doi:10.1016/j.jmb.2007.10.018 *J. Mol. Biol.* (2007) **374**, 1319–1332

JMB

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Opsin Stability and Folding: Modulation by Phospholipid Bicelles

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Received 19 June 2007; received in revised form 28 September 2007; accepted 10 October 2007
Available online
13 October 2007

Integral membrane proteins do not fare well when extracted from biological membranes and are unstable or lose activity in detergents commonly used for structure and function investigations. We show that phospholipid bicelles provide a valuable means of preserving alpha-helical membrane proteins *in vitro* by supplying a soluble lipid bilayer fragment. Both 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/3-[(cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (Chaps) and DMPC/L-α-1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) bicelles dramatically increase the stability of the mammalian vision receptor rhodopsin as well as its apoprotein, opsin. Opsin is particularly unstable in detergent solution but can be directly purified into DMPC/Chaps. We show that opsin can also be directly purified in DMPC/DHPC bicelles to give correctly folded functional opsin, as shown by the ability to regenerate rhodopsin to ~70% yield. These well-characterised DMPC/DHPC bicelles enable us to probe the influence of bicelle properties on opsin stability. These bicelles are thought to provide DMPC bilayer fragments with most DHPC capping the bilayer edge, giving a soluble bilayer disc. Opsin stability is shown to be modulated by the *q* value, the ratio of DMPC to DHPC, which reflects changes in the bicelle size and, thus, proportion of DMPC bilayer present. The observed changes in stability also correlate with loss of opsin secondary structure as determined by synchrotron far-UV circular dichroism spectroscopy; the most stable bicelle results in the least helix loss. The inclusion of Chaps rather than DHPC in the DMPC/Chaps bicelles, however, imparts the greatest stability. This suggests that it is not just the DMPC bilayer fragment in the bicelles that stabilises the protein, but that Chaps provides additional stability either through direct interaction with the protein or by altering the DMPC/Chaps bilayer properties within the bicelle. The significant stability enhancements and preservation of secondary structure reported here in bicelles are pertinent to other membrane proteins, notably G-protein-coupled receptors, which are unstable in detergent solution.

Edited by J. E. Ladbury

Keywords: rhodopsin; opsin; membrane protein folding; stability; bicelles

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Abbreviations used: BTP, bis-tris-propane; DDM, *n*-dodecyl-β-D-maltoside; DHPC, L-α-1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; GPCR, G-protein-coupled receptor; Meta, metarhodopsin; ROS, rod outer segment; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide.

Introduction

Membrane proteins represent approximately one-third of the proteome and yet much less is known about their structure and function than for water-soluble proteins. One reason is that solubilisation of the hydrophobic membrane proteins in aqueous environments is a necessary step for detailed *in vitro* molecular characterisation. While detergents are predominately employed for preserving membrane proteins in a water-soluble form, detergent micelles are a radical departure from the structure of a native lipid bilayer environment.

Membrane proteins are often unstable in detergent micelles and frequently denature or become inactivated. The difference in chemical and structural support provided by detergent micelles as compared to membrane lipid bilayers may lead to exposure of previously buried surfaces of the protein, formation of nonnative contacts and protein aggregation. Several alternatives to classical detergents such as peptergents[1,2] and amphipols[3] have been engineered for the stabilisation of membrane proteins.[4] Certain mixtures of long-chain [e.g., 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC)] and short-chain [e.g., L-α-1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC)] phospholipids assemble as bilayer discs where a DMPC bilayer fragment is solubilised by a rim of DHPC.[5] There is a high degree of segregation between DMPC in the bilayer region of the bicelle and DHPC around the outside. These bicelles are a promising alternative to standard detergents for the stabilisation of membrane proteins. The bicelle morphology makes them attractive bilayer model systems, which are both soluble in water and likely to stabilise the correct membrane protein fold. Phospholipid bicelles have been well characterised and have many useful properties. In particular, the radius of the bilayer fragment can be increased (or decreased) by increasing (or decreasing) the ratio of long-chain lipid to short-chain lipid in solution, known as the q ratio (q = [long-chain lipid]/[short-chain lipid]).

Bicelles are emerging as very useful solubilising systems that promise to advance membrane protein research, particularly G-protein-coupled receptor (GPCR) research. In particular, bicelles can overcome several major obstacles in membrane protein work: refolding, stability and crystallisation. Two bicelle systems, DMPC/DHPC and a similar (although less well characterised) system, DMPC/3-[(cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate (Chaps) (composed of DMPC and the zwitterionic fused-ring detergent Chaps), have demonstrated their usefulness in the handling of membrane proteins *in vitro*. Bacteriorhodopsin, a bacterial seven transmembrane light-driven proton pump, can be refolded from an SDS-unfolded apoprotein state into DMPC/Chaps[6] and DMPC/DHPC.[7] Further, the DMPC/DHPC q ratio was shown to modulate bacteriorhodopsin refolding rates and yields. DMPC/Chaps/cholesterol hemisuccinate mixtures have been used to solubilise a GPCR, the 5-HT_{4a} receptor, from urea-solubilised inclusion bodies in *Escherichia coli*.[8] DMPC/Chaps is also compatible with membrane protein crystallisation,[9] and the stability the bicelles impose on membrane proteins, particularly receptor proteins, means it is becoming an increasingly used approach. Recently, the transmembrane domain of a β-barrel membrane protein, OmpA (20 kDa), was successfully reconstituted into bicelles and made to align in a magnetic field, the largest protein to do so to date.[10]

The prototypical GPCR, rhodopsin, is relatively stable in solution and was the first GPCR for which a crystal structure was solved.[11] Rhodopsin is a seven-α-helical, 40-kDa membrane protein that binds an 11-*cis* retinal cofactor covalently. In contrast to the stability of rhodopsin, the apoprotein opsin denatures rapidly in detergent solution, losing its ability to bind 11-*cis* retinal and regenerate rhodopsin.[12] A breakthrough in handling opsin *in vitro* was, however, achieved with the finding that opsin can be solubilised and purified directly from membranes into DMPC/Chaps and is stable for several days at room temperature.[13]

In light of the current understanding of bicelle properties as bilayer mimics and the ability of these bicelles to support the folding and stability of membrane proteins, we investigate the stability of rhodopsin and opsin solubilised in bicelles. In particular, we use the well-defined DMPC/DHPC bicelle system to investigate the effect of bilayer lipid chain length and q value (i.e., ratio of long- to short-chain lipid). We also compare stability with that in DMPC/Chaps and detergent micelles.

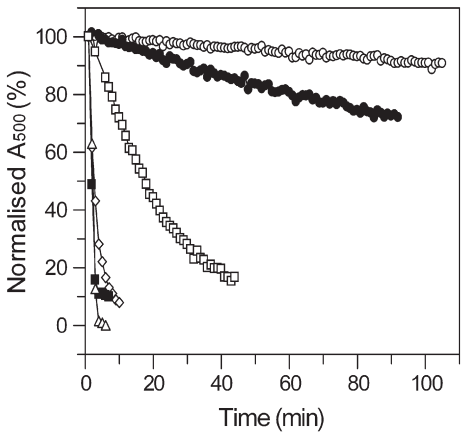

Fig. 1. Thermal stability of rhodopsin in detergents and bicelles. Decay of 500 nm rhodopsin absorbance over time at 55 °C in Buffer A. Rhodopsin ($Rh_{bicelles}$) in (○) DMPC/Chaps and (●) DMPC/DHPC, as well as rhodopsin in individual detergents: (□) 0.1% DDM, (◇) 1% Chaps, (■) 1% DHPC and (Δ) 0.2% LDAO. The initial A_{500} was taken after 5 min preincubation at 55 °C. A_{500} is plotted as a percentage of the initial A_{500} *versus* time. Data are for 1% DMPC/1% Chaps (0.52 mol fraction Chaps; q = 0.9) and 1% DMPC/1% DHPC (0.6 mol fraction DHPC; q_{eff} = 0.98). Rhodopsin concentration was 1.5 μM.

Results

In this investigation opsin was prepared by two different methods:

(a) Solubilisation and purification of opsin directly from native rod outer segment (ROS) membranes (termed $opsin_{ROS}$) into either 1% DMPC/1% Chaps or 1% DMPC/1% DHPC (0.6 mol fraction DHPC).[13] It is of note that opsin cannot be purified from ROS into detergents, as it is too unstable in detergent to survive the purification.

(b) Photobleaching rhodopsin to give $opsin_{Rh}$. This provides a more convenient preparative method and can also be used with detergents. In this case, rhodopsin was purified from ROS into 0.1% *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) (this is termed Rh_{LDAO}) and then transferred into DMPC/Chaps or DMPC/DHPC (termed $Rh_{bicelle}$ or more specifically $Rh_{DMPC/}$Chaps and $Rh_{DMPC/DHPC}$) by rapid dilution of LDAO to ~0.002% (below the critical micelle concentration of ~0.034%) in a method adapted from Ref. 14. These $Rh_{bicelle}$ samples were then photobleached and incubated at room temperature at pH 6.0 in the light for >1 h for the metarhodopsin (Meta) II (and III) intermediates to decay to give $opsin_{Rh}$.

In order to establish the effects of bicelles on rhodopsin and opsin stability as well as the photobleaching pathway of rhodopsin, rhodopsin or opsin was characterised in each solubilising system according to three experimental criteria:

(1) The thermal bleaching kinetics of rhodopsin at 55 °C (Fig. 1)
(2) The absorbance species formed following rhodopsin photobleaching (Figs. 2 and 3)
(3) The ability of both types of opsin, $opsin_{ROS}$ and $opsin_{Rh}$, to regenerate rhodopsin in the presence of 11-*cis* retinal (Fig. 4).

The solubilising systems tested were (a) 1% DMPC/1% Chaps, (b) 1% DMPC/1% DHPC, (c) 0.1% *n*-dodecyl-β-D-maltoside (DDM), (d) 1% DHPC, (e) 0.2% LDAO and (f) 1% Chaps.

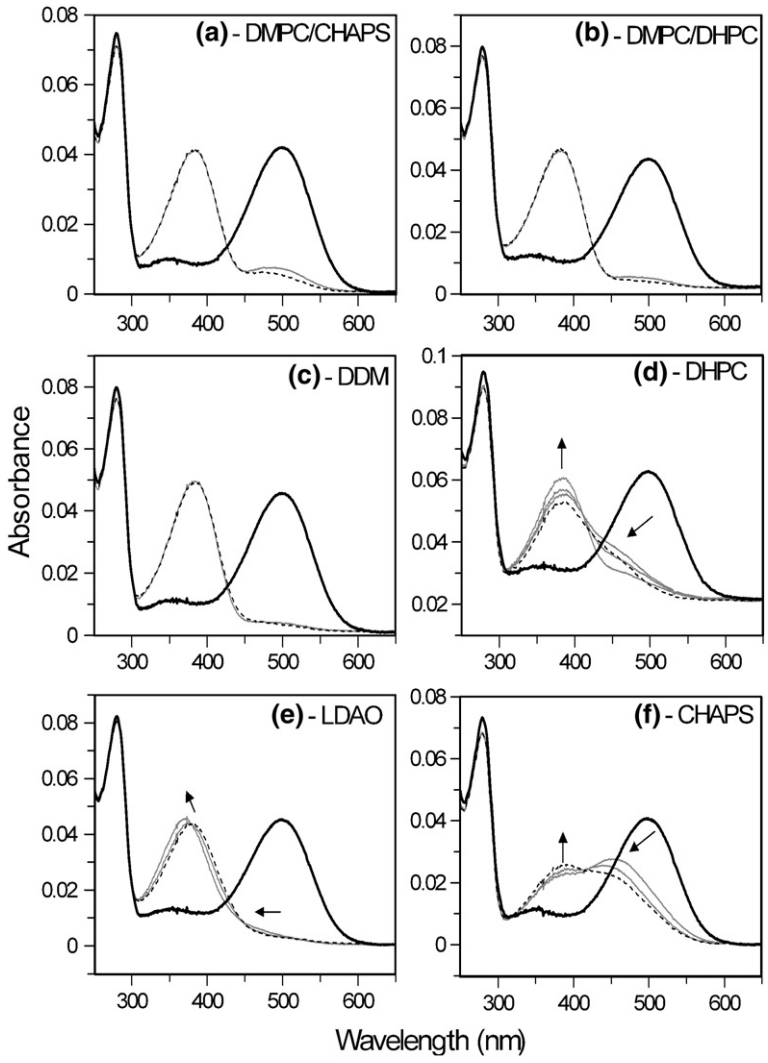

Fig. 2. Photobleaching properties of rhodopsin in detergents and bicelles. (a) $Rh_{DMPC/Chaps}$, (b) $Rh_{DMPC/DHPC}$, (c) Rh_{DDM}, (d) Rh_{DHPC}, (e) Rh_{LDAO}, (f) RhChaps. Rhodopsin was diluted from Rh_{LDAO} into bicelles or detergents (final rhodopsin concentration was 1.5 μM) and samples were photobleached for 15 s using a >495-nm long-pass filter; spectra were taken immediately (thick continuous black line with 500-nm absorbance band). For (a–c), samples were then bleached for a further 2 min before the final spectra (black dashed line) were recorded. For (d–f), spectra were taken every minute after the initial 15-s photobleach (black dashed line being latest time point). Grey lines represent spectra at intermediate times. Arrows indicate direction of changes of absorption bands over time.

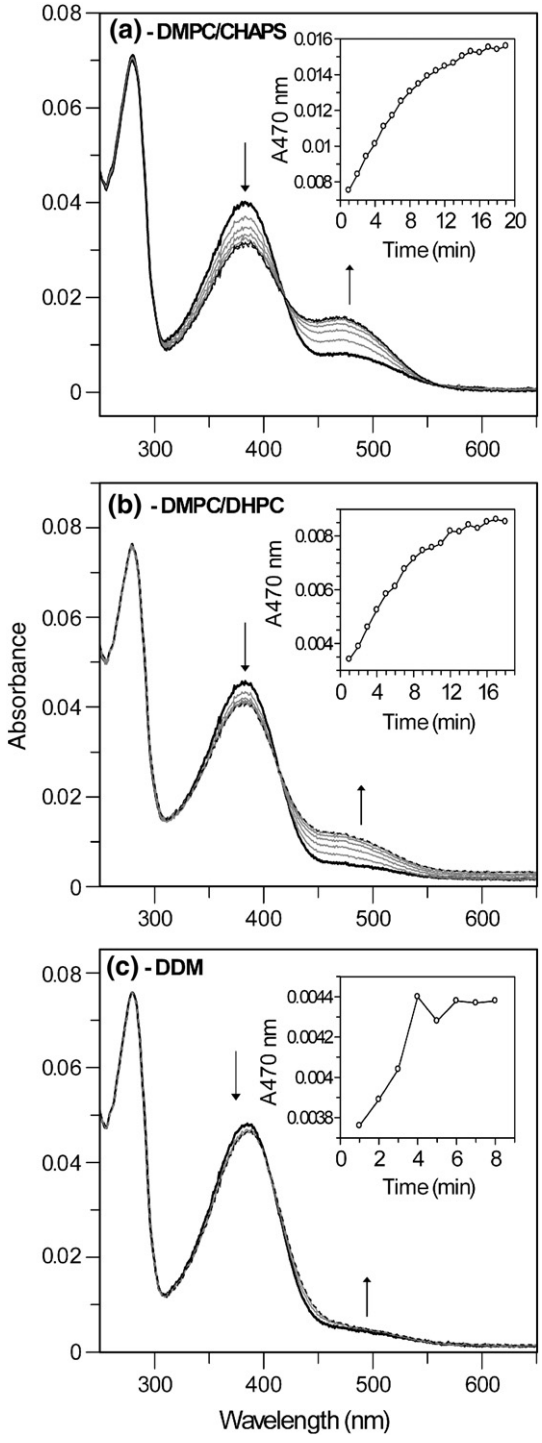

Fig. 3. Meta III formation following rhodopsin photobleaching in DMPC/Chaps, DMPC'/DHPC and DDM. Rhodopsin ($Rh_{bicelles}$) samples in (a) DMPC/Chaps, (b) DMPC/DHPC and (c) Rh_{DDM} were photobleached at 25 °C for 2 min and then spectra were recorded every minute for 20 min. Thick continuous black line (with little 470-nm absorbance) is the first spectrum, dashed black line the final spectrum, and grey lines the spectrum at intermediate times. Arrows indicate the direction of change of the 380-nm band (decay) and 470-nm band (increase) over time. Inset figures: plots of absorbance at 470 nm over time, with data points joined for convenience. Rhodopsin concentration was 1 μM.

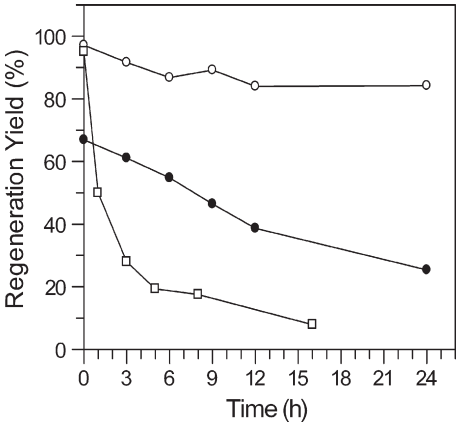

Fig. 4. Stability of opsin purified directly into DMPC/Chaps and DMPC/DHPC, as shown by the ability to regenerate rhodopsin. Ability of opsin ($opsin_{ROS}$) to regenerate rhodopsin, immediately after purification from ROS and over the following 24 h in (○) DMPC/Chaps or (●) DMPC/DHPC at room temperature (concentrations 1% as in Fig. 1). (□) Regeneration yield for rhodopsin photobleached to $opsin_{Rh}$ in 0.1% DDM (Rh_{DDM}) is shown as a comparison. At the times shown on the graph, samples were incubated in the dark with 1.5 molar equivalents of 11-*cis* retinal (for 2 h) at room temperature and then the regeneration yield was determined. Purified opsin samples were at a concentration between 1 and 2 μM.

The properties of rhodopsin in different solubilising environments

Rh_{LDAO} was diluted into the different detergent/lipid systems to give $Rh_{bicelles}$, Rh_{Chaps} etc. Rh_{DDM} was used as a comparative standard for the properties of solubilised rhodopsin as this has been investigated previously.[12,15,16] Figure 1 shows that rhodopsin thermal stability, as measured by the loss of the characteristic rhodopsin absorbance at 500 nm over time at 55 °C, decreases in the order DMPC/Chaps > DMPC/DHPC ≫ DDM ≫ LDAO = Chaps = DHPC (with DMPC/Chaps being the most stable). The absorbance spectra of photobleached rhodopsin samples in each detergent and lipid/detergent system are shown in Fig. 2. $Rh_{bicelle}$ and Rh_{DDM} (Fig. 3a, b and c) exhibit "normal" bleaching behaviour, where upon photobleaching, the maximum absorbance shifts from 500 to 380 nm (corresponding to Meta II) with a small absorbing contribution at ~485 nm (from Meta I). Following this, the 380-nm species decays and an increase in absorbance at 470 nm is observed, which corresponds to the storage intermediate of rhodopsin, Meta III[17] (see Fig. 3). Meta III subsequently decays, losing retinal and forming opsin. The yield of Meta III formation is highest in DMPC/Chaps (Fig. 3a) and lowest in DDM (Fig. 3c). All three of these solubilising systems (DMPC/Chaps, DMPC/DHPC and DDM) allowed regeneration of rhodopsin from opsin in the presence of 11-*cis* retinal (see below and Fig. 4).

Figure 2d, e and f show that rhodopsin in either Chaps, DHPC or LDAO does not undergo normal bleaching behaviour. Upon photobleaching in Chaps, two absorption bands are observed, with maxima at about 455 and 390 nm; over time the 455-nm band decreases, while there is a slight increase at 390 nm. The 455-nm band may represent a denatured yet protonated Schiff base light-activated species (since the absorbance maximum of protonated Schiff base retinal free in solution is ~440 nm[18]), and the 390-nm absorbance species a deprotonated, covalently attached retinal/opsin species, or free retinal. Rh_{DHPC} photobleaches in a similar but less pronounced manner to RH_{Chaps}, with an intermediate at 450 nm, which decays to a 390-nm species (Fig. 2d). In contrast to DMPC/Chaps, DMPC/DHPC and DDM, rhodopsin photobleached in Chaps, DHPC or LDAO did not undergo conversion to the Meta III state. Furthermore, the Chaps, DHPC and LDAO solubilising systems did not support any regeneration of rhodopsin from opsin.

Together, these studies indicate a clear divide between those solubilising systems that can support stabilisation of rhodopsin structure, the normal photobleaching pathway and an opsin conformation that is functional for the binding of 11-*cis* retinal (DMPC/Chaps, DMPC/DHPC and DDM) and those that cannot (Chaps, DHPC and LDAO). They also show that diluting Rh_{LDAO} (so that LDAO is well below the critical micelle concentration) into DMPC/Chaps or DMPC/DHPC is an effective method to transfer the protein into the latter bicelles, in which it exhibits properties characteristic of the bicelle, rather than LDAO.

Opsin activity in DMPC/Chaps and DMPC/DHPC

Correctly folded, functional opsin can spontaneously bind its cofactor 11-*cis* retinal to generate rhodopsin, as shown by formation of the characteristic rhodopsin 500-nm absorption band. The yield of regenerated rhodopsin from opsin shows what percentage of opsin is correctly folded, active and able to bind 11-*cis* retinal.[12] Thus, rhodopsin regeneration is a measure of opsin stability against conformational denaturation in a given solubilising system. Figure 4 shows the regeneration yields of freshly purified opsin ($opsin_{ROS}$) directly from ROS into DMPC/DHPC and DMPC/Chaps and treated either immediately ($t=0$ h) with 11-*cis* retinal or after aging of the eluted opsin at room temperature over time. The regeneration yields of $opsin_{Rh}$ left to age in DDM after photobleaching Rh_{DDM} are shown for a stability comparison (opsin is not stable enough in DDM to be prepared directly from ROS membranes).

Figure 4 shows that opsin is stabilised over DDM, in DMPC/DHPC and to an even greater extent in DMPC/Chaps. Note that the "initial" rhodopsin regeneration yield in DMPC/DHPC is ~67%, compared to ~98% in DMPC/Chaps ($t=0$ h). Direct purification of opsin from ROS takes of the order of 4 h and some opsin denatures over this time in DMPC/DHPC. Opsin prepared directly from ROS ($opsin_{ROS}$) or from photobleaching rhodopsin ($opsin_{Rh}$) exhibited similar stability in DMPC/DHPC, with $opsin_{Rh}$ being marginally more stable. Thus, Fig. 4 ($t=0$ h) shows a regeneration yield of 67% for $opsin_{ROS}$, which has been in DMPC/DHPC for ~4 h during purification. This compares reasonably well with the yield of ~76% obtained for 3-h-old $opsin_{Rh}$ (see Table 1 for carbon chain length 14; DMPC) and 6-h-old $opsin_{Rh}$ that has a regeneration yield of ~70% [see Fig. 6 for DHPC mole fraction 0.6, which is equivalent to 1% DMPC/1% DHPC (w/v)].

Opsin also showed less propensity to aggregate in bicelles as compared to DDM. Aggregation was followed by the increase in light scattering over time, after rhodopsin had been photobleached to give $opsin_{Rh}$, as shown in Fig. 5. The increase in light scattered at 650 nm shows aggregation was rapid in 0.1% LDAO (shown for 4 μM protein as a control). Opsin was less prone to aggregate in 0.1% DDM, with aggregation of 6 μM opsin in DDM occurring at a slower rate than in LDAO and no aggregation being observed at concentrations of 1 or 3 μM protein in DDM. Opsin in bicelles showed no evidence of aggregation even at 6 μM protein.

Opsin stability in DMPC/DHPC is sensitive to bilayer lipid chain length and mole fraction of DHPC

The length of the long-chain diacylPC lipid was varied in the mixed PC lipid bicelles, while keeping the mole fraction of DHPC constant at 0.6. $Opsin_{Rh}$ stability in each system was measured by the ability to regenerate rhodopsin. Table 1 shows that the highest regeneration yield (76%), and thus the most stable opsin, is found for C14 (i.e., DMPC/DHPC bicelles).

The effect of the ratio of DMPC to DHPC, which alters the bicelle radius, on $opsin_{Rh}$ stability was

Table 1. Opsin stability as shown by ability to regenerate rhodopsin as a function of the lipid chain length in diacylPC/DHPC bicelles

Carbon chain length	Regeneration yield (%)[a]
12	14±8
13	56±14
14	76±1
15	63±1
16	33±23[b]

[a] Rh_{LDAO} diluted into each bicelle condition was photobleached to give $opsin_{Rh}$, which was left to age for 3 h at 25 °C in the light. All regeneration yields are after a further incubation with 1.5 molar equivalents of 11-*cis* retinal for 2 h. Data are the average and standard error of two independent measurements.

[b] The relatively large error for D16PC/DHPC is due to all experiments being done at 25 °C, while the phase transition of D16PC is ~41 °C, and thus it (D16PC) alone would be in the gel phase at 25 °C.

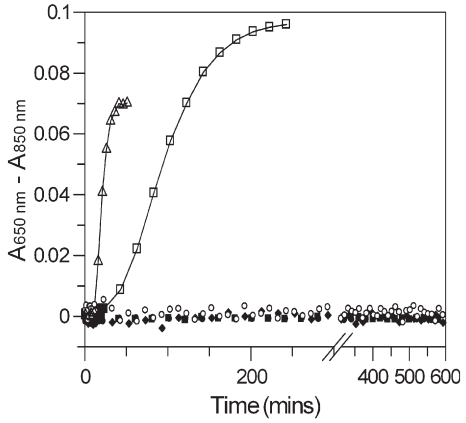

Fig. 5. Opsin aggregation monitored by changes in light scattering at 650 nm. Rhodopsin was photobleached to give opsin at different concentrations in different solubilising systems: (Δ) 4 μM in 0.1% LDAO, (♦) 1 μM in 0.1% DDM, (■) 3 μM in 0.1% DDM, (□) 6 μM in 0.1% DDM, (○) 6 μM in 1% DMPC/1% Chaps and (●) 1% DMPC/1% DHPC. The change in absorbance at 650 nm (where there is no absorbance contribution from the rhodopsin chromophore), relative to that at 850 nm (where light scattering is negligible), was measured over time at 25 °C. An increase in the 650-nm absorbance corresponds to an increase in light scattering.

monitored. $Opsin_{Rh}$ in DMPC/DHPC bicelles with different mole fractions of DHPC (but 2% total lipid, i.e., DMPC plus DHPC) was left to age for different lengths of time before regenerating rhodopsin by addition of 11-*cis* retinal. Figure 6 shows that optimum regeneration, and thus opsin stability, are obtained with 0.5 or 0.6 mol fractions of DHPC, while both higher and lower DHPC mole fractions lead to lower regeneration.

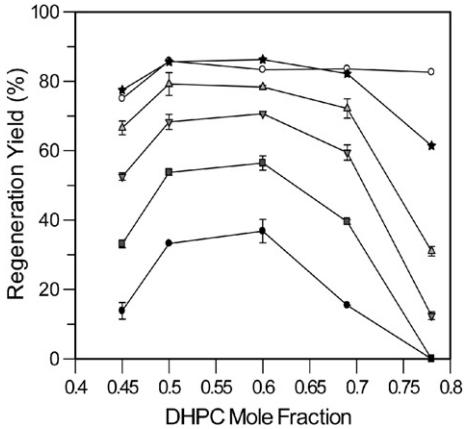

Fig. 6. Opsin stability over time in DMPC/DHPC bicelles with different mole fractions of DHPC, as shown by the ability of opsin to regenerate opsin. $Rh_{DMPC/.DHPC}$ samples were photobleached for 15 s to induce Meta II formation and decay to give $opsin_{Rh}$ and left to age for (○) 0, (★) 1, (Δ) 3, (▽) 6, (■) 12 and (●) 24 h at 25 °C. All regeneration yields are after incubation with 1.5 molar equivalents of 11-*cis* retinal for a subsequent 2 h. Initial rhodopsin concentration was 1.5 μM.

The effect of altering the total lipid concentration of DMPC/DHPC bicelles on opsin stability was also investigated. Figure 7a shows that increasing the total lipid concentration from 2% [i.e., 1% (w/v) of each lipid] to 8% changes the optimal regeneration yield (and thus opsin stability) from 0.6 DHPC mol fraction at 2% to a lower mole fraction of 0.45 DHPC with 8% total lipid. This change in optimal stability point can be explained if mole fraction DHPC is converted into q value:

$$q = \frac{[\mathrm{DMPC_{total}}]}{[\mathrm{DHPC_{total}}]} \quad (1)$$

Additionally, there is evidence that in DMPC/DHPC mixtures, a considerable population of DHPC exists

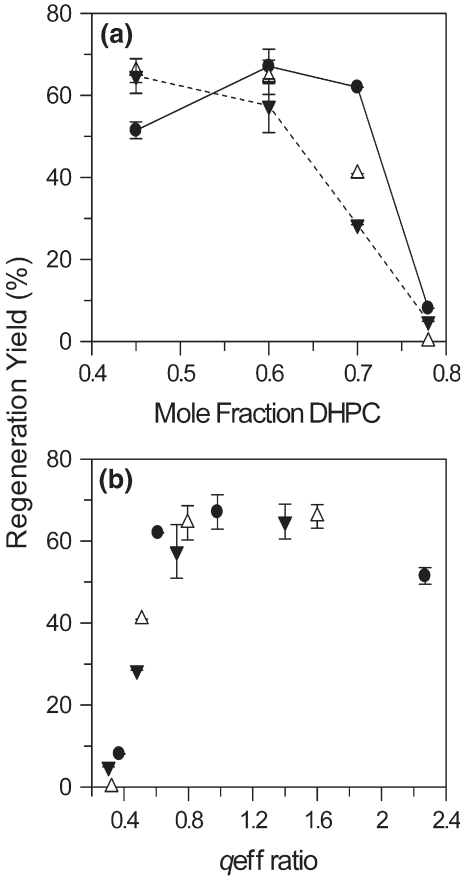

Fig. 7. Dependence of opsin stability, as shown by ability to regenerate rhodopsin, on DHPC and q_{eff} in DMPC/DHPC bicelles of different lipid concentration. $Rh_{DMPC/.DHPC}$ samples were photobleached to give $opsin_{Rh}$ and left to age for 3 h at 25 °C in the light: (●) 2% (w/v) total lipid (i.e., as in Fig. 6), (Δ) 4% (w/v) total lipid and (▼) 8% (w/v) total lipid. Dependence of regeneration yield (after 2 h incubation with 1.5 molar equivalents of 11-*cis* retinal) on (a) DHPC mole fraction and (b) q_{eff}, where q_{eff} is calculated with Eq. (2). Data points are joined in (a) to highlight trends for 2% and 8% total lipids. For 2% total lipid (●), the q_{eff} values relate to DHPC mole fraction as follows: 0.45 mol fraction is a q_{eff} of 2.27; 0.6 mol fraction is a q_{eff} of 0.98; 0.78 mol fraction is a q_{eff} of 0.37. Initial rhodopsin concentration was 1.5 μM.

as free monomers at a concentration of ~7 mM that do not associate in bicelle aggregates, giving an effective q value:[19]

$$q_{\text{eff}} = \frac{[\text{DMPC}_{\text{total}}]}{[\text{DHPC}_{\text{total}}] - [\text{DHPC}_{\text{free}}]} \qquad (2)$$

At 2% DMPC/DHPC, the effective concentration of DHPC left to associate with DMPC in bicelles after the 7 mM free monomer population is taken into account results in a higher q_{eff} value than at 8% DMPC/DHPC. When q ratios are corrected as q_{eff} ratios to take this free DHPC concentration into account, the same dependence of opsin stability with regard to q_{eff} is seen, independent of total lipid concentration (see Fig. 7b).

Loss of opsin secondary structure depends on DHPC mole fraction

The secondary structural changes associated with opsin denaturation were monitored by circular dichroism over time for opsin_{Rh} in 2% DMPC/DHPC bicelles with DHPC mole fractions of 0.45 [q=1.24/q_{eff}=2.27], 0.6 [q=0.67/q_{eff}=0.98] and 0.78 [q=0.29/q_{eff}=0.37] (Fig. 8). In each case, a slight decrease in helicity was observed as a consequence of aging opsin in DMPC/DHPC. The loss in secondary structure content is indicated by decreases in both the negative 222-nm band and the positive 195-nm band. Structure loss over 6 h was least for 0.6 mol fraction DHPC (Fig. 8b), the most stabilising condition (q_{eff}=0.98, cf. Fig. 7b). In 0.45 and 0.78 mol fraction DHPC (q_{eff}=2.27 and 0.37, respectively) secondary structure loss was greater over 1, 3 and 6 h.

These CD experiments were performed at a higher opsin concentration of 6 μM, as opposed to 1.5 μM used for most other experiments here. No difference in stability was found for these two opsin concentrations.

Opsin is more stable in DMPC/Chaps and sensitive to the mole fraction of Chaps

The Chaps mole fraction also affects opsin stability in DMPC/Chaps bicelles. Figure 9 shows opsin stability in DMPC/Chaps on the basis of the q ratio (note that all Chaps is thought to be in the DMPC/Chaps bicelle,[20] with no free Chaps, and thus q and q_{eff} are the same). Opsin stability was monitored by the ability to regenerate rhodopsin from opsin_{Rh} aged for 6 h in DMPC/Chaps. Regeneration yield and thus opsin stability increase with increasing q_{eff} ratio up to about 1, above which ~100% of opsin can regenerate rhodopsin. Also shown are the regeneration yields for DMPC/DHPC for comparison (for which opsin_{Rh} was aged for 3 h). The yields, and thus opsin stability, are lower in DMPC/DHPC than in DMPC/Chaps (in agreement with Fig. 4), indicating the greater stabilising effect of Chaps over DHPC in the bicelles. The yield also seems to decrease at q_{eff} higher than ~1.5 (i.e., high DMPC) in DMPC/DHPC.

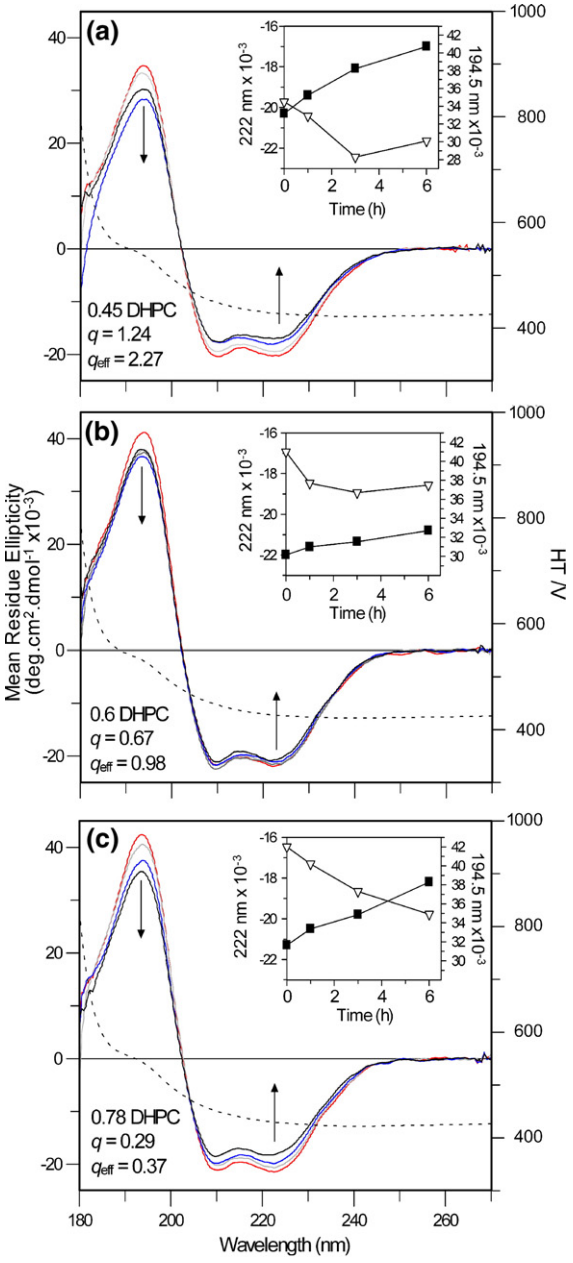

Fig. 8. CD spectra of rhodopsin and opsin in DMPC/DHPC bicelles with different mole fractions of DHPC: (a) 0.45 mol fraction DHPC (q=1.24, q_{eff}=2.27), (b) 0.6 mol fraction DHPC (q=0.67, q_{eff}=0.98), (c) 0.78 mol fraction DHPC (q=0.29, q_{eff}=0.37). Rhodopsin was purified from ROS into DMPC/DHPC bicelles and photobleached to give opsin. Spectra shown are for rhodopsin (red) and for opsin at room temperature aged for 1 h (light grey), 3 h (blue) and 6 h (black). The high tension voltage (HT) is shown as a dashed trace for the rhodopsin sample in each bicelle condition. Inset figures: plots of ellipticity at (■) 222 nm and (▽) 194.5 nm over time. All samples are in Buffer E to avoid buffer absorption in the far UV. Initial rhodopsin concentration was 6 μM.

Discussion

Opsin is unstable in detergent solution and undergoes irreversible denaturation within hours to a form that can no longer react with 11-*cis* retinal to regenerate rhodopsin.[21–23] The correctly folded opsin structure can be stabilised in native ROS membranes[24] and when solubilised in DMPC/Chaps lipid/detergent mixtures.[13] We find that opsin is also stabilised in DMPC/DHPC bicelles, offering a well-characterised system to study this stabilising influence of bicelles on opsin. The properties of DMPC/DHPC bicelles can be manipulated by altering the ratio (known as the q value) of long-chain (DMPC) to short-chain (DHPC) lipid.[19,25–27] We show that opsin stability is sensitive to this ratio, with stability increasing with increasing DMPC mole fraction in both DMPC/DHPC and DMPC/Chaps systems. DMPC/DHPC bicelles have been better characterised than DMPC/Chaps in terms of the q (and q_{eff}) value and their size. Additionally, DMPC/DHPC enables higher quality protein CD spectra to be collected into the far UV (180 nm), thus providing good insight into secondary-structure changes. We find that opsin loses only small amounts of secondary structure in DMPC/DHPC bicelles over time, with the most stabilising composition (0.6 mol fraction DHPC, $q=0.67$, $q_{eff}=0.98$) showing the smallest loss of protein secondary structure.

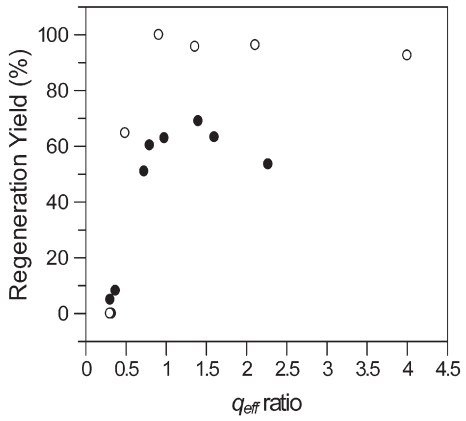

Fig. 9. Dependence of opsin stability on q_{eff} values in DMPC/Chaps and DMPC/DHPC bicelles as shown by the ability to regenerate rhodopsin. $Opsin_{Rh}$ in (○) DMPC/Chaps and (●) DMPC/DHPC. $Rh_{DMPC/Chaps}$ or $Rh_{DMPC/DHPC}$ samples were photobleached for 15 s and left to age for 3 h (DMPC/DHPC) and 6 h (DMPC/Chaps) in Buffer A in the light at 25 °C (with 2% total DMPC and DHPC, or DMPC and Chaps). (Note different opsin aging times.) All regeneration yields are after incubation with 1.5 molar equivalents of 11-*cis* retinal for 2 h. q_{eff} calculated for DMPC/DHPC bicelles, assuming a free DHPC monomer population of 7 mM (as for Fig. 7b). Since all Chaps is thought to be in DMPC/Chaps bicelles, with no "free" Chaps monomers, $q=q_{eff}=$[DMPC]/[Chaps]. Initial rhodopsin concentration was 6 μM.

Rhodopsin and opsin stability

Our results show that bicelles provide a stabilising environment for both rhodopsin and opsin. DMPC/DHPC, like DMPC/Chaps but in sharp contrast to DDM, is sufficiently stabilising to allow the direct purification of opsin from ROS membranes. However, DMPC/Chaps remains the superior stabilising system. DMPC/Chaps stabilises opsin with >80% activity remaining at 24 h, long after opsin becomes completely denatured in DDM (at ~12 h; see Fig. 4). Lower opsin stability is found in DMPC/DHPC bicelles compared to DMPC/Chaps, but the former are still considerably more stabilising than DDM detergent micelles. Opsin stability, in terms of the ability to bind 11-*cis* retinal and regenerate rhodopsin, decreases in the order (most stabilising system first) DMPC/Chaps>DMPC/DHPC>DDM, while opsin cannot regenerate in Chaps, DHPC or LDAO. The detergent/lipid systems also stabilise the rhodopsin structure against thermal denaturation in the same order (most stabilising system first): DMPC/Chaps>DMPC/DHPC≫DDM. In contrast, rapid denaturation of rhodopsin occurs in the individual detergents Chaps, DHPC or LDAO. Equally, a similar solubilising system order is apparent in terms of rhodopsin photobleaching behaviour, with rhodopsin in DMPC/Chaps, DMPC/DHPC and DDM photobleaching efficiently to a 380-nm absorbing species corresponding to Meta II, while rhodopsin in Chaps, DHPC or LDAO has "abnormal" photobleaching. The bicelles systems also reduce opsin aggregation.

DMPC/DHPC bicelle properties

DMPC/DHPC bicelles have found considerable use in NMR structural studies of peptides;[28] as a result, the majority of physical studies of DMPC/DHPC bicelles have focussed on bicelles of large q value at high lipid concentrations that can be aligned in a magnetic field (higher concentrations than used here). However, a study by Glover *et al.*[19] on the morphology of small, "fast-tumbling," nonorientable DMPC/DHPC bicelles has direct implications for our work. In this study, low-concentration [total lipid concentration=2.5% (w/v)] DMPC/DHPC aggregates at q values from 0.05 to 0.5 were evaluated by complimentary physical techniques (i.e., similar to our conditions of 2–8% total lipid and q values of 0.17 to 2.01). By a combination of NMR, dynamic light scattering and electron microscopy, distinct chemical environments were found for the phosphate groups of DMPC and DHPC molecules. The hydrodynamic radii for 2.5% (total lipid) bicelles over a q ratio range of 0.5 to 2.0 were 4 to 10 nm, respectively. Electron microscopy visualisation clearly showed disc-shaped aggregates. Their findings support the view that a DMPC bilayer fragment solubilised by a rim of DHPC is formed under the conditions used here.

Several groups have attempted to describe the radius of a bicelle in relation to its q value.[19,26,27]

These have generally built on the simple geometric expression given by Vold and Prosser.[29]

$$q = \frac{2\pi R^2}{\pi h[\pi R + h]} \quad (3)$$

where q = molar ratio of DMPC/DHPC, h = the total lipid bilayer thickness and R = the radius of the bicelle disc. Eq. (3) thus enables R to be determined from q. This equation also fits reasonably well with the experimentally determined bicelle radii given in Glover *et al.*[19] Thus, while the equation can be further modified (e.g., to take into account the different physical volumes of DMPC and DHPC[25]), we use it in this form to estimate R, since our experimental conditions match those used in Glover *et al.*[19] Table 2 shows the range of bicelle radii, R, and surface areas for the range of DHPC concentrations used here, calculated from Eq. (3). The values highlighted in grey are those used to emphasise the q_{eff} relationship as well as the opsin secondary structure changes over time (see Figs. 7 and 8). We can also further determine (a) the number of DMPC lipids per bicelle and (b) the number of bicelles per rhodopsin molecule (see Table 3, e.g., bicelle radii). By taking the cross-sectional area of a DMPC molecule in a pure DMPC bilayer based on X-ray scattering data as 0.606 Å^2,[30] the expected number of bicelles per rhodopsin molecule can be determined under our experimental conditions [1.2 μM rhodopsin reconstituted in 2% (total lipid w/v) DMPC/DHPC bicelles], to be or on the order of about 10 bicelles per rhodopsin.

Knowledge of the bicelle size enables us to estimate the relative sizes of rhodopsin and the bicelle by using the crystal structure of rhodopsin[11] to derive dimensions for the protein. When distances are measured across rhodopsin in the plane of the hypothetical bilayer, the greatest distance is between M49 and V209, at 40.6 Å The other distances are F88–L266 (29.2 Å) and A166–F294 (24.0 Å). This gives an approximate cross-sectional area of 1200 Å^2, or 12 nm^2, for monomeric rhodopsin. Similar values are given in other structural reports.[31] Atomic force microscopy of native rhodopsin in intact disc membranes[32] revealed rhodopsin forming rows of dimers with an average packing density of 48,000 monomers per 1 μm^2. This is equivalent to a 20-nm^2 area of bilayer occupied per molecule. In the context of the current experimental conditions, the area of rhodopsin and the predicted areas of 0.45 (q_{eff} = 0.37), 0.60 (q_{eff} = 0.98) and 0.78 (q_{eff} = 2.27) mol fraction DHPC in DMPC/DHPC bicelles are shown to scale in Fig. 10. This shows that in the most stabilising bicelle with a q_{eff} of 0.98 (middle blue circles, Fig. 10), a rhodopsin protein would be well within a DMPC bilayer disc of ~76 Å radius. In contrast, for a destabilising q_{eff} of 0.37, the disc is not much larger than rhodopsin.

Modulation of opsin stability by altering the bicelle *q* value in DMPC/DHPC bicelles

Opsin stability is dependent on the q_{eff} ratio of DMPC/DHPC bicelles; increasing sharply (over a q_{eff} range of ~0.4–0.8) to an optimum (as seen by ~65–70% rhodopsin regeneration, Fig. 7b) at q_{eff} values of ~0.8–1.6, above which it decreases slightly (to ~50% regeneration). At the low q_{eff} values (<0.5) there is little DMPC present and opsin is mostly in DHPC micelles that contain only a few DMPC molecules. Since opsin is unstable in DHPC micelles and cannot regenerate rhodopsin, poor regeneration yields are found at low q_{eff} values. At high q_{eff} values over ~1.6, the bicelle radius enlarges and the DMPC portion of the bicelle begins to approximate to a pure DMPC bilayer as far as the opsin molecule is concerned (see relative sizes in Fig. 10). The slight reduction in opsin stability at high q_{eff} could be due to the fact that DMPC bilayers are not an optimal stabilising environment for opsin or rhodopsin; the protein functions better in bilayers containing a mixture of lipids and are less rigid than DMPC (see below). Photobleaching of rhodopsin and regeneration of opsin in DMPC vesicles have shown that in DMPC, Meta II formation is almost completely inhibited. Nevertheless, regeneration can be achieved to good yield (76%) in DMPC (although still lower than

Table 2. Predicted bilayer radii and surface areas for DMPC/DHPC bicelles calculated using Eq. (3)

DHPC (Mole fraction)	DMPC/DHPC q	DMPC/DHPC eff[a] q_{eff}	Radius (nm)[b]	Radius eff[a] (nm)[b]	Area (nm)^2[b]	Area eff[a] (nm)^2[b]
0.33	2.01	5.5	14.5	37.6	656.89	4430
0.39	1.56	3.31	11.5	23.1	414.93	1680
0.45	1.24	2.27	9.4	16.2	275	827
0.5	1	1.66	7.8	12.2	189	466
0.55	0.82	1.26	6.5	9.5	133	284
0.6	0.67	0.98	5.5	7.6	94.5	182
0.65	0.55	0.77	4.6	6.2	67.9	120
0.69	0.45	0.61	3.9	5.1	48.8	80.3
0.74	0.36	0.48	3.3	4.2	34.8	54.2
0.78	0.29	0.37	2.8	3.4	24.5	36.4
0.82	0.22	0.28	2.3	2.8	16.9	24
0.86	0.17	0.21	1.9	2.2	11.2	15.3

[a] Taking into account 7 mM monomeric DHPC in solution.[19]
[b] Using bicelle thickness of 42 Å. From Ref. 26.

Table 3. The number of bicelles per rhodopsin molecule for exemplar bicelle radii

Radius[a] (nm)	Surface area (nm^2)	DMPC per bicelle[b]	DHPC (mol fraction)[c]	Rh molecules[d]	DMPC lipids per Rh	Bicelles per Rh
4	50.2	8290				96
6	113	18,700	0.45	7.20×10^{14}	16,700	43
8	201	33,200				24
10	314	51,800				15
4	50.2	8290				74
6	113	18,700	0.6	7.20×10^{14}	12,400	33
8	201	33,200				19
10	314	51,800				12
4	50.2	8290				44
6	113	18,700	0.78	7.20×10^{14}	7360	20
8	201	33,200				11
10	314	51,800				7

[a] Assumed, for example, bicelle radii between 4 and 10 nm, in the range used in the experiments.
[b] The cross-sectional surface area of DMPC in a pure DMPC bilayer is taken as 0.606 Å^2.[30]
[c] DHPC bicelles (0.45, 0.6 and 0.78 mol fraction) as used in experiments (see Figs. 7 and 8).
[d] As in experimental conditions [2% total lipid (w/v) DMPC/DHPC and 1.2 μM rhodopsin].

in ROS membranes or DMPC/Chaps, where regeneration is almost 100% efficient).[24] Other factors that could influence the stability of opsin at high q_{eff} include protein aggregation or a change in bicelle morphology induced by the larger proportion of DMPC. We anticipate these factors to be negligible. In all our experiments we have an excess of bicelles over protein (See Table 3), which reduces the likelihood of protein aggregation, nor do we observe any aggregation (see, e.g., Fig. 5). Moreover, increasing the bicelle:opsin ratio further by altering the total lipid concentration [from 2% to 8% (w/v)] has no effect on the influence of q_{eff} on opsin stability, which provides further evidence for the lack of protein aggregation under any of these lipid concentrations. We have also selected lipid concentrations, including our highest q_{eff}, which are known to form disc-shaped bicelles. (Preliminary static light-scattering measurements, not shown, also confirmed that the average hydrodynamic radius of the bicelles agreed with the earlier reports.) While we cannot rule out that the incorporation of protein slightly alters the radius or bicelle shape, the estimated radius of the protein-free bicelle nevertheless provides a good empirical guide for optimal protein stability (as shown in Figs. 7 and 9). In terms of chain length, DMPC appears to be the optimal lipid chain length for opsin stability in bicelles (see Table 1). The DMPC/DHPC bilayer thickness has been calculated as 42 Å,[26] while the thickness of a ROS disc membrane bilayer measured by atomic force microscopy is roughly comparable, being approximately 37 ± 2 Å.[33] The apparent opsin stability may also reflect differences in association of each long-chain lipid with DHPC in bicelle aggregates.

An implication of our data (Fig. 9) is that there may be two different denaturation processes occurring depending on the hydrophobic environment. In bicelles of low q_{eff} value, DHPC solubilisation of opsin results in one kind of denaturation related to "looseness" of the DHPC micelle structure. In bicelles of high q_{eff} value, the solely DMPC bilayer does not provide an optimal lipid environment for opsin and hinders regeneration of rhodopsin, possibly by being a too rigid environment for the necessary protein conformational changes. At inter-

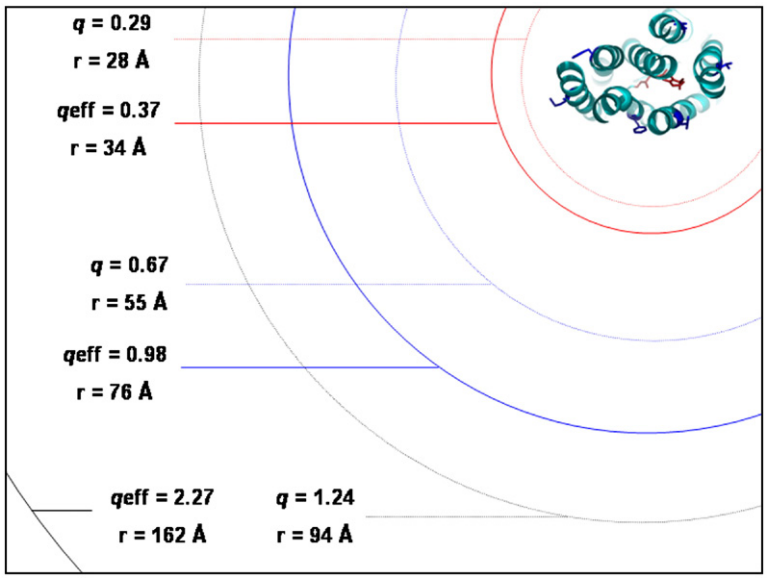

Fig. 10. Predicted radii of DMPC/DHPC bicelles in comparison with rhodopsin, drawn to scale. Radii were calculated using Eq. (3). Circles in faint, dotted lines are based on q values for 0.78 (red, 28 Å, circle), 0.6 (blue, 55 Å, circle) and 0.45 (black, 94 Å, circle) for DHPC mole fractions of 0.29, 0.67 and 1.24, respectively. Larger bold circular lines denote bicelle radius estimates if a free 7 mM monomeric DHPC population exists, using q_{eff}, in which case 0.78, 0.6 and 0.45 mol fraction bicelles would have considerably larger radii of 34, 76 and 162 Å, respectively.

mediate q_{eff} values (~1–2), the properties of the too low or high DMPC situations are balanced out and optimal stability of opsin is observed. This optimal stability seems to arise from opsin being surrounded by a small DMPC bilayer, which protects it from a large destabilising influence of DHPC, but the DHPC imparts a certain degree of flexibility on the DMPC bilayer.

Influence of Chaps

DMPC/Chaps stabilises opsin more than DMPC/DHPC. The DMPC/Chaps system has been less well characterised than DMPC/DHPC. Electron microscopic imaging of bacteriorhodopsin in DMPC/Chaps has revealed discoidal structures that suggest a DMPC bilayer fragment component is present in these mixtures, suggesting a bicelle.[34] It has also been shown that Chaps has an ordering effect on the flexibility of DMPC hydrocarbon chains, which affects the overall bicelle size. At an identical q_{eff} value, DMPC/Chaps mixtures are smaller in diameter than DMPC/DHPC, implying tighter packing of the lipid chains.[20] In addition, all of the Chaps seems to associate with DMPC into these bicelles, while a monomeric population of DHPC exists free in solution.[19]

Interestingly, the stabilities of opsin in DMPC/DHPC and DMPC/Chaps do not converge as the mole fraction of DHPC or Chaps is decreased, when one might imagine that opsin is in a similar, largely DMPC environment. Opsin maintains stability and activity with high relative amounts of DMPC in DMPC/Chaps micelles, whereas in contrast, opsin stability drops off once the amount of DMPC is above a q_{eff} of ~2 in DMPC/DHPC bicelles and the stability (i.e., regeneration yield is lower than in DMPC/Chaps; see Fig. 9). This could be, in part, related to the bicelle size, since to attain similar bicelle sizes, higher amounts of DMPC and thus q_{eff} are required for DMPC/Chaps bicelles. An additional explanation to account for the different properties of DMPC-rich bicelles in the presence of Chaps or DHPC is that Chaps associates specifically with opsin within a bilayer environment. Such Chaps association could directly stabilise the opsin conformation or prevent denaturing interactions. Alternatively, Chaps may mix to a greater extent with DMPC bilayers than DHPC, altering bulk DMPC bilayer properties in a way that favours preservation of opsin structure, such as the lipid-ordering properties of Chaps.[20] Chaps is also structurally related to cholesterol, which has been resolved in bilayer cryoelectron crystallographic studies of rhodopsin[35] and has been proposed to bind directly to rhodopsin from fluorescence studies.[36]

Comparison with rhodopsin photobleaching properties in different environments

The effects of the lipid/detergent environment on the formation of absorbance intermediates (Meta I, II and III) in the photobleaching pathway of rhodopsin have led to an enriched understanding of how local environment can influence membrane protein function. In lipid membranes, flexibility tends to encourage the extended conformational transitions required to form Meta II. The polyunsaturated docosahexaenoic acid abundant in ROS membranes,[37,38] unsaturated hydrocarbon chains[24] and temperatures above the gel-to-fluid phase transition[24] all catalyse the formation of Meta II. Rigidity within the membrane restricts this transition, resulting in buildup of Meta I. This can be created by doping membranes with cholesterol[39] or by using saturated (e.g., DMPC) and short-chain lipids.[24] Furthermore, lateral pressure within the bilayer has also been invoked in the behaviour of rhodopsin during photobleaching in membranes.[40] In detergent micelles, unordered aliphatic detergent chains such as those of DDM promote Meta II formation, whereas bile salt derivatives such as "fused-ring" CHAPSO, a derivative of Chaps, are believed to increase packing interactions and favour Meta I.[41] The stability of opsin (against loss of 11-*cis* retinal binding ability) is also detergent sensitive and is dramatically improved upon addition of phospholipids.[12,13,42] The loss of protein–lipid interactions has been measured to be a considerable factor in the destabilisation of opsin upon solubilisation in detergent.[21] Thus, reintroducing lipids as in bicelles offers a favourable environment.

In conclusion, the findings of this work indicate that a true bilayer environment may not be required to stabilise opsin. Rather, the bilayer discs of simpler DMPC/Chaps and DMPC/DHPC bicelles stabilise functional opsin *in vitro*. DMPC/DHPC bicelles provide a tunable system for modulating opsin stability *in vitro* and this work highlights the impact of the q_{eff} value of bicelles on the stability of a membrane protein. Our results point to an optimal bicelle size, and thus DMPC bilayer size, in the stabilisation of opsin by DMPC/DHPC bicelles. Although a pure DMPC bilayer is not itself optimal for rhodopsin or opsin function, our data indicate that the presence of Chaps in DMPC/Chaps bicelles can compensate for this, with opsin being very stable in these bicelles. Opsin may be further stabilised by direct opsin–Chaps interactions within the DMPC core of these bicelles.

Materials and Methods

Materials

11-*cis* Retinal was a gift from R. Crouch (Medical University of South Carolina and the National Eye Institute, National Institutes of Health, USA). Bovine ROS were from G. Schertler (LMB, Cambridge, UK) and prepared as described.[43] 1D4 anti-rhodopsin monoclonal antibody was from University of British Columbia, USA. Sepharose 4B and concanavalin A–Sepharose were from Amersham Biosciences. 1D4 antibody was coupled to Sepharose 4B matrix according to the manufacturer's instructions. C9 elution peptide TETSQVAPA corres-

ponding to the C-terminus of rhodopsin was synthesised by G. Bloomberg (University of Bristol, UK). DDM and LDAO were from Anatrace Inc. (Maumee, OH). DiacylPC lipids with C12, C13, C14 (DMPC), C15 and C16 saturated chains as well as DHPC were from Avanti Polar Lipids Inc (Alabaster, AL). Chaps was from Calbiochem. All other reagents were from Sigma-Aldrich.

Buffers

The following buffers were used: Buffer A, 10 mM bis-tris-propane (BTP), pH 6.0, 140 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$; Buffer B, 0.1% DDM, 10 mM Tris–HCl, pH 8.0; Buffer C, 0.1% DDM, 10 mM sodium phosphate, pH 7.0, 140 mM NaCl; Buffer D, 0.1% DDM, 10 mM BTP, pH 6.0; solubilisation buffer, 10 mM BTP, pH 6.0, 140 mM NaCl, 1 mM PMSF, 10% (w/v) sucrose, 5 mM ATP, 5 mM $MgCl_2$; Buffer E, 10 mM sodium phosphate, pH 6.0, 100 mM NaF.

Bicelle preparation

All lipid powders were used directly from Avanti Polar Lipids without further purification. A 2% (w/v) DMPC solution was made by suspending the powder in Buffer A and vortexing, followed by heating to 42 °C and then cooling to room temperature. DHPC or Chaps solutions (2%) were also prepared in Buffer A (all percentages are w/v). Volumes of DMPC and DHPC (or Chaps) were added together to give the required mole fraction of DHPC in a final 2% (w/v, total lipid) DMPC/DHPC or 2% (w/v, lipid and detergent combined) DMPC/Chaps solution. For higher concentrations of bicelles, higher starting concentrations were used and mixed in the same proportions. Mixtures were vortexed briefly and then left to stir at room temperature for 1 h or until samples became clear. All bicelle samples were used within 36 h.

Purification and preparation of samples

Rhodopsin purification from ROS into LDAO was modified from Ref. 44. Briefly, ROS membranes were solubilised in 1% LDAO for 1 h. The supernatant was collected by centrifuging at 35,000*g* and rhodopsin was purified by concanavalin A–Sepharose chromatography in 0.1% LDAO in 50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$. The bound protein was eluted in 300 mM methyl α-D-mannoside. Eluted rhodopsin was concentrated through an Amicon PM 10 membrane to 3 mg mL^{-1}.[4] This concentrated rhodopsin is termed Rh_{LDAO}.

Opsin was prepared either by bleaching rhodopsin, $opsin_{Rh}$ (with 300 W>495 nm, as indicated below and in the text), or by direct purification from ROS membranes, $opsin_{ROS}$. $Opsin_{ROS}$ was purified directly from ROS into DMPC/Chaps or DMPC/DHPC mixtures in Buffer A by an adaptation of the existing method.[13] Briefly, ROS was photobleached using a 300-W projector lamp with a >495-nm long-pass filter for 30 min in the presence of 50 mM hydroxylamine on ice. ROS was then solubilised in 1% DMPC/1% Chaps or 1% DMPC/1% DHPC in solubilisation buffer for 1 h at 4 °C before centrifuging at 39,000*g* to collect the supernatant. The supernatant was bound to 1D4-Sepharose 4B and washed with 50 bed volumes of 1% DMPC/1% Chaps or 1% DMPC/1% DHPC in Buffer A. Bound material was eluted with 100 μM C9 elution peptide in 1% DMPC/1% Chaps or 1% DMPC/1% DHPC in Buffer A. All procedures were carried out at 4 °C.

For CD spectroscopy, rhodopsin was purified from ROS into DMPC/DHPC, avoiding LDAO. Rhodopsin was purified as above for opsin, but DDM was used instead of DMPC/DHPC to solubilised rhodopsin for expense reasons and efficiency of solubilisation (additionally no photobleaching step was required); all steps were carried out in the dark at room temperature. 1D4-Sepharose bound rhodopsin was washed in 0.05% DDM and then thoroughly detergent exchanged with 50 bed volumes of DMPC/DHPC. Rhodopsin was then purified directly into DMPC/DHPC of varying mole fractions of DHPC in Buffer E, again by elution with 100 μM C9 peptide.

Photobleaching, regeneration yields and UV/visible spectroscopy

UV/visible absorption spectra were recorded using a Varian Cary 300 UV/vis spectrophotometer equipped with water-jacketed cuvette holders connected to a circulating water bath. All spectra were recorded at 25 °C between 250 and 700 nm with bandwidths of 2 nm, a 0.1-s integration time and a scan speed of 600 nm min^{-1}.

Samples were photobleached for 15 s (unless otherwise stated) by use of a 300-W projector lamp with a >495-nm long-pass filter and absorption spectra were taken immediately after photobleaching. The samples were incubated for varying lengths of time at 25 °C in the light. For $opsin_{Rh}$ preparation, $Rh_{bicelle}$ samples were photobleached for 15 s and incubated at room temperature at pH 6.0 in the light for >1 h for the Meta II (and III) intermediates to decay to give $opsin_{Rh}$.

The regeneration yield was determined by adding 1.5 molar equivalents of 11-*cis* retinal to each sample (approximately 1 μL of a 1 mM solution in ethanol) and recording absorption spectra after 2 h when regeneration was complete. The molar extinction coefficients of rhodopsin (ε_{500}) and 11-*cis* retinal (ε_{380}) were taken as 40,600 and 24,935 M^{-1} cm^{-1}, respectively,[45,46] and a 1-cm path length cell was used for all measurements. In general, opsin was aged for 3 h in DMPC/DHPC or 6 h (due to the increased stability) in DMPC/Chaps, prior to addition of 11-*cis* retinal for rhodopsin regeneration (with a subsequent 2-h incubation for regeneration); times are given in the text.

Data were normalised by $\Delta A_t/\Delta A_i$, where ΔA_t is the change in absorbance at 500 nm relative to 650 nm at any time t and ΔA_i is the absorbance of the original rhodopsin sample at 500 nm relative to 650 nm before bleaching. For thermal bleaching studies, rhodopsin diluted from Rh_{LDAO} into different detergents in Buffer A were incubated in a temperature-controlled cuvette at 55 °C. Initial spectra were taken after 5 min equilibration at 55 °C. Spectra were recorded between 250 and 700 nm at regular time intervals. The ΔA_{500} was plotted against time and normalised against the "initial" spectra recorded 5 min after equilibration at 55 °C. Kinetic data were analysed using GraFit 5 software. Experimentally determined rate constants were obtained by fitting ΔA_{500} *versus* time to a sum of exponential equations.

Circular dichroism spectroscopy

Samples were stored at room temperature in the dark until use and were used within 48 h. CD spectra were recorded at Station 12.1 of the Synchrotron Radiation

Source (SRS) at Daresbury Laboratories, UK. Spectra were recorded at 25 °C between 170 and 270 nm in 0.5-nm intervals with a 0.5-s integration time. The bandwidth was 1 nm and a 0.02-cm path length was used to minimise light scattering. Data were analysed using CDtool software.[47]

Acknowledgements

This work was supported by grants from the MRC (studentship to C.M.), Wellcome Trust (072075) and the EU (European Membrane Protein Consortium). We thank Gebhard Schertler for very generous supplies of ROS membranes and rhodopsin as well as continued support of our work. We are also grateful to Andy Miles for advice with initial SRCD experiments.

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Note added in proof: It is of note that the recent high resolution crystal structure reported for the beta adrenergic receptor used a bicelle crystallisation method. Rasmussen, S. G. F., Choi, H. -J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnala, V. R. P., Sanishvili, R., Fischetti, R. F., chertle, G. F. X., Weis, W. I. & Kobilka, B. K. (2007). Nature. doi:10.1038/nature06325.