ELSEVIER

Biochimica et Biophysica Acta 1466 (2000) 260–266

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Newly isolated archaerhodopsin from a strain of Chinese halobacteria and its proton pumping behavior

Qingguo Li *, Qingan Sun, Wei Zhao, Hui Wang, Deqiang Xu

Department of Physiology and Biophysics, School of life Science, Fudan University, Shanghai 200433, China

Received 11 November 1999; received in revised form 29 February 2000; accepted 9 March 2000

Abstract

A strain of extremely salt-loving halobacteria *Halobacterium species xz515* from a salt lake in Tibet was isolated. SDS-polyacrylamide gel electrophoresis shows that there is only one protein on claret membrane, which is the same membrane fraction as purple membrane from *Halobacterium salinarum*, with a molecular weight close to bacteriorhodopsin (br). The purified retinal containing protein from xz515 has an absorption peak at around 550 nm. These facts indicate that it is a br-like protein. The partial sequence determination [H. Wang et al., Chin. Sci. Bull., 45 (2000)] shows that this br-like protein belongs to the archaerhodopsin family. The measurements of light-induced medium pH change in intact cells and cell envelope vesicles of xz515 suggest that this type of archaerhodopsin has a proton pumping function. However, the study about the dynamics of pumped protons across the membrane reveal that the proton release and proton uptake is in reverse order compared to br. The probable reason, attributing to regulating the rate of proton release is discussed.

Keywords: Halobacteria; Archaerhodopsin; Bacteriorhodopsin-like protein; Proton release rate

1. Introduction

Bacteriorhodopsin (br) is the first retinal protein, found in extremely salt loving halobacteria, *Halobacterium salinarum* (formerly *halobium*) in the sixties of the last century [1]. It functions as a light-driven proton pump [2]. Since then, the second retinal protein, halorhodopsin (hr) [3], which is a light-driven chloride pump, and two other sensory-rhodopsins, slow rhodopsin (sr) [4] and phoborhodopsin (or slow rhodopsin II, sr II) [5], that are photoreceptors and help bacteria to orientate in medium properly to light, were found in succession. The strain *H. salinarum* is kept in many laboratories in the whole world and its membrane protein br has been studied for many years in order to study the mechanism of its function as light-driven proton pump. From the fact, that several kinds of retinal proteins are found even in one strain, we can imagine that bacteria retinal proteins, widely distributed in nature, very easily suffered variation and differentiation and they should form a big bacterial rhodopsin family in a long process of evolution. Another strain of halobacteria, settled in different ecological habitats may synthesize another kind of retinal proteins, or the retinal proteins carrying the same ion pump function but with a different amino acid sequence, more or less, from *H. salinarum*. To find these new halophiles and study

* Corresponding author. Fax: +86-21-65340149;
E-mail: liqnguok@online.sh.cn

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00188-7

the behavior of their ion pumps, as well as compare them to corresponding proteins of *H. salinarum* is a reasonable way to understand the relationship between the structure and function of these ion pumps.

Mukohata et al. [6], Otomo et al. [7,8] have described several strains of halobacteria isolated from Australia and Mexico. The behavior of ion pumps and the gene structure of retinal proteins of these strains were studied as well. The retinal proteins of these new found halobacteria were named archaerhodopsin by Mukohata [9]. This trend of study started a new field of research, namely the comparative biochemistry and protein chemistry of bacterial rhodopsins that diverged in nature [10].

In this paper we report a newly isolated archaerhodopsin found in an extremely salt-loving *Halobacterium*, *H.sp.xz515*, isolated from a salt lake in Tibet. According to our identification, it is a br-like protein and has a proton pumping function. However, some aspects of its proton pumping behavior are different from br, namely the proton release and uptake is in reversed order of the proton pump of br. The archaerhodopsin gene of xz515 encoding the protein from helix C to helix G has been identified and reported earlier [11]. The proton pumping behavior of xz515 is also discussed here in this paper, considering the primary structure of the amino acid sequence of this protein.

2. Materials and methods

2.1. Isolation of strain H.sp.xz515

The strain of *H.sp.xz515* was isolated from crude solar salts, which were a general gift from a team of geological prospecting and originated from the Zhebai Salt Lake of Tibet. This salt was first dissolved in a complex medium used for growth of *H. salinarum* [12]. After culturing with shaking vigorously for a week, the diluted culture was spread over solidified agar plates. Single colonies were obtained through repeatedly streaking. One colony, tinged with translucent pink and stable in morphology, was collected. The strain xz515 was inoculated from this colony. The cells of xz515 appear globular or oval shaped with a diameter close to 1 μm in the stationary growth phase, under optical microscopy.

2.2. Identification of ion pumps

The intact cells of xz515, as the sample for measurements of light-induced medium pH change, were collected through centrifuging and washed with 4 mol NaCl for three times, then suspended in the same high salt solution for measurements.

The cell envelope vesicles of xz515 were prepared from intact cells according to MacDonald and Lanyi [13]. The prepared envelope vesicles were suspended in 4 mol NaCl for pH change measurement in the medium.

Claret-colored membrane was a membrane fraction from strain xz515, which was obtained by the same procedure as the isolation of the purple membrane from *H. salinarum* [12].

The light-induced pH change in medium of intact cells and envelope vesicles was monitored with a combined glass pH electrode connected to a pH meter. The actinic light was a 150 W halogen lamp, which shone the sample through an orange cut-off filter to pass the wavelength of light $\lambda > 530$ nm. The sample was contained in a water-jacketed glass vessel. Temperature regulation was achieved by circulating water at constant temperature. The size of pH change was calibrated by adding a standard aliquot of HCl. The flash-induced ΔpH change in a millisecond time range was inferred by measuring the absorption change of pH indicator dye, pyranine, in a kinetic spectrophotometer, which was manufactured in our lab [14] according to the model described by Govindjee et al. [15]. The photoproducts of M412 and O640 were also measured with this instrument. The actinic flash was provided from a camera photoflash, the half-bandwidth of which was about 0.2 ms.

Isolation of br-like protein from the strain xz515 was performed using the method, which was described by Ogurusu et al. [16] for isolating hr. This method can be referred as a general method for isolating a intrinsic membrane protein. The main procedure shortly is as following: the membranes containing archaerhodopsin were dissolved with the aid of Triton X-100 and then applied to a series of octyl-Sepharose columns chromatography for fractionation. The whole process and spectral analysis for every step was reported in another paper [17].

The protein contents on the isolated claret mem-

brane were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). In our method 10% acrylamide gel was taken as separate gel and 5% for stacking gel.

3. Results and discussion

3.1. Light-induced pH change in the medium of intact cells and cell envelope vesicles of strain xz515

The trace in Fig. 1 is the profile recorded from a pH electrode, following the light-induced medium pH change of intact cells of xz515. It shows that the suspension of intact cells of xz515 has, on illumination, a transient small alkalization in the medium followed by an acidification. The pH changes are reversed in the dark, but a small transient acidification occurs immediately after the light has been shut off before the system relaxes to the original dark pH value. This profile resembles the typical trace of flash-induced pH change of intact cells of *H. salinarum* [18,19].

Cell envelope vesicles, which are more simple than intact cells, for the cell contents have been removed, but most of them retain the membrane orientation of intact cells [20], should be more accurately follow the light reaction of the ion pump on the membrane. We observed a steady state pH decrease in the suspension of cell envelope vesicle under actinic light (Fig. 2). The recorded curve in Fig. 2 illustrates that vesicles, as intact cells, acidify the medium, similar to envelope vesicles of *H. salinarum*, the proton pumping behavior of which has also been recorded

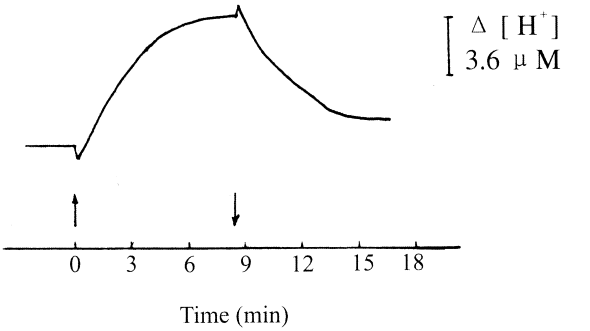

Fig. 1. Light-induced medium H^+ change of intact cells of strain xz515, measured with a combined pH electrode, in 4 mol NaCl, 37°c, pH = 6.8. The arrows indicate the start and end of actinic illumination.

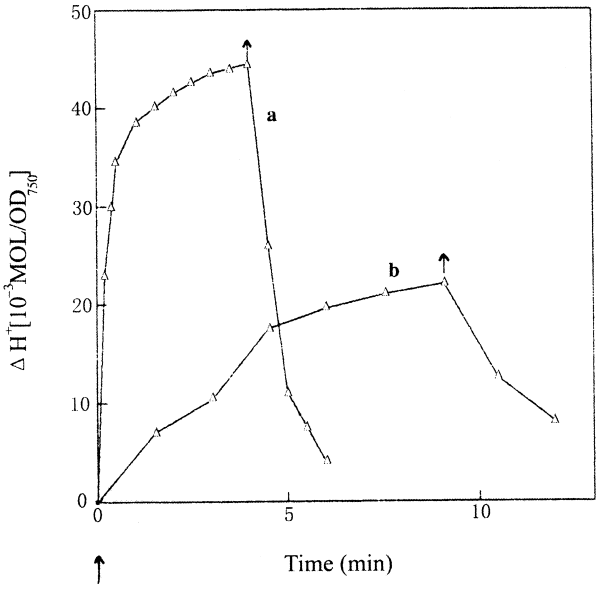

Fig. 2. Light-induced medium H^+ change in cell envelope vesicles of *H. salinarum* (a) and *xz515* (b) in 4 mol NaCl, pH = 6.8. The ordinate is normalized according to its light scattering density at 750 nm, which should be proportional to the amount of vesicles. Arrows indicate light on and off.

and shown in Fig. 2. Both profiles of Fig. 2 are normalized according to their optical scattering density at 750 nm, which should be proportional to the amount of vesicles. Much higher efficiency and rate of pH change of *H. salinarum* than that of *xz515* is evident by comparing both curves.

Light-induced medium acidification seen in intact cells (Fig. 1) and in cell envelope vesicles (Fig. 2) of xz515 can be explained by the light-induced proton pumping function of their retinal containing proteins which pump protons outwards the cell, similar to the proton pump of br in *H. salinarum*. The other possibility causing the medium pH change may occur by another retinal protein, hr, that functions as an inward directed chloride pump with passive proton transport into the cell [21]. However, we have measured the difference spectrum of the isolated membrane fraction, in which the hr should be included, from xz515 at different pH [22]. We did not find any obvious absorption change around 410 nm at pH > 7.4 relative to pH 6.5, that would be the sign for the contents of hr [21]. So, we are certain that halophiles of xz515 do not synthesize hr in a noticeable amount.

3.2. The optical and biochemical analysis of the claret membrane of xz515

The absorption spectrum of the claret colored membrane from xz515 is shown in Fig. 3a. It features by three peaks in the region 450–550 nm, that is the sign for the content of bacterioruberin. The absorption of retinal protein was immersed in the background of these peaks. In order to get a pure absorption spectrum of archaerhodopsin of xz515 and study its optical property, it is necessary to get out of the contamination from this membrane fraction, i.e. carotenoid pigment, cytochrome etc. By using the method mentioned in Section 2, we got an absorption spectrum of purified archaerhodopsin of xz515, which has an absorption peak at about 550 nm, as shown in Fig. 3b. The weak absorption seen in the Fig. 3b curve in the region at 400 nm may be from slim contamination of cytochrome pigment.

The protein content on the claret membrane was analyzed by SDS-PAGE. The SDS-PAGE pattern as shown in Fig. 4 indicates that only one protein with a molecular weight close to br exists on the membrane. This situation resembles the purple membrane that has also only one protein br.

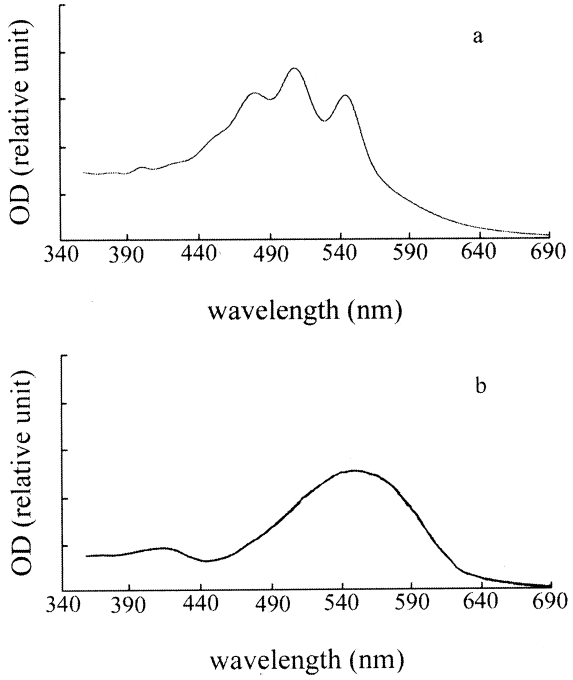

Fig. 3. Absorption spectrum of claret membrane of xz515 in water (a) and its purified archaerhodopsin in sodium of cholatic acid after octyl-Sepharose column chromatography (b).

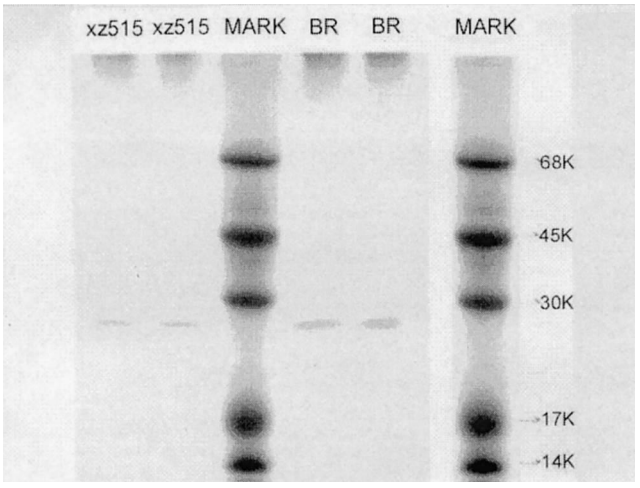

Fig. 4. SDS-PAGE pattern of claret membrane of H.sp.xz515 and the purple membrane of *H. salinarum*.

3.3. The photochemical reaction of the claret membrane

We recorded the photoflash-induced absorption change of claret membrane in water suspension at 410 and 660 nm, corresponding to M412 and O640 photoproducts of br in photocycle, respectively. As shown in Fig. 5, the photoproduct of M412 of xz515 is similar to that of br. The M412 decay, as in br, is biphasic with two composites, M_f and M_s. However, the decay process in xz515 is faster than in br. While the half time decay of total M412 in br is 4.2 ms, it reaches only 3.3 ms in xz515.

The O640 products of both samples are also similar. The arising phase of O640 appears in the time decay of M_f. The succeeding appearance of O640 after M_f [23] in xz515 is another evidence to show that the archaerhodopsin in xz515 is a br-like protein.

3.4. The dynamics of pumped protons across the membrane in xz515

The dynamic process of flash-induced protons across the membrane, pumped by retinal protein xz515, can be followed by the absorption change of pH-sensitive dye, pyranine, in the kinetic spectrophotometer. The proton concentration change in the me-

dium of suspension of purple membrane (Fig. 6a), as well as of claret membrane (Fig. 6b) caused by flash were recorded. The downward curve (reducing the absorption of dye) means that protons are released out of the membrane, that acidifies in turn the medium. The upward curve (increasing the absorption of the dye, pyranine) indicates that protons are taken up from the medium and the medium is alkalized in the same time.

The results of Fig. 6 mean that in the purple membrane the protons are released first on the ultracellular side, then proton uptake was followed on the cytoplasmic side. However, the proton release and uptake for the claret membrane of xz515 is in reversed order, compared to the purple membrane, i.e. the proton uptake precedes proton release. We have recorded the proton pumping curve of xz515 in higher salt concentration (1 mol Na_2SO_4), the order of proton release and uptake does not return to the order of the purple membrane (results not shown). This implies that screening the negative charge on the surface of membrane by high salt concentration does not remove the reversed order of proton release and uptake. Thus, retardation of proton release in xz515 is not totally caused by the increased negative surface potential on the membrane, which would affect the proton release from the negatively charged membrane surface. Though the order of proton release and uptake is reversed, the proton release is more likely still at the ultracellular side. As we have shown by the experiments with intact cells (Fig. 1) and cell envelope vesicles (Fig. 2), that always pump protons out of the cell and acidify the medium under light.

In the scope of proton pumps by retinal protein, we observed reversed order of proton release and uptake in several cases according to the literature. It suggests that blocking of the normal proton release pathway may not be a serious defect. Zimanyl et al. [24] mentioned that the proton pump of br would delay its proton release at the ultracellular side until proton uptake has happened at the cytoplasmic side, when the medium pH was lower than

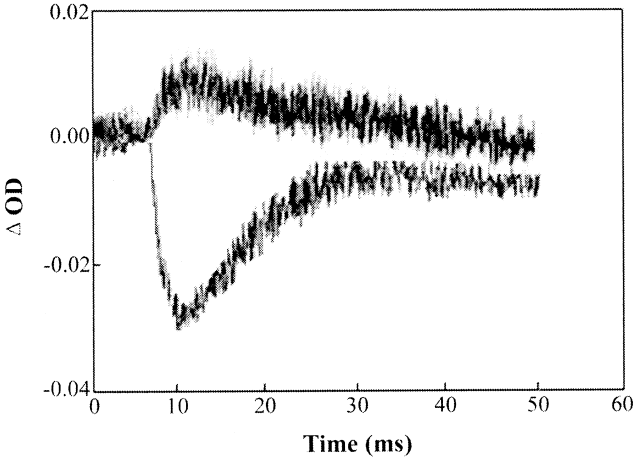

Fig. 6. Photoflash induced absorption change of pH-sensitive dye, pyranine, by proton pumping in purple membrane in water (bottom) and in claret membrane of *xz515* in 0.2 M Na_2SO_4 (top). The downward curve implies acidification of the medium.

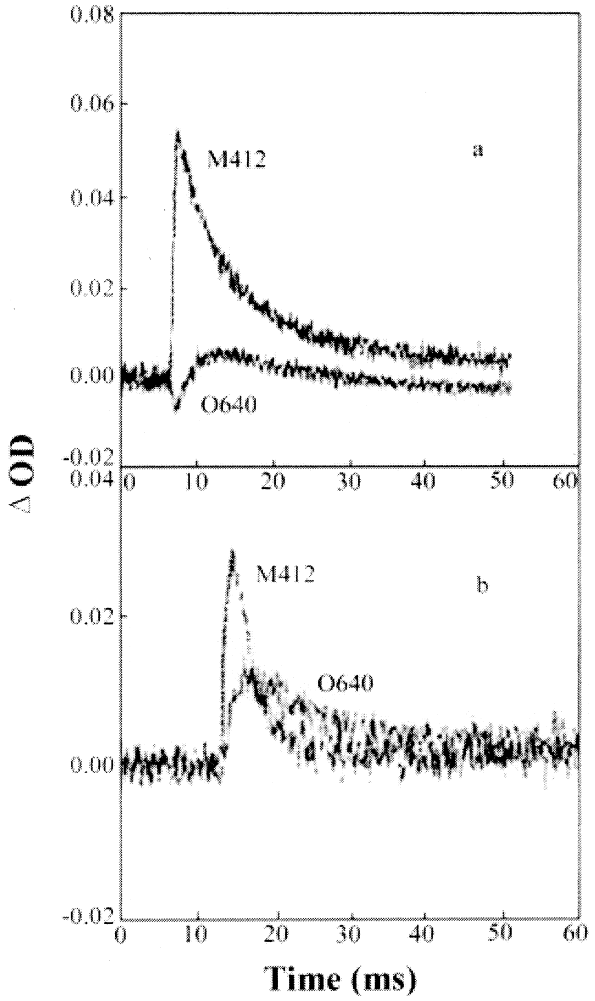

Fig. 5. The photoflash-induced M412 and O640 photoproducts in photocycle of purple membrane (a) and claret membrane of *xz515* (b) in water suspension.

5.8, the pK_a of the proton release group [XH] in photocycle. (It was suggested that the intrinsic pK_a of XH in the ground state is about 8.2 [25].) Lukashev et al. [26] found that the proton pump of archaerhodopsin of strain ar-1 was in reversed order, and mentioned that point mutation at Lys-129 may be involved in this phenomenon, for Lys-129 is replaced with His in this protein. Govindjee et al. [27] recently reported that directed point mutation of Lys-129 in br reversed the order of proton release and uptake. They mentioned that Lys-129 in br was involved in adjustment of the pK_a of the proton release group. The recent reports [28,29] have shown that in the proton pump of br the Glu-204 and water molecule, hydrogen-bonded with it, should be in the proton release pathway. Affecting the pK_a of Glu-204 should have a strong effect on the rate of proton release. We have identified the archaerhodopsin gene encoding the helix C to helix G of the retinal protein in xz515 [11] and found Lys-129 not mutated in this protein. Therefore, we can not attribute the reverse order of proton release and uptake in the strain xz515 to the mutation of Lys-129. We expect that the pK_a of Glu-204 in xz515 has been modified through some other way. One point mutation we found [11] in xz515, Ile203Val (Ile was replaced with Val), might be involved in the regulation of the pK_a of the proton release group. Because the Ile-203 is the closest neighbor to Glu-204 in the primary structure, its point mutation should have the effect to influence the three dimensional structure, in which the Glu-204 joins. In addition, the residue Ile is more hydrophobic than residue Val. So the mutation Ile203Val should have the effect to influence the hydrophobicity around Glu-204. These might be the reasons that the point mutation Ile203Val changes the interrelation between Glu-204 and its bound water molecule and raises the pK_a of Glu-204 in photocycle and in turn, retards proton release rate.

Acknowledgements

We thank to Prof. Zhang, Z.H. and Prof. Huang, W.D. for critical comments and warm discussion. This work was supported by the National Natural Science Foundation of China (39730150).

Appendix

In reviewing of this article, one reviewer offered another explanation for the observed reversed order of proton release and uptake in the proton pump of *xz515*. We highly appreciate this point of view and adapted it here as an appendix to this paper. The author strengthened the role of Ser-193 in salinarum br in the proton release function. The Ser-193 is replaced with Thr in archaerhodopsin ar-1, ar-2 and xz515. Since Ser-193 is hydrogen-bonded to both Glu-194 and Glu-204 [29], residues clearly involved in proton release, its replacement might influence proton release. However, this is obviously not the case in ar-1, which also lacks proton release. The blocking of proton release pathway in ar-1 might not caused by the mutation Ser-193, but there the culprit might be Lys-129.

References

[1] D. Oesterhelt, W. Stoeckenius, Rhodopsin-like protein from the purple membrane of *halobacterium halobium*, Nature 233 (1971) 149–152.
[2] J. Lanyi, Bacteriorhodopsin as a model for proton pump, Nature 375 (1995) 461–463.
[3] A. Matsuno-Yagi, Y. Mukohata, Two possible roles of bacteriorhodopsin; a comparative study of strains of *H. Halobium* differing in pigmentation, Biochem. Biophys. Res. Commun. 78 (1977) 237–243.
[4] R. Bogomolni, J. Spudich, Identification of a third rhodopsin-like pigment in phototactic *Halobacterium halobium*, Proc. Natl. Acad. Sci. USA 79 (1982) 6250–6254.
[5] T. Takahashi, H. Tomioka, N. Kamo, Y. Kobatake, A photosystem other than PS370 also mediates the negative phototaxis of *Halobacterium halobium*, FEMS Microbiol. Lett. 28 (1985) 161–164.
[6] Y. Mukohata, K. Ihara, K. Uegaki, Y. Miyasita, Y. Sugiyama, Australian halobacteria and their retinal protein ion pumps, Photochem. Photobiol. 54 (1991) 1039–1045.
[7] J. Otomo, H. Tomioka, H. Sasabe, Bacterial rhodopsins of newly isolated halobacteria, J. Gen. Microbiol. 138 (1992) 1027–1037.
[8] J. Otomo, Y. Urabe, H. Tomioka, H. Sasabe, The primary structure of helices A to G of three new bacteriorhodopsin-like retinal proteins, J. Gen. Microbiol. 138 (1992) 2389–2396.
[9] Y. Mukahata, Y. Sugiyama, K. Ihara, M. Yoshida, An Australian *halobacterium* contains a novel proton pump retinal protein: Archaerhodopsin, Biochem. Biophys. Res. Commun. 151 (1988) 1339–1345.

[10] Y. Mukohata, Comparative studies on ion pumps of the bacterial rhodopsin family, Biophys. Chem. 50 (1994) 191–201.
[11] H. Wang, S. Zhan, Q. Li et al., The primary structure of helix A to helix G of a new retinal protein of H.sp.xz515, Chin. Sci. Bull. 45 (2000).
[12] D. Oesterhelt, W. Stoeckenius, Isolation of the cell membrane of *H. halobium* and its fractionation into red and purple membrane, Metods Enzymol. 31 (1974) 667–678.
[13] R. Macdonald, J. Lanyi, Light-induced leucine transport in *H. halobium* envelope vesicles: a chemiosmotic system, Biochemistry 14 (1975) 2882–2889.
[14] N. Wang, Q. Li, The microcomputer-controlled kinetic spectrophotometer in millisecond time scale and its application to the observation of the bacteriorhodopsin photocycle, Acta Biophys. Sin. 2 (1986) 32–36.
[15] R. Govindjee, T. Ebrey, A. Crofts, The Quantum efficiency of proton pumping by the purple membrane of *H. Halobium*, Biophys. J. 30 (1980) 231–242.
[16] T. Ogurusu, A. Maeda, T. Yoshizawa, Absorption spectral properties of purified halorhodopsin, J. Biochem. 95 (1984) 1073–1082.
[17] Qingguo Li, Hui Wang, Dajie Song, The isolation and purification of archaerhodopsin from H.Sp.xz515, Acta Biochim. Biophys. Sin. 29 (1997) 517–520.
[18] D. Oesterhelt, W. Stoeckenius, Functions of a new photoreceptor membrane, Proc. Natl. Acad. Sci. USA 70 (1973) 2853–2857.
[19] R. Bogomolni, R. Baker, R. Lozier, W. Stoeckenius, Light-driven proton translocation in *Halobacterium halobium*, Biochim. Biophys. Acta 440 (1976) 68–88.
[20] Qingguo Li, R. Govindjee, T. Ebrey, A proton release site on the C-terminal site of Bacteriorhodopsin, Photochem. Photobiol. 44 (1986) 515–518.
[21] J. Lanyi, Photoreaction and chloride translocation in halorhodopsin, in: Yu. Ovchinnikov (Ed.), Retinal Proteins, 1987, pp. 356–361.
[22] Wei Zhao, Minqiang Chai, Fenghua Hai, Qingguo Li, Photochemical and proton pumping properties of archaerhodopsin in H.xz515, Acta Biophys. Sin. 14 (1998) 543–547.
[23] W. Eisfeld, C. Pusch, R. Diller, R. Lohrmann, M. Stockburger, Resonance Raman and optical transient studies on the light-induced proton pump of bacteriorhodopsin reveal parallel photocycles, Biochemistry 32 (1993) 7196–7215.
[24] L. Zimanyi, G. Varo, M. Chang, B. Ni, R. Needleman, J. Lanyi, Pathways of proton release in the bacteriorhodopsin photocycle, Biochemistry 31 (1992) 8535–8543.
[25] M. Mono, S. Misra, T. Ebrey, PH dependence of light-induced proton release by bacteriorhodopsin, FEBS Lett. 331 (1993) 31–34.
[26] E. Lukashev, R. Govindjee, M. Kono, T. Ebrey, Y. Sugiyama, Y. Mukohata, pH dependence of the absorption spectra and photochemical transformation of the archaerhodopsins, Photochem. Photobiol. 60 (1994) 69–75.
[27] R. Govindjee, E. Imasheva, S. Misra, S. Balashov, T. Ebrey, N. Chen, D. Menick, R. Crouch, Mutation of a surface residue, Lys-129, reverses the order of proton release and uptake in bacteriorhodopsin; Guanidine hydrochloride restores it, Biophys. J. 72 (1997) 886–898.
[28] E. Pebay-Pyroula, E. Rummel, J. Rosenbusch et al., X-ray structure of bacteriorhodopsin at 2.5 Å from microcrystal grown in lipidiccubic phases, Science 277 (1997) 1676–1680.
[29] H. Luecke, H. Richter, J. Lanyi, Proton transfer pathways in bacteriorhodopsin at 2.3 angstrom resolution, Science 280 (1998) 1934–1937.