ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(10): 691–696 **CN** 31-1940/Q

Studies on the Temperature Effect on Bacteriorhodopsin of Purple and Blue Membrane by Fluorescence and Absorption Spectroscopy

Lan-Ying CHENG[1,2], Yue ZHANG[3], Shi-Gui LIU[1], Kun-Sheng HU[3], and Kang-Cheng RUAN[2]*

[1] *College of Life Science, Sichuan University, Chengdu 610064, China;*
[2] *State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China;*
[3] *Institute of Biophysics, Academia Sinica, Beijing 100101, China*

Abstract Fluorescence and absorption spectra were used to study the temperature effect on the conformation of bacteriorhodopsin (bR) in the blue and purple membranes (termed as bRb and bRp respectively). The maximum emission wavelengths of tryptophan fluorescence in both proteins at room temperature are 340 nm, and the fluorescence quantum yield of bRb is about 1.4 fold higher than that of bRp. As temperature increases, the tryptophan fluorescence of bRb decreases, while the tryptophan fluorescence of bRp increases. The binding study of extrinsic fluorescent probe bis-ANS indicated that the probe can bind only to bRb, but not to bRp. These results suggest that significant structural difference existed between bRb and bRp. It was also found that both kinds of bR are highly thermal stable. The maximum wavelength of the protein fluorescence emission only shifted from 340 nm to 346 nm at 100 ºC. More interestingly, as temperature increased, the characteristic absorption peak of bRb at 605 nm decreased and a new absorption peak at 380 nm formed. The transition occurred at a narrow temperature range (65 ºC–70 ºC). These facts indicated that an intermediate can be induced by high temperature. This phenomenon has not been reported before.

Key words bacteriorhodopsin; purple membrane; blue membrane; fluorescence and absorption spectroscopy

Purple membrane is the membrane fragment found in *Halobacterium halobium*. Each purple membrane fragment contains more than hundreds of thousands of molecules of bacteriorhodopsin (bR), the only integrated membrane protein. Each bR contains 248 amino acids, having seven transmembrane α-helixes, and has a molecular weight of 26 kD. There are eight tryptophan and 11 tyrosine residues in bR. bR functions as light-driven proton pump, is able to photocycle and carry out photon-electronic reactions. At pH lower than 2 or after the removal of surface bivalent cations (mainly Mg^{2+} and Ca^{2+}), the color of the purple membrane becomes blue (referred to as blue membrane). The blue membrane does not undergo photocycles nor does it have any light-driven proton pump functions. To date, the structure, photocycles and photochoromic effect of the purple membrane have been extensively studied. The unique physical properties of the purple membrane suggested that bR could be useful in photoelectronic detection, neural network, biochip, etc. [1–14]. However, the comparison of the conformation of bR in purple and blue membrane has rarely been studied by fluorescence spectroscopy. In this paper, we used fluorescence and absorption spectroscopy to study the temperature effect on the conformation of bR proteins in the purple and blue membrane. For description convenience, bR in the purple and blue membrane will be referred to as bRp and bRb, respectively.

Received: June 20, 2006 Accepted: August 2, 2006
This work was supported by the grants from the National Natural Science Foundation of China (No. 30370303) and the National Key Basic Research and Development Program (No. 2002CB713802)
*Corresponding author: Tel, 86-21-54921168; Fax, 86-21-54921011; E-mail, kcruan@sibs.ac.cn

Materials and Methods

Preparation of bRp and bRb

Halobacterium halobium culture, isolation and purification of the purple membrane were carried out accord-

DOI: 10.1111/j.1745-7270.2006.00219.x

ing to Wang *et al*. [3–5]. The obtained purple membrane was dissolved in 50 mM Tris-HCl buffer (pH 7.1) and stored at 4 ºC. The light-adaption of the purple membrane was carried out by illuminating the sample for 1 h with a 25 W lamp. The blue membrane was obtained from the purple membrane by adding 10 mM EDTA to pR solution of deionized water with resistance of 18 MΩ/cm. The concentrations of bRp and bRb were kept at 4.0 μM for all fluorescence and absorption measurements.

Fluorescent probe bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid, dipotassium salt) was purchased from Molecular Probe Incorporation (Invitrogen, Eugene, USA). *L*-tryptophan was obtained from Sigma (Sigma, St Louis, USA). Unless specified, all chemicals are AR grade.

Fluorescence and absorption measurements

Fluorescence and absorption measurements were carried out on Hitachi F-4010 fluorescence spectrophotometer and Hitachi U-3010 UV-visible spectrophotometer. All the fluorescence spectra were corrected by using the software provided in the instrument. In the temperature effect experiments, samples were incubated at each target temperature for 5 min before the measurement.

Results and Discussion

Temperature effect on intrinsic fluorescence of bRp and bRb

When bRp and bRb were excited at 295 nm, only tryptophan residues were excited. As shown in **Fig. 1**, the fluorescence spectra of both bRp and bRb had a maximum emission peak at 340 nm, suggesting that the tryptophan residues in two kinds of bR are in a similar hydrophobic environment [15–19]. However, the fluorescence intensity of bRb was 2.4 times as much as that of bRp at 25 ºC. These data indicate that the fluorescence efficiency of tryptophan in bRb increases with the state of transition form purple to blue. The fluorescence efficiency of tryptophan is related with the hydrophobic characteristics, the number and sort of nearby quenching group. In the case of bR, it seems that some nearby quenching groups moved away upon the transition from bRp to bRb, resulting in an increase in the fluorescence efficiency of tryptophan in bRb. However, there is one more possibility. It has been proposed that some but not all tryptophan residues are involved in energy transfer between Trp residues and retinal chromophore for bR [20]. The energy transfer efficiency between Trp residues and the retinal chromophore is dependent on the spectral overlap integral between the emission spectrum of Trp and the absorption spectrum of retinal, as well as their mutual distance and orientations. The transfer efficiency (E) is strongly dependent on the distance between donor and acceptor (r) as E is inversely proportional to r^6. As seen in **Fig. 1**, the fluorescence spectra of bRp and bRb were identical, indicating the spectral overlap mentioned above in these two kinds are very similar. This suggested that the decrease in E of bRb is probably caused by the variation of the distance between Trp residues and retinal chromophore. All of these possibilities implied that bR undergo pronounced conformational change upon the state transition from purple to blue.

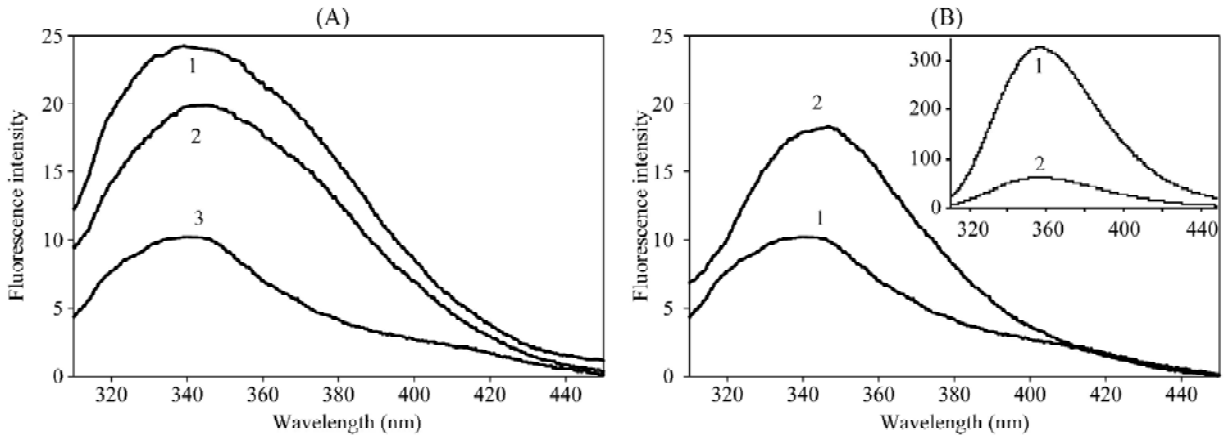

Fig. 1 Effect of temperature on tryptophan fluorescence spectra of bRp and bRb

(A) The tryptophan fluorescence spectra of bRb at 25 ºC (curve 1) and at 100 ºC (curve 2); curve 3, the tryptophan fluorescence spectrum of bRp at 25 ºC. (B) The tryptophan fluorescence spectra of bRp at 25 ºC (curve 1) and at 100 ºC (curve 2); inset, the fluorescence spectra of free tryptophan at 25 ºC (curve 1) and at 100 ºC (curve 2). All concentrations of bRp and bRb and tryptophan were 4.0 μM; the excitation wavelength was 295 nm.

It is also shown in **Fig. 1** that the maximum emission wavelength (λ_{max}) of the tryptophan in both bRp and bRb slightly shifted to red as the temperature increased. However, even at 100 ºC, the λ_{max} of the proteins only shifted from 340 nm to 346 nm, but not to 352 nm, the latter is the maximum emission wavelength of the free tryptophan in water or tryptophan residues in a completely denatured protein [see inset in **Fig. 1(B)**]. This suggested that both the bRp and bRb were only partial unfolded at a temperature as high as 100 ºC. These results indicated that bR in the purple and blue membranes are thermal stable, which is consistent with those reported by Heyes *et al.*[2] and Zhang *et al.*[8].

Although the spectral red shift of bRp and bRb induced by the temperature were very similar, the changes in fluorescence intensity were different. As shown in **Fig. 1(A)**, the fluorescence intensity of bRb decreases with the increase in temperature, while the fluorescence intensity of bRp increases with the increase in temperature. Actually, the fluorescence intensity at 100 ºC has a 16% decrease for bRb and a 69% increase for bRp compared with that at 25 ºC. In general, the fluorescence intensity of tryptophan decreased with the increased temperature as shown in the inset of **Fig. 1(B)**. Therefore, the enhancement of bRp fluorescence intensity implies a mechanism which can increase the fluorescence efficiency of tryptophan to overcome the fluorescence reduction caused by higher temperature. One possibility of this phenomenon is that some quenching groups, which originally locate near to tryptophan residues in bRp, moved away during the unfolding process caused by higher temperature, resulting in weakening of the quenching effect. No matter what the mechanism is, the different changes in fluorescence intensity of bRb and bRp with temperature indicate that conformation around tryptophane residues of bR in the blue and purple membrane is different, which is consistent with the conclusion obtained from the comparison of tryptophan fluorescence efficiency at room temperature.

Effect of temperature on absorption spectra of bRb

As shown in **Fig. 2(A)**, the absorbance of the bRb at 605 nm decreases as temperature increases, and a new absorption peak at 380 nm formed and gradually increases. **Fig. 2(B)** shows the temperature dependence of the absorption of bRb at 380 nm and 605 nm. There was no any significant change below 60 ºC. However, when temperature was higher than 60 ºC, the obvious decrease at 605 nm and increase at 380 nm were observed simultaneously. The sharp transition occurs at about 70 ºC. The absorption change is so large that, the temperature effect can be observed unaided during the experiments. The color of the solution changed from blue to colorless at the transition temperature (70 ºC). Since the absorption spectrum characteristic is related to various intermediates of bR, these observations demonstrated that the state of retinal chromophore of bR in the blue membrane is highly temperature-dependent.

The formation of the 380 nm absorption peak might be the result of two possibilities. Luecke *et al.* reported that the formation of protonated Schif's base at the M-intermediate state resulted in absorption at 380 nm [21]. Popp *et al.* [22] and Fischer *et al.* [23] reported the formation of the Q intermediate state resulted in an absorption peak at 380 nm from bRb by a slow thermal reaction. Analysis of the two possibilities indicated that the transition in **Fig. 2(A,B)** most likely belong to the latter. However, in Popp's and Fischer's work, the Q state was obtained from acid-induced bRb in the presence of glycerol by a temperature change from 15 ºC to 35 ºC and incubation for 3 h, very different from our experimental condition in **Fig. 2**. In our experiment, there is no obvious change at 380 nm and 605 nm from 20 ºC to 60 ºC. Actually, even at 10 ºC, the absorption of bRb was very similar with that at 20 ºC (data not shown). Therefore, our results implied that the temperature change from 60 ºC to 70 ºC might be a new way to induce the transition of bRb into a Q state. More interestingly, Heyes *et al.* found that the phase transition in bRb was at about 65 ºC [2], close to the temperature of 70 ºC in our observation, implying that there would be some relationship between phase transition and the loss of retinal chromophore. It was also found that the absorption change of bRb in **Fig. 2** is irreversible. The characteristic absorption of bRb at 605 nm could not be restored and the enhanced absorption at 380 nm remained even when the temperature slowly returned to room temperature.

Effect of temperature on the absorption spectrum of bRp

The effect of temperature on the absorption spectra of bRp was shown in **Fig. 2(C)**. Unlike bRb, the absorption of bRp from 350 nm to 650 nm continuously decreased as temperature increased. The decrease of the 570 nm peak is quite pronounced, however, the 380 nm peak was not observed [**Fig. 2(C)**]. In **Fig. 2(D)**, it is clear that the absorption of bRp at 380 nm and 570 nm were almost synchronically decreased as the temperature increased. This indicates that the temperature effect on the retinal chromophore in bRb and bRp is different, implying that the characteristics of the chromophore could relate with the conformation of bR.

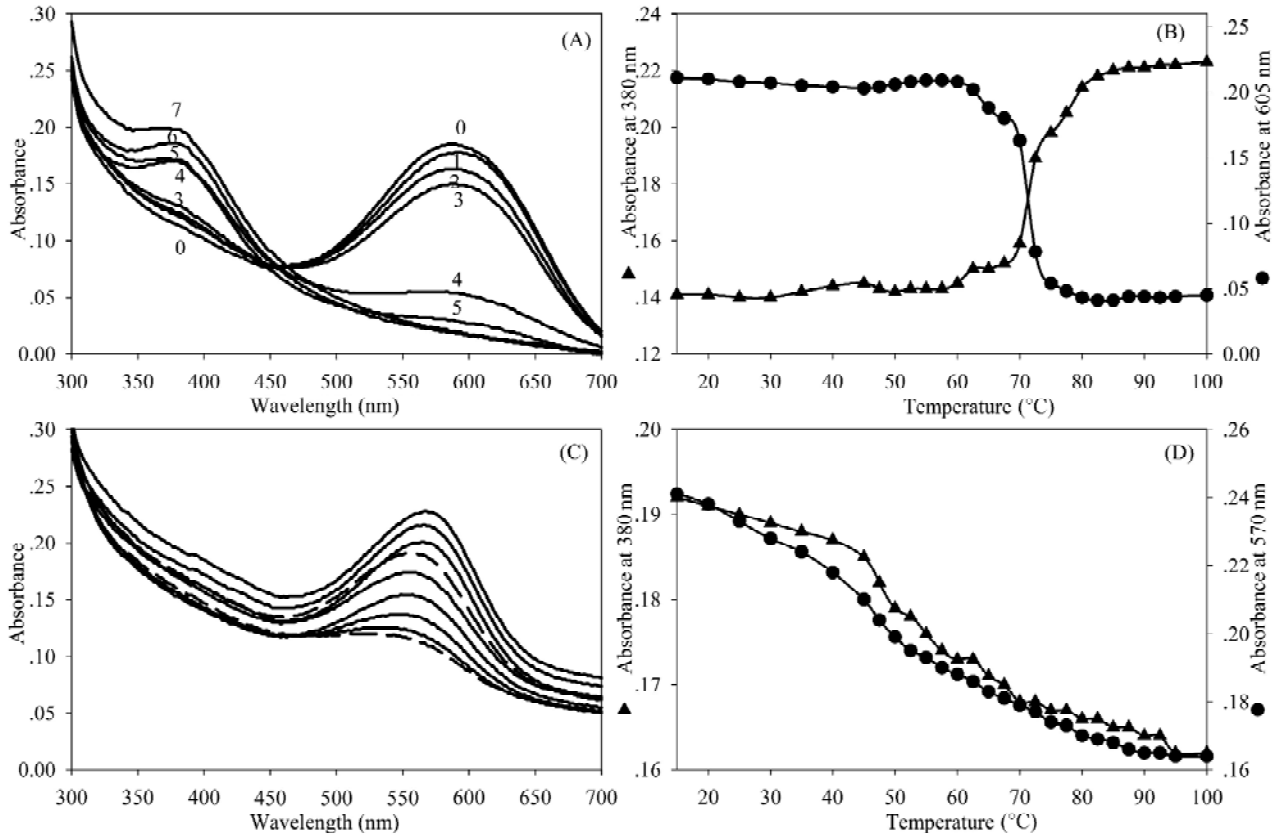

Fig. 2 Effect of temperature on absorption spectrum of bRb and bRp

(A) The absorption spectra of bRb at 25 ºC, 60 ºC, 67.5 ºC, 70 ºC, 72.5 ºC, 75 ºC, 80 ºC and 100 ºC (0–7). (B) The temperature dependence of bRb absorption at 605 nm and 380 nm. (C) The absorption spectra of bRp at 25 ºC, 35 ºC, 40 ºC, 60 ºC, 65 ºC, 70 ºC, 75 ºC, 80 ºC and 100 ºC (from top to bottom). (D) The temperature dependence of bRp absorption at 570 nm and 380 nm. The concentrations of bRb and bRp were kept at 4.0 μM.

The binding of bis-ANS to bRb, but not bRp

Bis-ANS is a non-polar fluorescent probe which is almost non-fluorescent in water and becomes highly fluorescent upon its binding to the protein hydrophobic sites. The maximum fluorescence emission also shifts to blue upon binding. With these specific characteristics, bis-ANS has been extensively used to study conformational changes of proteins [24–28]. **Fig. 3** shows the fluorescence spectra of bis-ANS in the presence (curve 1) and absence (curve 3) of bRb. With the addition of bRb, the fluorescence intensity of bis-ANS has an obvious increase and the spectrum is blue-shifted from 517 nm to 480 nm, indicating that the bis-ANS can bind to bRb. However, with the same experimental condition, on the addition of bRp there is no obvious fluorescence intensity increase of bis-ANS, suggesting bis-ANS does not bind to bRp (see inset in **Fig. 3**) [27–31]. These facts indicated that the hydrophobic area accessible to bis-ANS only exist in bRb but

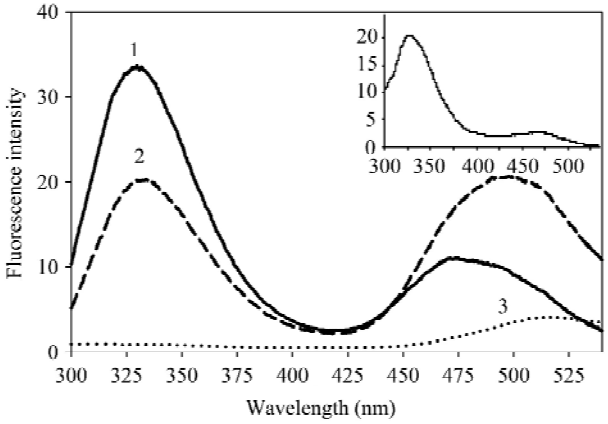

Fig. 3 The effect of temperature on the binding of bis-ANS to blue and purple membranes

Curve 1, the fluorescence spectrum of bis-ANS bound to bRb at 25 ºC; curve 2, the fluorescence spectrum of bis-ANS bound to bRb at 100 ºC; curve 3, the fluorescence spectrum of free bis-ANS at 25 ºC. Inset, the fluorescence spectrum of bis-ANS in the presence of bRp at 25 ºC. All concentrations of bRb and bRp were 4.0 μM; the bis-ANS concentration was 10 μM.

not in bRp, implying that the protein conformation has significant changes upon the removal of bivalent cations from the surface of bRp, which result in the exposure of hydrophobic areas that are originally buried in bR to bis-ANS or in the formation of a new hydrophobic area which is accessible by bis-ANS. In either case, these observations indicated that the conformation of bR in the blue and purple membranes is different from each other, which is consistent with the conclusion obtained in intrinsic fluoresce experiments described above.

More interestingly, when the temperature increases, the fluorescence intensity of bis-ANS bound to bRb becomes stronger (**Fig. 3**). At 100 ºC, the fluorescence intensity is almost two-fold higher than that at 25 ºC accompanied with a red shift from 480 nm to 500 nm. The red shift implies that the binding site of bis-ANS in the protein becomes less hydrophobic [27–29], which is consistent with the partial denaturation of bRb caused by high temperature mentioned above. It is reasonable for a protein that the hydrophobic characteristics have some changes during denaturation. As to the increase in fluorescence intensity, it might be attributed to more bis-ANS bound to bRb at a high temperature. A detailed study will be carried out in the future. In the case of bRp, no obvious binding of bis-ANS can be observed either at room or higher temperature, even at 100 ºC.

In this paper, we used intrinsic fluorescence, bis-ANS binding and absorption spectra to study the conformation of bR in the blue and purple membranes and temperature effect on it. All the results obtained from both the intrinsic fluorescence and extrinsic fluorescence probe at various temperatures lead to a conclusion: the removal of bivalent cations from the surface of bR in purple membrane not only influences the retinal chromophore state, but also changes the local environments of tryptophan residues and the condition of the hydrophobic area of the protein. In other words, during the transition from the purple to blue membrane, the bR conformation has a fundamental change. The high thermal stability of bR protein in both the blue and purple membrane was also revealed by these studies. One more significant finding in this study is that a higher temperature can promote the chromophore in bRb, but not in bRp, to form a new absorption peak at 380 nm accompanied with the absorption decrease at 605 nm. This finding would be useful to study the potential intermediates of the chromophore and its relationship with the protein part of bR. Further studies on the dynamics of the conformation change during the transition from purple to blue of bR and the mechanism of the absorption increase at 380 nm will be carried out in the future.

References

1 Okumura H, Murakami M, Kouyama T. Crystal structures of acid blue and alkaline purple forms of bacteriorhodopsin. J Mol Biol 2005, 351: 481–495

2 Heyes CD, Wang J, Sanii LS, El-Sayed MA. Fourier transform infrared study of the effect of different cations on bacteriorhodopsin protein thermal stability. Biophys J 2002, 82: 1598–1606

3 Wang J, El-Sayed MA. The effect of metal cation binding on the protein, lipid and retinal isomeric ratio in regenerated bacteriorhodopsin of purple membrane. Photochem Photobiol 2001, 5: 564–571

4 Wang G, Wang AJ, Hu KS. Tryptophan fluorescence quenching by alkaline earth metal cations in deionized bacteriorhodopsin. J Photochem Photobiol B 2000, 59: 38–41

5 Wang J, Sayed MAE. The effect of protein conformation change from α_{II} to α_{I} on the bacteriorhodopsin photocycle. Biophys J 2000, 78: 2031–2036

6 Heyn MP, Dudda C, Otto H, Seiff F, Wallat I. The purple to blue transition of bacteriorhodopsin is accompanied by a loss of the hexagonal lattice and a conformational change. Biochemistry 1989, 23: 9166–9172

7 Tokunaga F, Ebrey T. The blue membrane: the 3-dehydroretinal-based artificial pigment of the purple membrane. Biochemistry 1978, 17: 1915–1922

8 Zhang HT, Tan MQ. The effect of temperature on the structure of bacteriorhodopsin of purple membrane. Acta Biophys Sin 1990, 6: 540–544

9 Wan FY, Hu KS. Progress on the structure and function of bacteriorhodopsin. Prog Biochem Biophys 2001, 28: 342–347

10 Zhao W, Li QG. The interaction of neutral red with deionized purple (blue) membrane of *H. halobium*. Acta Biochim Biophys Sin 1995, 1: 31–36

11 Higashiyama H, Tokunaga F. Cause of the blue shift of the absorption spectrum of tetranitromethane-treated bacteriorhodopsin. FEBS Lett 1987, 218: 287–291

12 Mowery PC, Lozier RH, Chae Q, Tseng YW, Taylor M, Stoeckenius W. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. Biochemistry 1979, 18: 4100–4107

13 Ohtani H, Tsukamoto Y, Sakoda Y, Hamaguchi H. Fluorescence spectra of bacteriorhodopsin and the intermediates O and Q at room temperature. FEBS Lett 1995, 359: 65–68

14 Kamiya N, Ishikawa M, Kasahara K, Kaneko M, Yamamoto N, Ohtani H. Picosecond fluorescence spectroscopy of the purple membrane of halobacterium halobium in alkaline suspension. Chem Phys Lett 1997, 265: 595–599

15 Zhang F, Gao J, Weng J, Tan C, Ruan KC, Xu C, Jiang D. Structural and functional differentiation of three groups of tyrosine residues by acetylation of *N*-acetylimidazole in manganese stabilizing protein. Biochemistry 2005, 44: 719–725

16 Li J, Chi CW, Ruan KC. Conformation nearby Trp residues of APIA and APIB modulates the inhibitory specificity of the protease. Acta Biochim Biophys Sin 2002, 4: 494–497

17 Qin L, Ling J, Ruan KC, Liu WY. Relationship between the three enzymatic activities of camphorin and its single tryptophan residue. Prog Biochem Biophys 1995, 22: 177–178

18 Zheng L, Li MY, Zhou GM, Cui DF, Ruan KC. Fluorescence spectroscopy study on the conformation of human gastric inhibitory polypeptide in solution. Pharmaceutical Biotechnology 1997, 4: 204–207

19 Hu LY, Ruan KC. The interaction between trypsin and fluorescence probe ANS. Chinese Journal of Biochemistry and Molecular Biology 1998, 14: 567–572

20 Singh AK, Aruna RV. Fluorescence studies of tryptophan and tryptophan-retinal Schiff base in reverse micellar matrix. J Photoch Photobio A 1995, 89: 247–250

21 Luecke H, Richter HT, Lanyi JK. Proton transfer pathways in bacteriorhodopsin

at 2.3 angstrom resolution. Science 1998, 280: 1934–1937
22 Popp A, Wolperdinger M, Hampp N, Bruchle C, Oesterhelt D. Photochemical conversion of the O-intermediate to 9-*cis*-retinal-containing products in bacteriorhodopsin films. Biophys J 1993, 65: 1449–1459
23 Fischer UC, Towner P, Oesterhelt D. Light induced isomerisation at acidic pH initiates hydrolysis of bacteriorhodopsin to bacterio-opsin 9-*cis*-retinal. Photochem Photobiol 1981, 33: 529–537
24 Rosen CG, Weber G. Dimer formation from 1-amino-8-naphthalenesulfonate catalyzed by bovine serum albumin. A new fluorescent molecule with exceptional binding properties. Biochemistry 1969, 8: 3915–3920
25 Stryer L. The interaction of a naphthalene dye with apomyoglobin and apohemoglobin, a fluorescent probe of non-polar binding sites. J Mol Bio1 1965, 13: 482–495
26 Zheng L, Ruan KC, Lin NQ. A study on the neutral phospholipase A_2 from the venom of *Agkistrodon halys* Pallas by fluorescence spectroscopy. Acta Biophys Sin 1993, 9: 347–352
27 Parasad AR, Luduena RF, Horowitz PM. Detection of energy transfer between tryptophan residues in the tubulin molecule and bound bis (8-anilinonaphthaleno-1-sulfonate), an inhibitor of microtubule assembly, that binds to a flexible region on tubulin. Biochemistry 1986, 25: 3536–3540
28 Ruan KC. The interaction between fluorescence probe bis-ANS with triose phosphate isomerase. Acta Biophys Sin 1992, 8: 563–568
29 Takashi R, Tonomura Y, Morales MF. 4,4′-bis (1-anilinonaphthalene 8-sulfonate) (bis-ANS): a new probe of the active site of myosin. Proc Natl Acad Sci USA 1977, 74: 2334–2338
30 Das KP, Surewicz WK. Temperature-induced exposure of hydrophobic surfaces and its effect on the chaperone activity of α-crystallin. FEBS Lett 1995, 369: 321–325
31 Horowitz PM, Hua S, Gibbons DL. Hydrophobic surfaces that are hidden in chaperonin Cpn60 can be exposed by formation of assembly-competent monomers or by ionic perturbation of the oligomer. J Biol Chem 1995, 270: 1535–1542

Edited by
Chun-He XU