Effects of Mutations of Lys41 and Asp102 of Bacteriorhodopsin

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Bacteriorhodopsin (BR) is a retinal protein that functions as a light-driven proton pump. In this study, six novel mutants including K41E and D102K, were obtained to verify or rule out the possibility that residues Lys41 and Asp102 are determinants of the time order of proton release and uptake, because we found that the order was reversed in another retinal protein archaerhodopsin 4 (AR4), which had different 41th and 102th residues. Our results rule out that possibility and confirm that the pKₐ of the proton release complex (PRC) determines the time order. Nevertheless, mutations, especially D102K, were found to affect the kinetics of proton uptake substantially and the pKₐ of Asp96. Compared to the wild-type BR (BR-WT), the decay of the M intermediate and proton uptake in the photocycle was slowed about 3-fold in D102K. Hence those residues might be involved in proton uptake and delivery to the internal proton donor.

Key words: bacteriorhodopsin; point mutant; proton pump; M decay

BR is a retinal-containing protein acting as a light-driven proton pump. It was found in the purple membrane of the extremely halophilic archaeabacterium Halobacterium salinarum.1) BR has been investigated extensively over the past four decades.2–18) Upon photon absorption, the retinal is isomerized from the all-cis to the 13-cis configuration. This initiates a photocycle in which a series of photointermediates are formed. They are distinguished by their absorption maxima:

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BR_{570} \rightarrow K_{610} \rightarrow L_{550} \rightarrow M_{412}^I \rightarrow M_{412}^O \rightarrow N_{560} \rightarrow O_{640} \rightarrow BR_{570}
\]

The photocycle is coupled to the directional transfer of a proton across the membrane. Illumination induces proton transfer from the nitrogen of the Schiff base linking retinal and Lys-216 to Asp-85 during the L-to-M transition, which leads, on a submillisecond timescale, to prompt proton release from the proton release complex (PRC or XH) to the extracellular surface at neutral pH.19–21) After proton release, another proton is taken up from the cytoplasmic side in the N

Site-specific mutagenesis is used extensively to illustrate the relationship of function and structure for various proteins.22–24) It has been found that carboxylic residues in the proton-conducting cytoplasmic and extracellular half-channels, D96, D85, E194, and E204 are involved in the photocycle and proton transfer. Negatively charged D85 is part of the counterion to the protonated Schiff base and proton acceptor. Buried in the cytoplasmic domain, neutral D96 serves as an internal proton donor for the Schiff base in the M intermediate. Two glutamates at the extracellular surface, E194 and E204, and some interacting water molecules are thought to comprise PRC.25–27) While much progress has been made in finding key residues in BR, only about 58 residues have been examined to date while the functional significance of a majority of the residues in BR remains undetermined. Especially the key residues in the cytoplasmic side are less investigated than the extracellular side, where proton release takes place and the PRC is located. The mutants located on the cytoplasmic side including D36N, D38N, D102N, and D104N, were expressed in E. coli. They presented a slightly decreased time of M decay,28) but proton pumping rates similar or higher than the BR-WT.29) Among the other mutants expressed in Halobacterium salinarum L33, with D36, D38, D102, and D104 replaced by cysteine or arginine, D36 was found to act as a proton attractor at the orifice of the proton-conducting channel.30) D38 was found to influence the late steps in the functional photocycle, such as the occurrence of intermediates N and O.31) and D104C lowered the transient proton binding capacity of the cluster that consists of D104, E161, and E234.32) Hints from comparison of the BR sequence to those of other archaeal and bacterial retinal proteins is helpful in searching for residues involved in proton pumping.7) In the present study we examined effects of mutating Lys-41 (K41) and Asp-102 (D102), two charged residues on the cytoplasmic side of the light-driven proton pump. These two amino acid residues were selected for a comparison of BR and AR4.

AR4 was found in a strain of the halobacterium Halorubrum species xz515 isolated from a salt lake in

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Abbreviations: BR, bacteriorhodopsin; AR4, archaerhodopsin 4; PRC, proton release complex; BR-WT, wild-type BR expressed in L33
Tibet in 2000. Its amino acid sequence has been determined. In BR, AR4 functions as a light-driven proton pump. In contrast to BR, it exhibits a reversed order of proton release and uptake at neutral pH, even though both BR and AR4 have key residues E194 and E204, acting as PRC. It also exhibits several other features (faster decay of the M intermediate and higher pKa of the counterion). We have found that the inversion of the order of proton release and uptake is caused by the elevated pKa of the PRC in the M intermediate (from 5.6 in BR to 8.3 in AR4). The higher pKa of the PRC in the M intermediate of AR4 might be caused by replacement of nearby or distant residues. Several polar or charged residues located in the extracellular side are conserved in BR and AR4, including R7, E9, and K129. A few polar or charged residues at the cytoplasmic side are different in these pigments. Especially, K41 in helix B and D102 in loop-linking helixes C and D (the CD loop) are interesting. Their locations are schematically presented in Fig. 1. In AR4, these two residues are replaced by E and K respectively.

In this study, we examined the consequences of mutations of the two residues (K41 and D102) on the cytoplasmic surface of BR. A series of point mutants of BR, including K41D, D102K, D102E, K41E/D102K, and K41D/D102E, were constructed by the same method. All of the primers used to construct the mutants are shown as the primers 3 to 10 in Supplemental Table S1 (see Biosci. Biotechnol. Biochem. Web site) in supporting information.

The pXLoN-r plasmid does not contain the gene fragment of bacterio-opsin in contrast with the pXLoN-r-bop plasmid. This plasmid and the PCR amplified products of the bop-(K41E) gene were digested with BamHI and HindIII restriction enzymes and ligated together. The transformants grew on plates containing 1 µg/mL of novobiocin for 2 weeks, and then were inoculated into a liquid culture medium for large-scale culture in the presence of 1 µg/mL of novobiocin. The resulting purple membranes were isolated by the same procedure as previously.

Mass spectra. Mass spectra of BR-WT and the series of mutants were measured in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Applied Biosystems Voyager System 4310). The ice-dried protein samples were dissolved with pure acetic acid at a concentration of 10 mg/mL. Then the matrix, 2,5-dihydroxybenzoic acid (DHB), was dissolved with methanol and chloroform (v/v 2:1) at a concentration of 10 mg/mL. Finally, 2 µL of sample solution and 5 µL of matrix solution were mixed thoroughly and dropped on the sample plate for MALDI-TOF measurement.

Circular dichroism spectra. Circular dichroism (CD) spectra in the ultraviolet (UV) region were measured with a spectropolarimeter (J-715, Jasco, Tokyo) at 20 °C. The samples were dissolved in Milli-Q water at a concentration of 20 µg/mL. The path length of the measured cells was 1 cm. The molar ellipticity (deg cm² dmol⁻¹) was determined on a mean-residue basis.

Absorption spectra. The absorption spectra of the samples were measured on a Shimadzu UV-3150 spectrophotometer (Shimadzu, Ohtsu, Japan). For light adaptation, a 200 W tungsten lamp with a 500 nm cut-off filter was employed. Dark adaptation of samples was achieved when necessary at 25 °C for 24 h. The rate constant of dark adaptation was determined by measuring the absorption changes in the light-adapted samples at 570 nm at intervals of 1 min in the dark environment. The curve was fitted with one-component exponential decay. Samples were prepared in 0.2 mol/L phosphate buffer saline (PBS) solution at pH 7.0.

Flash-induced photocycle intermediates and proton pump behaviors. Traces of flash-induced proton release and uptake were detected in a spectrophotometer, which we manufactured in our lab. A photoflash for light-adapted samples was used for excitation. The M intermediate was monitored at 412 nm. In order to monitor flash-induced proton release and uptake by BR, absorption changes in the

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**Materials and Methods**

BR-R/M1 and AR4-xz515. Natural Halobacterium salinarum strain R/M1 and xz515 and gene engineering strain L33 standard procedures following a previous report. Retinal proteins BR-R/M1 and AR4-xz515 and the gene engineering proteins expressed in L33 WT and other mutants were isolated. BR-R/M1 was the only protein in the purple membrane isolated from strain R/M1, and AR4-xz515 was the only protein in the claret membrane isolated from strain xz515. The claret membrane contains some carotenoid bacterioruberin, which was hard to separate from AR4 by our purification method. This difficulty has also been reported for other archaerhodopsins. Point mutation via gene engineering. The reagents needed for gene engineering, including the restricted nuclease, T4 ligase and Taq DNA polymerase were purchased from BioRad (Hercules, CA). The oligonucleotide primers for the mutants were synthesized and purified with oligonucleotide purification cartridge (OPC) from Applied Biosystems (USA).

Gene engineering BR-WT and mutant proteins were expressed in H. salinarium strain L33. This stain is negative for the synthesis of bacterio-opsin (bop), but positive for the synthesis of retinal. Expression of the BR mutant proteins in strain L33 was done by the method of Ni et al. The expressing plasmid for the K41E mutant, Lys41 replaced by Glu, was derivative from plasmid pXLNov-r-bop, a gift from Dr. Richard Needleman. Primer 1: 5'-GGATCCCGTGAAGATGGGCC-3' (BanHI site, underlined); primer 2: 5'-AAGCTTCTGATACGTCGTCAGG-3' (HindIII site, underlined); Primer 3: 5'-GCAAGGAGTTTCTAGCCATCGACGCG-3', and Primer 4: 5'-TGATAACCTTTTGCATCGGGCGAG-3' were used in order to produce the bop gene sequence with K41E. A new 1.2-kb DNA sequence was obtained and, was named bop-(K41E), with BanHI and HindIII sites. After the sequence of the bop-(K41E) fragment was checked, fractions produced by PCR amplification were ligated to the pSK plasmid and analyzed for identification.

Other point mutants of BR, including K41D, D102K, D102E, K41E/D102K, and K41D/D102E, were constructed by the same method. All of the primers used to construct the mutants are shown as the primers 3 to 10 in Supplemental Table S1 (see Biosci. Biotechnol. Biochem. Web site) in supporting information.

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Flash-induced photocycle intermediates and proton pump behaviors. Traces of flash-induced proton release and uptake were detected in a spectrophotometer, which we manufactured in our lab. A photoflash for light-adapted samples was used for excitation. The M intermediate was monitored at 412 nm. In order to monitor flash-induced proton release and uptake by BR, absorption changes in the
pH-sensitive dye pyranine were detected at 456 nm, at which pyranine has maximum absorption while no BR intermediates have significant absorption. The signal without pyranine was subtracted from that with dye. All the measurements were done at 25°C.

Results

Identification of mutants

The results for gene sequences of the mutant fragments (Figs. S1–S6 in supporting information) and the mass spectra of the mutant (Figs. S7–S8) confirmed that the pre-designed mutants were successfully obtained. The CD spectra of the mutants in the UV region (Fig. S9) were similar to that of BR-WT, suggesting that the secondary structure did not change significantly in those mutants and confirming that the mutants might also have seven transmembrane α-helixes. As the visible absorption spectra in Figs. S10–S11 indicate, the chromophore was not perturbed significantly. The rates of dark adaptation of the BR mutants indicate that the replacements of amino acids at the cytoplasmic side did not significantly affect the isomerization of the retinal chromophore (Fig. S12).

Flash-induced proton pumping and M formation and decay

The proton pumping kinetics of the AR4 and BR analogs at pH 7.0 are presented in Fig. 2a. The samples were suspended in a salt solution with 0.1 mol/L of NaCl and 0.02 mol/L of KCl. Proton-pumping was traced with the pH-sensitive dye pyranine. An increase in dye absorbance at 456 nm reflected proton uptake by the protein, and a decrease in absorbance corresponded to proton release into the medium. As Fig. 2a shows, the BR mutants exhibited proton-pumping activity with proton release preceding proton uptake, similarly to BR-WT.

These results confirm not only that the mutant maintained the basic biological function as a light-driven proton pump, but also that the replacement mutations did not alter the temporal order of proton release and uptake. The decrease in the rate of proton uptake presented most prominent in the D102K mutant, which suggest D102 is involved in this process, perhaps acting as a proton antenna in the cytoplasmic side.

Flash-induced changes in absorption at 412 nm shown in Fig. 2b were due mostly to the M intermediate. The lifetime of M decay of the pigment was obtained by exponential fitting. The fitting results are summarized in Fig. 3, along with the delay times of proton uptake as calculated by fitting the data in Fig. 2a based on a single exponential equation. The characteristic times of M decay and proton uptake were consistent with each other. Compared to the wide-type BR, expressed in strain L33, every BR mutant exhibited slower M decay. Two mutants, D102K and K41E/D102K showed 3-fold increases in M lifetime.

pH dependence of M decay

The kinetics of the M decay of BR-WT and all the mutants were monitored at a pH range of 4 to 10. As shown in Fig. 4, the M decay of BR-WT was monophasic at neutral pH and became biphasic at higher pH, as fitted with two components, $\tau_{fast}$ and $\tau_{slow}$. Biphasic decay was also observed at pH levels below 7. The K41E/D102K mutant exhibited biphasic decay at all pH levels. The fraction of fast M decay in the mutant was smaller than in the WT, suggesting that the mutation on the surface of the protein had an effect on the internal proton transfer from Asp96 to the Schiff base. About 3-fold elongation of the slow component of M decay in the mutant was observed from pH 4 to 8, but at higher pH levels the difference with the BR-WT was smaller, indicating the possibility that it was caused by the
charge on Lys-102, which was deprotonated at higher pH levels. Similar results were found for D102K (Fig. S13).

The pH dependence of the formation and decay of the BR-WT and mutant proteins at neutral pH measured at 660 nm are shown in Fig. S14. The pH dependence of the O intermediate was used to estimate the pK\textsubscript{a} of D96, the internal proton donor to the Schiff base in the photocycle. Figure S15 describes the change in maximum absorption at 660 nm relative to the change maximum absorption at 412 nm during the N-to-O process in the photocycle. The transition in the acidic pH region corresponded to the larger accumulation of the O intermediate along with the decrease in the rate of its decay, and reflects pK\textsubscript{a} of the PRC in the O intermediate, and the transition in the alkaline region was caused by a slowing of proton uptake and reflected the pK\textsubscript{a} of D96 in the N intermediate. 45) All of the pK\textsubscript{a} values in the alkaline region of D96 of the AR4, BR-R\textsubscript{1}M\textsubscript{1}, BR-WT and BR mutants are summarized in Fig. 5. These data illustrate that the amount of the O intermediate and its pK\textsubscript{a} strongly decreased in the K41E/D102K mutant, consistently with slowing of M decay and proton uptake in that mutant.

The pK\textsubscript{a} of PRC in the M intermediate

Our group recently developed an efficient photo-electrochemical approach 14) to study the pH dependence of proton release and uptake of retinal proteins and to determine the pK\textsubscript{a} of PRC in the photocycles. In this study, we employed this approach to investigate the BR mutants. Figure 6a showed photo-response profiles from a K41E/D102K film deposited on an ITO electrode in an electrolyte solution of 100 mmol/L NaCl and 20 mmol/L KCl at various pH. The amplitude and polarity of the observed signals changed with the medium pH. The signals were negative at pH levels above 6, but became positive at pH levels below 5. A transition thus took place between 5 and 6. The pH

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**Fig. 5.** pK\textsubscript{a} Values of D96 in Proteins Expressed in Gene-Engineering Strain L33 as Well as Wild-Type Proteins from Strains R1M1 and xz515.

Values were obtained by fitting the pH dependence of the maximum light-induced absorption change at 660 nm (O intermediate) divided by maximum absorption change at 410 nm (M state) at the corresponding pH by the equation \( y = A_1 + (A_2 - A_1)/[1 + 10^{(pK - x)/C138}] \) in the alkaline regime, as shown in Fig. S13 in supporting information.

**Fig. 3.** Histogram of the Characteristic Times of (a) Proton Uptake and (b) M Decay of the BR, BR Mutants and AR4 at Neutral pH.

**Fig. 4.** pH Dependence of M Decay of BR-WT and Mutants. (a) The kinetics of M decay of BR-WT and its mutant K41E/D102K at pH 7.0 and 10.0; (b) The lifetime of the M intermediate of BR-WT and K41E/D102K fitted with a bi-exponential equation. The decay of BR-WT is monophasic, with similar \( t_{fast} \) and \( t_{slow} \) at neutral pH, while it is biphasic at the other indicated pH levels. K41E/D102K presented biphasic at all pH levels examined.
dependence of the time constants of proton release and uptake are presented in Fig. 6b. Based on the fraction of fast proton release at a series of pH levels, we determined the pK_a of PRC of K41E/D102K (Fig. 6c).

The photo-response profiles and the pK_a of PRC of the other mutants are presented in Fig. S16 in supporting information. Figure 7 summarizes all the pK_a values of the mutants of BR and AR4. The resulting pK_a values of PRC of all the BR mutants after illumination were lower than 7, like that of BR-WT (pK_a 6.3) and that of BR-R1M1 (pK_a 5.7). Thus the temporal order of proton release and uptake of mutants at neutral pH were the same as that of BR-WT.

Discussion

Our mutagenesis of K41 and D102 in BR was initially done to interpret the reversed time order of proton release and uptake of AR4. Govindjee et al. found that mutation of a surface residue, Lys-129 of BR, on the extracellular side reversed the temporal sequence of proton release and uptake, and thus the replacement of one charged residue might alter the time order. Hence, we devoted attention to charged residues on the cytoplasmic side where a proton is taken up in the photocycle. Two charged residues, K41 and D102, which are different between BR and AR4, became candidates. The present study rules out the possibility that amino acids Lys41 and Asp102 are determinants of the time order of proton release and uptake. The residues determining the different time orders of proton release and uptake (a much higher pK_a for PRC in archaerhodopsin) remain to be identified, and further examination is called for.

Although mutagenesis did not reverse the order of proton release and uptake, the replacements resulted in significant changes in certain photo-response properties of BR, including the decay time of the M intermediate, the time constant of proton uptake, and the proton affinity of D96 in the photocycle. As shown in Fig. 3, M-intermediate decay was about 3-fold slowed down in D102K and the corresponding double mutant K41E/D102K, as compared to BR-WT. Thus, the replacement of Asp at site 102 with Lys affected the efficiency of proton collection from the cytoplasmic surface. This is especially interesting considering that D102 is located in the CD loop.

Our results agree with those of Riesle et al., who proposed that polar or charged residues on the cytoplasmic side of BR might participate in or affect re-protonation of the Schiff base and proton uptake. In their studies, M decay was prolonged strongly by the mutant of D2102,104K2, in which two aspartates were replaced by arginines. However, another group found that replacement of D102 with a neutral Asn did not cause substantial changes, and hence questioned the role of this Asp residue in proton collection. Due to
our novel mutations, the pH₅₀ of D96 of BR-WT decreased from 7.9 to D102K (6.5) and K41E/D102K (6.9) (Fig. 5). The lowered pH₅₀ of D96 apparently weakens the association of a proton with aspartic acid, especially at pH levels higher than that of pH₅₀. Proton transfer from the cytoplasmic surface to D96 was 3-fold slowed at pH levels below 9, which is consistent with the slowing of the decay of M intermediate, since it is in equilibrium with the N intermediate. The decay of the latter is straightforwardly related to proton uptake. 6) Thus, the present study suggests that the acidic 102 site of BR is involved in the process of proton uptake from the cytoplasmic side and donation of it to Asp96, since replacement of Asp102 with Lys interfered with de-protonation and re-protonation of Asp96, and even with re-protonation of the Schiff base. Using the photoelectrochemical approach recently developed by us, 14) the pH₅₀ values of the PRC of BR mutants after illumination were measured. While the mutation at the cytoplasmic side might have disturbed the charge distribution of the cytoplasmic surface of BR and have influenced the pH₅₀ of PRC, the values were still far lower than that of AR4. Hence those mutations of BR do not lead to the reversed time order of proton release and uptake.

In summary, we performed gene engineering on BR and examined the effects of the K41 and D102 mutations on the functions of the light-driven proton pump. These replacements did not alter the basic spatial structure of the protein, and the charged amino acids affected proton uptake on the cytoplasmic side. Hence the two residues are not key to the temporal order of proton release and uptake of BR and AR4. Nevertheless, these replacements influenced the photocycle by affecting the proton affinity of D96 and the rate of proton delivery to it through the proton channel. The mutants containing D102K prolonged the lifetime of the M intermediate about 3-fold. They were intended to make the cytoplasmic surface of BR more similar to that of AR, but the results did not show earlier M decay or proton uptake, which are characteristic of AR. On the contrary, the rates of these processes were slowed in the mutations.

Acknowledgments

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