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Membrane-protein stability in a phospholipid-based crystallization medium

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Abstract

Protein stability is a crucial factor to consider when attempting to crystallize integral membrane proteins. Cubic phase, or in meso, lipid-bilayer crystallization media are thought to provide native-like environments that should facilitate membrane protein crystallization by helping to stabilize the native protein conformation for the duration of the crystallization process. While excellent crystals of bacteriorhodopsin (bR) and other Halobacterial rhodopsins have been obtained in lipid-bilayer gels formed with monoglycerides, success remains elusive in the general application of such media to other membrane proteins. Additionally, we have noted that some mutants of bR are highly unstable in gels formed with monoolein. Phosphatidylethanolamines (PE) and derivatives of PE represent another class of lipids that can form connected-bilayer gels. When wildtype bR and a labile bR mutant were reconstituted into this phospholipid gel, spectroscopy showed that the protein is both more stable and has improved conformational homogeneity as compared to gels formed using monoolein. In addition, we demonstrate that well-diffracting crystals of bR can be grown from a PE-based crystallization medium. Since most proteins lack a stability-indicating chromophore and other structure-based analytical techniques are poorly compatible with the lipid gel, we developed a generally-applicable spectroscopic technique based on the intrinsic fluorescence of tryptophan residues. This fluorescence assay makes possible the rapid evaluation of lipid gels as media for the crystallization of membrane proteins.
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1. Introduction

While protein stability is a crucial factor to consider when working with any protein, maintaining long-term stability is especially critical when handling detergent-solubilized membrane proteins. The fact that the half-life for detergent-solubilized membrane proteins may be as short as days or even hours is of particular concern in the context of membrane-protein crystallization.

One approach that has been developed for the crystallization of detergent-solubilized membrane proteins involves the use of lipid cubic phase, or in meso, crystallization media (Caffrey, 2003; Landau and Rosenbusch, 1996). It is generally thought that the lipid-bilayer environment of such media could, upon incorporation of the detergent-solubilized protein, stabilize the native protein conformation for the duration of the crystallization process, which may extend from days to weeks.

Cubic phase gels formed by hydrated monoglycerides have been very successful for crystallizing bacteriorhodopsin and other members of the Halobacterial rhodopsin family (Gordeliy et al., 2002; Kolbe et al., 2000; Landau and Rosenbusch, 1996; Lanyi and Schobert, 2002; Luecke et al., 1999, 2001; Rouhani et al., 2001). This research has significantly contributed to our detailed understanding of structural events that define reaction pathways of ion-pumping

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and transmembrane signaling (Facciotti et al., 2004). Few examples exist, however, of other membrane proteins for which well-diffracting crystals have been grown from monoglyceride gels. The two examples of other structures that could be solved at high-resolution are a photosynthetic reaction center complex (Katona et al., 2003) and a eubacterial sensory rhodopsin (Vogeley et al., 2004). Recently, Misquitta et al. (2004) have reported success in the crystallization of the outer membrane transporter, BtuB, in both monoolein (MO) and in a second, rationally designed monoglyceride, which resulted in protein diffraction to 4 Å.

In the course of previous structural studies of intermediates in the bacteriorhodopsin photocycle (Facciotti et al., 2001, 2003; Rouhani et al., 2001), we found that some mutants of bacteriorhodopsin are actually less stable when reconstituted into MO. This observation contradicted the generally accepted hypothesis that incorporation of the solubilized protein into the MO bilayer might lead to a more favorable crystallization environment. Rather, this experience suggested that hydrated monoglyceride gels may in some cases actually destabilize membrane proteins, and this could account for the scarcity of other membrane proteins that have been crystallized from these media.

Since the reconstitution of membrane proteins into lipid bilayers is nevertheless known to confer considerable stability to membrane proteins, it seemed worthwhile that connected-bilayer gels based on diacylglycerophospholipids, rather than monoglycerides, should be explored. In fact, there already is an example of a hydrated phosphatidylcholine-based gel (Nieh et al., 2002) from which it has been demonstrated that bacteriorhodopsin can be crystallized (Faham and Bowie, 2002).

Phosphatidylethanolamines (PE) and derivatives of PE also represent a class of lipid that are known to make lipid-bilayer gels (Haque et al., 2001; Johnsson and Edwards, 2001; Keller et al., 1996; Koynova et al., 1997), but these have not previously been investigated as media for the crystallization of membrane proteins. Given the abundance of PE in natural membrane lipids, and that they are sometimes implicated as being required for the proper function of membrane proteins, PE-based bilayer gels present an attractive alternative as a crystallization medium.

In this paper, we first show that even wildtype bacteriorhodopsin (wt bR), and not just its more labile mutants, is structurally perturbed when reconstituted into the MO cubic-phase gel. We then demonstrate that the visible-light absorption spectra of both wt bR and an extremely labile triple-mutant of bR are much less perturbed when the protein is reconstituted into a hydrated gel formed with monomethyl-DOPE plus 5% DOPE-mPEG350. In addition, we show that well-diffracting crystals of wt bR can be grown from this PE-based lipid bilayer medium. (The development of this and other PE lipid-gel media and a description of methods used for screening conditions that produce the growth of protein crystals is detailed in Rouhani et al., in preparation). Finally, we describe a more general technique to monitor the stability of membrane proteins in various lipid-gel environments, which is based upon the fact that intrinsic tryptophan fluorescence is sensitive to protein denaturation and permits the evaluation of lipid gels as media for the crystallization of membrane proteins.

2. Materials and methods

2.1. Lipid gel formation

Phosphatidylethanolamine lipids and their conjugates were purchased from Avanti Polar Lipids (Alabaster, Al). Monoolein was purchased from Nu-Check-Prep (Elysian, MN). The D96G/F171C/F219L mutant was kind gift from Dr. J. Tittor and Dr. D. Oesterhelt.

Chloroform stocks of host and fusogenic lipids were mixed according to the desired mass-ratio of lipid and then dried. Note that fusogen percentages reported are percentages of total lipid, not total gel volume post-hydration. To form the lipid bilayer gel, as described by Rouhani et al. (in preparation), the dry lipid was mixed with protein solution to a final aqueous concentration of 67% (w/w) using the syringe mixing apparatus described previously (Cheng et al., 1998; Rouhani et al., 2002). MO gel was prepared in syringes by hydrating dry MO to a final aqueous concentration of 40% (w/w) with protein solution.

2.2. Protein crystallization and diffraction measurements

Wildtype bR was purified as previously described (Facciotti et al., 2001; Rouhani et al., 2001). In this paper, wildtype bR refers to the protein produced by *Halobacterium salinarum* strain ET1001. Briefly, bR was solubilized from sucrose-gradient purified membranes in 1.2% octylglucoside in phosphate buffer at 20:1 detergent:protein mass ratio for 18–24 h. The protein was concentrated up to 30 mg/ml using Millipore Ultrafree concentrators with 30 kD MWCO.

Crystallization screens were carried out in 96 well plates. In brief, 0.2–1 μl drops of the gel were dispensed in the bottom of each well using the syringe-dispensing apparatus. The gel drops were then covered with 5 mm glass coverslips (Bellco Glass, Vineland, NJ) and overlaid with 50 μl of crystallization solution. The plate was sealed using Clear Seal tape (Hampton Research, Aliso Viejo, CA). Crystallization was carried out at room temperature (22 °C).

2.3. Spectroscopy

Absorbance and fluorescence measurements were performed by flattening the gel to a thickness which is appropriate for optimal detection depending upon protein concentration, type of gel medium, and instrument detection limits. A uniform thickness of gel was obtained by either squashing the gel between a coverslip and the bottom of the well using a Teflon washer as a spacer (125 or 500 μm thick), or within a detachable-window quartz cell assembly (200 μm

pathlength) (Hellma Cells, Plainview, NY). Alternatively, centrifuging a microplate so that the gel spreads flat across the well-bottom works well when processing many samples. In this case, a centrifuge with microplate rotor (Eppendorf 5804, A2DWP rotor) was used at 2000*g* for 1–3 min. It was important to dispense the gel into wells that are close to the centrifuge spindle, as gel in those wells farther away becomes wedge-shaped instead of uniformly flat.

A variety of instruments were used for absorbance and fluorescence measurements including a Beckman 640DU spectrophotometer, a SpectraMax Plus absorbance plate reader & Gemini XS fluorescence plate reader (Molecular Devices, Sunnyvale, CA), and a Safire plate reader (Tecan US, Research Triangle Park, NC). Absorbance spectra were scaled and their backgrounds were adjusted to permit comparison of relevant features of the spectra. Absolute absorbance intensity has little meaning for these samples since variations in gel turbidity, gel thickness, and protein concentration strongly affect the output. In contrast, relative changes to the waveforms remain very informative when scaled to one another.

The intrinsic fluorescence assay was performed by dispensing gel (usually 5 μl) into a 384-well plate (Corning #3710, Acton, MA) which was chosen for its small well footprint, negligible autofluorescence, and adequate resistance to heat. Addition of 50 μl of buffer before heating is important to prevent desiccating the gel. The instrument was set for excitation at 280 nm and the emission spectrum was recorded from 300 to 400 nm. Once this first measurement was taken, tape was applied to seal the microplate and the gel was heated at 90 °C for 5 min in a microplate heating block, allowed to cool, sealing tape was removed, and then another fluorescence measurement was taken. Changes in the spectra that indicate protein denaturation after heating include changes in fluorescence intensity, λ_{max} shifts, and other waveform changes.

3. Results

3.1. Bacteriorhodopsin and its mutants show improved stability in a PE-based crystallization medium

During previous structural studies of intermediates in the bacteriorhodopsin photocycle, we found that some mutants of bR were quite unstable when reconstituted into the MO cubic-phase gel. In addition to the F219L mutant, which we were able to crystallize by the addition of excess retinal (Rouhani et al., 2002), these included the D85N/F42C double mutant and the D96G/F171C/F219L triple mutant. Although the F219L mutant could be stabilized in the MO gels by the addition of excess retinal, even this strategy failed to prevent bleaching of the others, which occurs on the time scale of minutes to hours after reconstitution, depending upon the mutant and the buffer conditions. No crystals were obtained in cases where the visible purple color of the protein was lost after reconstitution into the MO gel.

Following up on the observation that mutants of bR are prone to denature in MO bilayers, we decided to look closely at wildtype bR itself. The color of such gels appeared "by eye" to be similar to native purple membranes and to detergent-solubilized bR, and remained so without apparent bleaching for weeks. However, absorbance spectra now show that a substantial fraction of the protein, in both the detergent-solubilized state (octylglucoside) and when reconstituted into MO, is spectroscopically perturbed relative to the native protein in the membrane.

As seen in Fig. 1, absorbance spectra give information about the structural state of bR. Purple membranes exhibit a peak at 568 nm while bR which is detergent-solubilized or incorporated into MO exhibits a peak shifted to 560 nm or lower. The spectrum of detergent-solubilized wt bR shows that a fraction of the protein is denatured. Upon inserting the protein into MO, however, it is clear that the main peak is considerably broadened towards shorter wavelengths than it is in the detergent-solubilized state, and that a secondary peak appears at 410 nm. The peak broadening and appearance of the 410 nm species shows that the structure of bacteriorhodopsin is actually quite heterogeneous after reconstitution into the MO gel. Heat denaturing the protein while in MO results in a single peak at 380 nm, as would be expected for completely denatured protein.

These results confirmed that the degree of structural homogeneity of bR in MO is significantly less than that

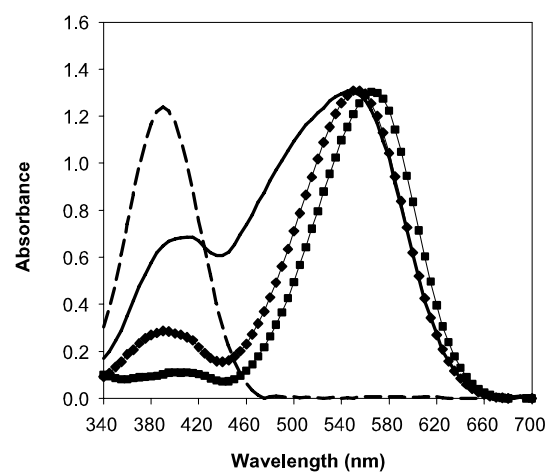


Fig. 1. The absorption spectrum of bacteriorhodopsin (bR) in three different environments: native purple membrane, detergent-solubilized (octylglucoside), and reconstituted into monoolein (MO). Purple membranes exhibit a λ_{max} at 568 nm while bR which is detergent-solubilized or incorporated into MO exhibits a peak shifted to 560 nm or lower. Additionally, when in MO, the peak is considerably broadened toward shorter wavelengths and there is an additional peak at 410 nm, implying the presence of a species with a deprotonated Schiff base. When in detergent, bR has an additional peak at 380 nm which is due to free retinal that is released from denatured protein. Essentially all of the protein is denatured (retinal is released) when the protein is heated while in the MO gel. The spectral curves have been adjusted for background and scaled to facilitate comparisons of their respective waveforms. The spectra are identified as: purple membranes (-■-), detergent-solubilized bR (-◆-), bR in MO (—), heat-treated bR in MO (- - -).

in native membranes. It is possible that large alterations in the spectrum of bR can result from very small structural changes in the protein. Even so, any structural heterogeneity that is detectable during crystallization is of concern, as nonuniform proteins are less likely to consistently form the favorable protein–protein contacts needed for crystal formation. As a result, we were motivated to investigate whether other lipids, which can also form connected-bilayer gels, might provide a better environment for retaining a more native conformational state of the protein.

The PE gels were next evaluated for their effect on protein stability. The triple mutant of bR, D96G/F171C/F219L, is especially stringent as a test protein as it is the most labile mutant that we have worked with to date. In this case, the protein was introduced directly into the PE gel in the form of purified purple membrane, since solubilization with detergent already leads to the rapid onset of denaturation.

Fig. 2 shows absorption spectra of the D96G/F171C/F219L triple mutant and wt bR after reconstitution into a gel of monomethyl-DOPE, which was formed with 5% DOPE-mPEG350 as a fusogenic catalyst. When the triple mutant is reconstituted into MO, the color rapidly bleaches, indicating denaturation of the protein as seen in the spectrum which shows a single peak at 380 nm (Fig. 2A). In contrast, triple mutant is dramatically more stable and retains its purple color in a PE-based gel. Although the visible spectrum is broader than it is while in the isolated (mutant) purple membrane, the spectrum still shows far less perturbation than occurs in MO.

The data in Fig. 2B show that the absorption maximum of wt bR in the PE gel is close to 568 nm, the value found in native membranes, rather than being blue-shifted as it is for detergent-solubilized protein. Improvement of the absorption spectra of wt bR in the PE gel versus the detergent solubilized bR and bR in MO reinforces the point that even wt bR is structurally disordered in the MO gel.

As with reconstitution using detergent-solubilized protein, the method of adding intact purple membrane during the formation of a hydrated gel permits the protein to dissolve and disperse into the lipid gel. The fact that the triple mutant of bR was dispersed into the lipid bilayers of the gel when added as purple membranes was confirmed by heating the gel to 80 °C for 5 min. This heat-treatment does not affect the visible spectrum of the triple mutant when the protein is still in purple membranes, but when membranes have been used to form the PE-lipid gel the 560 nm peak shifts predominantly to 440 nm after heating, indicating the presence of a partially denatured bR state (Fig. 3). These spectra demonstrate that the bR must have dissolved into the PE lipid matrix, thereby permitting the protein to become sensitive to denaturing heat.

It is notable that the triple mutant, as well as wt bR (Fig. 2B), appear to be only partially denatured when heated in PE gels, since the spectrum is shifted to approximately 440 nm (indicating a perturbed opsin-shift for the retinal chromophore). In contrast, heat treatment of wt bR in MO results in a complete peak shift to 380 nm (Fig. 1).

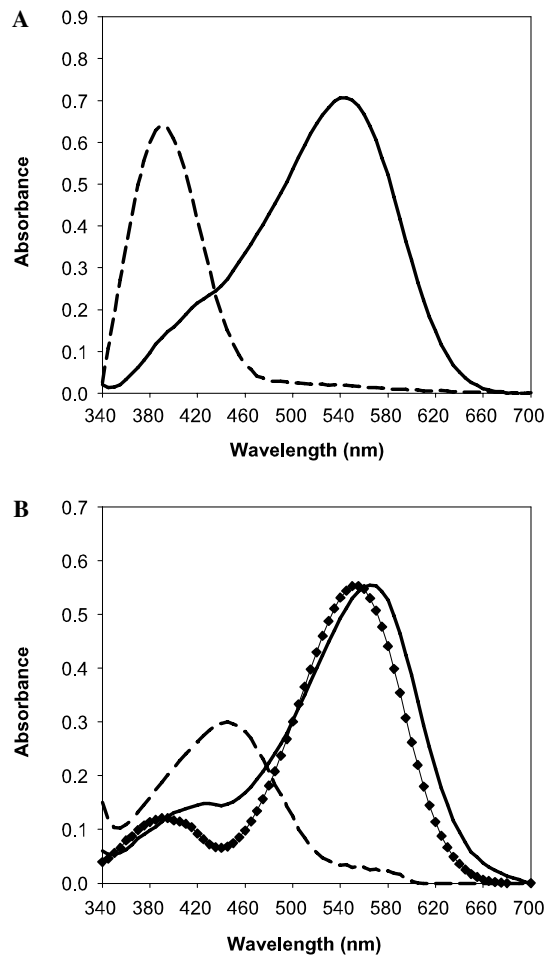


Fig. 2. Absorption spectra of wt bR and a triple mutant of bR (D96G/F171C/F219L) demonstrate that both are in a more native conformation in a PE-based gel. (A) The very labile triple mutant of bR is rapidly denatured when incorporated into MO (- - -) but is highly stabilized against denaturation in the PE gel (—). (B) Even wildtype bR exhibits more native character in PE (—) than in detergent (-◆-) or MO (Fig. 1) as seen by absence of a 380 nm peak, a smaller 410 nm peak, and λ_{max} at 568 nm which is most similar to bR in native membrane. Heat-denatured bR in PE exhibits a peak which is shifted from 568 to 440 nm (- - -). The spectral curves have been adjusted for background and then scaled to facilitate comparisons of their respective waveforms.

3.2. PE-based lipid gels support the growth of diffraction-quality crystals of bacteriorhodopsin

Even though protein stability is a requirement for crystal formation, by itself stability is not a guarantee that crystals will nucleate and grow. It was thus important to determine whether wildtype bacteriorhodopsin crystals could be grown from this medium. To test this point, monomethyl-DOPE with 5% DOPE-mPEG350 was hydrated with solubilized wildtype bR to a protein concentration of 18 mg/ml. Aliquots of this gel were dispensed into the bottom of a microplate well, flattened with a small coverslip, and overlaid with crystallization solution.

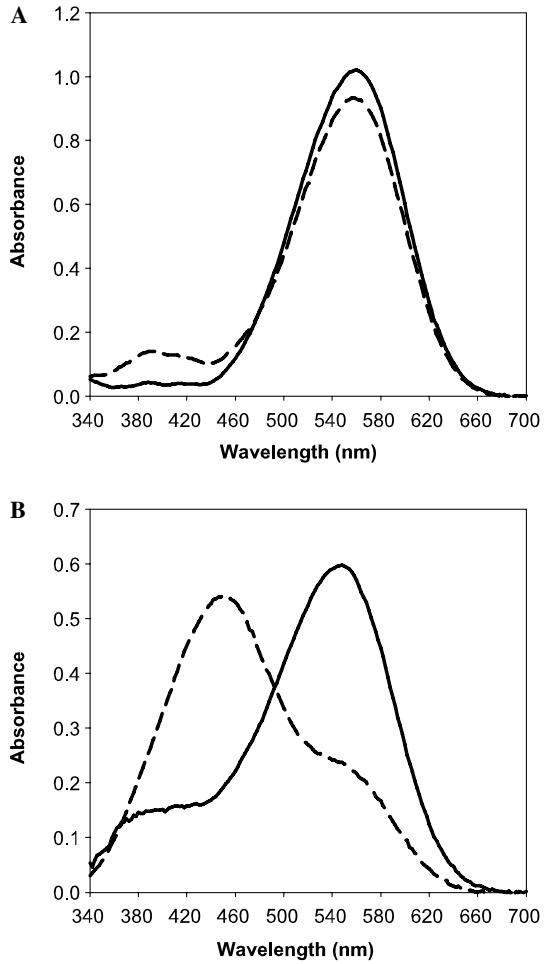


Fig. 3. Absorption spectra of bR triple mutant before and after a heat-treatment. (A) Triple mutant membranes before (—) and after (- - -) heating at 80 °C show minimal difference, since this temperature is not high enough to denature the mutant protein of bR when it is still in the highly stabilizing native membranes. (B) Hydrated lipid bilayer gel, prepared by mixing triple mutant membranes with PE lipid, before (—) and after (- - -) heating at 80 °C shows the 560 nm peak shift predominantly to 440 nm indicating a perturbed opsin-shift for the retinal chromophore. The different sensitivity to heating seen for the mutant bR incorporated into the PE gel indicates that the protein has dissolved into the lipid matrix. The spectral curves have been adjusted for background and then scaled to facilitate comparisons of their respective waveforms.

Crystals of wt bR were discovered to grow when 2.5 M sodium potassium phosphate buffer pH 5.6 was overlaid on the gels, and the crystals displayed the expected hexagonal morphology (Fig. 4). A variety of crystal sizes were observed, ranging from 5 to 80 μm across. The crystals were harvested, frozen without cryoprotectant, and evaluated at beamline 8.3.1 of the Advanced Light Source (ALS) at the Lawrence Berkeley National Laboratory. Diffraction patterns collected from crystals grown in PE gels are well-ordered and display isotropic diffraction, in one instance producing detectable spots at a resolution of 1.5 Å (Fig. 5). This result confirmed that the PE-based crystallization medium supports the growth of diffraction-quality crystals as well as providing an environment that is favorable for greater protein stability.

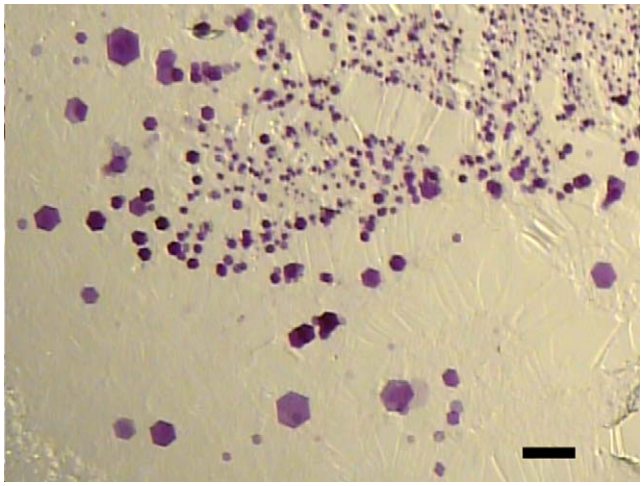


Fig. 4. Crystals of bR grown in PE-based lipids. Detergent-solubilized wildtype bR was reconstituted into gels of monomethyl-DOPE with 5% DOPE-mPEG350. The crystallization solution was 2.5 M sodium potassium phosphate, pH 5.6. The scale bar is 100 μm.

3.3. Intrinsic fluorescence assay for protein stability

The discovery that membrane proteins might actually be perturbed or denatured by reconstituting them into the various lipid bilayer gels has made us aware that it is useful to include a spectroscopic assay for protein stability as a part of our crystallization experiments. We have therefore developed a method for using the intrinsic fluorescence of tryptophan residues to monitor whether denaturation has occurred. The objective is to monitor a membrane protein's structural integrity in the various environments encountered in the course of crystallization experiments, including the detergent-solubilized state, shortly after insertion into the bilayer gel matrix, and after exposure to crystal-inducing precipitant solutions.

The assay that we have developed works by measuring the change in fluorescence that occurs after the protein is exposed to denaturing levels of heat. This assay is performed in a microplate format, so it is well suited for screening a large number of crystallization conditions. If there is no measurable change in the intrinsic fluorescence spectrum after heating the sample to 90 °C for 5 min, the result is scored as indicating that the protein was already denatured *before* the sample was heated. In this case, the particular step in question would be rejected as being unsuitable for crystallization of that particular protein. We have found that the method is well-suited to give semi-quantitative comparisons.

As an example, we show that the intrinsic fluorescence assay for retention of native structure confirms, as expected from previous observations of the visible absorption spectra, that the native structure of bacteriorhodopsin is more fully retained in the PE-based gels than it is in MO. Fig. 6A shows that the intrinsic fluorescence of the triple mutant is hardly changed after heating when the protein is in MO, demonstrating that it was already denatured before heating.

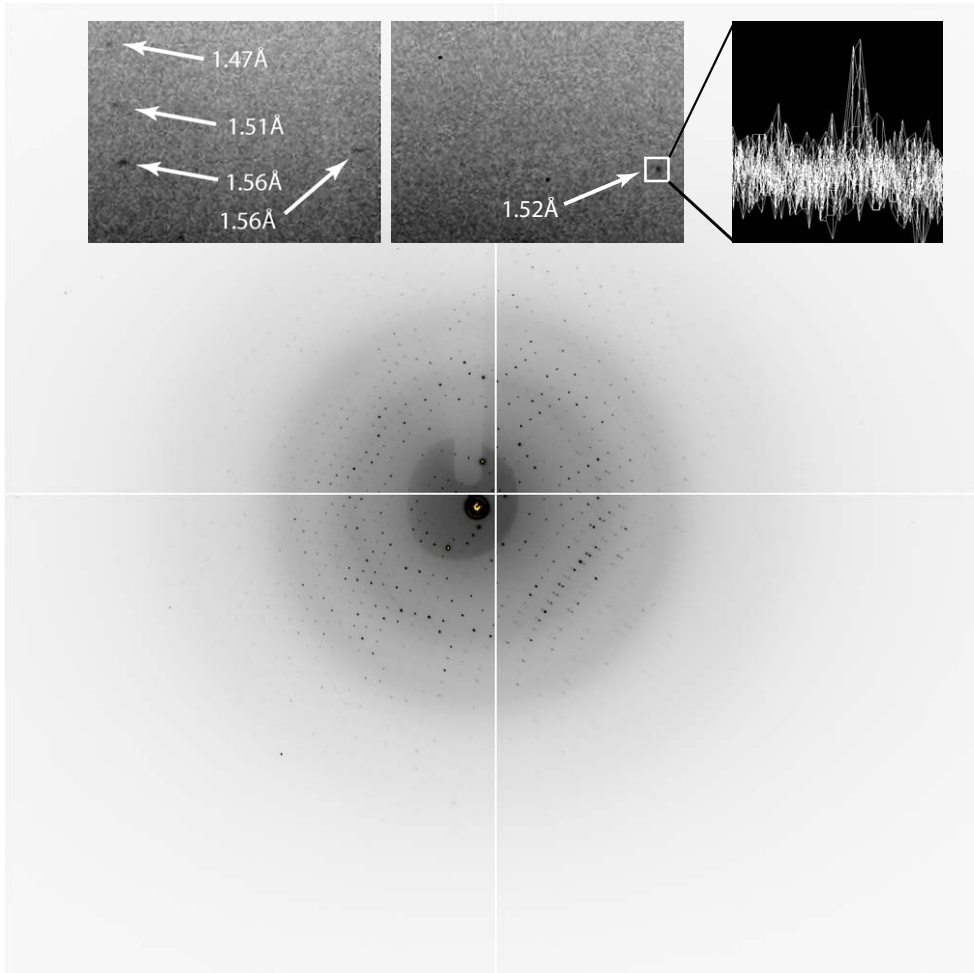


Fig. 5. Diffraction pattern of bR crystal grown in PE-based lipids. Insets are magnifications of sections from near the top-center of the pattern, and show the diffraction spots around 1.5 Å. The trace shows the signal-to-noise ratio of the selected diffraction spot (sample-to-detector distance, 100 mm; exposure, 60 s).

The 112% ratio of relative fluorescent units (RFU) before and after heating protein in MO falls within experimental variability. Fig. 6B, however, shows that there is a large change in the fluorescence after heating when the triple mutant is in PE gel, demonstrating that the protein was sensitive to heating and was therefore initially in a more native-like state. The visible spectrum of triple mutant in PE (Fig. 3B) confirms that denaturation occurs as a result of heating.

The intrinsic fluorescence of wildtype bR was also tested in MO and PE (data not shown). Upon heating, a large change in intensity was produced in both media as expected, because we already knew (from the visible spectra in Figs. 1 and 2B) that wt bR remains largely in the native state in MO as well as in PE. However, in its current state the intrinsic fluorescence assay was not able to clearly show the structural differences between native (non-heated) wt bR in MO versus PE that are reflected in the visible absorbance spectrum.

4. Discussion

As experience in the expression, purification and crystallization of membrane proteins accumulates, the number of membrane protein structures determined to high resolution has increased significantly in the last few years (Iwata and Kaback, 2004). What is notable in many of these successful crystallizations is that either a ligand (or a conformation-specific Fab) was used, suggesting that a specific protein conformation was stabilized during crystallization. Alternatively, a functionally stalled mutant was found to be significantly more amenable to crystallization. Furthermore, the retention of lipids during solubilization, or the addition of lipids during crystallization, has been shown to stabilize membrane proteins and improve crystallization. This trend indicates that crystal formation is more likely to occur when the protein can be stabilized in a structurally homogeneous state over the period of time required for crystal growth to occur. It is for this reason that we believe it is important to know that the PE-based crystallization medium favors protein stability, and in addition, that it is a medium which permits the formation of protein crystals.

The various structurally perturbed (as well as predominantly native) forms of wildtype bacteriorhodopsin species that are seen in MO presumably coexist in a reversible

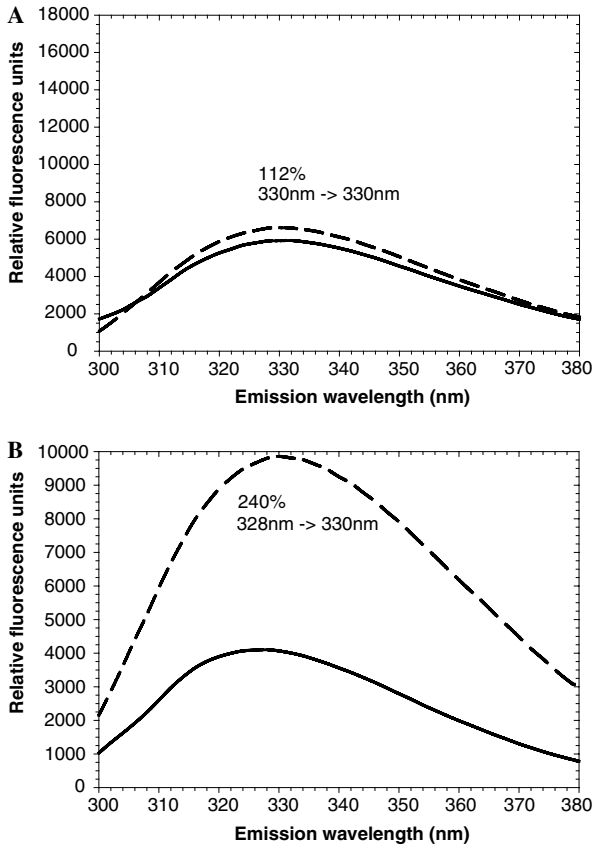
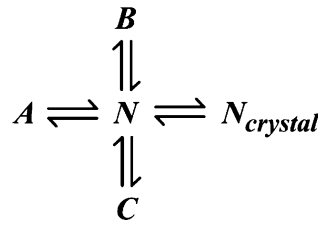


Fig. 6. The intrinsic tryptophan fluorescence (280 nm excitation) of bR triple mutant is compared for protein after reconstitution in MO (A) and PE-based gels (B). The fluorescence spectra show that the mutant protein is hardly changed after heating when in monoolein, demonstrating that it was already denatured *before* heating. In contrast, a large change is observed after heating when the protein was reconstituted in PE gel, demonstrating that in this case the protein was sensitive to heating and was therefore initially in a more native-like state. The percentage indicated in the figure refers to the ratio of the fluorescence intensities of the same sample measured before and after heating, while the wavelength value refers to the amount of shift, if any, in the position of λ_{max}. The spectra are identified as: before heating (—) and after heating (- - -).

equilibrium. Indeed, wt bR has a notoriously robust ability to refold even after having been completely unfolded. The native color and proton-pumping activity were recovered, for example, when proteolytic fragments were fully denatured in sodium dodecylsulfate and organic solvent, reconstituted into separate liposomes, combined by fusion of those liposomes, and finally regenerated by the addition of the retinal ligand (Huang et al., 1981; Liao et al., 1984). However, not all of the bR mutants are such robust folders, nor can one expect most other membrane proteins to be so.

If a membrane protein can coexist in two or more conformations that are in true equilibrium, then there is a possibility that favorable crystal-packing forces will select only one conformational state that is compatible with translational (i.e. crystal-lattice) symmetry. Restating this as an equation, there is a possibility that the following would occur:



In this model, N represents the "native" (meaning crystallizable) conformation, while A, B, and C are some of the perturbed or denatured conformations. Alternatively, A, B, and C might each represent a truly native, different conformation that occurs at different stages of a biochemical cycle. The basic point is that, in a favorable case, the formation of a crystal of N, designated as $N_{crystal}$, would draw the equilibrium to the right. As a result, a well-ordered crystal consisting of a single conformational state could still be grown in the presence of a heterogeneous mixture of protein conformations.

This model is, in fact, what we believe must occur when wildtype bR crystallizes from hydrated gels of MO. Spectra of single crystals of bR reveal that the protein has a high degree of structural homogeneity and is very similar to that of native membranes (Royant et al., 2001). The measured spectrum of crystalline bR thus supports the idea that the variety of bR structural states observed in MO are in reversible equilibrium with the native, crystallizable form of the protein.

We also believe that this model describes the crystallization of the F219L mutant of bacteriorhodopsin, which occurs only when excess retinal is added. This particular mutant is quite stable in octylglucoside but most of the purple color is bleached when the solubilized protein is then incorporated into an MO gel. The addition of excess retinal prevents this bleaching and the protein also crystallizes (Rouhani et al., 2002). The stability and crystallization behavior of the F219L mutant is described by the equation above if excess retinal is included with the various states A, B, and C, and also with the crystallization solution, thereby driving the equilibrium toward N and $N_{crystal}$.

On the basis of the spectra reported here, we conclude that MO does not stabilize either wt bR or its more labile mutants relative to when they are in detergent, contrary to what we had previously supposed. Instead the environment of the hydrated MO cubic phase appears to exert a destabilizing influence. The PE-based gel, on the other hand, appears superior to MO for maintaining a more native and structurally more homogeneous population of protein. Because the triple mutant of bR displays substantially reduced stability compared to wildtype, it serves as a more stringent—and possibly more typical—model for evaluating different crystallization media. Even so, more extensive testing with other types of membrane proteins will be needed to determine the generality of the results obtained with bacteriorhodopsin and its triple mutant.

The lipid formulation used in this study was selected as one which was likely to form a hydrated gel in which the

lipid bilayers were connected to each other in three dimensions, and also to support protein stability by favoring a native, structurally homogeneous population of protein. Although the lipids used in this study are not "natural" membrane lipids, they are diacylglycerophospholipids which are more similar to lipids in native membranes than are monoglycerides. Lipid formulations of the type investigated here may stabilize different membrane proteins to a greater or lesser extent, depending upon each protein in hand. We thus anticipate that the stability of any new protein will have to be evaluated in a range of different lipid formulations, for example by using the intrinsic fluorescence assay described here, to determine which lipid formulation is best suited to screen for crystallization.

In the absence of analytical techniques to monitor whether structural perturbations have occurred, it will not be clear whether the failure to crystallize is due to a perturbed structural state of the protein and whether or not any of these perturbations can be associated with the lipid gel composition. Therefore, to intelligently evaluate the usefulness of hydrated MO and PE-based gels as media for the crystallization of other membrane proteins, we have developed a fluorescence-based assay to assess structural changes to the protein. As stated earlier, the assay evaluates the structural state of a protein based on its sensitivity to denaturing levels of heat.

The intrinsic tryptophan fluorescence assay can be used for proteins both in solution and in the viscous lipid gels. A key reason why it is difficult to use other spectroscopic techniques to monitor protein stability is that hydrated-lipid gels are difficult to transfer from the syringe to other vessels without introducing bubbles and air pockets. In addition, the PE gels can be turbid due to the presence of multiple phases as well as tiny air bubbles formed during the initial mixing of dry lipid and protein. These factors rule out the use of techniques that are sensitive to artifacts from light scattering.

We found that by developing a fluorescent assay in a microplate format these issues could be minimized. In a microplate, only a small volume of gel is required (1–10 μl) and the plate can be spun in a centrifuge to evenly flatten the gel. This process generates a thin layer of the sample that should accept nearly 100% of the exciting light and emit fluorescence without interference. The fluorescent microplate reader uses a bifurcated light guide that delivers excitation light and simultaneously receives emission light. This architecture further minimizes possible artifacts from turbid samples by collecting light from the same face as it illuminates.

High levels of heat are applied to the gel to denature the protein and create a reference point for fluorescence intensity. Since the hydrated lipid is sensitive to the percentage of water in the gel, it is critical to prevent dessication of the lipid gel upon heating. This is accomplished by overlaying the flattened gel with the appropriate buffer and then sealing the microplate with tape. Also, a very favorable aspect of the assay is that multiple gel formulations can be assessed simultaneously, which improves the accuracy of analysis and makes a large number of comparisons feasible.

An excellent correlation was obtained between the result of the intrinsic fluorescence assay and the measurement of the visible absorption spectrum as a way of monitoring protein stability (or denaturation) after the protein was reconstituted into the lipid gels. Since the measurement compares the two spectra obtained from the same well, it is robustly immune to the large number of factors that can give variations in the fluorescence intensity. The assay was able to discriminate the differences in structural state between the already denatured bR triple mutant in MO versus native bR triple mutant in PE. The assay also showed a similar large change in intensity upon heating for wildtype bR in PE and MO.

It is notable that the intrinsic-fluorescence assay can be sensitive to modest changes in tertiary structure, since small differences in tryptophan position and orientation should result in changes to the fluorescence waveform. As long as the protein contains at least one tryptophan, this assay should provide information on the protein's structural state independent of protein type or function.

In summary, the PE-based medium has been shown to be a favorable environment for maintaining the stability of membrane proteins and we have demonstrated that diffraction-quality crystals of bacteriorhodopsin can be grown from this medium. More extensive testing will be needed to determine the generality of these results for a broad range of membrane proteins. However, the preliminary results obtained with bR and an especially labile bR mutant indicate that a connected-bilayer matrix based on diacylglycerophospholipids, i.e. lipids that are more similar to those with which membrane proteins interact in their native membranes, could improve the success of crystallization efforts for numerous membrane proteins.

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References

Caffrey, M., 2003. Membrane protein crystallization. J. Struct. Biol. 142, 108–132.

Cheng, A., Hummel, B., Qiu, H., Caffrey, M., 1998. A simple mechanical mixer for small viscous lipid-containing samples. Chem. Phys. Lipids 95, 11–21.

Facciotti, M.T., Cheung, V.S., Nguyen, D., Rouhani, S., Glaeser, R.M., 2003. Crystal structure of the bromide-bound D85S mutant of bacteriorhodopsin: principles of ion pumping. Biophys. J. 85, 451–458.

Facciotti, M.T., Rouhani, S., Burkard, F.T., Betancourt, F.M., Downing, K.H., Rose, R.B., McDermott, G., Glaeser, R.M., 2001. Structure of an early intermediate in the M-state phase of the bacteriorhodopsin photocycle. Biophys. J. 81, 3442–3455.

Facciotti, M.T., Rouhani-Manshadi, S., Glaeser, R.M., 2004. Energy transduction in transmembrane ion pumps. Trends Biochem. Sci. 29, 445–451.

Faham, S., Bowie, J.U., 2002. Bicelle crystallization: a new method for crystallizing membrane proteins yields a monomeric bacteriorhodopsin structure. J. Mol. Biol. 316, 1–6.

Gordeliy, V.I., Labahn, J., Moukhametzianov, R., Efremov, R., Granzin, J., Schlesinger, R., Buldt, G., Savopol, T., Scheidig, A.J., Klare, J.P., Engelhard, M., 2002. Molecular basis of transmembrane signalling by sensory rhodopsin II–transducer complex. Nature 419, 484–487.

Haque, M.E., McIntosh, T.J., Lentz, B.R., 2001. Influence of lipid composition on physical properties and peg-mediated fusion of curved and uncurved model membrane vesicles: "nature's own" fusogenic lipid bilayer. Biochemistry 40, 4340–4348.

Huang, K.S., Bayley, H., Liao, M.J., London, E., Khorana, H.G., 1981. Refolding of an integral membrane protein. Denaturation, renaturation, and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. J. Biol. Chem. 256, 3802–3809.

Iwata, S., Kaback, H.R., 2004. Membranes: A new era in membrane protein studies. Curr. Opin. Struct. Biol. 14, 387–389.

Johnsson, M., Edwards, K., 2001. Phase behavior and aggregate structure in mixtures of dioleoylphosphatidylethanolamine and poly(ethylene glycol)-lipids. Biophys. J. 80, 313–323.

Katona, G., Andreasson, U., Landau, E.M., Andreasson, L.E., Neutze, R., 2003. Lipidic cubic phase crystal structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.35 Å resolution. J. Mol. Biol. 331, 681–692.

Keller, S.L., Gruner, S.M., Gawrisch, K., 1996. Small concentrations of alamethicin induce a cubic phase in bulk phosphatidylethanolamine mixtures. Biochim. Biophys. Acta 1278, 241–246.

Kolbe, M., Besir, H., Essen, L.O., Oesterhelt, D., 2000. Structure of the light-driven chloride pump halorhodopsin at 1.8 angstrom resolution. Science 288, 1390–1396.

Koynova, R., Tenchov, B., Rapp, G., 1997. Low amounts of PEG-lipid induce cubic phase in phosphatidylethanolamine dispersions. Biochim. Biophys. Acta 1362, 167–170.

Landau, E.M., Rosenbusch, J.P., 1996. Lipidic cubic phases—a novel concept for the crystallization of membrane proteins. Proc. Natl. Acad. Sci. USA 93, 14532–14535.

Lanyi, J., Schobert, B., 2002. Crystallographic structure of the retinal and the protein after deprotonation of the schiff base: the switch in the bacteriorhodopsin photocycle. J. Mol. Biol. 321, 727–737.

Liao, M.J., Huang, K.S., Khorana, H.G., 1984. Regeneration of native bacteriorhodopsin structure from fragments. J. Biol. Chem. 259, 4200–4204.

Luecke, H., Schobert, B., Lanyi, J.K., Spudich, E.N., Spudich, J.L., 2001. Crystal structure of sensory rhodopsin II at 2.4 angstroms: insights into color tuning and transducer interaction. Science 293, 1499–1503.

Luecke, H., Schobert, B., Richter, H.T., Cartailler, J.P., Lanyi, J.K., 1999. Structure of bacteriorhodopsin at 1.55 angstrom resolution. J. Mol. Biol. 291, 899–911.

Misquitta, L.V., Misquitta, Y., Cherezov, V., Slattery, O., Mohan, J.M., Hart, D., Zhalnina, M., Cramer, W.A., Caffrey, M., 2004. Membrane protein crystallization in lipidic mesophases with tailored bilayers. Structure 12, 2113–2124.

Nieh, M.P., Glinka, C.J., Krueger, S., Prosser, R.S., Katsaras, J., 2002. SANS study on the effect of lanthanide ions and charged lipids on the morphology of phospholipid mixtures. Small-angle neutron scattering. Biophys. J. 82, 2487–2498.

Rouhani, S., Cartailler, J.P., Facciotti, M.T., Walian, P., Needleman, R., Lanyi, J.K., Glaeser, R.M., Luecke, H., 2001. Crystal structure of the D85S mutant of bacteriorhodopsin: Model of an O-like photocycle intermediate. J. Mol. Biol. 313, 615–628.

Rouhani, S., Facciotti, M.T., Woodcock, G., Cheung, V., Cunningham, C., Nguyen, D., Rad, B., Lin, C., Lunde, C.S., Glaeser, R.M., 2002. Crystallization of membrane proteins from media composed of connected-bilayer gels. Biopolymers 66, 300–316.

Royant, A., Edman, K., Ursby, T., Pebay-Peyroula, E., Landau, E.M., Neutze, R., 2001. Spectroscopic characterization of bacteriorhodopsin's L-intermediate in 3D crystals cooled to 170 K. Photochem. Photobiol. 74, 794–804.

Vogeley, L., Sineshchekov, O.A., Trivedi, V.D., Sasaki, J., Spudich, J.L., Luecke, H., 2004. Anabaena sensory rhodopsin: a photochromic color sensor at 2.0 Å. Science 306, 1390–1393.