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# An improved tripod amphiphile for membrane protein solubilization

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**Abstract**

Intrinsic membrane proteins represent a large fraction of the proteins produced by living organisms and perform many crucial functions. Structural and functional characterization of membrane proteins generally requires that they be extracted from the native lipid bilayer and solubilized with a small synthetic amphiphile, for example, a detergent. We describe the development of a small molecule with a distinctive amphiphilic architecture, a "tripod amphiphile," that solubilizes both bacteriorhodopsin (BR) and bovine rhodopsin (Rho). The polar portion of this amphiphile contains an amide and an amine-oxide; small variations in this polar segment are found to have profound effects on protein solubilization properties. The optimal tripod amphiphile extracts both BR and Rho from the native membrane environments and maintains each protein in a monomeric native-like form for several weeks after delipidation. Tripod amphiphiles are designed to display greater conformational rigidity than conventional detergents, with the long-range goal of promoting membrane protein crystallization. The results reported here represent an important step toward that ultimate goal.

**Keywords:** crystallization; delipidation; membrane protein; micelle; solubilization; synthetic amphiphile

Intrinsic membrane proteins represent a large proportion of the proteins produced by living organisms. These proteins mediate material and information transfer between cells and their environment (Wallin & Von Heijne, 1998). Membrane proteins often are difficult to work with, and high-resolution structural analysis, therefore, lags far behind that of soluble proteins. Difficulties arise because the natural environment for a membrane protein is a lipid bilayer, but structural analysis requires extraction from the bilayer. Attempts to isolate membrane proteins from the native bilayer frequently result in protein aggregation and loss of function (Stein, 1986). Membrane proteins are typically isolated and purified as protein–detergent complexes in aqueous solution. The detergent molecules shield the large hydrophobic patches on the protein surface from the aqueous medium, mimicking the action of membrane lipids (Tanford & Reynolds, 1976; Moller & le Maire, 1993). Detergents cannot always provide a native-like environment, however; they often denature membrane proteins instead (Lau & Bowie, 1997).

All high-resolution structures of membrane proteins reported to date were obtained with crystals grown from protein–detergent complexes. These crystals contain large amounts of detergent, and the detergent is highly disordered (Roth et al., 1989; Timmins et al., 1994). Detergent is essential for generating a monomeric form of a membrane protein in solution, which is considered to be the ideal starting condition for crystal growth; however, the high intrinsic flexibility of conventional detergents seems likely to disfavor the formation of an ordered crystal lattice. Identification of a suitable detergent is often a critical step in the crystallization of a membrane protein (Michel, 1983; Garavito et al., 1986, 1996; Kühlbrandt, 1988; Sowadski, 1994; Ostermeier & Michel, 1997), which suggests that there is a delicate balance between the favorable and unfavorable features of conventional detergents with regard to crystal nucleation and growth.

Detergents influence crystallization of membrane proteins in several ways (Kühlbrandt, 1988; Sowadski, 1994). (1) Protein

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*Abbreviations:* CHAPSO, (3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate); BR, bacteriorhodopsin; Rho, rhodopsin; LDAO, lauryl dimethyl-aminoxide; Con-A, concanavalin-A; ROS, rod outer segment; CD, circular dichroism; PIPES, piperazine-*N*,*N*″-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; CMC, critical micelle concentration; DOC, deoxycholate; THF, tetrahydrofuran; *m*CPBA, *m*-chloroperbenzoic acid; MALDI-TOF, matrix assisted laser desorption ionization–time of flight; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

stability: because high-quality crystals often require weeks for growth, the detergent must be able to maintain the native protein conformation for this length of time. In general, detergents with nonionic polar head groups are considered to be optimal for protein stability (Anathapadmanabhan, 1993). (2) Interprotein contacts: if the micellar layer around a protein is too large, protein–protein contacts necessary for crystallization can be inhibited. It has been suggested that there is an optimal size and an optimal shape of the micelle, neither too large nor too small, for a given crystal lattice (Michel, 1991). (3) Phase separation: when the micelles formed from nonionic detergents are sufficiently concentrated, dehydration of the headgroups can occur, causing the detergent to separate from aqueous solution as a liquid crystalline phase. Phase separation during crystallization is detrimental to high-quality crystal growth, but some authors suggest that crystallization is promoted under conditions that approach those of phase separation (Michel, 1991).

Despite the importance of membrane protein structural data and the centrality of detergents in membrane protein crystallization, relatively few efforts have been made to investigate amphiphiles with nontraditional structures as aids to membrane protein crystallization (Schafmeister et al., 1993; Tribet et al., 1996). We are trying to develop new amphiphiles that can replace conventional detergents for solubilization and ultimately crystallization of membrane proteins. (A complementary approach would be to engineer a more stable form of the protein, as recently discussed by Zhou & Bowie, 2000.) The new amphiphiles contain relatively rigid hydrophobic units rather than the highly flexible alkyl chains common to many biochemical detergents. Our hypothesis is that amphiphiles with diminished flexibility will be more prone to become part of an ordered crystal lattice than are conventional detergents. Excessive amphiphile rigidity, however, may prevent structural adjustments necessary to maximize protein–protein contacts essential for crystallization (Kühlbrandt, 1988; Welte & Wacker, 1991). Steroid-based detergents (e.g., CHAPS; Hjelmeland et al., 1983) have very rigid skeletons and are excellent for stabilizing membrane proteins in solution, but these rigid detergents have not given rise to high quality crystals.

We recently introduced "tripod amphiphiles," which are built around a tetrasubstituted carbon atom that has three hydrophobic substituents and one hydrophilic substituent (McQuade et al., 2000). The presence of a tetrasubstituted carbon intrinsically limits conformational flexibility (Alder et al., 1990; Hoffmann et al., 1998), and additional rigidification can be achieved via choice of the hydrophobic substituents. The versatile synthetic route we developed for tripod amphiphiles allows us to vary the shape and flexibility in the hydrophobic and hydrophilic subunits as we seek to optimize physical characteristics.

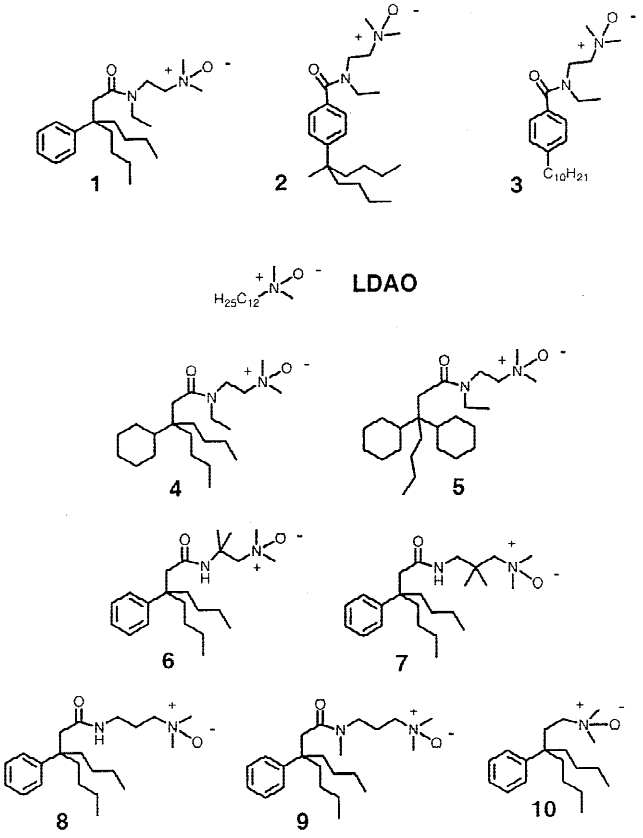
A preliminary paper (McQuade et al., 2000) focused on tripod amphiphile **1** and isomers **2** and **3**, the topologies of which approach that of a conventional detergent. We showed that the unique topology of **1** within this isomer set is critical for extraction of a test membrane protein, BR, from its native membrane in a properly folded form. Both **2** and **3** denature BR upon attempted solubilization, as does the conventional detergent LDAO under the conditions we used. Additional structure–activity studies (McQuade, 1998) showed that BR stabilization depends upon the nature of the hydrophobic tripod substitutents. Tripod **4**, in which the phenyl ring of **1** is replaced by a cyclohexyl ring, failed to solubilize BR from the native membrane. Tripod **5**, with two cyclohexyl rings, was insoluble in water.

Here, we describe variation of the tripod's hydrophilic group, which we have pursued in an effort to understand the relationship between amphiphile structure and membrane protein solubilization. Among the new amphiphiles (**6**–**10**; Scheme 1), we discovered another excellent solubilizing agent (**8**), which displays enhanced chemical stability relative to **1**. We report detailed characterization of the soluble complexes of **8** with BR and with bovine Rho. The stability of the delipidated complexes should be sufficient for crystallization trials. In addition, tripod amphiphiles may prove to be useful for exploration of membrane protein stability (Lau & Bowie, 1997; White & Wimley, 1999).

## Materials and methods

### *Materials*

Dark-adapted bovine retinas were purchased from Schenk Packaging Co. (Stanwood, Washington). Concanavalin (Con)-A sepharose and Q-sepharose were purchased from Sigma (St. Louis, Missouri). All other reagents were purchased as the highest grade from Fisher Scientific (Springfield, New Jersey), Aldrich (Milwaukee, Wisconsin), or Sigma, and used without further purification. Ultraviolet/visible absorbance measurements were performed on a Perkin-Elmer Lamda 20, a Shimadzu Biospec-1601, or a Spectramax 190 microplate spectrophotometer. Circular dichroism was measured on an Aviv 62 A-DS spectropolarimeter, and fluorescence spectra were obtained from a Hitachi F-4500 fluores-




**Scheme 1.**

cence spectrophotometer. NMR spectra were acquired on Bruker AM-250 or AM-300 spectrometers. Mass spectra were obtained using a Kratos MS-80 mass spectrometer. Fast Atom Bombardment (FAB) mass spectra were obtained on a VG Analytical ZAB-2F spectrometer. MALDI-TOF mass spectra were obtained on a Bruker Reflex II Instrument. Analytical ultracentrifugation data were collected using a Beckman XL-A centrifuge with an AN60Ti rotor and dual sector cells.

*Synthesis of tripod amphiphile **8** (Scheme 2)*

*Compound **12***

This was synthesized by the modified method of Cope et al. (1941). 5-Nonanone (**11**, 12.1 mL, 70.3 mmol) was dissolved in benzene (25 mL) containing acetic acid (3.21 mL, 56.4 mmol) and ammonium acetate (1.08 g, 14.1 mmol). To this stirring solution was added malononitrile (4.43 mL, 70.3 mmol), and the round-bottom flask holding the mixture was fitted with a Dean-Stark trap. The solution was refluxed for 4 to 6 h until no more water was being collected by the trap. The mixture was cooled, and 50 mL of 1 N aqueous NaOH was added. The organic layer was separated and washed with 1 N aqueous NaOH until no color was observed in the aqueous layer. The organic layer was dried with $MgSO_4$ and concentrated by rotary evaporation providing 13.38 g (100%) of the desired product as a yellow oil. $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 2.57 (t, 4H), 1.54 (m, 4H), 1.42 (m, 4H), 0.96 (t, $J$ = 7, 6H); $^{13}$C NMR ($CDCl_3$, 75.4 MHz) $\delta$ 186.5 (C), 111.7 (C), 85.3 (C), 35.3 ($CH_2$), 29.8 ($CH_2$), 22.4 ($CH_2$), 13.4 ($CH_3$); EI-MS $m/z$ ($M^+$) calcd for $C_{12}H_{18}N_2$ 190.1470, obsd 190.1467.

*Compound **13***

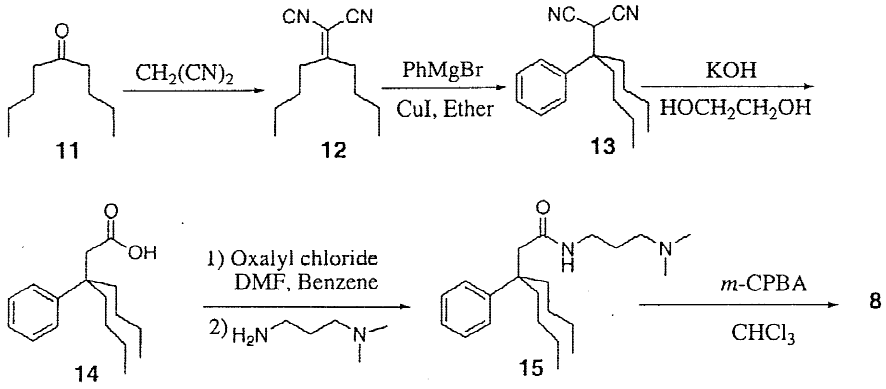
This was synthesized by a modified version of a reported method (Davis & Orchard, 1993). To 100 mL THF containing Mg (1.97 g, 81.2 mmol) was added phenyl bromide (6.2 mL, 59 mmol), and the heterogeneous mixture was allowed to stir at room temperature until the solution became homogeneous. The reaction mixture was then heated to reflux for 1 h. After the reaction mixture had been cooled to 3 °C, CuCN (2.58 g, 28.8 mmol) was added, which resulted in formation of thick precipitate that caused stirring to cease. A solution of compound **12** (5 g, 26.2 mmol) in THF (100 mL) was added to the congealed reaction mixture, and the flask was agitated by hand. The precipitate dissolved after 15 min of agitation. After 30 min of stirring, the reaction mixture was poured into an ice-cold saturated aqueous ammonium chloride solution and stirred overnight. The mixture was then extracted with 150 mL of diethyl ether. The organic layer was dried with $MgSO_4$, concentrated by rotary evaporation and purified by silica gel column chromatography (9.5/1.5 ethyl acetate/hexane) providing 3.0 g (55%) of the desired product as a yellow oil. $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 7.34–7.44 (m, 5H), 3.90 (s, 1H), 2.03 (m, 4H), 1.38 (m, 4H), 1.23 (m, 4H), 0.93 (t, $J$ = 7, 6H); $^{13}$C NMR ($CDCl_3$, 75.4 MHz) $\delta$ 139.1 (C), 128.7 (CH), 127.8 (CH), 126.3 (CH), 111.7 (C), 46.7 (C), 33.8 (CH), 32.9 ($CH_2$), 25.6 ($CH_2$), 22.4 ($CH_2$), 13.7 ($CH_3$); EI-MS $m/z$ ($M^+$) calcd for $C_{18}H_{24}N_2$ 268.1939, obsd 268.1933.

*Compound **14***

This was synthesized by a modified version of a reported method (Patai & Dayagi, 1962). Compound **13** (3.0 g, 11.2 mmol) was mixed with ethylene glycol (50 mL) and potassium hydroxide (3.3 g, 58.8 mmol). The mixture was refluxed for three days. The reaction mixture was then cooled to room temperature and diluted with 50 mL of water. The solution was poured into 100 mL of ice-cold aq. HCl (~20% v/v), and the resulting milky solution was extracted with four 50 mL portions of ether. The organic layers were combined, dried with $MgSO_4$, and concentrated by rotary evaporation to yield a slightly yellow oil. The oil was crystallized in hexane to afford 2.4 g (90%) of the desired product as colorless crystals. $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 7.18–7.33 (m, 5H), 2.74 (s, 2H), 1.78 (t, $J$ = 7, 4H), 0.96–1.28 (m, 8H), 0.83 (t, $J$ = 7, 6H); $^{13}$C NMR ($CDCl_3$, 75.4 MHz) $\delta$ 178.0 (C), 145.6 (C), 127.9 (CH), 125.9 (CH), 125.5 (CH), 43.0 (C), 40.7 ($CH_2$), 38.2 ($CH_2$), 25.6 ($CH_2$), 23.0 ($CH_2$), 13.8 ($CH_3$); EI-MS $m/z$ ($M+H^+$) calcd for $C_{17}H_{28}O_2$ 262.1933, obsd 262.1941.

*Compound **15***

Compound **14** (300 mg, 1.1 mmol) was dissolved in 5 mL of benzene, and a drop of DMF was added. The solution was cooled with an ice bath and oxalyl chloride (0.5 mL, 5.72 mmol) was added dropwise, resulting in a vigorously bubbling solution. The reaction was allowed to stir for 3 h, and the volatiles were then removed under vacuum. The resulting oil was dissolved in dry ether (15 mL), cooled to 0 °C, and *N,N*-dimethylpropylenediamine (0.29 mL, 2.2 mmol) in ether (15 mL) was added dropwise, resulting in a white precipitate. After overnight stirring, 1 N NaOH




**Scheme 2.**

(25 mL) was added to the reaction mixture to dissolve the precipitate. The ether layer was separated, washed twice with 25 mL portions of 1 N NaOH, washed with saturated NaCl solution, and dried with $MgSO_4$. The ether solution was concentrated to yield 0.29 g (86%) of the desired product in light yellow oil. $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 7.17–7.34 (m, 5H), 5.53 (t, $J = 6$, 1H), 3.06 (q, $J = 6$, 3H), 2.49 (s, 2H), 2.14 (s, 6H), 2.08–2.12 (m, 2H), 1.74–1.84 (m, 4H), 1.37 (p, $J = 7$, 2H), 1.02–1.32 (m, 8H), 0.86 (t, $J = 7$, 6H); $^{13}$C NMR ($CDCl_3$, 75.4 MHz) $\delta$ 170.6 (C), 146.6 (C), 128.1 (CH), 126.2 (CH), 125.6 (CH), 57.6 ($CH_2$), 46.2 ($CH_2$), 45.2 ($CH_3$), 43.0 (C), 38.0 ($CH_2$), 36.7 ($CH_2$), 23.1 ($CH_2$), 13.8 ($CH_3$); MALDI-TOF ($M+H^+$) calcd for $C_{23}H_{41}N_2O$ 361.59, obsd 361.32.

*Compound **8***

Compound **15** (5.2 g, 16.9 mmol) was dissolved in 5 mL of chloroform. The solution was cooled to −10 °C, and *m*-chloroperoxybenzoic acid (*m*CPBA, 57–86%) (14.6 g, 84.5 mmol) was added. The reaction was stirred for 3 h, and the reaction mixture was then poured onto an alumina column pre-equilibrated with chloroform. The column was eluted first with chloroform (one bed volume) and then with chloroform containing 10% methanol. The fractions containing product were combined and concentrated. The resulting oil was dissolved in water and passed through a 0.22 $\mu$m syringe filter to remove alumina. The filtrate was lyophilized. The resulting white powder was further purified by recrystallization from dichlormethane/ether affording 5.0 g (85%) of the desired product as a white solid. m.p. 105 °C; $^1$H NMR ($CDCl_3$, 300 MHz) $\delta$ 7.10–7.34 (m, 5H), 6.55 (t, $J = 4$, 1H), 3.08–3.12 (m, 10H), 2.48 (s, 2H), 1.75–1.91 (m, 6H), 1.08–1.40 (m, 8H), 0.86 (t, $J = 7$, 6H); $^{13}$C NMR (MeOH, 75.4 MHz) $\delta$ 171.1 (C), 146.5 (C), 127.8 (C), 126.1 (CH), 125.3 (CH), 68.1 ($CH_2$), 58.5 ($CH_3$), 45.5 ($CH_2$), 42.8 (C), 36.4 ($CH_2$), 25.4 ($CH_2$), 23.1 ($CH_2$), 22.9 ($CH_2$), 13.7 ($CH_3$), 13.4 ($CH_3$); FAB-MS *m/z* ($M+H^+$) calcd for $C_{23}H_{41}N_2O_2$ 377.3, obsd 377.3.

*Decomposition kinetic studies of **1** and **8***

Tripod amphiphile (**1** or **8**) was dissolved in 0.1 M $K_2DPO_4$ buffer (pH 6.8) to a final amphiphile concentration of 0.2 M. This solution (2 mL) was transferred to a glass capillary tube (diameter 1.5 mm), and the tube was sealed with high temperature flame. The sealed capillary tube containing the sample was immersed in an oil bath with preset temperature (117, 126, 133, or 142 °C) to initiate decomposition. After a predetermined time (45, 90, 150, or 210 min), the tube was transferred to an ice bath to stop the decomposition reaction. The capillary tube was placed inside an NMR tube (5 mm), which was filled with $CDCl_3$ containing 0.005% *para*-dinitrobenzene (external reference). $^1$H NMR spectra were recorded (16 scans, 5 s relaxation delay) on a Bruker AM-300; the decomposition reaction was followed by observing the reduction of methylene proton signals (~3 ppm). The decomposition rate constant *k* was deduced from the slope of a reaction time vs. ln (amphiphile concentration) plot, and the activation energy for the decomposition reaction was estimated from the slope of $\ln k$ vs. $1/T$. Additional details are reported elsewhere (Hackenberger, 1999).

*Critical micelle concentration (CMC) measurements (Chattopadhyay & London, 1984)*

Tripod amphiphile solutions (1 mL, 0.06 to 25 mM amphiphile) containing 25 mM sodium phosphate (pH 6.9) were prepared and transferred to screw-capped vials, and 1 $\mu$L of a 1,6-diphenylhexatriene solution (3.125 mM in THF) was added. The solutions were vortexed at room temperature for 30 s. The fluorescence spectra of the solutions were obtained (excitation 358 nm, emission 430 nm), and the fluorescence intensity of each sample was plotted against amphiphile concentration. The CMC was determined from the intersection of the two lines formed by linear regression calculated for the concentrations that show low fluorescence and those that show high fluorescence.

*Membrane preparation*

Purple membrane was purified from the *Halobacterium salinarum* as described (Oesterhelt & Stoeckenius, 1974). A stock suspension of purple membrane that contained 150 to 200 $\mu$M BR (based on absorbance at 570 nm) was prepared in double distilled water containing 0.025% (w/v) sodium azide. Rod outer segment (ROS) disk membranes were isolated from dark-adapted bovine retinas by a reported procedure (Papermaster, 1982). A stock suspension containing 150 to 250 $\mu$M Rho (based on absorbance at 500 nm) was prepared in 10 mM Tris-acetate, pH 7.4.

*Solubilization of purple membrane and ROS disk membrane*

For purple membrane solubilization, a suspension of purple membrane (40 $\mu$L, 150 $\mu$M BR) was added to amphiphile solution (800 $\mu$L) buffered with 10 mM sodium phosphate (pH 5.9), to a final amphiphile concentration of 0 to 40 mM. The concentration of amphiphile was at least 1,000 times that of BR in all the samples except the 0 mM sample. These mixtures were stirred in the dark at room temperature for 30 min, and their absorbances at 550 nm were measured. The samples were centrifuged at 300,000 × *g* at 4 °C for 40 min, and their absorbances at 550 nm were immediately measured. The percent solubilization of BR was calculated from the ratio of the spectral absorbances before and after centrifugation.

Solubilization of ROS disk membrane preparations was performed either in the dark or under dim red light because of the light sensitivity of Rho. A suspension of disk membrane preparation (30 $\mu$L, 200 $\mu$M Rho) was added to amphiphile solutions (800 $\mu$L) buffered with 10 mM sodium phosphate (pH 6.9) to a final amphiphile concentration of 0 to 40 mM. Total amount of Rho was estimated from the spectral absorbance difference at 500 nm (4.5 mM **2**) before and after 15 min bleaching under white light. The amount of Rho solubilized by **2** was estimated from the centrifuged (300,000 × *g*, 40 min) samples by measuring the difference in absorbance at 500 nm before and after bleaching. In some samples, bleaching was performed in the presence of excess hydroxylamine to ensure entrapment of released retinal; however, the spectral properties of Rho-containing solutions were not affected by the presence of hydroxylamine.

*Dissolution of purple membrane monitored by exciton coupling decay*

Purple membrane stock suspension was dissolved in amphiphile solution (Triton X-100 or **8**; containing 25 mM sodium phosphate, pH 6.9) to a final amphiphile concentration of 12.5, 25, or 37.5 mM, and a final BR concentration of 22.6 $\mu$M. These solutions were immediately placed into a 1 cm pathlength quartz cuvette. The circular dichroism (CD) measurement was performed at room temperature, and data were obtained every 2 nm with a bandwidth of 1 nm and averaging time of 10 s. With these settings, each wavelength scan took ~30 min.

*Analytical ultracentrifugtion of BR*

The aggregation state of BR in tripod amphiphile solutions was examined by analytical ultracentrifugation at 4 °C. The typical sample contained 7 $\mu$M BR in 10 mM sodium phosphate pH 5.9 and tripod amphiphile **8** at 3, 8, or 10 mM. These three amphiphile concentrations were used in different cells within the rotor. The samples were spun at 3,000 rpm to acquire initial data and then equilibrated at 15,600 and 18,000 rpm. The samples attained equilibrium within 20 h, and the data set at 550 nm (absorbance) was obtained when the absorbance vs. radial distance curves became invariant over time. The data were initially analyzed by a nonlinear regression fitting method (Hansen et al., 1994; McQuade, 1998). This method took into account the possible existence of multimeric species and baseline variation. The best fit was achieved with a single-species model; the residuals of nonlinear fitting were minor and random without any systematic deviation. The data were also analyzed according to a single-species model following a reported method (Reynolds & Stoeckenius, 1977). In this latter analysis, the reduced molecular weight of the BR–lipid–detergent complex is deduced from the slope of $\ln c$ vs. $r^2$ plot ($c$ is BR concentration and $r$ is radial distance from the center of the rotor) as the sum of the reduced molecular weights of individual components (Equation 1).

$$M(1-\phi\rho) = M_p(1-v_p\rho) + M_d(1-v_d\rho) + M_l(1-v_l\rho)$$
$$= M_p[(1-v_p\rho) + \delta_d(1-v_d\rho) + \delta_l(1-v_l\rho)] \quad (1)$$

where $M$ is the molecular weight of BR–lipid–detergent complex, $\phi$ is the partial specific volume of this complex, and $M_p$, $M_d$, and $M_l$ are the molecular weights of BR, detergent, and lipid, respectively. The partial specific volume of BR, detergent, and lipid are designated as $v_p$, $v_d$, and $v_l$, and $\rho$ is the density of the solution. $\delta_d$ is the ratio of the total weight of detergent within the BR–detergent–lipid complex to the molecular weight of BR. $\delta_l$ is the ratio of the total weight of lipid within the BR–detergent–lipid complex to the molecular weight of BR. The $v_p$ (0.75) and $v_l$ (0.98) values were obtained from published data (Reynolds & Stoeckenius, 1977) and $v_d$ (0.973) was calculated from a reported table as described (Durchschlag & Zipper, 1994). For the calculation of $\delta_d$ (3.5), the total weight of detergent in the BR–detergent–lipid complex was replaced with the molecular weight of a pure micelle **8**, which was deduced from a separate sedimentation equilibrium experiment with **8** alone at 7 mM.

*Delipidation of bacteriorhodopsin and rhodopsin*

The following manipulations were conducted under a dim red light and at 4 °C unless otherwise stated. For BR delipidation, we employed a modified version of a reported method (Schertler et al., 1993). A suspension of purple membrane preparation (800 $\mu$L, 200 $\mu$M BR) was dissolved in 5 mL of solution **A** (10 mM **8**, 10 mM sodium acetate, pH 5.8). After 40 min of stirring at room temperature, the sample was subjected to centrifugation (300,000 $\times$ $g$) for 30 min at 4 °C. The supernatant was immobilized onto 1.5 mL of swollen Q-sepharose anion-exchange resin previously equilibrated with 15 mL of solution **A** at 4 °C. The resin was washed with ~100 mL of solution **A**, and the BR was then eluted with 10 mL of 10 mM **8** (pH 5.8) containing 250 mM sodium acetate. The flow rate was fixed at 5 mL/h throughout the experiment, and 2 mL fractions were collected. In the Rho delipidation experiment, all experimental conditions were the same as those for BR except the following (DeGrip, 1982a). The buffer solution (solution **B**) contained 40 mM PIPES, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.2 mM EDTA, 300 mM NaCl, and 10 mM tripod amphiphile **8**. Rho solubilized in solution **B** was immobilized onto 1.5 mL of swollen Con-A sepharose resin that was previously washed with 10 mL of solution **B** containing 2 mM $MnCl_2$. Delipidated Rho was eluted with 10 mL of solution **B** containing 250 mM $\alpha$-methyl mannoside. The protein content of each fraction was obtained from optical absorbance at 550 nm for BR or at 500 nm for Rho. Phospholipid content of each fraction was analyzed via phosphomolybdate complex formation as previously described except that 1 mL of sample (from the elution fraction), 0.3 mL of perchloric acid and 3 mL of molybdate reagent were used (McClare, 1971; Miercke et al., 1989). The perchloric acid digestion was performed at 195 °C for 2 h, and the color development at 95 °C for 45 min.

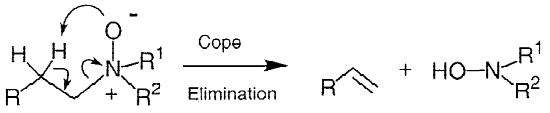
*Long-term stability tests*

For each protein, three samples were prepared from a stock membrane suspension with 4.4, 8.8, and 17.6 mM **8**. Delipidated samples with 10 mM **2** were prepared using column chromatography as above and were used directly for the stability test. The BR sample with 8.8 mM **8** was prepared as follows. A suspension of purple membrane preparation (200 $\mu$L, 150 $\mu$M BR) was dissolved in 1.5 mL of 10 mM **2** (containing 10 mM sodium acetate pH 5.9). This solution was stirred in the dark for 30 min and centrifuged at 300,000 $\times$ $g$ for 30 min at 4 °C. The supernatant containing solubilized BR was divided into five separate 1.5 mL eppendorf tubes and stored in the dark at 4 °C. The stability was assessed by monitoring absorbance of BR chromophore (550 nm) over 20 days at five-day intervals. Each sample was centrifuged at 300,000 $\times$ $g$ for 30 min just prior to the absorbance measurement. Similar experimental procedures were used for the long-term stability test of Rho; however, because of light sensitivity, all Rho experiments were performed under a dim red light.

## Results and discussion

*Tripod amphiphile optimization: Hydrophilic group variation and effects on BR solubilization*

There were two motivations for our examination of hydrophilic group variants of tripod amphiphile **1**. First, we wanted to explore structure–activity relationships among tripod amphiphiles, with a long-term goal of learning how to generate new amphiphiles that display specific properties via rational design. Second, we wanted to enhance thermal stability, which is important for practical applications. N-oxides can decompose via Cope elimination if there are protons on a carbon $\beta$ to the nitrogen (Scheme 3). Two of the




**Scheme 3.**

new tripod amphiphiles examined here, **6** and **7** (Scheme 1), have methyl groups on the $\beta$-carbon, which precludes Cope elimination. These methyl groups should also diminish the conformational flexibility of the polar unit, relative to the other analogues. Amphiphiles **8–10** were synthesized to investigate the significance of the amide group within the polar unit: **8** has a secondary amide, **9** has a less hydrophilic tertiary amide, and **10** lacks an amide group. Critical micelle concentration (CMC) values for the new amphiphiles are summarized in Table 1; they all fall in a relatively narrow range, 1–8 mM.

The polar group variations among **6–10** exert profound effects with regard to BR solubilization, despite the similarities among the CMC values. Amphiphiles **6**, **7**, **9**, and **10** all denatured BR during attempted solubilization from the native membrane ("purple membrane"). Similar behavior was observed with the conventional N-oxide detergent LDAO under the conditions we used. In contrast, **8** solubilized BR from purple membrane without significant denaturation.

### *Thermal stability of amphiphiles*

During our investigation of **1** and related compounds, we observed qualitatively that the N-oxide head group can decompose via Cope elimination (McQuade et al., 2000). Therefore, we quantitatively assessed the thermal stabilities of **1** and **8** in aqueous solution by measuring their decomposition rates at elevated temperatures (116–146 °C). The activation energies for the decomposition reactions were determined, and the half-lives of **1** and **8** at room temperature were estimated by extrapolation to be $\leq$229 days and $\geq$446 days, respectively. These results confirm that the stability of **8** is enhanced with respect to **1**. Both of these tripod amphiphiles are sufficiently stable for membrane protein manipulation.

### *Solubilization of BR and Rho from native membranes*

BR and bovine Rho are useful as test membrane proteins for evaluation of an amphiphile's solubilization properties because both of these proteins contain a retinal chromophore, and the spectral properties of protein preparations provide a sensitive and easily monitored indicator of structural integrity (Khorana, 1993). The interaction of conventional detergents with both proteins has been studied in detail (DeGrip, 1982b). High-resolution crystal structures of BR have recently been reported (Luecke et al., 1999). BR occurs naturally in a two-dimensionally crystalline protein–lipid array (purple membrane), and the high degree of order in this assembly renders BR resistant to solubilization by many detergents. The purple membrane is, therefore, an ideal system with which to test the solubilizing power of new tripod amphiphiles. Rho is a G protein-coupled receptor and represents a large class of important signaling proteins (Sakmar, 1998). A three-dimensional crystal structure of Rho at 2.8 Å resolution was reported while this paper was under review (Palczewski et al., 2000).

The ability of tripod amphiphile **8** to extract BR and Rho from their native membranes was evaluated as a function of amphiphile concentration. Figure 1 shows % solubilization of BR and of Rho, determined in separate experiments from the ratio of retinal absorbances (550 nm for BR or 500 nm for Rho) before and after 300,000 $\times$ *g* centrifugation, as a function of the concentration of **8**. The CMC of **8** (4.5 mM) is located within the transition regions of both solubilization plots, which suggests that it is the amphiphile's ability to form micelles that is responsible for effective protein solubilization from the native membranes. The solubilization behavior of **8** is that of an ideal detergent, according to published criteria of Hjelmeland and Chrambach (1984), because both purple membrane and ROS disk membranes are readily solubilized by **8** above its CMC, and the proteins are stable even at high amphiphile concentrations (up to 40 mM). The data in Figure 1 were obtained after 30 min mixing between **8** and the membrane preparations. In contrast, the conventional detergent reported to be most effective for extracting BR from purple membrane, Triton X-100, requires 20 h for complete protein solubilization (Dencher & Heyn, 1978). More than 95% of the protein embedded in purple membrane or ROS disk membrane is successfully extracted by **8** above the CMC.

### *Circular dichroism and sedimentation equilibrium studies with BR*

To show that tripod amphiphile **8** can solubilize membrane proteins in a monomeric state, we performed CD measurements and

**Table 1.** *CMC values of tripod amphiphiles* [a]

| Amphiphile | CMC (mM) |
|---|---|
| 1 [b] | 3.0 |
| 6 | 1.2 |
| 7 | 7.5 |
| 8 | 4.5 |
| 9 | 3.0 |
| 10 | 2.5 |

[a] Determined by fluorescent dye solubilization, as described in the text.
[b] McQuade et al. (2000)

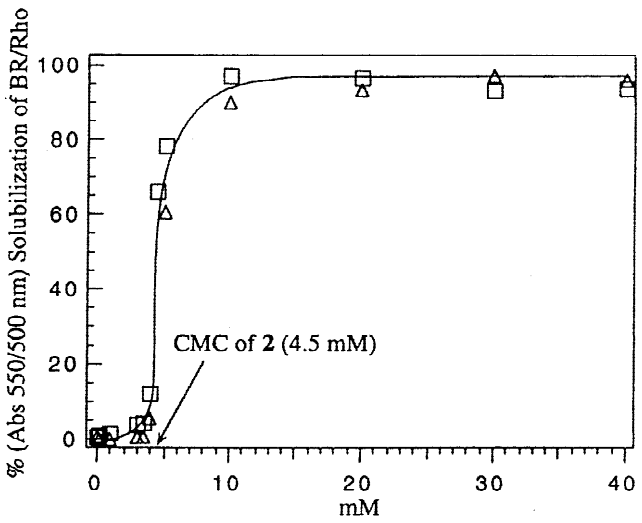



**Fig. 1.** Solubilization of BR from purple membrane (squares) and Rho from ROS disk membrane (triangles) as a function of the concentration of tripod amphiphile **8**. Amphiphile to protein ratios (mol amphiphile/mol protein) were above 1,000 for all except 0 mM samples. After mixing the membranes with solutions containing various amounts of **8**, samples were incubated in the dark for 30 min at room temperature. The percent of solubilized protein was estimated from the ratios of retinal absorbances (BR: 550 nm, Rho: 500 nm) measured before and after 40 min 300,000 $\times$ *g* centrifugation. The curve is arbitrary.

sedimentation equilibrium studies. BR occurs in the purple membrane as a trimeric cluster, and achieving a monomeric state in aqueous solution without protein denaturation can be difficult. It has been demonstrated that generation of monomeric BR from the purple membrane with Triton X-100 can be followed by the decrease in the asymmetric sigmoidal (exciton coupling) CD signal of BR between 450 and 700 nm (Heyn et al., 1975). Exciton coupling arises for native purple membrane because of the close spacing and specific arrangement among neighboring chromophores. Using the CD method, we found that at least 5 h is required to generate monomeric BR with Triton X-100 (data not shown). In contrast, when the purple membrane was dissolved with tripod amphiphile **8**, complete loss of exciton coupling occurred in less than 30 min (the limit of our CD measurement). No appreciable loss in the absorbance at 550 nm was observed during the experiment, indicating that BR monomers remained largely in the native tertiary structure during solubilization. The very rapid generation of monomeric BR by **8** seems to suggest that this amphiphile is "harsh," like conventional N-oxide detergents; however, unlike conventional N-oxide detergents (e.g., LDAO), the tripod amphiphile does not disrupt BR tertiary structure under these conditions. As will be discussed below, the rapidity with which the tripod amphiphile replaces native membrane lipids is auspicious with regard to removing lipids or exchanging detergents from the solubilized protein.

Although the CD studies indicate that **8** rapidly disrupts the regular array of BR molecules in the purple membrane, analytical ultracentrifugation is required to determine whether BR is monomeric when solubilized by this tripod amphiphile. Samples of solubilized BR were prepared in 3, 8, or 10 mM **8** (recall that the CMC of **8** is 4.5 mM). The sedimentation equilibrium data were analyzed by nonlinear regression analysis and by a linear fitting method, both of which suggested that the 8 and 10 mM samples contained predominantly monomeric species. Figure 2 shows $(\ln c)/\omega^2$ vs. $r^2$ plots ($\omega$ is angular velocity, $c$ is BR concentration, and $r$ is radial distance from the center of the rotor) for the 8 and 10 mM samples at two different centrifugation speeds, 15,600 and 18,000 rpm. All of the 8 and 10 mM plots show a linear relationship between $(\ln c)/\omega^2$ and $r^2$ with similar slopes, indicating the presence of monodisperse protein–micelle complexes. The reduced molecular weight was calculated to be 10,420 ± 31 for these samples. In contrast, samples containing 3 mM tripod amphiphile (below the CMC) showed large amounts of aggregated protein, which sedimented to the bottom of the cell during the experiment.

The absolute molecular weight of BR in the BR-**8** complex was deduced from the sedimentation equilibrium results as previously described (Reynolds & Stoeckenius, 1977). In this analysis, the reduced molecular weight determined from sedimentation equilibrium measurements is taken as the sum of the BR, amphiphile, and lipid molecules that comprise the aggregate (Equation 1). This analysis requires one to know the number of lipid and amphiphile molecules bound to BR, which is not easily determined in a direct way. The number of protein-bound amphiphile molecules is commonly assumed to be equal to the aggregation number of the amphiphile in pure micelles (Reynolds & Stoeckenius, 1977). We performed separate analytical ultracentrifugation experiments with pure amphiphile **8** and obtained an aggregation number of 255 for a micelle. Using this value, and with the contribution from lipids ignored, the absolute molecular weight of BR in the protein–micelle complex was calculated to be 29,800 for both 8 and 10 mM

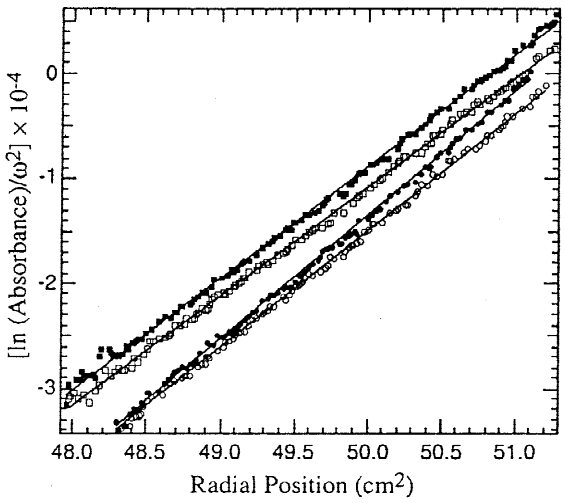



**Fig. 2.** Sedimentation equilibrium data for BR solubilized with tripod amphiphile **8**. The concentration of BR was ~7 $\mu$M for all samples. The concentration of **8** was 8 mM (squares) or 10 mM (circles). The open symbols indicate data obtained at 15,600 rpm, and the filled symbols indicate data obtained at 18,000 rpm. The solid lines are linear square fits to the data. Each set was fit independently; the fact that 8 and 10 mM data display nearly parallel slopes at two different speeds supports the validity of this analysis (e.g., that micelle compressibility is not significant). From the slopes of these plots, reduced molecular weights of BR-amphiphile complexes were calculated to be 10,420 ± 31.

**8** samples. When the contribution from the lipid was included by assuming that the lipid-to-protein ratio of the complex is the same as that of the purple membrane (0.315 g/g; maximum lipid content), the absolute molecular weight calculated for BR in the sedimenting complex decreased slightly to 28,800. The molecular weight of BR calculated from amino acid composition is ~27,100. Thus, the results from our sedimentation equilibrium studies suggest that the protein–micelle complex is a single, nonassociating entity with one BR molecule per complex. The CD and sedimentation equilibration analyses, together, indicate that tripod amphiphile **8** rapidly disrupts the purple membrane and solubilizes BR in a monomeric state.

### *Delipidation of BR and Rho*

Solubilization of a membrane protein from its native membrane typically results in the formation of a protein–amphiphile complex that contains some lipid. The stability of a solubilized membrane protein in a delipidated state is a critical test of an amphiphile's solubilization properties. In a practical sense, delipidated protein is useful for chemical and biochemical studies of membrane proteins because such studies often require reconstitution of membrane structures by adding exogenous lipids to the protein. Systematic membrane protein crystallization studies could also benefit from delipidation. Excess lipid adds complexity to the system, potentially altering the amphiphile phase diagram or leading to formation of precipitate during crystallization. However, complete removal of lipids may not be advantageous because some lipid may be specifically bound to the protein and necessary for the protein's native conformational stability (De Grip et al., 1992).

Delipidation of solubilized membrane proteins is commonly performed using solid supports. We used the a method (Schertler

et al., 1993) in which an anion-exchange resin is used to immobilize BR, and lipids are then removed by washing the immobilized protein with aqueous amphiphile solution. This method can also be used to exchange detergents or to concentrate protein, both of which are important operations in preparing samples for membrane protein crystallization.

Figure 3 summarizes delipidation results for BR and Rho. BR solubilized with **8** was immobilized on a quaternary ammonium anion-exchange resin (Q-sepharose). The resin was washed with 8–15 mM aqueous **8**, and the BR was then eluted with a solution containing **8** and 250 mM sodium acetate. Figure 3A shows the elution profiles of BR and phospholipid. During the initial wash process, part of the immobilized BR (20–40%) was eluted, indicating the existence of a population of protein–detergent complex with low affinity for the resin. Schertler et al. recorded similar observations and reported that the lower affinity fraction contained BR isoforms with truncated C-termini (Schertler et al., 1993). However, when we performed SDS-PAGE analysis on the eluted proteins, the proteins from the two elutions showed similar molecular weight. In addition, the amount of the lower affinity fraction increased as the concentration of the amphiphile in the wash solution was increased. Recently, the observation of two protein bands during BR purification with phenylsepharose columns has been described and attributed to different BR aggregation states in

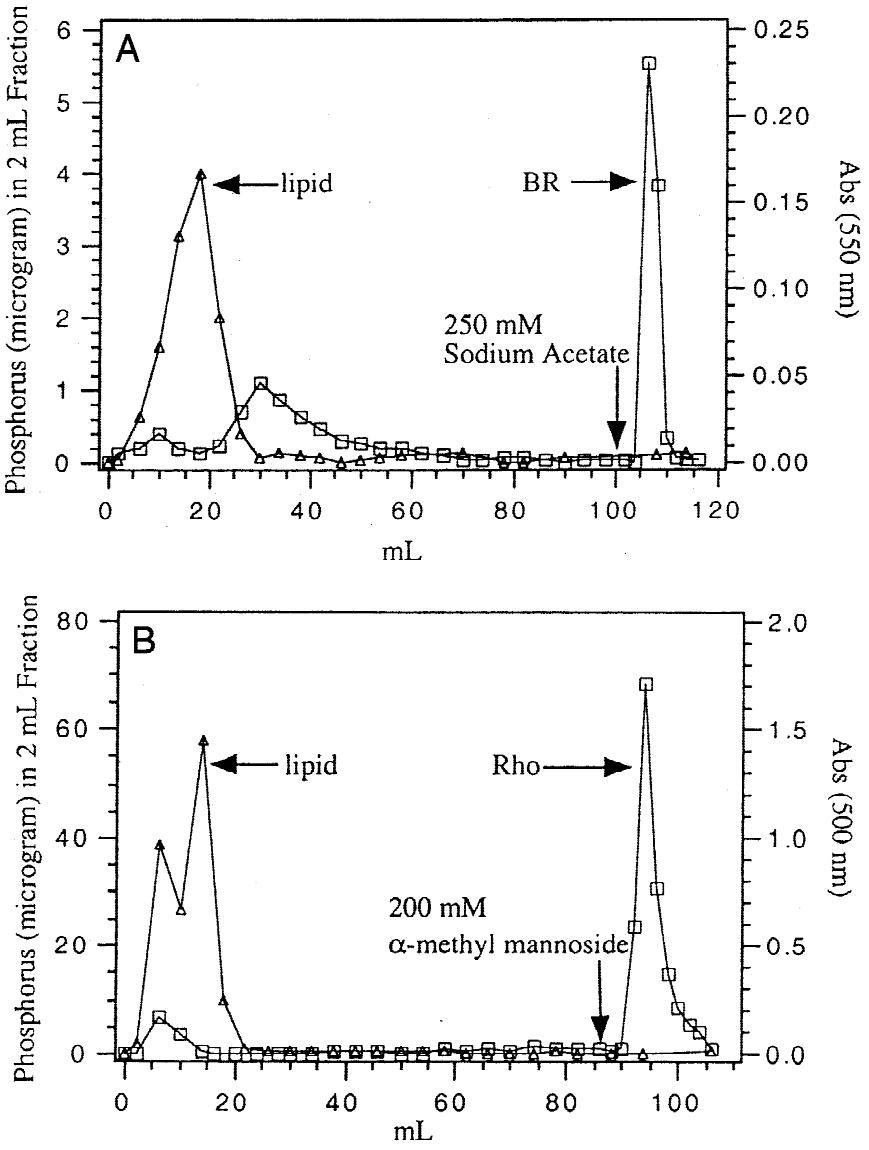



**Fig. 3.** Delipidation of (**A**) BR and (**B**) Rho by chromatography. **A:** BR was purified with Q-sepharose anion-exchange resin, and the delipidated protein was eluted with 250 mM of sodium acetate. **B:** Rho was purified with Con-A sepharose resin, and delipidated Rho was eluted with 250 mM of $\alpha$-methylmannoside. All the solutions used in the column purifications contained 10 mM **8**. Flow rate was fixed at 5 mL/h throughout the experiment, and 2 mL fractions were collected. Protein content (squares) of each fraction was obtained from optical absorbances at 550 nm for BR or at 500 nm for Rho. Phospholipid content (triangles) was determined from the phosphomolybdate complex, as previously described (McClare, 1971; Miercke et al., 1989).

BR–detergent complexes (Lopez et al., 1999). However, our analytical ultracentrifugation results suggest that most the BR is in a monomeric state under these conditions. Observation of two protein bands in column elutions has also been reported for halorhodopsin during purification with phenylsepharose columns (Corcelli et al., 1996). The precise origin of the low affinity protein fraction in our delipidation studies is not clear, but we suspect that variations in BR's affinity for the ion-exchange resin result from differences in the lipid content of protein–detergent–lipid complexes. No loss of retinal from BR occurred during the chromatography, as judged by our failure to detect free retinal (absorbance at 380 nm) in any of the column elutions.

Phospholipid analysis of BR solubilized from purple membrane before ion exchange chromatography indicated the presence of about 10 mol of phosphorus per mole of BR. This value is close to the reported phosphorus content of the purple membrane (Bakker & Caplan, 1978; Miercke et al., 1989). As can be seen from the elution profile (Fig. 3A), most of the phospholipid was eluted during the first 30 mL wash. The purified BR fractions contained less than 1% of the phospholipid originally present in the purple membrane.

In the case of Rho, Con-A sepharose resin was used for affinity column purification following a reported method (DeGrip, 1982a). The elution profiles of phospholipid and Rho (Fig. 3B) are similar to those seen for the BR purification. Most of the phospholipid was eluted in the first 10 mL wash, and the purified Rho, eluted by a solution containing 250 mM $\alpha$-methyl mannoside and **8**, contained $<$0.1% of its original lipid content.

Successful delipidation can be attributed to the high exchangeability and nondenaturing characteristics of tripod amphiphile **8**. A "mild" detergent such as digitonin is not suitable for this type of delipidation, presumably because the exchange of amphiphiles between the pure micelle and resin bound micelle is too slow (DeGrip, 1982a). On the other hand, "harsh" conventional detergents can denature BR and/or Rho under these conditions.

### *Long-term stability of solubilized BR and Rho*

Protein crystals often require weeks to grow, and long-term maintenance of the native state in soluble form is crucial for obtaining high quality crystals of membrane proteins. We have assessed the long-term stability of BR and Rho solubilized by **8** by monitoring the change in absorbance of the retinal chromophore over a 20-day period. The protein stability determined in this fashion is the "functional stability" relevant for crystallization trials. The stability studies were conducted at pH 5.8 for BR and pH 6.9 for Rho. Three different amphiphile concentrations were used (4.4, 8.8, or 17.6 mM), and all solutions were stored at 4 °C in the dark. The results for BR and Rho are shown in Figure 4A and 4B, respectively. Both BR and Rho are highly stable in the presence of 8.8 and 17.6 mM **8**. Less than 10% of the original retinal absorbance is lost over 20 days. Both proteins were less stable in 4.4 mM **8** with more than 30% of initial absorbance lost over 20 days. This lowest concentration is very close to the CMC of **8** (4.5 mM), and this result suggests that fully micellar amphiphile is required for maximum protein stability. Figure 4 (filled symbols) shows that removal of lipids from the protein–micelle complexes has almost no effect on the protein stability. This behavior indicates that the tripod amphiphile is completely competent for maintaining BR and Rho in soluble form.

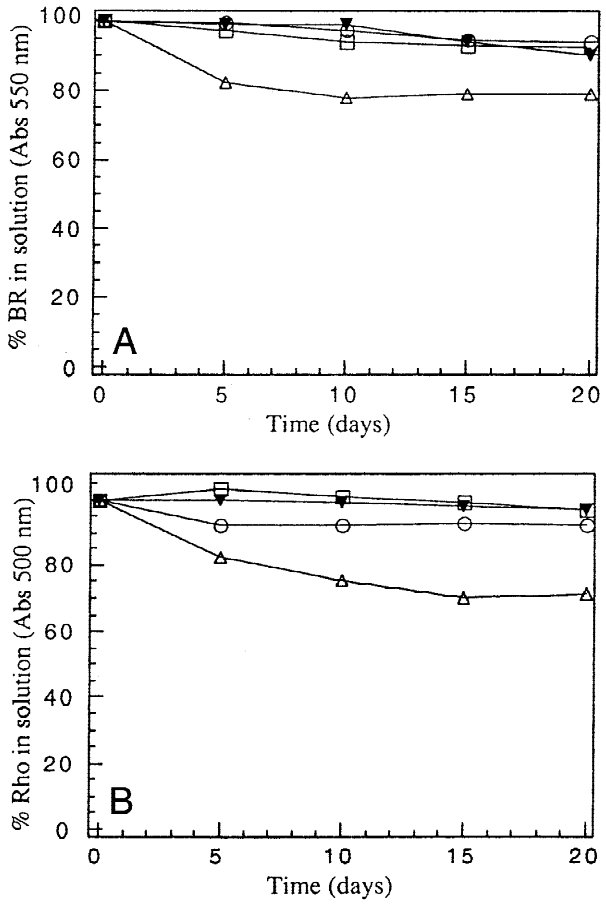



**Fig. 4.** Long-term stability of (**A**) BR and (**B**) Rho in the presence of tripod amphiphile **8**. Long-term stability of BR and Rho was studied in solutions containing 4.4 mM (open triangles), 8.8 mM (open circles), or 17.6 mM (open squares) **8**. These samples were prepared by direct solubilization of proteins from their native membranes and contain lipids from the membranes. Stability of delipidated samples of BR and Rho (in 10 mM **8**, column elutions from Fig. 3) are also shown (filled inverted triangles). All samples were stored in the dark at 4 °C, and % active protein was determined at five-day intervals from retinal absorbances of separate samples. All spectroscopic measurements were acquired immediately after 30 min centrifugation (300,000 $\times$ $g$) at 4 °C, to minimize background scattering.

## Conclusion

There have been relatively few documented attempts to create unconventional amphiphiles for membrane protein manipulation; exploratory efforts of this type are important because development of new amphiphile architectures (e.g., tripod amphiphiles) could expand the range of membrane proteins for which high quality crystals can be grown. We have shown that tripod amphiphile **8** is a good candidate for membrane protein crystallization by demonstrating that **8** can (1) dissolve BR and Rho in monomeric form, (2) be used in conjunction with ion-exchange or affinity column chromatography for delipidation or detergent exchange purposes, and (3) maintain BR and Rho in stable, delipidated, native-like forms for long periods of time.

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