Correlation Between Surfactant/Micelle Structure and the Stability of Bacteriorhodopsin in Solution

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ABSTRACT The rate of solubilization and isothermal bleaching of bacteriorhodopsin (bR) in a series of nine alkylammonium surfactants is studied by using time-resolved optical spectroscopy. The surfactant series $RN^+R'_3$ covers a range in tail length (R = $C_{12}H_{25}$, $C_{14}H_{29}$, or $C_{16}H_{33}$) and headgroup size and hydrophobicity (R' = CH_3, C_2H_5, or C_3H_7). The rate of bleaching increases initially with increasing surfactant concentration but decreases at higher concentrations. Possible explanations for this behavior are discussed. The kinetic data are consistent with the penetration of the surfactant into the protein interior. Interaction of the surfactants with the protein is a complicated, multistep process, and the rate curves are a function of at least four variables: 1) the micellar environment, 2) the length of the surfactant tail, 3) the size of the headgroup, and 4) the hydrophobicity of the headgroup. Our data provide new insights into the molecular characteristics that help define the performance of surfactants in the solubilization and denaturation of membrane-bound proteins.

INTRODUCTION

Micellar solubilization is a standard procedure in the stabilization and purification of membrane proteins (Hjelmeland, 1990; Marston and Hartley, 1990; Neugebauer, 1990). In addition, the regeneration of functionalized proteins is carried out extensively by using micellar media. Despite the importance of these endeavors in protein research, the choice of an optimal surfactant remains a trial and error process. Numerous studies on the effect of solubilization on the activity of proteins have been carried out (Endo and Oya, 1989; Fendler and Fendler, 1975; Kijima et al., 1990), but the mechanistic details have not been resolved. The present series of papers is aimed at unveiling the specific molecular interactions that are relevant to protein solubilization with the hope that these data will lead to a more comprehensive understanding of this important process. Ultimately it is hoped that new surfactants can be designed that are optimized not only for protein solubilization but also for maintaining functionality. Our studies are carried out on bacteriorhodopsin, a membrane-bound protein that exhibits an unusual sensitivity to micelle environment and for which the solubilization and denaturation process can be followed with quantitative precision by using time-resolved optical methods.

Bacteriorhodopsin (molecular mass, 26 kDa), the only protein in the purple membrane (PM) of *Halobacterium halobium* (also called *Halobacterium salinarium*), forms a two-dimensional hexagonal lattice in the membrane (Jonas et al., 1990). The bR polypeptide spans the membrane seven times via α-helical segments separated by small loop regions (Henderson et al., 1990; Henderson and Unwin, 1975). The characteristic λ_{max} of bR at 568 nm is due to the retinal moiety, which is located in a pocket in the interior of the membrane. The PM is made up of 25% lipids (Oesterhelt and Stoeckenius, 1971), of which 60% are phospholipids and 30% are glycolipids (Kushwada et al., 1976), with a ratio of about 10 lipids/bR (Glaesser et al., 1985). Bacteriorhodopsin by itself is extremely hydrophobic (Engelman and Zaccai, 1980). It is soluble in water only in the presence of its native lipids or some other external solubilizing agent (Miercke et al., 1989; Stoeckenius and Bogolmoni, 1982). Micelles have been used extensively to mimic the lipid bilayer environment of bR and may provide mechanistic details of hydrophobic and electrostatic interactions in the membrane. Bacteriorhodopsin has been successfully incorporated into a variety of micelles, including Triton X-100 (Dencher and Heyn, 1978; Milder et al., 1991; Seigneuret et al., 1991), OEGDE (Milder et al., 1991), DM (Milder et al., 1991), CHAPS (Seigneuret et al., 1991), CHAPSO (Milder et al., 1991), OG (Dencher and Heyn, 1978; Milder et al., 1991; Seigneuret et al., 1991), and OTG (Seigneuret et al., 1991). Although bR is stable in its native lipids, bleaching occurs even in the most stable micellar environments studied to date (Massotte and Aghion, 1991; Naito et al., 1981). The bleaching process is enhanced by light (Massotte and Aghion, 1991; Naito et al., 1981). (We note that the term "bleaching" as used here describes the isothermal denaturation of bacteriorhodopsin as monitored by a blue shift in the chromophore absorption band that accompanies extrac-

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Abbreviations used: The alkylammonium surfactants are abbreviated by C_n-R, where *n* is the number of carbon atoms in the aliphatic tail and R references the three identical hydrocarbon groups (Me = methyl, Et = ethyl, Pr, Pro = propyl) attached to the nitrogen atom in the headgroup. Thus, C_{14}-Et is tetradecyl triethyl ammonium [$C_{14}H_{29}$-$N(CH_2CH_3)_3$]. ATR, all-*trans* retinal; bR, light-adapted bacteriorhodopsin; CMC, critical micelle concentration; PM, purple membrane containing bacteriorhodopsin; OEGDE, octyl ethylene glycol dodecyl ether; DM, dodecyl maltoside; CHAPSO, 3-[(3 cholamidopropyl) dimethylammonio]-2-hydroxy-1-propane sulfonate; OG, octyl glycopyranoside; OTG, octylthio glucopyranoside; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; SDS, sodium dodecyl sulfate; OPEO, octyl phenol ethylene oxide.

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tion of the solvated chromophore from the binding site. This surfactant-induced bleaching process should not be confused with the photochemical process that follows light activation of the vertebrate visual pigment rhodopsin.) Solubilization in micelles has also been found to be a useful method for delipidating bR. For example, 90% lipid-free bR can be prepared by dialysis of bR solubilized in dodecyl trimethyl ammonium bromide at pH 3 (Happe and Overath, 1976). A similar extent of delipidation can be achieved by using successive incubations in CHAPS and DM at pH 5 (Seigneuret et al., 1991). Micelles have also been used in the renaturation of bacterioopsin (protein minus chromophore) and gave variable yields according to the type of micelles/mixed micelles used (Renthal et al., 1990a,b). For example, regeneration of up to ϵ_{550} = 40,000 M^{-1} cm^{-1} was obtained in 2.3 mM or 6.5 mM CHAPS with 6.9 mM SDS, 4.5 mM dihexanoyl-phosphotidylcholine in 0.16 M NaCl at pH 6, but ϵ_{550} was only 10,000 M^{-1} cm^{-1} for an equimolar mixture of CHAPS and SDS at the same pH (Renthal et al., 1990a,b).

Bacteriorhodopsin is an excellent candidate for this study because 1) the λ_{max} for light-adapted bR is 568 nm, which is not complicated by the absorbance for free and solubilized retinal (λ_{max} = 381(EtOH) and 390 nm, respectively); 2) bR exhibits a large absorptivity (ϵ_{568} = 64,000 M^{-1} cm^{-1} for the light-adapted state), which allows for the use of very low concentrations of the protein ($\sim\mu$M) that will essentially not perturb the intrinsic micellization properties of the surfactant; 3) the change in A_{568} can be easily monitored spectrophotometrically, and the kinetics can be related to the stability of the protein. In this paper, a series of alkylammonium surfactants was chosen for study, as they can be prepared in high purity and the size of the headgroup and length of the long hydrocarbon chain can be varied systematically. Space-filling models of all-*trans* retinal (ATR), the chromophore that is bound to the protein via a protonated Schiff base linkage, and selected surfactants are shown in Fig. 1.

MATERIALS AND METHODS

The trialkylamines were purchased from Fluka (purum, catalog nos. 92260, 90342, and 93240). The long-chain alkyl bromides were purchased from Aldrich (catalog nos. B6, 555-1; 19, 533-2; and 23, 445-1) and were of pure grade. Ethanol (Aldrich) and ether (Fluka) used in the preparation of the surfactants were reagent grade and anhydrous. Millipore deionized and distilled water was used throughout the kinetic experiments described in this paper.

Preparation of bacteriorhodopsin

The bacteriorhodopsin protein was isolated from two "overproducing" variants of *Halobacterium halobium*. One of the variants (strain SP-9) was kindly provided to us by John Spudich. A different overproducing variant developed by J. A. Stuart was also used for comparison purposes with identical results. Whereas the wild-type bacteria will only produce bacteriorhodopsin in its native form under the appropriate physiological conditions (anaerobic conditions in the presence of steady illumination), the above variants produce large quantities of purple membrane with high concentrations of dissolved oxygen and without illumination. Growth conditions followed those used previously to minimize the formation of non-fully developed protein (see discussion by Stuart et al., 1995). The bacteria were grown at 40°C, and the growth medium was prepared by adding the following reagents per liter of solution in the order given: NaCl (250 g), $MgSO_4$ (9.77 g), KCl (2 g), NH_4Cl (5 g), sodium citrate·$2H_2O$ (3 g), glycerol (1 ml), KH_2PO_4 (100 mg), anhydrous $CaCl_2$ (0.2 g), and Oxoid bacteriological peptone L-37 (Oxoid Ltd., Basingstoke, Hampshire, England) (10 g). The broth was adjusted to a pH between 7.0 and 7.4 by adding solid NaOH pellets. After the fermenter equilibrated to a temperature of 40°C, it was inoculated with 200–250 ml of the variant bacterial culture. The broth was continuously aerated at 3.0 liters/min, and rotational

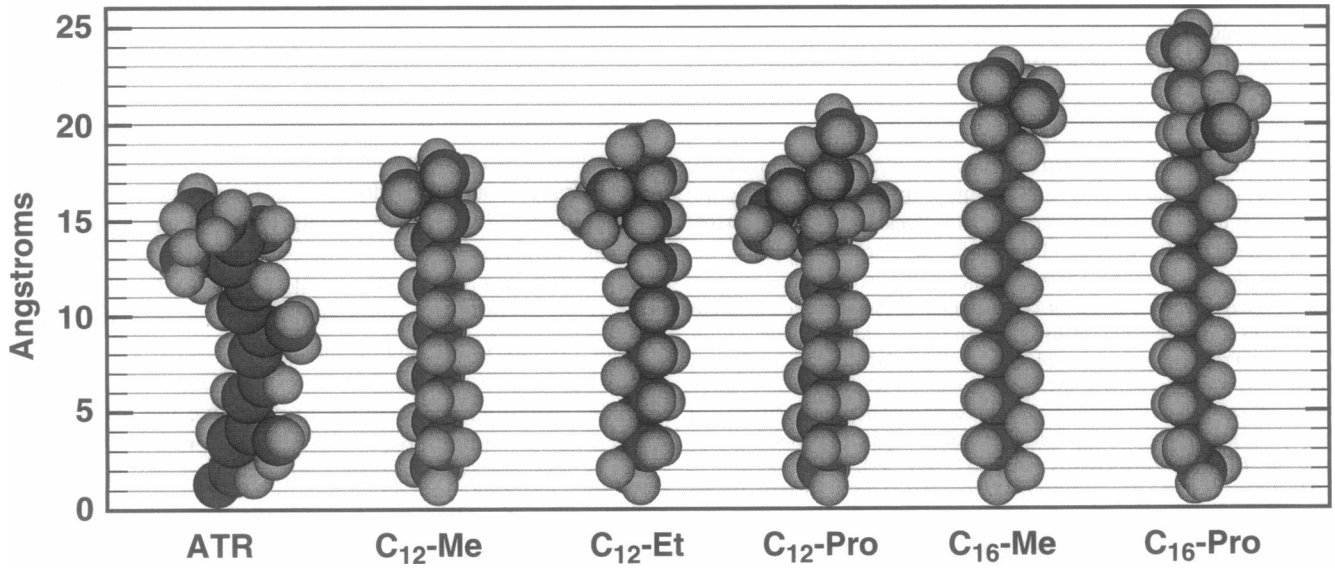

FIGURE 1 Structure of all-*trans* retinal (ATR) and selected surfactants studied in this paper. The C_{16}-Me surfactant is the most potent in the bleaching of bR, whereas C_{12}-Et provides the most stable solubilization environment for the protein.

agitation at about 300 rpm was maintained throughout the ~7-day growth cycle. Antifoam A (Dow Corning) was used as needed to control foaming. Growth of the bacteria was monitored by observing the absorbance at 660 nm, and the purple membrane production was monitored by observing the absorbance at 570 nm. Cell harvesting was performed when the culture reached the pre-stationary growth phase (at an absorbance at 570 nm of about 3.0 absorbance units). The protein (purple membrane) was isolated and purified by the following nine-step process recommended to us by Gregory Dewey (University of Denver), with modifications recommended by J. A. Stuart (manuscript to be published): 1) The cells were spun down by centrifuging the culture broth at 10,000 rpm for 10 min, the pellet was washed with 4 M NaCl and resuspended in a minimal amount of 4 M NaCl; 2) the resuspended pellet was adjusted to 1 mM in $MgSO_4$, 0.1 mg/ml DNase I was added, and the solution was stirred for ~15 h at room temperature; 3) the resulting solution was then dialyzed against distilled water at 4°C for 24 h, and the water was changed a minimum of six times; 4) the dialysate was spun down at 5000 rpm for 15 min, the pellet at the bottom (cell debris) was thrown out, and the supernatant (suspended purple membrane) was pelleted at 15,000 rpm for 20 min; 5) the pellet (which now contains the purple membrane) was resuspended in minimal distilled water, and 7-ml portions of the concentrated sample were placed on a sucrose gradient with the following profile (4 ml: 20% sucrose; 13 ml: 36% sucrose; 9 ml: 40% sucrose; 4 ml: 46% sucrose) and ultracentrifuged using a swinging bucket rotor for 18 h at 20,000 rpm at 4°C; 6) the purple membrane residing in the 40% sucrose layer was collected, and care was taken to strip off the orange band of carotenoids in the upper 36% sucrose layer; 7) the purple membrane was concentrated by pelleting it at 40,000 rpm for 1 h at 4°C and was resuspended in a minimal amount of distilled water; 8) the solution was dialyzed against distilled water at 4°C for 24 h (changing the water six times); 9) the dialysate was pelleted at 43,000 rpm for 1 h at 4°C and resuspended in a minimal amount of distilled water. After the sucrose density gradient, the purple membrane was occasionally stored in the ~40% sucrose before carrying out steps 7 and 8. We have found that the purple membrane is stable in ~40% sucrose for extended periods of time (over 6 months).

Preparation of surfactants

The procedure for the preparation of the alkylammonium surfactants is well established (Venable and Nauman, 1964). A twofold molar excess of the appropriate trialkylamine was mixed with the long-chain alkyl bromide of choice suspended in ethanol and refluxed for a minimum of 36 h. The solution was then concentrated by evaporating the solvent off by using a rotary evaporator (model RE-121; Buchi). Ether was added to precipitate the product. Sometimes the mixture had to be chilled to ensure precipitation. This process was usually required for surfactants with bigger headgroups. The white crystalline products were recrystallized at least three times from ethanol-ether and kept under vacuum overnight in a desiccator before use. Further recrystallization did not alter the kinetic results described in this paper.

Kinetic studies

All kinetic data were collected by using a Shimadzu UV-Vis-NIR spectrophotometer (model UV-3101 PC). The surfactant solution (1.2 ml and above the critical micellar concentration (CMC)) in 0.1 M Tris-HCl buffer, pH 7.20) was introduced into a 1-cm cuvette with built-in side chambers that provide temperature control via water circulation. The solution was left to stand for about 15 min to thermally equilibrate with the circulating water at (25 ± 0.1)°C. Then 0.1 ml of the stock aqueous bR, which was continually exposed to light and separately thermally equilibrated to the same temperature as the micellar solution, was injected with a microsyringe (Hamilton) accurate to ±1 μl, followed by rapid stirring with a glass rod to ensure homogeneous mixing of the solution. The rate of bleaching of bR was followed at 568 nm for at least five half-lives. Kinetic data were checked for all of the surfactants by doing the measurements for two separate syntheses of the compounds.

RESULTS

The alkylammonium surfactants used in this paper induced relatively rapid bleaching of bR in aqueous solutions, as shown in Fig. 2. After the injection of bR into the micellar solution, a rapid decrease of about 30–50% in the molar absorptivity at 568 nm (A_{568}) was observed for all of the surfactants studied, with a concomitant shift in the absorbance maximum from 568 nm (characteristic of light-adapted native bR) to lower wavelength, finally reaching a λ_{max} value of 555 nm. The longer the surfactant, the faster

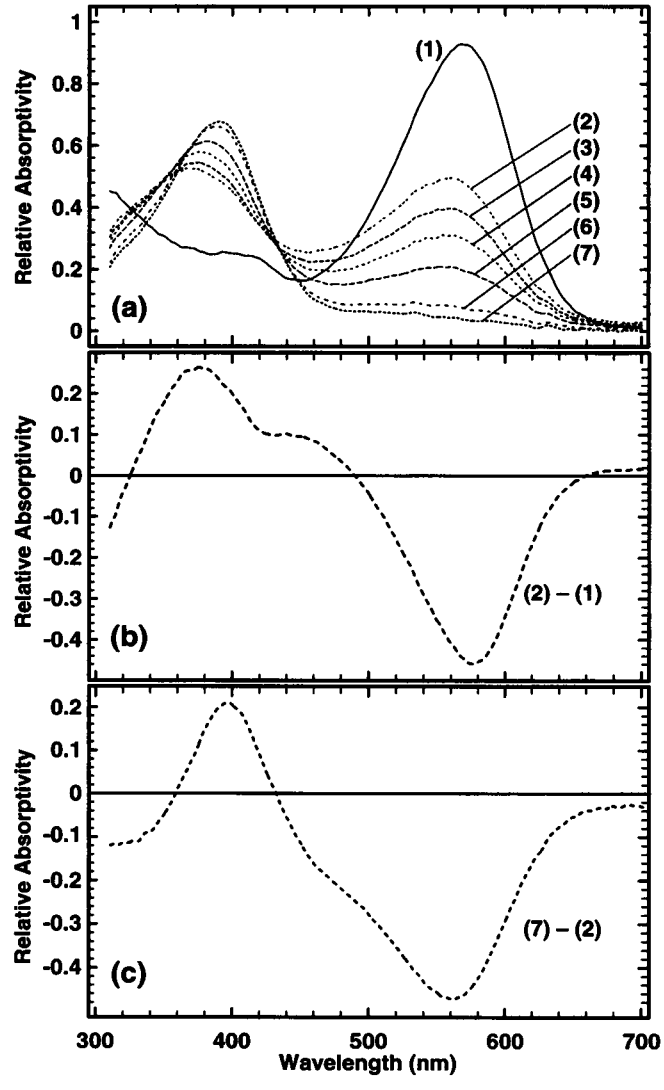

FIGURE 2 (*a*) Spectra of 1.2 μM bR in 0.138 M C_{12}-Me micellar solution (0.1 M Tris-HCl buffer, pH = 7.20, T = 25°C) as a function of time. The spectra were taken at approximately 10 s (2), 70 s (3), 200 s (4), 400 s (5), 1000 s (6), and 1500 s (7) after mixing. A reference spectrum of 1.2 μM bR in the same buffer but no surfactant is shown in (1). *b* and *c* present difference spectra with reference to the data presented in *a*. (*b*) Spectrum (2) minus spectrum (1). (*c*) Spectrum (7) minus spectrum (2).

the initial drop in absorbance, indicating more rapid solubilization of the protein for a bigger micelle. For the C_{16} surfactants it was not possible to detect the early portion of the curve with our methods because of the speed at which A_{568} decays. (Bacteriorhodopsin solubilized in micelles has absorbance maxima in the region of 550 to 555 nm. For example, the λ_{max} values for light-adapted bR solubilized in Triton X-100, OG, and OPEO are 555, 554, and 551 nm, respectively (Massotte and Aghion, 1991).) There is no shift in the absorbance maximum for bR in aqueous cetyltrimethylammonium solutions below the CMC (Padros et al., 1984). In addition, bR regenerated in micelles has ϵ_{max} values that are about 40% lower than for native bR (Renthal et al., 1990a,b; Seigneuret et al., 1991). For example, by comparing the visible absorbances of micellized bR with the near-UV spectra for the same samples denatured in SDS, ϵ_{max} for bR in Triton X-100, OTG, and DM was estimated to be 44,000, 43,000, and 41,000 M^{-1} cm^{-1}, respectively (Seigneuret et al., 1991). We conclude that bR is solubilized in the alkylammonium micellar solutions, as would be expected, because high concentrations of the surfactants were used (ratio of surfactant:bR ranged from 4000 to 398,000). The absorbance at 568 nm eventually decays to the baseline, with corresponding complete recovery of free retinal absorbance at 390 nm. This observation, when considered together with the constant magnitude of A_{390} for a fixed concentration of bR (irrespective of the type of surfactant used), indicates the nonreversible nature of the bleaching process. This new absorbance maximum (390 nm) is characteristic of solubilized free retinal (London and Khorana, 1982) and is indicative of the extraction of the chromophore from the binding site. Our results are different from those for the regeneration of bR in the presence of non-ionic or zwitterionic mixed micelles, where regeneration of the protein is indicated by an increase in the absorbance of the solubilized protein at ~550 nm (Renthal et al., 1990a). Absorbance at 550 nm for bR in the quaternary ammonium micelles upon completion of the bleaching process gives only a baseline, indicating that these micelles, in the absence of extraneous surfactants/compounds, are unable to regenerate or sustain the native protein structure. Addition of excess free retinal to the bleached protein solution did not regenerate the purple color of the protein. This observation suggests that the bleaching process is not due solely to a direct displacement of the retinyl chromophore and indicates denaturation of the protein configuration. After the initial decrease, the decay of A_{568} follows first-order kinetics, as exemplified by the linear $\ln[-(A_\infty - A_t)]$ versus time plots (see Fig. 3). The observed first-order rate constants (k_{obs}) were determined from the slopes of the plots in the usual manner. Kinetic rates determined from the rise in A_{390} give values that are within experimental error of those determined at 568 nm, and together with the monoisosbestic nature of the bleaching of solubilized bR, suggest the existence of only two species of the protein, solubilized bR and solubilized bleached protein. Although the fits for C_{16}-Me and C_{14}-Me were not quite as good (±10%), because of

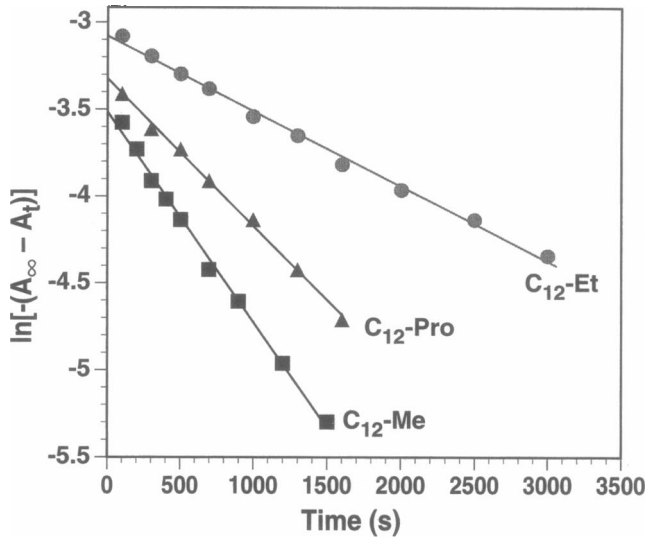

FIGURE 3 First-order plots for the isothermal bleaching of 1.2 μM bR in 92.0 mM C_{12}-R micellar solutions (0.1 M Tris-HCl buffer, pH = 7.20, T = 25°C.).

rapid bleaching, the k_{obs} values could still be calculated with confidence. Rates determined at 555 nm and 568 nm were identical to within experimental error for the same initial conditions. Kinetic analyses could not be carried out at very low surfactant concentrations because the solutions turned turbid (fine white particles) upon standing. The solutions cleared up with the addition of higher surfactant concentrations, indicating rapid solubilization of the bleached compound (apoprotein) into the micellar phase. Because bR and its apoprotein have about the same surface potential (Ehrenberg and Meiri, 1983) and molecular weight (the difference being the retinal moiety), it is a reasonable assumption that they will experience similar solubilization. The kinetic studies reported in this paper were carried out by using surfactant concentrations for which there was no detectable turbidity (no increase in the baseline at 800 nm). This observation provides further support that bR is actually solubilized before bleaching.

The rate-versus-concentration profiles for the bleaching of bR in micelle solution follow a similar trend for all of the surfactants studied in this paper (see Figs. 4–7). The rate increases initially with surfactant concentration, but decreases after a certain concentration has been exceeded, giving a characteristic rate maximum value (k_{max}) at a certain concentration (c_{max}). Trends in the maximum values are evident (see Table 1). For the C_n-Me surfactants, a longer surfactant gives a larger k_{max} value at a lower concentration of the surfactant (c_{max}). This trend is also observed for the C_n-Et surfactants; however, as the size of the headgroup is increased to tripropyl ammonium, the tetradecyl surfactant becomes most potent in the bleaching process (Table 1). The relative k_{max} values are also of interest. The tripropyl ammonium headgroup gives the most stabilizing

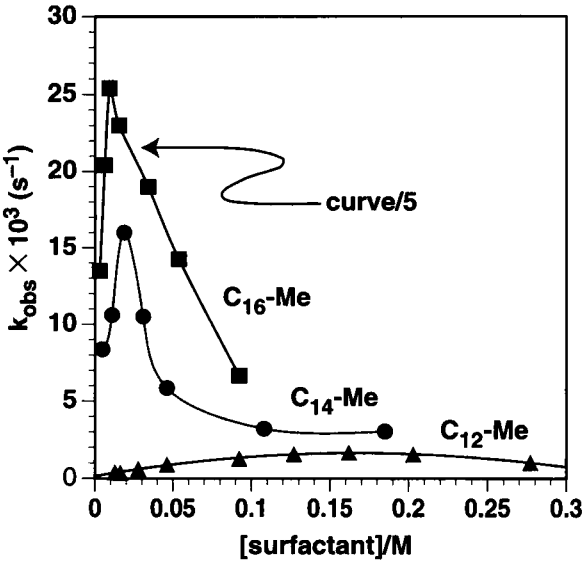

FIGURE 4 Rate-versus-concentration profiles for the isothermal bleaching of 1.2 μM bR in C_n-Me micellar solutions (0.1 M Tris-HCl buffer, pH = 7.20, T = 25°C). The curve for C_{16}-Me is scaled to one-fifth of its actual value in the rate axis as indicated by the curved arrow. The curved lines connecting the data points are drawn to aid visualization of the data.

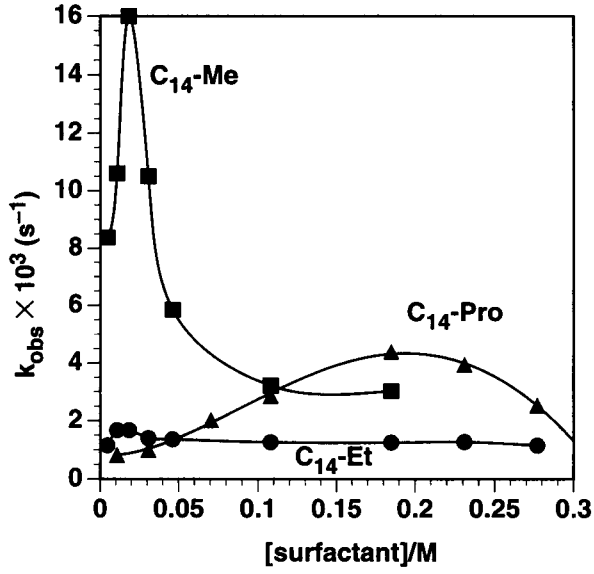

FIGURE 6 Rate-versus-concentration profiles for the isothermal bleaching of 1.2 μM bR in C_{14}-R micellar solutions (0.1 M Tris-HCl buffer, pH = 7.20, T = 25°C). The curved lines connecting the data points are drawn to aid visualization of the data.

solubilization environment for the C_{16} surfactants; however, as the tail length is decreased there is a gradual increase in the relative rate for the tripropyl ammonium headgroup. For example, we note that $k_{max}(C_{16}\text{-Pr}) < k_{max}(C_{16}\text{-Et}) \ll k_{max}(C_{16}\text{-Me})$, but $k_{max}(C_{12}\text{-Pr}) > k_{max}(C_{12}\text{-Me}) > k_{max}(C_{12}\text{-Et})$.

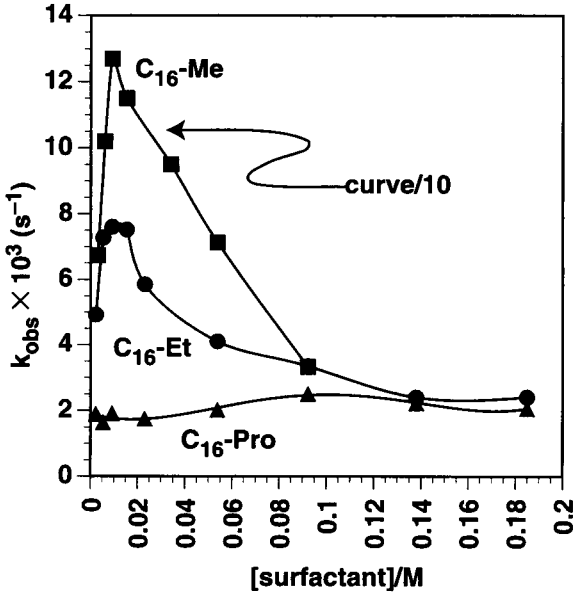

FIGURE 5 Rate-versus-concentration profiles for the bleaching of 1.2 μM bR in C_{16}-R micellar solutions (0.1 M Tris-HCl buffer, pH = 7.20, T = 25°C). The curve for C_{16}-Me is scaled to one-tenth of its actual value in the rate axis. The curved lines connecting the data points are drawn to aid visualization of the data.

DISCUSSION

The chemical structure of bacteriorhodopsin offers a great advantage in the study of the effects of surfactant/micelle on proteins. To a first approximation, bR can be considered as a probe (retinyl chromophore) within a protein, and thus changes in the protein structure or penetration of chemicals into the protein can be detected by time-resolved visible measurement of the probe. Because of the variation in the CMC and the aggregation number of the surfactants, the rate

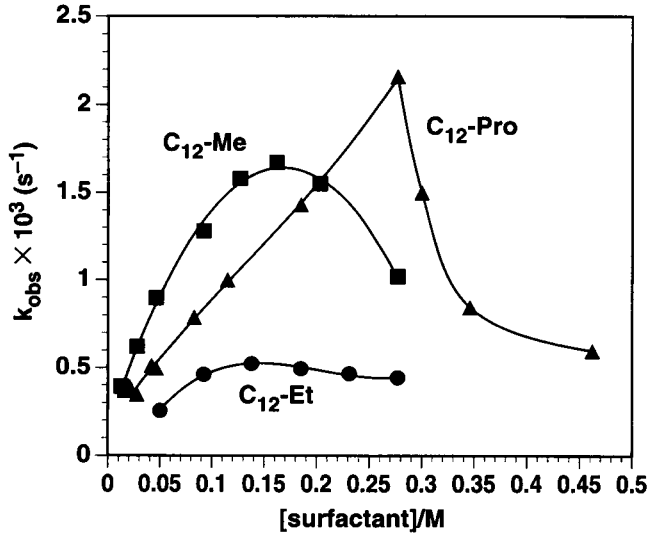

FIGURE 7 Rate-versus-concentration profiles for the isothermal bleaching of 1.2 μM bR in C_{12}-R micellar solutions (0.1 M Tris-HCl buffer, pH = 7.20, T = 25°C). The curved lines connecting the data points are drawn to aid visualization of the data.

TABLE 1 Comparison between selected micellar properties and the kinetic constants for the bleaching of 1.2 μM bR in micellar solution

Surfactant	CMC* (mM)	$N^{\ddagger}$	c_{max} (mM)	$k_{max} \times 10^3$ (s^{-1})
C_{12}-Me	14.4	53	150	1.65
C_{12}-Et	13.6	—	140	0.53
C_{12}-Pr	—	—	270	2.15
C_{14}-Me	3.6	65, 68	15	16
C_{14}-Et	3.2	—	15	1.8
C_{14}-Pr	2.1	—	85	4.3
C_{16}-Me	0.90	90	10	127
C_{16}-Et	0.73	—	10	7.7
C_{16}-Pr	0.46	—	90	2.6

Micellar solution was in 0.1 M Tris-HCl buffer, pH 7.20, at T = 25°C.
*Critical micelle concentration taken from Buckingham et al. (1993).
‡Aggregation number (N) at 0.05 M surfactant concentration taken from Lianos and Zana (1981).

of micellar reactions is best described in terms of the c_{max} and k_{max} values rather than micelle concentration.

The behavior of the k_{obs} values is typical for reactants solubilized into the micellar phase (Bunton and Robinson, 1969; Gobbo et al., 1984; Jagt and Engberts, 1977; Lim et al., 1993; Menger and Portnoy, 1967; Van Senden and Koningsberger, 1966), where the initial increase in the rate is followed by a fall in k_{obs} beyond a certain concentration (c_{max}) of the surfactant. The rate-versus-concentration profiles for the decomposition of bR in the alkylammonium micellar solutions are shown in Figs. 4–7. The increase in the rate before $\{c_{max}, k_{max}\}$ can be ascribed to an increase in micelle concentration and is thus easily understood. In contrast, the subsequent fall in k_{obs} after the surfactant concentration passes c_{max} is not easily explained. Nevertheless, this phenomenon is common in micellar catalysis and has been observed in a number of other systems (Baumrucker et al., 1973; Berndt et al., 1979; Bunton and Robinson, 1969; Gobbo et al., 1984; Jagt and Engberts, 1977; Lim et al., 1993; Menger and Portnoy, 1967; Romsted, 1984; Van Senden and Koningsberger, 1966). Previous studies on systems other than bacteriorhodopsin have yielded the following mechanistic interpretations of the decrease in the rate constant for surfactant concentrations above c_{max}: a) the substrate becomes more deeply buried in a micelle of a larger size and becomes more stable (Bunton and Robinson, 1969); b) the substrate is diluted into a larger micelle (Romsted, 1984); c) the reaction is inhibited by that surfactant counter-ion as the concentration increases (Baumrucker et al., 1973; Berndt et al., 1979); d) the structure of the micelle changes to yield a more stabilizing environment (Berndt et al., 1979); e) a secondary species is partitioned between the bulk phase and the micellar phase (Berndt et al., 1979); and f) the reaction site is sterically shielded (Van Senden and Koningsberger, 1966). As we discuss below, some of the above mechanisms may be relevant to the present experimental data.

Bacteriorhodopsin is negatively charged and has a net charge of −1 (pH 7.4), −4 (pH 6.1), and −6 (pH 6.5) in 10 mM HEPES buffer, as determined by electrophoretic light scattering (Packer et al., 1984), the partitioning of a positively charged spin label (Carmeli et al., 1980), and the change in the apparent pK_a of membrane-bound neutral-red dye (Li and Ni, 1988), respectively. Thus the bromide counter-ion for the surfactants in this paper is not likely to stabilize bR through direct binding with the protein at near-neutral bulk pH values. Anions bind with bR only in very acidic medium (~pH 2) (Renthal et al., 1990b). However, it may induce changes in the micelle size and packing of the surfactant monomers in the micelle. Micelles become larger (Berr et al., 1992; Lianos et al., 1984) and more compact (Lianos et al., 1984; Tanford, 1972) at high detergent concentrations. The same effect can be induced by the addition of salt, because of the screening of the charges of the surfactant headgroup by the salt, which reduces electrostatic repulsion and allows closer packing of the detergent monomers (Shinitzky et al., 1971; Venable and Nauman, 1964). Bacteriorhodopsin cannot be regenerated from bacterioopsin and all-*trans* retinal in SDS, but the addition of CHAPS improves reconstitution significantly (Renthal et al., 1990a). The improved stability of regenerated bR in the mixed micelles of SDS/CHAPS could be due to an increase in the viscosity of the interior of the micelles (microviscosity). It has been observed that the addition of cholesterol to alkylammonium micelles increases the microviscosity of the micelle interior, as indicated by greater polarization in the fluorescence of perylene in the micelles (Shinitzky et al., 1971). Cholesterol-like detergents like sodium taurocholate were also found to have much greater microviscosity than for micelles of SDS (Chen et al., 1975). Thus, the drop in k_{obs} at high surfactant concentrations is consistent with an increasing stability of bR due to a more closely packed micelle structure. Presumably, the more closely packed micelle structure provides a density and intramembrane-like pressure more comparable to the native hexagonal lattice of the purple membrane.

Although a more closely packed micelle may enhance the stability of the protein at high surfactant concentration, other effects are also important. The use of buffers in micellar solutions has been examined previously (Bunton and Minch, 1974; Bunton and Wolfe, 1973). Bunton and Wolfe showed that the relative concentration and reactivity of the buffer species may differ between the micellar and solvent phases (Bunton and Wolfe, 1973). Incorporation of acids into the interior of micelles may increase the dissociation constant of the hydronium ion for the former (Bunton and Minch, 1974), and thus the effective acidity in the micelles may be different from that of the bulk solvent. Solubilized bR decomposes more slowly at low pH (Seigneuret et al., 1991). Thus, the partitioning of the deprotonated buffer species into the micellar phase and consequently into the protein, at high surfactant concentrations, may help increase the concentration of hydronium ions in the vicinity of bR (to maintain charge stability of the protein) and result in a slowdown of the bleaching. However, by titrating the micellar solution to pH 7.00 ± 0.04 with μM

NaOH, in the absence of buffer, we observed the same trend in the rate-versus-concentration profile, and thus the presence of the buffer species is not required for the subsequent fall in the rate constant. Steric shieding of the reaction site may also contribute to the bleaching kinetics. Increased shielding, especially at high detergent concentrations, for example, could make penetration of the surfactants into the protein more restricted (see below).

The concentration at which the rate maximum occurs (c_{max}) increases as the chain length of the surfactant decreases (c_{max} = 0.01, 0.02, and 0.15 M for C_{16}-Me, C_{14}-Me, and C_{12}-Me, respectively) (see Fig. 4). Furthermore, the k_{max} peaks are sharper for the longer surfactants. Both of these observations are likely due to micellar effects and are reminiscent of the micelle-catalyzed effects of the hydrolysis of *p*-nitrophenyldiphenylphosphate in copper-containing micelles of different sizes (Lim et al., 1993).

Length of the surfactant tail

The rate-versus-concentration profiles for the bleaching of bR for surfactants with the methyl headgroup are shown in Fig. 4. The longer surfactant has a higher rate, with an increase of about 8.5 times in the rate maximum (k_{max}) for every addition of two carbons in the long aliphatic chain. This result is not surprising, because bR is extremely hydrophobic (Engelman and Zaccai, 1980) and bigger micelles have greater hydrophobic character. This characteristic is indicated from the lower CMCs for the longer surfactants (CMCs for aqueous C_{16}-Me, C_{14}-Me, and C_{12}-Me are 0.92 mM (Fendler, 1982), 3.51 mM (Venable and Nauman, 1964), and 15 mM (Fendler, 1982), respectively). Longer surfactants give greater rates because of a combination of three effects: a) higher extent of micellization, b) better solubilizing ability for a bigger micelle, and c) better penetration of the surfactant into the protein (further discussed later). "Higher extent of micellization," a term that is frequently used in micellar chemistry, indicates the formation of micelles at a lower concentration of the surfactant, which helps increase the rate, because more surfactants are in the form of micelles. As is established above, solubilization of the protein (reaction of the protein with the micelle, not with the free surfactant) is a prerequisite for the effective bleaching of the protein. Bigger micelles have greater solubilization ability, hence the increase in the rate of bleaching for a longer surfactant. Penetration of the detergent molecule into the protein has been suggested for the bleaching of bR in the presence of SDS micelles (Massotte and Aghion, 1991). The retinal is actually washed away from the protein, which led some investigators to suggest that the surfactant initially occupies a position in the protein that is close to the retinyl chromophore (Massotte and Aghion, 1991). In addition, the purple color of the protein can be regenerated from the blue-colored cation-depleted form of bR (the blue membrane) upon addition of C_{16}-Me below the CMC (Padros et al., 1984). Thus, it appears that the surfactant can enter the protein and the headgroup can occupy (or doubly occupy) one of the cation-binding sites near the chromophore. The importance of local charges in mediating the protein color is well documented (Lugtenberg et al., 1986; Spudich et al., 1986; Stuart et al., 1995). In agreement, our studies suggest that the surfactant may penetrate into the interior of the protein, gain access to the binding site, and interact directly with the chromophore. This chromophore:surfactant interaction is probably dominated by dispersion forces, and can result in the preferential stabilization of the chromophore by the surfactant relative to the stabilization afforded by the protein-binding site. When this latter mechanism is important, the chromophore migrates out of the binding site to a lower free energy environment within the micelle. An analysis of the effect of the headgroup on the bleaching process supports this proposal (see below).

The solution viscosity can be increased by using a) a higher surfactant concentration and/or b) a longer surfactant tail. However, k_{max} is larger for the longer C_n-Me surfactants, in contradiction to the predictions from the effects of solution viscosity on the collisional frequencies of the protein with the micelle. Thus, we conclude that the rate is not limited by the viscosity of the solution.

Size of the surfactant headgroup

The rate profiles for the C_{16} surfactants (Fig. 5) show that a smaller headgroup gives a higher k_{max} value. Although the micellar structure may cause a decrease in the k_{obs} at high surfactant concentrations (see above), the relative rates for the surfactants are not likely due only to a cooperative effect of the whole micelle. This conclusion follows from the following five observations. First, the CMC for a detergent with a propyl headgroup is lower than that observed for a methyl headgroup attached to a tail group with the same chain length. For example, CMCs for C_{16}-Me and C_{16}-Pr are 0.92 mM (Fendler, 1982) and 0.57 mM (Venable and Nauman, 1964), respectively. However, c_{max} for C_{16}-Pr is 0.09 M, which is significantly higher than for C_{16}-Me and C_{16}-Et ($c_{max} \approx 0.01$ M). Second, solubilization of benzene for all of the surfactants with the same chain length is nearly the same under dilute conditions. In contrast, the propyl micelle solubilizes more effectively than the methyl micelle for the same tail length in concentrated solutions. These results were obtained in an excess of benzene (Venable and Nauman, 1964), where solubilization into the interior of the micelle is expected (Eriksson and Gillberg, 1966). The same trend was observed for the solubilization of orange-OT in decyltrialkylammonium micelles (Jacobs and Anacker, 1973). If the influence of the total micellar structure is important in the solubilization and hence bleaching of bR, k_{max} for C_{16}-Pr should be similar or higher than that observed for C_{16}-Me. This comparison should follow the same relationship as the effect of the hydrophobicity of the surfactant tail on the rate maximum. Third, the surfactant with

a smaller headgroup is expected to form a more compact micelle (Tanford, 1972), based on aggregation number (Jacobs and Anacker, 1973; Venable and Nauman, 1964). Because bR may be more stable in a viscous micelle (see discussion above), C_{16}-Pro should be the most potent bleaching surfactant (see Fig. 5). Fourth, micellar properties of alkylammonium surfactants with the same chain length usually vary in a continuous fashion with the size of the headgroup. For example, the aggregation number for the propyl micelle is lower than for the methyl micelle for both C_{10} (Jacobs and Anacker, 1973) and C_{14} (Venable and Nauman, 1964). The CMC for C_{14}-Pr is lower than for C_{14}-Me (Venable and Nauman, 1964), and the trend is also observed for C_{16} (Venable and Nauman, 1964) and C_{10} (Jacobs and Anacker, 1973). The degree of counter-ion dissociation for C_{14}-R increases almost linearly with the increase in the size of R from CH_3 to C_4H_9 (Zana, 1980). However, the relative k_{max} values for the headgroups differ when surfactants with the same tail size are compared (see Figs. 5–7). Fifth, bleaching of bR occurs even at surfactant concentrations below the CMC (Padros et al., 1984), however, at a much slower rate.

The effect of the headgroups in Fig. 5 can be explained in terms of steric hindrance. The hydrated radius (r_H) of the surfactant headgroups can be qualitatively assigned by reference to the corresponding symmetrical quaternary ammonium salt (without the long chain). The r_H values for Me_4N^+, Et_4N^+, and $(n\text{-}Pr)_4N^+$ are 3.67, 4.00, and 4.52 Å, respectively (Nightingale, 1959). There is no definitive measurement of the size of the bacteriorhodopsin proton channel, but neutron diffraction studies (figure 4 of Heyn et al., 1988) and electron cryo-microscopy (figure 18 of Henderson et al., 1990) suggest that the radius of the broader channel is probably close to 5 Å. This value is comparable in size to the r_H of the detergent headgroups. It is thus reasonable to expect steric hindrance to play an important role in limiting access of the surfactant to the retinal binding site. Furthermore, the propyl headgroup is anticipated to experience significant steric hindrance. Thus, the observation that C_{16}-Me exhibits the highest k_{max} follows from the relative ease with which the smaller headgroup surfactant can enter the proton channel to interact with the chromophore. Conversely, the much higher relative c_{max} values for the propyl headgroup surfactants are consistent with the importance of steric interactions in slowing surfactant penetration into the binding site.

Hydrophobicity of the headgroup

The increase in the headgroup size creates two effects: i) increase in radial size and ii) higher hydrophobic character. The surfactant bleaching rates indicate that headgroup hydrophobicity plays a subtle role in determining the rate of bleaching. The rate-versus-concentration profiles for C_{14}-R and C_{12}-R (Figs. 6 and 7, respectively) show an interesting difference when compared to those observed for C_{16}-R (Fig. 5). For example, $k_{obs}[C_{14}\text{-Pr}]$ is lower than $k_{obs}[C_{14}\text{-Et}]$ at concentrations below 0.045 M, but $k_{obs}[C_{14}\text{-Et}] > k_{obs}[C_{14}\text{-Pr}]$ at higher detergent concentrations. The k_{max} for C_{14}-Pr is actually 2.4 times higher than the k_{max} observed for C_{14}-Et. The relative increase in the k_{max} for the propyl surfactant is even more pronounced for C_{12}, the k_{max} for C_{12}-Pr being 1.3 times that for C_{12}-Me and 4.0 times higher than that for C_{12}-Et. It is also observed that the ratios of the k_{max} values as shown in Table 2 become progressively smaller as the chain length decreases.

A comparison between the values of the CMC and aggregation number (N) of the detergent molecules with c_{max} and k_{max} is given in Table 1. Although c_{max} increases with a decrease in the CMC, there is no simple relationship between the two parameters. There is also no apparent trend relating the k_{max} values to the CMCs of the surfactants, indicating the insignificance of the latter in the determination of the rate maximum for our experiments, where high surfactant concentrations were used. The micelle concentration can be estimated by using Eq. 1 (Menger and Portnoy, 1967):

$$[M] = \frac{(C - CMC)}{N}, \quad (1)$$

where [M] = micelle concentration, C = total concentration of detergent, and N is the aggregation number.

The CMC is invariant, as indicated by the constant surface tension for micellar solutions above CMC. By using Eq. 1, [M] is estimated to be 2.6 and 0.10 mM at c_{max} for C_{12}-Me and C_{16}-Me, respectively; however, $k_{max}[C_{16}\text{-Me}]$ is about 80 times higher than $k_{max}[C_{12}\text{-Me}]$. At 0.10 mM C_{12}-Me micelle, k_{max} is $\sim 5 \times 10^{-4}\ s^{-1}$, as compared to 127 s^{-1} for C_{16}-Me at the same micelle concentration. These results indicate that micellar properties play a more significant role than micelle concentration in determining the k_{max}. The rate maximum increases by close to an order of magnitude with each addition of two carbon atoms in the surfactant tail.

If one assumes that the hydrophobic character is proportional only to the number of carbon atoms in the chain, then the relative hydrophobic influence of the headgroup as compared to the detergent tail will be described simply by the ratio of the number of carbon atoms in the headgroup over those in the tail. (The ratios for C_{12}-Pr, C_{14}-Pr, and C_{16}-Pr are 0.75, 0.64, and 0.56, respectively.) Based on this simple approach, C_{12}-Pr can be expected to be more strongly influenced by the headgroup as compared to C_{14}-Pr

TABLE 2 Ratios of the k_{max} values for the bleaching of bR in alkylammonium micellar solutions

No. of carbon atoms in surfactant tail	$\frac{k_{max}(Me)}{k_{max}(Et)}$	$\frac{k_{max}(Et)}{k_{max}(Pr)}$	$\frac{k_{max}(Me)}{k_{max}(Pr)}$
16	16.5	3.0	48.8
14	8.9	0.42	3.72
12	3.1	0.25	0.77

Experimental conditions as given in the captions to Figs. 4–7.

and C_{16}-Pr. Thus the relative increase in the k_{max} for the propyl headgroup as the chain length decreases is consistent with the greater hydrophobic influence of the headgroup. The smaller k_{max} ratios for shorter surfactants in Table 2 reflect the lower significance of the surfactant tail and greater control of the headgroup in the bleaching process. In light of the above effects, the reversal of the relative rates for C_{14}-Et and C_{14}-Pr can be attributed to a concentration-dependent headgroup mechanism. At low surfactant concentrations, the headgroup size represents the principal variable determining the rate of bleaching, whereas at high surfactant concentrations, the effect of the hydrophobicity of the headgroup becomes more significant.

Before we accept the above mechanistic interpretation, however, an alternative mechanism should be examined. It is possible that the headgroup occupies specific cation-binding sites within the protein that enhance incorporation of the surfactant into the inner channel of the protein. If one or more of these sites are near the retinyl chromophore, such binding would enhance the bleaching process. At least one and possibly two cation-binding sites are near the retinyl polyene (Chang et al., 1985; Stuart et al., 1995). Furthermore, these sites appear to be quite lenient in terms of charge (monovalent, bivalent, and trivalent ions are accommodated) and size (Chang et al., 1985; Stuart et al., 1995). If this mechanism were operative and had a significant effect on the bleaching rates, we would anticipate that bleaching by C_{12}-Pr would be inhibited in the presence of $(Me)_4N^+$ and relatively unaffected by the addition of $(Et)_4N^+$. The data shown in Table 3, however, indicate that the rate of bleaching for C_{12}-Pr is insensitive to the type and concentrations of the added alkyl quaternary ammonium salts. This observation rules out the possibility that the bleaching rates are influenced in a significant way by specific binding of the headgroups. Furthermore, the tail of the surfactant plays the key role in chromophore stabilization and/or extraction. We have observed that bacteriorhodopsin is stable in aqueous R_4N^+, where R = CH_3, C_2H_5, or C_3H_7. Thus the hydrophobic tail group is a key component in the bleaching process and prompts our suggestion that the primary mechanism of chromophore:surfactant interaction is dispersive.

From the data above, we conclude that the primary factors that are important for the stability of solubilized bR are the structure of the micelle and the ability of the surfactant to penetrate into the protein interior. Ideally, the surfactant headgroup has to be large, to prevent the complete insertion of the detergent molecule into the protein but yet able to provide a micelle structure that is compact. This explanation helps rationalize the choice of non-ionic surfactants (for example, Triton X-100), which are usually preferred in the extraction of proteins. Non-ionic detergents form more compact micelles because of the absence of an intrinsic charge at the headgroup, which allows the surfactants to more closely approach each other in the micelle. In addition, the effective headgroup area is usually much larger as compared to ionic surfactants.

Effective bleaching is possible only when the protein is solubilized into the micelle. The kinetic results also indicate that the rate of bleaching is affected by the penetration of the surfactant into the interior of the protein. Thus a possible mechanism of the interaction of the surfactants with bR involves the symbiotic action of the solubilizing micelle and the penetrated surfactants. Surfactants within the protein may help unravel and hence bleach the protein by compensating the native hydrophobic and electrostatic interactions that are relevant to the stabilization of the native protein structure.

TABLE 3 Rate constants for the bleaching of 1.2 μM bR in aqueous C_{12}-Pr with added simple quaternary ammonium salts

Type of salt	[salt] (mM)	[C_{12}-Pr] (mM)	$k_{max} \times 10^4$ (s^{-1})
Me_4NBr	16.7	83.0	7.88
Et_4NBr	16.7	83.0	8.13
Pro_4NBr	16.7	83.0	7.88
Me_4NBr	100	41.7	5.24
Et_4NBr	100	41.7	4.97
Pro_4NBr	100	41.7	5.12

Conditions: 0.1 M Tris-HCl buffer, pH 7.20, T = 25°C.

SUMMARY AND CONCLUSIONS

The effect of alkylammonium surfactants on bR is typical for reactions in micellar solution. The correlation between detergent structure and stability of solubilized bR is complicated and involves an interplay of at least four effects: the micellar environment, length of the surfactant tail, the size of the headgroup, and the hydrophobicity of the headgroup. The rate of solubilization is faster for a longer surfactant because of the higher hydrophobic character for a larger micelle. The surfactant C_{12}-Et gives the best combination of the above effects and provides the most stable environment for the solubilization of bR of the surfactants studied. In contrast, C_{16}-Me is the most denaturing surfactant studied. We attribute this observation to the hydrophobicity of the long-chain tail group and the relatively high ratio of the number of carbon atoms in the aliphatic tail to those in the headgroup. This molecular combination leads not only to the rapid solubilization of the protein but, more importantly, to effective penetration of the surfactant into the protein-binding site and effective solvation of the chromophore. The end result is a competition with respect to chromophore stabilization between the binding site of the protein and the solvating characteristics of the surfactant. Contrary to our expectation, the surfactants with the closest structural similarity to the chromophore (C_{12}-Me and C_{12}-Et) are not the most effective at bleaching the protein. Thus, we conclude that solvation of the chromophore rather than competition for the binding site is more important in destabilizing the protein-binding site and extraction of the chromophore from the protein.

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