BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Influence of Halide Binding on the Hydrogen Bonding Network in the Active Site of *Salinibacter* Sensory Rhodopsin I

Louisa Reissig,[†] Tatsuya Iwata,[‡,§] Takashi Kikukawa,[#] Makoto Demura,[#] Naoki Kamo,[∥] Hideki Kandori,[‡] and Yuki Sudo*[,†,⊥]

[†]Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, 464-8602, Japan

[‡]Department of Frontier Materials, Nagoya Institute of Technology, Showa-ku, Nagoya, 466-8555, Japan

[§]Center for Fostering Young and Innovative Researchers, Nagoya Institute of Technology, Showa-ku, Nagoya, 466-8555, Japan

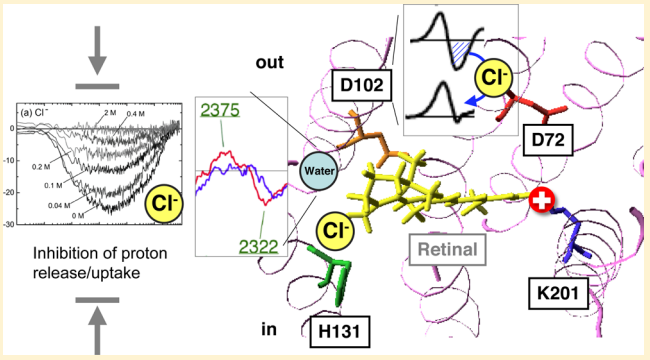
[#]Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810, Japan

[∥]College of Pharmaceutical Sciences, Matsuyama University, Matsuyama, Ehime 790-8578, Japan

[⊥]PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho Kawaguchi, Saitama, 332-0012, Japan

Supporting Information

ABSTRACT: In nature, organisms are subjected to a variety of environmental stimuli to which they respond and adapt. They can show avoidance or attractive behaviors away from or toward such stimuli in order to survive in the various environments in which they live. One such stimuli is light, to which, for example, the receptor sensory rhodopsin I (SRI) has been found to respond by regulating both negative and positive phototaxis in, e.g., the archaeon *Halobacterium salinarum*. Interestingly, to date, all organisms having SRI-like proteins live in highly halophilic environments, suggesting that salt significantly influences the properties of SRIs. Taking advantage of the discovery of the highly stable SRI homologue from *Salinibacter ruber* (*Sr*SRI), which maintains its color even in the absence of salt, the importance of the chloride ion for the color tuning and for the slow M-decay, which is thought to be essential for the phototaxis function of SRIs, has been reported previously [Suzuki, D., et al. (2009) *J. Mol. Biol. 392*, 48−62]. Here the effects of the anion binding on the structure and structural changes of SRI during its photocycle are investigated by means of Fourier transform infrared (FTIR) spectroscopy and electrochemical experiments. Our results reveal that, among other things, the structural change and proton movement of a characteristic amino acid residue, Asp102 in *Sr*SRI, is suppressed by the binding of an anion in its vicinity, both in the K- and M-intermediate. The presence of this anion also effects the extent of chromophore distrotion, and tentative results indicate an influence on the number and/or properties of internal water molecules. In addition, a photoinduced proton transfer could only be observed in the absence of the bound anion. Possible proton movement pathways, including the residues Asp102 and the putative Cl binding site His131, are discussed. In conclusion, the results show that the anion binding to SRI is not only important for the color tuning, and for controlling the photocycle kinetics, but also induces some structural changes which facilitate the observed properties.



Salinibacter ruber is a rod-shaped, motile eubacterium, which requires a high salt concentration (above 150 g/L) for growth.[1] It was the first well studied halophilic eubacterium[1,2] and was successfully isolated in 2002 from crystallizer ponds of the salterns of Santa Pola in Mallorca. This eubacterium was of great interest, as up until that point it was believed that only microorganisms from the archaeal domain play a significant role in the biology of highly salt-rich environments. Interestingly, *S. ruber* achieves its adaptation to life at high salt concentrations with a strategy similar to archaea of the order Halobacteriales. Its cell contains an extremely high concentration of K^+ and Cl^- ions in its cytoplasm, used to osmotically balance the high NaCl concentration in its surrounding medium. This is in contrast to other known halophilic and halotolerant aerobic bacteria, which show high concentration of organic osmotic solutes.[1,3] In accordance, the amino acid composition of its bulk protein has a high content of acidic amino acids and serine, while a low abundance of basic or hydrophobic amino acids, necessary for protein solubility at such high ionic strengths.[1,4] In contrast, its membrane lipid composition, which is modified to ensure membrane integrity at high salt concentrations, is more typical of Bacteria, with complex adaptive changes in both headgroup and acid composition in response to changes in salinity.[1]

Received: July 18, 2012
Revised: October 12, 2012
Published: October 14, 2012

ACS Publications © 2012 American Chemical Society

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

One of the most striking features of many halophilic organisms is their red color. As early as 2700 B.C., a red coloring of lakes or rivers was reported which was triggered by the increasing salt concentration upon evaporation of water. This color has been shown to originate from dense communities of halophilic microorganisms, which are often pigmented.[3] Some of these pigments were found to be of great interest, and among them, the most studied ones are the rhodopsin proteins having retinal as their chromophore, which is bound to a conserved lysine residue in the protein *via* a protonated Schiff base linkage. After excitation by light a photoisomerization of the retinal chromophore from all-*trans* to 13-*cis* initializes the photocycle of the rhodopsin protein, which is followed by an array of structural relaxations and a proton transfer from the Schiff base to its counterion at a pH higher than the pK_a of this counterion.[5−7] The architecture of all such microbial rhodopsins (type 1 rhodopsins) comprises seven transmembrane alpha helices but is only of slight similarity to type 2 rhodopsins, which can be found in, e.g., the vision of vertebrates.[8] Despite their high similarity in structure, microbial rhodopsin molecules can perform a range of function, which can be, in general, divided into two main groups: (1) light-driven ion transporters and (2) photosensors. The most studied ion transporter is the proton pump bacteriorhodopsin (BR), which was discovered in the 1970s[9] and creates a proton electrochemical potential used for, e.g., the ATP synthesis in the archaeon *Halobacterium salinarum*.[5−7] In contrast, sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII) from *H. salinarum* are involved in light sensing for phototaxis, attracting the cells to light of such wavelengths which can activate, e.g., ion transporters, while avoiding harmful radiation, such as near-UV light.[10] It should be noted that SRI and its homologous proteins (e.g., SRI-like proteins from *S. ruber* (*Sr*SRI) and *H. vallismortis* (*Hv*SRI)) have, so far, all been isolated from highly halophilic organisms, and salt has been shown to affect their properties.[11]

The genome of *S. ruber* was shown to encode four rhodopsin molecules,[4] and from the sequence similarity, two of those were suggested to be SRI-like proteins and the others a putative chloride pumping rhodopsin like halorhodopsin (HR) and a proton pumping rhodopsin (named xanthorhodopsin, XR).[4,12] XR has been shown to be expressed in *S. ruber* cells, and this unique light-harvesting complex which uses a carotenoid antenna (salinixanthin) to extend its absorption range, has been well characterized in the past few years.[12−14] In 2008 we succeeded in cloning and expressing one of the SRI-like proteins (SRU_2511 or *Sr*SRI) as a recombinant protein in *Escherichia coli* cells,[15] enabling not only the studies of mutant proteins, but also due to its high stability, even in the absence of salt, the preparation and study of large amounts of protein. Thus, *Sr*SRI as well as its complex with its transducer protein have been well characterized over the last years,[11,15−20] though the function of *Sr*SRI has never been demonstrated in *Salinibacter* cells because of the lack of the functional assay. Therefore, at this stage, *Sr*SRI should be called a putative sensory rhodopsin I protein.

The peptide sequence of *Sr*SRI is distantly related to the well-characterized SRI from *H. salinarum* (*Hs*SRI) (~40% identity) and contains most of the amino acid residues identified as necessary for its function of *Hs*SRI.[15] In addition, *Sr*SRI has a putative chloride binding site at the His131 residue which is located close to the β-ionone ring of the retinal chromophore and which is conserved among SRIs.[10,11] This has been based on (i) the absorption spectrum of *Sr*SRI being altered by the anion binding in direction (red shift) which could be explained by the location of His131 close to the β-ionone ring, (ii) the effects of the anion binding to *Sr*SRI being completely suppressed by the H131A and H131F mutations.[11] Similar results have been obtained in another SRI-like protein *Hv*SRI.[21] The binding affinity of anions depends on the environment of the protein and follows the Hofmeister series, with, e.g., a K_m of 0.31 M for Cl^- in DDM micelles.[11] Upon binding with salt the absorption maximum of *Sr*SRI is red-shifted from about 542 to 558 nm depending on the protein environment. Salt has been shown to not only influence the absorption maximum of the respective protein forms but also the lifetimes of the intermediates, which suggests that the incorporation of the Cl^- ion induces some structural changes which facilitate the observed properties.[11]

The structural changes upon formation of the K-intermediate and the M-intermediate have been previously studied only in the absence of salt.[16,18] The results confirmed the proton transfer between the Schiff base and its counterion Asp72 upon formation of the M-intermediate. Interestingly, this has been shown in 2011 to be accompanied by, for sensory rhodopsin I proteins unique, proton release from the widely conserved aspartic acid residue Asp102, which is located close to the β-ionone ring of the retinal chromophore, and therefore, also close to the chloride binding site His131. Furthermore, the mutation of Asp102 to Glu102, i.e., making the residue slightly longer, shows a structural perturbation of the cysteine residue Cys130 which is located next to the chloride binding site.[18] All these residues are located around the otherwise hydrophobic β-ionone ring. Therefore, the binding of chloride is also expected to attract water molecules, or to change the hydrophobic character of this environment. Already, in the absence of salt, the structure and the structural changes of water molecules have been reported.[16]

In this study, the influence of salt on the structure and structural changes during the photocycle of *Sr*SRI were studied using low temperature FTIR spectroscopy. Although the scattering of the sample hampers the experiment, we succeeded in the preparation of highly stable films of *Sr*SRI reconstituted in PG liposomes with an ion binding of nearly 80%. Among other things, it could be shown that the binding of ions, such as Cl^-, Br^- and NO_3^-, suppresses the unique change in protonation state of Asp102 upon formation of the M-intermediate. In addition the results indicate the existence of new water molecules in the presence of anions, as expected, as such water molecules would occupy sites inside the protein that may be energetically unfavorable, such as the mainly hydrophobic β-ionone ring region, close to which the ion binding site lies. Furthermore, using indium tin oxide (ITO) electrodes, the movements of protons in *Sr*SRI were studied, which were greatly affected by the presence of ions in the medium. These results allowed us to estimate the affinities of a range of anions to the protein reconstituted into liposomes, and to identify the contribution of some charged residues in the protein moiety to the proton transfer reactions. Thus, we concluded that the anion binding on SRI is important not only for the color tuning and for controlling the photocycle kinetics, but also for the maintenance of the structure and structural changes. The implications are discussed.

MATERIALS AND METHODS

Sample Preparation. The wild-type and D102E mutant expression plasmids were constructed as previously described.[15,18] The preparation of crude membranes and the purification of the proteins were performed using essentially the same method as previously described.[17] In short, proteins which have a six-histidine tag at the C-terminus were expressed in *E. coli* BL21 (DE3) cells as recombinant proteins (*Sr*SRI). They were solubilized by DDM and purified using a Ni^{2+} affinity column. The samples were concentrated and exchanged using an Amicon Ultra filter (Millipore, Bedford, MA, USA), and the purified proteins were reconstituted into PG or PC liposomes (*Sr*SRI:PG or *Sr*SRI:PC molar ratio of 1:50), from which DDM was removed with Bio-Beads (SM-2, Bio-Rad). The PG-reconstituted samples were washed three times with a 2 mM phosphate buffer (pH 7.0) of a range of salt concentration (NaCl 0 mM, 50 mM, 100 mM or 200 mM, NaBr and $NaNO_3$ 100 mM, Na_2SO_4 33 mM). The PC-reconstituted samples were washed several times with distilled water to remove all salts and buffering agents, and then they were suspended in the final experimental media.

UV−Visible Spectroscopy. UV−visible spectra of the samples in solution were recorded using a UV2450 spectrophotometer with an ISR2200 integrating sphere (Shimazu, Kyoto, Japan) at 25 °C. UV−visible spectra of the films used for the FTIR measurements were recorded using a V-550DS (JASCO) spectrometer at room temperature ($T \approx 22$ °C). For baseline correction, a clean BaF_2 disk was used. The spectra were recorded in the dry and hydrated state. No difference was found for spectra recorded before or after the FTIR measurements (once the salt binding was completed; see UV−vis results section), and the films were stable over several days. The spectra were normalized for comparison.

FTIR Spectroscopy. FTIR spectroscopy was performed with 2-cm^{-1} resolution, as described previously.[16,18] A droplet of each sample of about 60 μL, with a protein concentration of about 10 mg/mL, was dried under a vacuum onto a BaF_2 window with a diameter of 18 mm, resulting in a homogeneous circular film of a diameter of about 8 mm. This film was hydrated with a trace of H_2O, D_2O or $D_2{}^{18}O$, sealed and inserted into a Bio-Rad FTS-7000 spectrometer, which was equipped with an Oxford Optistat-DN cryostat. In the case of D_2O and $D_2{}^{18}O$ the hydration process was repeated at least two times to ensure sufficient exchange against H_2O.

For the measurement of the K-intermediate, the sample was cooled to equilibrate at 77 K. The sample was illuminated with light of $\lambda \approx 450$ nm (filter KL45, AGC Techno Glass, Japan) for 2 min, and light of $\lambda > 600$ nm (filter R62, AGC Techno Glass, Japan) for 1 min to convert the sample to $SrSRI_K$ and *Sr*SRI, respectively.[16] In the case of the M-intermediate, the sample was stabilized at 260 K. By irradiating the sample with light of $\lambda > 500$ nm (filter Y52, AGC Techno Glass, Japan) through a glass filter (AGC Techno Glass Y-52, Japan) for 2 min, the sample was converted to $SrSRI_M$, after which a subsequent 1 min illumination with UV light changed $SrSRI_M$ back to *Sr*SRI.[16,18] In both cases, after cooling the samples to the temperature of the measurement, the sample was left for over 1 h to stabilize until the baseline was of sufficient quality (see Results).

The difference spectrum was calculated by subtracting the obtained spectrum (128 interferograms) after the illumination from the one measured before the illumination. To increase the quality of the spectra, 24 difference spectra of $SrSRI_M$ minus *Sr*SRI or $SrSRI_K$ minus *Sr*SRI were averaged, removing the ones with strong baseline fluctuations (about two spectra per measurement). All spectra were normalized with respect to the C−C or C═C stretching vibration of the retinal chromophore. In between the measurements, the films were stored in a fridge in the absence of light. The films did not show any change in signal for several days, and the results could be reproduced using separately prepared films/samples.

To increase the quality of the analysis of the spectra in the region associated with internal water molecules (see Figure 4 and Figures SI3 and SI4), an additional detailed analysis, as previously shown to be applicable by ref 22, was performed.

ITO Measurements. To investigate the movement of protons in *Sr*SRI, a photoelectrochemical cell was used.[23,24] The ITO electrode has been shown to work as a time-resolving pH electrode. Because of the weak attachment of the *Sr*SRI proteins reconstituted in PG lipids to the ITO surface, *Sr*SRI reconstituted in PC was used in this measurement. The sample was washed several times with distilled water to remove all salts and buffering agents. It was then diluted with distilled water until the absorbance at 550 nm, originating from *Sr*SRI, was reduced to about 0.1. A droplet of 100 μL of this suspension was placed onto an ITO surface and dried under weakly reduced pressure, resulting in a sample of about 10 mm in diameter. The membrane suspension was washed by water to remove any weakly bound parts. The ITO disk was placed into the electrochemical cell, into which a buffer solution was added. The pH of the used buffer consisting of a mixture of six buffering agents (0.89 mM citric acid, 0.89 mM MES, 1.07 mM TES, 0.78 mM TAPS, 1.11 mM CHES, 0.33 mM CAPS) was adjusted with H_2SO_4 and NaOH. The sample was illuminated with a 7 ns laser pulse (532 nm, 0.6 mJ/pulse) from a Q-switched Nd:YAG laser (Minilite I, Continuum), and the ITO signal was measured in the μs-s time range after the illumination by using a homemade amplifier (response time ~ 20 μs) which was equipped with a 0.033 Hz low cut filter for the elimination of the baseline drift. To improve the S/N ratio, 30−100 laser pulses were used. The time course was measured for different salt concentrations and different pHs. After any changes in the buffer solution, the sample was left for a few minutes to equilibrate. All ITO measurements were performed at room temperature. The samples did not show any change in signal for several days, and the results could be reproduced using separately prepared samples.

RESULTS

Influence of the Film Formation on the Absorption Properties of *Sr*SRI. In the FTIR measurements performed to study the structural changes of *Sr*SRI upon illumination, a good stability of the hydration state of the films is required, which was reported to be made difficult by the addition of salt. The estimated K_m values of *Sr*SRI have been reported to be on the order of 0.1−1 M,[11] depending on the anion type and the protein environment. Thus, high salt concentrations are required in solution to ensure full ion binding. However, in the case of a film condensed onto a BaF_2 window, the ion binding was greatly increased (see Figure 1), which could be explained by the effective increase in salt concentration upon evaporation of water. Therefore, the experiments could be carried out with sample solutions of salt concentrations of about 100 mM, which resulted in fairly stable films, in which a large degree of proteins bound an ion.

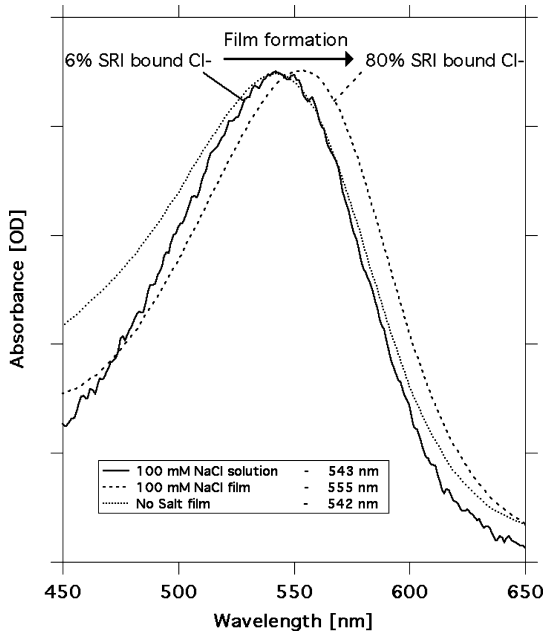


Figure 1. Absorption spectra of *Sr*SRI under various conditions. The film formation shifts the absorption maximum of *Sr*SRI from 543 to 555 nm, which was interpreted as an increase in the degree of ion binding from about 6% to about 80%.

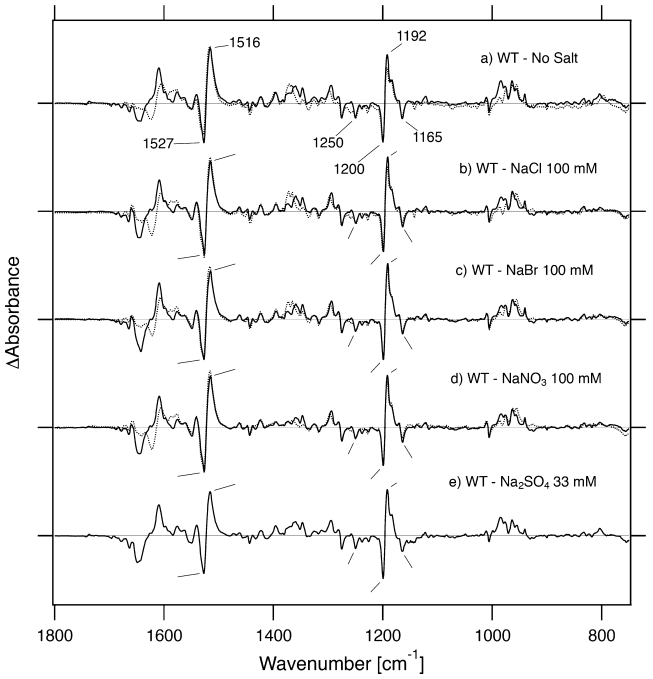


Figure 2. The dependence on the type of anion bound to *Sr*SRI of the changes in the $SrSRI_K$ minus *Sr*SRI difference infrared spectra, in the 1800−800 cm^{-1} region, for WT *Sr*SRI in the absence of salt (a), and in the presence of 100 mM NaCl (b), 100 mM NaBr (c), 100 mM $NaNO_3$ (d) and 33 mM Na_2SO_4 (e). The samples were hydrated with H_2O (solid line) or D_2O (dotted line), and measured at 77 K at pH 7.0. The spectra were normalized with respect to the C−C or C═C stretching vibration of the retinal chromophore. The spectra of *Sr*SRI hydrated with H_2O in the presence of NaCl, NaBr, $NaNO_3$ and Na_2SO_4 were multiplied by 1.5, 0.9, 0.9, and 1.4 for comparison, respectively. The spectra of *Sr*SRI hydrated with D_2O in the presence of NaCl and $NaNO_3$ were multiplied by 1.2 for comparison. The spectra in the absence of salt were reproduced from ref 16. One division of the *y*-axis corresponds to 0.008 absorbance units.

It should be noted that the increase in ion binding was also observed in the ITO experiments (see further Table 1 and its discussion), and therefore the deposition of *Sr*SRI reconstituted in liposomes onto a surface might also be an important factor. Furthermore, especially in the case of NaCl, the successive hydration, or longer equilibration times, were necessary to ensure complete ion binding, which is consistent with preliminary results in solution, which suggest this effect to be caused by the negative charge on the PG lipids (unpublished results). Once the samples had equilibrated, the UV−vis spectra were stable over prolonged time scales.

Interestingly, the ion binding seems to be greatest in films containing NaCl, compared to NaBr and/or $NaNO_3$ (see Figure SI1). The reduced shift in the absorption maximum is consistent with the FTIR results, in which the change in the spectrum, compared to the salt-free films, was most pronounced in the case of NaCl (see further Figure 2 and its discussion). Under these experimental conditions, the stability of the hydrated films was sufficient for the measurements of the K- and M-intermediate, especially the low temperature of the measurements for the K-intermediate facilitated the stability of the baseline in the region characteristic for water molecules (see Figure SI2).

Influence of Salt on the Structural Changes of *Sr*SRI during the Photocycle Studied by Low-Temperature FTIR Spectroscopy. The high stability of the films allowed the comparison of the structural changes upon formation of the K- and M-intermediate in the photocycle of *Sr*SRI in the presence and absence of a range of anions. All results were of high reproducibility, both for successive measurements after storage, as well as between separately prepared films/samples. It should be noted that the assignment of some tentatively assigned bands shown to be affected by the anion binding are the focus of the next study.

(1). K-Intermediate. Figure 2 shows the $SrSRI_K$ minus *Sr*SRI difference spectrum upon addition of 100 mM NaCl, $NaNO_3$ or NaBr (pH = 7.0, T = 77 K). To ensure that the changes in the spectra could be associated with the binding of salt to the protein, and not with changes in the ionic strength of the solution, a control experiment was performed in 33 mM Na_2SO_4, as sulfate ions have been reported to not affect the absorption spectrum and the photocycle kinetics of *Sr*SRI,[11] as well as SRI from *Haloarcula vallismortis* (*Hv*SRI).[21] The spectrum in the presence of sodium sulfate was, indeed, identical to the spectrum recorded in the absence of salt. The spectrum in the absence of salt is reproduced from ref 16, which could be repeated in the current experiment.

As shown in Figure 2, the changes in the 1800−700 cm^{-1} region were independent of the type of anion bound to *Sr*SRI. The only slight differences between the spectra could be attributed to baseline distortion as well as to the different degrees of ion binding in the samples, which could be, to a large extent, eliminated by subtracting a small percentage of the spectra in the absence of salt from that in the presence of salt.

The formation of the K-intermediate could be confirmed, both in the absence and presence of salt, by the appearance of the C−C stretching vibrations of the retinal chromophore at about 1250 (−), 1200 (−), 1192 (+) and 1165 (−) cm^{-1}, representing the retinal isomerization from the all-*trans* to 13-*cis* form. These bands were only slightly shifted by the addition of salt (<1 cm^{-1}) (see Figure 3A). The same is the case for the

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

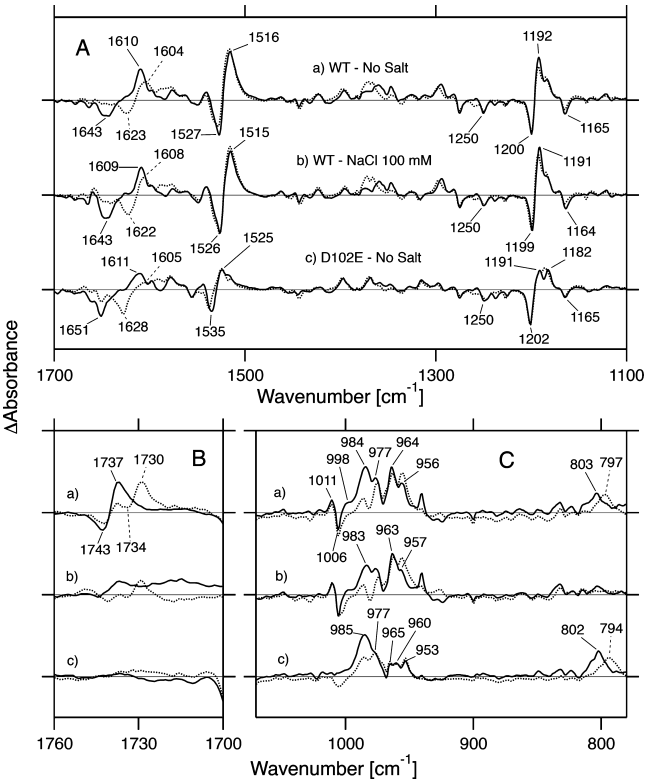


Figure 3. The effect of salt and of the D102E mutation on the *Sr*SRI$_K$ minus *Sr*SRI difference infrared spectra, in the 1700−1100 cm^{-1} region (A), the magnified 1760−1700 cm^{-1} region (B) and the 1070−780 cm^{-1} region (C). The spectra of WT *Sr*SRI were measured in the absence of salt (a) and in the presence of 100 mM NaCl (b), and that of the D102E mutant in the absence of salt (c). The samples were hydrated with H_2O (solid line) or D_2O (dotted line), and measured at 77 K at pH 7.0. The spectra of WT *Sr*SRI in the presence of NaCl were multiplied by 1.5 (1.2) in the cases of hydration with H_2O (D_2O). The spectra in the absence of salt were reproduced from ref 16. One division of the *y*-axis corresponds to 0.008 (a), 0.0004 (b), or 0.003 (c) absorbance units.

bands corresponding to the C=C stretching vibration of the retinal chromophore (1527 (−)/1516 (+) cm^{-1} in H_2O in the absence of salt). However, this small dependence on the binding of salt is surprising, as the observed shift between the original state and the K-intermediate is caused by the spectral red shift upon formation of the K-intermediate. This red shift has been reported to depend on the ion concentration (542 to 610 nm or 558 to 618 nm in the absence or presence of 1 M NaCl, respectively[11]), which was expected to result in a shift to smaller wavenumbers of ∼6 cm^{-1}. A similar reduced shift has been reported for proteorhodopsin,[25] in which the comparably small shift in the FTIR spectrum was explained by the reduction of the spectral red-shift at 77 K. Although the reason for the reduced shift is still unclear the amide-II vibrations of the peptide backbone might also make it difficult to recognize the proper position of the C=C stretch due to its overlapping.

Interestingly, a change in the spectrum around 1742 cm^{-1} could be observed (see Figure 3B) which was also the case in the M-intermediate (see further Figure 5). The bands in the absence of salt, which have been tentatively associated with Asp102 (1743 (−)/1737 (+) cm^{-1} in H_2O and 1734 (−)/1730 (+) cm^{-1} in D_2O),[16] were greatly reduced by the addition of salt. It should be noted that this band was also observed, in reduced intensity, in *Hs*SRI at alkaline conditions in the presence of 300 mM NaCl, as well as in *Hv*SRI in the absence of salt.[16,21]

To confirm the assignment of this band, the spectrum of the D102E mutant was measured (see Figure 3c). It should be noted that a spectrum of the D102N mutant was not measured as its low protein stability has been already previously reported to hamper the measurement, even in the absence of salt.[18] Also in the spectrum of the D102E mutant, the band associated with Asp102 disappears. However, instead of a disappearance of the band, a shift to shorter wavenumbers was expected, caused by the addition of the ethylene group to the protonated residue (as shown by the change in protonation state recorded in the M-intermediate).[18] The addition of the CH_2 group might have resulted in a slight geometry change, which reduced the effect on the glutamate vibration, especially at such low experimental temperatures. In general, the observed effect of the band previously associated with Asp102 by the D102E mutation supports this tentative assignment.[18] One should also note that this residue is conserved, to a large extend, in microbial rhodopsins such as BR or halorhodopsin (HR), and that similar bands have been observed in these proteins with similar band shapes upon formation of the K-intermediate.[26−28]

This mutation shows also extended changes on other parts of the spectrum, compared to the few changes upon formation of the M-intermediate,[18] similar to the trend observed upon the addition of salt (one should note that due to strong scattering of the films of the D102E mutant in the presence of salt, no detailed information of the effect of salt on the changes in the mutant could be obtained). Therefore, one can say that the structure, and structural changes, upon formation of the K-intermediate are affected by modifications close to the β ionone ring, despite the fact that the isomerization occurs around the Schiff base region, which is far from the β-ionone ring. Once the protein relaxes its structure to accommodate for the change in retinal conformation, the structural changes become again more similar. One should, however, note that the spectral blue shift from 540 to 529 nm, induced by the D to E mutation at position 102 of *Sr*SRI in the absence of salt,[18] resulted in a comparably large shift of the C=C stretching vibrations of the retinal to 1535 (−)/1525 (+) cm^{-1}.

Furthermore, the addition of salt to the sample could be shown to influence the tentatively assigned C=N stretching vibrations of the Schiff base (see Figure 3A). It is well-known that the difference in frequency between H_2O and D_2O is regarded as a marker of the hydrogen-bond strength of the Schiff base nitrogen. Thus, the decrease in the frequency difference of 6 cm^{-1} (1610−1604 cm^{-1}) in the K-intermediate to 1 cm^{-1} (1609−1608 cm^{-1}) in the presence of salt indicates that the binding of salt weakens the H-bond of the Schiff base in the K-intermediate. The observed changes in the presence of salt were similar to the spectra of *Hs*SRI in the presence of 300 mM salt, and of *Hv*SRI in the absence of salt, in which almost no shift in the K-intermediate has been observed.[16,21] The D102E mutation shows similar spectral features in the C=N vibrational range upon formation of the K-intermediate, with its hydrogen bond strength in the initial state being only slightly larger than that for the WT. It should be, however, noted that, for more precise conclusions, the spectra of samples having ^{15}N labeled Lys and/or C_{15}−H deuterated should be measured.

The influence of modifications around the β-ionone ring could also be seen in the region associated with the hydrogen-out-of-plane (HOOP) vibration of the retinal chromophore (see Figure 3C). On the basis of detailed studies on BR and

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

SRII,[29−34] the positive bands in the absence of salt have previously been assigned as follows:[16] the bands at 998, 984, and 977 cm^{-1} have been assumed to originate from the C15-HOOP vibrations, close to the Schiff base, while the bands at 963 and 956 cm^{-1} have been associated with the C7═C8 and C11═C12 Au HOOP vibrations, respectively. These vibrational features have been interpreted as showing that the structural changes spread to the middle part of the retinal chromophore, similar to those of other signaling rhodopsin molecules.[28] The band at 1011 cm^{-1} has been tentatively assigned to the symmetric rocking of the methyl groups connected to the C9 and C13 atoms of the retinal.[28] In the presence of salt, the positions of several bands were slightly shifted (±1 cm^{-1}). Furthermore, the intensity of the bands associated with the C15-HOOP modes, therefore the vibrations close to the Schiff base, was decreased. This, together with the disappearance of the band at 803 cm^{-1}, which was tentatively proposed to be associated with the wagging vibrations of the retinal,[16] suggests a stiffening of the retinal chromophore upon ion binding.

On the other hand, the D102E mutation led to a strong decrease of the vibrations associated with the middle part of the protein, together with the disappearance of the bands at 1012 (+) and 1206 (−) cm^{-1}, indicating that the structural changes of the mutant, upon formation of the K-intermediate, are localized close to the Schiff base region of the chromophore, similar to ion-pumping rhodopsins.[16,30] In the future more detailed studies of this spectral region are necessary to fully charaterize the changes induced to the chromophore.

In a next step, the influence of the binding of anions on the vibrations of water molecules was studied using D_2O and $D_2{}^{18}O$ hydrated samples (see Figure 4 and Figures SI3 and SI4). The spectrum in the absence of salt was reproduced from ref 16 for comparison. One should note that, in contrast to the low frequency region (Figure 2), a dependence on the type of anion could be observed. However, a more detailed study of this anion dependency is needed, combined with additional isotope labeling and the study of mutants, for a complete assignment of the observed bands. One should also note that the spectrum of the sample containing NaBr was of poor quality in this region, due to structuring in the broad water peak (see Figure SI5).

In the absence of salt, a number of isotope dependent bands (exhibiting a spectral downshift of ∼10 cm^{-1}), which can, therefore, be assumed to originate from O−D stretching vibrations of water molecules, have been proposed[16] (light blue bands). Upon addition of NaCl or $NaNO_3$, an additional water band pair could be identified (2375 (+)/ 2322 (−) and 2384 (+)/ 2322 (−) cm^{-1} in the presence of NaCl or $NaNO_3$, respectively). Some of the bands seem to be affected by the type of anion used, suggesting that those water bands are originating from water molecules whose vibrations are directly affected by the anions. The appearance and/or alteration of internal water molecules in the presence of salt can be expected, as the anion would need to be hydrated in the otherwise hydrophobic region close to the β-ionone ring of the retinal chromophore. In the future a more detailed study of the water region is planned to confirm their precise assignment.

In this region, also the isotope independent bands previously tentatively assigned to the O−D stretch of Thr76 (2526 (−)/ 2467 (+) cm^{-1} in the absence of salt) and to the N-D stretches of the Schiff base (2066 (−)/ 2045 (−) cm^{-1} in the absence of salt) can be found.[16]

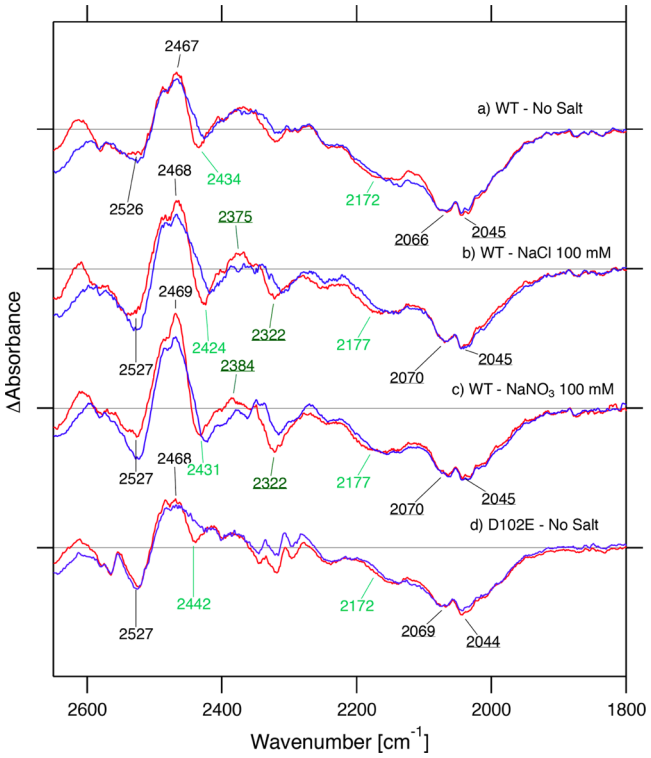


Figure 4. Indications of the appearance of new vibrations associated with bound water molecules upon addition of salt in the *Sr*SRI$_K$ minus *Sr*SRI difference infrared spectra, in the 2700−1800 cm^{-1} region, for WT *Sr*SRI in the absence of salt (a), and in the presence of 100 mM NaCl (b), and 100 mM $NaNO_3$ (c) and, for comparison, for the D102E mutant in the absence of salt (d). The samples were hydrated with D_2O (red line) or $D_2{}^{18}O$ (blue line) and measured at 77 K at pH 7.0. The spectra were normalized with respect to the C−C or C═C stretching vibration of the retinal chromophore. The spectra of *Sr*SRI hydrated with D_2O ($D_2{}^{18}O$) in the presence of NaCl and $NaNO_3$ were multiplied by 1.2 (1.4) and 1.4 (1.15) for comparison, respectively. The spectra in the absence of salt were reproduced from ref 16. One division of the *y*-axis corresponds to 0.00065 absorbance units.

(2). M-Intermediate. In a next step, the changes in the M minus initial state difference FTIR spectra in the 1800−700 cm^{-1} region of WT *Sr*SRI upon addition of 100 mM NaCl (pH = 7.0, T = 260 K) were studied. The spectrum in the absence of salt was reproduced from ref 16, which could be repeated in our measurement. Compared to the K-intermediate, the influence of the addition of salt on the structure and structural changes were small. In general, both spectra were of high similarity, though there were some significant differences. In both spectra, a shift to higher frequencies of the C═C stretching vibration of the retinal chromophore (1526 (−)/1565 (+) cm^{-1} and 1525 (−)/1564 (+) cm^{-1} in the absence and presence of NaCl, respectively) was observed. This shift is consistent with the spectral blue-shift observed upon formation of the M-intermediate (from 542 to 382 nm in the absence of salt and from 558 to 390 nm in the presence of salt).[11] As can be seen, the C═C stretching vibration, which is a good indicator of the visible absorption maximum,[35] was shifted by about −1 cm^{-1} upon the binding of Cl^-, which could be expected due to the Cl^- induced red-shift of the absorption in both the M-intermediate, as well as in the original state.

The formation of the M-intermediate in both samples was further confirmed by the reduced intensity of the positive bands of the C−C stretching vibration of the retinal chromophore

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

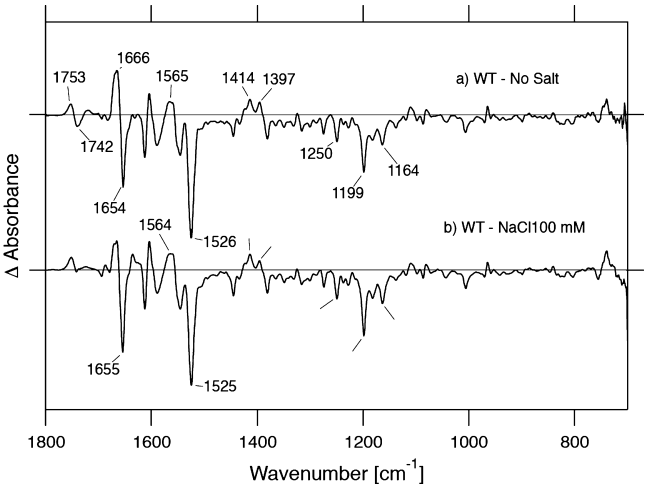


Figure 5. Influence of salt on the *Sr*SRI$_M$ minus *Sr*SRI difference infrared spectra in the 1800−700 cm^{-1} region, showing the disappearance of the negative band at 1742 cm^{-1} in the presence of salt, previously assigned to Asp102.[18] The samples were measured in the absence of salt (a) and in the presence of 100 mM NaCl (b) at 260 K at pH 7.0 and hydrated with H_2O. The spectra were normalized with respect to the C−C or C═C stretching vibration of the retinal chromophore. The spectrum in the presence of salt was multiplied by 0.7 for comparison. The spectra in the absence of salt were reproduced from ref 16. One division of the *y*-axis corresponds to 0.002 absorbance units.

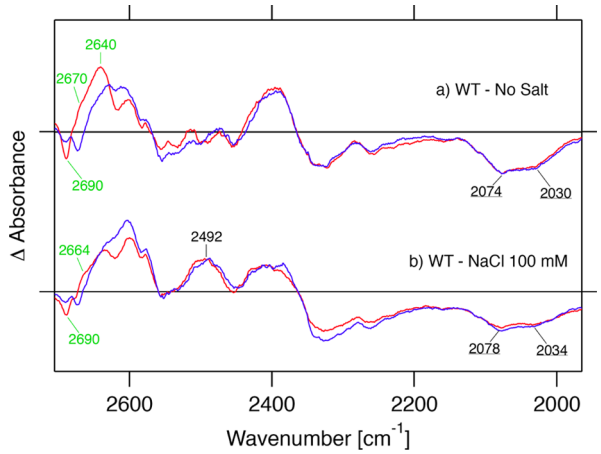


Figure 6. Influence of salt on the *Sr*SRI$_M$ minus *Sr*SRI difference infrared spectra in the 2750−1900 cm^{-1} region. The samples were measured in the absence of salt (a) and in the presence of 100 mM NaCl (b) at 260 K at pH 7.0 and hydrated with D_2O (red) or $D_2{}^{18}O$ (blue). The spectra were normalized with respect to the C−C or C═C stretching vibration of the retinal chromophore. The spectrum in the presence of salt in D_2O was multiplied by 0.94 for comparison. The spectra in the absence of salt were reproduced from ref 16. One division of the *y*-axis corresponds to 0.001 absorbance units.

associated with the proton transfer from the Schiff base to its counterion Asp72 (1753 cm^{-1}). Both this band, as well as the negative bands of the C−C stretches (1250, 1199, and 1164 cm^{-1}), were not affected by the addition of salt to the sample. The same is the case for the band at 1644 cm^{-1} (1624 cm^{-1} in D_2O), associated with the C═N stretching vibration (the signal in D_2O is only slightly less defined (results not shown)). Furthermore, the bands at 1666 (+)/1654 (−) cm^{-1}, attributed to the amide-I vibration of the α helix, are only slightly shifted for the original state 1666 (+)/1655 (−) cm^{-1}.

The "only" striking difference between the spectra was observed for the recently assigned negative band at 1742 cm^{-1} (1734 cm^{-1} in D_2O),[18] which is unique to *Sr*SRI, and which was associated with the deprotonation of Asp102. The disappearance of this band in the presence of NaCl suggests that the binding of Cl^- in the close proximity of Asp102 suppresses its change in protonation state upon formation of the M-intermediate. It should be noted that the positive corresponding band is expected in the region around 1400 cm^{-1} and was previously tentatively assigned to the band at 1414 cm^{-1}, which is also unique to *Sr*SRI.[16,18] However, no effect on this band could be seen upon addition of NaCl, while the nearby band at 1397 cm^{-1}, which is commonly observed among the studied sensory rhodopsin I proteins,[16,21] was slightly reduced in intensity. This suggests that the COO^- stretching vibration of Asp102 is rather observed around the latter frequency. Thus, one can say that the overall structure, and structural changes, are, in general, similar between the anion-bound and anion-free forms of *Sr*SRI.

Furthermore, using D_2O and $D_2{}^{18}O$ hydrated samples, the influence of salt on the vibrations of internal water molecules could also be studied (see Figure 6). The spectra in the absence of salt are reproduced from ref 16 for comparison. One should note that the quality of the spectra in the presence of salt is comparably weak, caused by the constant increase in water in the sample (see Figure SI2).

As can be seen, there are some distinct differences between the two spectra, especially in the region above 2400 cm^{-1}. Among other things, a new positive band occurs upon binding of Cl^- at 2492 cm^{-1}, originating from some X−D stretching vibrations. In addition, the previously assigned O−D stretching vibrational band at 2640 cm^{-1}, originating from a water molecule, disappeared upon binding of Cl^-. The relatively high frequency of those water bands suggest that they are rather located in a comparably hydrophilic environment, as would, e.g., be the Schiff base region.

A more detailed assignment of the high frequency region both in the K- and M-intermediate by attempting to increase the signal-to-noise ratio as well as by using isotope labeling and mutants is planned to confirm the precise assignment of the affected bands.

Proton Movement in *Sr*SRI during the Photocycle. As described above, one of the most characteristic differences between the FTIR spectra of the anion-free and anion-bound forms is the suppression of the structure and structural changes of the conserved residue Asp102 during the photocycle in the presence of salt. In a next step, the proton movement during the photocycle was investigated using the ITO method. In this method the proton release and uptake from some charged residue(s) upon photoillumination can be measured as a change in voltage, as described previously.[23,24] It should be noted that because of the low stability of the D102E mutant of *Sr*SRI during the ITO experiments, it was impossible to obtain a significant signal from the sample.

It should be noted that because PG-reconstituted *Sr*SRI was not well attached to the SnO_2 electrode, PC-reconstituted samples were used (see Materials and Methods). As can be seen, a clear signal in the potential change was recorded for varying concentrations of various salts (Figure 7), suggesting the movement of protons (uptake → release) upon photoillumination. This signal was greatly suppressed by the addition of monovalent anions (Cl^-, Br^- or NO_3^-). One should note

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

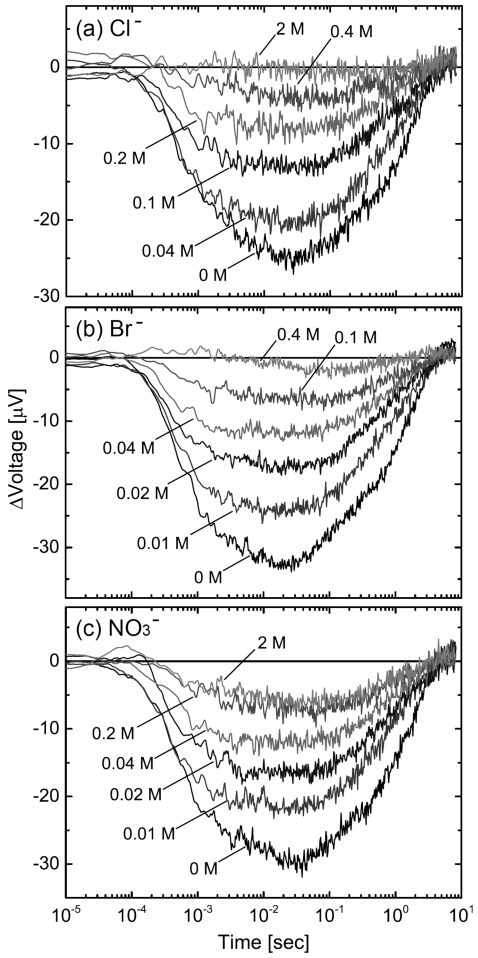


Figure 7. The decrease of the ITO signals caused by the addition of three monovalent anions. The downward (negative) deflection indicates the alkalization in the medium, i.e., the uptake of a proton by *Sr*SRI, while the upward shift indicates the proton release. The anions were added as sodium salts, and the ionic strength of the solutions were kept constant equivalent to the 4 M monovalent salt by adding Na_2SO_4. The medium pH was adjusted at 6.0 by a 6-mix buffer. In the case of Cl^- (a) and Br^- (b), the signal almost disappeared at concentrations above 2 M. In the case of NO_3^- (c), the signal leveled at about 15% of the initial signal, even at high concentrations.

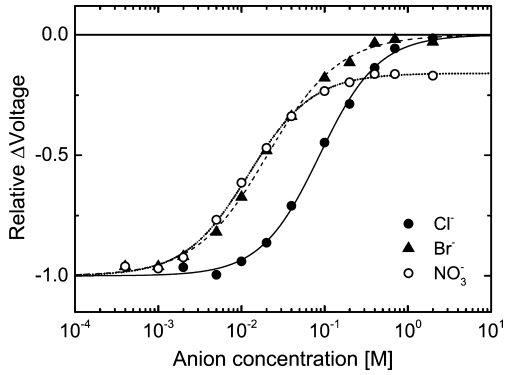


Figure 8. The anion-induced decrease of the H^+ uptake signal detected by ITO (the values represent relative values taken from Figure 7 at 25 ms). The signals were fitted using eq 1 (smooth lines).

that the signal showed also an initial dependence on changes in the ionic strength in the solution (see Figure SI6), and thus the ionic strength was kept constant by the addition of Na_2SO_4. Although the effect of the ionic strength is still unclear, the alteration of the lipids may be one possibility.

The anion-induced decrease of the H^+ uptake signal detected by the ITO electrode (the values represent relative values taken from Figure 7 at 25 ms) allowed us to estimate the affinities of the anions used (see Figure 8). The signals were fitted using eq 1 (smooth lines in Figure 8).

$$\Delta\text{voltage} = AK_d^n/(K_d^n + [\text{anion}]^n) + B \quad (1)$$

where K_d is the dissociation constant, n is the Hill coefficient, and the terms A and B are constants taking into account the negative shifts. For Cl^- and Br^-, B was fixed at zero. The voltage changes were plotted against the anion concentrations, and the K_d values of each anion were estimated as 86.6 ± 0.2 mM for the chloride ion, 20.4 ± 0.8 mM for the bromide ion, and 11.8 ± 0.4 mM for the nitrate ion (Table 1). The order of the K_d values for the used anions is the same as the Hofmeister series ($Cl^- > Br^- > NO_3^-$),[36] which represents their order of hydrophobicity and their tendency to stabilize structured low-density water. Though the affinities of the anions are 4−6-fold higher than those for the DDM-solubilized sample,[11] the order is the same. In addition, the Hill coefficients of the anions (1.3 for Cl^-, 1.0 for Br^- and 1.1 for NO_3^-) are close to 1, suggesting the noncooperative effect of the anion binding to *Sr*SRI. For both Cl^- and Br^-, the signal decreased with increasing salt concentration, until it completely disappears at concentrations above about 2 M (see Figure 7a,b, respectively). In contrast, in the case of NO_3^-, the signal leveled already at about 15% of the initial signal (see Figure 7c). This suggests that the nature of nitrate ions (maybe its different shape) cannot completely block the H^+ movement pathway.

To investigate which intermediate is involved in the proton movement, the time-resolved absorption changes on the membrane suspension were obtained and compared with the voltage changes. Figure 9 shows the time courses of the absorbance change at 390 nm for monitoring the M-intermediate and at 550 nm for the original state, without NaCl (1.33 M Na_2SO_4). The absorbance change at 620 nm is also shown in the figure to follow the red-shifted intermediates, such as the K- and/or O-state. In general, the time course of the voltage change follows the absorbance changes at 390 nm which describes the rise and decay of the M-intermediate, suggesting that the proton is taken up on formation of the M-intermediate, and released during the M-decay. However, the rise of the ITO signal appeared after the absorption change. One should note that Inoue et al. have previously reported the existence of three M states (M_1, M_2 and M_3) by using the transient grating (TG) method,[20] and, therefore, the result suggests that the proton uptake might occur at a middle M state (e.g., M_2).

Table 1. Dissociation Constants (K_d) and Hill Coefficients (n) Determined from the Fit Using eq 1 to the Anion-Induced Decreases of the H^+ Uptake Signal (see Figure 8)[a]

ion	K_d [mM]	n
Cl^-	86.6 ± 0.2	1.27 ± 0.04
Br^-	20.4 ± 0.8	0.99 ± 0.02
NO_3^-	11.8 ± 0.4	1.11 ± 0.02

[a]The data represent the mean ± SE (three experiments).

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

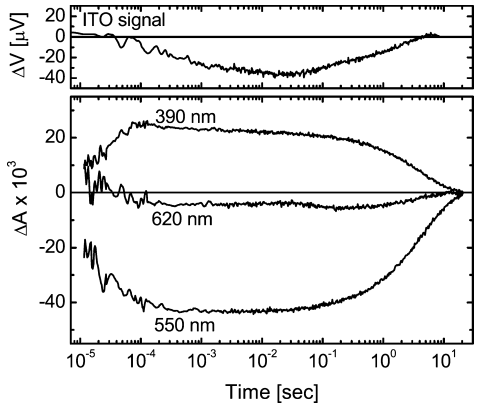


Figure 9. Comparison of the times courses between the flash-induced ITO signals (upper panels) and absorbance changes (lower panels) in the presence of 1.33 M Na_2SO_4. The medium pH was adjusted to 6.0 by a 6-mix buffer.

Furthermore, the light-induced pH changes at the ITO electrode surface, on which the PC-reconstituted *Sr*SRI proteins were adhered to, were measured under varying pH, as shown in Figure 10, to estimate the pK_a values of the charged residues involved. These changes were reversible (except for a pH of 10.0), indicating the structure of *Sr*SRI is maintained in this pH range. The changes in voltage, which represent pH changes induced during the *Sr*SRI-to-*Sr*SRI$_M$ transition, strongly depended on the pH of the electrolyte (see Figure 10a,b). Figure 10c shows the the recorded peak voltages plotted against the pH of the electrolyte. Alkalinization of the media was observed at pHs between 4 and 8, indicating a proton uptake, while the acidification was observed at pHs between 8.5 and 10 due to a proton release. The decrease of the signal below pH 5.0 is likely to correspond to the protonation of the counterion Asp72 of the Schiff base, as its pK_a value has been estimated to be 4.3.[11] This indicates that the deprotonation of Asp72 in the dark state is needed for the proton uptake and release during the photocycle. Furthermore, the proton release observed at high pH values (>8.5) is characteristic for many microbial rhodopsins,[24,37] and it can be thought to originate from the proton release of the Schiff-base proton into the highly basic medium. For the observed increase in the medium pH range, several mechanistic candidates exist, and the origin of these will be discussed below.

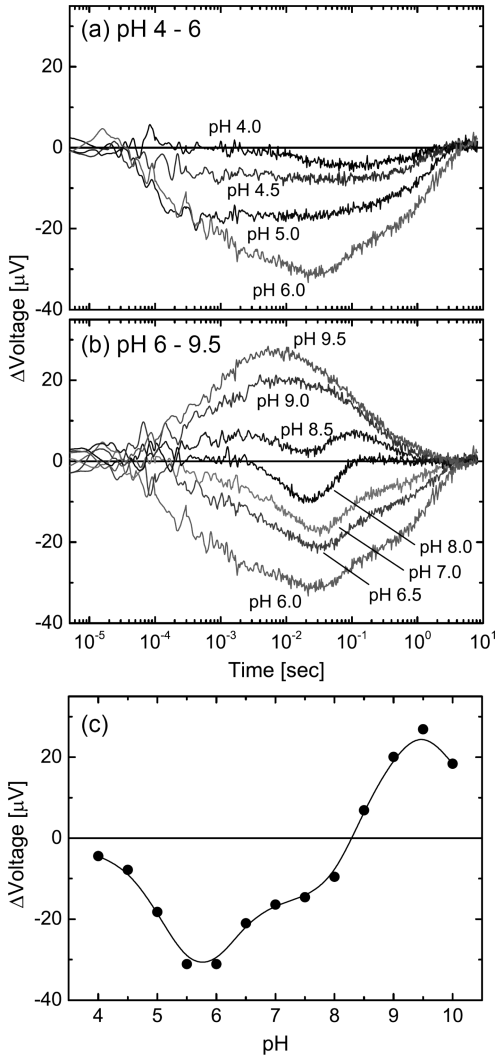


Figure 10. (a, b) Proton transfer of *Sr*SRI under various pH. The medium includes 1.33 M Na_2SO_4 and the pH was adjusted to the desired value with the 6-mix buffer. (c) Peak values in panels a and b plotted vs pH.

DISCUSSION

In this study, we analyzed the effects of anion-binding on the structure and structural changes of *Sr*SRI by using FTIR spectroscopic and electrochemical methods. On the basis of the experimental observations, a schematic model is proposed which describes the structure and structural changes close to the retinal chromophore during the photocycle in the presence and absence of salt (see Figure 11).

In the Absence of Chloride. In the dark state of *Sr*SRI, the conserved residue, Asp102, is protonated. This is reasonable because the environment around the β-ionone ring is highly hydrophobic and it is confirmed by the FTIR data. Upon formation of the K-intermediate, the frequency of the C=O stretching vibration of Asp102 is shifted from 1743 to 1737 cm^{-1}, indicating that the hydrogen bond of Asp102 with, e.g., a water molecule (as water511 in BR or water602 in SRII) and/or a neighboring hydrophilic residue (such as Thr90 in BR or Thr80 in SRII)[38−40] becomes stronger by the photoisomerization from all-*trans* to 13-*cis*. Furthermore, the hydrogen-out-of-plane (HOOP) vibrations of the retinal chromophore in the K-intermediate show that extended chromophore distortions take place in *Sr*SRI, similar to SRII, but in contrast to BR and HR, in which the distortions are localized in the Schiff base region. Upon formation of the multiple M-intermediates, Asp102 is deprotonated, and a proton is taken up from the bulk solution, as revealed by the ITO signal. This proton movement reaction (i.e., the proton uptake occurring before the proton release) should involve at least one other proton accepting residue. One of the candidates for this proton accepting residue is His131, because it is needed for charge balancing as the counterpart to the deprotonated Asp102. Furthermore, this proton uptake is delayed from the formation of the first M intermediate. Thus our speculation is that (1) Asp102 is deprotonated, and its proton is either kept to provide a charge balance in its vicinity (a), or taken up by His131 (b), leading to its protonation, (2) then a proton is taken up from the bulk to the still unprotonated His131 (a), or to the deprotonated Asp102 (b) at the M_2 state. (3) After this proton is released from His131 (a, b) or from the vicinity of Asp102 (a) to the bulk during the

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

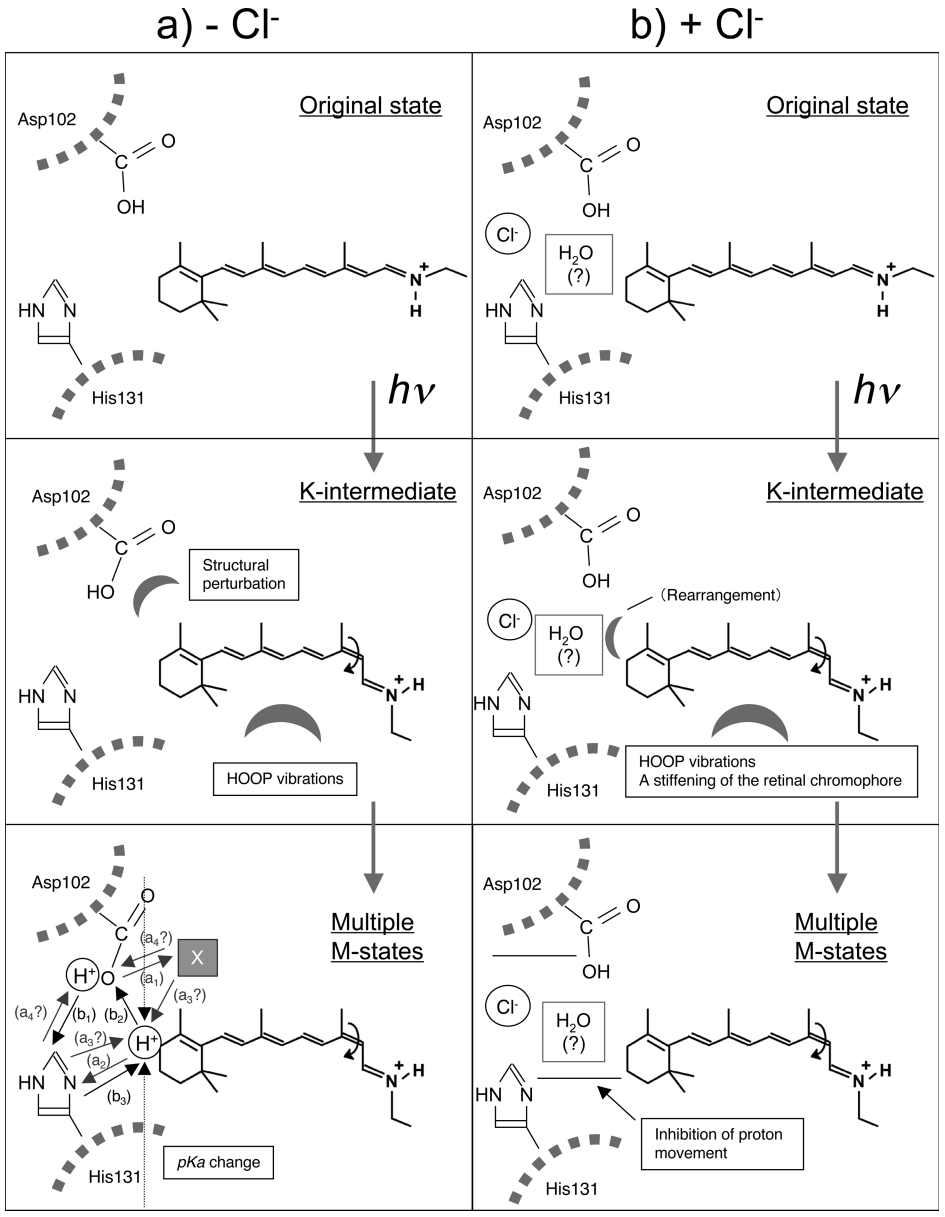


Figure 11. Schematic model of the effects of the anion binding to *Sr*SRI on its structure and structural changes during the photocycle. "X" means a proton acceptable molecule such as water, O−H or N−H groups.

M_3 state. (4) Finally in the case of (a) the still deprotonated Asp102 receives a proton from either its vicinity or from the protonated His131 in a later intermediate, such as N or O. These proton movements would be correlated with the large enthalpy changes both from the M_1 to M_2, and from the M_2 to M_3 transitions.[20] Thus, our data suggest that in addition to the Schiff base region, SRI is likely to have another proton transfer pathway around the β-ionone ring, where proton translocation or in proton circulation can occur. Interestingly, the recently reported crystal structure of channel rhodopsin has also suggested another proton transfer pathway constructed by TM2 and TM7.[41] Therefore, it is likely that microbial rhodopsins might have more proton transfer pathways than first thought.

In the Presence of Chloride. In general, the overall structure and structural changes of *Sr*SRI binding a chloride ion are similar to its chloride free form. However, the K minus original state difference spectrum revealed that at least one additional water molecule exists in the presence of anions, and that its hydrogen bond becomes weak upon formation of the K-intermediate. It is likely that these water molecules are needed for the hydration of the Cl^- ion, which is located around the β-ionone ring because (i) the recorded structural changes are localized around the retinal chromophore due to the restrictions caused by the cryogenic temperature of the experiment, (ii) the environment around the β-ionone ring is highly hydrophobic, causing the chloride ion to be unstable when it is located in its unhydrated form, (iii) the bands are observed only in the presence of anions, suggesting that the water molecule is related to the anion binding. Thus, we would like to tentatively assign the newly appearing band to a chloride coupled water molecule. Interestingly, upon formation of the K-intermediate, the frequency shift of Asp102 is strongly suppressed. This seems to be a characteristic feature of *Sr*SRI compared with other microbial rhodopsins, such as BR, HR and SRII, and might, therefore, be related to the chloride binding in

dx.doi.org/10.1021/bi3009592 | *Biochemistry* 2012, 51, 8802−8813

the vicinity of Asp102, which is also characteristic for SRIs. Also in the presence of chloride, extended chromophore vibrations are observed; however, a stiffening of the retinal chromophore is indicated. Upon formation of the M-intermediate, the deprotonation of Asp102 is suppressed by the binding of the chloride ion, indicating that only a small (if at all) pK_a change of Asp102 occurs during the formation of the multiple M-states. This suppression is the likely reason that no proton movement signal is observed.

In conclusion, we successfully performed FTIR spectroscopy, as well as ITO measurements on a SRI protein, both in the presence and absence of anions, by making use of the highly stable SRI protein from *S. ruber*. Our experiments suggest that the chloride binding to *Sr*SRI plays important roles for the structure and structural changes of, among other things, Asp102 and internal water molecules, as well as for the proton movement during its photocycle. These are characteristic features for SRI compared with the other microbial rhodopsins such as BR or SRII. How halophilic organisms adapt to life in extreme environments, especially in terms of their internal molecular structure, is an interesting question. As already mentioned, *S. ruber* uses an intracellular accumulation of high salt concentrations, which requires extensive adaptations of the entire intracellular enzymatic machinery to be functional in the presence of high ionic concentrations. Our results suggest that the SRI protein of *S. ruber* makes use of the high salt concentration available inside and outside the cell to optimize its structure and structural changes during its photocycle. However, in future, detailed studies on the function of *Sr*SRI in its native cells and on the effect of salt on this function are needed and planned, to verify the importance of the observed changes. Also, more detailed assignments both of the water bands and the vibrations (e.g., C=N stretch) of the retinal chromophore in the presence and absence of salt are planned by using additional isotope labeling, in combination with the replacement of certain amino acid residues located around the retinal chromophore by site-directed mutagenesis. Furthermore, the complex pH profile of the ITO signal in Figure 10c implies the contribution of several proton accepting and releasing residues. Further studies are needed to elucidate the detailed proton movement reaction.

■ ASSOCIATED CONTENT

Supporting Information

Figure SI1 shows the absorption spectra of *Sr*SRI in the presence of a range of anions. Figure SI2 shows the FTIR spectra of films of *Sr*SRI at 77 K and 260 K. Figures SI3 and SI4 show a more detailed analysis of the difference FTIR spectra in the 2600−1800 cm^{-1} region associated with internal water molecules. Figure SI5 shows the observed structuring in the water (D_2O) peak in the presence of NaBr. Figure SI6 shows the Na_2SO_4 concentration dependence of the ITO potential changes in *Sr*SRI. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel. +81-52-789-2993. Fax: +81-52-789-3001. E-mail: z47867a@cc.nagoya-u.ac.jp.

Funding

This work was financially supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology to H.K. (20108014) and to Y.S. (20050012 and 22018010).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Yukie Kawase for assistance in sample preparation. We also thank Dr. Jin Yagasaki for useful discussions.

■ ABBREVIATIONS USED

DDM, *n*-dodecyl-*β*-D-maltoside; SRI, sensory rhodopsin I; SRII, sensory rhodopsin II; *Sr*SRI, SRI from *Salinibacter ruber*; $SrSRI_K$, K-intermediate of *Sr*SRI; $SrSRI_M$, M-intermediate of *Sr*SRI; FTIR, Fourier transform infrared; ITO, indium tin oxide; PG, L-*α*-phosphatidyl glycerol; PC, L-*α*-phosphatidyl choline; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, 2-[(2-hydroxy-1,1-bis(hydroxyl-methyl)ethyl)amino]-ethanesulfonic acid; TAPS, [(2-hydroxy-1,1-bis-(hydroxymethyl)-ethyl)amino]-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; Nd:YAG, neodymium-doped yttrium aluminum garnet

■ REFERENCES

(1) Oren, A., Rodríguez-Valera, F., Antón, J., Benlloch, S., Rosselló-Mora, R., Amann, R., Coleman, J., andRussel, N. (2004) Red, extremely halophilic, but not archaeal: the physiology and ecology of salinibacter ruber, a bacterium isolated from saltern crystallizer ponds, in *Halophilic Microorganisms* (Ventosa, A., Ed.) pp 63−76, Springer-Verlag, Berlin, Germany.

(2) Antón, J., Oren, A., Benlloch, S., Rodriguez-Valera, F., Amann, R., and Rosselló-Mora, R. (2002) Salinibacter ruber gen. nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. *Int. J. Syst. Evol. Microbiol. 52*, 485−491.

(3) Oren, A. (2002) *Halophilic Microorganisms and Their Environments*, Kluwer Academic Publisher, Dordrecht.

(4) Mongodin, E. F., Nelson, K. E., Daugherty, S., Deboy, R. T., Wister, J., Khouri, H., Weidman, J., Walsh, D. A., Papke, R. T., Sanchez Perez, G., Sharma, A. K., Nesbø, C. L., MacLeod, D., Bapteste, E., Doolittle, W. F., Charlebois, R. L., Legault, B., and Rodriguez-Valera, F. (2005) The genome of Salinibacter ruber: convergence and gene exchange among hyperhalophilic bacteria and archaea. *Proc. Natl. Acad. Sci. U. S. A. 102*, 18147−18152.

(5) Heberle, J., Fitter, J., Sass, H. J., and Buldt, G. (2000) Bacteriorhodopsin: the functional details of a molecular machine are being resolved. *Biophys. Chem. 85*, 229−248.

(6) Lanyi, J. K. (2004) Bacteriorhodopsin. *Annu. Rev. Physiol. 66*, 665−688.

(7) Sudo, Y. (2012) Transport and sensory rhodopsins in microorganisms, in *CRC Handbook of Organic Photochemistry and Photobiology*, 3rd ed., pp 1173−1193 CRC Press, Boca Raton.

(8) Kouyama, T., and Murakami, M. (2010) Structural divergence and functional versatility of the rhodopsin superfamily. *Photochem. Photobiol. Sci. 9*, 1458−1465.

(9) Oesterhelt, D., and Stoeckenius, W. (1971) Rhodopsin-like protein from the purple membrane of Halobacterium halobium. *Nat. New Biol. 233*, 149−152.

(10) Spudich, J. L., Yang, C. S., Jung, K. H., and Spudich, E. N. (2000) Retinylidene proteins: structures and functions from archaea to humans. *Annu. Rev. Cell Dev. Biol. 16*, 365−392.

(11) Suzuki, D., Furutani, Y., Inoue, K., Kikukawa, T., Sakai, M., Fujii, M., Kandori, H., Homma, M., and Sudo, Y. (2009) Effects of Chloride Ion Binding on the Photochemical Properties of Salinibacter Sensory Rhodopsin I. *J. Mol. Biol. 392*, 48−62.

(12) Balashov, S. P., Imasheva, E. S., Boichenko, V. A., Anton, J., Wang, J. M., and Lanyi, J. K. (2005) Xanthorhodopsin: a proton pump with a light-harvesting carotenoid antenna. *Science 309*, 2061−2064.
(13) Luecke, H., Schobert, B., Stagno, J., Imasheva, E. S., Wang, J. M., Balashov, S. P., and Lanyi, J. K. (2008) Crystallographic structure of xanthorhodopsin, the light-driven proton pump with a dual chromophore. *Proc. Natl. Acad. Sci. U. S. A. 105*, 16561−16565.
(14) Slouf, V., Balashov, S. P., Lanyi, J. K., Pullerits, T., and Polivka, T. (2011) Carotenoid response to retinal excitation and photoisomerization dynamics in xanthorhodopsin. *Chem. Phys. Lett. 516*, 96−101.
(15) Kitajima-Ihara, T., Furutani, Y., Suzuki, D., Ihara, K., Kandori, H., Homma, M., and Sudo, Y. (2008) Salinibacter sensory rhodopsin: sensory rhodopsin I-like protein from a eubacterium. *J. Biol. Chem. 283*, 23533−23541.
(16) Suzuki, D., Sudo, Y., Furutani, Y., Takahashi, H., Homma, M., and Kandori, H. (2008) Structural changes of Salinibacter sensory rhodopsin I upon formation of the K and M photointermediates. *Biochemistry 47*, 12750−12759.
(17) Sudo, Y., Okada, A., Suzuki, D., Inoue, K., Irieda, H., Sakai, M., Fujii, M., Furutani, Y., Kandori, H., and Homma, M. (2009) Characterization of a signaling complex composed of sensory rhodopsin I and its cognate transducer protein from the eubacterium Salinibacter ruber. *Biochemistry 48*, 10136−10145.
(18) Irieda, H., Reissig, L., Kawanabe, A., Homma, M., Kandori, H., and Sudo, Y. (2011) Structural characteristics around the beta-ionone ring of the retinal chromophore in Salinibacter sensory rhodopsin I. *Biochemistry 50*, 4912−4922.
(19) Sudo, Y., Yuasa, Y., Shibata, J., Suzuki, D., and Homma, M. (2011) Spectral tuning in sensory rhodopsin I from Salinibacter ruber. *J. Biol. Chem. 286*, 11328−11336.
(20) Inoue, K., Sudo, Y., Homma, M., and Kandori, H. (2011) Spectrally silent intermediates during the photochemical reactions of Salinibacter sensory rhodopsin I. *J. Phys. Chem. B 115*, 4500−4508.
(21) Yagasaki, J., Suzuki, D., Ihara, K., Inoue, K., Kikukawa, T., Sakai, M., Fujii, M., Homma, M., Kandori, H., and Sudo, Y. (2010) Spectroscopic studies of a sensory rhodopsin I homologue from the archaeon Haloarcula vallismortis. *Biochemistry 49*, 1183−1190.
(22) Zhang, Y., Iwata, T., Yamamoto, J., Hitomi, K., Iwai, S., Todo, T., Getzoff, E. D., and Kandori, H. (2011) FTIR study of light-dependent activation and DNA repair processes of (6−4) photolyase. *Biochemistry 50*, 3591−3598.
(23) Sudo, Y., Iwamoto, M., Shimono, K., Sumi, M., and Kamo, N. (2001) Photo-Induced Proton Transport of Pharaonis Phoborhodopsin (Sensory Rhodopsin II) Is Ceased by Association with the Transducer. *Biophys. J. 80*, 916−922.
(24) Tamogami, J., Kikukawa, T., Miyauchi, S., Muneyuki, E., and Kamo, N. (2009) A tin oxide transparent electrode provides the means for rapid time-resolved pH measurements: application to photo-induced proton transfer of bacteriorhodopsin and proteorhodopsin. *Photochem. Photobiol. 85*, 578−589.
(25) Yoshitsugu, M., Yamada, J., and Kandori, H. (2009) Color-changing mutation in the E-F loop of proteorhodopsin. *Biochemistry 48*, 4324−4330.
(26) Kandori, H., Shimono, K., Sudo, Y., Iwamoto, M., Shichida, Y., and Kamo, N. (2001) Structural Changes of pharaonis Phoborhodopsin upon Photoisomerization of the Retinal Chromophore: Infrared Spectral Comparison with Bacteriorhodopsin. *Biochemistry 40*, 9238−9246.
(27) Shibata, M., Muneda, N., Sasaki, T., Shimono, K., Kamo, N., Demura, M., and Kandori, H. (2005) Hydrogen-bonding alterations of the protonated Schiff base and water molecule in the chloride pump of Natronobacterium pharaonis. *Biochemistry 44*, 12279−12286.
(28) Sudo, Y., Furutani, Y., Spudich, J. L., and Kandori, H. (2007) Early photocycle structural changes in a bacteriorhodopsin mutant engineered to transmit photosensory signals. *J. Biol. Chem. 282*, 15550−15558.
(29) Earnest, T. N., Roepe, P., Braiman, M. S., Gillespie, J., and Rothschild, K. J. (1986) Orientation of the bacteriorhodopsin chromophore probed by polarized Fourier transform infrared difference spectroscopy. *Biochemistry 25*, 7793−7798.
(30) Maeda, A., Sasaki, J., Pfefferle, J. M., Shichida, Y., and Yoshizawa, T. . (1991) Fourier transform infrared spectral studies on the Schiff base mode of all-trans bacteriorhodopsin and its photointermediates, K and L. *Photochem. Photobiol. 54*, 911−921.
(31) Smith, S. O., Braiman, M. S., Myers, A. B., Pardoen, J. A., Courtin, J. M. L., Winkel, C., Lugtenburg, J., and Mathies, R. A. (1987) Vibrational Analysis of the All-trans Retinal Chromophore in Light-Adapted Bacteriorhodopsin. *J. Am. Chem. Soc. 109*, 3108−3125.
(32) Maeda, A., Balashov, S. P., Lugtenburg, J., Verhoeven, M. A., Herzfeld, J., Belenky, M., Gennis, R. B., Tomson, F. L., and Ebrey, T. G. (2002) Interaction of internal water molecules with the schiff base in the L intermediate of the bacteriorhodopsin photocycle. *Biochemistry 41*, 3803−3809.
(33) Furutani, Y., Sudo, Y., Wada, A., Ito, M., Shimono, K., Kamo, N., and Kandori, H. (2006) Assignment of the hydrogen-out-of-plane and -in-plane vibrations of the retinal chromophore in the K intermediate of pharaonis phoborhodopsin. *Biochemistry 45*, 11836−11843.
(34) Shim, S., Dasgupta, J., and Mathies, R. A. (2009) Femtosecond time-resolved stimulated Raman reveals the birth of bacteriorhodopsin's J and K intermediates. *J. Am. Chem. Soc. 131*, 7592−7597.
(35) Shimono, K., Furutani, Y., Kandori, H., and Kamo, N. (2002) A pharaonis phoborhodopsin mutant with the same retinal binding site residues as in bacteriorhodopsin. *Biochemistry 41*, 6504−6509.
(36) Zhang, Y., and Cremer, P. S. (2006) Interactions between macromolecules and ions: the Hofmeister series. *Opin. Chem. Biol. 10*, 658−663.
(37) Kikukawa, T., Shimono, K., Tamogami, J., Miyauchi, S., Kim, S. Y., Kimura-Someya, T., Shirouzu, M., Jung, K. H., Yokoyama, S., and Kamo, N. (2011) Photochemistry of Acetabularia rhodopsin II from a marine plant, Acetabularia acetabulum. *Biochemistry 50*, 8888−8898.
(38) Kandori, H., Shimono, K., Shichida, Y., and Kamo, N. (2002) Interaction of Asn105 with the retinal chromophore during photoisomerization of pharaonis phoborhodopsin. *Biochemistry 41*, 4554−4559.
(39) Luecke, H., Schobert, B., Richter, H. T., Cartailler, J. P., and Lanyi, J. K. (1999) Structure of bacteriorhodopsin at 1.55 A resolution. *J. Mol. Biol. 291*, 899−911.
(40) Luecke, H., Schobert, B., Lanyi, J. K., Spudich, E. N., and Spudich, J. L. (2001) Crystal structure of sensory rhodopsin II at 2.4 angstroms: insights into color tuning and transducer interaction. *Science 293*, 1499−1503.
(41) Kato, H. E., Zhang, F., Yizhar, O., Ramakrishnan, C., Nishizawa, T., Hirata, K., Ito, J., Aita, Y., Tsukazaki, T., Hayashi, S., Hegemann, P., Maturana, A. D., Ishitani, R., Deisseroth, K., and Nureki, O. (2012) Crystal structure of the channelrhodopsin light-gated cation channel. *Nature 482*, 369−374.