Influence Exercised by Histidine-95 on Chloride Transport and the Photocycle in Halorhodopsin

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ABSTRACT: The anion pumping mechanism of halorhodopsin was studied using site-directed mutagenesis. Comparison of the amino acid sequence revealed that the B−C interhelix loop segment was highly homologous in all known halorhodopsins. Especially a basic residue, histidine-95, was conserved in all halorhodopsins. Using the expression-vector plasmid carrying the *bop* promoter, two His-95 mutants (H95R, H95A) were successfully expressed in *Halobacterium salinarium*. The expression levels of these halorhodopsin mutants were slightly lower than that for the wild-type halorhodopsin. In addition, these mutants were unstable under illumination compared with the wild-type. It suggested that His-95 is probably important for stabilizing the structure of halorhodopsin. The absorption maxima of these mutants are approximately 15 nm blue-shifted compared with the wild-type, suggesting that His-95 interacts with the retinal Schiff base. At low chloride concentrations, the light-induced chloride pumping activity of these mutants was more than 20 times lower than that for the wild-type. Only under physiological conditions, the chloride pumping activity was detected. Even at a high chloride concentration (1 M NaCl), the HR520 intermediate could not be detected for these mutants. These results clearly indicate that His-95 has a crucial role in the chloride transport of halorhodopsin.

Halorhodopsin (HR)[1] can function as a light-driven chloride pump. HR was found in extremely halophilic archaebacteria, like bacteriorhodopsin (BR) (Matuno-Yagi & Mukohata, 1977). Extensive site-directed mutagenesis studies for BR have detected several amino acid residues crucial for the proton pumping mechanism; however, few site-directed mutagenesis studies have been undertaken so far for the chloride pump function of HR. Recently, a transformation system for halobacteria was developed (Needleman et al., 1991; Ferrando et al., 1993), and HR overexpression in halobacteria was successfully carried out using the promoter and signal peptide for bacterioopsin (Heymann et al., 1993). Subsequently to its overexpression, Arg-108 mutants for HR were expressed in halobacteria, and it was found that Arg-108 plays an essential role in chloride transport (Rudiger et al., 1995). A new HR from the halobacterial strain *shark* was also overexpressed using the same bacterioopsin promoter system, and its new halorhodopsin was successfully characterized as to chloride pumping activity and photoreaction cycle (Otomo & Muramatsu, 1995). Under extremely acidic conditions, it is known that the color of BR returns to purple from blue, and its BR might bind and transport chloride (Der et al., 1991). Recently, it was found that when Asp-85 was replaced with threonine, this point mutant BR had a pumping action on chloride ions at a neutral pH (Sasaki et al., 1995). In this case, however, its chloride affinity was lower than that for the wild-type HR.

Since the discovery of HR from *Halobacterium salinarium* (*halobium*), several new HRs have been also found in other halophilic archaebacteria (Lanyi et al., 1990; Otomo et al., 1992; Soppa et al., 1993; Otomo & Muramatsu, 1995). Comparison of their amino acid sequences clearly suggested that several positively charged residues are essential for chloride pumping. In the putative B−C interhelix loop, a positively charged residue, histidine, is conserved in all the known HRs. In the BRs, however, there are few identities in this B−C interhelix loop and no identical positively charged residue in it. Therefore, it was suggested that the histidine residue in the B−C loop is critical in the chloride pumping function (Otomo, 1995).

In the physiological condition (4 M NaCl), the absorption maximum among all known HRs was at approximately 578 nm. Removal of the chloride ion from salinarium HR blue-shifts its absorption maximum by 10 nm (Ogurusu et al., 1984). On the other hand, the absorption maximum of HRs from *Natronobacterium pharaonis* and the strain *shark* was found to be at approximately 600 nm in the chloride-ion-free condition (Scharf et al., 1994; Otomo & Muramatsu, 1995). The absorption maximum shift of pharaonis HR has been also extensively characterized, and it was found that the shift can be regulated by changes in the anions added and their concentration (Scharf et al., 1994).

The photoreaction cycle of salinarium HR has been reported by several groups (Oesterhelt et al., 1985; Tittor et al., 1987; Zimanyi et al., 1989; Cao et al., 1995). All-trans to 13-cis photoisomerization of the retinal initiates its photocycle, and the HR600 intermediate arises a few picoseconds. Then the HR520 intermediate is produced in about 1 μs and decays to the HR640 intermediate. Within a few milliseconds HR640 regenerates the ground state of HR578. The rise and decay times of both HR520 and HR640 strongly depend on the anions added and their concentration. Resonance Raman and FTIR studies have

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[1] Abbreviations: BR, bacteriorhodopsin; HR, halorhodopsin; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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also indicated that both HR520 and HR640 are key intermediates for elucidation of the mechanism of the HR chloride pumping function (Ames et al., 1992; Braiman et al., 1994). Recently, the photochemical cycle of pharaonis HR was also studied, and differences between the two HR were reported (Varo et al., 1995).

In this study, His-95 point mutants were overexpressed in halobacteria; their chloride pumping function and photocycle reactions are described. The results in this report show that His-95 is one of the essential residues for chloride transport in HR.

MATERIAL AND METHODS

Microbial Strains and Cell Culture. H. salinarium strain HN5 (BR and HR negative) and *Escherichia coli*−*Halobacteria* shuttle vector plasmid (pEF191) were generous gifts from Dr. Dieter Oesterhelt. Salinarium HR overexpression strain JO17, described by Otomo and Muramatsu (1995), was used for the characterization of the wild-type HR. Competent cells of *E. coli* JM109 were obtained from Takara Shuzo Co., Ltd., Japan. All halobacterial strains were grown in the standard peptone medium described by Oesterhelt and Stoeckenius (1974).

Mutagenesis of Halorhodopsin. The fusion gene for HR overexpression described by Otomo and Muramatsu (1995) was used for expression of His-95 mutants [the position of the amino acid numbered as in Blanck and Oesterhelt, (1987)]. Construction of the genes for the mutants was performed by the recombinant PCR method, using synthetic oligonucleotide primers. *H. salinarium* strain HN5 (BR and HR negative) was used as the host expressing the mutants.

Preparation of Cell-Envelope Vesicles and Anion Pumping Analysis. After 7 day cultivation at 40 °C, the cells were collected by centrifugation and cell-envelope vesicles were prepared by the freeze−thaw method. The envelope vesicles were washed with a P-buffer (4 M NaCl, 25 mM PIPES/NaOH at pH 7.0) by centrifugation. The light-induced ion pump activity was performed by the same apparatus as described previously (Otomo & Muramatsu, 1995).

Spectroscopic Analysis. Absorption spectra were recorded on a Hitachi U-3210 spectrophotometer. Kinetic flash-spectroscopic measurements were performed with a flash-spectroscopic apparatus (model USP-500, Unisoku Scientific Instruments Corp., Japan). The monitoring beam provided by a 12-V, 100-W tungsten−halogen lamp was passed through a monochromator. The monitoring beam was guided to the sample by a glass fiber. After passing though the sample and an additional monochromator, the beam was focused onto a photomultiplier. The photomultiplier output was fed into a current−voltage converter, digitized by an A/D converter, and transferred to a microcomputer. The actinic flash from a xenon lamp, filtered by an orange filter, O-57 (>570 nm) was delivered at a 90° angle to the monitoring beam. All measurements were performed at 20 °C.

RESULTS

Using the expression-vector plasmid carrying the *bop* promoter, wild-type HR as well as two His-95 mutants (H95R, H95A) was successfully expressed in *H. salinarium*. Their cell-envelope vesicles were prepared and the expression levels of the mutant HRs were monitored by a means of SDS−PAGE analysis (Figure 1). The vesicles from the mutants were light purple-blue in contrast to the wild-type which was clear purple. As shown in Figure 1, mutant HRs were the main product in total membrane proteins, the same as for the wild-type. However, the densities of the HR bands for both mutants were slightly lower than that for the wild-type, showing that their expression levels were relatively lower than that for the wild-type.

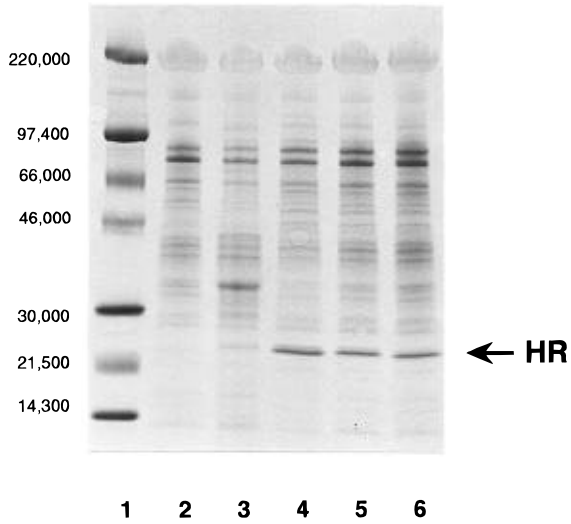

FIGURE 1: SDS−PAGE analysis of total membrane fractions of strain HN5 (lane 2), L33 (lane 3), wild-type (lane 4), H95R (lane 5), and H95A (lane 6). 10 mg of the total membrane protein was loaded. Lane 1 is a marker protein and allows correspondence to halorhodopsin.

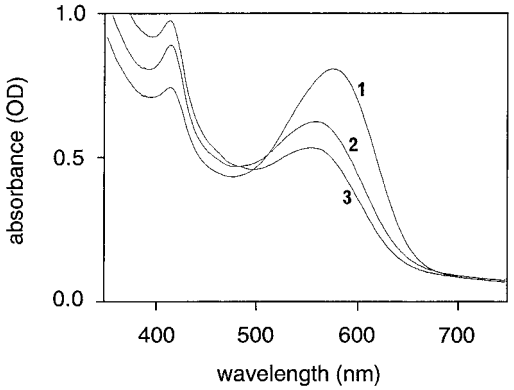

FIGURE 2: Absorption spectra of the total membrane fraction for wild-type (1), H95R (2), and H95A (3). The sample containing 2.5 mg of total membrane protein/mL was suspended in 1 M NaCl, 20 mM PIPES (pH 7.0). Spectra were measured immediately after illumination with orange light for 1 min (light-adapted). The absorption maxima are as follows: wild-type HR (576 nm); H95R mutant HR (559 nm); H95A mutant HR (555 nm).

Membrane fractions were prepared by dialysis from their cell-envelope vesicles, and their absorption spectra were monitored. Figure 2 shows the absorption spectra of the total membrane fractions for the wild-type, the H95R mutant, and the H95A mutant in the presence of 1 M sodium chloride. The absorption maxima of these mutants were approximately 17 nm (H95R) and 21 nm (H95A) blue-shifted compared with that for the wild-type. In the absence of sodium chloride, the absorption maximum of the wild-type HR was known to be approximately 10 nm blue-shifted. However, no blue-shift was observed for both mutants after removal of chloride. The absorbance of both mutants was lower than that for the wild-type, showing that the expression levels of

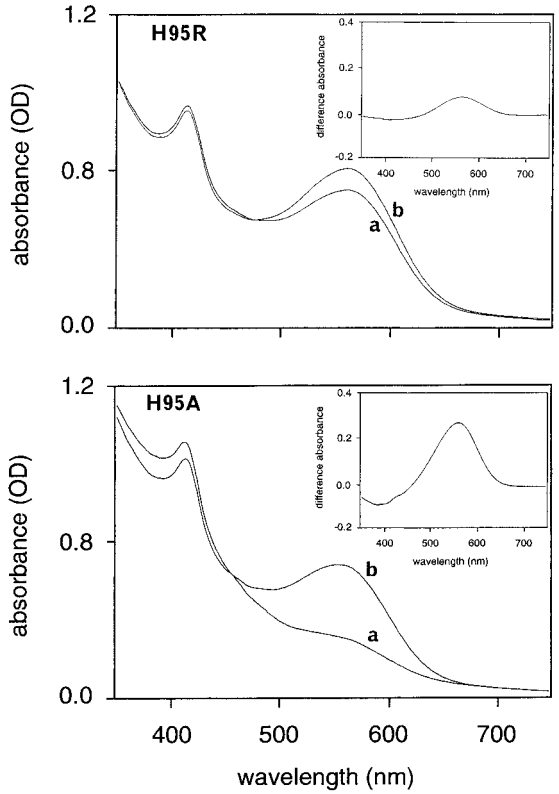

FIGURE 3: Absorption spectra of the total membrane fraction for H95R and H95A before (b) and after (a) bleaching. The sample conditions were the same as for Figure 2. Bleaching was performed by illumination with orange (O54 filter) light for 1 h at 4 °C. Inset : Difference absorption spectra between the spectra before and after bleaching. The absorption maxima for the difference spectra are as follows: H95R mutant HR (565 nm); H95A mutant HR (561 nm).

these mutants were lower than that for the wild-type, if the absorption coefficients for the mutants are assumed identical to that for the wild-type. In addition, the level for the H95A was lower than that for the H95R.

Although the wild-type HR in the total membrane fraction is stable under illumination, the mutant HRs were unstable under the same illumination conditions. Figure 3 shows absorption spectra of the mutants before and after bleach by illumination. In the H95A mutant, almost all of the HR was bleached by 1 h illumination. Addition of retinal to their bleached membrane fraction did not bring about the reconstitution of the HR, showing that the protein had become denatured. A slight absorbance decrease was observed for the H95R mutant by the same illumination. The result indicates that these mutants, particularly H95A, are unstable.

Chloride pumping activity was monitored using the light-induced passive proton uptake of the cell-envelope vesicles in the presence of 50 μM CCCP. Figure 4 shows the pumping activity for the wild-type and the mutants in terms of their dependence on chloride concentration. In 0.2 M chloride concentration, the pumping rate for the wild-type was approximately 400 nmol/min. In comparison, little pumping activity for either mutants was observed under the same chloride concentration. The pumping rates for both mutants were more than 20 times smaller than that for the wild-type in a chloride concentration of 0.5 M. These data clearly indicate that the histidine residue in the position 95 for HR plays a role in chloride transport in millimolar chloride concentrations. Nitrate pumping activity was also

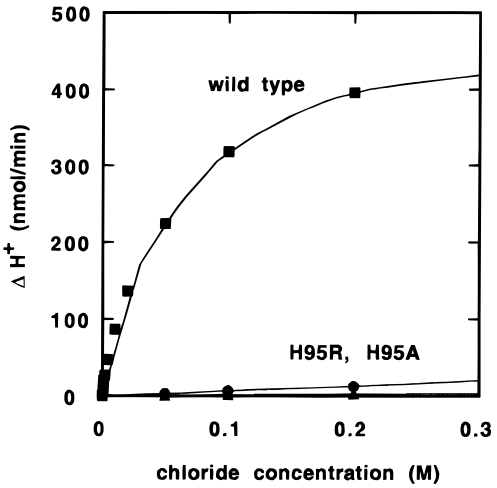

FIGURE 4: Light-induced chloride transport by the cell-envelope vesicles for wild-type HR (■), H95R mutant HR (●) and H95A mutant HR (▲) in the chloride concentration range of 0−0.3 M. The rate of the light-driven chloride was plotted as a function of the added chloride concentrations. The rate was determined by measuring the initial slope of pH change during the illumination. The sample (1 mg of protein/mL) was suspended in 1.5 M sodium sulfate, 1 mM PIPES (pH 7.0), 50 μM CCCP. A projector lamp (300 W) was used as a light source. The light was passed through a heat-absorbing water bath, an infrared cut filter, and an orange filter (O54).

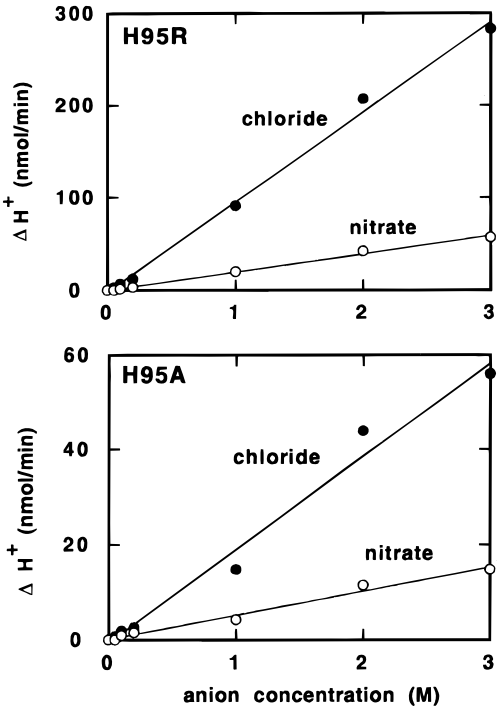

FIGURE 5: Light-induced anion [chloride (●) and nitrate (○)] transport by the cell envelope vesicles for H95R mutant HR and H95A mutant HR in the anion concentration range of 0−3 M. The rate of the light-driven anion pumping was plotted as a function of the added chloride and nitrate concentrations. The measuring conditions were the same as for Figure 4.

detected for this overexpressed wild-type HR (Otomo & Muramatsu, 1995). The activity was about 3-fold lower than that for chloride pumping (data not shown). Its anion selectivity was the same as the wild-type HR from *H. salinarium* strain L33.

At high concentrations of chloride and nitrate, light-induced pumping activity for both mutants was also monitored (Figure 5). Although the pumping rate of the wild-

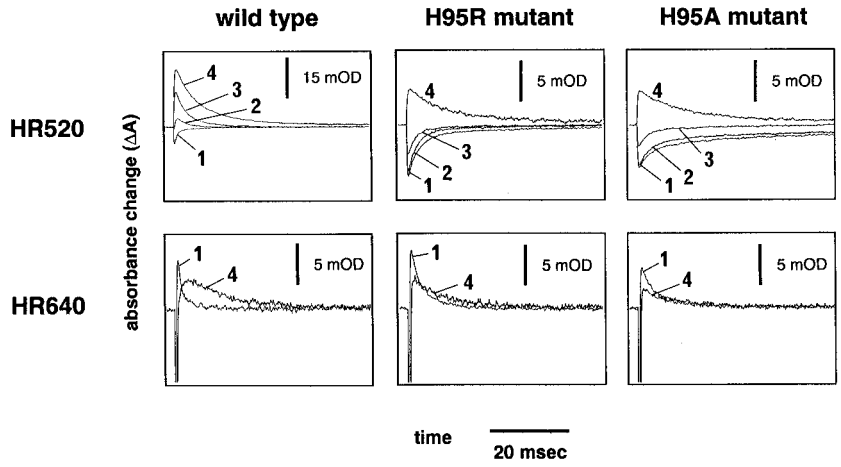

FIGURE 6: Flash-induced absorption changes of HRs for wild-type, H95R, and H95A with sodium chloride concentrations of 50 (1), 200 (2), 1000 (3), and 3000 mM (4). For detection of the HR520 intermediate, absorption changes were monitored at 500 nm. For the HR640 intermediate, absorption changes were monitored at 660 nm. The samples containing 5.0 mg of total membrane protein/mL were suspended in 20 mM PIPES (pH 7.0).

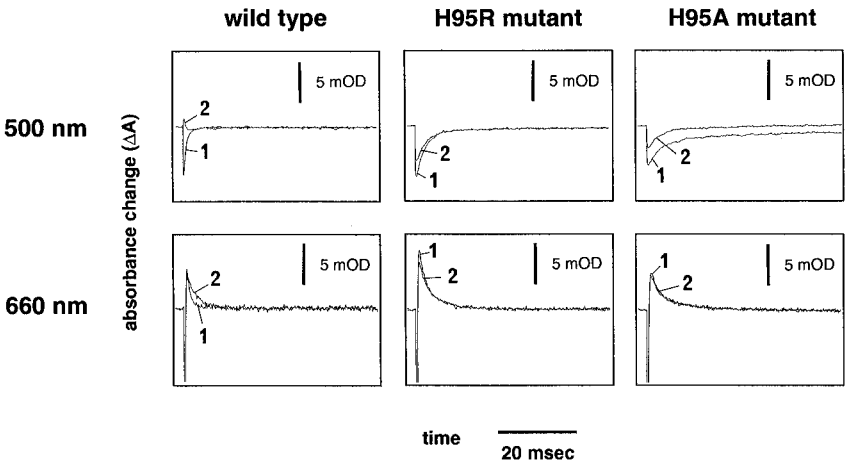

FIGURE 7: Flash-induced absorption changes at 500 and 660 nm of HRs for wild-type, H95R, and H95A with sodium nitrate concentrations of 200 (1) and 3000 mM (2). The samples containing 5.0 mg of total membrane protein were suspended in 20 mM PIPES (pH 7.0).

type was almost saturated above the chloride concentration of approximately 0.5 M, the pumping rate for both mutants increased in proportion to the chloride concentrations. The chloride pumping rate for the H95R mutant was approximately 300 nmol/min in a 3 M chloride solution. The expression level of its mutant was estimated, from the relative absorbance of the membrane fraction, to be about two-thirds that of the wild-type (Figure 2). Therefore, the pumping activity of the wild-type and the H95R mutant at the physiological condition was almost the same. In the presence of nitrate, an increase in pumping rate was also observed for both mutants. In addition, the selectivity between chloride and nitrate for both mutants was also found to be almost the same as that for the wild-type.

The photoreaction cycle of both mutants was analyzed from flash-induced absorption changes. The formation and depletion of the intermediates (HR520 and HR640) were monitored in the presence of sodium chloride at 50, 200, 1000, and 3000 mM (Figure 6). In the wild-type, the HR520 intermediate was detectable even in 200 mM of sodium chloride, and its formation increased in proportion to the concentration of sodium chloride. By comparison, the HR520 intermediate was not detectable even in 1000 mM of sodium chloride for both mutants. Only in the presence of 3000 mM of sodium chloride could the formation of its intermediate be monitored for both mutants. The HR640 intermediates of both mutants were also monitored. In contrast with the formation of the HR520 intermediate, there was little difference between the wild-type and the mutants. Only the HR640 decay time of the wild-type in 3000 mM of sodium chloride was relatively slower than that for the mutants. In the presence of sodium nitrate, the flash-induced absorption changes at 500 and 660 nm were also monitored (Figure 7). In the wild-type HR, a small absorption increase at 500 nm was detected in the presence of 3000 mM of sodium nitrate, whereas no increase was detectable for both mutants. The changes at 660 nm were almost identical for the wild-type and the two mutants in the presence of both 200 and 3000 mM of sodium nitrate.

The flash-induced absorbance changes at 410 and 540 nm for the wild-type and both mutants were monitored at high and low concentrations of chloride (Figure 8). In the H95A mutant, an absorption increase at 410 nm and an absorption decrease at 500 nm were detected in the presence of low chloride concentration (50 mM). Their half-life times were found to be greater than 100 ms, much slower than the decay times for the HR520 and the HR640 intermediates. These absorption changes were very similar in effect to that for the so-called azide effect, which was first found by Hegemann et al. (1985). These absorption changes decreased in

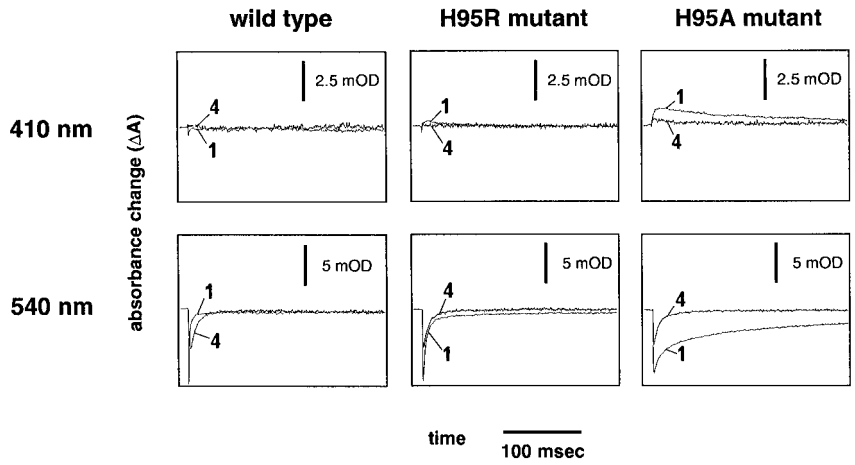

FIGURE 8: Flash-induced absorption changes at 410 and 540 nm of HRs for wild-type, H95R, and H95A with sodium chloride concentrations of 50 (1) and 3000 mM (4). The samples containing 5.0 mg of total membrane protein/mL were suspended in 20 mM PIPES (pH 7.0).

proportion to the chloride concentration and were not detected in the presence of 3000 mM of sodium chloride. In the H95R mutant, small but similar changes were detected in the presence of 50 mM of sodium chloride, whereas no such changes could be detected even at 50 mM of sodium chloride for the wild-type. The increase in absorbance at 410 nm was known to be caused by deprotonation of the retinal Schiff base. These results therefore suggested that the His-95 substitution affected de- and reprotonation of the retinal Schiff base.

DISCUSSION

Comparison of the amino acid sequences revealed that a histidine residue in the B−C interhelix loop was conserved in all known halorhodopsins. In this report, therefore, His-95 point mutants were produced, and their functions were characterized. Although an HR overexpression vector was used for the production of the mutants, the expression levels of the mutants were lower than that for wild-type. Absorption spectra of the total membrane fractions of the mutants showed that the expression level of the H95A mutant was relatively lower than that for the H95R mutant. In addition, other mutant genes (H95K, H95D, and His-95 deletion) were also constructed, and the HR negative strain was transformed with these genes in the same way. Only the H95K mutant transformant was obtainable by color selection of colonies. However, no transformant with genes containing substitution to a positively charged residue or deletion expressed no protein characterized by purple-blue color (data not shown). From these results, we suspect that a positive charge in position 95 of the HR was vital to expression and/or protein folding in the cell membrane. The photobleaching experiments also clearly indicated that both mutants were unstable compared to the wild-type. In addition, the neutrally charged substitution (H95A) was more effective for protein denaturation than the positively charged substitution (H95R), suggesting that the positive charge of this histidine was crucial in stabilizing the protein structure.

Ames et al. (1992) have suggested that the HR520 intermediate arises from the K-intermediate (HR600) during the movement of chloride to the vicinity of the retinal Schiff base to act as a counter ion. The formation of the HR520 was known to depend on the chloride concentration of the solution. In the wild-type HR, the HR520 formation was detectable at chloride concentrations of greater than 200 mM (Figure 6). On the other hand, HR520 was not detectable for either mutants, even in a 1 M chloride solution, showing that the movement of the chloride ion to the vicinity of the protonated Schiff base was not able to occur. This photoreaction at low chloride concentrations was consistent with the chloride pumping activity using the cell-envelope vesicles containing mutants (Figure 4). These results indicate that substitution of histidine-95 affects the formation of the HR520 intermediate as well as the chloride pumping activity.

At a high chloride concentration, both mutants formed the HR520 intermediate and it was possible to detect light-induced chloride pumping activity. In addition, nitrate pumping activity for both mutants was also present, and the pumping selectivity between chloride and nitrate for both mutants was almost identical to that for the wild-type. It is therefore indicated that anion pumping activity of the mutants recovered at high chloride concentrations. The histidine-95 in the B−C interhelix loop plays most likely a role in increasing the binding affinity of the anion for the transport.

Similar absorbance changes, caused by the azide-effect for the wild-type HR (Hegemann et al., 1985), were detected for the H95A mutant at low chloride concentrations. It is known that the HR410 intermediate, similar to the BR M-intermediate is produced in the presence of azide and decays to the ground state after more than 100 ms. This slow decaying intermediate at the wavelength of 410 nm probably arises from deprotonation of the retinal Schiff base. In the H95A mutant, this intermediate at 410 nm was detected in the solution without azide. Therefore, these similar effects indicated that the histidine residue in the B−C loop is probably near the Schiff base and also affects the protonated state of the retinal Schiff base.

In BR, a small α-helical segment was observable in the B−C interhelix loop from a three-dimensional structure analysis at a near-atomic resolution, suggesting that this B−C loop has some static structure (Henderson et al., 1990). Three-dimensional structural analysis of HR at a 7 Å resolution (Havelka et al., 1995) revealed that seven α-helical polypeptides spanned the membrane, almost the same as in BR. Interhelix loop segments of HR, however, have not been yet identified in this analysis. From the density map of the extracellular surface the area surrounded by the helices ABCG seems to be wider than that of the cytoplasmic side,

in HR as well as BR (Havelka et al., 1995). The B−C loop of the HR is 10 residues longer than that for BR, and His-95 is found to be located almost at the center of this loop. Likewise, proline (Pro-92), which is conserved in all known HRs as with His-95, is present in the position three residues before His-95 (Otomo & Muramatsu, 1995). Therefore, it is possible that the B−C loop is folded into the area of the helices ABCG, bringing it closer to the retinal Schiff base. A previously proposed structure of the potassium channel showed the H5 segment (22 amino acid residues between helix 5 and helix 6) folding into the ion pore, contributing to the formation of constructions or potassium binding sites (Yool & Schwarz, 1991; Jan & Jan, 1994). This also supports the premise that the B−C loop, as well as His-95 in HR, is essential for anion transport.

The human red and green color vision pigments, as well as iodopsin, are known to bind the chloride ion and to undergo a large red-shift in their absorption maxima (Shichida et al, 1990; Wang et al., 1993). Mutations of all positively charged residues in the green pigment clearly indicate that His-197 was one of the chloride binding sites (Wang et al., 1993). This histidine is also located in the extracellular loop (D−E interhelix loop), equivalent to the B−C loop in HR. Moreover, the H197A mutant of the green pigment had an approximately 20 nm red-shifted absorption maximum, almost the same absorption shift as in the H95A HR mutant. In both cases, it is possible to speculate that histidine located in the extracellular loop is crucial for binding of chloride and that it interacts with the retinal Schiff base.

In this study, I found that His-95 in the putative B−C loop was one of the chloride binding sites and was also crucial for the anion pumping function. It is still unknown at this time whether the interaction of this histidine to the retinal Schiff base is direct or not, due to the lack of structural information on the B−C loop in HR. It should become clear after determination of the HR 3-D structure at an atomic resolution in the future.

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