8. Bhutta, Z. A., Black, R. E., Brown, K. H. et al., Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: Pooled analysis of randomized controlled trials, J. Pediatr., 1999, 135: 689.
9. Black, R. E., Therapeutic and preventive effects of zinc on serious childhood infectious diseases in developing countries, Am. J. Clin. Nutr., 1998, 68: 476 S.
10. Hambidge, K. M., Human zinc deficiency, J. Nutr., 2000, 130: 1344 S.
11. Kikafunda, J. K., Walker, A. F., Allen, E. F. et al., Effect of zinc supplementation on growth and body composition of Ugandan pre-school children: A randomized, controlled intervention trial, Am. J. Clin. Nutr., 1998, 68: 1261.
12. Eggar, N., Sandstead, H. J., Alcock, N. et al., Zinc supplementation improves growth in Mexican-American children, FASEB J., 1999, 13: A246 (abs.).
13. Penland, J., Sandstead, H., Eggar, N. M. et al., Zinc, iron and micronutrients supplementation effects on cognitive and psychomotor function of Mexican-American school children, FASEB J., 1999, 13: A921 (abs.).
14. Bentley, M. E., Caulfield, L. E., Ram, M. et al., Zinc supplementation affects the activity patterns of rural Guatemalan infants, J. Nutr., 1997, 127: 1333.
15. Sandstead, H. H., Penland, J. G., Alcock, N. W. et al., Effects of repletion with zinc and other micronutrients on neuropsychologic performance and growth of Chinese children, Am. J. Clin. Nutr., 1998, 68: 470 S.
16. Bremner, I., Nutritional and physiological significance of metallothionein, Experintia Supplementum, 1987, 52: 81.
17. Nielson, K. B., Atkin, C. L., Winge, D. R., Distinct metal-binding configurations in metallothionein, J. Biol. Chem., 1985, 260: 5342.
18. Nettesheim, D. G., Engesth, H. R., Otovos J. D., Products of metal exchange-reactions of metallothionein, Biochemistry, 1985, 24: 6744.
19. Otvos, J. D., Engeseth, H. R., Wehrli, S., Preparation and Cd-113 NMR-studies of homogeneous reconstituted metallothionein reaffirmation of the 2-cluster arrangement of metals, Biochemistry, 1985, 24: 6735.
20. Zhou, Y. J., Li, L. Y., Ru, B. G., Expression, purification and characterization of β domain and β domain dimer of metallothionein, *Biochim. Biophys. Acta*, 2000, 25(32): 1.
21. Zhang, X. Y., Li, W., Zhang, J. et al., Construction of high efficient expression vector of metallothionein mutant in plant and its expression in tobacco, Chin. J. Biochem. Mol. Biol., 1999, 15: 888.
22. Shi, S. H., Zhang, Q., Chen, Y. Q. et al., A simple method for isolation of total RNA and DNA from silicagel-dried and fresh leaves of plants, Acta Sci. Nat. Univ. Sunyatseni, 1996, 35: 103.
23. Porebski, S., Bailey, L. G., Baum, B. R., Modification of a CTAB DNA extraction protocol for plants containing high Polysaccharide and Polyphenol components, Plant Mol. Biol. Rep., 1997, 15: 8.
24. Baker, A. J. M., Brooks, R. R., Terrestrial higher plants which hyperaccumulate metallic elements: A review of their distribution, ecology and phytochemistry, Biorecovery, 1989, 1: 81.
25. Zhang, X. Y., Zuo, X. F., Xiao, C. Y. et al., Expression of the *mouse metallothionein mutant αα-cDNA improving cadmium resistance* in transgenic tobacco, Chin. J. Biochem. Mol. Biol., 2000, 16: 631.
26. Michael, W. P., David, E. S., Possible molecular mechanisms involved in nickel, zinc and selenium hyperaccumulation in plants, Biotechnol. Genet. Eng. Rev., 2000, 17: 389.
27. Grotz, N., Fox, T., Connolly, E. et al., Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency, Proc. Natl. Acad. Sci. USA, 1998, 95: 7220.

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The interaction between purple membrane and membrane lipid

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Abstract **Bacteriorhodopsin in purple membrane was reconstituted into different lipid vesicles. The effect of three different lipids on the structure and function of bacteriorhodopsin in lipid vesicles was studied by the observation on freeze-fracture eletron microscopy, the rotational diffusion of bacteriorhodopsin in lipid vesicles, the measurement of absorption spectrum, and the absorbance change with time. For these prepared samples, the results showed that DMPC was the stable lipid environment of bacteriorhodopsin; egg-pc causeed the loss of retinal chromophore of bacteriorhodopsin and it was not reversible change, cholesterol could stabilize the bacteriorhodopsin in lipid environment, but it caused the aggregation of bacteriorhodopsin.**

Keywords: purple membrane, bacteriorhodopsin (BR), lipid vesicle.

Bacteriorhodopsin (BR) is the only protein component in the purple membrane from *Halobacterium halobium*, which possesses the ability to convert light energy to electrochemical energy[1]. BR is one of the well-known proteins. Research on the structure and function of BR and the application of molecular electronics are hot spots on biological membrane[2]. The native purple membrane is an integral membrane protein and its trimer is arranged in a two-dimentional hexagonal crystal lattice[3]. This structure results in the stability of purple membrane. In purple membrane, the main constituents of lipid are phosphatidyl glycerophosphate, glycolipid, phophatidyl glycerosulphate, phosphatidyl glycerol, and several other neutral lipids. The changes of lipid in purple membrane will affect the structure and function of BR[4]. Its study will help elucidate the relationship between structure and function and it will be possible for the application of BR. In this report, the effects of three different lipids on BR were studied.

1 Materials and methods

(i) Preparation of DMPC/BR, egg-PC/BR, DMPC+ egg-PC/BR, and egg-PC + cholesterol/BR vesicles. BR-containing purple membrane (12 mg) was solubilised through suspension in sodium acetate buffer (100 mol/L, pH 5.0, 48 mL) containing sodium azide (0.02%) and Triton X-100 (48 mg) for more than 24 h in the dark at room temperature. DMPC (from Sigma), egg-PC (from Sigma), DMPC + egg-PC, and egg-PC + cholesterol were added to

the suspension and the Triton was removed by dialysis for about 1 week. The reconstituted BR vesicle was purified by sucrose density gradient centrifugation (15%—40%) to remove remaining free lipid and protein. The protein and lipid concentration were determined by standard methods.

(ii) Measurement of rotational diffusion. The rotational diffusion of BR in vesicles was measured by observing flash-induced fransient dichroism[5]. The sample was excited by a vertically-polarized 532 nm laser pulse. Measurements of vertically and horizontally polarised transient intensity changes at 570 nm were obtained simultaneously with two photomultipliers $A_{//}(t)$ and $A_{\perp}(t)$. The anisotropy parameter $\gamma(t)$ was calculated as follows

$$\gamma(t)=\frac{A_{//}(t)-A_{\perp}(t)}{A_{//}(t)+2A_{\perp}(t)}. \quad (1)$$

In the case of multiple rotating species, monomers are the main constituents of BR in vesicles; $\gamma(t)$ can be analyzed by the general double-exponential equation

$$\gamma(t)=A_1\exp(-t/T_1)+A_2\exp(-t/T_2)+A_3, \quad (2)$$

where A_1, A_2, and A_3 are constants which depend on the orientation of the transition dipole moment. A_3 is the fraction of immobile molecules, and T_1 and T_2 are the rotational relaxation time.

(iii) The observation of electron microscopy. Samples were mounted on the gold disks and frozen in liquid nitrogen as quickly as possible. Freeze-fracturing was carried out in a Blazeris BAF-400D apparatus, and specimens were coated with Pt/C at a vacuum of 1×10^{-4} Pa and picked onto copper grids of 400 mesh. Electron micrographs of replicas were taken with JEM-100CX.

2 Results and discussion

(i) Observation of electron microscopy. The freeze-fracture morphology of DMPC/BR vesicles at room temperature is shown in fig. 1(a). DMPC/BR mole ratio was 161, and the concentration of BR was 1.5×10^{-5} mol/L. From the freeze-fracture morphology in fig. 1(a), the diameter of vesicles was about 0.7 μm. Two vesicles appeared to be fused. Incorporation of BR was demonstrated by existence of particles. These particles were randomly distributed in the plane of membrane. Most of the particles appeared to be in the monomeric state of BR. The freeze-fracture morphology of egg-pc/BR vesicles at room temperature is shown in fig. 1(b). Egg-pc/BR mole ratio was 120, and the concentration of BR was 2×10^{-5} mol/L. The diameter of vesicles was about 0.2 μm, smaller than that of DMPC/BR vesicles. The particles were randomly distributed in the vesicles in the monomeric state.

(ii) Measurement of rotational diffusion. The rotational diffusion of BR in three vesicles of DMPC, DMPC + egg-pc, and egg-pc + cholesterol is shown fig. 2. The measurement condition of DMPC/BR vesicles is the same as in fig. 1(a).

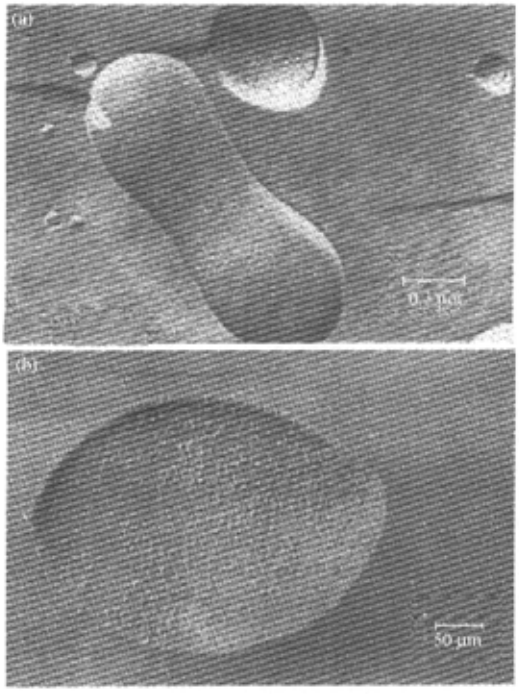

Fig. 1. Freeze-fracture electron micrographs for reconstituted BR in DMPC (a) and egg-pc (b) vesicles.

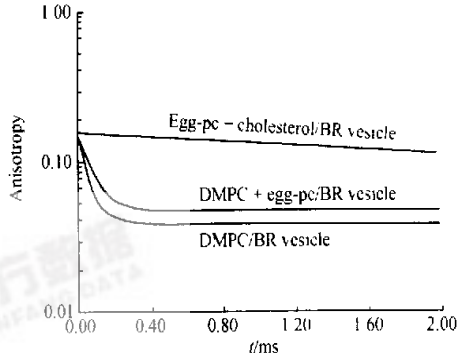

Fig. 2. The rotational diffusion of BR in DMPC/BR (a), DMPC + egg-pc/BR (b) and egg-pc + cholesterol/BR vesicles.

In the vesicles of DMPC + egg-pc/BR, BR : DMPC : egg-PC = 1 : 4.2 : 7 (w/w), the concentration of BR being 1.05×10^{-5} mol/L. In the vesicles of egg-pc + cholesterol/BR, BR : egg-PC : cholesterol = 1 : 3.5 : 2.5 (w/w), the concentration of BR being 1.2×10^{-5} mol/L. The measurement temperature is 30℃.

According to the analysis and calculation[6], the rotational relaxation time of BR in DMPC/BR vesicles was

22.0 μs (T_1), 255.0 μs (T_2), A_1 = 0.111, A_2 = 0.030. The fraction of immobile molecules was 20.2%. In the DMPC + egg-pc/BR vesicles, the results of measurement did not show obvious difference. The rotational relaxation time of BR was 39.8 μs (T_1) , 261.6 μs (T_2), A_1 = 0.091, A_2 = 0.022. The fraction of immobile molecules was 38%. For the vesicles of egg-pc + cholesterol/BR, the rotational diffusion was very slow and these molecules were immobile in the time scale of measurement.

(iii) Measurement of absorption spectrum. In the egg-pc/BR reconstituted vesicles as the same condition of DMPC reconstituted vesicles, the samples were not stable and the purple colour became colorless during the reconstitution and measurement. We reconstituted the colorless sample with all-trans retinal using the usual method[7]. The absorption spectra of BR of egg-pc/BR vesicles and all-trans retinal reconstituted vesicles are shown in fig. 3. From fig. 3, the 570 nm absorption peak disappeared, which means that BR was denatured.

If the vesicles were reconstituted by using egg-pc + cholestrol with BR, from the absorption spectrum of fig. 3, the absorption peak is 570 nm. If the retinal chromophore was removed, the sample can be reconstituted with all-trans retinal to obtain the 570 nm absorption peak (not shown in fig. 3), which means that this is reversible.

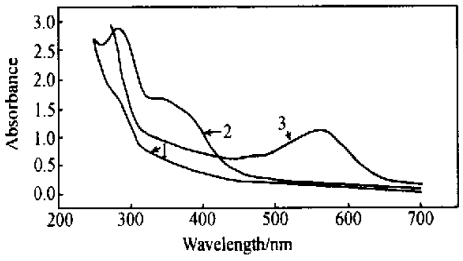

Fig. 3. The absorption spectra of BR in the vesicles of the egg-pc (1), all-trans retinal reconstitution (2), and egg-pc + cholesterol (3).

The 570 nm absorbance changes of BR in egg-pc + cholesterol vesicles with time at different temperatures are shown in fig. 4. Photocycle of BR in vesicles can be found and the life time of intermediate M_{412} is 6.4 ms at 18℃, 3.8 ms at 30℃ and 3.1 ms at 38℃, respectively.

The effects of different lipids on BR is different. In DMPC, BR is very stable in the monomeric state and can maintain its properties for more than one year at room temperature. Measurement of rotational diffusion and flash kinetic spectrum showed that BR in DMPC vesicles had the function of proton pump and can rotate in the membrane. In egg-pc, the chromophore of BR lost and the property of struture changed. BR in egg-pc vesicles had no function of photocycle or proton pump. The reconstitution using all-trans retinal was not successful. In DMPC-added egg-pc the structure and function of BR in these vesicles were recovered. Cholesterol in egg-pc vesicles

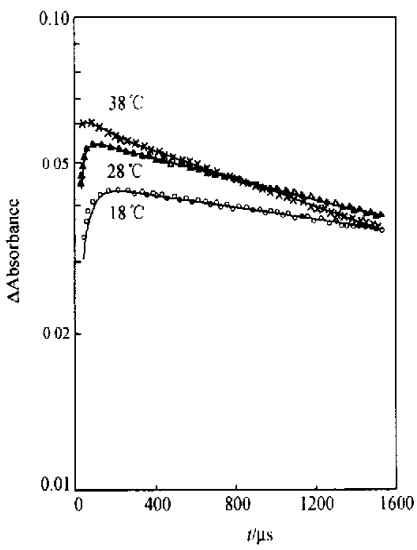

Fig. 4. The absorbance change of BR with time in egg-pc + cholesterol vesicles at different temperatures.

caused the aggregation of BR. The rotational diffusion became very slow. It cannot be measured in the scale of resolution, but BR in these vesicles was stable and had the function of photocycle and proton pump.

In conclusion, DMPC was the stable lipid environment of BR vesicles. Egg-pc caused the loss of chromophore, which is a non-reversible change. Cholesterol can stabilize the BR in egg-pc vesicles but caused the aggregation of BR. The mechanism of effect of lipid environment on BR will be studied further.

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References

1. Lanyi, J. K., Proton understanding structure and function in the light-driven proton pump bacteriorhodopsin, J. Struct. Biol., 1998, 124: 164.
2. Hampp, N., Heat-proof proteins, Nature, 1993, 366: 12.
3. Hartmut, L., Brigitte Schobert, Hans-Thomas Richter et al., Structural changes in bacteriorhodopsin during ion transport at 2 angstrom resolution, Science, 1999, 286: 255.
4. Hu, K. S., Sun, Y., Chen, D. L. et al., The effect of lipid environment in purple membrane on bacteriorhodopsin, Journal of Photochemistry and Photobiology B, 2000, 58: 163.
5. Hu, K. S., Wang, A. J., Ian, E. G., Electron microscopic observation and rotational diffusion measurement of bacteriorhodopsin in lipid vesicles, Science in China, Ser. B, 2001, 44(6): 663.
6. Hu, K. S., Dufton, M. J., Cherry, R. J. et al., Interaction of bee venom melittin with bacteriorhodopsin in lipid vesicles: Protein rotational diffusion measurement, Biochem. Biophys. Acta, 1985, 816(2): 358.
7. Hu Kunsheng, The effect of reconstitution of Bacteriorhodopsin on the structure and mobility, Acta Biophysica Sinica (in Chinese), 1987, 3(4): 346.

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