Biophysical Journal Volume 67 September 1994 1187-1191

Met-145 Is a Key Residue in the Dark Adaptation of Bacteriorhodopsin Homologs

Kunio Ihara, Tomohiro Amemiya, Yukiya Miyashita, and Yasuo Mukohata
Department of Biology, School of Science, Nagoya University, Nagoya 464-01, Japan

ABSTRACT Composition of retinal isomers in three proton pumps (bacteriorhodopsin, archaerhodopsin-1, and archaerhodopsin-2) was determined by high performance liquid chromatography in their light-adapted and dark-adapted states. In the light-adapted state, more than 95% of the retinal in all three proton pumps were in the all-*trans* configuration. In the dark-adapted state, there were only two retinal isomers, all-*trans* and 13-*cis*, in the ratio of all-*trans*:13-*cis* = 1:2 for bacteriorhodopsin, 1:1 for archaerhodopsin-1, and 3:1 for archaerhodopsin-2. The difference in the final isomer ratios in the dark-adapted bacteriorhodopsin and archaerhodopsin-2 was ascribed to the methionine-145[1] in bacteriorhodopsin. This is the only amino acid in the retinal pocket that is substituted by phenylalanine in archaerhodopsin-2. The bacteriorhodopsin point-mutated at this position to phenylalanine dramatically altered the final isomer ratio from 1:2 to 3:1 in the dark-adapted state. This point mutation also caused a 10 nm blue-shift of the absorption spectrum, which is similar to the shift of archaerhodopsin-2 relative to the spectra of bacteriorhodopsin and archaerhodopsin-1.

INTRODUCTION

Archaerhodopsins (aRs) are light-driven proton pumping retinal proteins that are found in extremely halophilic archaea collected in Western Australia (Mukohata et al., 1988). Sequence analyses of aR-1 (Sugiyama et al., 1989) and aR-2 (Uegaki et al., 1991) revealed that: (i) the amino acid sequences of aR-1 and aR-2 were, respectively, 59 and 56% identical with that of bacteriorhodopsin (bR) (Dunn et al., 1981) and 88% identical to each other; (ii) the 123 amino acids conserved among three proton pumps include those residues assigned to the proton channel and the retinal pocket (Henderson et al., 1990), except for the Met-145 in the retinal pocket in bR and aR-1, which is replaced with phenylalanine in aR-2; and (iii) these conserved amino acid residues are largely concentrated in C (91%) and G (85%) helices, or in the region where the helices face one another longitudinally.

Some physicochemical properties of the three proton pumps are different from one another, such as the molecular extinction coefficients, the mode of spectral red shift caused by the removal of bound cations, and photochemical reaction kinetics (Mukohata et al., 1991, 1992).

We studied the dark adaptation process and found that the three proton pumps have different ratios of retinal isomers in the dark-adapted state (Mukohata et al., 1992). Dark adaptation is a thermodynamic isomerization process of retinal in the retinal pocket in conditions of darkness. The amino acid residues in the retinal pocket are considered to be responsible for the rate of the process and for the final ratio of retinal isomers. In the retinal pocket, Met-145 in bR is substituted by Phe in aR-2 (Fig. 1). Therefore, this Met/Phe was assumed to be a main cause for the different isomer ratios between bR and aR-2 in conditions of darkness. To verify this assumption, the bR gene was mutated at Met-145 to Phe and expressed in *Halobacterium salinarium* (formerly *halobium*) to produce bR(M145F). The dark adaptation process of bR(M145F) and the final isomer ratios were compared with the three native pumps. The rate constants of retinal isomerization were also analyzed at various temperatures for all four proton pumps.

MATERIALS AND METHODS

Halobacterium spp. aus-1[2] and aus-2, and *Halobacterium salinarium* R_1M_1 (for bR) and L33 (host) were grown aerobically in a polypeptone medium as described previously (Matsuno-Yagi and Mukohata, 1977).

The purple membranes (for bR and bR(M145F)) were isolated from *H. salinarium* R_1M_1, and the claret membranes were isolated from *H.* sp. aus-1 (for aR-1) and aus-2 (for aR-2). All of the membranes were purified as in Sugiyama et al. (1989). After sucrose density gradient centrifugation, the membranes were washed with a phosphate buffer (10 mM Na-Pi, 50 mM NaCl, 5 mM $MgCl_2$, pH 7.0) and suspended in the same buffer solution.

The isomer compositions of retinal were determined by the procedure reported by Scherrer et al. (1989): Briefly, 100 μl of 10–50 μM retinal protein was mixed for 2 min with 250 μl of ice-cold ethanol and then with 250 μl of ice-cold hexane for another 2 min. After centrifugation in microcentrifuge for 1 min, the hexane phases were analyzed by HPLC, using a C18 column (Ultrasphare-ods, 4.6 × 250 mm, Beckman) with 4% diethyl ether in hexane as a solvent.

The absorption spectra were measured with a Shimadzu UV-300 spectrophotometer. Dark adaptation kinetics were followed by the absorption

Received for publication 3 May 1994 and in final form 20 June 1994.

Address reprint requests to Prof. Yasuo Mukohata, Department of Biology, School of Science, Nagoya University, Nagoya, Japan 464-01. Fax: 81-52-789-2968; E-mail: i45032a@nucc.cc.nagoya-u.ac.jp.

Abbreviations used: aR, archaerhodopsin; bR, bacteriorhodopsin.

In this paper, we called the purple membrane with bR for bR and the claret membrane with aR-1 and aR-2 for aRs for short.

[1] Numbering is according to bR after the probable alignment of the three proton pumps.

0006-3495/94/09/1187/05 $2.00

[2] Recent phylogenetic analysis has revealed that these strains group together with *Halobacterium sodomense*. *Halobacterium saccharovorum* and *Halobacterium lacusprofundi* and constitute one distinct halobacterial genus, *Halorubra* (Mukohata, 1994).

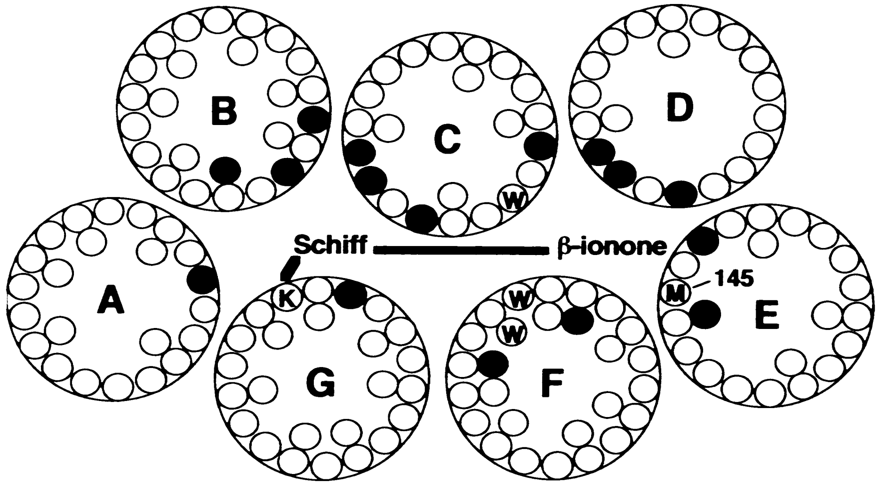

FIGURE 1 The retinal pocket in the helical wheel model of bacteriorhodopsin. The helical wheel (Schiffer and Edmundson, 1967) shows the positions of amino acid residues projected from the cytosol side of the bacteriorhodopsin molecule. The positions of 21 amino acid residues assigned for those constructing the retinal pocket (Henderson et al., 1990) are shown by the single letter notation (W86, M145, W182, W189, and K216) or are shadowed.

change at 585 nm for bR and aR-1, and at 575 nm for bR (M145F) and aR-2. The protein concentration was determined by the Lowry method using BSA as a standard for aRs.

Site-directed mutagenesis was carried out according to the procedure of Hutchinson et al. (1978), using a kit (ALTERED SITES *IN VITRO* MUTAGENESIS SYSTEM) from Promega Corp. (Milwaukee, WI). The mutagenic oligonucleotide for Met-145→Phe (ATG→TTC) was 5′-CACCG-CAGCGTTCCTGTACATCCT-3′. A 1.2-kilobase-long *EcoR*I- *Hin*dIII fragment containing the mutated bop gene was cloned into pWL102ΔH. Transformation of halobacteria was performed according to the method described by Cline et al. (1989).

RESULTS AND DISCUSSION

Final isomer ratios in the dark-adapted state

The difference spectrum between the dark- and the light-adapted state suggests that both the dark-adapted aR-1 and aR-2 contain higher amounts of all-*trans* retinal than does bR (Fig. 2). Retinal isomer compositions analyzed by HPLC show that in the light-adapted state, more than 95% of the retinal in aR-1 and aR-2 are in the all-*trans* configuration, as is also the case with bR (Table 1). In the dark-adapted state, the retinal was found to be a mixture of two isomers, all-*trans* and 13-*cis* just as for bR, but their compositions are considerably different from one another (Table 1).

Our results confirm the results of Scherrer et al. (1989), who reported the 1:2 ratio of all-*trans*:13-*cis* isomer for the dark-adapted bR. Furthermore, our results show that aR-1 and aR-2, naturally occurring proton pumps, have different isomer compositions in the dark-adapted state. A different problem that remains to be solved is why the species and ratios of retinal isomers in individual dark-adapted rhodopsin differ greatly from those of retinal in solution.

Correlation between the retinal isomer ratios and difference spectra in the dark-adaptation process

The isomerization of retinal in bR in conditions of darkness reflects on the absorbance decrease and the blue-shift of the absorption maxima, because the visible absorption maximum and its extinction coefficient of bR with 100% 13-*cis*

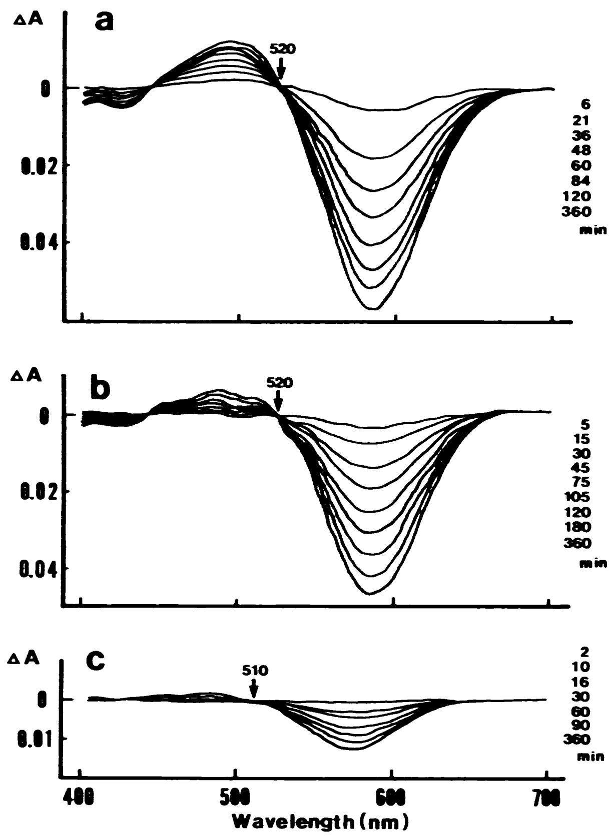

FIGURE 2 Difference spectra between light-adapted and dark-adapting states of bR, aR-1, and aR-2. Spectra were recorded at given times after the light-adapted bR (*a*), aR-1 (*b*), and aR-2 (*c*) were each placed in the conditions of darkness at 30°C.

retinal are, respectively, 555 nm and 52,000 M^{-1} cm^{-1} (Mukohata et al., 1992); whereas for bR with 100% all-*trans* retinal, the visible absorption maximum and its extinction coefficient are 568 nm and 63,000 M^{-1} cm^{-1}, respectively (Rehorek and Heyn, 1979). In the case of aR-1 and aR-2, the light-dark difference spectra include some ripples caused by coexisting bacterioruberin that is slightly bleached in the light. Even so, the isosbestic points at 520 ± 5 and

TABLE 1 Retinal isomer ratios of four proton pumps in light-adapted and dark-adapted states

	LA 13-*cis* : all-*trans*	DA 13-*cis* : all-*trans*
bR	5:95	67:33
aR-1	3:97	52:48
aR-2	3:97	23:77
bR(M145F)	3:97	25:75

510 ± 5 nm for aR-1 and aR-2, respectively (Fig. 2), suggest that the light/dark adaptation processes of aR-1 and aR-2 are a two-state transition, a finding that is further supported by the above HPLC results. In practice, the absorbance difference at 585 nm in aR-1 and at 575 nm in aR-2 fit well with the determined ratios of retinal isomer (data not shown). Therefore, the isomer compositions can be estimated from the difference spectra normalized to the amount of retinal protein. This procedure was used for rapid isomerization process at high temperatures, where direct determination of retinal was difficult.

Dark-isomerization process

The different isomer ratios of retinal in aR-1, aR-2, and bR in the dark-adapted state can be ascribed to the differences in the isomerization rate constants, k_1 (from all-*trans* to 13-*cis* isomer) and k_{-1} (from 13-*cis* to all-*trans* isomer). To obtain these two rate constants by ordinary analysis, retinal was extracted and analyzed in the course of the dark adaptation process (Fig. 3). Each of the dark adaptation processes of three pumps at 30°C (with additional data from difference spectra) was well described by a single exponential equation, and the values for k_1 and k_{-1} were readily obtained. The higher content of 13-*cis* retinal in the dark-adapted bR was caused by a larger rate constant from all-*trans* to 13-*cis* retinal (Table 2). The higher content of all-*trans* retinal in the dark-adapted aR-2 was caused by a larger rate constants from 13-*cis* to all-*trans* retinal. The k_1 and k_{-1} values of aR-1 were almost the same and, therefore, yielded a 1:1 ratio of two isomers.

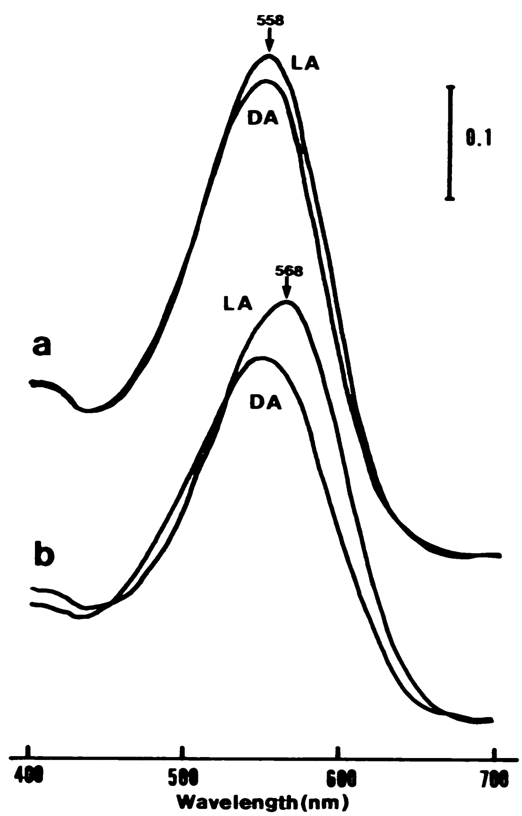

FIGURE 3 Light-adapted and dark-adapted absorption spectra of bR-(M145F) (*a*) and bR (*b*). These spectra were recorded after 1 min in the light (LA) and after 96 h in the conditions of darkness (DA).

TABLE 2 Rate constants of retinal isomerization from all-*trans* to 13-*cis* (k_1) and vice versa (k_{-1}) in four proton pumps at 30°C

	k_1 (10^{-3} min^{-1})	k_{-1} (10^{-3} min^{-1})
bR	12.1	6.9
aR-1	4.6	4.2
aR-2	5.3	15.4
bR(M145F)	2.3	5.8

A key amino acid residue for the blue-shift of absorption spectrum and the final retinal isomer ratio

The differences of k_1 and k_{-1} values among three retinal proteins could be ascribed to some amino acid residues that interact differently with retinal. Among the 21 amino acid residues assigned for the retinal pocket (Henderson et al., 1990), only one, Met-145 in bR, is substituted by Phe in aR-2 (Fig. 1). This substitution, although conservative, alters the bulkiness of the side chain and, thus, might affect isomerization of the retinal molecule. This assumption was tested by site-directed mutagenesis.

The absorption spectrum of bR(M145F) was blue-shifted by 10 nm with respect to that of bR (Fig. 2), as was the difference spectrum between the light- and the dark-adapted bR(M145F). The difference spectrum of aR-2 was also blue-shifted by about 10 nm with respect to the spectra of bR and aR-1 (Fig. 2), which suggests that the absorption spectrum was also blue-shifted (only the difference spectra could be compared because of coexisting bacterioruberin). The blue-shift of the absorption band of aR-2 as well as of bR(M145F), therefore, might be mostly because of the Phe-145 in the retinal pocket, where the amino acid residues are identical.

As expected from the small spectral shift in the dark-adapted bR(M145F) (Fig. 4), the retinal isomer ratio of 3:1 (13-*cis*:all-*trans*) was confirmed by direct HPLC analysis of retinal isomers (Table 1). The substitution of Met-145 for Phe in bR, as predicted, actually altered the isomerization equilibrium of retinal from that of bR to aR-2. The space of the retinal pocket should be wide enough to exchange the

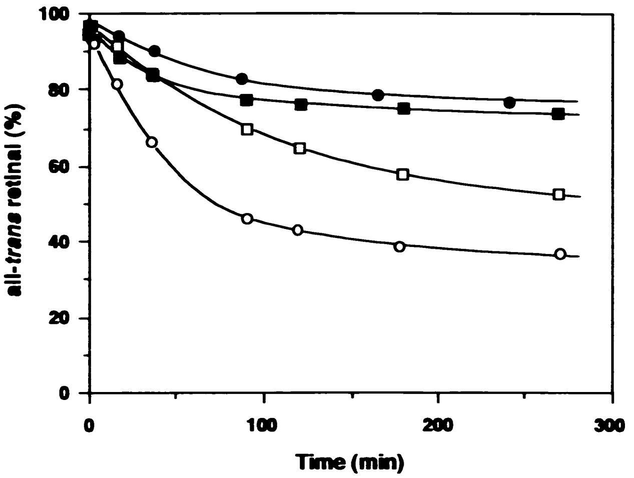

FIGURE 4 The contents of all-*trans* retinal in bR, bR(M145F), aR-1, and aR-2 in the course of dark adaptation. After bR (○), bR(M145F) (●), aR-1 (□), and aR-2 (■) were illuminated for 1 min (before time 0) (light adaptation), the retinal isomers (a mixture of all-*trans* and 13-*cis* retinal) were analyzed by HPLC. The solid curves were calculated by first-order kinetics curve fitting.

chromophore with the various kinds of retinal analogs (Balogh-Nair and Nakanishi, 1982). However, in that case there would be a narrow neck for retinal at the position near the 9th carbon of retinal, which is surrounded by three Trp residues (Trp-137, Trp-138, and Trp-182 in the retinal pocket; Fig. 1). If a retinal molecule moves with this position as its fulcrum, the β-ionone ring would move when the Schiff base at the other end of the molecule moves, when the retinal isomerizes. This movement of the β-ionone ring would be regulated or restricted by its surrounding amino acid residues. The bulkiness of the 145th residue, which is close to the β-ionone ring would thus have effects on the dark adaptation process. Such effects are also observed in the photocycles of both aR-2 and bR(M145F) (results to be published), although the detailed 13-*cis* isomerization processes are different between photoreaction and dark adaptation (Smith et al., 1987; Fodor et al., 1988). In this connection, investigation with bR(M145W) is now under way by the authors. Here we analyzed the effect of natural substitution (M145F).

Site-directed mutagenesis in the retinal pocket should disturb the structural delicacy of the pocket, and more or less affect the isomer ratio of the dark-adapted bR, and spectroscopic properties, such as L93A (Subramaniam et al., 1991), Y185F (Sonar et al., 1993), and D85N (Turner et al., 1993). The difference in the rates and the final isomer ratios between bR and aR-1, both of which hold the identical composition of the retinal pocket, remain to be explained. The explanation might lie (at least partially) in the indirect interaction of amino acid residues near the retinal pocket as reported with R82A (Balashov et al., 1993). Such indirect interaction might also explain the difference in the isomerization rates between bR(M145F) and aR-2.

Temperature dependency

The dark adaptation process of all three retinal proteins and bR(M145F) between 20 and 50°C follow first-order kinetics, and the Arrhenius plots show linear relationships (Fig. 5). The activation energies from all-*trans* retinal/opsin complex to an active intermediate complex were determined to be 23.9, 22.7, 22.6, and 18.1 kcal/mol, and those from 13-*cis* complex to the active intermediate complex to be 25.6, 22.1, 18.7, and 17.8 kcal/mol for bR, aR-1, aR-2, and bR(M145F), respectively.

In bR and bR(M145F), the all-*trans* retinal contents were almost constant at temperatures up to 50°C and increased rapidly above that. By contrast, in aR-2 the content of 13-*cis*

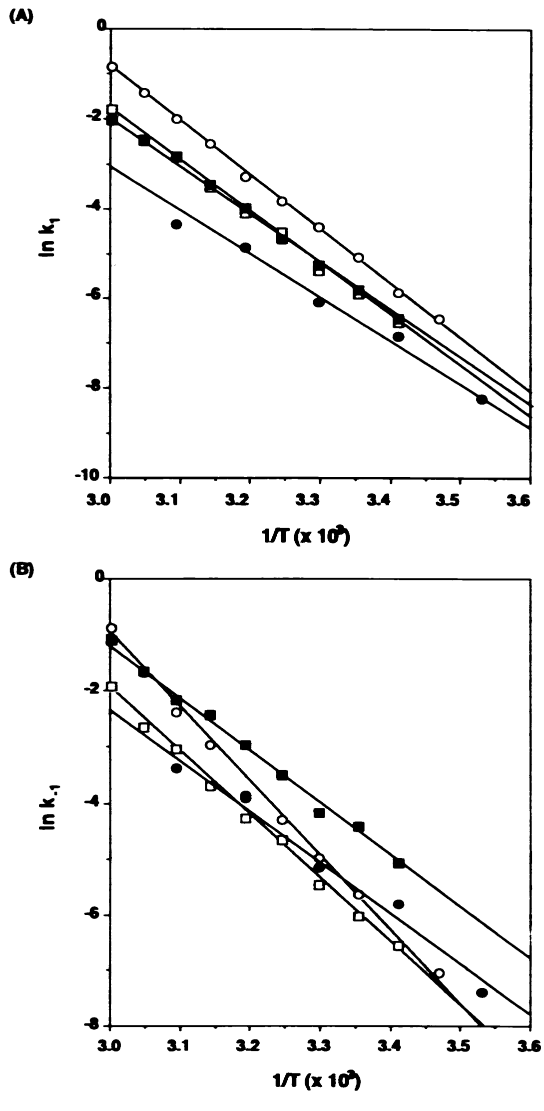

FIGURE 5 Temperature dependence of the rate constants from all-*trans* to 13-*cis* retinal (*A*) and vice versa (*B*). The rate constants of bR (○), bR(M145F) (●), aR-1 (□), and aR-2 (■) were calculated by first-order kinetics. The straight lines were drawn by the least-square method. At temperatures above 35°C, the time course of the absorbance changes at 585 nm for bR and aR-1, and at 575 nm for bR(M145F) and aR-2, were recorded, and the absorbance decrease during the initial loss time after turning off the light was estimated by extrapolation to $t = 0$.

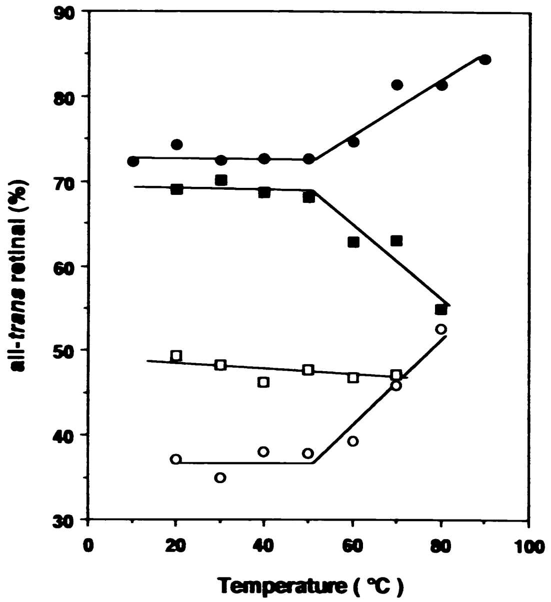

FIGURE 6 Temperature dependence of all-*trans* retinal content in the dark-adapted proton pumps. bR (○), bR(M145F) (●), aR-1 (□), and aR-2 (■).

retinal increased with temperature, whereas in aR-1 the content was almost constant in the temperature range observed (Fig. 6). The difference of temperature dependency between bR and aRs has also been observed in the thermal stability of intermediates in the photocycle (personal communication from K. Gerwert, Max-Planck-Institut). This may be caused by bacterioruberin in tightly interaction with aR-1 and aR-2 in the claret membranes (Mukohata et al., 1991). The effects of surrounding lipids remain to be studied.

We wish to thank Dr. Richard Needleman of Wayne State University for providing us with his *Halobacterium-E. coli* shuttle vector pWL102ΔH and a plasmid pALTER-1 containing the bop gene. We also would like to thank Dr. Y. Sugiyama of Nagoya University for his helpful comments and technical support during the course of this research.

This work was supported in part by a grant-in-Aid for Scientific Research in the Priority Areas (#05266103) to Y. Mukohata from the Ministry of Education, Science and Culture of Japan.

REFERENCES

Balashov, S. P., R. Govindjee, M. Kono, E. Imasheva, E. Lukashev, T. G. Ebrey, R. K. Crouch, D. R. Menick, and Y. Feng. 1993. Effect of arginine-82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release, and the photochemical cycle. *Biochemistry.* 32:10331–10343.

Balogh-Nair, V., and K. Nakanishi. 1982. Synthetic analogs of retinal, bacteriorhodopsin and bovine rhodopsin. *Methods Enzymol.* 88:496–506.

Cline, S. W., W. L. Lam., R. L. Charlebois, L. C. Schlkwyk, and W. F. Doolittle. 1989. Transformation methods for halophilic archaebacteria . *Can. J. Microbiol.* 35:148–152.

Dunn, R., J. McCoy, M. Simsek, A. Majumdar, S. H. Chang, U. L. Rajbhandary, and H. G. Khorana. 1981. The bacteriorhodopsin gene. *Proc. Natl. Acad. Sci. USA.* 78:6744–6748.

Fodor, S. P. A., J. B. Ames, R. Gebhard, D. B. E. M. M. Van, W. Stoeckenius, J. Lugtenburg, and R. A. Mathies. 1988. Chromophore structure in bacteriorhodopsin's N intermediate: implications for the proton-pumping mechanism. *Biochemistry.* 27:7097–7101.

Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.* 213:899–929.

Matsuno-Yagi, A., and Y. Mukohata. 1977. Two possible roles of bacteriorhodopsin: a comparative study of strains of *Halobacterium halobium* differing in pigmentation. *Biochem. Biophys. Res. Commun.* 78:237–243.

Mukohata, Y. 1994. Comparative studies of ion pumps of the bacterial rhodopsin family. *Biophys. Chem.* 50:191–201.

Mukohata, Y., K. Ihara, Y. Miyashita, T. Amemiya, T. Taguchi, M. Tateno, and Y. Sugiyama. 1992. Comparative studies on bacteriorhodopsin-type pumps: basic physicochemical data for proton pumps. *In* Structure and Functions of Retinal Proteins. Vol. 221. Jean-Louis Rigaud, editor. John Libbery Eurotext. 101–104.

Mukohata, Y., K. Ihara, K. Uegaki, Y. Miyashita, and Y. Sugiyama. 1991. Australian *Halobacteria* and their retinal-protein ion pumps *Photochem. Photobiol.* 54:1039–1045.

Mukohata, Y., Y. Sugiyama, K. Ihara, and M. Yoshida. 1988. An Australian halobacterium contains a novel proton pump retinal protein archaerhodopsin. *Biochem. Biophys. Res. Commun.* 151:1339–1345.

Reborek, M., and M. P. Heyn. 1979. Binding of all-*trans* retinal to the purple membrane: evidence for cooperativity and determination of the extinction coefficient. *Biochemistry.* 18:4977–4983.

Schiffer, M., and A. B. Edmundson. 1967. Use of helical wheel to represent the structures of proteins and to identify segments with helical potential. *Biophys. J.* 7:121–135.

Scherrer, P., M. K. Matthew, W. Sperling, and W. Stoeckenius. 1989. Retinal isomer ratio in dark-adapted purple membrane and bacteriorhodopsin monomers. *Biochemistry.* 28:829–834.

Smith, S. O., G. H. J. M. De, R. Gebhard, J. M. L. Courtin, J. Lugtenburg, J. Herzfeld, and R. G. Griffin. 1989. Structure and protein environment of the retinal chromophore in light and dark-adapted bacteriorhodopsin studied by solid-state NMR. *Biochemistry.* 28:8897–8904.

Sonar, S., M. P. Krebs, H. G. Khorana, and K. J. Rothschild. 1993. Static and time-resolved absorption spectroscopy of the bacteriorhodopsin mutant Tyr-185 → Phe: evidence for equilibrium between bR570 and an O-like species. *Biochemistry.* 32:2263–2271.

Subramaniam, S., D. A. Greenhalgh, P. Rath, K. J. Rothschild, and H. G. Khorana. 1991. Replacement of leucine-93 by alanine or threonine slows down the dacay of the N and O intermediates in the photocycle of bacteriorhodopsin: implication for proton uptake and 13-*cis*-retinal → all-*trans*-retinal reisomerization. *Proc. Natl. Acad. Sci. USA.* 88:6873–6877.

Sugiyama, Y., M. Maeda, M. Futai, and Y. Mukohata. 1989. Isolation of a gene that encodes a new retinal protein archaerhodopsin from *Halobacterium* sp. *aus-1. J. Biol. Chem.* 264:20859–20862.

Turner, G. J., L. J. W. Miercke, T. E. Thorgeirsson, D. S. Kliger, M. C. Betlach, and R. M. Stroud. 1993. Bacteriorhodopsin D85N: three spectroscopic species in equilibrium. *Biochemistry.* 32:1332–1337.

Uegaki, K., Y. Sugiyama, and Y. Mukohata. 1991. Archaerhodopsin-2, from *Halobacterium* sp. aus-2 further reveals essential residues for light-driven proton pumps. *Arch. Biochem. Biophys.* 286:107–110.