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Tandem Facial Amphiphiles for Membrane Protein Stabilization

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Abstract: We describe a new type of synthetic amphiphile that is intended to support biochemical characterization of intrinsic membrane proteins. Members of this new family displayed favorable behavior with four of five membrane proteins tested, and these amphiphiles formed relatively small micelles.

Membrane proteins (MPs) play crucial roles in biology, but these proteins are difficult to handle and analyze because of their physical properties.[1] The native conformations of MPs display extensive nonpolar surfaces, which is necessary for residence in a lipid bilayer but leads to denaturation and/or aggregation in an aqueous medium. Detergents, such as dodecyl-β-D-maltoside (DDM), are typically employed to render MPs soluble by coating nonpolar protein surfaces.[2] However, not all MPs can be maintained in native-like conformations when solubilized with conventional detergents.[3] Moreover, even when a native conformation can be achieved, the MP−detergent complex may manifest unfavorable properties with regard to structural analysis (inability to crystallize and/or too large for NMR). Since our understanding of membrane protein structure and function remains poorly developed relative to understanding of soluble proteins, there is a persistent need for new amphiphilic "assistants" that can promote solubilization and manipulation of MPs.[4]

Several groups have reported creative implementations of the "facial amphiphile" concept for the design of novel agents that display favorable behavior with selected membrane proteins.[5] McGregor et al., for example, have reported lipopeptides that are intended to match the width of a lipid bilayer and to form a sheath around nonpolar surfaces of MPs.[5c] Zhang et al. have developed cholate-based amphiphiles in which hydrophilic maltose units project from one side of the rigid and hydrophobic steroidal skeleton.[5d] Here we disclose the design of "tandem facial amphiphiles" (TFAs), which contain a pair of maltose-functionalized deoxycholate units. Unlike previous cholate-based designs, the TFAs are long enough to match bilayer width,[6] and unlike lipopeptides, the TFAs are readily synthesized in large quantities. We show that one TFA forms micelles containing only six molecules and that simple TFAs can be used to maintain a variety of MPs in native-like states in aqueous solution.

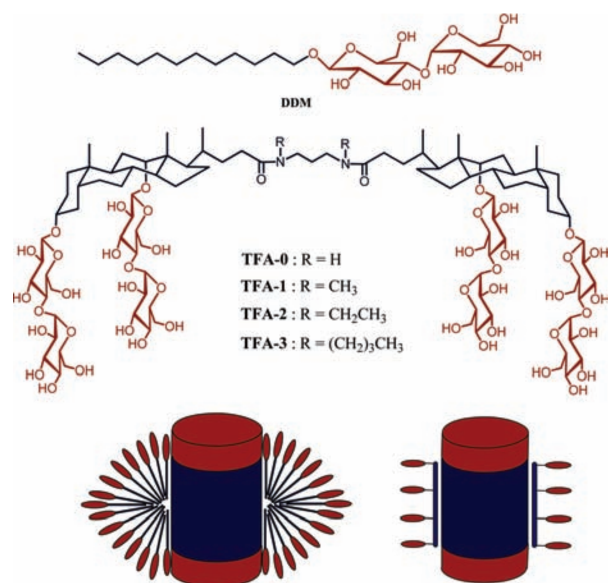

Figure 1. Chemical structures of DDM (*top*), tandem facial amphiphiles (TFAs, *middle*), and schematic representation of membrane proteins interacting with DDM (*bottom left*) and TFAs (*bottom right*).

A set of four TFAs was generated from a deoxycholate-bis-maltoside building block via linkage with a diaminopropane unit (Figure 1). Molecular mechanics calculations suggest that an extended conformation of the TFA backbone has a length that is comparable to the width of a typical lipid bilayer (~30 Å).[6] These TFAs vary in the appendage on the amide nitrogen atoms. Each amphiphile could be obtained in excellent purity (>98%) and good overall yield (~65%) in five straightforward synthetic steps with two chromatographic purifications.[6] Multigram quantities are readily available.

The TFAs displayed interesting behavior in water. TFA-0 forms a hydrogel at concentrations >0.4 wt %, and this compound was not studied further. The other three TFAs are soluble to 5−10 wt % in aqueous media. Critical micelle concentrations (CMCs) were determined by monitoring solubilization of a hydrophobic fluorescent dye, dicyclohexatriene,[7] and the hydrodynamic radii (R_h) of the micelles were determined via dynamic light scattering (DLS).[6] Table 1 compares the data for TFAs with those for DDM, a conventional detergent that is very widely used for MP applications; DDM and our TFAs share maltose as their hydrophilic moieties. CMC values of the three TFAs are smaller than that of DDM, whether the CMC is measured in units of mM or wt %. The micelles

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Table 1. Critical Micelle Concentration (CMC) of TFAs and Hydrodynamic Radii (R_h) of Their Micelles (Mean ± SD, $n = 3$)

	MW[a]	CMC (μM)	CMC (wt %)	R_h (nm)[b]
TFA-1	2148.4	13 ± 1.4	0.0028 ± 0.00030	1.9 ± 0.08
TFA-2	2176.5	13 ± 1.8	0.0028 ± 0.00039	2.0 ± 0.03
TFA-3	2232.6	7 ± 2.3	0.0016 ± 0.00051	3.3 ± 0.12
DDM	510.1	170	0.0087	3.4 ± 0.03

[a] Molecular weight of detergents. [b] Hydrodynamic radius of micelles measured by dynamic light scattering.

Table 2. Detailed Characterization of TFA-1 (Mean ± SD, $n = 6$) and DDM Micelles (Mean ± SD, $n = 8$)

	MW[a]	N[b]	R_h (nm)	IV[c]	M_w/M_n[d]	dn/dc[e]
DDM	89 982 ± 663	176.4 ± 1.3	3.42 ± 0.04	0.028 ± 0.009	1.01 ± 0.00	0.130
TFA-1	13 279 ± 157	6.2 ± 0.07	1.96 ± 0.01	0.036 ± 0.005	1.00 ± 0.00	0.173

[a] Molecular weight of micelles. [b] Aggregation number of micelles. [c] Intrinsic viscosity. [d] Weight-averaged molecular weight divided by number-averaged molecular weight. [e] Specific refractive index increment.

formed by TFA-1 and TFA-2 ($R_h \approx 2.0$ nm) are somewhat smaller than those formed by DDM, while micelles formed by TFA-3 are comparable to those of DDM ($R_h \approx 3.4$ nm).

Micelles formed by DDM or by TFA-1 in pH 7.0 buffer (20 mM HEPES, 150 mM NaCl) were further characterized by gel filtration using a triple-detector system[8] (light scattering, refractive index, and differential pressure) (Table 2). In both cases the micelles are globular and monodisperse. TFA-1 micelles contain only 6 molecules, which contrasts with the ~175 molecules in a DDM micelle. TFA-2 (R = ethyl) seems to behave similarly to TFA-1 (R = methyl), given the similarity in R_h, but TFA-3 (R = butyl) forms larger micelles. A related trend was observed among lipopeptides, with increasing length of the alkyl appendages leading to increasing micelle size.[5c]

Bacteriorhodopsin (bR) has been widely employed for assessment of new amphiphiles because this membrane protein is readily available, and stability can be assessed via spectrophotometry (absorbance at 554 nm). Following standard protocols,[9] we used 2.0 wt % octyl-β-D-thioglucoside (OTG) to extract bR from the native purple membrane. After removal of insoluble debris via ultracentrifugation, the bR solution was diluted with amphiphile-containing solutions to generate samples containing 0.2 wt % OTG + 0.8 wt % TFA. A control sample had OTG added to give a total of 1.0 wt %. Figure 2a shows that all three TFA-containing samples were much more effective at maintaining native bR absorbance over 20 days relative to the sample containing only OTG. The bR was almost completely denatured by day 10 in the OTG-only sample but ~80% intact at day 20 when solubilized with TFA-1.[10,11]

The promising results with bR stabilization led us to investigate a more challenging system, the photosynthetic superassembly formed by the light harvesting I (LHI) and reaction center (RC) complexes from *Rhodobacter capsulatus*.[12] This superassembly contains 30−40 protein molecules (five different components), and maintenance of native quaternary structure can be assessed via spectrophotometry. The LHI-RC superassembly was extracted from native membranes with 1.0 wt % DDM and purified with DDM at its CMC (0.009 wt %). This preparation was diluted 20-fold with solutions containing TFA-1, TFA-2, or TFA-3, so that residual DDM was far below its CMC (0.0004 wt %). The final TFA concentrations were 0.043 wt % (well over the CMC in each case). A control sample had DDM added to a total concentration of 0.049 wt % (all samples were CMC + 0.04 wt %). Figure 2b shows that

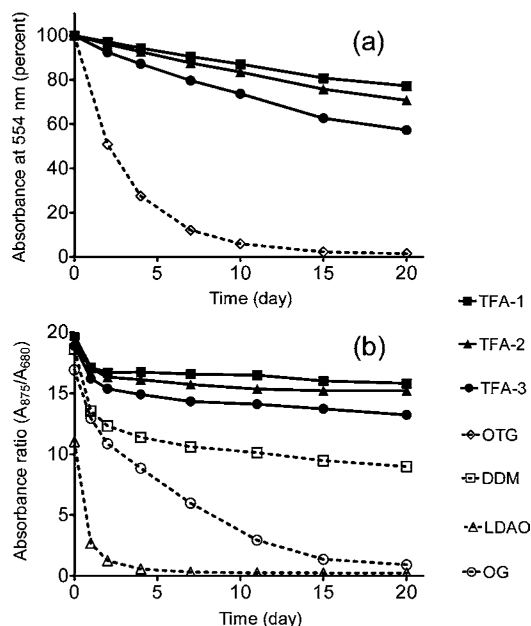

Figure 2. Time course of the stability of bR (a) and *R. capsulatus* superassembly (b) at room tempearture. Detergents were tested at 0.2 wt % OTG + 0.8 wt % TFA and CMC + 0.04 wt % for bR and *R. capsulatus* superassembly, respectively.

the LHI-RC superassembly solubilized with any of the TFAs was more stable over 20 days than was the superassembly solubilized by DDM. Controls involving other common biochemical detergents (lauryldimethylamine oxide or octyl-β-D-glucoside) showed rapid degradation of the superassembly.[10]

Each membrane protein (such as bR) or membrane protein assembly (such as LHI-RC) has unique requirements for maintenance in a native-like state in aqueous solution; therefore, it is important to assess the capabilities of new amphiphiles in multiple systems, in order to establish the breadth of their utility. We turned next to cytochrome bo_3 ubiquinol oxidase (Cyt bo_3), the structural stability of which was assessed at elevated temperature (40 °C) with a reactive probe, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM).[13] This maleimide derivative reacts with the thiol groups of sterically accessible Cys side chains. The coumarin moiety of CPM is internally quenched by the maleimide unit, but thiol reaction causes the unit to become fluorescent. CPM can therefore be used to detect thermally induced protein unfolding, via an increase in fluorescence, if the protein contains Cys residues that are buried in the native state but accessible upon unfolding. Cyt bo_3 was initially extracted from the native membrane with DDM and then diluted to generate solutions containing 0.043 wt % TFA-1, TFA-2, or TFA-3 (residual DDM = 0.0008 wt %). A control sample had DDM added to a total concentration of 0.049 wt % (CMC + 0.04 wt % for each amphiphile). Figure 3a shows that TFA-solubilized Cyt bo_3 samples were more resistant to thermal denaturation than was the DDM-solubilized control.

The wild type of bacterial leucine transporter (LeuT WT) was examined because the functional state of this membrane protein is readily assessed by using a scintillation proximity assay (SPA)[14] to monitor binding of radiolabeled leucine. LeuT was initially extracted with DDM and then diluted with amphiphile-containing solutions to generate final TFA concentrations of 0.04 or 0.2 wt % (residual DDM = 0.005 wt %). Control samples had 0.05 or 0.2 wt % DDM (overall, the final concentrations were CMC + 0.04 wt % or CMC + 0.2 wt %). At the lower amphiphile concentrations, DDM was slightly better than the TFAs at maintaining LeuT WT

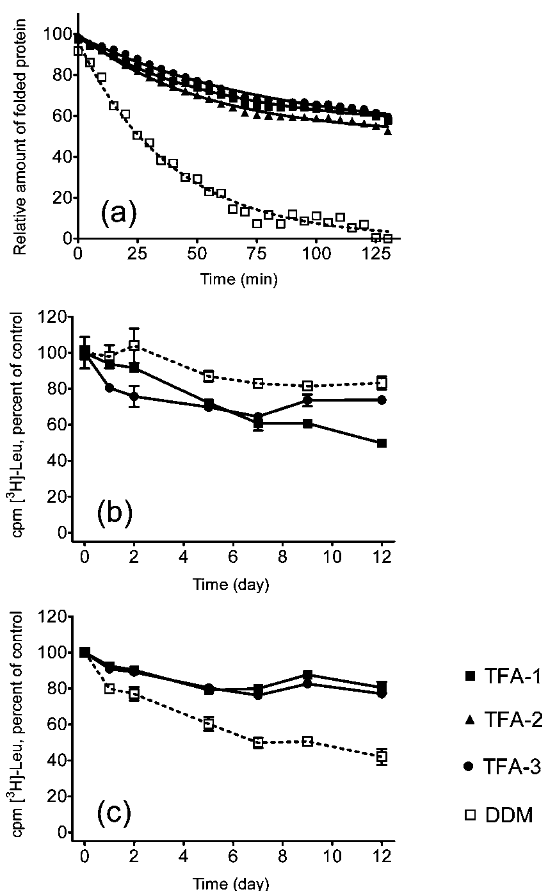

Figure 3. Time course of changes in stability of solubilized Cyt bo_3 and activity of LeuT WT. (a) CPM assay for Cyt bo_3 was performed at 40 °C for 130 min using CMC + 0.04 wt % amphiphile. LeuT WT was kept at room temperature up to 12 days in the presence of CMC + 0.04 wt % (b) or CMC + 0.2 wt % (c) amphiphile before determining binding activity by SPA.

function over 12 days (Figure 3b), but the TFAs were clearly superior at the higher concentrations (Figure 3c). TFA-1 and TFA-3 at the higher concentration matched DDM at the lower concentration in maintaining LeuT WT activity over the time period.

As a final test, we examined the TFAs for the ability to stabilize a GPCR, the human β_2 adrenergic receptor (β_2AR).[15] This assay employs a β_2AR-T4-lysozyme fusion protein (β_2AR-T4L) complexed to the inverse agonist carazolol; stability is assessed by following the fluorescence emission maximum of carazolol, which shifts from 341 nm in the bound state to 356 nm in aqueous solution (i.e., after release upon β_2AR denaturation). Monitoring the 341:356 nm peak intensity ratio upon heating yields cooperative denaturation data. In this assay, the TFAs proved to be inferior to DDM.

We have introduced a new class of molecules, "tandem facial amphiphiles", that contain two deoxycholate-derived subunits and that are sufficiently long to span a lipid bilayer.[6] These molecules can be easily prepared on a scale that would support biochemical research. One of the new amphiphiles, TFA-1, was shown to form small, discrete micelles in water (MW ≈ 13 kD). In contrast, DDM, a popular biochemical detergent, forms much larger micelles (MW ≈ 90 kD). Three TFAs have been evaluated for the ability to maintain intrinsic membrane proteins or protein assemblies in native-like forms in aqueous solution. In four of the five cases we examined, the TFAs proved to be comparable or superior to a conventional detergent for stabilizing the membrane proteins. Given the great variation in structure and physical properties among membrane proteins, no single amphiphile or amphiphile family will be maximally effective for every case. Because the TFAs manifest favorable solubilization/stabilization behavior with several diverse membrane protein systems, relative to widely used conventional detergents (DDM or OTG), and because this new amphiphile class can form small assemblies, it seems likely that TFAs will be valuable tools for characterization of membrane proteins, perhaps including high-resolution structural analysis.[3b]

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Supporting Information Available: Experimental procedures for characterizations of new compounds, micelle characterization, and membrane protein stability assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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