Electron microscopic observation and rotational diffusion measurement of bacteriorhodopsin in lipid vesicles

HU Kunsheng(胡坤生)[1], WANG Aojin (王敖金)[1], Ian E. G. Morrison[2] & Richard J. Cherry[2]

1. Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China;
2. Department of Biological Science, University of Essex, UK
Correspondence should be addressed to Hu Kunsheng (email: huks1401@sun5.ibp.ac.cn)

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Abstract The morphology of bacteriorhodopsin reconstituted into dimyristoylphosphatidylcholine and egg-phosphatidylcholine vesicles was observed by freeze-fracture electron microscopy. The rotational diffusion of bacteriorhodopsin at different concentrations of melittin was measured by observing flash-induced transient dichroism in dimyristoylphosphatidylcholine vesicles. In the presence of melittin, bacteriorhodopsin molecules in dimyristoylphosphatidylcholine vesicles were aggregated into large particles or patches, and the ability of rotational diffusion of bacteriorhodopsin in vesicles was decreased. This suggests that melittin produces its effect via direct electrostatic interaction with bacteriorhodopsin. Low temperature-induced aggregation of bacteriorhodopsin was also observed in dimyristoylphosphatidylcholine vesicles. Low temperature may cause phase separation. Bacteriorhodopsin was also successfully reconstituted into egg-phosphatidylcholine vesicles, but low temperature-induced aggregation of bacteriorhodopsin in dimyristoylphosphatidylcholine cannot appear in egg-phosphatidylcholine vesicles. This suggests that different lipids have different effects on bacteriorhodopsin in vesicles.

Keywords: bacteriorhodopsin, melittin, electron microscopy, rotational diffusion.

Bacteriorhodopsin (BR) is the single protein in the purple membrane (PM) from halobacterium halobium. Wild-type BR contains a single-chain polypeptide of 248 amino acid residues and a retina attached to lys216 of the peptide via a Shiff base[1]. BR is an integral membrane protein (MW=26 kd) whose seven transmembrane α-helical segments span the bilayer lipid membrane of PM, and its trimer is arranged in a two-dimensional hexagonal crystal lattice.

Under illumination, BR embarks a photocycle with a series of intermediates and transports a proton from the cytoplasmic side to the extracellular medium, and thereby the resulting pH gradient generates a proton-motive force which was used by halobium to synthesize ATP from inorganic phosphate and ADP for the bacteria to survive[2,3].

Melittin is the principal protein component of bee venom. It has 26 residues and different secondary tertiary and quaternary structures depending upon the physical environment[4]. Melittin has amphiphilic character and a high degree of positive charge[5]. The effects of melittin on membrane lipids have been intensively studied in red blood cell and model membrane systems[6]. The possibility that melittin-membrane protein interactions are involved has received little attention.

Since BR is an well-investigated and relatively simple membrane protein, research on its interaction with lipids and protein can improve out insights into the mechanisms of interaction and the state of membrane protein. We have done some work about the influences of melittin on the rotational diffusion of BR, the photoelectric response and the photocycle of PM[7—9], and suggested that there are electrostatic interaction and other kinds (especially hydrophobic) of interactions between them. Here we report the electron microscopic observation of bacteriorhodopsin in dimyristoylphosphatidylcholine (DMPC) and egg-phosphatidylcholine(egg-pc) vesicles, at meantime we measure the rotational diffusion of BR at the same conditions in DMPC vesicles, and these results further confirm our former suggestion.

Protein aggregation affects the structure and function of membrane proteins and other proteins[10]. The oligomeric state of membrane proteins is a fundamental property. Membrane proteins extracted into detergent micelles often exist as dimers or higher oligomers, but this does not prove their association-state in the membrane. Electron microscopic observation can provide a morphological evidence of the state of protein in the membrane.

1 Materials and methods

1.1 Preparation of DMPC/BR and egg-pc/BR vesicles

For DMPC/BR vesicles, bacteriorhodopsin-containing purple membranes (12 mg) were isolated from halobacterium halobium (strain R_1M_1) and solubilized through suspension in sodium acetate buffer (100 mmol/L, pH 5.0, 48 mL) containing sodium azide (0.02%) and Triton X-100 (48 mg) for 24—30 h in the dark at room temperature. After centrifugation to remove unsolubilized residue, DMPC was added and the Triton was removed by dialyzing against sodium acetate buffer (100 mmol/L, pH 5.0) containing sodium azide (0.02%) for about one week. The reconstituted sample was purified by sucrose density gradient centrifugation (15%—40%) to remove remained free lipid and protein. For egg-pc/BR vesicles, the main procedure is the same as DMPC/BR vesicles. The difference is that the dialysis lasted for 9 d with the first 7 d at 4℃ then at 18℃ for 2 d in the dark. Only these vesicles were collected at 20000—25000 r/min by centrifugation and used to do observation. Protein content was determined by the method of Lowry et al. and corrected for bacteriorhodopsin as described by Rehorek et al.[11]. Lipid content was determined by phosphorus analysis[12].

1.2 Purification of melittin

Melittin, low in phospholipase A_2 activity, was generously provided by Prof. Hider. Throughout the study, melittin concentration was determined according to a molar extinction coefficient of 5600 cm^{-1} at 280 nm. The incorporation of melittin in vesicles was monitored by centrifuging the vesicles and determining the melittin remained in the supernatant. No melittin was detected in the supernatant. Thus all the added melittin is associated with the vesicles.

1.3 Electron microscopy

Samples were mounted on the gold disks and frozen in liquid nitrogen as quickly as possible at a temperature of about −190℃. Freeze-fracturing was carried out in a Blazer's BAF-400D apparatus, and specimens were coated with pt/c at a vacuum of 1×10^{-4} Pa and picked on copper grids of 400 mesh. Electron micrographs of replicas were taken with JEM-100 CX.

1.4 Rotational diffusion measurement

The rotational diffusion of bacteriorhodopsin in DMPC vesicles was measured by observing flash-induced transient dichroism in Prof. Cherry's lab in U.K.

$$r(t)=\left[\frac{r_0}{A_1+A_2+A_3}\right]\left[A_1\exp(-t/\Phi_{11})+A_2\exp(-4t/\Phi_{11})+A_3\right],$$

where $r(t)$ is the anisotropy parameter, r_0 is the anisotropy at $t=0$, A_1 and A_2 are constants, Φ_{11} is the rotational relaxation time of BR, and A_3 is the fraction of immobile molecules.

2 Results and discussion

The freeze-fracture morphology of reconstituted vesicles at room temperature is shown in fig.1(a). DMPC/BR mole ratio is 109, and the concentration of BR is 1.68×10^{-5} mol/L. When DMPC/BR mole ratio is 231, and the concentration of BR is 0.54×10^{-5} mol/L, the freeze-fracture morphology of reconstituted vesicles at room temperature is shown in fig. 1(b). Fig. 1(c) shows the electron micrograph of reconstituted vesicles frozen at 4℃, DMPC/BR mole ratio of 120, and the concentration of BR of 1.09×10^{-5} mol/L. From the freeze-fracture morphology, the vesicles appear to be unilamellar with diameters in the order of 0.3—1 μm. Incorporation of BR is demonstrated by existence of particles. From fig.1(a), the particles were randomly distributed in the plane of the membrane, since monomeric BR molecule's dimensions are 3.5 nm×2.5 nm and 2 nm thickness of replica. In fig.1(a) the diameter of most particles is less than 5 nm, so most of the particles appear to be the monomeric state of BR. At high lipid/protein mole ratio (fig. 1(b)), it showed the same as fig. 1(a) except that the BR particles are less than the low lipid/protein ratio. If the reconstituted vesicles were frozen at 4℃ (low temperature), and the diameter of particles is more than 10 nm, it is not monomeric state of BR, so the BR molecules in the lipid vesicles were aggregated into large particles (fig.1(c)).

When 5×10^{-6} mol/L melittin was added to the suspension of BR-lipid vesicles, and the experimental conditions were as for fig.1(a), the freeze-fracture morphology is shown in fig. 2(a). The diameter of BR particles is more than 10 nm, and some BR molecules in the vesicles were aggregated into larger particles. When the melittin concentration was increased to 5×10^{-5} mol/L (fig. 2(b)), the most diameter of BR particles was more than 10 nm and the diameter of some BR particles was up to 40 nm, so most of BR molecules were aggregated into large particles or patches.

The previous studies[7,9] showed clearly that the degree of bacteriorhodopsin immobilization is determined by the melittin/protein ratio rather than the melittin/lipid ratio and melittin and its site-specific mutants differentially delay the slow-decaying component of the photocycle intermediate M_{412} of BR in the purple membrane. We suggested that there is a direct interaction between melittin and bacteriorhodopsin. In the case of bacteriorhodopsin, the molecule protrudes from both sides of the bilayer, one side bearing an overall charge of 1- in N-terminal and the other side of 7- in C-terminal. In the absence of melittin, bacteriorhodopsin could be dispersed in the vesicle membrane by a degree of anionic repulsion between these charged regions. The major hydrophilic segment of melittin is the C-terminal segment of six residues that bears a cluster of four positive charges. Another previous study[13] showed that removal of the C-terminal peptide of BR affects the interaction between BR and melittin, and BR of removal of the 5- in C-terminal has less effect between BR and melittin. A plausible mechanism of bacteriorhodopsin aggregation in DMPC vesicles is the cationic C-terminus of melittin crosslinks bacteriorhodopsin by electrostatic binding to anion residues. Some degrees of hydrophobic interaction between the intramembrane portions of bacteriorhodopsin and melittin are also possible, but this would probably be secondary to the ionic aspect.

From the morphology of DMPC/BR reconstituted vesicles frozen at 4℃, the previous study[14] and our results showed that BR molecules were also aggregated into large particles or patches. The mechanism of low temperature-induced BR aggregation is different from the melittin-induced BR aggregation. In this case, low temperature may cause phase separation.

It has been found that the melittin causes the aggregation of membrane protein in human erythrocyte[15]. Now we can directly provide the evidence of melittin-induced and low temperature-induced BR aggregation in DMPC vesicles from freeze-fracture electron micrographs. Despite the differences between bacteriorhodopsin and band 3, both can be readily aggregated by melittin. Thus the protein aggregation property is relatively nonspecific. BR aggregation induces the decrease of rotational mobility of BR or no rotation diffusion can be observed in the experimental time range. Low temperature also causes the increase of membrane viscosity, and induces the decrease of rotational mobility of BR, but BR aggregation can also lead to the latter.

BR has only been reconstituted using saturated lipid such as dipalmitoylphosphatidylcholine (DPPC) and DMPC. Attempts to reconstitute BR into unsaturated lipid vesicles resulted in loss of the important retinal chromophore, presumably indicating a degree of denaturation. Some proteins, such as band 3, have been successfully reconstituted into vesicles made from unsaturated lipids, such as egg-pc and DOPC. The oligomeric state of the erythrocyte anion transporter, band 3, has been the subject of many debates, with different investigators favoring either a dimeric or tetrameric state. If we plan to use BR as a calibration protein in lipid vesicles for the oligomeric state of the other membrane proteins based on measurement of rotational diffusion, we must reconstituted different proteins into the same lipid. So we try to reconstitute BR into egg-pc vesicles. From the freeze fracture morphology (fig.4), we have successfully reconstituted BR into egg-pc

vesicles; BR molecules exist in egg-pc vesicles in the most of monomeric state. We have measured the photocycle of BR in egg-pc vesicles and found the form and decay of intermediate M_{412} (unpublished).

In the morphology, no much difference can be found from figs. 4(a) and (b). Low temperature-induced BR aggregation in DMPC vesicles cannot appear in egg-pc vesicles. It means that different lipids have different effects on BR in vesicles.

3 Conclusions

(i) Melittin produces its effect via direct electrostatic interaction with bacteriorhodopsin. In the presence of melittin, bacteriorhodopsin molecules in DMPC vesicles were aggregated into large particles or patches, and the ability of rotational diffusion of BR in vesicles was decreased.

(ii) Low temperature-induced aggregation of bacteriorhodopsin appears in DMPC vesicles.

(iii) Bacteriorhodopsin was successfully reconstituted into egg-pc vesicles.

(iv) Low temperature-induced aggregation of bacteriorhodopsin in egg-pc vesicles cannot appear, and different lipids have different effects on bacteriorhodopsin in vesicles.

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