

18. DeBiasio, R. L., Wang, L.-L., Fisher, G. W. & Taylor, D. L. *J. Cell Biol.* **107**, 2631–2645 (1988).
 19. Small, J. V. *J. Cell Biol.* **91**, 695–705 (1981).
 20. Wang, Y. -L. *J. Cell Biol.* **101**, 597–602 (1985).
 21. Fan, J., Mansfield, S. G., Redmond, T., Gordon-Weeks, P. R. & Raper, J. A. *J. Cell Biol.* **121**, 867–878 (1993).
 22. Holmes, T. J. *J. Opt. Soc. Am. A* **5**, 666–673 (1988).
 23. Carrington, W. A. *Soc. Photo-opt. Instrumentation Engng Proc.* **1205**, 72–83 (1990).
 24. Podilchuk, C. I. & Mammon, R. J. *J. Opt. Soc. Am. A* **7**, 517–521 (1990).
 25. Koshy, M., Agard, D. A. & Sedat, J. W. *Soc. Photo-opt. Instrumentation Engng Proc.* **1205**, 64–71 (1990).
 26. Preza, C., Miller, M. I., Thomas, L. J. Jr., & McNally, J. G. *J. Opt. Soc. Am. A* **9**, 219–228 (1992).
 27. Kogelnik, H. & Li, T. *Proc. Instn Electr Electron. Engrs* **54**, 1312–1329 (1966).

ACKNOWLEDGEMENTS. This research is supported by the NSF directly, and through the Science and Technology Centers program. We thank D. A. Pane for assistance with computerized instrument control and image acquisition, and J. Montibeller, R. DeBiasio and Y. H. Wang for help with the preparations of labelled proteins and cells.

Stabilization of the membrane protein bacteriorhodopsin to 140 °C in two-dimensional films

Yi Shen^{*†}, Cyrus R. Safinya^{*†}, Keng S. Liang[†],
A. F. Ruppert[†] & Kenneth J. Rothschild[§]

* Materials and Physics Departments, and the Materials Research Laboratory, University of California, Santa Barbara, California 93106, USA

† Exxon Research & Engineering Co., Annandale, New Jersey 08801, USA

§ Physics and Physiology Departments, Boston University, Boston, Massachusetts 02215, USA

‡ To whom correspondence should be addressed

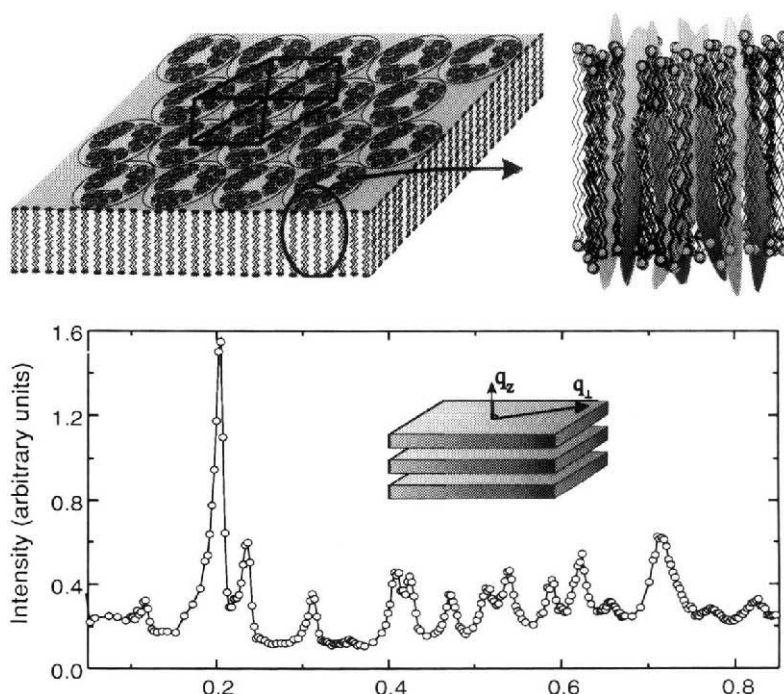
TWO-DIMENSIONAL assemblies of membrane proteins (see ref. 1, for example) such as bacteriorhodopsin are of current interest because of their potential application in technological areas as diverse as molecular electronics and optical switching², molecular sieves^{3,4} and the lithographic fabrication of nanometre-scale

FIG. 1 Top, Schematic top view of the hexagonal lattice of the purple membrane composed of bacteriorhodopsin (bR) trimers with a blow-up of a side view (redrawn from refs 14, 15) of one bR molecule showing the seven α -helices that span the membrane. The purple colour resulting from the absorption of light by the purple membrane, which in its light-adapted form has an absorption maximum near 570 nm, is due to retinal covalently attached to bR (not drawn), found also in the visual pigment rhodopsin^{7–9}. High-resolution cryoelectron diffraction²⁵ and spectroscopic techniques²⁶ have led to a more detailed 3-dimensional structure of bR and its retinal chromophore. Bottom, A typical X-ray intensity scan in reciprocal space along a direction parallel to the membrane plane through the many ordered peaks ((1, 0) through (4, 4)) of the 2-dimensional hexagonal lattice at 27 °C and relative humidity 60%. The inset shows the scattering geometry with respect to the multilayer orientation. Purple membrane was prepared from *Halobacterium halobium* R1M1 (ref. 27). A suspension of lyophilized purple membrane in distilled water (5 mg ml⁻¹) was centrifuged and 1 mg was spread on a thin 25- μ m (hydrophilically prepared) silicon wafer transparent to X-rays and covering an area of diameter about 10 mm. The wet membrane/substrate preparation was then placed in a closed container and brought into equilibrium with deionized water at 100% relative humidity. This sample procedure yielded highly oriented \sim 10- μ m-thick multilayer samples with a mosaic spread of the layer normals of \sim 15 deg measured through a standard crystallographic 'rocking curve' X-ray scan. The X-ray scattering experiments were carried out using both an 18 KW rotating anode X-ray generator, and at higher

patterns^{5,6}. Here we report that bacteriorhodopsin^{7–9} can retain its folded native structure to temperatures as high as 140 °C when incorporated in multilayer structures of self-assembled, ordered films. Synchrotron X-ray scattering reveals that, under hydrated conditions, the two-dimensional lattice in multilayer films exhibits a reversible solid-liquid transition at about 69 °C, followed by irreversible denaturing of the bacteriorhodopsin at about 90 °C. But in dry films the melting transition and denaturation are suppressed up to 140 °C. These results suggest that it may be feasible to use multilayer assemblies of functional proteins and enzymes^{10,11} in high-temperature applications.

The pioneering work of Blaurock, Oesterhelt and Stoekenius^{7–9} in the early 1970s led to the elucidation of the biological function of the integral membrane protein bacteriorhodopsin (bR). This protein functions as a light-driven proton pump and is involved in photosynthesis carried out by *Halobacterium halobium*, an extreme halophile (living in high salt environments) and a member of Archaeobacteria^{12,13} which thrive under conditions inhospitable to common eubacteria. Archaeobacteria also include the high-temperature extreme thermophiles¹², which undergo optimal growth at around 90 °C and are stable to about 110 °C (ref. 12). To our knowledge, the structural stability of self-assembled bR at 140 °C, which we report here, is unprecedented for a folded protein, including those of the extreme thermophiles¹².

Unlike water-soluble globular proteins, membrane proteins¹ can be incorporated into a two-dimensional planar lipid membrane. In its native form, bR self-assembles into a two-dimensional crystalline purple membrane which is part of the plasma membrane of the bacterium. The initial X-ray scattering work⁹ showed that the purple membrane, which has a diameter of \sim 0.5 μ m and bilayer thickness of 49 Å, consists of trimers of bR which self-assemble in a hexagonal lattice with a spacing of 61.6 Å, with the remaining space filled by lipid (Fig. 1, top). Using electron microscopy, Henderson and Unwin^{14,15} showed that each bR molecule (shown schematically in Fig. 1, top right) consists of a polypeptide chain that folds through the membrane to form seven α -helices; this is consistent with infrared dichroism measurements (see ref. 16, for example).



resolution, the synchrotron source at the National Synchrotron Light Source. Details of the experimental set-up have been described^{28,29}.

Figure 1 shows a typical X-ray intensity scan as a function of q_{\perp} in reciprocal space parallel to the membrane at room temperature, through the numerous sharp in-plane peaks of the ordered hexagonal lattice of the purple membrane, with lattice spacing $a=61.6 \text{ \AA}$. This highly developed two-dimensional ordering is in sharp contrast to the commonly occurring ordered L_{β} phases of membranes which comprise lipids for which normally only one or two low-order diffraction peaks are observed¹⁷.

The temperature-dependent behaviour of multilayer stacks of purple membrane at high relative humidity is shown in Fig. 2. A $(0, 0, q_z)$ scan (Fig. 2A) along the layer normal direction through the first six harmonics of the structure factor shows a very large interlayer spacing for the multilayer structure, with $d=2\pi/q_1=196 \text{ \AA}$. Figure 2B includes plots of three in-plane scans: plot (a) shows the sharp (10), (11) and (20) peaks of the ordered phase below the melting transition at $T=65.8 \text{ }^{\circ}\text{C}$, which are replaced by a broad peak of the liquid structure factor indicative of short-range positional order just above T_c in the disordered phase at $T\approx 69.2 \text{ }^{\circ}\text{C}$ (plot b). Upon further heating to $T=99 \text{ }^{\circ}\text{C}$ (plot c), no remnant of the broad liquid peak is seen in the disordered phase. The melting transition of the two-dimensional bR lattice, which we found to be fully reversible, occurs at $T_c\approx 69.0 \text{ }^{\circ}\text{C}$. The slight shift in the position of the liquid peak towards lower q_{\perp} values is consistent with a slightly less dense disordered phase with short-range positional order of the trimers of bR which make up the ordered phase. In contrast, the disappearance of the liquid peak for temperatures at around $99 \text{ }^{\circ}\text{C}$, its irreversible behaviour when the liquid peak does not reappear

upon cooling from this temperature, and the bleaching of the purple colour of the multilayer sample, are all consistent with the onset of denaturation of bR.

The behaviour under low-humidity conditions is entirely different and quite unexpected. The left panels of Fig. 3 show plots of the X-ray intensity under flowing nitrogen conditions, for scans along the in-plane direction (Fig. 3a), the q_z direction through the edge of the $q_{\perp}=(1, 1)$ scattering peak (Fig. 3b), and the multilayer stacking direction (Fig. 3c), both at room temperature and at $T=132 \text{ }^{\circ}\text{C}$. The behaviour for temperatures within this range is essentially identical. The $(0, 0, q_z)$ scans (Fig. 3c) show that the multilayer structure is unchanged from room temperature to very high temperatures at about $132 \text{ }^{\circ}\text{C}$. The order-disorder transition, which occurs around $T=69 \text{ }^{\circ}\text{C}$ at high humidities (Fig. 2B), is suppressed entirely under these dry conditions as the membrane layers are forced to stack closely to one another.

Figure 3d compares high-resolution plots of the X-ray intensity under vacuum, for scans along the in-plane direction at $T=76 \text{ }^{\circ}\text{C}$ and $140 \text{ }^{\circ}\text{C}$. The observation of sharp in-plane peaks of the q_{\perp} scans of Fig. 3a and d is remarkable and shows that under dry conditions, the ordered hexagonal lattice remains intact up to temperatures as high as $140 \text{ }^{\circ}\text{C}$. Furthermore, the fact that the X-ray structure factor intensities of Fig. 3a-d are essentially unchanged from room temperature to $140 \text{ }^{\circ}\text{C}$ indicates that bacteriorhodopsin remains in its tightly coiled α -helix conformation, with bR trimers self-assembled in the hexagonal lattice. We have observed a reduction in the degree of rehydration of multilayers previously exposed to these extreme temperatures of about $140 \text{ }^{\circ}\text{C}$. This suggests that the membrane surface charge density associated with the hydrophilic amino-acid sections of bR protruding between layers is affected by these high temperatures. Although the self-assembled lattice is stable at these temperatures over several days, we found that at temperatures approaching $160 \text{ }^{\circ}\text{C}$, bR denatures irreversibly over a period of a few hours, with the loss of the sharp diffraction peaks observed at $140 \text{ }^{\circ}\text{C}$ and of the purple colour.

Figure 3e shows, the visible absorption spectrum of three dry purple membrane samples measured at room temperature: curve 1 is for the sample at $23 \text{ }^{\circ}\text{C}$ ($\lambda_{\text{max}} 560 \text{ nm}$), whereas the other two samples were cycled through $140 \text{ }^{\circ}\text{C}$ (curve 2, $\lambda_{\text{max}} 567 \text{ nm}$), which resulted in a reduced intensity, broadening and slight red shift, and $160 \text{ }^{\circ}\text{C}$ (curve 3), where the sample bleached irreversibly and showed a flat visible band. The retention of the visible absorption at $\sim 570 \text{ nm}$ of all the dehydrated samples heated up to $140 \text{ }^{\circ}\text{C}$ contrasts sharply with hydrated purple membrane and

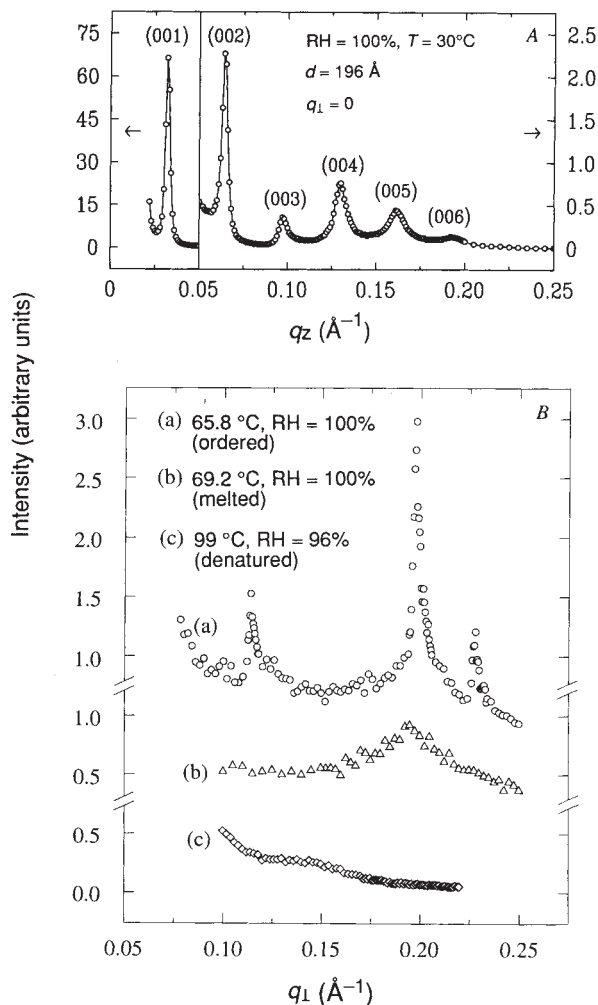
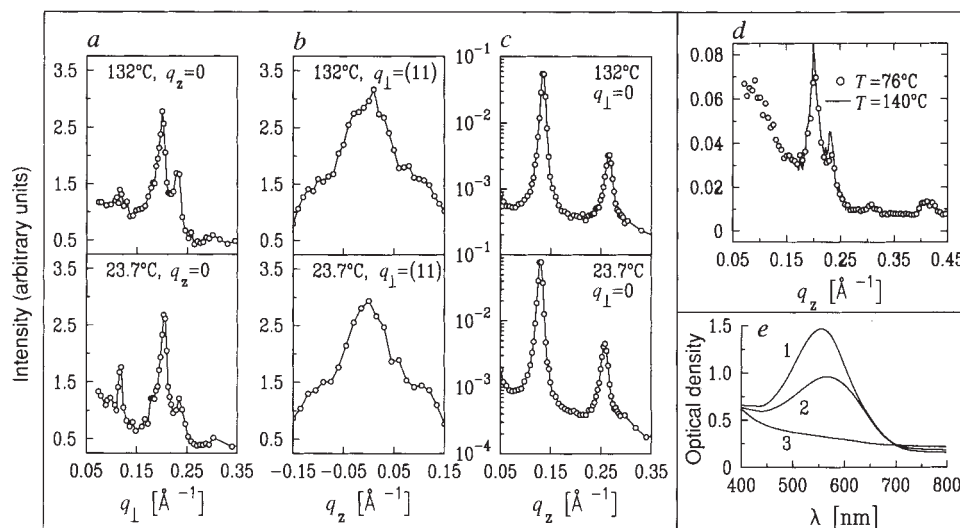


FIG. 2 High-resolution X-ray intensity scans on multilayers at high humidity (sealed in an X-ray capillary containing deionized water). A, a $(0, 0, q_z)$ scan normal to the layers through the first six harmonics of the structure factor of the multilayer, which shows the large interlayer spacing with $d=2\pi/q_1=196 \text{ \AA}$ at 100% relative humidity (RH) B, In-plane scans parallel to the membrane plane: (a) the ordered sharp peaks below the melting temperature of the 2-dimensional hexagonal lattice ($T_c=69 \text{ }^{\circ}\text{C}$); (b) the broad peak of the liquid structure factor of the disordered phase just above T_c ; and (c) the absence of the liquid peak at higher temperatures when bacteriorhodopsin (bR) begins to denature and the membrane purple colour bleaches. Once denaturation (the coil-random transition of the α -helices) has set in, the behaviour is irreversible upon cooling. The broad peak around 0.13 \AA^{-1} is from the tail of the $(0, 0, 1)$ stacking peak which extends down to the q_{\perp} plane because of the very large misorientation of the multilayer under these high humidity and temperature conditions. The scattering profiles are vertically shifted for clarity. Temperature control was about $\pm 0.1 \text{ }^{\circ}\text{C}$. These data are consistent with differential-scanning calorimetry studies of purple membrane dispersed in water basal salts or phosphate buffers²³, which indicated a transition of $\sim 70 \text{ }^{\circ}\text{C}$ associated with the lattice melting²⁴, followed by a broad denaturation transition starting at $\sim 90 \text{ }^{\circ}\text{C}$.

FIG. 3 Low-humidity (dry), low- and high-temperature data from multilayers of bacteriorhodopsin (temperature control was about $\pm 0.1^\circ\text{C}$ for nitrogen-dried samples, and $\pm 0.5^\circ\text{C}$ for samples under vacuum). *a*–*c*, X-ray intensity scans under flowing dry nitrogen at 23.7 and 132°C : *a*, in-plane scans through the first four peaks ((10), (11), (20) and (21)) of the hexagonal lattice; *b*, q_z scans through the $q_\perp = (11)$ peak, showing a width much broader than those of the in-plane scan (*a*), which is indicative of the lack of interlayer positional correlations and arises entirely from the molecular form factor. This suggests that the suppression of the in-plane melting transition is not a result of the lock-in of interlayer positional correlations of neighbouring hexagonal lattices and a cross-over from 2-dimensional to 3-dimensional behaviour; *c*, (0, 0, q_z) scans along the layer normal are indicative of the well-defined multilayer nature of the samples, with interlayer spacing $d = 48.5 \text{ \AA}$ at 23°C and 46.4 \AA at 132°C . *d*, X-ray intensity scans along the in-plane direction under vacuum ($P \approx 10^{-8}$ torr) conditions at 76 and 140°C . Because of the sample holder configuration in our vacuum chamber, scans were recorded with the incident beam at grazing angles to the multilayer film. This resulted in the excess small-angle scattering originating from the substrate which swamped the (10) peak but allowed us to see the strong (11), (20), (21) and higher order peaks of the hexagonal lattice clearly. The sharp in-



plane peaks (*a* and *d*) result from the highly ordered hexagonal lattice of bacteriorhodopsin which retains its structure to these very high temperatures ($T = 140^\circ\text{C}$). *e*, Visible absorption spectrum of three dry purple membrane samples measured at room temperature. Curve 1: sample was held at 23°C ; curve 2: sample cycled through and maintained at 140°C for 2 d; curve 3: sample cycled through and maintained at 160°C for 4 h, which resulted in bleaching. Samples were exposed to room light and then measured in a Varian 2390 spectrophotometer; measuring time was 45 min.

with bacteriorhodopsin reconstituted in various lipids and detergents, which exhibit an irreversible structural denaturation below 100°C , that is characterized by a loss of visible absorption^{18–20}. The proton-pumping activity of bacteriorhodopsin has not been measured in the high-temperature films, but the retention of the purple colour at 140°C , together with the X-ray data, is strong evidence that the overall structure of the protein, the protonated Schiff-base bond attaching the retinal chromophore to the lysine residue at position 216, and the protein–chromophore interactions^{2,7–9}, are all maintained. The partial red shift and broadening in the absorption of the high-temperature films is not unexpected and may be due to a shift in equilibrium of bR towards the 'O' intermediate, the last in the bR photocycle², which absorbs near 640 nm. A similar effect has been observed in purple membrane when the temperature is raised to 35°C (ref. 21) and also in the mutant Tyr 185→Phe at room temperature²².

Although it is not clear how the removal of water, which results in the forced stacking of the membrane sheets and the hindrance of out-of-plane fluctuations, suppresses the temperature-induced order–disorder transition in purple membrane, it is likely that the absence of the melting transition enhances the stability of the protein structure at these unusually high temperatures around 140°C . Our data suggest that the dehydrated two-dimensionally ordered phase of bR results in more extensive inter-protein interactions and a larger pressure of the ordered phase up to 140°C , relative to that of the corresponding liquid phase of bR found by us and others^{23,24} above 70 – 80°C in hydrated multilayers. This then enhances the bacteriorhodopsin folding forces by exerting steric constraints that prevent protein structural changes, which otherwise would disrupt the bR α -helical bundle at these high temperatures. This is in agreement with previous results^{18,20} which indicate that elimination of the crystalline lattice lowers the thermal stability of bR.

Aside from the direct potential applications to bacteriorhodopsin, we believe that our finding should lead to the stabilization of the structure of many other proteins and enzymes: that is, by artificially preparing multilayers of ordered arrays of

macromolecules^{10,11}, significant high-temperature stability should be generated. Such stability will allow the investigation of the structure of a folded protein under these extreme temperature conditions. □

Received 12 July; accepted 21 September 1993.

1. *Electron Microscopy at Molecular Dimensions: State of the Art and Strategies for the Future* (eds Baumeister, W. & Vogell, W.) (Springer, Berlin and New York, 1980).
2. Birge, R. R. *Rev. Phys. Chem.* **41**, 683–733 (1990).
3. Steyr, U. B. & Sara, M. *Appl. Microbiol. Biotechnol.* **25**, 83–90 (1986).
4. Baumeister, W. & Lembcke, G. *J. Bioenerg. Biomembr.* **24**, 567–575 (1992).
5. Douglas, K., Clark, N. A. & Rothschild, K. J. *Appl. Phys. Lett.* **56**, 692–694 (1990).
6. Douglas, K., Devaud, G. & Clark, N. A. *Science* **257**, 642–644 (1992).
7. Oesterhelt, D. & Stoekenius, W. *Nature New Biol.* **233**, 149–152 (1971).
8. Oesterhelt, D. & Stoekenius, W. *Proc. Natn. Acad. Sci. U.S.A.* **70**, 2853–2857 (1973).
9. Blaurock, A. E. & Stoekenius, D. *Nature New Biol.* **233**, 152–155 (1971).
10. Uzgiris, E. E. & Kornberg, R. D. *Nature* **301**, 125–129 (1983).
11. Blankenburg, R., Meller, P., Ringsdorf, F. & Saless, C. *Biochemistry* **28**, 8214–8221 (1989).
12. Brock, T. D. & Madigan, T. in *Biology of Microorganisms* 6th edn (Prentice Hall, New Jersey, 1991).
13. Woese, C. R. *Sci. Am.* **244**, 98–122 (1981).
14. Henderson, R. & Unwin, P. N. T. *Nature* **257**, 28–32 (1975).
15. Unwin, P. N. T. & Henderson, R. *J. molec. Biol.* **94**, 425–440 (1975).
16. Braiman, M. S. & Rothschild, K. J. *A. Rev. Biophys. Chem.* **17**, 541–570 (1988).
17. Smith, G. S., Sirota, E. B., Safinya, C. R., Plano, R. J. & Clark, N. A. *J. chem. Phys.* **92**, 4519–4529 (1990).
18. Brouillette, C. G., McMicheas, R. B., Stern, L. J. & Khorana, H. G. *Proteins: Struct. Funct. Genet.* **5**, 38–46 (1989).
19. Kresheck, G. C. et al. *J. Photochem. Photobiol. B* **7**, 289–302 (1990).
20. Kahn, T. W., Sturtevant, J., Engelman, D. M. *Biochemistry* **31**, 8829–8839 (1992).
21. Fukuda, K. & Kouyama, T. *Biochemistry* **31**, 11740–11747 (1992).
22. Sonar, S., Krebs, M. P., Khorana, H. G. & Rothschild, K. *Biochemistry* **32**, 2263–2271 (1993).
23. Jackson, M. B. & Sturtevant, J. M. *Biochemistry* **17**, 4470–4474 (1978).
24. Hiraki, K., Hamanaka, T., Mitsui, T. & Kito, Y. *Biochim. biophys. Acta* **647**, 18–28 (1981).
25. Henderson, R. et al. *J. molec. Biol.* **213**, 899–929 (1990).
26. Rothschild, K. J., Braiman, M. S., Mogi, T., Stern, L. J. & Khorana, H. G. *FEBS Lett.* **250**, 448–452 (1989).
27. Oesterhelt, D. & Stoekenius, W. *Meth. Enzym.* **31**, 667–678 (1974).
28. Safinya, C. R. et al. *Phys. Rev. Lett.* **57**, 2718–2721 (1986).
29. Safinya, C. R., Sirota, E. B., Roux, D. & Smith, G. S. *Phys. Rev. Lett.* **62**, 1134–1137 (1989).

ACKNOWLEDGEMENTS. We thank S. Sonar and N. Patel for help with purple membrane preparation; R. Bruinsma, N. Clark, P. Pincus, T. Lubensky, D. Morse, D. Nelson, and S. Sonar for discussion; and D. Thirumalai for critically reading the manuscript. The National Synchrotron Light Source (NSLS) is supported by the US Department of Energy. The Materials Research Laboratory at Santa Barbara is supported by the NSF. C.R.S. acknowledges the NSF for a biomolecular materials grant, and the Exxon Research and Engineering Company for a research grant; K.J.R. thanks the NSF and the ARO for support.