Organisms utilize light as energy sources and as signals. Rhodopsins, which have seven transmembrane \( \alpha \)-helices with retinal covalently linked to a conserved Lys residue, are found in various organisms as distant in evolution as bacteria, archaea and eukarya. One of the most notable properties of rhodopsin molecules is the large variation in their absorption spectrum. Sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII) function as photo-sensors, and have similar properties (retinal composition, photocycle, structure and function) except for their \( \lambda_{\text{max}} \) (SRI, ~560 nm; SRII, ~500 nm). An expression system utilizing \( E. \) coli and the high protein stability of a newly found SRI-like protein, \( \text{SrSRI} \), enables studies of mutant proteins. To determine the residue contributing to the spectral shift from SRI to SRII, we constructed various SRI mutants, in which individual residues were substituted with the corresponding residues of SRII. Three such mutants of \( \text{SrSRI} \) showed a large spectral blue-shift (>14 nm) without a large alteration of their retinal composition. Two of them, A136Y and A200T, are newly discovered color tuning residues. In the triple mutant, the \( \lambda_{\text{max}} \) was 525 nm. The inverse mutation of SRII (F134H/Y139A/T204A) generated a spectral shifted SRII towards longer wavelengths, although the effect is smaller than in the case of SRI, which is probably due to the lack of anion-binding in the SRII mutant. Thus, half of the spectral shift from SRI to SRII could be explained by only those three residues taking into account the effect of Cl\(^{-}\) binding.

Light-absorbing photoactive proteins having their cognate chromophore within the protein moiety exist in various organisms, and show characteristic colors originating from an energy gap between the ground and excited states, which lead to changes in the absorption maximum (\( \lambda_{\text{max}} \)). It is important to know the molecular mechanism of these color changes, not only to understand the functions of the proteins, but also to utilize such proteins as tools in fields such as optogenetics (1).

Seven-transmembrane proteins having retinal (vitamin A aldehyde) as a chromophore (supplemental Fig. S1) are called “\( \sim \)rhodopsin” (2-6). Rhodopsins react to light of their corresponding specific wavelengths, although they all share the same basic structure (3,6). They are covalently bound to an \( 11\text{-cis} \) (mammalian type) or an \( \text{all-trans} \) (microbial type) retinal chromophore at a conserved lysine residue on the G-helix via a protonated Schiff base (PSB) bond (supplemental Fig. S1). One of the most notable properties of rhodopsins is the large variation in their absorption spectrum depending on their interaction between the opsin (apoprotein) and the retinal chromophore, which is called the opsin-shift (7). The \( \lambda_{\text{max}} \) corresponds to its most probable transition from the ground to the excited state. Empirical and theoretical studies of the spectral tuning in organic solvents have suggested several mechanisms. They include the following: (i) an alteration...
in the strength of the electrostatic interaction between the PSB and its counter ion or hydrogen bond acceptor (8-10); (ii) an alteration in the polarity or polarizability of the environment of the chromophore-binding site, caused by the arrangement of polar or aromatic residues (10,11); and (iii) an isomerization around the 6-S bond connecting the polyene chain to the β-ionone ring (9,10,12). By the combinations of these effects, microbial (type-1) rhodopsins show various absorption maxima ranging from 485 to 590 nm. However, it is still unclear how to tune their λmax.

Type-1 rhodopsins are widespread in the microbial world in prokaryotes (bacteria and archaea) and in eukaryotes (fungi and algae) (3,4). A striking characteristic of these rhodopsins is their wide range of seemingly dissimilar functions. Some are light-driven transporters, such as the proton pumps bacteriorhodopsin (BR) in haloarchaea, Xanthorhodopsin (XR) in halophilic bacteria, proteorhodopsin (PR) in marine bacteria, Leptosphaeria rhodopsin (LR) in fungi, and halorhodopsin (HR) in haloarchaea and in bacteria (3,13-17). These proteins generate an electrochemical membrane potential upon light activation, which is utilized by ATP-synthase to produce ATP (18). Other rhodopsins are light sensors, such as the phototaxis receptors sensory rhodopsins I (SRI) and II (SRII) in haloarchaea (19). Recently, BR and HR have been utilized by neuroscientists to control neural cells by light (1). SRII, functioning as a negative phototaxis receptor with the cognate transducer protein HtrII, absorbs light of shorter wavelengths, compared to ion pumping rhodopsins including BR and HR (19). This is reasonable because light contains harmful UV radiation. On the other hand, SRI mediates attractant motility responses to light of green/orange wavelengths which are used by the ion pumps BR and HR (20). Thus, for all rhodopsin molecules, it is essential to tune the color to their specific function.

Although all microbial rhodopsins are quite similar in their primary and tertiary structures (21), especially in the chromophore-binding site, the λmax value of SRII (~500 nm) is remarkably different from that of BR, HR and SRI (560–590 nm) (supplemental Fig. S1) (22). In previous studies aiming to determine the key residue(s) contributing to the color change of SRII, several SRII mutants were analyzed in which each residue was replaced by its corresponding residue in BR (23-25). However, SRII has only all-trans retinal as a chromophore and has a slow photocycle (~seconds) (24,26), whereas BR has both 13-cis (~50%) and all-trans (~50%) retinal in the dark and has a fast photocycle (~10 milliseconds) (27). Thus SRII differs from BR not only in its color but also in its function, retinal composition and photoreaction. One should note that the Hydrogen-Out-of-Plane (HOOP) vibration of the retinal chromophore of SRII is similar to that of SRI, but not to BR and HR (28). In addition to that, SRI, similar to SRII, has only all-trans retinal, a slow photocycle (~seconds) and works as a phototaxis receptor (2,20). Thus, it is expected that comparative studies between SRI and SRII would provide useful information. However, the instability of SRI from H. salinarum (HsSRI) hampered such studies in the past.

Recently we found a new SRI-like protein from Salinibacter ruber (SrSRI) which has its λmax at 557 nm (supplemental Fig. S1) similar to HsSRI (29). In addition to its high stability, the expression system utilizing E. coli makes it possible to prepare large amounts of protein and enables studies of mutant proteins (29). Moreover, new genes encoding the sensory rhodopsin proteins have been recently discovered through the genome sequencing of various microbes (4). On the basis of these advances, and according to the amino acid sequence alignment of SRI and SRII, including newly found sensory rhodopsins, we identified nine candidates for the color tuning residues (Figure 1). Using mutational analysis, we have now characterized the contribution of the conserved residues to the color tuning.

Experimental Procedures
Protein expression and purification - The expression plasmid of SrSRI was constructed as previously described (29). The mutant genes of SrSRI and SRI from N. pharaonis (NpSRII) were constructed by PCR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) as described previously (30). All constructed plasmids were analyzed using an automated sequencer (ABI 3100) to confirm the expected nucleotide sequences.

Cells were grown in LB medium supplemented with ampicillin (final concentration of 50 µg/ml). E. coli BL21(DE3) cells harboring the plasmid were grown to an OD660 of 0.3-0.4 in a 30°C incubator, followed by the addition of 0.5 mM IPTG and 5 µM all-trans retinal. Cells harvested 8 hrs post-induction at 18°C by centrifugation at 4°C, were resuspended in buffer (50 mM MES, pH 6.5) containing 1 M NaCl, and were disrupted by sonication as previously described (31). Cell debris was removed by low speed centrifugation (5,000 x g, 10 min, 4°C). Crude membranes were collected by centrifugation (100,000 x g for 30 min at 4°C) and were washed with buffer (50 mM MES, pH 6.5) containing 1 M NaCl. To solubilize the membranes, 2% (w/v) n-dodecyl-β-D-maltoside (DDM) was added and the suspension was incubated for 30 min at 4°C. The solubilized membranes were isolated by high speed centrifugation (100,000 x g for 30 min at 4°C) and the supernatant was applied to a Ni-affinity column (HisTrap, GE Healthcare, Uppsala, Sweden) at 4°C in the dark. Thereafter, the column was washed extensively in the dark with buffer (50 mM MES, pH 6.5) containing 1 M NaCl. To avoid denaturation, the spectra shown were measured immediately after purification. λmax values (main absorption) and retinal absorptions were calculated from areas of the peaks monitored at 360 nm. The assignment of each peak was performed by comparing it with the HPLC pattern from retinal oximes of authentic all-trans- and 13-cis-retinals.

RESULTS

Absorption spectra of mutants with a single substitution - Figure 2 shows visible absorption spectra obtained for the wild-type (WT) and for various mutants of SrSRI in which a single amino acid residue was replaced by its corresponding residue in SRII (I19T, F53L, V113A, H131F, A136Y, G165V, L166V, Y209L and A200T). To avoid denaturation, the spectra shown were measured immediately after purification.
configurations of the mutants are shown in Figure 3a and supplemental Fig. S2, and listed in Table 1. For I19T on helix-A, F53L on helix-B, V113A on helix-D, G165V and L166V on helix-F and Y209L on helix-G, essentially no or only small (<8 nm) shifts of λmax without significant changes in retinal composition were observed, indicating that those residues do not mainly contribute to the difference in λmax between SRI and SRII. On the other hand, for H131F on helix-E, the λmax was 533 nm, which is 24 nm (808 cm⁻¹) blue-shifted compared to that of the WT, as reported previously (33). Also for the A136Y and A200T mutants (replacements that changed the polarity) the λmax showed relatively large blue-shifts of 14 nm (463 cm⁻¹) and 17 nm (565 cm⁻¹), respectively. To investigate whether the spectral changes were caused by differences in retinal configuration, we performed HPLC analysis (Figure 3a), since it is well known that an increase in the 13-cis retinal isomer causes a spectral blue-shift in microbial rhodopsins (33). As shown in Figure 3 and Table 1, SrSRI single mutants contain more than 91% all-trans retinal with a small proportion of 13-cis retinal as do SrSRI (98.2%) and SRII (94.5%) also, indicating that the spectral blue-shift was not caused by a change in retinal configuration.

To investigate whether these recombinant proteins have a photocycling activity, flash spectroscopy was employed. Figure 3b shows the flash-induced absorbance changes of the WT and the H131F, A136Y and A200T mutants of SrSRI at selected wavelengths (390 nm or 380 nm for the M state and 550, 530 or 540 nm for the unphotolyzed state). The M-decay rate of WT SrSRI has been estimated to be 6.99 s⁻¹ by a single exponential equation as previously reported (29). This slow photocycle is particularly important because a key difference between transport and sensory rhodopsins is the much slower kinetics of the photochemical reaction cycle of the sensors (2). The ion-pumping rhodopsins have been optimized for fast photocycling rates to make them efficient pumps. In contrast, the sensory rhodopsins SRI and SRII have slow photocycles because of the existence of a long-lived M-intermediate, which allows the transient accumulation of long-lived signaling states of the receptors to catalyze a sustained phosphorylation cascade controlling flagellar motor rotation (34). The M-decay rates of I19T (6.85 s⁻¹), G165V (5.15 s⁻¹) and V113A (9.69 s⁻¹) determined by a single exponential equation are similar to that of the WT (6.99 s⁻¹), whereas those of H131F (1.38 s⁻¹), A136Y (0.08 s⁻¹) and A200T (0.61 s⁻¹) are significantly smaller (Figure 3b), indicating that these residues are involved not only in the color tuning, but also in the structure and/or structural changes around the chromophore.

**Effects of chloride ion-binding to SrSRI on its absorption maximum and photocycle** - Figure 4 shows the effects of Cl⁻ on the absorption maxima (a) and photocycle kinetics (b) in the WT and in various mutants of SrSRI. As reported in a previous study (32), the absorption maximum of WT SrSRI is shifted from 557 to 544 nm (430 cm⁻¹) in a Cl⁻-dependent manner. The M-decay rate is also increased 5-fold in the absence of Cl⁻ (black lines in Figure 4b), compared to a 1M NaCl solution (gray lines in Figure 4b) (32). In the case of the SRI homologue from *Haloarcula vallismortis* (*HvSRI*), the M-decay rates also increased 3.4-fold at pH 5.0, and 1.7-fold at pH 8.5 in the absence of Cl⁻, compared to those in the 1 M NaCl solution (35). These data strongly suggest that Cl⁻ binding to SRI is widely conserved among the SRI protein family, in contrast to SRII, and is assumed to be an important property for the functional differences between SRI and SRII. As reported, the H131F mutant did not alter the absorption maximum and photocycle upon addition of Cl⁻, and the λmax of the mutant (533 nm) was similar to that of Cl⁻ free SrSRI (544 nm) (Figure 4), indicating that His131 of SrSRI participates in the Cl⁻ binding site (32). The residual shift from 544 to 533 nm suggests that His131 itself is also involved in the color tuning. Although the values were different from those of the WT, similar Cl⁻ effects on
λmax and the M-decay were also observed in I19T, G165V, V113A, A136Y and A200T (Table 1 and Figure 4), indicating the Cl- binding to the mutant proteins. Thus the spectral blue-shifts of the A136Y and A200T mutants are not caused by the lack of their Cl- binding ability.

Absorption spectra of multiple mutants containing H131F, A136Y and A200T - To further characterize the effects of these residues, double and triple mutants of SrSRI were constructed, and the absorption spectra were measured (Figure 5). These multiple SrSRI mutants contain more than 90% all-trans retinal with a small proportion of 13-cis retinal, similar to SrSRI and SRII (Table 1), indicating that the spectral blue-shift is not caused by a change in retinal configuration. The three double mutants, H131F/A136Y, H131F/A200T and A136Y/A200T, had their λmax at 530, 529 and 531 nm, respectively. The lowest panel in Figure 5 shows the absorption spectrum of the triple mutant (H131F/A136Y/A200T) where the λmax is located at 525 nm. The shift of the triple mutant from WT SrSRI was 1094 cm⁻¹, which corresponds to half of the difference between SRII and SRI (2127 cm⁻¹). The absorption spectrum of the triple mutant could not be obtained in the absence of Cl⁻ because of its inactivation. This triple mutant does not have the vibrational fine structure seen in SRII (spectral shoulder at 460 nm in SRII seen in Figure 6), indicating that the retinal molecule is not restricted, according to the interpretation of fine structures by Takahashi et al. (22). We should, however, be aware that it seems that the shoulder of the vibrational fine structure of SRII disappears when the main absorption band shifts to longer wavelengths (23-25).

The inverse mutations of SRII - To further investigate the role of these residues, the inverse mutants of SRII were constructed and the absorption spectra shown in Figure 6 were measured in the same buffer condition. For the F134H, Y139A and T204A single mutants, the λmax were 502, 499 and 507 nm, respectively, which are 4 nm (160 cm⁻¹), 1 nm (40 cm⁻¹) and 9 nm (356 cm⁻¹) red-shifted from the λmax of the wild-type SRII. Furthermore, for the triple mutant (F134H/Y139A/T204A) the λmax showed a relatively large red-shift of 12 nm (472 cm⁻¹). This shift (472 cm⁻¹) is smaller than in the case of SrSRI (1094 cm⁻¹). We measured the absorption spectra of the wild-type, and the F134H and F134H/Y139A/T204A mutants of SRII in the presence (dotted lines) and absence (solid lines) of Cl⁻ (supplemental Fig S3). Essentially no shift of λmax was observed, indicating that Cl⁻ does not bind to SRII even in the mutants, and that other residue(s) are involved in the Cl⁻ binding in SRI in addition to His131.

DISCUSSION

In this study, a variety of SrSRI mutants were constructed to examine effects on λmax, including 9 single substitutions (I19T, F53L, V113A, H131F, A136Y, G165V, L166V, Y209L and A200T), 3 double substitutions (H131F/A136Y, H131F/A200T and A136Y/A200T) and 1 triple substitution (H131F/A136Y/A200T). The absorption spectra of mutants with a single substitution revealed that 3 of them (His131, Ala136 and Ala200) are involved in the spectral tuning in SrSRI (Figure 2). His131 forms a Cl⁻ binding site and affects the absorption maximum (Figure 4) as reported previously (32), while Ala136 and Ala200 are newly identified color tuning residues.

Computer model (supplemental Fig. S4) shows the positions of the three substituted residues relative to the all-trans retinal chromophore. This model was generated by only changing the positional numbers of the marked amino acids to those in SrSRI in the previously reported theoretical 3-D structural model of SRI from Halobacterium salinarum (HisSRI) (36). The residues important for the color tuning are located at helices E (His131 and Ala136) and G (Ala200). This is consistent with a previous mutational and chimeric analysis of SRII reported by Shimono et al. (25), which revealed the involvement of helices D, E, F and G in the color tuning of SRII.
In the case of the A200T mutant, a 17 nm blue-shift was observed. The position of Ala200 in S/SRI corresponds to Thr204 in SRII and to Ala215 in BR, a neighbor of the Schiff base (supplemental Fig. S4). The replacement of Thr204 in SRII by alanine and of Ala215 in BR by threonine caused a 9 nm red-shift from 498 to 508 nm (Figure 6) and a 10 nm blue-shift from 560 to 550 nm (37), respectively. The importance of Thr204 in SRII was also reported in theoretical studies (38,39). Thus, the addition of the hydroxyl group in SRI may change the electrostatic field near the Schiff base, which might cause the ground state to become more stable. As reported previously, Thr204 in SRII is important not only for the color tuning, but also for the negative phototaxis function of SRII (40), which suggests it is an important property for the functional differentiation between SRI and SRII.

Ala136 in S/SRI is a novel color-tuning residue. In the predicted structural model for HsSRI, Ala136 orients its side chain towards the transmembrane region (supplemental Fig. S4). In fact, the side chains of the corresponding residues of BR and SRII in the X-ray crystal structures show outward-facing conformations (41-43), and the Y139A mutant of SRII shows only a 1 nm spectral shift (Figure 6). Interestingly, the A136Y mutant of S/SRI affects not only the visible spectrum (~14 nm), but also the photocycle (Figure 3b), whereas the inverse mutation (Y139A) of SRII shows only a 10 nm spectral shift (Figure 6). In the latter case, the environment of Ala178 is distant from the retinal chromophore (~25 Å), the molecular mechanism of the unusual mutation effect on color tuning is intriguing. Thus the shift is caused by a long-range effect in some indirect manner. The Ala136 of S/SRI is relatively far from the chromophore compared to Ala200 and His131 (supplemental Fig. S4), which suggests some long-range effect(s).

In the case of the H131F mutant, a 24 nm blue-shift including the Cl\(^-\) binding effect was observed as reported previously (32). Similar Cl\(^-\) effects on \(\lambda_{\text{max}}\) and the M-decay were also observed in the cases of the A136Y and A200T mutants (Table 1 and Figure 3) but not in the mutants having the H131F mutation. Thus, the Cl\(^-\) sensitivity is a phenomenon independent of the role of Ala136 and Ala200 on the color tuning.

If the effects of these three amino acid residues, His131, Ala136 and Ala200, are additive (sum of the shifts of single mutants; 1836 cm\(^{-1}\)), the replacements of the three residues may be sufficient to explain the difference of \(\lambda_{\text{max}}\) between SRI and SRII (2127 cm\(^{-1}\)). However, the opsin shifts of the multiple mutants, H131F/A136Y, H131F/A200T, A136Y/A200T and H131F/A136Y/A200T are 914, 879, 950 and 1094 cm\(^{-1}\), respectively, which are smaller than the sum of the shifts of the single mutants (1271, 1028, 1373 and 1836 cm\(^{-1}\)) (Table 1 and Figure 5). This indicates that His131, Ala136 and A200T are responsible for the color tuning by an overlapping effectiveness including the stabilization of the ground state and the destabilization of the excited state. It can be assumed that this lack of additivity can be explained by similar effects as stated in the introduction for the
spectral tuning in organic solvents, such as the alteration of the strength of the electrostatic interaction or of the polarity/polarizability, isomerization around the 6-S bond in the vicinity of the retinal chromophore. The overlapping effectiveness and roles of His131, Ala136 and Ala200 of SrSRI would be further investigated in the future by structural and theoretical studies.

In the inverse triple mutant of SRII, the \( \lambda_{\text{max}} \) shifts from 498 to 510 nm, and the shift value (472 cm\(^{-1}\)) is much smaller than in the case of SrSRI (1094 cm\(^{-1}\)). However, as mentioned above, the independently caused shift of \( \lambda_{\text{max}} \) by Cl\(^-\) binding has to be taken into account. On the basis of the results of SrSRI, the expected shift value of SRII by Cl\(^-\) binding is approximately ~430 cm\(^{-1}\). The sum of these values (430+472=902 cm\(^{-1}\)) is similar to the difference in the value between the triple mutant of SrSRI and SRII (1033 cm\(^{-1}\)). Another possibility is the effect of other unconserved residues in the amino acid sequences of sensory rhodopsins (Figure 1). Further studies will give us a better understanding of the color tuning.

Acknowledgement - We thank Yukie Kawase for technical assistance in sample preparation. We also thank Dr. Kunio Ihara for his encouragement of the study.

REFERENCES
Sci U S A 102, 6879-6883
Biophys Acta 1515, 92-100
30. Sudo, Y., Yamabi, M., Kato, S., Hasegawa, C., Iwamoto, M., Shimono, K., and Kamo,
32. Suzuki, D., Furutani, Y., Inoue, K., Kikukawa, T., Sakai, M., Fujii, M., Kandori, H.,
Biol 291, 899-911

FOOTNOTES

*This work was financially supported in part by a Grants-in-Aid for Scientific Research (KAKENHI) on a Priority Area (Area No. [477]) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. This work was also supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology to MH and to YS.

1The abbreviations used are: SRI, Sensory rhodopsin I; SRII, Sensory rhodopsin II; SrSRI, SRI from Salinibacter ruber; NpSRII, SRII from Natronomonas pharaonis; BR, bacteriorhodopsin; DDM, n-dodecyl-β-D-maltoside; HPLC, High Performance Liquid Chromatography; PSB, protonated Schiff base.

FIGURE LEGENDS

Fig. 1. Alignment of the putative amino acid sequences of SRIs and SRIIs reported so far. Names of archaeal and bacterial sensory rhodopsins and sources from the top are: SRU_2511(SrSRI), Salinibacter ruber strain DSM 13855 (3852586); SRU_2579, Salinibacter ruber strain DSM 13855(3852268); sop_Hs_F5R, Halobacterium salinarum strain Flx5R (L05603); sop_Hs_Gl, Hb. sp. strain GGI sR (X70290); sop_Hm, Haloarcula marismortui strain DSM 3752 (Q5UXM9); sop_Hv (HvSRI), Haloarcula vallismortis strain DSM 3756 (Q48334); sopII_Np (NpSRII), Natoronomonas pharaonis sensory rhodopsin II (P15647); sopII_Hl, Halorubrum lacusprofundi strain DSM 5036 (B9LQT9); sopII_Hs_F15, Halobacterium salinarum strain Flx5R (U62676); sopII_Hm, Haloarcula marismortui strain DSM 3752 (Q5V5V3). Amino acid residues marked with asterisks are the sites of attention in this study. Microbial rhodopsins, including SRI and SRII, have 7-transmembrane α-helices shown as A, B, C, D, E, F and G-helix.

Fig. 2. Visible absorption spectra of SRI mutants from S. ruber (SrSRI) with single residue substitutions. One division of the y-axis corresponds to 0.1 absorbance units. The retinal configurations of the mutants determined by HPLC are indicated in each panel, as is the absorption maximum. The dotted lines show the absorption spectrum of WT SrSRI for comparison. The solutions contained 1 M NaCl and 0.05% DDM with pHs adjusted to 7.0 with 50 mM Tris-HCl. The putative secondary structure of SrSRI is shown in the middle of the figure. The membrane normal is roughly in the vertical plane of this figure, and the top and bottom regions correspond to the cytoplasmic and extracellular sides, respectively. The white letters indicate the highly conserved residues among microbial rhodopsins, Asp72 and Lys201.

Fig. 3. a) Chromophore configurations extracted from WT, V113A, H131F, A136Y, L166V and A200T mutants of SrSRI. The detection beam was set to 360 nm. Ts, Ta, 13s and 13a stand for all-trans 15-syn retinal oxime, all-trans 15-anti retinal oxime, 13-cis 15-syn retinal oxime and 13-cis 15-anti retinal oxime, respectively. The molar composition of each retinal isomer was calculated from the areas of the peaks in the HPLC patterns, and the estimated molar compositions are listed in Table 1. b) Flash-induced kinetic data of the WT, and the H131F,
A136Y and A200T mutants of SrSRI at 390 or 380 nm representing the M-decay, and at 550, 530 or 540 nm representing the recovery of the original SrSRI. Gray lines show the fits of the curves with single exponential equations. The temperature was kept at 25°C.

Fig. 4. Chloride effects on the absorption maximum and the photocycle of the WT, and the H131F, A136Y and A200T mutants of SrSRI. a) Absorption spectra of SrSRI with 1 M NaCl (dotted line) and without NaCl (solid line) over the spectral range from 480 to 600 nm. The ionic strength was kept constant using 333 mM Na₂SO₄ at pH 7.0 adjusted with 50 mM Tris-H₂SO₄. The samples contain 0.05% DDM as a detergent. b) Flash-induced kinetic data of SrSRI with 1 M NaCl (gray lines) or without NaCl (333 mM Na₂SO₄) (black lines) at 390 or 380 nm representing the M-decay, and at 550, 530 or 540 nm representing the recovery of the original SrSRI. The temperature was kept at 25°C.

Fig. 5. Visible absorption spectra of double and triple mutants of SrSRI. The experimental conditions are the same as described for Figure 3. Dotted lines show the absorption spectrum of the WT SrSRI. In this lowest panel, the absorption spectra of WT SRII from *Natronomonas pharaonis* and of WT SRI from *Salinibacter ruber* obtained in the same buffer solution as the triple mutant (1 M NaCl, 50 mM Tris-HCl, pH 7.0 and 0.05% DDM) are shown in the figure for comparison.

Fig. 6. Visible absorption spectra of the SRII mutants from *N. pharaonis*. The experimental conditions are the same as described for Figure 3. Dotted lines show the absorption spectrum of the WT SRII for comparison.

Fig. 7. The diagram of the spectral changes of the mutants of SRI and SRII. From the right, the absorption maxima of WT SrSRI, I19T, Y209L, F53L, G165V, L166V, V113A, A136Y, A200T, H131F, A136Y/A200T, H131F/A136Y, H131F/A200T and H131F/A136Y/A200T were plotted. From the left, the absorption maxima of WT SRII, Y139A, F134H, T204A, F134H/T204A and F134H/Y139A/T204A were plotted. We assumed that the difference between H131F/A136Y/A200T of SRI and F134H/Y139A/T204A of SRII is caused by Cl⁻ binding.
Table 1. Absorption maximum, opsin shift, retinal configuration of the WT and of various SrSRI mutants, M-decay rate and effect of the Cl⁻ ion. ND, not determined.

<table>
<thead>
<tr>
<th>Opsin type</th>
<th>( \lambda_{\text{max}} ) [nm]</th>
<th>( \Delta \nu ) [cm(^{-1})]</th>
<th>all-trans [%]</th>
<th>Cl [cm(^{-1})]</th>
<th>Cl effect on M-decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (SrSRI)</td>
<td>557</td>
<td>-</td>
<td>98.2</td>
<td>430</td>
<td>Yes</td>
</tr>
<tr>
<td>I19T</td>
<td>557</td>
<td>0</td>
<td>98.3</td>
<td>463</td>
<td>Yes</td>
</tr>
<tr>
<td>Y209L</td>
<td>555</td>
<td>65</td>
<td>91.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F53L</td>
<td>553</td>
<td>130</td>
<td>93.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G165V</td>
<td>553</td>
<td>130</td>
<td>96.7</td>
<td>333</td>
<td>Yes</td>
</tr>
<tr>
<td>L166V</td>
<td>550</td>
<td>228</td>
<td>92.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>V113A</td>
<td>549</td>
<td>262</td>
<td>95.8</td>
<td>304</td>
<td>Yes</td>
</tr>
<tr>
<td>A136Y</td>
<td>543</td>
<td>463</td>
<td>96.3</td>
<td>241</td>
<td>Yes</td>
</tr>
<tr>
<td>A200T</td>
<td>540</td>
<td>565</td>
<td>97.5</td>
<td>243</td>
<td>Yes</td>
</tr>
<tr>
<td>H131F</td>
<td>533</td>
<td>808</td>
<td>93.5</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>A136Y/A200T</td>
<td>531</td>
<td>879</td>
<td>90.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H131F/A136Y</td>
<td>530</td>
<td>914</td>
<td>91.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H131F/A200T</td>
<td>529</td>
<td>950</td>
<td>91.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H131F/A136Y/A200T</td>
<td>525</td>
<td>1094</td>
<td>90.1</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>SRII (pR)</td>
<td>498</td>
<td>2127</td>
<td>94.5</td>
<td>40</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 2. Absorption maximum, opsin shift, and retinal configuration of the WT and of various SRII mutants. ND, not determined.

<table>
<thead>
<tr>
<th>Opsin type</th>
<th>( \lambda_{\text{max}} ) [nm]</th>
<th>( \Delta \nu ) [cm(^{-1})]</th>
<th>all-trans [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (SRII)</td>
<td>498</td>
<td>-</td>
<td>94.5</td>
</tr>
<tr>
<td>Y139A</td>
<td>499</td>
<td>40</td>
<td>98.6</td>
</tr>
<tr>
<td>F134H</td>
<td>502</td>
<td>160</td>
<td>98.4</td>
</tr>
<tr>
<td>T204A</td>
<td>507</td>
<td>356</td>
<td>95.9</td>
</tr>
<tr>
<td>F134H/T204A</td>
<td>508</td>
<td>395</td>
<td>97.2</td>
</tr>
<tr>
<td>F134F/Y139A/T204A</td>
<td>510</td>
<td>472</td>
<td>93.8</td>
</tr>
<tr>
<td>SrSRI</td>
<td>557</td>
<td>2127</td>
<td>98.2</td>
</tr>
</tbody>
</table>
FIGURE 1

(A-helix)  *119T
(B-helix)

F53L *

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)

G165V  

(A-helix)  

SRI

SRI

V113A *

H131F  *  A136Y *(E-helix)
FIGURE 2

[Graphs and diagrams showing wavelength and absorption values for different genotypes.

- WT
- [1] L19T
- [4] H131F
- [9] Y209L
- [2] F53L
- [3] V113A
- [7] L166V
- [8] A200T]
FIGURE 4

(a) Absorbance vs. Wavelength [nm]

(b) Absorbance change [mOD] vs. Time [s]
FIGURE 5

Absorbance [OD]

WT

H131F/A136Y

H131F/A200T

A136Y/A200T

Absorbance [OD]

H131F/A136Y/A200T

SR1

SR1I

Wavelength [nm]
FIGURE 6