Purification of a Membrane Protein with Conjugated Engineered Micelles

Guy Patchornik,*† Dganit Danino,‡ Ellina Kesselman,§ Ellen Wachtel,§ Noga Friedman,‖ and Mordechai Sheves†

†Department of Biological Chemistry, Ariel University, 70400, Israel
‡Department of Biotechnology and Food Engineering, Technion, Haifa, 32000, Israel
§Chemical Research Infrastructure Unit, Weizmann Institute of Science, 76100, Rehovot, Israel
‖Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, 76100, Rehovot, Israel

ABSTRACT: A novel method for purifying membrane proteins is presented. The approach makes use of engineered micelles composed of a nonionic detergent, β-octylglucoside, and a hydrophobic metal chelator, bathophenanthroline. Via the chelators, the micelles are specifically conjugated, i.e., tethered, in the presence of Fe²⁺ ions, thereby forming micellar aggregates which provide the environment for separation of lipid-soluble membrane proteins from water-soluble proteins. The micellar aggregates (here imaged by cryo-transmission electron microscopy) successfully purify the light driven proton pump, bacteriorhodopsin (bR), from E. coli lysate. Purification takes place within 15 min and can be performed both at room temperature and at 4 °C. More than 94% of the water-soluble macromolecules in the lysate are excluded, with recovery yields of the membrane protein ranging between 74% and 85%. Since this approach does not require precipitants, high concentrations of detergent to induce micellar aggregates, high temperature, or changes in pH, it is suggested that it may be applied to the purification of a wide variety of membrane proteins.

INTRODUCTION

Purification of membrane proteins is essential for their structural determination and commonly requires extraction from biological membranes using detergent molecules.¹,² The detergents disrupt the membrane and, in parallel, surround and cover the hydrophobic domains of the protein, leading, under favorable conditions, to a water-soluble [detergent-lipid–protein] complex which is then purified by chromatography. However, identification of the particular detergent that will preserve the membrane protein in its functional native state is laborious as dozens of detergents are available and the ideal choice can only be determined experimentally.³ Once an appropriate detergent is identified, membrane proteins can be purified, similarly to soluble proteins, but in the presence of a detergent. Purification can be accomplished either via classical chromatographic methods (e.g., gel filtration, ion exchange chromatography) or by genetically engineered affinity-tags which greatly simplify the isolation process and are commonly placed at the amino- or carboxy-terminal end.⁴–⁷ Affinity tags may include His-tag,⁸ Flag-tag,⁹ HA-tag,¹⁰ glutathione S-transferase, GST-tag,¹¹,¹² or maltose binding protein-tag¹³ and generally lead to a highly purified protein preparation within a single chromatographic step.⁵ Among the different affinity-tags, His-tag is probably the most widely used¹⁴ as it possesses sufficient affinity and specificity toward metal chelated resins (e.g., Ni-NTA), as well as requiring only mild elution conditions.¹⁵ Since affinity-tags may alter the properties of the membrane protein, they are removed at the end of the purification process.¹⁶ The presence of detergents throughout the purification process deserves special emphasis. Detergents, being amphiphatic molecules, are driven by the hydrophobic effect to assemble spontaneously in water into noncovalent assemblies (micelles) when the concentration exceeds a broad threshold called the critical micelle concentration (cmc).²,¹⁷–²⁰ Further addition of detergent above the cmc ideally increases only the micelle concentration while keeping the free detergent concentration constant.¹⁰ Micelles may undergo major structural alterations in the presence of an additional detergent, lipid, or protein¹¹ and will generally not interact with one another in dilute aqueous suspension.¹² However, micelles may cluster once physical or chemical modifications of the medium are induced. Such modifications include: (a) addition of polymeric or inorganic precipitants; (b) increase in ionic strength; or (c) temperature alteration.¹,²,²² Under these conditions, the solution can become turbid and a transient state called the cloud point,²³ containing micellar aggregates, is reached. Further micellar aggregation results in phase
The detergent rich phase is then subjected to an approach relying on the preferential partitioning of membrane proteins into the detergent rich phase due to their hydrophobic character whereas hydrophilic proteins do not. This phenomenon is used for purification of membrane proteins from watersoluble proteins and is called cloud point extraction. The approach relies on the preferential partitioning of membrane proteins into the detergent rich phase due to their hydrophobic character. The detergent rich phase is then subjected to an additional chromatographic step to allow purification of the target membrane protein from other membrane proteins that may be present. The key to success, therefore, relies on our ability to induce phase separation under conditions that preserve the functionality of the detergent solubilized membrane proteins. This, however, is a nontrivial task as many detergents used in membrane protein biochemistry phase separate only at (i) very high concentrations or (ii) elevated temperatures, both of which may be deleterious to the protein. However, such barriers may sometimes be successfully suppressed when high concentrations of water-soluble polymers (e.g., dextran, PEG-4000) or precipitants (e.g., ammonium sulfate) are added to the medium. Under these conditions, the detergent cloud point temperature is lowered and purification can be accomplished while preserving protein functionality. The absence of other nonionic detergents that could be used similarly encouraged us to seek a general solution that would allow utilization of a broad range of nonionic detergents regardless of their concentration or cloud point temperature.

We recently demonstrated that micelles composed of nonionic detergents and hydrophobic metal chelators, referred to as engineered micelles, can be tethered specifically with various divalent metal cations serving as mediators in the aqueous phase, and lead to micellar aggregates (Figure 1A). The fact that such aggregates are generated at room temperature or at 4 °C suggested that they could potentially serve as purification scaffolds for membrane proteins. It was further speculated that gentle agitation of these aggregates with membranes containing the target protein might result in partitioning of the target into the aggregates whereas hydrophilic proteins would be excluded (Figure 1B).

**EXPERIMENTAL PROCEDURES**

**Materials.** Bathophenanthroline, octyl β-D-glucopyranoside (OG), NaCl, FeSO₄, and ZnCl₂ were obtained from Sigma-Aldrich (St. Louis, MO).

**Preparation of Purple Membranes.** Halobacterium salinarum was grown from the S9 strain, and purple membranes containing bacteriorhodopsin were isolated as previously described.

**Preparation of Conjugated Engineered Micelles.** Conjugated engineered micelles are prepared by adding 10 μL of 20 mM bathophenanthroline in methanol at room temperature to 22 μL of 200 mM OG in water with continuous, vigorous vortexing for 10–15 s. Water (68 μL) is added immediately with continuous vortexing (10 s) and 45 μL aliquots of the resulting mixture are mixed with identical volumes of 1 mM FeSO₄ and 400 mM NaCl in water (kept on ice).

**Purification of Bacteriorhodopsin (bR) with Conjugated Engineered Micelles.** Freshly prepared tethered engineered micelles (90 μL) were allowed to stand at room temperature for 0–3 min, and only then, were E. coli lysate (10–25 μL) and purple membranes containing bR at 6.25 mg/mL (1–2 μL) slowly added without vortexing. The system was
gently agitated with an orbital shaker (300 rounds per minute) for 15 min at 4 °C/room temperature in the dark. Samples were then briefly centrifuged (2 min at 985 × g) and the majority of the supernatant (90 μL) was carefully discarded. The resulting red pellet was briefly washed with cold 200 mM NaCl (90 μL), an additional spin (2 min at 3935 × g) was applied, the supernatant was discarded, and the pellet composition was analyzed by SDS-PAGE.

**Light Microscopy.** Light microscope images were obtained using an Olympus CX40 microscope.

**UV Spectroscopy.** Absorption measurements were performed using the HP 8453 UV−Vis spectrophotometer.

### Cryo-Transmission Electron Microscopy (Cryo-TEM)

The cryo-TEM images were obtained using an FEI T12 G2 Cryo-TEM, operating at 120 kV. Images were recorded under low dose conditions as described previously. 

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**RESULTS**

**Preparation and Analysis of Red Oily Globules.** When the hydrophobic metal chelator, bathophenanthroline (1 mM), was added to an aqueous suspension containing 22 mM OG in 200 mM NaCl, red oily globules appeared a few minutes after addition of Fe^{2+} (0.5 mM) (Figure 2A). The diameter of the globules ranged between 0.1 and 0.01 mm (Figure 2A); the color indicated that they contained the red [(bathophenanthroline)_3:Fe^{2+}] complex. Analysis of these globules at higher resolution was achieved by cryo-TEM (Figure 2B–D). The results show that conjugation of OG micelles leads to the formation of densely packed, entangled, thread-like micelles with a defined boundary between the micellar aggregate and the aqueous phase (Figure 2B–C). In the absence of the [(bathophenanthroline)_3:Fe^{2+}] complex, no red oily globules were observed by light microscopy (data not shown) and cryo-TEM analysis detected the presence of numerous dispersed and independent OG micelles (Figure 2D). Additional control experiments indicated that micellar aggregates are not generated when either the chelator or the metal are absent; only individual, dispersed micelles were obtained (data not shown).

**Purification of Bacteriorhodopsin (bR).** Purification of the target membrane protein (bR) was performed in two steps: (i) preparation of conjugated engineered micelles composed of [OG:bathophenanthroline:Fe^{2+}]; (ii) addition of purple membranes containing bR and E. coli lysate (serving as an artificial background) to the preformed micellar aggregate. Efficient partitioning of bR into the detergent aggregate occurred either at room temperature (Figure 3A) or at 4 °C (Figure 3B) and required the presence of both the hydrophobic metal chelator (bathophenanthroline) and the metal ion (Fe^{2+}).

**Spectroscopic Characterization of Partially Purified bR.** To determine whether bR was affected by the high detergent concentration present within the micellar aggregates, the latter were resuspended at a concentration of 50–100 mM OG and the absorption spectrum of the protein was compared to that of bR present within its native, purple membrane environment. Purification of bR was performed as described above, but, in the presence of Zn^{2+}, so as to avoid the red color of the [(bathophenanthroline)_3:Fe^{2+}] complex which was found to overlap the absorption maximum of bR at 568 nm. No significant difference could be found between the absorption spectrum of bR within its native membrane and that of the protein which had partitioned into the micellar aggregate (Figure 3C). Similarly, no major spectroscopic changes were identified when bR was kept within the micellar aggregate overnight at 4 °C (Figure 3C).

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**DISCUSSION**

The nonionic detergent β-octylglucoside (OG) was chosen as the test detergent since it is extensively used in the purification, reconstitution, and crystallization of integral membrane proteins. Preparation of the [OG:batho:Fe^{2+}] tethered, engineered micelles, led to the formation of red oily globules, as detected by light microscopy (Figure 2A). Imaging of these red oily globules by cryo-TEM demonstrated that micellar aggregates were formed only in the presence of the [(bathophenanthroline)_3:Fe^{2+}] complex (Figure 2B–C). The field was covered with micrometer-sized regions of electron-dense material alternating with amorphous ice. The boundaries between these regions and the bulk solvent background were sharp and distinct, showing the presence of thread-like micelles at the edges of the large micellar aggregates (black arrows in Figure 2B–C). In the absence of the [(batho)_3:Fe^{2+}] complex, such well-defined electron-dense regions did not form, even when the OG surfactant concentration was raised 5-fold. A control sample contained only small spherical micelles (Figure 2D).

These findings paved the way for purification trials with purple membranes containing the light driven proton pump, bacteriorhodopsin (bR) from *Halobacterium salinarum*.41,42 bR is a small integral membrane protein (26 kDa) containing a
retinal chromophore covalently bound via a protonated Schiff-base to Lys216. The protein utilizes light energy for the active transport of protons from the cytosol to the extracellular side of the membrane and uses the generated proton gradient for the synthesis of ATP by ATP synthase. Following micellar aggregate formation, the E. coli lysate (serving only as an artificial background and not as an expression host), and purple membranes were added to the aqueous dispersion and were incubated either at room temperature or 4 °C. The presence of the target membrane protein, bR, in the centrifuge pellet was clearly identified by SDS-PAGE (Figure 2A, see asterisk); most water-soluble molecules (>94%, by densitometry) were excluded. These results are consistent with our working hypothesis as they show that a membrane protein does indeed partition into the micellar aggregate, whereas most water-soluble proteins present in the lysate do not. Recovery yields for bR as measured by densitometry ranged between 74% and 85%.

The absolute requirement for the [(batho)3:Fe2+] complex was demonstrated by repeating the process in the absence of either the chelator (Figure 3A) or the metal ion (Figure 3A). The results clearly indicate that both are critical components for the process. An unexpected band with a very low molecular weight appeared in both lanes 3 and 4, and was shown to be composed of the [detergent:chelator:metal] complex (i.e., [OG:batho:Fe2+] since it could be generated in the absence of bR or the lysate (Figure 3A). The fact that similar results were observed when purification was performed at 4 °C (Figure 3B) suggests that (a) the process may be used with temperature-labile membrane proteins; and (b) purification of membrane proteins via phase separation may now be performed with other detergents of the alkyl-glucoside family in spite of the elevated temperatures (up to 100 °C) generally required to induce their phase separation.32,43

The purity of the bR protein is not very high since the method can only separate water-soluble and membrane proteins. Additional bands in the gel pictured in lane 3 of...
Figure 3A,B are due to lipophilic macromolecules from the *E. coli* lysate which also partitioned into the micellar aggregates. However, the fact that more than 94% of the background due to the lysate is excluded, and the volume of the sample is reduced ~20-fold should simplify the final polishing step. Pellets containing the partially purified bR could then be dissolved in small volumes (30–75 μL) of 50–100 mM OG in 200 mM NaCl and applied directly to a chromatographic column for the final purification step. However, the fact that bR encountered high detergent concentrations also required demonstration of its integrity. To measure the absorption spectrum of bR, purification was performed in the presence of the colorless [(batho)2:Zn2+] complex (not shown) instead of the red [(batho)2:Fe2+] complex as the latter overlaps the absorption maximum of bR at 568 nm. No significant changes in the absorption spectra of purified bR were observed (Figure 3C), indicating that bR is stable following the dissolution process and its integration into the micellar aggregate. Moreover, the exceptional stability of bR44 was apparent when samples containing purified bR were kept overnight in 100 mM OG and 200 mM NaCl at 4 °C (in the dark); again, major changes in the absorption spectrum were not observed (Figure 3C, red line).

These results demonstrate several advantages which seem to be inherent to the approach presented here. They include the following: (a) promoting phase separation under very mild conditions—in this study, OG was induced to undergo phase separation at room temperature or 4 °C and at a concentration (22 mM) close to its critical micelle concentration (18–19 mM);45 (b) the absence of precipitants (e.g., ammonium sulfate) or water-soluble polymers (e.g., dextran) from the process—no such additives were required to induce phase separation, thus reducing the system complexity; (c) extraction of the target membrane protein by micellar aggregates—bR partitioned into the micellar aggregates without any prior disruption of the membrane with detergents; (d) simple detection of the detergent-rich phase—the red color of the [(batho)2:Fe2+] complex serves as a convenient indicator for the detergent rich phase containing the target protein. In addition, this feature provides a practical means for evaluating precipitation efficiency and loss of protein during pellet washing.

The method may have three potential limitations. (i) Although micellar conjugation is induced at low detergent concentration, the significantly higher concentration in the conjugated micelles may, in some cases, cause protein denaturation. This difficulty, however, is common to all purification strategies employing detergent phase separation. (ii) Purification of His-tagged proteins in the presence of a hydrophobic metal chelator may require defining conditions for efficient protein binding to the immobilized metals present on chromatographic media. (iii) The hydrophobic chelator should be removed from the protein. This may be accomplished in a similar manner to the way detergents are exchanged for other detergents while the target protein is bound to chromatographic media.

**CONCLUSIONS**

The utility of tethered *engineered micelles* composed of β-octylglucoside and the chelator:metal complex [(batho)2:Fe2+] in the purification of bacteriorhodopsin was demonstrated. The fact that other nonionic detergents undergo phase separation under similar mild conditions37 implies that they may be used similarly for the successful isolation of diverse membrane proteins. This, however, remains to be shown.

**AUTHOR INFORMATION**

*Corresponding Author*  
E-mail: guyp@ariel.ac.il, Tel: 972-3-9755806, Fax: 972-3-9066634.

**Notes**  
The authors declare no competing financial interest.

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**ABBREVIATIONS**

batho, bathophenanthroline; bR, bacteriorhodopsin; cmc, critical micelle concentration; cryo-TEM, cryo transmission electron microscopy; OG, β-D-glucoside; PEG, poly ethylene glycol; Ni-NTA, nickel nitritetriaic acid

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