Engineered-membranes: A novel concept for clustering of native lipid bilayers

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1. Introduction

Since the introduction of the fluid mosaic model more than four decades ago [1] as the fundamental architecture of lipid bilayers in living organisms, major efforts were directed toward developing model systems to characterize their chemical, electrical, thermodynamic, and physiological properties. X-ray diffraction and calorimetric studies were first applied to small unilamellar vesicles produced by sonication of large multilamellar liposomes as these provided excellent model systems for biomembranes [2].

In the early 1980s, however, a novel approach for the production of lipid bilayers on solid supports (e.g., glass, quartz, and silicon) was introduced [3]. These so-called supported phospholipid bilayers are stable, possess planar geometry, and thus allowed direct measurement of lateral diffusion of membrane components by fluorescence recovery after bleaching (FRAP) [4] and single particle tracking (SPT) [5]. Additional applications of these supported bilayers include the study of the following: (i) secondary structure and orientation of membrane proteins and lipophilic peptides [6], (ii) interactions between helices in signaling proteins [7], (iii) kinetics of ligand binding to membrane embedded receptors [8], (iv) protein induced vesicle fusion in viruses [9], (v) exocytosis [10], and (vi) as biosensors for different target molecules [11–13].

We postulated that the possibility of creating natural biomembranes with large surface area has the potential of providing a native environment for membrane proteins while at the same time allowing an acceptable signal-to-noise ratio for spectroscopic studies since large numbers of proteins could be accommodated. Therefore, as a first step toward this goal, we sought to develop a specific and mild process for interbilayer clustering that would preserve the integrity of embedded integral membrane proteins.

The clustering process depicted in Fig. 1A relies on the formation of strong complexes between hydrophobic chelators embedded within the lipid bilayer and metal cations in the aqueous phase, capable of binding two (or more) chelators simultaneously. We used this approach with purple membranes containing the light driven proton pump protein bacteriorhodopsin (bR) and showed that patches of purple membranes cluster into mm sized aggregates and that these are stable for months when incubated at 19 °C in the dark. The strategy may be general since four different hydrophobic chelators (1,10-phenanthroline, bathophenanthroline, Phen-C10, and 8-hydroxyquinoline) and various divalent cations (Ni²⁺, Zn²⁺, Cd²⁺, Mn²⁺, and Cu²⁺) induced formation of membrane clusters. Moreover, the absolute requirement for a hydrophobic chelator and the appropriate metal cations was demonstrated with light and atomic force microscopy (AFM); the presence of the metal does not appear to affect the functional state of the protein. The potential utility of the approach as an alternative to assembled lipid bilayers is suggested.

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protein in its native environment and functional state. Our method satisfies these requirements.

2. Materials and methods

2.1. Materials

Bathophenanthroline, 1,10-phenanthroline, 1,10-phenanthroline-5-amine, PEG-6000, NaCl, NiBr₂, ZnCl₂, CdCl₂, MnCl₂, and CaCl₂ were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Synthesis of a N-(1,10-phenanthrolin-5-yl)decanamide (Phen-C₁₀)

Phen-C₁₀ was synthesized according to Patchornik et al.[16].

2.3. Preparation of purple membranes

H. salinarum was grown from the S9 strain, and purple membranes containing bacteriorhodopsin were isolated as previously described [17].

2.4. Transforming purple membranes into the corresponding Engineered-membranes

Freshly prepared purple membranes (3.5 µl, OD = 6.6) were first diluted in 6 µl of double distilled water (DDW), which was followed by the addition of 0.5 µl of either 20 mM 1,10, phenanthroline, 20 mM bathophenanthroline or 20 mM Phen C₁₀ (all in methanol). The slow chelator addition was performed with constant vortexing. The resulting mixture was incubated for 15 min at 4 °C in the dark prior to the addition of divalent cations.

2.5. Engineered-membranes containing 8-hydroxyquinoline

The identical protocol that was used with the phenanthroline derivatives was utilized to transform purple membranes into Engineered-membranes containing the hydrophobic chelator 8-hydroxyquinoline. Thus, the slow addition of 0.5 µl of 20 mM 8-hydroxyquinoline (in methanol) to the membrane suspension was followed accordingly by 15 min incubation at 4 °C in the dark.

2.6. Clustered Engineered-membranes with or without a precipitant

Engineered-membranes were clustered on siliconized cover slides (Hampton Research, Aliso Viejo, CA) by the addition of 1 µl of the prepared Engineered-membranes to a medium containing: 1 µl of either 20 mM NiBr₂, ZnCl₂, CdCl₂, FeSO₄ or MnCl₂ in DDW and 2 µl of 9% PEG-6000 in 200 mM NaCl. The resulting mixture was incubated in VDX™ plates (Hampton Research, Aliso Viejo, CA) and equilibrated against 9% PEG-6000 in 200 mM NaCl at 19 °C in the dark. An identical protocol was employed for clustering of Engineered-membranes in the absence of the precipitant, PEG-6000.

2.7. The effect of EDTA or catechol on the clustering process

1 µl of 500 mM EDTA (pH 7.5) or 500 mM catechol (DDW) was added to samples containing 4 µl of clustered Engineered-membranes. Incubation in VDX™ plates was as described above.

3. Methods

3.1. Light microscopy

Images of hanging drops were obtained using an Olympus CX-40 light microscope equipped with an Olympus U-TV1X-2 digital camera.

3.2. Absorption spectroscopy of clustered purple membranes

Absorption measurements of native and Engineered-PM were performed using the HP 8453 UV–Vis spectrophotometer.

3.3. Atomic force microscopy (AFM) measurements

The samples for AFM imaging were prepared on freshly-cleaved mica. A suspension (8 µl) containing purple membranes with or

Fig. 1. Illustration depicting the formation of clusters of Engineered-membranes. (A) Native membranes are transformed into the corresponding Engineered-membranes by the addition of a hydrophobic chelator. Further addition of metal cations, capable of binding up to three chelators simultaneously, induces membrane assembly. (B) Chemical structures of the hydrophobic chelators and metals studied.
without 1,10-phenanthroline (as described above) was added to a water droplet (12 μl) on the mica which contained 3.3 mM ZnCl₂. After 5 min of incubation at 25 °C, excess water was drawn away from the side of the sample with filter paper. The sample was subsequently dried in a stream of dry nitrogen and immediately transferred to the AFM for imaging. All imaging was performed on a Bruker MMAFM instrument using a J scanner, and Olympus AC240TS probes with nominal frequency of 70 kHz and spring constant of 2 N/m. Agglomerations of the membrane could be observed optically as dark patches. AFM imaging was done near, but not within these regions, starting with scans of approximately 40 μm² to identify regions of interest and then zooming in. The raw data were processed only by leveling.

3.4. Flash photolysis

A sample of purple membranes in the presence of Zn²⁺ and 1,10-phenanthroline was irradiated with a 532 nm, 10 nS ND:YAG pulsed laser. Absorbance changes were recorded at several delay times.

4. Results and discussion

Since bathophenanthroline (Fig. 1B) binds diverse metal cations with different affinities [18] and was shown to embed itself at the membrane interface [19], it represented a potential hydrophobic chelator for membrane clustering. A series of three hydrophobic chelators which included, in addition to bathophenanthroline, also 1,10-phenanthroline and Phen C-10 were tested. The last was synthesized with the intention of comparing the effect of a 10 carbon aliphatic tail in Phen C-10 with the two aromatic phenyl groups in bathophenanthroline (Fig. 1B).

We found that a brief incubation (15 min. 4 °C, in the dark) of each of these three hydrophobic chelators at 1 mM with native membranes containing bR led to the formation of Engineered-membranes that clustered into extended pink-purple layers in the presence of each of four metal cations (Fig. 2A–C) and were stable for months when kept at 19 °C in the dark.

The fact that no such clustering phenomenon occurred with native membranes alone suggested the participation of the hydrophobic chelators in the clustering process (Fig. 2D). Direct evidence for the partitioning of bathophenanthroline into the membrane is provided when the cation is Fe²⁺ (Fig. 3A). Fe²⁺ forms a red complex with bathophenanthroline and therefore can serve as an indicator for their location in the membrane medium or both. The microscope image (Fig. 3A) shows that most of the [(bathophenanthroline)₃:Fe²⁺] complex has partitioned into the conjugated membranes, with prominent, well-defined boundaries between the membrane aggregate and the aqueous phase (see arrows in Fig. 3A).

Additional evidence for the mandatory requirement for a hydrophobic chelator became apparent when experiments were...
Conducted in the presence of the water-soluble chelator, catechol. Catechol forms strong complexes with Ni²⁺, Zn²⁺, and Mn²⁺ [20] in a 2:1 stoichiometric ratio, but failed to induce membrane clustering in the presence of Ni²⁺ ions (Fig. 3B) and Zn²⁺ or Mn²⁺ (not shown), presumably due to its high water solubility which precluded significant partitioning into the lipid bilayers. The dependence of the clustering process on appropriate metal cations, capable of binding two chelators (or more), was shown by...

**Fig. 3.** Dependence of clustering on hydrophobic chelators and metal cations. Light microscope images of purple membranes incubated with (A) bathophenanthroline and FeSO₄, (B) catechol and NiBr₂, and (C) 1,10-phenanthroline in the absence of divalent metal cations (I); with Mg²⁺ ions (II) or in the presence of EDTA prior to the addition of Zn²⁺ ions (III); and (D) 8-hydroxyquinoline in the presence of indicated metal cations (I–III); in the absence of divalent cations (IV); in the presence of Mg²⁺ ions (V). Arrows (in A) point to the boundary between clustered membranes and the aqueous medium. The scale bars represent 0.5 mm.
repeating the experiments with phenanthroline (i) in the absence of Ni²⁺, Zn²⁺, Cd²⁺, Mn²⁺ (Fig. 3C-I), (ii) in the presence of Mg²⁺ ions – which cannot bind two phenanthroline moieties (Fig. 3C-II), or (iii) in the presence of the water-soluble chelator, EDTA [21]. EDTA was expected to compete for the metal ions with the phenanthroline derivatives embedded within the membrane and thus suppress membrane (clustering). We have found that inclusion of 100 mM EDTA prior to the addition of Engineered-membranes to the aqueous medium containing the divalent cations inhibited the process when Zn²⁺ ions (Fig. 3C-III) or other metals were present (e.g., Ni²⁺ and Mn²⁺, not shown). These findings supported process specificity and its dependence on strong [hydrophobic chelator + metal] complexes where m = 2 or 3 and n = 1.

It was also possible to demonstrate the viability of the process with other hydrophobic chelators, which are not phenanthroline derivatives. Therefore, Engineered-membranes containing the hydrophobic chelator 8-hydroxyquinoline [22] were prepared accordingly and incubated with several different cations. These conditions led to the formation of large pink/purple layers (Fig. 3D IV and V) and thus were consistent with those involving the phenanthroline analogs (Fig. 3C I and II).

Two particularly notable results were as follows: (i) the formation of similar extended membranes in the absence of any precipitant (i.e., PEG-6000, not shown), which emphasized the reliance of the clustering process on the [metal : chelator] complexes rather than on the effect exerted by the precipitant; and (ii) the lateral dimensions of the resulting purple layers were in the mm range (Fig. 2A–C, see bar) representing surface areas which are orders of magnitude larger than those of individual native purple membranes [23].

Although clustered Engineered-membranes did preserve their pink/purple color for several months when kept at 19 °C in the dark (not shown), we also assessed the potentially deleterious effect of the metal, chelator, or both on the native state of the protein, embedded in the Engineered-membranes, by measuring its absorption spectrum (Fig. 4).

No significant alteration in the absorption spectrum was found when the chelator (phenanthroline) or the metal ion (Zn²⁺) was incubated independently with bR, whereas a decrease in absorption intensity and a small blueshift to 565 nm occurred when both were present (Fig. 4). Further incubation at 4 °C for an additional 13 days (in the dark) did not alter the spectrum. The intensity decrease and the small blueshift may be due to membrane aggregation. However, it is evident that the bR protein did not experience any significant conformational alteration due to the clustering process, nor did damage accumulate during prolonged incubation in the presence of the chelator and the metal ion (Fig. 4).

Direct evidence for bR functionality after clustering was derived from a flash-photolysis experiment demonstrating formation of the characteristic photochemically induced intermediate M₄₁₂ which absorbs at 412 nm (Fig. 5).

Analysis of Engineered-membranes by atomic force microscopy (AFM) provided additional support for the specificity and efficiency of the clustering mechanism. When purple membranes were incubated with the hydrophobic chelator 1,10-phenanthroline and Zn²⁺, assembly of membranes was observed (Fig. 6A).

These assemblies appeared to be constructed from smaller units of approximately 0.5 μm width and 5 nm height. The overall dimensions of the membrane assemblies were tens of μm in width and up to 250 nm in thickness (Fig. 6A). In the presence of metal, but absence of the chelator (Fig. 6B), many single isolated patches with widths in the range of 0.5 μm were clearly identified (see arrows 1–3 in Fig. 6B) whereas assemblies were much more limited in size than with chelator. These findings provided additional evidence for the participation and mandatory requirement for a hydrophobic chelator in the clustering process. Due to the 3-dimensional nature of the assemblies, membrane fusion could not be identified at the molecular level. Isolated patches, as seen in 6B, were not observed within the aggregated system where even the thinner patches at the edge of the assemblies are extensively linked to one another.

To suppress free, non-chelated metal which may be present in the medium, water-soluble chelators were added after Engineered-membranes formed clusters. Surprisingly, no significant disintegration of membrane assemblies occurred even when high concentrations (e.g., 100 mM) of strong chelators such as EDTA or catechol were present at a molar excess of 400:1 relative to that of 1,10-phenanthroline. Moreover, long-term incubation (days) at 19 °C in the dark (due to the sensitivity of the bound retinal chromophore) did not result in any major dissolution (not shown). These results imply that inclusion of water-soluble chelators following membrane assembly can be used to sequester excess metal ions while keeping clustered membranes intact. Such a property is of great practical importance as proteins and membrane proteins...
may be affected structurally and functionally by non-specifically bound transition metal ions.

5. Conclusion

A specific process for clustering of native lipid bilayers containing a membrane protein has been presented. The process is based on the introduction of hydrophobic chelators into the lipid bilayers (Engineered-membranes), which are then capable of clustering into membrane aggregates in the presence of the appropriate divalent metal cations. The potential generality of the approach derives from its demonstration with four hydrophobic chelators and five transition metals. It is suggested that such a strategy may promote the formation of extended membrane structures which could provide a natural alternative environment to assembled lipid bilayers on solid supports. However, since the cluster of engineered bilayer membranes, as described here, likely has a large concentration of defects, various possible applications would require extending the conjugation mechanism to induce fusion and self-sealing, perhaps by promoting lateral diffusion of the chelator molecules.

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References