slightly above the detergent's cmc. However, while the b_6f complex inactivates rapidly in solution of C12-b-M when the concentration is raised above the cmc (Breyton et al., 1997), it is only marginally affected in PCC-a-M even at 5mM concentration (Fig 4).

Crystallization in presence of PCC-a-M occurred within a few days, under similar conditions as in C12-b-M. The crystals obtained were isomorphous and with a diffraction quality similar to the best crystals obtained in C12-b-M. The structure was solved by difference Fourier techniques (see Supplementary information). As expected, the overall fold of the complex is almost identical to that in C12-b-M (Stroebel et al., 2003) (Fig 5), albeit with a weaker electron density for the mobile domain of the Rieske protein. The latter is due to a lower occupancy of the Q_0 site by the inhibitor tridecylstigmatellin which is probably not related to the use of PCC-a-M, but to the handling and batches of the inhibitor.

Major differences in the electron density maps obtained in the presence of PCC-a-M vs. C12-b-M are located at the protein-lipid interface. They were attributed to PCC-a-M molecules according to the following criteria: (1) Positive densities in the difference Fourier map between the two sets of data, and (2) thicker densities as expected for cyclohexyl groups, which are bulkier than the alkyl chains of lipids or of C12-b-M. While these criteria allowed the identification of eight PCC-a-M molecules, only two of them show well defined densities for maltosyl groups. Seven PCC-a-M molecules are facing the stroma

(supplementary figure S2a) and only one the lumen (supplementary figure S2b). On the stromal side, PCC-a-M molecules are located near or in the large cleft that lies between the two monomers. They can be divided into two sets: The first one contains five PCC-a-M molecules interacting mainly with the amphiphilic helix α (Stroebel et al., 2003; Zhang, 1998) of cytochrome b_6 , which crosses the cleft and connects the two monomers. Two of these molecules have well defined headgroups and substitute for two C12-b-M molecules. One of them exhibits only minor positional and conformational changes, while the other exhibits a drastic reorientation of its headgroup as compared to C12-b-M putatively due to the α -glycosidic linkage. The three remaining molecules of this set have less well defined electron densities for the maltosyl group. In the second set, two molecules are further away from the cleft and interact with the transmembrane segments of the Rieske protein. The single PCC-a-M molecule visible on the luminal face of the membrane interacts with the C-terminal end of Subunit 4 and with a lipid molecule.

Discussion

The surfactants stabilize membrane proteins against temperature-induced denaturation

For structural work on labile membrane proteins, improving thermostability has been shown to be advantageous (Warne et al., 2008). Detergent-solubilized membrane proteins are more thermolabile than their membrane-embedded forms. For the turkey β_1 -adrenergic receptor in C12-b-M, the apparent T_{50} is 30 °C while in other surfactants like LDAO or β -octylglucoside, which are more suitable for crystallizing small membrane proteins, T_{50} values could not be determined, because the receptor was inactivated immediately upon solubilisation (Warne et al., 2009). In membranes from birds or mammals, one would expect for labile proteins a minimal T_{50} of 40°C. The low T_{50} values observed in micellar solution thus are in accord with the general experience that many membrane proteins are destabilized upon detergent solubilization. One approach to solve this problem is to stabilize the membrane protein by suitable mutations. For example, in the turkey β_1 -adrenergic receptor, a combination of six point mutations increased the T_{50} by 21°C compared to the wild-type receptor, which allowed its crystallization and structure determination to 2.7 Å resolution (Warne et al., 2008).

Another approach, as adopted here, is to search for milder surfactants. We hypothesized that conformationally restricted groups in the hydrophobic part lead to mild surfactants. Combinations of cyclohexyl rings and aromatic rings were tried. The cone-like shape of a phenyl-cyclohexyl group as in PoPC-a-M, PPC-b-M and BPC-b-M was expected to increase the curvature and thereby to reduce the size of the micelles, while the brick-like shape of the cyclohexyl-cyclohexyl group was expected to act in the opposite way. This was indeed observed by SANS and SAXS (Table 1). Because conformationally restricted groups cannot be well-packed into a spheroidal micelle core, propyl or butyl groups were attached to the end of the hydrophobic moiety, resulting in an overall hydrophobic length comparable to that of C12-b-M.

Conventional “alkylic” surfactants are not only used as a micellar solvent for proteins, but also as detergents, i.e. to solubilize biomembranes. It is therefore noteworthy that the semi-rigid surfactants studied here and in (Schleicher et al., 1987) in our hands did not dissolve membranes, even at high concentrations. A detergent has to be used for initial solubilization and then exchanged for one of the surfactants by affinity chromatography.

In order to assess the properties of the surfactants and to compare them with the widely used detergent C12-b-M, thermostability measurements and crystallization experiments were undertaken. Two GPCRs, turkey β_1 -adrenergic receptor and hSmo, were both thermostabilized in PCC-a-M by about 12°C compared to C12-b-M (Table 2, Fig. 2, Figure

3a). In PPC-b-M and BPC-b-M, however, the GPCRs show the same or lower T_{50} values than in C12-b-M. However, the latter compounds stabilize the hPtc receptor (Table 2, Figure 3b), with an apparent T_{50} of 36° C in PPC-b-M compared to 23°C in C12-b-M. This seems to indicate that aromatic π -electron systems stabilize some but destabilize other proteins. Destabilization is conceivable if there is an aromatic network in the transmembrane region (Haeger et al., 2010). Edge-to-face aromatic-aromatic interactions (Burley and Petsko, 1986) could then affect the native structure of the protein. This weak electrostatic interaction of detergent molecules may in other cases be beneficial because it generates a lateral interaction between surfactant molecules which should contribute to the ESFE.

The surfactants achieve temperature stabilizations of the β_1 -adrenergic receptor by about 10°C, which is about half of the values reported for a six-residue mutant used for structure analysis (Serrano-Vega et al., 2008; Warne et al., 2008; Shibata et al., 2009). The design concept thus can be expected to lead to compounds which are instrumental for crystallizing labile membrane proteins.

PCC-a-M was therefore tested for crystallization of the the b_6f complex from *C. reinhardtii*. This complex is quite unstable in conventional surfactants which induce dissociation of the dimeric complex, associated with loss of the Rieske protein (Breyton et al., 1997). The structure explains this observation by the swapping of the soluble part of the flexible Rieske protein between the two monomers and by the presence of a large hydrophobic cleft separating the two monomers. This cleft is important for the catalytic mechanism of the complex since it permits plastoquinone exchange between the Q_o and Q_i sites located on opposite sides of the membrane. *In situ*, the cleft has to be filled with lipids of sufficient flexibility to avoid impairment of this exchange. The localization of several well packed surfactant molecules in the cleft and near the transmembrane helix of the Rieske protein contributes to explaining how detergents may disrupt the complex, as observed with C12-b-M at concentrations above the cmc. To overcome this problem, several approaches have been used in the past: working at concentrations near the cmc and rapid addition of lipids (Pierre et al., 1995) to counterbalance the effect of detergent (Zhang et al., 2003). The mildness of PCC-a-M made it possible to work under similar conditions as with C12-b-M, but without the burden of an extremely high sensitivity to changes in surfactant concentration, a crucial issue for the crystallization of membrane proteins.

Packing differences between hydrophobic micellar and bilayer cores which are relevant to the design of the surfactants

According to our hypothesis, alkyl chains interact less strongly in micelles than in lipid bilayers. This is supported by experiments comparing enthalpies of alkyl chain methylene groups in micelles and bilayers. Heerklotz and Eppand, 2001, compared enthalpies of melting of hydrocarbon chains in bulk oil and in lipid bilayers which had been determined by Philipps et al., 1969. For the transition of a methylene group from a liquid crystalline lipid bilayer into a molten bulk hydrocarbon phase a $\Delta\Delta H$ value of approximately +2 kJ/mol per methylene group was found. This value suggests stronger mutual interactions in the bilayer as compared to bulk oil, putatively caused by partial alignment of the alkyl chains in the former.

In a micelle, the order of the hydrocarbon chains formed by an alkylic surfactant will differ from both of these (Dill and Flory, 1981; Menger and Doll, 1984) but should resemble more the bulk oil than the bilayer (Tanford, 1974).

This supports our concept that hydrocarbons in an alkylic micelle core mediate a lower ESFE at the micelle-protein interface than a liquid crystalline bilayer and is in accord with ESFE estimations by Marsh, 2007.

Membrane proteins may also be adapted to the increasing order of methylene groups towards the polar headgroup in lipids (Seelig and Seelig, 1974; Petrache et al., 2000), which is possibly opposite in micelles (Néry et al., 1986). Preferential rigidity of a surfactant in the apolar part adjacent to its polar headgroup thus could also contribute to mildness.

The concept to use rigid groups to enhance ESFE in other surfactants

The reduced chain order and increased enthalpy in micelles as compared to bilayers suggests that alkylic detergents have a lower ESFE than bilayer lipids. Rigid groups in the hydrophobic moiety of surfactants may lead to an increase in ESFE by a combination of improved lateral packing in the bulk of the micelle core and reduced ability to adopt conformations complementary to the protein or protein-lipid surface (supplementary figure 3).

Another recently reported family of mild surfactants, hemifluorinated surfactants (HF), may be based on a similar principle. Perfluoroalkyl chains are both bulkier and more rigid than hydrocarbon chains so that gauche conformations are more restricted. Moreover they are more hydrophobic than alkyl chains and not miscible with the latter (Krafft and Riess, 1998). Due to the gauche restrictions, amphiphiles with a perfluoroalkyl chain form rather large micelles (Shepherd and Holzenburg, 1995). This led to the design of HF surfactants (Breyton et al., 2010; Popot, 2010) which is reminiscent of the design explored here. The cytochrome *b₆f* complex and hSmo show excellent temporal stability in some HF surfactants (Breyton et al., 2010; Nehmé et al., 2010). This suggests that the rigidity and the low polarizability of the perfluoroalkyl group also enhances the ESFE at the hydrophobic protein surface.

Conclusions

Our work suggests that surfactants with an increased ESFE may help to retain functionality of labile eukaryotic integral membrane proteins in micellar solution. According to our hypothesis, rigid groups in the hydrophobic moieties should increase van der Waals interactions of surfactants in the bulk of the micelle and decrease interactions with the hydrophobic surface of membrane proteins, increasing the ESFE. In addition, weak interactions between the hydrophobic moieties of a surfactant as for instance aromatic edge-to-face interactions, can also be expected to increase ESFE.

Furthermore, such compounds can be expected to show a reduced activity to displace tightly bound lipids from the protein surface, further contributing to a mild micellar environment. The general concept of rigidityESFE concept proved of value here, offers many opportunities to devise mild surfactants which may be instrumental for in-vitro functional, as well as crystallographic studies of labile membrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Dr. Eike Poetsch and Dr. Alfred Blume helped at various stages of the work with stimulating discussions. RN wasMost of us were supported by the European Community Specific Targeted Research Project grant "Innovative Tools for Membrane Protein Structural Proteomics" (STREP-IMPS). Part of this work was supported by the foundation France Cancer, the Conseil Général des Alpes Maritimes.

We thank David Pauron and the UMR Interactions Biotiques et Santé Végétale (INRA/CNRS/Université de Nice Sophia Antipolis, France) for the access to the Biacore 3000, and Marielle Bauzan (Unité de fermentation IMM/

IFR88 CNRS Marseille, France) for fermentor cultures. We are grateful to the synchrotron SOLEIL for providing beamtime and to Pierre Legrand for assistance in data collection on the beamline Proxima I. We also acknowledge the ESRF for provision of synchrotron radiation facilities and we would like to thank Petra Pernot and Adma Round for assistance in using beamline ID14-eh3.

We gratefully acknowledge the donation of some resynthesized PCC-a-M resynthesized by Dr. C. Kolar, Glycon GmbH, Luckenwalde, Germany, for late control experiments.

Abbreviations

GPCR	G Protein-Coupled Receptor
cmc	critical micelle concentration
hSmo	Smoothed
MAP	Multitag Affinity Purification
HA	hemagglutinin
C12-b-M	dodecyl- β -D-maltoside
DHA	[3 H]dihydroalprenolol
SPR	surface plasmon resonance
RU	resonance units
SHh	Sonic Hedgehog
Ptc	Patched
ESFE	excess surface free energy
CPN	cyclopamine
HF	hemifluorinated surfactants
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
LDAO	lauryldimethylamineoxide
PoPC-a-M	4-(4'-propoxyphenyl)- <i>trans</i> -cyclohexyl- α -D-maltoside
PCC-a-M	<i>trans</i> -4-(<i>trans</i> -4'-propylcyclohexyl)cyclohexyl- α -D-maltoside
PPC-b-M	<i>trans</i> -4-(4'-propylphenyl)cyclohexyl- β -D-maltoside
BPC-b-M	<i>trans</i> -4-(4'-butylphenyl)cyclohexyl- β -D-maltoside

References

- Baldwin PA, Hubbell WL. Effects of Lipid Environment on the Light-Induced Conformational Changes of Rhodopsin. 1. Absence of Metarhodopsin II Production in Dimyristoylphosphatidylcholine Recombinant Membranes. *Biochemistry*. 1985; 24:2624–2632. [PubMed: 4027217]
- Breyton C, Tribet C, Olive J, Dubacq JP, Popot JL. Dimer to monomer conversion of the cytochrome *b₆f* complex. Causes and consequences. *J Biol Chem*. 1997; 272:21892–21900. [PubMed: 9268322]
- Breyton, C.; Pucci, B.; Popot, J-L. Amphipols and fluorinated surfactants: two alternatives to surfactants for studying membrane proteins *in vitro*. In: Mus-Veteau, I., editor. *Heterologous expression of membrane proteins: Methods and protocols*. Vol. 601. The Humana Press; Totowa, NJ: 2010. p. 219-245.

- Brown GM, Dubreuil FM, Ichhaporla FM, Desnoyers JE. Synthesis and properties of some a-D-alkyl glucosides and mannosides: apparent molal volumes and solubilization of nitrobenzene in water at 25° C. *Can J Chem*. 1970; 48:2525–2531.
- Burley SK, Petsko GA. Dimerization Energetics of Benzene and Aromatic Amino Acid Side Chains. *J Am Chem Soc*. 1986; 108:7995–8001.
- Dill KA, Flory PJ. Molecular organization in micelles and vesicles. *Proc Natl Acad Sci USA*. 1981; 78:676–680. [PubMed: 16592970]
- Focher B, et al. Micelles of 1-Alkyl Glucoside and Maltoside: Anomeric Effects on Structure and Induced Chirality. *Chem Phys Lett*. 1989; 158:491–494.
- Garavito RM, Ferguson-Miller S. Surfactants as Tools in Membrane Biochemistry. *J Biol Chem*. 2001; 276:32403–32406. [PubMed: 11432878]
- Haeger S, et al. An intramembrane aromatic network determines pentameric assembly of Cys-loop receptors. *Nat Struc Biol*. 2010; 70:90–99.
- He LZ, Garamus V, Niermeyer B, Helmholz H, Willumeit R. Determination of micelle structure of octyl- β -glucoside in aqueous solution by small angle neutron scattering and geometric analysis. *J Mol Liquids*. 2000; 89:239–249.
- Heerklotz H, Epand RM. The Enthalpy of Acyl Chain Packing and the Apparent Water-Accessible Apolar Surface Area of Phospholipids. *Biophys J*. 2001; 80:271–279. [PubMed: 11159400]
- Holler F, Callis JB. Conformation of the Hydrocarbon Chains of Sodium Dodecyl Sulfate Molecules in Micelles: An FTIR Study. *J Phys Chem*. 1989; 93:2053–2058.
- Hunte C, et al. Structure of a Na⁺/H⁺ antiporter and insights into mechanism of action and regulation by pH. *Nature*. 2005; 435:1197–1202. [PubMed: 15988517]
- Israelachvili JN, Mitchell DJ, Ninham BW. Theory of Self-Assembly of Hydrocarbon Amphiphiles into Micelles and Bilayers. *J Chem Soc Faraday Transactions II*. 1976; 72:1525–1568.
- Iwata, S. *Methods and Results in Crystallization of Membrane Proteins*. Int. University Line; LaJolla, Ca: 2003.
- Joubert O, Nehmé R, Fleury D, De Rivoyre M, Bidet M, Polidori A, Ruat M, Pucci B, Mollat P, Mus-Veteau I. Functional studies of membrane-bound and purified human Hedgehog receptor Patched expressed in yeast. *Biochim Biophys Acta*. 2009; 1788:1813–1821. [PubMed: 19463780]
- Krafft MP, Riess JG. Highly fluorinated amphiphiles and colloidal systems, and their applications in the biomedical field. A contribution. *Biochimie*. 1998; 80:489–514. [PubMed: 9782389]
- Marsh D, Horvath LI. Structure, dynamics and composition of the lipid-protein interface. Perspectives from spin-labelling. *Biochim Biophys Acta*. 1998; 1376:267–296. [PubMed: 9804973]
- Marsh D. Lateral Pressure Profile, Spontaneous Curvature Frustration, and the Incorporation and Conformation of Proteins in Membranes. *Biophys J*. 2007; 93:3884–3899. [PubMed: 17704167]
- Menger FM, Doll DWJ. On the Structure of Micelles. *J Am Chem Soc*. 1984; 106:1109–1113.
- Michel H, Oesterhelt D. Three-dimensional crystals of membrane proteins: Bacteriorhodopsin. *Proc Natl Acad Sci USA*. 1980; 77:1283–1285. [PubMed: 6929485]
- Michel H. Crystallization of membrane proteins. *TIBS*. 1983; 8:56–59.
- Nehmé R, Joubert O, Bidet M, Lacombe B, Polidori A, Pucci B, Mus-Veteau I. Stability Study of the Human G-Protein Coupled Receptor. Smoothened *Biochim Biophys Acta*. 2010; 1798:1100–1110.
- Néry H, Söderman O, Canet D, Walderhaug H, Lindman B. Surfactant Dynamics in Spherical and Nonspherical Micelles. A Nuclear Magnetic Resonance Study. *J Phys Chem*. 1986; 90:5802–5808.
- Petrache HI, Dodd SW, Brown MF. Area per Lipid and Acyl Length Distributions in Fluid Phosphatidylcholines Determined by ²H NMR Spectroscopy. *Biophys J*. 2000; 79:3172–3192. [PubMed: 11106622]
- Phillips MC, Williams RM, Champan D. On the nature of hydrocarbon chain motions in liquid crystals. *Chem Phys Lip*. 1969; 3:234–244.
- Pierre Y, Breyton C, Kramer D, Popot JL. Purification and characterization of the cytochrome *b₆f* complex from *Chlamydomonas reinhardtii*. *J Biol Chem*. 1995; 270:29342–29349. [PubMed: 7493968]

- Popot J-L. Amphipols, nanodiscs, and fluorinated surfactants: three non-conventional approaches to studying membrane proteins in aqueous solutions. *Ann Rev Biochem.* 2010; 79:737–775. [PubMed: 20307193]
- Rosenbusch, JP.; Garavito, RM.; Dorset, DL.; Engel, A. Structure and Function of a pore-forming transmembrane protein: High resolution studies of a bacterial porin. In: Peeters, H., editor. *Protides of the biological fluids*. Pergamon Press; Oxford: 1981. p. 171-174.
- Schleicher A, Hofmann KP, Finkelmann H, Welte W. Deoxylysolecithin and a New Biphenyl Surfactant as Solubilizing Agents for Bovine Rhodopsin. Functional Test by Formation of Metarhodopsin II and Binding of G-Protein. *Biochemistry.* 1987; 26:5908–5916. [PubMed: 3118952]
- Seelig A, Seelig J. The Dynamic Structure of Fatty Acyl Chains in a Phospholipid Bilayer Measured by Deuterium Magnetic Resonance. *Biochem.* 1974; 13:4839–4845. [PubMed: 4371820]
- Senak L, Davies MA, Mendelsohn R. A Quantitative IR Study of Hydrocarbon Chain Conformation in Alkanes and Phospholipids: CH₂ Wagging modes in Disordered Bilayer and HII Phases. *J Phys Chem.* 1991; 95:2565–2571.
- Serrano-Vega MJ, Magnani F, Shibata S, Tate CG. Conformational thermostabilisation of the β -adrenergic receptor in a surfactant-resistant form. *Proc Natl Acad Sci USA.* 2008; 105:877–882. [PubMed: 18192400]
- Shepherd FH, Holzenburg A. The Potential of Fluorinated Surfactants in membrane Biochemistry. *Anal Biochem.* 1995; 224:21–27. [PubMed: 7710073]
- Shibata Y, et al. Thermostabilization of the Neurotensin Receptor NTS1. *J Mol Biol.* 2009; 390:262–277. [PubMed: 19422831]
- Stroebel D, Choquet Y, Popot J-L, Picot D. An atypical heme in the cytochrome *b₆f* complex. *Nature.* 2003; 426:413–418. [PubMed: 14647374]
- Szleifer I, Ben-Shaul A. Chain organization and thermodynamics in micelles and bilayers. II Model calculations. *J Chem Phys.* 1985; 83:3612–3620.
- Szundi I, Lewis JW, Kliger DS. Effect of Digitonin on the Rhodopsin Meta I–Meta II Equilibrium. *Photochem Photobiol.* 2005; 81:866–873. [PubMed: 15819603]
- Tanford C. Theory of Micelle Formation in Aqueous Solutions. *J Phys Chem.* 1974; 78:2469–2479.
- Tanford C, Reynolds JA. Characterization of Membrane Proteins in Surfactant Solutions. *Biochim Biophys Acta.* 1976; 457:133–170.
- Tate, CG. Practical Considerations of Membrane Protein Instability during Purification and Crystallization. In: Mus-Veteau, I., editor. *Heterologous Expression of Membrane Proteins: Methods and Protocols*. Vol. 601. Humana Press; Totowa, NJ: 2010. p. 187-203. *Methods in Molecular Biology*
- Timmins PA, Leonhard M, Weltzien HU, Wacker T, Welte W. A physical characterization of some surfactants of potential use for membrane protein crystallization. *FEBS Lett.* 1988; 238:361–368.
- Varjosalo M, Taipale J. Hedgehog: functions and mechanisms. *Genes Dev.* 2008; 22:2454–2472. [PubMed: 18794343]
- Walz L, Haase W, Eidenschink R. The crystal and molecular structures of Four homologous, mesogenic trans,trans-4,4'-dialkyl-(1 α ,1' -bicyclohexyl)-4B-carbonitril (CCN's). *Mol Cryst Liq Cryst.* 1989; 168:169–182.
- Warne T, Chirnside J, Schertler GF. Expression and purification of truncated, nonglycosylated turkey beta-adrenergic receptors for crystallization. *Biochim Biophys Acta.* 2003; 1610:133–140. [PubMed: 12586387]
- Warne T, et al. Structure of a β_1 -adrenergic G-protein coupled Receptor. *Nature.* 2008; 454:486–491. [PubMed: 18594507]
- Warne T, Serrano-Vega MJ, Tate CG, Schertler GFX. Development and crystallization of a minimal thermostabilised G protein coupled receptor. *Prot Exp Purif.* 2009; 65:204–213.
- Xie G, Timasheff SN. The thermodynamic mechanism of protein stabilization by trehalose. *Biophys Chem.* 1997; 64:25–43. [PubMed: 9127936]
- Zhang Z, et al. Electron transfer by domain movement in cytochrome *b c₁*. *Nature.* 1998; 392:677–684. [PubMed: 9565029]

Zhang H, Kurisu G, Smith JL, Cramer WA. A defined protein-surfactant-lipid complex for crystallization of integral membrane proteins: The cytochrome *b₆f* complex of oxygenic photosynthesis. *Proc Natl Acad Sci USA*. 2003; 100:5160–5163. [PubMed: 12702760]

Fig. 1. Representatives of the three surfactant families with rigid ring systems tested in this work. 4-(4'-propoxyphenyl)-*trans*-cyclohexyl- α -D-maltoside (PoPC-a-M), (1); *trans*-4-(4'-propylphenyl)cyclohexyl- β -D-maltoside (PPC-b-M), (2); *trans*-4-(*trans*-4'-propylcyclohexyl)cyclohexyl- α -D-maltoside (PCC-a-M), (3).

Figure 2.

Thermostability of $\beta_1\text{AR}_{34-324}$ in mild surfactants.

The receptor was partially purified in C12-b-M by Ni^{2+} -affinity chromatography with the surfactant exchange performed in the final column washes. The receptor was eluted in the relevant surfactant and the thermostability was immediately assessed by heating for 30 minutes, quenching on ice and measuring the amount of functional receptor remaining using a ^3H -DHA binding assay (Supplementary information): red squares, PCC-a-M, $T_{50} = 40^\circ\text{C}$; yellow triangles, C12-b-M, $T_{50} = 30^\circ\text{C}$; green inverted triangles, BPC-b-M, $T_{50} = 25^\circ\text{C}$; blue diamonds, PPC-b-M, $T_{50} = 21^\circ\text{C}$. The results shown are from a single representative experiment performed in duplicate with error bars representing the s.e.m. Other experiments performed using the surfactants above, but not all simultaneously, gave similar apparent T_{50} -s.

Fig. 3.

Thermostability of the human receptors of the Hedgehog pathway, hPtc and hSmo, in mild surfactants.

Thermostability of purified hPtc and hSmo. Samples of hPtc or hSmo purified in C12-b-M, PCC-a-M, PPC-b-M or BPC-b-M were heated 30 minutes at increasing temperatures ranging from 4 to 70°C prior to injection on the reference flow cell (Fc1) and measure flow cell (Fc2) (coupled with N-SHh for hPtc and with CPN for hSmo experiments).

Sensorgrams were recorded and the percentage of remaining native hSmo (a) and hPtc (b) was determined (SI). Red squares correspond to PCC-a-M; yellow triangles to C12-b-M; green inverted triangles to BPC-b-M and blue diamonds to PPC-b-M. The results shown are

from a single representative experiment performed in duplicate with error bars representing the s.e.m.. Several other experiments were performed and gave similar apparent T_{50} -s.

Figure 4.

Stability of cytochrome b_6f exposed to low (0.2 mM) or high (5 mM) concentrations of either C12-b-M or PCC-a-M. *Top.* Migration of the b_6f complex in sucrose gradients (10-30% w/w) prepared in 20 mM Tris/HCl buffer, pH 8.0, containing either 0.2 or 5 mM of either C12-b-M or PCC-a-M. Freshly prepared b_6f (4.9 μ M) in 0.2 mM C12-b-M (Stroebel et al., 2003) was layered on the top of the gradients and ultracentrifuged for 4 h at 4°C at 55,000 rpm ($260,000 \times g$) in a Beckman TLS 55 rotor. Monomerization, as observed in the presence of 5 mM C12-b-M, correlates with the inactivation of the complex (see refs. (Breyton et al., 1997; Popot, 2010)). *Bottom.* Kinetics of inactivation of the b_6f complex in the presence of either 0.2 or 5 mM of either C12-b-M (circles, diamonds) or PCC-a-M (squares, triangles). The dimer bands were collected from the gradients and their electron-transfer activity assayed as described (Pierre et al., 1995) immediately after collection ($t=0$) and after storage at 4°C in the dark for up to 10 days/three weeks.

Figure 5. Crystal structure of the dimer of cytochrome *b₆f* with the localisation of the structured PCC-a-M molecules (8 per monomer) seen from the membrane in CPK representation. The stromal side is at the top.

Table 1

Physicochemical data of new surfactants and of C12-b-M.

	PoPC-a-M	BoPC-a-M	PPC-b-M	BPC-b-M	PCC-a-M	BCC-a-M	C12-b-M
Molecular weight [g/mol]	570.63	572.65	540.29	554.31	548.67	562.70	510.6
CMC [mM] room temp.	0.41	0.21	1.18	0.43	0.036	0.0069	0.17
CMC [mM] 50°C			0.93	0.42	0.027		
Micellar mass [kDa]	36.0 ± 1.7 [§]				62.7 ± 3.5 [*]	84.9 ± 4.5 [§]	66
Micellar radius [Å]	23.2 ± 1.2 [§]		34.4 ^{&}	38.0 ^{&}		31.9 ± 1.5 [§]	

^{*} SAXS;[§] SANS, ...[&] Dynamic Light Scattering, assuming radial symmetry

Table 2T₅₀ values (°C)

	$\beta_1\text{AR}_{34-324}$	hSmo	hPtc
C12-b-M	30	40	23
PCC-a-M	40	52	32
PPC-b-M	21	31	36
BPC-b-M	25	40	26