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Photoreaction of *Anabaena* sensory rhodopsin and Creation of a New Function

*Anabaena* sensory rhodopsin の光反応研究と新規機能の創成

2009

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# CONTENTS

**Chapter 1**  
Introduction and Background ................................................................. 3

**Chapter 2**  
2-1 FTIR Spectroscopy of the all-\textit{trans} form of \textit{Anabaena} sensory rhodopsin at 77 K: Hydrogen bond of a water between the Schiff base and Asp75  
Introduction ........................................................................................................ 17  
Materials and Methods ...................................................................................... 18  
Results .................................................................................................................. 20  
Discussion ............................................................................................................ 34

2-2 FTIR study of the photoisomerization processes in the 13-\textit{cis} and all-\textit{trans} forms of \textit{Anabaena} sensory rhodopsin at 77 K  
Introduction ........................................................................................................... 43  
Materials and Methods ....................................................................................... 44  
Results ................................................................................................................. 45  
Discussion ............................................................................................................. 60

**Chapter 3**  
Photochromism of \textit{Anabaena} sensory rhodopsin  
Introduction ........................................................................................................... 69  
Materials and Methods ....................................................................................... 71  
Results and Discussion ....................................................................................... 72
Chapter 4

FTIR study of the L Intermediate of Anabaena sensory rhodopsin:
Structural changes in the cytoplasmic region

Introduction ................................................................. 91
Materials and Methods ................................................... 93
Results .......................................................................... 95
Discussion .................................................................... 107

Chapter 5

Engineering an inward proton pump from a bacterial sensor rhodopsin

Introduction ................................................................. 115
Materials and Methods ................................................... 117
Results and Discussion ................................................... 121

Chapter 6

Conclusion and perspectives ............................................ 131

Acknowledgements .......................................................... 137
Chapter 1

Introduction and Backgrounds

Photosynthesis is one of the most important chemical reactions in living cells because almost all energy spent by living things on the earth originates from it. Photosynthesis mainly works in chloroplasts of plants, where photoinduced electron transfer reaction first stores light energy, but eventually proton gradient is formed across the membrane. The proton gradient is used for the synthesis of ATP, because it is a driving force of an enzyme ATP-synthase. Some bacteria directly convert light energy into proton gradient through a proton pump. Light sensing is also important to optimize photosynthesis. They have to escape ultra-violet (UV) lights, because UV lights possibly kill our gene. In addition, if photosynthesis occurs under red light, it is better sense this color for photosynthetic function.

Four archaeal type rhodopsins [Bacteriorhodopsin (BR), Halorhodopsin (HR), Sensory rhodopsin I (SRI), and Sensory rhodopsin II (SRII); also called phoborhodopsin (pR)] were discovered in the cytoplasmic membrane of Halobacterium salinarum (1, 2). The former two (BR and HR) function as light-driven proton and chloride pumps, respectively, while the latter two (SRI and SRII) are responsible for attractive and repellent phototaxis, respectively (Figure 1-1). They have a retinal molecule as a chromophore, which forms a Schiff base linkage with a lysine residue of the 7th helix. All-trans form (all-trans, 15-anti) is the functional form in BR, HR, SRI and SRII, and absorption of light yields isomerization to the 13-cis, 15-anti form, which triggers protein structural changes for function. In the case of BR, a cyclic reaction comprises the series of intermediates, K, L, M, N, and O (Figure 1-2) (3, 4). During the photocycle, an ion is transported through membrane and signal transmit to transducer protein.

They have been extensively studied as model systems converting light energy
Figure 1-1
There are four archaeal rhodopsins in *Halobacterium salinarum*. Bacteriorhodopsin halorhodopsin, sensory rhodopsin I and II. All of them have seven transmembrane helices and an all-trans retinal as a chromophore.
Figure 1-2
The photocycle of Bacteriorhodopsin (PDB code: 1C3W). The reaction starts by light, returns to initial state through the various intermediate within 10 ms. During the photocycle, a proton transport from cytoplasmic to extracellular side.
to chemical potential or environmental signal. Although such archaeal type rhodopsins were considered to exist only in Archaea, the genome sequencing projects have revealed that archaeal rhodopsins also exist in Eukaryota and Bacteria during the last decade (Figure 1-3). In eucaryotes, archaeal rhodopsins were found in fungi (5), green algae (6, 7), dinoflagellates (8), and cryptomonads (9).

Eubacterial rhodopsins were found both in γ- and α-proteobacteria (10, 11) as well as in Anabaena (Notstoc) sp. PCC7120, a freshwater cyanobacterium (12), which was called as Anabaena Sensory Rhodopsin (ASR).

The gene encoding ASR, which is a membrane protein of 261 residues (26 kDa), and a smaller gene encoding a soluble protein of 125 residues (14 kDa) are under the same promoter in a single operon (12). The opsin expressed heterologously in Escherichia coli membranes bounds all-trans retinal to form a pink pigment (λ_max = 549 nm) with a photochemical reaction cycle of 110 ms half-life (pH 6.8, 18 °C) (12). The previous study revealed that co-expression with the 14 kDa protein increased the rate of the photocycle, indicating physical interaction with ASR and the possibility that ASR works as a photosensor protein (Figure 1-4) (12). It should be noted that SRI and SRII activate transmembrane transducer proteins (Figure 1-1). In this sense, ASR is closer to visual rhodopsins that activate soluble G-proteins.

According to the X-ray crystal structure of ASR, it was similar to those of other archaeal-type rhodopsins (Figure 1-5). ASR accommodates both all-trans and 13-cis retinal in the ground state, which can be interconverted between them by illumination with blue (480 nm) or orange (590 nm) light (Figure 1-4) (13). Such photochromic behavior has never been observed in other archaeal rhodopsins such as BR, HR, SRI and SRII, being characteristic to ASR. These results suggested that ASR could be a photochromic color sensor, whereas nothing has been known about its photochromic reactions.

Comparison of the amino acid sequences between ASR and BR reveals that some important residues for pumping protons are replaced in ASR. The proton donor to the Schiff base (Asp96 in BR) and one of proton release groups (Glu194 in
**Figure 1-3**

Phylogenetic tree of microbial rhodopsins. (from Dr. Kwang-Hwan Jung)
Proposed function of *Anabaena* sensory rhodopsin (1XIO). ASR is converted into two isomeric states, which have different interactions with 14kDa-protein (2II9). The 14kDa-protein controls the expression level of phycobilisome proteins (phycocyanin and phycoerythrin). Phycobilisome graphic is from Grossman et al.
Figure 1-5
(Left) X-ray crystallographic structures of ASR (1XIO (13)) (Right) The Schiff base region of ASR and BR (1C3W (16)), respectively. Each retinal molecule is fitted to compare the hydrogen-bonding networks by using Swiss-PdbViewer (17). Top and bottom regions correspond to the cytoplasmic and extracellular sides, respectively. Green spheres (Water 402) represent a water molecule which forms a hydrogen bond bridging between the protonated Schiff base and its counter ion, Asp75. Hydrogen-bonds (blue dashed lines) are deduced from the structure and the numbers are the hydrogen-bond distances in angstrom.
BR) are replaced to serine residues, Ser86 and Ser188, respectively (Figure 1-6).

Ten amino acid residues out of twenty-five which constitute the retinal binding site are different from that of BR, probably resulting in different absorption maximum and photochromic behavior of ASR. Among them, the most characteristic replacement is Pro206 locating at the position of Asp212 in BR, which is one of the counter ions of the Schiff base and a well conserved amino acid residue in archaeal type rhodopsins (Figure 1-7). The influence of Pro206 on the hydrogen bonds around the Schiff base should be studied precisely for elucidating the difference in the structural changes of retinal and protein between ASR and BR upon their activation.

As shown above, ASR is a unique archaeal-type rhodopsin. However, the molecular properties are much less known. Thus, in this doctoral thesis, I studied various properties of ASR mainly by use of spectroscopic techniques. Since ASR possesses visible absorption, UV-visible spectroscopy is the basic technique to study the property of this molecule. Low-temperature UV-visible spectroscopy was used to study the photochromism of ASR in Chapter 3. In the photochromic reaction, isomeric states of the retinal chromophore play important role, and HPLC analysis is the direct method to determine the isomeric composition of the retinal chromophore. I used this method in Chapter 2.

In this thesis, I mostly used low-temperature Fourier-transform infrared (FTIR) spectroscopy. Infrared frequencies cover the 4000-100 cm\(^{-1}\) region, which correspond to molecular vibrations. So infrared spectroscopy is a suitable experimental tool to study structural changes in protein. In particular, Kandori group has developed low-temperature FTIR spectroscopy to detect X-H and X-D (X = O, N) stretching vibrations (4000-1800 cm\(^{-1}\)) (14, 15). These vibrations can be direct indications of the hydrogen bonding network, including internal water molecules. In fact, comparison of the K intermediate (BR\(_K\)) minus BR difference spectra between hydration with D\(_2\)O and D\(_2^{18}\)O in the X-D stretching region (2700-1800 cm\(^{-1}\)) enabled us to assign the O-D stretching vibrations of water molecules not only with a weak
Comparison of amino acid sequences of ASR and BR. The transmembrane topology is based on the crystallographic three-dimensional structures. The sequence alignment was done using CLUSTAL W (18) with the default settings. Single letters in a circle denote residues common to ASR and BR. The residues that are different in ASR and BR are denoted at the top and bottom of the circles, respectively. The residues forming the retinal binding site within 5 Å of the chromophore are shown by bold or filled circles.
The 25 amino acid sequence of archaeal-type rhodopsin around retinal. First, second, third, fourth and fifth categories show the families of bacteriorhodopsin, halorhodopsin, sensory rhodopsin I, sensory rhodopsin II and other archaeal-type rhodopsins, respectively. position S: near Schiff base, P: near polyene chain, β: around β-ionon ring.

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Figure 1-7
The 25 amino acid sequence of archaeal-type rhodopsin around retinal. First, second, third, fourth and fifth categories show the families of bacteriorhodopsin, halorhodopsin, sensory rhodopsin I, sensory rhodopsin II and other archaeal-type rhodopsins, respectively. position S: near Schiff base, P: near polyene chain, β: around β-ionon ring.
hydrogen bond (at >2500 cm\(^{-1}\)) but also with a strong hydrogen bond (at <2400 cm\(^{-1}\)).

I also tried to engineer ASR for another function. ASR has been reported to have no proton pump activity. Therefore, I attempted to produce a proton-pumping ASR by site-directed mutagenesis. In this case, “proton pump” means a normal outward pump. However, unexpectedly, what I produced was an inward proton pump. An inward proton pump is disadvantageous for living cells, because it competes the function of ATP-synthase. Therefore, an inward proton pump has been neither created naturally nor artificially. The topic will be presented in Chapter 5.
REFERENCES

Chapter 2

2-1 FTIR spectroscopy of the all-trans form of Anabaena sensory rhodopsin at 77 K: Hydrogen bond of a water between the Schiff base and Asp75

Introduction
Comparison of the amino acid sequences of ASR and BR shows that some important residues for the proton pump in BR are replaced in ASR (Figure 1-6). The proton donor to the Schiff base (Asp96 in BR) and one of proton release groups (Glu194 in BR) are replaced to serine residues, Ser86 and Ser188, respectively. These replacements are probably responsible for the slow photocycle of ASR on the analogy of pharaonis phoborhodopsin (ppR), whose corresponding residues are Phe86 and Pro183, respectively (1). Ten amino acid residues out of 25 constituting the retinal binding site are different from those of BR, which probably yields a different absorption maximum and photochromic behavior of ASR. Among them, the most characteristic replacement is Pro206 at the corresponding position of Asp212 in BR, Asp212 counterions a counterion complex of the Schiff base in BR, and the aspartate is highly conserved among archaeal rhodopsins. How is the hydrogen bonding network around the Schiff base modified in ASR by the presence of Pro206?

We have developed low-temperature Fourier transform infrared (FTIR) spectroscopy to detect X-H and X-D (X = O, N) stretching vibrations in the midinfrared region (4000-1800 cm⁻¹) (2, 3). These vibrations are direct indications of the hydrogen bonding network, including internal water molecules. In fact, comparison of the K intermediate (BRK) minus BR difference spectra between hydration with D₂O and D₂¹⁸O in the X-D stretching region (2700-1800 cm⁻¹) enabled us to assign the O-D stretching vibrations of water molecules not only with a weak
hydrogen bond (at >2500 cm⁻¹) but also with a strong hydrogen bond (at <2400 cm⁻¹). A mutation study showed that one of the O-D stretches (2171 cm⁻¹) originates from a bridge water molecule between the Schiff base and its counterion (Asp85) (4). Hydration switch of the water plays an important role in the proton transfer reaction in BR (5). In addition, interestingly, comprehensive studies of BR mutants and other rhodopsins have revealed that strongly hydrogen-bonded water molecules are only found in the proteins exhibiting proton pump activities (6). This suggests that a strongly hydrogen bonded water molecule that bridges the Schiff base and its counterion is essential for proton pumping. In terms of this idea, the FTIR study of ASR is intriguing, because ASR possesses a bridged water like BR, but does not pump protons (7).

In this study, I applied low-temperature FTIR spectroscopy to the all-trans form of ASR, and compared the difference spectra at 77 K with those of BR. The K intermediate minus ASR difference spectra show that the retinal isomerizes from the all-trans to the distorted 13-cis form like BR. The N-D stretching of the Schiff base was observed at 2163 (-) and 2125 (-) cm⁻¹, while the O-D stretchings of water molecules were observed in the >2500 cm⁻¹ region. These results indicate that the protonated Schiff base forms a strong hydrogen bond with a water molecule, which is connected to Asp75 with a weak hydrogen bond. This result with ASR supports our working hypothesis about the strong correlation between the proton pump activity and the existence of strongly hydrogen bonded water molecules in archaeal rhodopsins. In this chapter, I discuss the structural reason the bridged water molecule does not form a strong hydrogen bond in ASR.

Materials and Methods

Sample Preparation of ASR. Samples for this spectroscopic analysis were prepared as described previously (7, 8). Briefly, E. coli strain BL21 (Stratagene) was transformed by introducing pMS107 derivative plasmids (7) which encode the Anabaena opsin and were grown in 2xYT medium in the presence of ampicillin (50
µg/mL) at 38 °C. Three hours after IPTG induction with 10 µM all-trans retinal, pink-colored cells were sonicated, solubilized with 1% DM, and purified with a Ni²⁺-NTA column. The purified ASR protein was then reconstituted into PC liposomes by dialysis to remove the detergent with Bio-Beads, where the molar ratio of the added PC to ASR was 50:1. The ASR protein in PC liposomes was washed three times with a buffer [2 mM sodium phosphate (pH 7.0)]. A 60 µL aliquot was deposited on a BaF₂ window 18 mm in diameter, and the sample was dried in a glass vessel that was evacuated with an aspirator.

**FTIR Spectroscopy.** FTIR spectroscopy was performed as described previously (8). Since the all-trans form is most abundant for the dark-adapted ASR (9), ASR films were kept in the dark for 3 days. Completely dark-adapted ASR was hydrated with H₂O, D₂O, or D₂¹⁸O before measurements. Then, the sample was placed in a cryostat (DN-1704, Oxford) mounted on the cell for the FTIR spectrometer (FTS-40, Bio-Rad). The cryostat was equipped with a temperature controller (ITC-4, Oxford), and the temperature was regulated with 0.1 K precision. All the experimental procedures were performed in the dark or under dim red light (>670 nm) before the spectroscopic measurement.

Photoreactions of the all-trans and 13-cis forms strongly depend on the illumination wavelength of ASR. In this study, it is required that we reduce the extent of the photoreaction of the 13-cis form as much as possible. By using a maker band in the fingerprint (1200-1100 cm⁻¹) region (see the Results), I established the following illumination conditions at 77 K, where difference spectra depict the photoreaction of the 13-cis form at <20%. Illumination with 543 nm light at 77 K for 1 min converted ASR to ASRK. ASRK was reconverted to ASR upon illumination with >590 nm light for 1 min, as evidenced by a mirror image of the difference spectra. Each difference spectrum was calculated from two spectra constructed from 128 interferograms taken before and after the illumination. Twenty-four (H₂O and D₂O) or forty-eight (D₂¹⁸O) difference spectra were obtained and averaged to produce the ASRK minus ASR spectrum. ASR molecules are randomly oriented in the liposome
film, which is confirmed by linear dichroism experiments, so we did not apply dichroic measurements using an IR polarizer. The obtained difference spectra were compared with those for BR with the window tilting angle of 53.5° in the polarized measurement, where all vibrational bands are observed in the highly oriented BR molecule.

Results

In spectroscopic studies of archaeal rhodopsins, it is important to separate the photocycles of the all-trans from the 13-cis forms. In the case of BR, a well-known light adaptation procedure leads to a complete all-trans form. On the other hand, Vogeley et al. reported that ASR has a maximal amount of the all-trans form in the dark (>75%), while light adaptation rather decreases the amount of the all-trans form (9). It was reproduced in this study, and hence, I used the dark-adapted ASR sample. The absorption maximum of the all-trans enriched ASR was located at 549 nm, which was the same value reported previously (9). Low-temperature UV-visible spectroscopy of ASR showed that the red-shifted intermediate (ASRk) is formed at 77 K. The difference absorption maximum was located at 593 nm, and I estimated the absolute absorption maximum of ASRk at 589 nm (data not shown).

Comparison of the Difference Infrared Spectra Obtained by the Photoreactions of Anabaena Sensory Rhodopsin (ASR) at 77 K with Those of Bacteriorhodopsin (BR). Figure 2-1 shows the ASRk minus ASR (a) and BRk minus BR spectra (b), which were measured at 77 K in the hydration with H2O (solid lines) and D2O (dotted lines). Unlike those of BR, the difference spectra of ASR contain a mixture from photoproducts of the all-trans and 13-cis form. However, I estimated that the contribution is less than 20% by use of the marker band (1178 cm⁻¹) under our illumination conditions (see below). All vibrational bands described in this chapter originate from the photoreaction of the all-trans form.

The negative band at 1537 cm⁻¹ corresponds to the ethylenic stretching vibration of the all-trans chromophore in ASR, which exhibits an absorption maximum at 549
nm (9). The frequency is in good agreement with the well-known linear correlation between the ethylenic stretching frequencies and absorption maxima for various retinal proteins (10). In the case of BR, the bands at 1530 (-) / 1514 (+) cm\(^{-1}\) correspond to the ethylenic stretching vibrations of the unphotolyzed and K intermediate (BR\(_K\)) states, respectively (Figure 2-1b). On the other hand, two positive bands appeared at 1545 and 1523 cm\(^{-1}\) for ASR (Figure 2-1a). According to the linear correlation between the ethylenic stretching frequencies and absorption maxima (10), I predicted the ethylenic stretch of ASR\(_K\) (589 nm) to be at 1525 cm\(^{-1}\). Therefore, the 1523 cm\(^{-1}\) band is likely to be the ethylenic band of ASR\(_K\), and the band at 1545 cm\(^{-1}\) can possibly be assigned to the amide II mode. A similar observation was gained for halorhodopsin (11), where the K intermediate exhibits two positive bands at 1538 and 1514 cm\(^{-1}\) with a negative band at 1525 cm\(^{-1}\).

Remarkable spectral differences between ASR and BR were seen in the 1500-1450 cm\(^{-1}\) region. Two negative bands at 1457 and 1451 cm\(^{-1}\) and a positive band at 1471 cm\(^{-1}\) were observed for ASR (Figure 2-1a). Among these three bands, the 1457 cm\(^{-1}\) band is insensitive to H-D exchange, whereas the bands at 1471 and 1451 cm\(^{-1}\) reduce the half intensity in D\(_2\)O. On the other hand, such strong bands are absent for BR (Figure 2-1b). This frequency region corresponds to the imide II vibrations of proline, and the assignment of these bands is now in progress.

Comparison of the Vibrational Bands of the Retinal Chromophore between ASR and BR. C-C stretching vibrations of the retinal in the 1250-1150 cm\(^{-1}\) region are sensitive to the local structure of the chromophore. In Figure 2-2b, the negative bands at 1217, 1169, 1254, and 1203 cm\(^{-1}\) were assigned to the C8-C9, C10-C11, C12-C13, and C14-C15 stretching vibrations of BR, respectively (12). These frequencies are characteristic of the all-\textit{trans} retinal protonated Schiff base, though the frequencies are higher because of the charge delocalization of the retinal molecule in BR. Upon formation of BR\(_K\), the retinal isomerizes to the 13-\textit{cis} form, resulting in the appearance of a strong positive band at 1194 cm\(^{-1}\), which is assigned to C10-C11 and C14-C15 stretching vibrations (13).
Figure 2-1
The ASR\textsubscript{R} minus ASR (a) and the BR\textsubscript{R} minus BR (b) spectra in the 1800-850 cm\textsuperscript{-1} region measured at pH 7 and 77 K upon hydration with H\textsubscript{2}O (solid line) and D\textsubscript{2}O (dotted line), respectively. In the hydrated film, ASR molecules are oriented randomly, while BR molecules are highly oriented. Spectrum in (b) is reproduced from Kandori et al. (39), where the sample window is tilted by 53.5°. One division of the y-axis corresponds to 0.005 absorbance units.
A more complex spectral feature was observed for ASR in the 1250-1150 cm$^{-1}$ region (Figure 2-2a). One reason is that the photoreaction of the 13-cis form to its photoproduct contributes to these spectra. It is known that a positive band at $\sim$1180 cm$^{-1}$ is a marker band of such a reaction in BR (14). Similarly, in this study for ASR, I found that the bands at 1183 (-) / 1178 (+) cm$^{-1}$ increase in intensity when illumination wavelengths are changed. Thus, I interpreted that the bands originate from the photoreaction of the 13-cis form in ASR as well as in BR. In other words, I established the illumination conditions to maximally reduce the bands at 1183 (-) / 1178 (+) cm$^{-1}$ in this study. The FTIR study of the 13-cis form will be published elsewhere.

In the case of the all-trans form of ASR, the negative bands at 1218, 1174 (and/or 1167), 1249, and 1196 cm$^{-1}$ were tentatively assigned to the C8-C9, C10-C11, C12-C13, and C14-C15 stretching vibrations, respectively (Figure 2-2a). These frequencies are similar to those of BR (each frequency difference is $<10$ cm$^{-1}$), supporting the fact that the retinal configuration of ASR in the dark-adapted state is all-trans. However, the relatively large difference in C12-C13 (6 cm$^{-1}$) and C14-C15 (7 cm$^{-1}$) stretching vibrations suggests that the retinal structure near the Schiff base region is somehow different in ASR and BR. In addition, the intensity of the band at 1218 cm$^{-1}$ is 3 times larger than that of BR, which also suggests different retinal structure around the C8-C9 bond. Upon formation of ASR$_k$, the retinal molecule is considered to isomerize to the 13-cis form in analogy to the case of BR. However, unlike BR, there are three positive bands at 1199, 1189, and 1149 cm$^{-1}$. The 1199 cm$^{-1}$ band is not sensitive to H-D exchange, suggesting the origin as a C-C stretching vibration in the polyene chain of the retinal molecule. The bands at 1189 and 1149 cm$^{-1}$ are upshifted upon hydration with D$_2$O, suggesting that they are influenced by the Schiff base vibration. The 1189 cm$^{-1}$ band can be assigned to the C14-C15 stretching vibration, while the 1149 cm$^{-1}$ band is difficult to identify at present. The downshift of the C14-C15 stretching vibration from 1196 to 1189 cm$^{-1}$ upon formation of ASR$_k$ suggests that the retinal configuration is 13-cis in ASR$_k$. Splitting into two
Figure 2-2
The ASR\textsubscript{K} minus ASR (a) and the BR\textsubscript{K} minus BR (b) spectra in the 1290-1100 cm\textsuperscript{-1} region, which correspond to C-C stretching vibrations and N-H in-plane bending vibrations of the retinal chromophore. The sample was hydrated with H\textsubscript{2}O (solid lines) or D\textsubscript{2}O (dotted lines). One division of the y-axis corresponds to 0.004 absorbance units.
negative bands at 1174 and 1167 cm$^{-1}$ may suggest the presence of a positive band at 1171 cm$^{-1}$, which can be assigned to the C10-C11 stretching vibration.

The H-D exchangeable band at 1255 cm$^{-1}$ was assigned to one of the modes containing the N-H in-plane bending vibration of the Schiff base of BR (15), while similar negative bands appear at 1249 cm$^{-1}$ in the spectra of ASR. The band disappearing upon hydration with D$_2$O can be assigned to the modes of the Schiff base. The neighboring negative band at 1237 cm$^{-1}$ is also sensitive to deuteration and seen only in ASR, but its origin remains unknown. The result suggests that the hydrogen bonding environment of the Schiff base of ASR is similar to that of BR.

The difference spectra in the 1110-890 cm$^{-1}$ region are expanded in Figure 2-3. Hydrogen-out-of-plane (HOOP), N-D in-plane bending, and methyl rocking vibrations are observed here, and the presence of strong HOOP modes represents the distortion of the retinal molecule at the corresponding position. The most intense HOOP band in the BR$\kappa$ minus BR difference spectra (Figure 2-3b) was observed at 957 cm$^{-1}$ (in H$_2$O) and 951 cm$^{-1}$ (in D$_2$O), which were assigned to the C15-H HOOP vibration of BR$\kappa$ (15). The origins of the bands at 941, 962, and 974 cm$^{-1}$ remain unknown, but they are considered to be able to be assigned to HOOP vibrations. On the other hand, the weak negative band at 911 cm$^{-1}$ was assigned to the C15-H and N-H HOOP vibrations of the original state of BR (16). These results have been interpreted as an increase in the retinal distortion around the Schiff base upon the retinal isomerization in BR. In the case of ASR, similar but slightly upshifted bands were observed. The positive bands at 1001, 973, 968, and 957 cm$^{-1}$ of ASR$\kappa$ (Figure 2-3a) possibly correspond to those at 974, 962, 957, and 941 cm$^{-1}$ of BR$\kappa$, respectively (Figure 2-3b). The negative bands at 932 and 927 cm$^{-1}$ have probably the same origin as that at 911 cm$^{-1}$ in BR, which was assigned to the C15-H and N-H HOOP vibrations (16).

The negative band at 976 cm$^{-1}$ and the positive band at 969 cm$^{-1}$ in Figure 2-3b were assigned to the N-D in-plane bending vibrations of BR and BR$\kappa$, respectively (15). The 1009 cm$^{-1}$ band is insensitive to H-D exchange and was assigned to the
Figure 2-3
The ASR$_k$ minus ASR (a) and the BR$_k$ minus BR (b) spectra in the 1110-890 cm$^{-1}$ region, which correspond to hydrogen-out-of-plane (HOOP) vibrations of the retinal chromophore. The sample was hydrated with H$_2$O (solid lines) or D$_2$O (dotted lines). One division of the y-axis corresponds to 0.002 absorbance units.
methyl rocking vibration of the retinal in BR. The band at 1006 cm$^{-1}$ in Figure 2-3a can also be assigned to the methyl rocking vibration in ASR similarly. On the other hand, the bands at 1088 (-), 1080 (+), and 1025 (-) cm$^{-1}$ are highly characteristic of the ASR$_{k}$ minus ASR difference spectra, and never observed in other archaeal-type rhodopsins such as BR, ppR, and NR (9, 15, 17). According to the literature, the antisymmetric NC$_3$ stretchings of tertiary amines appear in the 1250-1000 cm$^{-1}$ region (18). Thus, these bands may originate from the skeletal vibrations of Pro206 as well as those at 1471 (+), 1457 (-), and 1451 (-) cm$^{-1}$ (Figure 2-1a).

C=N stretching vibrations of the protonated retinal Schiff base are observed in the 1650-1600 cm$^{-1}$ region (Figure 2-4). The C=N stretching vibrations are sensitive to H-D exchange, and the difference in frequency has been considered as the probe for its hydrogen bonding strength (19, 20). Namely, the larger the difference is, the stronger the hydrogen bond is. The C=NH and C=ND stretching vibrations of BR were observed at 1641 and 1628 cm$^{-1}$, while those of BR$_{k}$ were at 1608 and 1606 cm$^{-1}$, respectively (21). The former difference in frequency is 13 cm$^{-1}$, and the latter is 2 cm$^{-1}$, suggesting that the protonated Schiff base forms a hydrogen bond in BR and is broken upon retinal isomerization. The C=N stretchings were observed at 1642 (C=NH) and 1624 cm$^{-1}$ (C=ND) in ASR, and its difference is 18 cm$^{-1}$, suggesting that the hydrogen bonding strength is stronger than that of BR. On the other hand, it is difficult to assign the positive bands because of the more complicated spectral feature. There are two sets of candidates for the C=N stretching vibrations of ASR$_{k}$. One set is the bands at 1621 (C=NH) and 1610 cm$^{-1}$ (C=ND), while another set is the bands at 1600 (C=NH) and 1595 cm$^{-1}$ (C=ND). The differences in frequency are 11 and 5 cm$^{-1}$ for the former and latter, respectively. If the former is the case, the hydrogen bond may not be broken upon retinal isomerization in ASR. Conclusive assignment of the C=N stretching of ASR$_{k}$ needs stable isotope labeling on the Schiff base, which is for our future study. On the other hand, the N-D stretching vibration of the Schiff base in D$_2$O provides another and more direct information about the hydrogen bond of the Schiff base as described below.
Figure 2-4
The ASR$_x$ minus ASR (a) and the BR$_x$ minus BR (b) spectra in the 1760-1570 cm$^{-1}$ region, most of which are ascribable for vibrations of the protein moiety. The underlined peaks are C=N stretching vibrations of the chromophore. The sample was hydrated with H$_2$O (solid lines) or D$_2$O (dotted lines). One division of the y-axis corresponds to 0.003 absorbance units.
Comparison of the C=O Stretching Vibrations of Carboxylate, Carbonyl, and Amide Groups of the Protein Moiety between ASR and BR. In the BR\textsubscript{x} minus BR difference spectra (Figure 2-4b), the bands at 1742 and 1733 cm\textsuperscript{-1} were assigned to the C=O stretching vibrations of the protonated Asp115, which are downshifted upon hydration with D\textsubscript{2}O (22). In contrast, there is no band in the same frequency region of the ASR spectra (Figure 2-4a), implying that Asp and Glu residues are located far from the retinal molecule even if they are protonated. ASR has a glutamine residue at the corresponding position of Asp115 in BR, whose vibrational bands are probably observed at 1698 (-) and 1694 (+) cm\textsuperscript{-1} (Figure 2-4a). Similar bands were also observed at 1704 (-) and 1700 (+) cm\textsuperscript{-1} in the difference spectra of ppR, which has an asparagine residue at the corresponding position (23). These observations suggest that the structural changes around Asp115 in BR are similar among ASR, BR, and ppR.

The band pairs at 1668 (-) / 1664 (+) cm\textsuperscript{-1} and at 1623 (+) / 1617 (-) cm\textsuperscript{-1} were assigned to the C=O stretching vibrations of amide I. The former was assigned to the amide I of \(\alpha\)\textsubscript{II} helix (24) and the latter to the amide I of Val49 (25). In the case of ASR, a band pair at 1679 (-) / 1673 (+) cm\textsuperscript{-1} could be similar in origin to the bands at 1668 (-) / 1664 (+) cm\textsuperscript{-1} in BR. It should be noted that the spectral changes of amide I vibrations at <1660 cm\textsuperscript{-1} are much smaller in ASR than in BR, which is clearly seen in D\textsubscript{2}O. This suggests that the structural changes of the peptide backbone in ASR are very small upon retinal isomerization. On the other hand, the structural perturbation of Pro206 was suggested for ASR.

Comparison of the X-D Stretching Vibrations between ASR and BR. X-D stretching vibrations of protein and water molecules appear in the 2750-1950 cm\textsuperscript{-1} region (Figure 2-5). A spectral comparison between the samples hydrated with D\textsubscript{2}O and D\textsubscript{2}\textsuperscript{18}O identifies O-D stretching vibrations of water molecules which change their frequencies upon retinal photoisomerization. Green-labeled bands in Figure 2-5 can be assigned to the O-D stretching vibrations of water because of the isotope shift. In BR, six negative peaks at 2690, 2636, 2599, 2321, 2292, and 2171 cm\textsuperscript{-1}
Figure 2-5
Comparison with the difference infrared spectra of ASR (a) and BR (b) hydrated with D$_2$O (red lines) or D$_2^{18}$O (blue lines) in the 2730-1950 cm$^{-1}$ region. Green-labeled frequencies correspond to those identified as water stretching vibrations. Purple-labeled frequencies are O-D stretches of Thr89 (29, 30), while the underlined frequencies are N-D stretches of the Schiff base (21). Spectrum in (b) is reproduced from Tanimoto et al. (5), where the sample window is tilted by 53.5°. One division of the y-axis corresponds to 0.0007 absorbance units.
were earlier assigned to vibrations of water molecules (Figure 2-5b) (5, 26). The bands are widely distributed over the possible frequency range for stretching vibrations of water. Since the frequencies of the negative peaks at 2321, 2292, and 2171 cm$^{-1}$ are much lower than those of fully hydrated tetrahedral water molecules (26), the hydrogen bonds of those water molecules must be very strong, possibly indicating their association with negative charges. Indeed, I assigned the 2171 cm$^{-1}$ band to the O-D group of a water molecule associated with deprotonated Asp85 (4). This water molecule, called water 402 in the crystal structure of BR (PDB entry 1C3W), is located between the Schiff base and Asp85 (Figure 1-5). A previous QM/MM calculation of the Schiff base region of BR also supported the existence of an extremely strong hydrogen bond between water 402 and Asp85 (27). Water stretching vibrations of BR$_{k}$ tend to be higher in frequency, implying that the overall hydrogen bonding becomes weaker upon photoisomerization.

In contrast, interestingly, only three negative peaks at 2690, 2640, and 2608 cm$^{-1}$ could be assigned to the O-D stretching vibrations of water in ASR (Figure 2-5a). The bands at 2701, 2653, and 2573 cm$^{-1}$ were assigned as water stretching vibrations of ASR$_{k}$. It should be emphasized that there are no water bands in the <2400 cm$^{-1}$ region, which is a significant difference from the published results for BR and ppR. In the case of ppR, two pairs of peaks were observed in the <2400 cm$^{-1}$ region, located at 2369 (+)/2307 (-) cm$^{-1}$ and at 2274 (+)/2215 (-) cm$^{-1}$ (28). Since ASR has a bridged water molecule between the Schiff base and Asp75 (Figure 1-5) as well as BR and ppR, one may expect similar water bands at <2400 cm$^{-1}$. However, that is not the case for ASR. I will discuss the structural reason for the lack of strongly hydrogen bonded water molecules below.

The frequency region shown in Figure 2-5 also contains X-D stretching vibrations other than water molecules. In the BR$_{k}$ minus BR spectrum, the bands at 2507 (-)/2466 (+) cm$^{-1}$ labeled in purple and the underlined bands at 2466 (+), 2171 (-), and 2124 (-) cm$^{-1}$ were assigned to the O-D stretching vibrations of Thr89 (29, 30) (the corresponding residue in ASR is Thr79) and the N-D stretching vibrations of the
Figure 2-6
The ASR$_k$ minus ASR spectra in the 2590-2500 cm$^{-1}$ (the upper panel) and 1890-1800 cm$^{-1}$ (the lower panel) region, which correspond to S-H and S-D stretching vibrations of cysteine residues, respectively. The sample was hydrated with H$_2$O (solid lines) or D$_2$O (dotted lines). One division of the y-axis corresponds to 0.0001 absorbance units.
retinal Schiff base (21), respectively. Thus, the negative 2171 cm\(^{-1}\) band contains both the O-D stretch of water and the N-D stretch of the Schiff base. In the ASR\(_k\) minus ASR spectrum, there are 10 bands other than water bands: 2547 (-), 2537 (+), 2508 (-), 2470 (-), 2446 (+), 2383 (+), 2336 (-), 2258 (-), 2163 (-), and 2125 (-) cm\(^{-1}\). The bands at 2547 (-) / 2537 (+) cm\(^{-1}\) are attributed to the H-D unexchangeable S-H stretching vibration of a cysteine residue as described below. The bands at 2508 (-) / 2470 (+) cm\(^{-1}\) can be assigned to the O-D stretching vibrations of Thr79 in analogy to BR. The O-D frequencies of Thr79 in ASR and ASR\(_k\) (2508 and 2470 cm\(^{-1}\)) are almost identical to those of Thr89 in BR and BR\(_k\) (2507 and 2466 cm\(^{-1}\)), respectively, indicating that the strength of hydrogen bonding between Thr79 and Asp75 is identical to that between Thr89 and Asp85 in BR.

Though not assigned directly by use of the labeled protein, the bands at 2163 and 2125 cm\(^{-1}\) are likely to originate from N-D stretching of the Schiff base, whose frequencies are very similar to those in BR (2171 and 2124 cm\(^{-1}\)). This fact indicates similar hydrogen bonding strengths between ASR and BR. The slightly lower frequency of the strong band (2163 cm\(^{-1}\) in ASR and 2171 cm\(^{-1}\) in BR) may correspond to the results obtained for the C=N stretching vibrations as shown before (Figure 2-4). The analysis of the C=N stretching vibrations of ASR\(_k\) suggested two possibilities for the hydrogen bonding strength of the Schiff base. Figure 2-5a clearly shows the presence of the negative bands at 2163 and 2125 cm\(^{-1}\), implying that the N-D stretch is upshifted in ASR\(_k\). I infer that one of the bands at 2470, 2446, and 2383 cm\(^{-1}\) can be assigned to the N-D stretch in ASR\(_k\). Thus, I can safely conclude that the hydrogen bond of the Schiff base in ASR becomes much weaker upon retinal photoisomerization as well as in BR.

**S-H Stretching Vibrations of the Cysteine Residues.** Figure 2-6 shows the ASR\(_k\) minus ASR spectra in the 2590-2500 cm\(^{-1}\) (top panel) and 1890-1800 cm\(^{-1}\) (bottom panel) regions, which correspond to S-H and S-D stretching vibrations of cysteine residues, respectively. There is a negative band at 2547 cm\(^{-1}\) and a positive band at 2538 cm\(^{-1}\), while no band is observed in the S-D stretching upon hydration with D\(_2\)O.
In fact, S-H stretching vibrations in D$_2$O are observed in Figure 2-5a (2547 and 2537 cm$^{-1}$). The S-H stretching frequency of cysteine appears in the 2580-2525 cm$^{-1}$ region. Thus, the frequency change from 2547 to 2538 cm$^{-1}$ suggests that the cysteine forms a considerably strong hydrogen bond upon retinal isomerization. The H-D unexchangeable nature of the cysteine S-H group presumably originates from either the hydrophobic environment or the strong hydrogen bond.

The lower-frequency shift in ASR is the opposite of the cysteine signal in the NR$_x$ minus NR spectra (17). In addition, the H-D exchange is different between ASR and NR. These facts suggest that the cysteine residues are located in different environments and their hydrogen bonds change differently. There are three cysteine residues in ASR, Cys134 and Cys137 in helix E and Cys203 in helix G. All of them are not conserved in archaeal-type rhodopsin, but Cys134 and Cys137 are located at a position similar to that of Cys170 in NR, which is conserved in halorhodopsin. The X-ray crystal structure of ASR also revealed that only the S-H group of Cys203 is directed to the inside of the protein. From these results, the observed band can be assigned to the S-H stretching of Cys203.

**Discussion**

In this study, I measured the ASR$_x$ minus ASR spectra by means of low-temperature FTIR spectroscopy. For this aim, ASR was expressed in E. coli, and the wild-type protein was reconstituted into PC liposomes. It is noted that the ASR molecule is not embedded in the native membrane, which could modify the FTIR spectra. For instance, H-D exchange may be different between PC liposomes and the native membrane, which should be elucidated in the future. However, this study focuses the structural changes near the retinal upon photoisomerization, and the light-induced difference FTIR spectra are not significantly affected by different lipid environments.

Despite the presence of the 13-$cis$ form, the obtained spectra predominantly originate from the photoreaction of the all-$trans$ form, and the spectra were compared
with those of BR. These results clearly show that the all-\textit{trans} to 13-\textit{cis} photoisomerization takes place in ASR like in BR, though the C-C stretching and HOOP vibrations are somehow different. The protonated Schiff base forms a strong hydrogen bond in ASR presumably with the bridged water (Figure 1-5), and the hydrogen bond is cleaved by the rotation of the N-H (N-D) group, as in BR. I also observed S-H stretches of a cysteine residue which is insensitive to hydration with D$_2$O. I observed the small bands of amide I, and large bands that can be ascribed to imide II [1471 (+), 1457 (-), and 1451 (-) cm$^{-1}$] and NC$_3$ [1088 (-) and 1080 (+) cm$^{-1}$] stretchings of proline residues. Previous resonance Raman spectroscopic study showed that the imide II vibration of the X-Pro bond appears at around 1450 cm$^{-1}$ (31).

BR has three Pro residues in the transmembrane region, Pro50, Pro91, and Pro186 (Figure 1-6). The previous FTIR study suggested that the environment around these proline residues changes upon retinal isomerization via observation of the isotope effect of $[^{15}\text{N}]$proline in the 1450-1420 cm$^{-1}$ region (32). It should be noted that spectral changes are much smaller in BR than in ASR in this frequency region. In the case of ASR, there is an additional proline to the conserved three Pro residues (Figure 1-6). It is Pro206, a corresponding residue of Asp212 in BR (Figure 1-5). Figure 1-5 shows that the peptide C=O group of Pro206 forms a hydrogen bond with the peptide amide (N-H group) of Lys210, which connects a retinal chromophore. Thus, retinal photoisomerization strongly perturbs the peptide C-N bond of Pro206 in ASR, presumably leading to the appearance of these unusually intense bands in the 1500-1450 cm$^{-1}$ region. It should be noted, however, that I can conclude this argument only when these bands are assigned by use of $[^{15}\text{N}]$proline-labeled ASR, which is now in progress.

A significant difference was seen for water bands between ASR and BR. I have so far observed the O-D stretching vibrations of water molecules under strongly hydrogen bonded conditions in the BR$_k$ minus BR and ppR$_k$ minus ppR difference spectra (5, 26, 28). The X-ray crystal structures of BR and ppR reported the presence of a bridged water molecule between the Schiff base and its counterion.
(Asp85 in BR and Asp75 in ppR) (33-35). Therefore, the hydrogen bond of the water is expected to be strong, and such strongly hydrogen bonded water molecules were observed in the FTIR studies. The water molecules possess O-D stretches at 2400-2100 cm\(^{-1}\) in D\(_2\)O (2, 6). Since ASR has a bridged water molecule between the Schiff base and Asp75 (Figure 1-5) as well as BR and ppR, one may expect similar water bands at <2400 cm\(^{-1}\). However, that was not the case for ASR. Therefore, the structural reason for the lack of strongly hydrogen bonded water molecules has to be explained on the basis of the structural background. Since both structures of ASR and BR are known (Figure 1-5), I will try to explain the reason here.

Our analysis of the Schiff base mode (C=N stretch and N-D stretch) in ASR showed that the hydrogen bonding strength of the Schiff base is similar in ASR and BR. This observation is consistent with the similar distance between the Schiff base nitrogen and water oxygen (2.6 Å for ASR and 2.9 Å for BR). A slightly stronger hydrogen bond in ASR than in BR is also consistent with the distance that is shorter in ASR. In contrast, water bands in ASR were entirely different from those in BR, although the distance between the water oxygen and the oxygen of the counterion are similar (2.7 Å for ASR and 2.6 Å for BR). The O-D stretch of the bridged water in BR is located at 2171 cm\(^{-1}\) (Figure 2-5b), whereas that in ASR is probably one of the bands at 2690, 2640, and 2608 cm\(^{-1}\) (Figure 2-5a). How is such difference observed between ASR and BR? It may be explained by the difference in the geometry of the hydrogen bond. Figure 2-7 shows that the N-O\(_{\text{water}}\) -O\(_{\text{Asp75}}\) (the Schiff base nitrogen, the water oxygen, and the oxygen of Asp75, respectively) angle in ASR is 83°. The corresponding N-O\(_{\text{water}}\) -O\(_{\text{Asp85}}\) angle in BR is 106° (Figure 2-7). As the consequence, if the water oxygen fully accepts the hydrogen bond of the Schiff base, the O-H group of water points toward the oxygen of Asp85 in BR, but not toward that of Asp75 in ASR (Figure 2-7). Such a small difference in angle possibly determines the hydrogen bonding strength of water molecules.

On the basis of our FTIR studies of BR mutants and other rhodopsins, I have found an interesting correlation between strongly hydrogen bonded water molecules
and proton pump activity. Among various BR mutant proteins I have studied, only D85N and D212N lack strongly hydrogen bonded water molecules. Other BR mutants possess their O-D stretches at <2400 cm$^{-1}$, which include T46V, R82Q, R82Q/D212N, T89A, D96N, D115N, Y185F, and E204Q (36). Among these mutants, only D85N and D212N do not pump protons. Therefore, strongly hydrogen bonded water molecules are only found in the proteins exhibiting proton pumping activities. The correlation between proton pumping activity and strongly hydrogen bonded water molecules is true not only for BR mutants but also for various rhodopsins. Whether rhodopsins possess strongly hydrogen bonded water molecules has been examined systematically. I found that BR and pharaonis phoborhodopsin (5, 26, 28), both of which pump protons, possess such water molecules (O-D stretch at <2400 cm$^{-1}$ in D$_2$O). In contrast, strongly hydrogen bonded water molecules were not observed for halorhodopsin (37), Neurospora rhodopsin (17), and bovine rhodopsin (38). It is known that none of them pumps protons. Such comprehensive studies of archaeal and visual rhodopsins have thus revealed that strongly hydrogen bonded water molecules are only found in the proteins exhibiting proton pumping activities. Taken together, these results for ASR suggest that the strong hydrogen bonds of water molecules and their transient weakening may be essential for the proton pumping function of rhodopsins.
Figure 2-7
Schematic drawing of hydrogen bonds of the water molecule locating between the protonated Schiff base and its counterion. A part of all-trans retinal are depicted and β-ionon ring and ethylenic part from C6 to C12 are omitted. The numbers are the angle of the N-O-O atoms derived from the crystal structures of ASR and BR (PDB entries are 1XIO and 1C3W, respectively). Hydrogen bonds are indicated by the dashed lines with their strength.
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2-2 FTIR study of the photoisomerization processes in the 13-cis and all-trans forms of Anabaena sensory rhodopsin at 77 K.

**Introduction**

To understand the details of light-induced structural changes of ASR, we have recently applied low-temperature FTIR spectroscopy to the all-trans form of ASR (AT-ASR) and compared the difference spectra at 77 K with those of BR in Figure 2-1 (1). The K intermediate minus AT-ASR difference spectra showed that the retinal isomerizes from all-trans to distorted 13-cis form similar to BR. On the other hand, a remarkable difference between AT-ASR and BR was revealed in water bands. Although ASR possesses a water molecule between the Schiff base and its counterion Asp75 similar to BR, the O-D stretching bands of water molecules were observed only in the >2500 cm⁻¹ region for AT-ASR (1). We interpreted that the weak hydrogen bond of the bridged water in ASR originates from its unique geometry. Since ASR does not pump protons and the direction of the proton movement is toward cytoplasmic side from the characteristics of the photoelectric signal (2), the results support the working hypothesis that the existence of strongly hydrogen-bonded water molecules is essential for proton pumping activity in archaeal rhodopsins (3).

Here, I extended the low-temperature spectroscopic study at 77 K to the 13-cis, 15-syn form of ASR (13C-ASR). HPLC analysis revealed that light-adapted ASR with light >560 nm at 4 °C possesses 78% 13C-ASR, while dark-adapted ASR has AT-ASR predominantly (97%). Then, I established the illumination conditions to measure the difference spectra between 13C-ASR and its K state without subtracting the difference between AT-ASR and its K state. Spectral comparison between 13C-ASR and AT-ASR provided useful information on structure and structural changes upon retinal photoisomerization in ASR. In particular, previous X-ray crystallographic study of ASR reported the same protein structure for 13C-ASR and
AT-ASR (4), whereas the present FTIR study revealed that protein structural changes upon retinal photoisomerization were significantly different between 13C-ASR and AT-ASR. The differences were seen for HOOP modes of the retinal chromophore, amide I, cysteine S-H stretch, the Schiff base N-D stretch, and water O-D stretch modes. These must trigger different global protein structural changes in each photoreaction cycle leading to the observed photochromic behavior.

**Materials and Methods**

*Sample Preparation.* Samples for the present spectroscopic analysis were prepared as shown in Chapter 2-1. [ζ-15N]Lysine labeled ASR was prepared as was done for [ζ-15N]lysine labeled *pharaonis* phoborhodopsin (5).

*HPLC Analysis.* HPLC analysis was performed as described previously (6). A high-performance liquid chromatograph was equipped with a silica column (6.0x 150 mm; YMC-Pack SIL). The solvent was composed of 12% (v/v) ethyl acetate and 0.12% (v/v) ethanol in hexane, and the flow rate was 1.0 mL/min. Extraction of retinal oxime from the sample was carried out by hexane after denaturation by methanol and 500 mM hydroxylamine at 4 °C (7). The molar composition of retinal isomers was calculated from the areas of the peaks in the HPLC patterns. Assignment of the peaks was performed by comparing them with the HPLC pattern from retinal oximes of authentic all-trans and 13-cis retinals. Three independent measurements were averaged.

*FTIR Spectroscopy.* FTIR spectroscopy was performed as described previously (8). The ASR film sample was hydrated with 1 μL of H₂O, D₂O, or D₂¹⁸O before the measurements. Then, the sample was placed in a cryostat (DN-1704; Oxford) mounted in the FTIR spectrometer (FTS- 40; Bio-Rad). The cryostat was equipped with a temperature controller (ITC-4; Oxford), and the temperature was regulated with 0.1 K precision. To accumulate 13C-ASR, I established the following conditions for the light adaptation. Hydrated films were illuminated with >560 nm light (O-58 cutoff filter; Toshiba) from a 1 kW halogen-tungsten lamp for 1 min at 4 °C.
analysis showed that the light-adapted ASR possesses 78\% 13-cis and 22\% all-trans forms. The sample was cooled for 3 min after the light adaptation to allow for the complete decay of photoproducts.

Light-adapted ASR contains both all-trans and 13-cis forms, thus, its photoreactions strongly depend on the illumination wavelength. In this study, I established the following illumination conditions to obtain the K intermediate of 13C-ASR (13C-ASR<sub>k</sub>) minus 13C-ASR spectra at 77 K. Illumination with 501 nm light for 1 min first converted 13C-ASR to 13C-ASR<sub>k</sub> together with the conversion of AT-ASR to the K intermediate of AT-ASR (AT-ASR<sub>k</sub>). Nevertheless, subsequent illumination at >560 nm (O-58 cutoff filter; Toshiba) reverted only 13C-ASR<sub>k</sub> to 13C-ASR, whereas no photoreversion was found for AT-ASR<sub>k</sub>. In chapter 2-1 for AT-ASR, I illuminated AT-ASR<sub>k</sub> at >590 nm for the photoreversion to AT-ASR (1). The present result indicates that illumination at >560 nm does not change the photoequilibrium between AT-ASR and AT-ASR<sub>k</sub>. Subsequent illuminations by 501 nm light and >560 nm light do not induce the spectral features of AT-ASR<sub>k</sub> minus AT-ASR. Each difference spectrum was calculated from the two spectra constructed from 128 interferograms taken before and after the illumination. Twenty-four (H<sub>2</sub>O and D<sub>2</sub>O) or forty-eight (D<sub>2</sub>18O) difference spectra were obtained and averaged to produce the 13C-ASR<sub>k</sub> minus 13C-ASR spectrum. ASR molecules are randomly oriented in the liposome film as confirmed by linear dichroism experiments, so I did not apply dichroic measurements using an IR polarizer.

**Results**

Dark-adapted ASR is predominantly in the all-trans form, while the light adaptation process increases concentration of the 13-cis form (4, 9). This is in contrast to the case of BR, where light adaptation leads to a complete conversion into the all-trans form (10). In this study, I attempted to establish the illumination conditions to accumulate the 13-cis form for DM-solubilized and PC-liposome-based ASR samples, using HPLC column chromatography.
Figure 2-8
HPLC of chromophores extracted from ASR in DM micelles (a) and in PC liposomes (b). The detection beam was set at 360 nm. After the extraction, retinal oxime exists in 15-*syn* and 15-*anti* form. In the shown range of retention times, only the 15-*syn* form appears. We used area of both 15-*syn* and 15-*anti* forms for calculation of isomeric ratios. Dark-adapted ASR (solid lines) is in the all-*trans* form predominantly (AT-ASR; 95.5 ± 0.8 % in (a) and 97.1 ± 0.1 % in (b)), while light-adapted ASR (dotted lines) possesses more of the 13-*cis* form (13C-ASR; 78.1 ± 1.2 % in (a) and 77.9 ± 1.7 % in (b)).
Panels a and b of Figure 2-8 show that the dark-adapted ASR (solid lines) possesses 95.5% and 97.1% all-trans form for the DM-solubilized and PC-liposome-based samples, respectively. On the other hand, illumination of ASR with >560 nm light for 1 min at 4 °C results in accumulation of 13C-ASR. HPLC analysis showed that the light-adapted ASR possesses 78.1% and 77.9% of the 13-cis form for the DM-solubilized and PC-liposome-based samples, respectively. Thus, the isomeric composition was not influenced by the reconstitution. Dark adaptation was a slow process, with half-time >1 h at 4 °C (data not shown).

A hydrated film of ASR in PC liposomes was light-adapted as described above and then cooled to 77 K, followed by illumination at 501 nm. Figure 2-9a shows the light minus dark difference FTIR spectra of the light-adapted ASR. Vibrational bands at 1218 (-), 1199 (+), 1196 (-), and 1189 (+) cm⁻¹ also appear in the AT-ASRₖ minus AT-ASR (Figure 2-9c) (1), indicating that the conversion of AT-ASR to AT-ASRₖ is involved in the spectrum of Figure 2-9a. On the other hand, Figure 2-9a possesses additional strong peaks at 1184 (-) and 1178 (+) cm⁻¹, suggesting the involvement of the photoreaction of 13C-ASR. In the previous study for AT-ASR, I illuminated AT-ASRₖ at >590 nm for the photoreversion to AT-ASR (1). In the present study, subsequent illuminations at >560 and 501 nm yielded the spectra shown in Figure 2-9b (dotted and solid lines, respectively). Lack of the bands at 1218, 1199, 1196, and 1189 cm⁻¹ strongly suggests that the spectra do not contain the photoreaction of AT-ASR. In other words, the solid line in Figure 2-9b corresponds to the 13C-ASRₖ minus 13C-ASR spectrum. In fact, the spectrum of Figure 2-9a is well constructed from the solid lines in Figure 2-9b (data not shown). In this way, I obtained the 13C-ASRₖ minus 13C-ASR difference FTIR spectra without any subtraction of spectral contribution from AT-ASR. It is likely that the photoequilibrium between AT-ASR and AT-ASRₖ is not changed between illuminations at 501 nm and at >560 nm, so that further illumination with 501 nm and >560 nm in Figure 2-9b yielded the difference spectra between 13C-ASR and 13C-ASRₖ.
Figure 2-9
Difference FTIR spectra in the 1240-1130 cm\(^{-1}\) region measured at 77 K (in H\(_2\)O), where the spectra before illumination were subtracted from those after illumination. Light-adapted ASR that contains 13C-ASR (78 %) and AT-ASR (22 %) was first illuminated with 501 nm light for 1 min (a). Then, illumination at >560 nm for 1 min converted a part of the photoproducts in (a) to the original state (dotted line in b). Subsequent illumination with 501 nm light yields the difference spectrum (solid line in b), which is a mirror image of the dotted spectrum. Repeated illuminations at >560 nm and at 501 nm yield the identical spectra. (c) The AT-ASR\(_k\) minus AT-ASR spectra are reproduced from Furutani et al. (15).
Comparison of the Difference Infrared Spectra of the Photoreactions of 13C-ASR and AT-ASR at 77 K in the 1770-870 cm\(^{-1}\) Region. Figure 2-10 shows the 13C-ASR\(_k\) minus 13C-ASR (a) and the AT-ASR\(_k\) minus AT-ASR spectra (b), which were measured at 77 K upon hydration with H\(_2\)O (solid lines) and D\(_2\)O (dotted lines). In Figure 2-10a, the negative band at 1539 cm\(^{-1}\) corresponds to the ethylenic stretching vibration of the 13-cis chromophore in ASR, which exhibits the absorption maximum at 537 nm (4). The frequency is in good agreement with the well-known linear correlation between the ethylenic stretching frequencies and absorption maxima for various retinal proteins (11). Illumination results in the spectral downshift to 1527 cm\(^{-1}\), indicating formation of the red-shifted K intermediate (13C-ASR\(_k\)).

C-C stretching vibrations of the retinal in the 1300-1150 cm\(^{-1}\) region are sensitive to the local structure of the chromophore. In the 13C-ASR\(_k\) minus 13C-ASR spectrum in H\(_2\)O, peaks are observed at 1277 (+), 1258 (-), 1204 (+), 1184 (-), 1178 (+), and 1161 (-) cm\(^{-1}\) (Figure 2-10a, solid line). In the case of the 13-cis form of BR, appearance of a peak pair at 1185 (-) and 1177 (+) cm\(^{-1}\) was regarded as a marker of the formation of the all-trans photoprodut (12). Similar bands at 1184 (-) and 1178 (+) cm\(^{-1}\) for 13C-ASR strongly suggest that 13C-ASR\(_k\) possesses the all-trans chromophore produced by photoisomerization of the C13=C14 bond. As in the case of BR, the 1184 (-) / 1178 (+) cm\(^{-1}\) bands are insensitive to the H-D exchange (Figure 2-10a, dotted line), being thus assignable to C10-C11 stretching vibration (12). Strong positive peaks at 1277 and 1204 cm\(^{-1}\) in H\(_2\)O and at 1231 cm\(^{-1}\) in D\(_2\)O were also observed for the 13-cis form of BR, where positive peaks at 1205 cm\(^{-1}\) in H\(_2\)O and at 1234 cm\(^{-1}\) in D\(_2\)O were assigned to be C14-C15 stretching vibrations (12). Therefore, corresponding peaks at 1204 cm\(^{-1}\) in H\(_2\)O and at 1231 cm\(^{-1}\) in D\(_2\)O are assignable to the C14-C15 stretching vibration of 13C-ASR\(_k\). Spectral coincidence between BR and ASR implies similar chromophore structures of their 13-cis forms and respective K states. Hydrogen-out-of-plane (HOOP), N-D in-plane bending, and methyl rocking vibrations are observed in the 1110-890 cm\(^{-1}\) region, and the presence of strong HOOP modes represents distortions of the retinal molecule (13).
Figure 2-10
The 13C-ASR<sub>k</sub> minus 13C-ASR (a) and the AT-ASR<sub>k</sub> minus AT-ASR (b) spectra (pH 7) in the 1800-800 cm<sup>-1</sup> region measured at 77 K upon hydration with H<sub>2</sub>O (solid line) and D<sub>2</sub>O (dotted line), respectively. The spectra in panel b are reproduced from Furutani et al. (15). One division of the y-axis corresponds to 0.007 absorbance units.
The AT-ASR<sub>k</sub> minus AT-ASR spectra exhibit two strong peaks at 968 and 957 cm<sup>-1</sup> (Figure 2-10b). In contrast, many positive bands were observed in the 13C-ASR<sub>k</sub> minus 13C-ASR spectra, whose frequencies are at 1002, 991, 981, 971, 965, and 957 cm<sup>-1</sup> (Figure 2-10a). This observation suggests that the chromophore of 13C-ASR<sub>k</sub> is more distorted along the polyene chain than that of AT-ASR<sub>k</sub>.

Figure 2-11 shows the 13C-ASR<sub>k</sub> minus 13C-ASR (a) and the AT-ASR<sub>k</sub> minus AT-ASR spectra (b) in the 1750-1550 cm<sup>-1</sup> region. Amide I vibrations appear in this frequency region together with the C=N stretching vibration of the protonated retinal Schiff base. In general, the former is insensitive to the H-D exchange, whereas the latter exhibits downshift in D<sub>2</sub>O. In the case of AT-ASR, a prominent peak pair at 1642 (-) and 1621 (+) cm<sup>-1</sup> is assignable to the C=N stretchings of AT-ASR and AT-ASR<sub>k</sub>, respectively, because of the spectral shifts to 1624 (-) and 1610 (+) cm<sup>-1</sup> in D<sub>2</sub>O (Figure 2-11b). In fact, I observed the downshift of the bands at 1642 (-) and 1621 (+) cm<sup>-1</sup> by 10 cm<sup>-1</sup> for [ζ-<sup>15</sup>N]lysine-labeled ASR, indicating that they originate from the C=N stretching vibrations (data not shown). It should be noted that the spectral changes of amide I vibrations at 1660-1630 cm<sup>-1</sup> are small in AT-ASR<sub>k</sub> minus AT-ASR, which is clearly seen in D<sub>2</sub>O (Figure 2-11b, dotted line), suggesting that no structural changes of the peptide backbone occur in AT-ASR upon retinal isomerization. The spectral features are quite different for 13C-ASR. Figure 2-11a shows the presence of the H-D exchange independent bands in the 1660-1630 cm<sup>-1</sup> region, located at 1669 (+), 1662 (-), 1655 (-), 1649 (-), 1644 (+), 1634 (-) and 1628 (+) cm<sup>-1</sup>. This suggests perturbation of the peptide backbone upon retinal photoisomerization of 13C-ASR. In particular, the peaks at 1662, 1655, and 1649 cm<sup>-1</sup> are ascribable to the amide I vibrations of the α-helix. Helical perturbation may be correlated with many peaks of the HOOP vibrations in 13C-ASR<sub>k</sub>.

Unlike in AT-ASR (Figure 2-11b), the 13C-ASR<sub>k</sub> minus 13C-ASR spectra (Figure 2-11a) do not show H-D exchange dependent bands clearly. This indicates that the C=N stretching vibrations are not clearly observed in the spectra. Reproducible differences between H<sub>2</sub>O and D<sub>2</sub>O samples in Figure 2-11a suggest that the C=N
The $^{13}$C-ASR$_k$ minus $^{13}$C-ASR (a) and the AT-ASR$_k$ minus AT-ASR (b) spectra (pH 7) in the 1750-1550 cm$^{-1}$ region, mostly representing vibrations of the protein moiety. The sample was hydrated with $\text{H}_2\text{O}$ (solid lines) or $\text{D}_2\text{O}$ (dotted lines). One division of the y-axis corresponds to 0.0025 absorbance units.
stretching vibrations are present in this frequency region. In fact, bands at 1640-1620 cm$^{-1}$ were sensitive to [$$^{15}$N]lysine labeling (not shown). However, the absence of clear peaks of the C=N stretch requests spectral analysis using double difference spectra. The C=N stretching vibrations have been regarded as an important marker, because the difference in frequency between H$_2$O and D$_2$O samples probes hydrogen-bonding strength of the Schiff base (14, 15). In the present study, however, I discuss the hydrogen-bonding strength of the Schiff base by use of the N-D stretching in D$_2$O (see below), which is the more direct probe (16).

In the carboxylic C=O stretching frequency region (>1700 cm$^{-1}$), there are no bands for 13C- and AT-ASR (Figures 2-10 and 2-11). This implies that Asp and Glu residues are located far from the retinal even if they are protonated. In the BR minus BR difference spectra, the bands at 1742 (-) and 1733 (+) cm$^{-1}$ were assigned to the C=O stretching vibrations of the protonated Asp115 (17). ASR has a glutamine residue (Gln109) at the corresponding position, whose vibrational bands are probably observed at 1698 (-) and 1693 (+) cm$^{-1}$ for AT-ASR (Figure 2-11b). Similar bands were also observed at 1704 (-) and 1700 (+) cm$^{-1}$ in the difference spectra of ppR, which has an asparagine residue at the corresponding position (18). Therefore, it can be suggested that the structural changes occurring around Asp115 in BR are common for ASR, BR, and ppR. Figure 2-11a shows the bands at 1694 (+) and 1692 (-) cm$^{-1}$ for 13C-ASR, which can be assigned to the C=O stretch of Gln109. It is likely that the C=O stretching vibrations of Asp115 in BR are dependent on the isomeric form as well.

_s-H Stretching Vibrations of Cysteine Residues._ Figure 2-12 shows the 13C-ASR minus 13C-ASR (upper panel) and AT-ASR minus AT-ASR (lower panel) spectra in the 2580-2500 cm$^{-1}$ region, which corresponds to S-H stretching vibration of cysteine. As I already reported, there is a negative band at 2547 cm$^{-1}$ and a positive band at 2538 cm$^{-1}$ for AT-ASR (Figure 2-12b). In contrast, no spectral changes were observed for 13C-ASR, indicating that the 13-cis to all-trans isomerization in ASR does not alter the local structure of cysteines (Figure 2-12a).
Figure 2-12
The $^{13}$C-ASR$_{k}$ minus $^{13}$C-ASR (a) and the AT-ASR$_{k}$ minus AT-ASR (b) spectra (pH 7) in the 2580-2500 cm$^{-1}$ region, which correspond to S-H stretching vibrations of cysteine residues. The sample was hydrated with H$_2$O. One division of the y-axis corresponds to 0.0003 absorbance units.
There are three cysteine residues in ASR, Cys134 and Cys137 in helix E and Cys203 in helix G. None of them is conserved in archaeal-type rhodopsins. The cysteines were replaced by alanines, producing proteins with absorption maxima at 546 nm for the wild type, 548 nm for C134A, 546 nm for C137A, and 553 nm for C203A (measured for DM-solubilized ASR at pH 7.0; unpublished data). The X-ray crystal structure of ASR also revealed that only the S-H group of Cys203 points to the inside of the protein in the Schiff base region consistent with the fact that the mutation at this position influences the absorption maximum. I suggested previously that the observed vibrational bands may be assignable to the S-H stretching of Cys203 (1).

Assignment of the N-D Stretching Vibrations in 13C-ASR and AT-ASR. X-D stretching vibrations of protein and water molecules appear in the 2750-2000 cm\(^{-1}\) region for the films hydrated with D\(_2\)O (Figure 2-13). The solid line of Figure 2-13c shows the AT-ASR\(_k\) minus AT-ASR spectrum reported earlier (1). On the other hand, the 13C-ASR\(_k\) minus 13C-ASR spectrum (solid line of Figure 2-13a) is obtained for the first time. Since the N-D stretching vibrations of the Schiff base should be present in this frequency region, I then attempted to assign them by use of the \([\zeta-^{15}\text{N}]\text{lysine-labeled ASR sample. Figure 2-13a compares the 13C-ASR}_k\) minus 13C-ASR spectra between \([\zeta-^{15}\text{N}]\text{lysine-labeled (dotted line) and unlabeled (solid line) ASR. Clear isotope-induced spectral downshift was observed for intense positive and negative bands at 2376 and 2165 cm\(^{-1}\), respectively. Other bands are identical between \([\zeta-^{15}\text{N}]\text{lysine-labeled and unlabeled 13C-ASR. Thus, I am able to conclude that the N-D stretching vibrations of the Schiff base are present in this frequency region. It should however be noted that the strong positive peak at 2376 cm\(^{-1}\) probably contains other vibrations because the isotope effect was observed in the broad range of 2370-2320 cm\(^{-1}\) (Figure 2-13a). In fact, the AT-ASR\(_k\) minus AT-ASR spectra contain such peak at 2383 cm\(^{-1}\) as well (Figure 2-13c), which may originate from amide A vibrations. By use of double difference spectra from the data shown in Figure 2-13a, I determined that the N-D stretching vibration of the
Figure 2-13
The $^{13}$C-ASR$_x$ minus $^{13}$C-ASR (a) and the AT-ASR$_x$ minus AT-ASR (c) spectra (pH 7) in the 2750-2000 cm$^{-1}$ region for $\left[\zeta^{-{^{15}}N}\right]$lysine-labeled (dotted line) and unlabeled (solid line) ASR. Double difference spectra in (a) and (c) (solid line minus dotted line) are shown in (b) and (d), respectively. The samples were hydrated with D$_2$O, and spectra were measured at 77 K. One division of the y-axis corresponds to 0.0007 absorbance units.
Schiff base in 13C-ASRK is located at 2351 cm⁻¹ (Figure 2-13b).

Figure 2-13c compares the AT-ASRₖ minus AT-ASR spectra between [ζ⁻¹⁵N]lysine-labeled (dotted line) and unlabeled (solid line) ASR. Clear isotope-induced spectral downshift was observed for the two negative bands at 2163 and 2125 cm⁻¹, indicating that the bands originate from N-D stretching vibrations of the Schiff base in AT-ASR. Additionally, the positive spectral feature at 2470 cm⁻¹ exhibits isotope shift from [ζ⁻¹⁵N]lysine labeling as well. By use of double difference spectra from the data shown in Figure 2-13c, I determined that the N-D stretching vibration of the Schiff base in AT-ASRₖ is located at 2483 cm⁻¹ (Figure 2-13d). The positive peak at 2470 cm⁻¹ probably contains other vibrations such as the O-D stretching vibrations of Thr79. In BR, the O-D frequencies of Thr89, the homologue of Thr79 in ASR, are 2507 and 2466 cm⁻¹ for BR and BRₖ, respectively (19). A similar positive band was also observed at 2476 cm⁻¹ for 13C-ASR (Figure 2-12a).

Thus, by use of [ζ⁻¹⁵N]lysine-labeled ASR, I identified the N-D stretching vibrations of the Schiff base at 2163 and 2125 cm⁻¹ for AT-ASR and at 2165 cm⁻¹ for 13C-ASR. This indicates that the hydrogen-bonding strength is very similar for the two isomeric forms, being slightly stronger in AT-ASR. The X-ray crystallographic structure reported the presence of a water molecule in contact with the Schiff base, making it a good candidate for the hydrogen-bonding acceptor (4). Similarity of the hydrogen bonding in AT-ASR and 13C-ASR is consistent with the X-ray structure.

I also identified the N-D stretching vibration of the Schiff base at 2483 cm⁻¹ for AT-ASRₖ and at 2351 cm⁻¹ for 13C-ASRₖ. Upshifted N-D frequencies indicate that retinal isomerization weakens the hydrogen bond of the Schiff base for both AT-ASR and 13C-ASR. Nevertheless, unlike in the unphotolyzed states, the difference in frequencies for the K states implies the different isomerization outcomes for AT-ASR and 13C-ASR. In case of AT-ASR, the upshift of the frequency is >300 cm⁻¹, indicating that the hydrogen bond is significantly weakened (or broken) in AT-ASRₖ, presumably because of the rotational motion of the Schiff base.
Figure 2-14
The $^{13}$C-ASR$_{x}$ minus $^{13}$C-ASR (a) and the AT-ASR$_{x}$ minus AT-ASR (b) spectra (pH 7) in the 2750-2520 cm$^{-1}$ region measured at 77 K. Sample was hydrated with D$_2$O (red line) or D$_2^{18}$O (blue line). Green-labeled frequencies correspond to those identified as water stretching vibrations. One division of the y-axis corresponds to 0.0004 absorbance units.
In contrast, the upshift of the frequency is about 200 cm\(^{-1}\) for 13C-ASR. This fact suggests that the rotational motion of the Schiff base that accompanies retinal isomerization is smaller in 13C-ASR than in AT-ASR.

**O-D Stretching Vibrations of Water in 13C-ASR and AT-ASR.** A spectral comparison between the samples hydrated with D\(_2\)O and D\(_2\)\(^{18}\)O identifies O-D stretching vibrations of water molecules which change their frequencies upon retinal photoisomerization. I previously reported the absence of the water O-D stretch at <2500 cm\(^{-1}\) for AT-ASR (1). This observation was entirely different from the case of BR, being consistent with the correlation between strongly hydrogen-bonded water molecules and proton pumping activity (3).

In this study, I also looked for the water bands in the 13C-ASR\(_k\) minus 13C-ASR spectrum, but no water bands were found at <2500 cm\(^{-1}\) similar to AT-ASR (data not shown). This fact indicates that the bridged water molecule between the protonated Schiff base and Asp75 forms a weak hydrogen bond for both the all-\textit{trans} and 13-\textit{cis} form. Figure 2-14 shows difference FTIR spectra in the 2750-2520 cm\(^{-1}\) region, where weakly hydrogen-bonded water molecules are observed. Green-tagged bands in Figure 2-14 are assignable to the O-D stretching vibrations of water because of the isotope shift. Figure 2-14b shows that three negative peaks at 2690, 2640, and 2608 cm\(^{-1}\) were assignable to the O-D stretching vibrations of water in AT-ASR, while the bands at 2701, 2653, and 2573 cm\(^{-1}\) were assigned as water stretching vibrations of AT-ASR\(_k\). The bands at 2547 (-) / 2537 (+) cm\(^{-1}\) are attributed to the H-D unexchangeable S-H stretching vibration of a cysteine residue as shown in Figure 2-12b. Figure 2-14a shows that the bands at 2660 (-) and 2645 (+) cm\(^{-1}\) exhibit isotope shift of water. In addition, clear isotope shift was seen for the positive band at 2589 cm\(^{-1}\). The negative band at 2553 cm\(^{-1}\) also contains water O-D stretch, though the small downshift suggests the presence of vibrations other than that of water. Therefore, two positive and two negative peaks can be assigned as O-D stretches of water in 13C-ASR.
Discussion

In this study, I compared the 13C-ASR_k minus 13C-ASR and AT-ASR_k minus AT-ASR spectra obtained by means of low-temperature FTIR spectroscopy. The present HPLC analysis revealed that the dark-adapted ASR is predominantly in the AT-ASR form (97%). Then, the optimal conditions of light adaptation to accumulate 13C-ASR were established, resulting in accumulation of 78% of the 13-cis form. This unique property of ASR raises several questions on how ASR relays the signal to its 14kDa transducer and the nature of the signaling state of ASR. If there is a structural difference between 13C-ASR and AT-ASR in the ground state, it might result in different binding affinity of the 14 kDa transducer for 13C-ASR and AT-ASR. But I cannot exclude a general mechanism in which the M state would be the signaling state as in other sensory rhodopsins. Although the light-adapted ASR contains AT-ASR, the appropriate illumination regime allowed us to obtain the 13C-ASR_k minus 13C-ASR spectra without any subtraction of the contribution of the all-trans form (Figure 2-9). The spectral comparison of 13C-ASR and AT-ASR upon the retinal isomerization at 77 K led to detection of the structural changes specific for each isomer. In addition, I revealed the hydrogen-bonding strengths of the Schiff base in each state using [ζ-15N]lysine-labeled ASR. Unphotolyzed State of 13C-ASR. I identified the N-D stretching vibration of the Schiff base at 2165 cm⁻¹ for 13C-ASR (Figure 2-13a). I also identified the N-D stretching vibration of the Schiff base at 2163 and 2125 cm⁻¹ for AT-ASR. The similar frequencies in 13C-ASR and AT-ASR indicate that the hydrogen-bonding strength of the Schiff base is nearly identical, being slightly stronger in AT-ASR. In the case of BR, the N-D stretching vibrations of the Schiff base were determined to be at 2171 and 2124 cm⁻¹ (16). X-ray crystallographic structures of ASR and BR reported the presence of a water molecule in contact with the Schiff base (4, 20). Therefore, similar hydrogen-bonding strength for 13C-ASR, AT-ASR, and BR suggests that the water molecule is a good hydrogen-bonding acceptor for the protonated Schiff base.

Interestingly, two peaks were observed for the N-D stretch of the Schiff base of
AT-ASR (Figure 2-13c) and BR (1), while only one peak was observed for that of 13C-ASR (Figure 2-13a). Origins of the two peaks in BR, ppR, and AT-ASR have not been well understood. Multiple vibrational modes or structural heterogeneity is a possible source of the two N-D stretches. A single peak of the 13-cis form in ASR may be useful for understanding of the nature of this mode.

I previously found that water vibrations are entirely different between AT-ASR and BR, though both possess a water molecule between the Schiff base and its counterion (Asp75 for ASR or Asp85 for BR) (4, 20). In the case of BR, I observed water O-D stretches at 2700-2150 cm\(^{-1}\) (21-23), whereas AT-ASR possesses water O-D stretches only at 2700-2600 cm\(^{-1}\) (Figure 2-14b) (1). I interpreted the absence of bands of strongly bound water molecules by the difference in geometry of the hydrogen bond. Namely, the N-O\(_{\text{water}}\)-O\(_{\text{counterion}}\) (the Schiff base nitrogen, water oxygen, and oxygen of the counterion) angle is 83° and 106° in ASR and BR, respectively. As the consequence, if the water oxygen fully accepts a hydrogen bond of the Schiff base, the O-H group of water points toward the oxygen of Asp85 in BR, but not toward that of Asp75 in ASR. Such a small difference in the angle can possibly determine the hydrogen-bonding strength of water molecules. I did not observe strongly hydrogen-bonded water molecules for 13C-ASR in this study (Figure 2-14a). This is consistent with the above argument, because the X-ray crystal structure of ASR provides a similar position of the Schiff base, the water, and Asp75 for both isomers at 2.0 Å resolution (4).

I observed water O-D stretches of 13C-ASR at 2660 and 2553 cm\(^{-1}\) (Figure 2-14a), which correspond to O-H stretches at 3592 and 3481 cm\(^{-1}\), respectively, from the spectral analysis of the O-H stretching vibrations in H\(_2\)O (not shown). The O-H stretches of AT-ASR corresponding to the O-D stretches at 2690, 2640, and 2608 cm\(^{-1}\) in Figure 2-14b are found at 3636, 3558, and 3530 cm\(^{-1}\), respectively. Since only the water bridging the Schiff base and Asp75 is located close to the chromophore, it is a reasonable postulation that two water bands of ASR originate from O-D (OH) stretches of this water molecule. In general, a water molecule has...
two O-H groups, and their frequencies are distributed in the wide 3700-2700 cm\(^{-1}\) region depending on their coupling and hydrogen-bonding strength (24). Gaseous water exhibits asymmetric and symmetric stretching modes at 3755 and 3657 cm\(^{-1}\), respectively, and the stretching frequency is lowered as its hydrogen bonding becomes stronger (25). It must be noted that the hydrogen bonding strengths of the two O-H groups are probably not equivalent in the restricted protein environment, which breaks the C\(_{2v}\)-type symmetry. In such C\(_s\)-type symmetry, one O-H is hydrogen bonded and the other O-H is unbonded, and their frequencies are widely split. That is the case for the bridged water of BR, where such decoupling of the two stretching modes occurs (23). Consequently, one O-D stretch of water is at 2171 cm\(^{-1}\), while another O-D stretch of water is at 2636 cm\(^{-1}\). We suggested that the former points toward Asp85, while the latter points toward Asp212 (23). Nonsymmetrical bonding of the water molecule in BR is presumably important for the function (26, 27).

In the case of 13C-ASR, the frequency difference between the O-D stretches is about 100 cm\(^{-1}\). Corresponding O-H stretches are also about 100 cm\(^{-1}\) different, being comparable to the gaseous water. Therefore, stretching vibrations of the water molecule are presumably coupled in 13C-ASR, where anti-symmetric and symmetric O-D stretches are located at 2660 and 2553 cm\(^{-1}\), respectively. The situation is probably similar for AT-ASR, where two out of the three bands at 2690, 2640, and 2608 cm\(^{-1}\) originate from the O-D stretches of the bridging water. The presence of the additional water band indicates involvement of a more distant water upon formation of AT-ASR\(_c\) (Figure 2-15).

Photoisomerization Process of 13C-ASR in Comparison with That of AT-ASR. Upon light absorption in 13C-ASR, photoisomerization probably takes place at the C13=C14 (double) bond, leading from the 13-cis,15-syn to the all-trans, 15-syn form. It is generally accepted that the primary K intermediate is a high-energy state for retinal proteins. Chromophore distortion is one of the characteristic features of such high energy state, and HOOP vibrations monitor the chromophore distortion. The
appearance of numerous HOOP modes in 13C-ASR<sub>k</sub> vs just two in AT-ASR<sub>k</sub> (Figure 2-10) implies that the chromophore distortion in 13C-ASR<sub>k</sub> is distributed more widely along the polyene chain. In other words, chromophore distortion is more localized in the Schiff base region for AT-ASR<sub>k</sub>. Such difference in HOOP modes is presumably correlated with the other observations including amide I, cysteine S-H stretch, the Schiff base N-D stretch, and water O-D stretch modes, as discussed below.

Amide I vibrations of the α-helix were clearly observed for the transition from 13C-ASR to 13C-ASR<sub>k</sub> as shown by the negative bands at 1662, 1655, and 1649 cm<sup>-1</sup> in Figure 2-11a. This is reasonable because the retinal chromophore is surrounded by α-helices. In addition, the bands at 1634 (-) / 1628 (+) cm<sup>-1</sup> are also ascribable to amide I vibration. In contrast, fewer structural changes reported by amide I vibrations were observed for the transition from AT-ASR to AT-ASR<sub>k</sub> as I showed previously (1). Instead, it was suggested that imide I vibration, possibly due to Pro206, was greatly altered (1). Several amide I changes observed only for 13C-ASR are consistent with the picture obtained from the HOOP analysis, suggesting that extensive structural changes take place in 13C-ASR<sub>k</sub>.

No structural perturbation was observed for S-H groups of cysteines in 13C-ASR, whereas there is a negative band at 2547 cm<sup>-1</sup> and a positive band at 2538 cm<sup>-1</sup> for AT-ASR (Figure 2-12). This indicates that only the all-trans to 13-cis isomerization leads to the alteration of the local structure of a cysteine in ASR. I previously suggested that among the three cysteines of ASR, Cys203 in helix G is the most likely candidate for this band. Cys203 is near Pro206 and close to the Schiff base region. Replacement of Cys203 by Ala results in a red-shifted λ<sub>max</sub> (553 nm) relative to the wildtype ASR (unpublished data). This suggests that the Schiff base region is more perturbed in AT-ASR<sub>k</sub> than in 13C-ASR<sub>k</sub>. The N-D stretching frequency of the Schiff base in 13C-ASR<sub>k</sub> (2351 cm<sup>-1</sup>) is lower than that in AT-ASR<sub>k</sub> (2483 cm<sup>-1</sup>), though they are similar between 13C-ASR and AT-ASR.
Retinal photoisomerization breaks the Schiff base H-bond in all-trans ASR, but not in 13-cis ASR, where structural changes are distributed widely along the polyene chain.

**Figure 2-15**
(Left) The X-ray structure around retinal Schiff base. Yellow retinal is all-trans form and blue retinal is 13-cis form. (Right) The diagram of the ASR<sub>k</sub> minus ASR infrared spectra in X-D vibration region. It shows only N-D stretch of the Schiff base.
I thus assume that the hydrogen bond of the Schiff base is broken in AT-ASR\textsubscript{κ} but not in 13C-ASR\textsubscript{κ}. Consequently, the hydrogen-bonding network is destabilized in AT-ASR\textsubscript{κ}, and protein structural changes proceed through the network, where the L, M (deprotonation of the Schiff base), and O states can be produced from AT-ASR. In contrast, structural perturbation of the Schiff base region is smaller in 13C-ASR\textsubscript{κ}, where the structural changes are distributed more widely.

The number of observed water bands was two for 13C-ASR and three for AT-ASR (Figure 2-14). As discussed above, the two water bands in 13C-ASR are assignable to the water molecule in the Schiff base region. The presence of an additional water band indicates involvement of a more distant water upon formation of AT-ASR\textsubscript{κ}. The second nearest water molecule in the X-ray structure is located 8.3 Å from the Schiff base nitrogen in the structure of AT-ASR and 8.0 Å in the structure of 13C-ASR (4). That water is located between Trp176 and Phe213 in the cytoplasmic region. The third nearest water molecule in the X-ray structure is located 9.2 Å from the Schiff base nitrogen in the structure of AT-ASR and 9.7 Å in the structure of 13C-ASR (4). That water is located near Arg72 in the extracellular region. No water molecules are present near the polyene chain. Thus, water signals may also be consistent with the above view that the chromophore of 13C-ASR\textsubscript{κ} is distorted more widely along the polyene chain than that of AT-ASR\textsubscript{κ}, which has larger changes in the Schiff base region.

In conclusion, ASR accommodates both all-\textit{trans} and 13-\textit{cis}, 15-\textit{syn} retinal in the ground state according to the X-ray crystal structure (4). On the other hand, the present FTIR study revealed that protein structural changes upon retinal photoisomerization were significantly different between 13C-ASR and AT-ASR. They must trigger global protein structural changes in each photoreaction cycle, resulting in the photochromic behavior. The photochromic signaling mechanism of ASR has not been found, but I should be able to reveal such mechanism if the AT-ASR and 13C-ASR states differ in the binding affinity of the 14kDa transducer. The other possibility is that the M state from the photocycle of AT-ASR, which is
conformationally changed, would be the signaling state similar to other sensory rhodopsins.
REFERENCE


Chapter 3

Photochromism of Anabaena sensory rhodopsin

Introduction
Rhodopsins convert light into signal or energy, and retinal is their chromophore molecule (1-3). The retinal forms a mostly protonated Schiff base linkage (C=NH+) with a lysine at the seventh helix. It is well-known that the protein environment of rhodopsins accommodates the retinal chromophore optimally for its functions. For example, the specific chromophore-protein interaction leads to wide color tuning in human visual pigments with a common chromophore (11-cis retinal) (4), and protein controls the highly efficient photoisomerization from 11-cis to the all-trans form in visual rhodopsins (5). Specific control of retinal photochemistry by protein can be also seen in rhodopsins from halophilic archaeabacteria such as the light-driven proton pump bacteriorhodopsin (BR) (5-7). Unlike visual rhodopsins, BR accommodates the retinal chromophore as the all-trans,15-anti (AT; BR_AT) and 13-cis,15-syn (13C; BR_13C) forms (Figure 3-1a) (8). BR_AT and BR_13C are in equilibrium in the dark, while only BR_AT possesses proton-pump activity (Figure 3-1b). Absorption of light by BR_AT yields isomerization to the 13-cis, 15-anti form, which triggers a cyclic reaction that comprises the series of intermediates, intermediates, K, L, M, N, and O (6, 7). During the photocycle, one proton is translocated from the cytoplasmic to extracellular side.

Photoexcitation of BR_13C partially converts it to BR_AT, which is called “light-adaptation”, but BR_AT is not converted into BR_13C photochemically. Photocycle of BR_AT with 100% yield is advantageous for repeating the proton-pumping cycle. This is also the case for other proton pumps found in eubacteria (proteorhodopsin) (9) and eucaryotes (Leptosphaeria rhodopsin) (10). In addition, haloarchaeal sensory rhodopsins possess only the AT chromophore in the dark, indicating that its
Figure 3-1
(a). The structure of the retinal chromophore of microbial rhodopsins in the dark. (b). Photo and thermal reaction scheme in a light-driven proton pump bacteriorhodopsin (BR). Only BR\textsubscript{AT} possesses proton-pump activity, and the reaction of BR\textsubscript{AT} is 100% cyclic without any branching reaction into BR\textsubscript{13C}. Dotted arrows represent thermal reaction in the dark, where BR\textsubscript{13C} is more stabilized than BR\textsubscript{AT}. (c). Photo and thermal reaction scheme in Anabaena sensory rhodopsin (ASR). While ASR\textsubscript{AT} is a predominant species in the dark (dotted arrow), photoexcitation of ASR\textsubscript{AT} and ASR\textsubscript{13C} yields the reaction of each species, either cyclic or branching, leading to the photocycle or photochromism, respectively. x and y are the branching ratio from ASR\textsubscript{AT} and ASR\textsubscript{13C}, respectively.
photocycle is important also for light-signal conversion (11, 12). Thus, the photocycle of the AT form with 100% yield has been the common mechanism for the functional processes of microbial rhodopsins.

Recently, a microbial rhodopsin has been discovered in Anabaena (Nostoc) PCC7120, which is believed to function as a photoreceptor for chromatic adaptation (13). In fact, the expected photochromism was found between the AT and 13C forms for Anabaena sensory rhodopsin (ASR) (14). These findings imply strongly branching reactions, from ASR<sub>AT</sub> to ASR<sub>13C</sub> and from ASR<sub>13C</sub> to ASR<sub>AT</sub> (Figure 3-1c), in striking contrast to what is known for microbial rhodopsins. Ideally, the conversion ratios should be unity for photochromic reactions (x = y = 1 in Figure 3-1c), but this is exactly the opposite of the properties of pump rhodopsins, such as BR. X-ray crystal structures reported similar chromophore structures and protein environments for ASR<sub>AT</sub> (15) and BR<sub>AT</sub> (16). Do photochromic reactions indeed take place for ASR<sub>AT</sub> and ASR<sub>13C</sub>? In this chapter, I determined the branching ratios (x and y values) for ASR<sub>AT</sub> and ASR<sub>13C</sub> by means of low-temperature UV-visible spectroscopy. Surprisingly, the obtained x and y values were unity, indicating that the photoreactions of ASR<sub>AT</sub> and ASR<sub>13C</sub> are completely photochromic. The complete photochromic reactions are highly advantageous for the chromatic sensor function of ASR.

Materials and Methods

The ASR protein with a histidine tag at the C-terminus was expressed in Escherichia coli, solubilized with 1.0% n-dodecyl-β-D-maltoside, and purified by a Ni<sup>2+</sup>-column (17, 18). The purified ASR sample was then reconstituted into L-α- phosphatidylcholine (PC) liposomes by the removal of the detergent with Bio-beads, where the molar ratio of the added PC to ASR was 50:1. The ASR protein in PC liposomes was washed three times with a buffer [2 mM sodium phosphate (pH 7.0)]. A 60 µL aliquot was deposited on a BaF<sub>2</sub> window of 18-mm diameter and dried in a glass vessel with evaporation by an aspirator.
The fully hydrated ASR film sample was used for low-temperature UV-visible spectroscopy, where three to five independent measurements were averaged. The UV-visible spectra were measured by a UV-visible spectrometer (V-550, JASCO) equipped with a cryostat (OptistatDN, Oxford). The cryostat was equipped with a temperature controller (ITC-4, Oxford), and the temperature was regulated with 0.1 K precision. A previous HPLC study showed that the completely dark-adapted ASR in PC liposomes is in the all-trans form predominantly (97.1 (±0.1%) (18). On the other hand, illumination of ASR with >560 nm light (O-58 cutoff filter, Toshiba) from a 1-kW halogen-tungsten lamp for 1 min at 277 K yields formation of 77.9 (±1.7)% 13-cis form (18).

Results and Discussion

Photoconversion of ASR_{AT} (1) Photoreaction at 170 K. I first examined the branching ratio of ASR_{AT} (x value in Figure 3-1c) because previous HPLC analysis revealed that the dark-adapted ASR in PC liposomes contains predominantly (97%) ASR_{AT} (18). Dark-adapted ASR was illuminated at 170 K, and then warmed to 277 K. The photoconversion yield of ASR_{AT} to its intermediates was calculated using the spectra at 170 K, which was compared with the conversion of ASR_{AT} to ASR_{13C} at 277 K. The black line in Figure 3-2a shows the absorption spectrum of the dark-adapted ASR at 170 K ($\lambda_{max} = 554$ nm). Illumination at >580 nm (red line) or 501 nm (blue line) resulted in reduction of the peak absorbance and increase of the shorter or longer wavelength tail, indicating the formation of the L and K photointermediates, respectively. Figure 3-2b shows the corresponding difference spectra, and positive peaks at 474 and 605 nm are characteristic absorption of the L and K intermediates, respectively. On the other hand, no positive band at about 400 nm indicates that the M intermediate is not formed at 170 K.

Since the red and blue spectra in Figure 3-2b contain contribution of the L and K intermediates, I next obtained the K minus ASR_{AT} and L minus ASR_{AT} spectra. The L minus ASR_{AT} spectrum was obtained by subtracting the blue spectrum from the red
one in Figure 3-2b, so that the spectral shape at about 600 nm coincides with that of the absolute spectrum of the dark-adapted ASR (black line in Figure 3-2a). The red spectrum in Figure 3-2c represents the L minus ASR$_{AT}$ spectrum thus obtained. Then, the L minus ASR$_{AT}$ spectrum was subtracted from the blue spectrum in Figure 3-2b so as to resemble that at 130 K (black dotted line in Figure 3-2c), where the photoproduct is only the K intermediate. The blue spectrum in Figure 3-2c represents the resulting K minus ASR$_{AT}$ spectrum. Isosbestic points are at 520 nm between ASR$_{AT}$ and L, and at 575 nm between ASR$_{AT}$ and K.

I then determined the absorption spectra of the K and L intermediates of ASR$_{AT}$ at 170 K. Absorption spectra of intermediates can be obtained from the difference spectrum and photoconversion ratio from ASR$_{AT}$ to the intermediate. Five colored lines in Figure 3-3a or b correspond to the calculated spectra of the K intermediate of ASR$_{AT}$ or the L intermediate of ASR$_{AT}$ at various percentages of conversion, respectively. The broken black line in Figure 3-3a represents the absorption spectrum of the K intermediate of ASR$_{AT}$ at 130 K, which was determined by illuminating ASR$_{AT}$ at two wavelengths as described in Figure 3-4. I regarded the red spectrum in Figure 3-3a as the absorption spectrum of the K intermediate of ASR$_{AT}$ at 170 K because the red one coincides well with the broken black line. On the other hand, the absorption spectrum of the L intermediate was determined from the spectral analysis of the second derivatives of the absorption spectra in Figure 3-3b. The second derivatives in Figure 3-3c show that the red spectrum coincides with the zero line at >588 nm. On the assumption that the L intermediate does not contain a spectral component in the second derivative at >588 nm, I regarded the red one in Figure 3-3b as the absorption spectrum of the L intermediate of ASR$_{AT}$ at 170 K.

Blue and red spectra in Figure 3-3d correspond to the absolute spectra of the K and L intermediates, respectively.
Figure 3-2

(a). Absorption spectra of the dark-adapted ASR (black line), illuminated ASR with >580 nm (red line) and 501 nm (blue line) light at 170 K. It should be noted that the dark-adapted ASR corresponds to ASR_{at}, because it contains negligible amount of ASR_{13C} (2.9 %) in the present sample conditions (18). (b). Light-minus-dark difference absorption spectra of ASR with >580 nm (red line) and 501 nm (blue line) light at 170 K. (c). L minus ASR_{at} (red line) and K minus ASR_{at} (blue line) difference absorption spectra at 170 K. Black broken line corresponds to the K minus ASR_{at} spectrum at 130 K, where only the K intermediate is formed. See text in detail.
**Figure 3-3**

Determination of the absorption spectra of the K and L intermediates of ASR$_{at}$ at 170 K. **(a).** Black solid line represents absorption spectrum of ASR$_{at}$ at 170 K. Absorption spectrum of the K intermediate can be obtained from the K minus ASR$_{at}$ difference spectrum (blue line in Figure 3-2c) and photoconversion ratio from ASR$_{at}$ to the K intermediate. Five colored lines correspond to the calculated spectra of the K intermediate of ASR$_{at}$ at various % of conversion (100-17 % from orange to blue; 32 % for the red line). Black broken line represents absorption spectrum of the K intermediate of ASR$_{at}$ at 130 K, which was determined by the method described in Figure 3-4. Since the red spectrum coincides well with the black broken line, we regarded the red one as the absorption spectrum of the K intermediate of ASR$_{at}$ at 170 K. **(b).** Black solid line represents absorption spectrum of ASR$_{at}$ at 170 K. Absorption spectrum of the L intermediate can be obtained from the L minus ASR$_{at}$ difference spectrum (red line in Figure 3-2c) and photoconversion ratio from ASR$_{at}$ to the L intermediate. Five colored lines correspond to the calculated spectra of the L intermediate of ASR$_{at}$ at various % of conversion (18-12 % from orange to blue; 14 % for the red line). **(c).** Second derivatives of absorption spectra in Figure 3-3b, where the corresponding spectra are shown by the same color. The second derivative of the ASR$_{at}$ spectrum (black line) coincides to the zero line at 588 nm. We assume that the L intermediate does not contain spectral component in the second derivative at >588 nm. Consequently, we regarded the red one in Figure 3-3b as the absorption spectrum of the L intermediate of ASR$_{at}$ at 170 K. **(d).** Absorption spectra of ASR$_{at}$ (black line), the K intermediate (blue line) and the L intermediate (red line) at 170 K. The spectra of the K and L intermediates are reproduced from the red spectra in Figure 3-3a and b, respectively.
Figure 3-4

Determination of the absorption spectra of the K intermediates of ASR_{AT} and ASR_{13C} at 130 K. (a) and (b). Black solid line represents absorption spectra of the dark-adapted (a) and light-adapted (b) ASR at 130 K. Blue, green, and red spectra are those by illumination with a 480, 548, and 577 nm light through interference filters (sharp colored peaks in the figure), respectively. (c) and (d). Light-minus-dark difference absorption spectra of the dark-adapted (c) and light-adapted (d) ASR by illumination with 480 (blue), 548 (green), and 577 (red) nm lights at 130 K. (e) and (f). K-minus-ASR_{AT} (e) and K-minus-ASR_{13C} (f) difference absorption spectra by illumination with 480 (blue), 548 (green), and 577 (red) nm lights at 130 K. These spectra were calculated for each illumination wavelengths from the spectra in c and d by taking account of the isomeric compositions of the dark-adapted (97 % ASR_{AT} and 3 % ASR_{13C}) and light-adapted (22 % ASR_{AT} and 78 % ASR_{13C}) ASR. Almost identical spectra between c and e indicate that the dark-adapted state can be regarded as ASR_{AT}. (g). To determine absorption spectrum of an intermediate, photoconversion ratio from the unphotolyzed state to the intermediate has to be obtained. Such ratio can be obtained by illuminations at two wavelengths if the quantum yields are independent of wavelength \( \phi \). For instance, ASR_{AT} is illuminated at 480 nm or 577 nm under photoequilibrium conditions,

\[
(1-x_1) \text{Abs}(\text{ASR}_{AT}, 480 \text{ nm}) = x_1 \phi \text{Abs}(\text{ASR}_{AT}(K), 480 \text{ nm})
\]

\[
(1- x_2) \text{Abs}(\text{ASR}_{AT}, 577 \text{ nm}) = x_2 \phi \text{Abs}(\text{ASR}_{AT}(K), 577 \text{ nm})
\]

where \( x_1 \) and \( x_2 \) are relative amount of ASR_{AT}(K) in the photosteady state mixture, \( \phi \) is the relative quantum yield of ASR_{AT}(K) to ASR_{AT}. \text{Abs}(\text{ASR}_{AT}, 480 \text{ nm}) and \text{Abs}(\text{ASR}_{AT}(K), 480 \text{ nm}) are the absorbance of ASR_{AT} and the K intermediate at 480 nm, respectively. On the other hand, the following equations are derived from the difference spectra before and after illumination:

\[
\Delta \text{Abs}(480 \text{ nm}) = x_1 (\text{Abs}(\text{ASR}_{AT}(K), 480 \text{ nm}) - \text{Abs}(\text{ASR}_{AT}, 480 \text{ nm}))
\]

\[
\Delta \text{Abs}(577 \text{ nm}) = x_2 (\text{Abs}(\text{ASR}_{AT}(K), 577 \text{ nm}) - \text{Abs}(\text{ASR}_{AT}, 577 \text{ nm}))
\]

where \( \Delta \text{Abs}(480 \text{ nm}) \) and \( \Delta \text{Abs}(577 \text{ nm}) \) are the difference absorbances at 480 and 577 nm, respectively. From the blue (480 nm) and red (577 nm) spectra in e, absorption spectrum of the K intermediate of ASR_{AT} can be determined by obtaining \( x_1 \) and \( x_2 \) values (red solid line in g). Red dotted line corresponds to the spectrum of the K intermediate of ASR_{AT} obtained from the green (548 nm) and red (577 nm) spectra in e. Red solid and dotted spectra in g are almost identical, implying that quantum yields are wavelength independent. Blue solid, dotted, and broken lines represent the spectra of the K intermediate of ASR_{13C} obtained from the blue and red, green and red, and blue and green spectra in f. We regarded the red and blue solid lines as the spectra of the K intermediates of ASR_{AT} and ASR_{13C}, respectively.
Photoconversion of ASR\textsubscript{AT} (2) Thermal relaxation by warming the sample from 170 K to 277 K. I reconstituted the experimentally obtained spectra (dotted black lines in Figure 3-5a and c) by use of the spectra in Figure 3-3d. For the illumination at >580 nm, the dotted black spectrum in Figure 3-5a is well coincident with the sum of 78\% ASR, 5\% K, and 17\% L (green line in Figure 3-5a), indicating the 22 (±2)\% conversion to intermediates at 170 K. On the other hand, for the illumination at 501 nm, the dotted black spectrum in Figure 3-5c is well coincident with the sum of 68\% ASR, 18\% K, and 14\% L (green line in Figure 3-5c), indicating the 32 (±5)\% conversion at 170 K.

I then warmed these states from 170 to 277 K so as to complete the thermal reactions of the K and L states to their end products, and calculated the conversion yield from the spectra. Dotted black lines in Figure 3-5b and d represent the spectra at 277 K after illumination at >580 and 501 nm, respectively, at 170 K. By the use of the absorption spectra of ASR\textsubscript{AT} and ASR\textsubscript{13C}, the percentage of conversions were calculated to be 23 (±2)\% and 34 (±4)\% in Figure 3-5b and d, respectively. The branching ratios (x in Figure 3-1) were thus determined to be 1.02 ± 0.13 and 1.10 ± 0.09 for illuminations at >580 and 501 nm, respectively. These values demonstrate that the K and L intermediates formed from ASR\textsubscript{AT} are completely converted into ASR\textsubscript{13C} without regaining the initial state in a photocyclic reaction.

By means of low-temperature FTIR spectroscopy, I had previously suggested that the primary photoproduct of ASR\textsubscript{AT} is the 13-cis, 15-anti form as in BR (Figure 3-6) (17). In BR, thermal isomerization takes place at the C13=C14 bond with virtually 100\% yield, recovering the original AT state. In contrast, in ASR thermal isomerization is likely to occur at the C15=N bond following photoisomerization of ASR\textsubscript{AT}, which converts to the 13C qstate with 100\% yield (Figure 3-6).
Figure 3-5
(a) and (c). Absorption spectra of the dark-adapted ASR before (black solid lines) and after (black dotted lines) illuminations with $>580$ nm (a) and 501 nm (c) lights at 170 K. Green lines represent the reconstituted spectra by use of those in Figure 3-2d. Under the present illumination conditions, 22 (± 2) and 32 (± 5) % portion were converted into the intermediates in a and c, respectively. (b) and (d). Black solid lines represent absorption spectra of the dark-adapted ASR at 277 K. Red and blue lines correspond to the calculated absorption spectra of ASR$_{AT}$ and ASR$_{13C}$ at 277 K, respectively, which were obtained from those of dark- and light-adapted ASR and the HPLC analysis (18). Black dotted lines represent absorption spectra at 277 K after illuminations with $>580$ nm (b) and 501 nm (d) lights at 170 K. Under the present illumination conditions, 23 (± 2) and 34 (± 4) % portion were converted from ASR$_{AT}$ to ASR$_{13C}$ at 277 K in b and d, respectively. From a-d, the branching ratio ($x$ in Figure 3-1c) is obtained to be 1.02 ± 0.13 and 1.10 ± 0.09 for illuminations at $>580$ nm and 501 nm, respectively, indicating complete branching reactions from ASR$_{AT}$ for both illumination conditions.
Figure 3-6
Structural changes of the all-trans, 15-anti chromophore during photoreactions. The all-trans, 15-anti form, either in BRAT or ASRAT, is first photoconverted to the 13-cis, 15-anti form, followed by thermal isomerization at C13=C14 or C15=N position in BR or ASR, respectively. Such thermal relaxations lead to 100% photocyclic and photochromic reactions for BRAT and ASRAT, respectively.
Photoconversion of \(\text{ASR}_{13c}\) (1) Relative photoconversion yields of \(\text{ASR}_{\text{AT}}\) and \(\text{ASR}_{13c}\) at 277 K. What is the branching ratio (\(y\) value in Figure 3-1c) from \(\text{ASR}_{13c}\)? Unlike \(\text{ASR}_{\text{AT}}\) that is present as nearly the only state in dark-adapted ASR, \(\text{ASR}_{13c}\) is present in a mixture with \(\text{ASR}_{\text{AT}}\). Therefore, I attempted to determine the branching ratio on the basis of relative photoconversion yields. A previous study showed that the dark-adapted or light-adapted ASRs in PC liposomes possess 97.1% \(\text{ASR}_{\text{AT}}\) and 2.9% \(\text{ASR}_{13c}\) or 22.1% \(\text{ASR}_{\text{AT}}\) and 77.9% \(\text{ASR}_{13c}\), respectively (18). Since \(\text{ASR}_{\text{AT}}\) has greater extinction than \(\text{ASR}_{13c}\) at 500-600 nm (Figure 3-5b and d), illumination of the dark-adapted ASR yields an absorption decrease in this wavelength region. In contrast, illumination of light-adapted ASR results in the increase of absorption at 500-600 nm, as reported previously (15). The isosbestic point of \(\text{ASR}_{\text{AT}}\) and \(\text{ASR}_{13c}\) is located at 496 nm (Figure 3-5b and d).

In Figure 3-7, I illuminated the dark-adapted and light-adapted ASR with a 496 nm light at 277 K, and the changes in absorbance at 569 nm (difference absorption maximum between \(\text{ASR}_{\text{AT}}\) and \(\text{ASR}_{13c}\) at 277 K) were plotted as the function of illumination time. Thermal conversion from \(\text{ASR}_{13c}\) to \(\text{ASR}_{\text{AT}}\) is negligible, because it takes 90 min (\(\tau_{1/2}\)) for ASR in PC liposomes at 277 K (data not shown). Absorbance at 569 nm decreases and increases for the dark-adapted and light-adapted ASR, respectively, and both curves eventually coincide after long illumination (Figure 3-7). The time courses are well fitted by single exponentials, and each photoconversion yield can be obtained from the initial slope (\(t = 0\)). By taking into account the contents of \(\text{ASR}_{\text{AT}}\) and \(\text{ASR}_{13c}\) in the dark-adapted and light-adapted forms, I determined the ratio between \(\text{ASR}_{13c}\)-to-\(\text{ASR}_{\text{AT}}\) and \(\text{ASR}_{\text{AT}}\)-to-\(\text{ASR}_{13c}\) to be 0.77 (±0.04):1. [Sineshchekov et al. estimated a similar photoconversion yield to be 0.3:1 from the HPLC analysis of the photosteady state mixture with white or >520-nm light illumination (14). While the accurate photoconversion yield is determined by the present method (from the initial slope after illumination at their isosbestic point), such a big difference (0.77-times vs 0.3-times) should be explained. I confirmed that the spectral analysis of the photosteady state, not initial slope, in Figure 3-7 yields
Figure 3-7
Absorption changes at 569 nm of the dark-adapted (open circles) and light-adapted (open squares) ASR after illumination at the isosbestic point of ASR$_{13C}$ and ASR$_{13C}$ (496 nm) at 277 K. Absorption changes were fitted by single exponentials (solid and broken lines), and the ratio of the initial slope (light-adapted ASR/dark-adapted ASR) was 0.40:1. By taking into accounts of the contents of ASR$_{13C}$ and ASR$_{13C}$ in each state, the ratio between ASR$_{13C}$-to-ASR$_{13C}$ and ASR$_{13C}$-to-ASR$_{13C}$ was determined to be 0.77 ($\pm$ 0.04).
the ratio to be similar (0.75:1). On the other hand, the ratio between ASR\textsubscript{13C}-to-ASR\textsubscript{AT} and ASR\textsubscript{AT}-to-ASR\textsubscript{13C} was significantly reduced by illumination at longer wavelengths, which is close to the value reported by Sineshchekov et al \cite{14}. Thus, the photoconversion yield depends on the illumination wavelength; 0.77 by the 496 nm illumination and about 0.3 by the illumination at >520 nm. I infer that under the photostationary conditions at >520 nm, the intermediate state of ASR\textsubscript{13C} is photoexcited, presumably forming the original ASR\textsubscript{13C}, while that of ASR\textsubscript{AT} (the M state) is not. Consequently, ASR\textsubscript{13C} is accumulated, and the ratio between ASR\textsubscript{13C}-to-ASR\textsubscript{AT} and ASR\textsubscript{AT}-to-ASR\textsubscript{13C} is apparently lowered.] Since the sample is illuminated at the isosbestic point, the ratio is directly correlated with the relative photoconversion yields. Although this value apparently shows a lower branching ratio for ASR\textsubscript{13C} than for ASR\textsubscript{AT} (x = 1), it should be noted that the photoisomerization quantum yields are not taken into account in this estimate. A lower photo-isomerization quantum yield of ASR\textsubscript{13C} may provide a lower value for ASR\textsubscript{13C}, and it was indeed the case.

\textit{Photoconversion of ASR\textsubscript{13C}} \cite{2} \textit{Relative photoisomerization quantum yields of ASR\textsubscript{AT} and ASR\textsubscript{13C} at 130 K.} I next compared the relative quantum yields for the photoisomerization of ASR\textsubscript{AT} and ASR\textsubscript{13C} by comparing the formation of their K intermediates at 130 K. Since the molar extinction coefficients of their K intermediates are required for the calculation, I determined the absorption spectra of the K-intermediates of ASR\textsubscript{AT} and ASR\textsubscript{13C} according to the procedure in Figure 3-4. Solid red and blue lines in Figure 3-8a represent absorption spectra of the K intermediates of ASR\textsubscript{AT} and ASR\textsubscript{13C}, respectively. Interestingly, the amplitude of the K state is decreased for ASR\textsubscript{AT} but increased for ASR\textsubscript{13C}. Together with the absorption of ASR\textsubscript{AT} greater than that of ASR\textsubscript{13C} (broken lines in Figure 3-8a), this suggests that the 13C \textit{trans} form has a large absorption in the protein pocket of ASR.

I then illuminated the dark-adapted and light-adapted ASR at 480 nm, the isosbestic point of ASR\textsubscript{AT} and ASR\textsubscript{13C}, at 130 K (Figure 3-8a). Figure 3-8b shows time-dependent absorbance changes at their difference absorption maxima (596 and
Figure 3-8
(a). Red and blue broken lines correspond to the absorption spectra of ASR\textsubscript{Ar} and ASR\textsubscript{13c} at 130 K, respectively, which were obtained from those of the dark- and light-adapted ASR and the HPLC analysis \((19)\). Isosbestic point is located at 480 nm. Red and blue solid lines represent absorption spectra of the K intermediates of ASR\textsubscript{Ar} and ASR\textsubscript{13c} at 130 K, which were obtained according to the procedure in Figure 3-4. (b). Time-dependent absorbance changes of the dark-adapted (open circles) and light-adapted (open squares) ASR. Each sample was illuminated at the isosbestic point at 130 K (480 nm; Figure 3-8a), and absorbance changes were monitored at 596 and 590 nm for the dark-adapted and light-adapted ASR, respectively.
590 nm) of the dark-adapted and light-adapted ASR. The increase of absorbance is greater for the light-adapted ASR, which contains more ASR\textsubscript{13C}, and originates also from the larger absorbance of the K intermediate of ASR\textsubscript{13C}. By considering the molar extinction coefficients of the K intermediates, the relative quantum yield for the photoisomerization of ASR\textsubscript{13C} and ASR\textsubscript{AT} was determined to be 0.73 (±0.07) : 1. From the data in Figure 3-5a and c, the branching ratio of ASR\textsubscript{13C} (y in Figure 3-1) was therefore determined to be 1.06 ± 0.11. This value demonstrates that the K intermediate formed from ASR\textsubscript{13C} is completely converted into ASR\textsubscript{AT} without regaining the initial state in a photocyclic reaction (Figure 3-9).

Functional optimization of photoconversions in rhodopsins. The present results reveal that the branching reactions take place with 100% efficiency, both from ASR\textsubscript{AT} and ASR\textsubscript{13C}. Although the present results were obtained for ASR in liposomes, not in native membranes, this characteristic is highly advantageous for a photochromic sensor. On the other hand, the AT form of BR has 100% photocyclic efficiency (Figure 3-6), which is important for the proton pump. Thus, it is concluded that ASR and BR have been optimized for their functions, presumably during evolution. It is intriguing that the structures of the chromophore and its binding pocket are similar between ASR (15) and BR (16), although their amino acid sequences are not highly homologous (60%). Our FTIR study revealed that hydrogen bond of the Schiff base is similarly strong in ASR and BR, and they are similarly cleaved after retinal photoisomerization (17). Replacement of aspartate (Asp212 of BR) by proline in ASR (Pro206) is one of the structural differences. Another difference is the hydrogen bonding strength of the water molecule near the Schiff base. BR possesses a strongly hydrogen-bonded water molecule between the Schiff base and its counterion (Asp85), which appears to be a prerequisite for proton-pump function (19). ASR possesses such a water molecule between the Schiff base and its counterion (Asp75) (15), but its hydrogen bond is much weaker (17). These small differences may be determinants for distinguishing photocyclic or photochromic reactions. Recently, Sudo and Spudich converted BR into a sensory receptor by
mutation of three hydrogen-bonding residues (20). This finding also suggests that distinct functions are determined by small differences. In addition, the M intermediate is formed during the photoreaction of ASR\textsubscript{AT} like BR, but Asp75 is not protonated (21), presumably because the proton is conducted toward the cytoplasmic domain (22). Further structural analysis of photoreaction intermediates will provide a better understanding of the mechanism for thermal relaxation of the photoisomerized chromophore.
Figure 3-9
The photoreaction of ASR. Both isomers (\(\text{ASR}_{\text{AT}}\) and \(\text{ASR}_{\text{13C}}\)) convert 100% yields to another isomer, respectively.
REFERENCES


Chapter 4

FTIR study of the L intermediate of Anabaena sensory rhodopsin: Structural changes in the cytoplasmic region

Introduction

Archaeal-type rhodopsins generally possess all-trans or 13-cis retinal as the chromophore in the dark, and ASR was suggested to function as a photochromic sensor existing in both isomeric forms (1, 2). However, functionally important states known so far were only derived from the all-trans form for archaeal-type rhodopsins, and all the photocycles of the all-trans forms have a common mechanism. For instance, in the light-driven proton pump of BR, the stable photoproduct at the end of the functional cycle of the all-trans form is 100% all-trans, i.e., it is truly cyclic. Surprisingly, our recent low-temperature UV-visible spectroscopy of ASR revealed that the stable photoproduct of the all-trans form is 100% 13-cis, and that of the 13-cis form is 100% all-trans (3). The complete photocycling for the proton pump in BR and the complete photochromism for the chromatic sensor of ASR are highly advantageous for their functions, and the unique photoreactions must have been acquired for each rhodopsin during evolution. One interesting aspect is that the protein structures are similar between ASR and BR (2), suggesting that distinct functions are determined by small differences.

To understand the details of light-induced structural changes of ASR, I applied low temperature FTIR spectroscopy to all-trans ASR, and compared the difference spectra at 77 K with those of BR (4). The K intermediate minus all-trans form ASR difference spectra showed that the retinal isomerizes from all-trans to distorted 13-cis form similar to BR. On the other hand, a remarkable difference between all-trans forms of ASR and BR was revealed in water bands. Although ASR possesses a water molecule between the Schiff base and its counterion Asp75 similar to BR (2),
the O-D stretching bands of water molecules were observed only in the >2500 cm\(^{-1}\) region for the all-\textit{trans} form of ASR (4). I interpreted that fact as a weak hydrogen bonding of the bridged water in ASR originating from its unique geometry. Since ASR does not pump protons and the direction of the proton movement is toward the cytoplasmic side as inferred from the sign of the photoelectric signal (5, 6), the results support the working hypothesis that the existence of strongly hydrogen-bonded water molecules is essential for proton pumping activity in archaeal-type rhodopsins (7).

The M intermediate with the deprotonated Schiff base is an important state in proton transport and signal transduction. It has been known that the Schiff base proton is transferred to the counterion (Asp85 in BR) if it is deprotonated. In this case, the proton transfer is toward extracellular side. On the other hand, the previous time-resolved FTIR study of ASR by Shi et al. reported the proton transfer to Asp217 in the cytoplasmic side (8), though Asp75 works as the counterion of the Schiff base in ASR. No proton transfer to Asp75 was also reported by Bergo et al. (9). This may be reasonable, because another aspartate (Asp212 in BR) is replaced by proline in ASR, and Asp212 plays an important role in the proton transfer in BR (10, 11). On the other hand, Sineshchekov et al. reported that the direction of proton transfer was dependent on the sample conditions, where the direction is toward cytoplasmic and extracellular side for C-terminal truncated and full-length ASR, respectively (12). According to these results, native full-length ASR in \textit{E. coli} cells exhibits proton transfer direction the same as in BR. Thus, the molecular mechanism of ASR activation remains yet unclear. In chapter 4, I applied low-temperature FTIR spectroscopy at 170 K to the dark-adapted ASR that has predominantly all-\textit{trans} retinal (97\%) (13). The obtained ASR\(_L\) minus ASR spectra were similar between the full-length and C-terminally truncated ASR, implying similar protein structural changes for the L state. The ASR\(_L\) minus ASR spectra were essentially similar to those of BR, but a unique spectral feature was observed in the carboxylic C=O stretching region. The bands at 1722 (\(+\)) and 1703 (\(-\)) cm\(^{-1}\) were
observed at pH 5, which was reduced at pH 7 and disappeared at pH 9. The mutation study successfully assigned the bands to the C=O stretch of Glu36. Interestingly, Glu36 is located at the cytoplasmic side, and the distance from the retinal Schiff base is about 20 Å (Figure 4-1). I also observed pH-dependent frequency change of a water stretching vibration, which is located near Glu36. Unique hydrogen-bonding network in the cytoplasmic domain of ASR will be discussed.

**Materials and Methods**

*Sample Preparation.* In the present study, I prepared C-terminally truncated and full-length ASR according to the method described previously (3, 5, 14). The E36Q and D217N mutants were designed based on the full-length ASR, which were produced by a two-step megaprimer PCR method (15), with two oligonucleotides (COSMO, Seoul, Korea): E36Q F-50-CA G TAC CAA TAC CTT GTG GCG A TG- 30 and D217N R-50-GT A AAT TCA GAA AAA CT A AAT C-30. The final PCR products were cloned into plasmid pKJ606 (16), derived from pMS107, by replacing the original insert with XbaI/NotI digestion. After ligation the plasmids were transformed in *E. coli* strain DH5R. All of the mutations were confirmed by DNA sequencing (COSMO, Seoul, Korea). *E. coli* strain BL21 (Stratagene) was transformed by introducing pMS107-derivative plasmid (5), which encodes the wild-type, E36Q and D217N opsin, and was grown in 2xYT medium in the presence of ampicillin (50 µg/ml) at 38 °C. Three hours after IPTG induction with addition of 10 µM all-trans retinal, pink-colored cells were harvested, sonicated, solubilized by 1% DM, and purified by a Ni²⁺-NTA column. The purified ASR was then reconstituted into PC liposomes by removing the detergent with Bio-Beads, where the molar ratio of the added PC to ASR was 30:1. The liposomes were washed three times with a buffer [2 mM sodium phosphate (pH 7.0)]. A 40 µL aliquot was deposited on a BaF₂ window of 18 mm diameter and dried in a glass vessel that was evacuated by an aspirator.
Figure 4-1
X-ray crystallographic structure of the cytoplasmic region of ASR (PDB entry 1XIO (16)). Top and bottom regions correspond to the cytoplasmic surface and retinal binding pocket, respectively. Green spheres represent water molecules in the cytoplasmic region. Hydrogen-bonds (yellow dashed lines) are inferred from the structure.
**FTIR Spectroscopy.** FTIR spectroscopy was performed as described previously (17). The ASR film sample was hydrated with 1 µL of H$_2$O, D$_2$O, or D$_2$$^{18}$O before the measurements. Then, the sample was placed in a cryostat (DN-1704, Oxford) mounted in the FTIR spectrometer (FTS-40, Bio-Rad). The cryostat was equipped with a temperature controller (ITC-4, Oxford), and the temperature was regulated with 0.1 K precision. All experimental procedures until setting the samples were performed in the dark or under dim red light (>670 nm). Illumination with >580 nm light at 170 K for 16 min converted ASR to ASR$_L$. Each difference spectrum was calculated from two spectra constructed from 128 interferograms taken before and after the illumination. Three difference spectra obtained in this way were averaged to produce the ASR$_L$ minus ASR spectrum. The BR$_L$ minus BR spectra were taken from Kandori et al. (17).

**Results**

*Comparison of the Difference Infrared Spectra of the L Intermediate of Full-length ASR and Truncated ASR in the 1800-800 cm$^{-1}$ Region.* The previous photoelectric measurements showed that the direction of charge movement of full-length ASR was different from that of C-terminally truncated ASR (truncated ASR) for the L and M intermediates, whereas both charge movements were similar for the K intermediate (12). This suggests that full-length and truncated ASR have different structural changes in the L and M intermediates. Therefore, I prepared both full-length and truncated ASR, and measured the difference FTIR spectra for the L intermediate. Figure 4-2 compares the full-length ASR$_L$ minus ASR (solid line) and the truncated ASR$_L$ minus ASR (dotted line) spectra at 170 K upon hydration with H$_2$O. As is clearly seen, the spectrum of the full-length ASR is very similar to that of the C-terminally truncated ASR. Thus, the present FTIR spectra for the L intermediate showed no effects of the C-terminal truncation. All data below are shown for the full-length ASR including the mutant proteins. It should be noted that I confirmed similarity of the spectra at 170 K between full-length and truncated ASR at acidic and
Figure 4-2
The full-length (solid line) and truncated (dotted line) ASR<sub>L</sub> minus ASR spectra (pH 7) in the 1800-900 cm<sup>-1</sup> region. The spectra are measured at 170 K upon hydration with H<sub>2</sub>O. One division of the y-axis corresponds to 0.004 absorbance units.
alkaline pH as well, though they could be different at room temperature.

Comparison of the Difference Infrared Spectra of the L Intermediate of ASR and BR in the 1800-800 cm$^{-1}$ Region. Figure 4-3 compares the ASR$_L$ minus ASR (a) and the BR$_L$ minus BR spectra (b) at 170 K. The samples were hydrated with H$_2$O (solid lines) and D$_2$O (dotted lines). In Figure 4-3a, the negative band at 1537 cm$^{-1}$ corresponds to the ethylenic vibration of all-trans retinal in ASR, which exhibits the absorption maximum at 549 nm (13). The ASR$_K$ minus ASR spectrum also showed the negative band at identical frequency (4). In the case of BR, the ethylenic vibration of the L intermediate is observed at higher frequency (1550 cm$^{-1}$) than that of the original state (1528 cm$^{-1}$), which corresponds to the blue-shifted absorption maximum of BR$_L$ (18). Similarly, illumination of ASR results in the spectral upshift to 1558 cm$^{-1}$. Blue-shifted visible absorption of ASR$_L$ is consistent with our low-temperature UV-visible analysis (3).

C-C stretching vibrations of retinal in the 1300-1150 cm$^{-1}$ region are sensitive to the local structure of the chromophore. Negative bands at 1255, 1216, 1202, and 1169 cm$^{-1}$ in Figure 4-3b were assigned to the C12-C13, C8-C9, C14-C15, and C10-C11 stretching vibrations of BR, respectively (19). These bands are typical to all-trans retinal protonated Schiff base but located at higher frequencies corresponding to charge delocalization in the retinal molecule in BR. BR$_L$ has a 13-cis retinal, resulting in the appearance of a strong positive band at 1192 cm$^{-1}$, which is assigned to C10-C11 and C14-C15 stretching vibrations (20). Essentially similar observation was obtained for ASR. From the similarity in frequency, negative bands at 1248, 1215, 1196, and 1174 (and/or 1167) cm$^{-1}$ can be assigned to C12-C13, C8-C9, C14-C15, and C10-C11 stretching vibrations of ASR (Figure 4-3a).

The difference spectra in the 1110-890 cm$^{-1}$ region are expanded in Figure 4-4, where hydrogen-out-of-plane (HOOP), N-D in-plane bending and methyl rocking vibrations appear. The presence of strong HOOP modes represents the distortion of the retinal molecule (21). The ASR$_L$ minus ASR spectra exhibit two positive peaks at 968 and 955 cm$^{-1}$, which possibly correspond to the bands at 968 and 951
Figure 4-3

The ASR$_l$ minus ASR (a) and the BR$_l$ minus BR (b) spectra in the 1800-900 cm$^{-1}$ region, which are measured at pH 7 and 170 K upon hydration with H$_2$O (solid line) and D$_2$O (dotted line), respectively. One division of the y-axis corresponds to 0.012 absorbance units.
cm\(^{-1}\) of BR\(_L\), respectively (Figure 4-4). The bands at 986 (+) and 976 (-) cm\(^{-1}\) were assigned to the N-D in-plane bending vibrations of BR\(_L\) and BR, respectively (22). On the other hand, the ASR\(_L\) minus ASR spectrum does not show clear H-D exchange dependent bands in this region. The 1009 cm\(^{-1}\) band in Figure 4-4b is insensitive to the H-D exchange and was assigned to the methyl rocking vibration of the retinal in BR. The band at 1005 cm\(^{-1}\) in Figure 4-4a is also assignable to the methyl rocking vibration in ASR. Thus, similar L spectra were observed for ASR and BR.

Amide-I vibrations appear in the 1700-1550 cm\(^{-1}\) region together with the C=N stretching vibration of the protonated retinal Schiff base (Figure 4-3). In general, the former is little sensitive to the H-D exchange, whereas the latter exhibits a downshift in D\(_2\)O. The bands at 1641 (-) and 1625 (+) cm\(^{-1}\) were assigned to the C=N stretching vibrations of BR and BR\(_L\), respectively (20). In the case of ASR, a prominent negative peak at 1644 cm\(^{-1}\) is assignable to the C=N stretch of ASR, because the D\(_2\)O-sensitive 1644 cm\(^{-1}\) band in the ASR\(_{\kappa}\) minus ASR spectra was identified by use of \(^{15}\)N-lysine labeled ASR (13). On the other hand, the C=N stretch of ASR\(_L\) is not obvious. The positive peak at 1625 cm\(^{-1}\) is a candidate, whereas the downshifted band was not clearly observed in D\(_2\)O (dotted line in Figure 4-3a). The H-D independent band at 1663 cm\(^{-1}\) presumably originates from amide-I vibration. The frequency suggests structural changes of a distorted \(\alpha\)-helix. Since the negative band at 1663 cm\(^{-1}\) is absent for the ASR\(_{\kappa}\) minus ASR spectra (4), the structural changes of \(\alpha\)-helix newly appear in ASR\(_L\).

**Comparison of the Difference Infrared Spectra of the L Intermediate in Protonated Carboxylic Acid (1800-1700 cm\(^{-1}\)) Region.** The infrared difference spectra in this frequency region mainly monitor the structural changes of protonated carboxylic acids. In the BR\(_L\) minus BR difference spectra, the bands at 1748 (+) and 1729 (+) cm\(^{-1}\) were assigned to the C=O stretching vibrations of the protonated Asp96 and Asp115, respectively, while large negative band at 1740 cm\(^{-1}\) contains the corresponding bands of Asp96 and Asp115 in the unphotolyzed state (Figure 4-3b).
Figure 4-4
The $\text{ASR}_l$ minus ASR (a) and the $\text{BR}_l$ minus BR (b) spectra in the 1030-900 cm$^{-1}$ region, which correspond to hydrogen-out-of-plane (HOOP) vibrations of the retinal chromophore. The sample was hydrated with $\text{H}_2\text{O}$ (solid lines) or $\text{D}_2\text{O}$ (dotted lines). One division of the y-axis corresponds to 0.0016 absorbance units.
The corresponding amino acids in ASR are Ser86 and Asn105, so that we did not expect any peaks in this frequency region. Nevertheless, Figure 4-3a shows a broad positive peak at 1722 cm\(^{-1}\) as well as a negative feature at 1703 cm\(^{-1}\), suggesting structural perturbation of carboxylic acids upon formation of ASR\(_L\). It should be noted that the bands do not originate from the contribution of ASR\(_M\), because UV-visible spectroscopy confirmed no formation of ASR\(_M\) at 170 K (3). The absence of a clear negative band at around 1400 cm\(^{-1}\), characteristic of COO\(^-\) stretching frequency of negatively charged carboxylates, suggests that appearance of the carboxylic C=O stretch at 1722 cm\(^{-1}\) in ASR\(_L\) is not due to the newly protonated species, but rather due to the frequency shift from 1703 cm\(^{-1}\) in ASR (Figure 4-3a).

To further examine the spectral feature in this region, I measured ASR\(_L\) minus ASR spectra at acidic (pH 5) and alkaline (pH 9) pH in addition to pH 7 (Figure 4-3a). I also measured the ASR\(_L\) minus ASR spectra of D217N and E36Q mutant proteins to identify the responsible carboxylic acid. Figure 4-5 clearly shows that ASR\(_L\) is formed at different pH values (5, 7, and 9 in a, b, and c, respectively), as well as for the D217N (d) and E36Q (e) mutants. Figure 4-6 highlights the carboxylic C=O stretching region, where all spectra were normalized by use of the negative 1196 cm\(^{-1}\) band (Figure 4-5). Although the positive peak at 1722 cm\(^{-1}\) was broad at pH 7 (Figure 4-6b), it was enhanced at pH 5 (Figure 4-6a). In contrast, the 1722 cm\(^{-1}\) band completely disappeared at pH 9 (Figure 4-6c). Spectral downshift to 1717 cm\(^{-1}\) in D\(_2\)O (Figure 4-6a) is typical for carboxylic C=O stretching vibrations. Thus, I identify the positive band at 1722 cm\(^{-1}\) in the ASR\(_L\) minus ASR spectra as a carboxylic C=O stretch, whose pKa was estimated between 6 and 7.

The negative band at 1703 cm\(^{-1}\) exhibits similar pH dependence to that of the 1722 cm\(^{-1}\) band, being enhanced at pH 5, but disappearing at pH 9 (Figure 4-6a-c). In addition, the 1703 cm\(^{-1}\) band is downshifted in D\(_2\)O (Figure 4-6a), though the shifted negative band was not clearly observable because of the strong peaks at 1695 (+) / 1687 (-) cm\(^{-1}\) (Figure 4-3a). Similar pH dependence strongly suggests that the bands at 1722 (+) / 1703 (-) cm\(^{-1}\) originate from the same carboxylic group.
Figure 4-5
The ASR₁ minus ASR infrared spectra of the wild type at pH 5 (a), pH 7 (b), and pH 9 (c), D217N at pH 5 (d) and E36Q at pH 5 (e) in the 1800-900 cm⁻¹ region. The spectra are measured at 170 K upon hydration with H₂O. One division of the y-axis corresponds to 0.009 absorbance units.
The absence of pH dependent bands at around 1400 cm\(^{-1}\) (Figure 4-5) also supports this interpretation.

Finally, the remaining question is the location of the carboxylic group responsible for this spectral feature. The previous time-resolved FTIR study observed a positive carboxylic C=O stretch at 1716 cm\(^{-1}\) in the ASR\(_m\) minus ASR spectra, and assigned the band to Asp217 located at the cytoplasmic region, because the band disappeared for D217N, but not for E36Q \((8)\). Interestingly, the 1716 cm\(^{-1}\) band in ASR\(_m\) was also pH-dependent, whose pKa was between 6 and 7, but the pH dependence was opposite to the present case. Namely, the positive band at 1716 cm\(^{-1}\) was observed at alkaline pH, but not at acidic pH, and the authors interpreted that Asp217 is protonated at acidic pH in the unphotolyzed state \((8)\). They did not observe frequency change of Asp217 at acidic pH, suggesting no structural changes of Asp217 at acidic conditions. Thus, there has been no information about the C=O stretching frequency of Asp217 at acidic pH, and Asp217 is a possible candidate for the bands at 1722 (+) / 1703 (-) cm\(^{-1}\) in the ASR\(_l\) minus ASR spectra. Here I also measured the spectra of the E36Q mutant, as Glu36 is located near Asp217 (Figure 4-1).

Figures 4-6d and 4-6e show the carboxylic C=O stretching region in the ASR\(_l\) minus ASR spectra of D217N and E36Q, respectively. Similar difference spectra in other frequency regions ensure the normal formation of ASR\(_l\) for these mutants (Figure 4-5). The bands at 1722 (+) / 1703 (-) cm\(^{-1}\) were reproduced in Figure 4-6d, indicating that they do not originate from Asp217. On the other hand, the bands at 1722 (+) / 1703 (-) cm\(^{-1}\) completely disappeared for E36Q (Figure 4-6e). Thus, I assigned the bands to Glu36. It is generally accepted that the C=O stretching vibrations appear at lower frequency for Glu than for Asp, but the frequency of Glu36 at 1703 cm\(^{-1}\) in ASR is particularly unusual. The frequency is very low, indicating that Glu36 forms a strong hydrogen bond in the unphotolyzed state, which is weakened by structural changes upon formation of ASR\(_l\) as shown by the upshift to 1722 cm\(^{-1}\).
Figure 4-6
The ASR$_l$ minus ASR infrared spectra of the wild type at pH 5 (a), pH 7 (b), and pH 9 (c), D217N at pH 5 (d) and E36Q at pH 5 (e) in the 1740-1700 cm$^{-1}$ region. The sample was hydrated with H$_2$O (solid lines) or D$_2$O (dotted lines). One division of the y-axis corresponds to 0.0008 absorbance units.
Comparison of the Difference Infrared Spectra of the L Intermediate in Water O-D Stretching Frequency (2750-2500 cm\(^{-1}\)) Region. The ASR\(_L\) minus ASR spectra clearly show hydrogen-bonding alteration of Glu36. Since there is a water cluster in the cytoplasmic region near Glu36 (Figure 4-1), detecting water signals in the ASR\(_L\) minus ASR spectrum is important. It was not easy, because ASR\(_L\) decays to the 13-cis form, not to the original all-trans form, and the sample was dark-adapted again before the next measurement. Nevertheless, I successfully measured the spectra in the frequency region of water O-D stretching vibrations in D\(_2\)O. Figures 4-7a, 4-7b, and 4-7c show the ASR\(_L\) minus ASR spectra of the wild type at pH 5, 7, and 9, respectively. The spectra show a negative peak at 2693 cm\(^{-1}\) and a positive peak at 2582 cm\(^{-1}\) at all pH. Since the peaks are downshifted upon hydration with D\(_2\)\(^{18}\)O, they originate from O-D stretching vibrations of water. The negative peaks at 2642 and 2628 cm\(^{-1}\) in Figure 4-7b similarly show the isotope shift of water, indicating that they originate from water O-D stretches. Interestingly, a single peak only exists at 2642 and 2628 cm\(^{-1}\) for the spectra at pH 5 (Figure 4-7a) and pH 9 (Figure 4-7c), respectively. This observation implies that the frequency of the water O-D stretch is pH-dependent, being downshifted at higher pH. The pKa is located at about 7, which is coincident with that of Glu36 (Figure 4-6). Similar pKa of the water O-D stretch at 2650-2620 cm\(^{-1}\) to that of Glu36 suggests that the water molecule is located near Glu36. This is indeed the case, because the pH dependence of the water O-D stretch is abolished in E36Q. Figure 4-7d shows identical spectra between the wild type (dotted line) and E36Q (solid line) at pH 5. However, the frequency shift of the negative band from 2642 cm\(^{-1}\) to 2628 cm\(^{-1}\) in the wild type at pH 9 was absent in E36Q (solid line in Figure 4-7e). This observation suggests that deprotonation of Glu36 at pH > 7 is correlated with the frequency shift of the water O-D stretch from 2642 cm\(^{-1}\) to 2628 cm\(^{-1}\). The straightforward interpretation is that the water directly interacts with Glu36. It should be noted that the water O-D stretch at 2650-2620 cm\(^{-1}\) represents a weak hydrogen bond, and I presumably monitor the free O-D stretch of a water molecule interacting with Glu36.
Figure 4-7
The ASR₆ minus ASR infrared spectra of the wild type at pH 5 (a), pH 7 (b), and pH 9 (c), E36Q at pH 5 (d) and pH 9 (e) in the 2750-2500 cm⁻¹ region. The spectra are measured at 170 K upon hydration with D₂O. One division of the y-axis corresponds to 0.0009 absorbance units.
Discussion

In this study, I report the ASR\textsubscript{L} minus ASR spectra measured by low-temperature FTIR spectroscopy. Although photoinduced current measurements of ASR\textsubscript{L} and ASR\textsubscript{tr} reported the different direction of charge signal between C-terminally truncated and full-length ASR (12), the present FTIR study showed almost identical ASR\textsubscript{L} and ASR spectra for them (Figure 4-2). While the spectral features were essentially similar to those for BR, a unique feature was obtained for the carboxylic C=O stretching frequency region for ASR. The pH-dependent bands were observed at 1722 (+) / 1703 (-) cm\textsuperscript{-1} in the ASR\textsubscript{L} minus ASR spectra, which were assigned to Glu36. pH-dependent water O-D stretching vibrations in D\textsubscript{2}O were also observed at 2642 and 2628 cm\textsuperscript{-1} for the unphotolyzed state of ASR at pH 5 and pH 9, respectively. These pKa were estimated to be between 6 and 7. According to the X-ray structure of ASR, Glu36 is located near the cytoplasmic surface (Figure 4-1), and the distances from the Schiff base nitrogen of the retinal chromophore to the side-chain oxygens of Glu36 are 19.3 and 20.2 Å (2). The present study clearly shows that formation of ASR\textsubscript{L} accompanies hydrogen-bonding alteration of Glu36. Since ASR\textsubscript{L} is formed at 170 K, this fact demonstrates structural alteration propagating over 20 Å at such low temperatures.

Spectral feature of the water signal in the ASR\textsubscript{L} minus ASR spectrum resembles that in the BR\textsubscript{L} minus BR spectrum at 2700-2500 cm\textsuperscript{-1} (24). In particular, an intense positive broadband at 2630-2550 cm\textsuperscript{-1} (O-H stretch at 3550-3450 cm\textsuperscript{-1}) has been regarded as a characteristic for the L state of BR (24). However, our recent time-resolved FTIR spectroscopy clearly showed the absence of the band for BR\textsubscript{L} at room temperature, and I concluded that such water signal is a low-temperature artifact, or a feature peculiar at low temperature (170 K) where L is stable (25). This may be also true for ASR. However, it should be noted that the cryotrapped L state is considerably relaxed to the original state in BR (26, 27), but decays to the subsequent intermediates in ASR by warming (3). This suggests different protein dynamics between ASR and BR, but room-temperature FTIR study of ASR is
required for further understanding.

**Hydrogen-Bonding Structures in the Cytoplasmic Domain of ASR and ASR<sub>L</sub>.**

The present study showed that the pKa of Glu36 is between 6 and 7 in ASR, and its hydrogen bonding is significantly altered upon formation of ASR<sub>L</sub>. Previous FTIR study reported the pKa of Asp217 being also between 6 and 7 in ASR (8). According to these observations, Glu36 and Asp217 are both protonated at low pH, while being both deprotonated at high pH. However, the latter may be unlikely, because Glu36 and Asp217 are located close to each other (Figure 4-1). Below we discuss a possible model for activation of ASR based on these observations and X-ray structure (2).

At low pH, Glu36 and Asp217 are both protonated in the unphotolyzed state. The hydrogen bond of Glu36 is remarkably strong (C=O stretch at 1703 cm<sup>-1</sup>), whose hydrogen-bonding donor may be Gln93 or a water molecule (Figure 4-1). Upon ASR<sub>L</sub> formation, the hydrogen bond of Glu36 is weakened (C=O stretch at 1722 cm<sup>-1</sup>). The absence of the bands for Asp217 in the ASR<sub>L</sub> minus ASR spectra (Figure 4-5) implies that no hydrogen-bonding alteration of this residue occurs. The hydrogen bond of a water molecule near by Glu36 is strengthened upon formation of ASR<sub>L</sub> as shown by the spectral shift from 2642 cm<sup>-1</sup> to 2582 cm<sup>-1</sup> (Figure 4-7a).

At high pH, Asp217 is probably deprotonated according to the previous results (8). ASR<sub>L</sub> formation does not change the hydrogen bond of Asp217, because there is no pH dependent signal at around 1400 cm<sup>-1</sup> (Figure 4-5). Then, protonated state of Glu36 may be more likely, because negatively charged Asp217 and Glu36 are energetically unfavorable. However, the two negative charges at carboxylates could be stabilized by a positively charged water cluster (H<sub>3</sub>O<sup>+</sup> or H<sub>7</sub>O<sup>3+</sup>). The water structure in Figure 4-1 may resemble the proton release group of BR, where water cluster stabilizes Glu194 and Glu204. Experimental evidence of protonated water cluster was first reported by the group of Dr. Gerwert as a continuum band at 2200-1800 cm<sup>-1</sup> in the room-temperature BR<sub>w</sub> minus BR spectra (28, 29), and we recently identified that the continuum band contains a water signal (25). I did not
observe such a continuum band in the ASR\textsubscript{L} minus ASR spectra at 170 K (data not shown), but this does not exclude the presence of a protonated water cluster, because the continuum band of BR is observed only at room temperature (25). Water-containing hydrogen bonding network in the cytoplasmic domain (Figure 4-1) must play important roles in the activation of the transducer protein by ASR.

**Characteristic Features of Photoreaction in ASR.** By use of low-temperature UV-visible spectroscopy, I recently revealed that the stable photoproduct of the all-\textit{trans} form is 100% 13-\textit{cis}, and that of the 13-\textit{cis} form is 100% all-\textit{trans} (3). This was entirely unique for archaeal-type rhodopsins, because functionally important states known so far were only derived from the all-\textit{trans} form, and the photocycle of the all-\textit{trans} form without branching into the 13-\textit{cis} stable states has been the common mechanism. The complete photocycling for the proton pump in BR and the complete photochromism for the chromatic sensor of ASR are highly advantageous for their functions. Although the protein structures are similar between ASR and BR (2), the present study suggests that the migration of protons to the cytoplasmic side is correlated with the unique photoreactions of ASR.

ASR has Asp75 as a counterion of the retinal chromophore, which corresponds to Asp85 in BR. Nevertheless, the Schiff base proton is transferred not to Asp75 (8, 9), but to Asp217 in the cytoplasmic region. What is the mechanism of proton transfer in the opposite direction? The present FTIR spectroscopy of the L intermediate revealed similar structural changes for the chromophores of ASR and BR, suggesting the importance of the surrounding protein moiety. It should be noted that Asp212 in BR is replaced by proline (Pro206) in ASR. Previous studies reported the important role of Asp212 during the M formation, and we proposed a hydration switch mechanism as the primary cause of proton transfer reaction in BR. In this mechanism, the bridged water molecule between the Schiff base and Asp85 forms a strong hydrogen bond transiently, which leads to the proton transfer to Asp85 (4). Lack of aspartate at position 206 would be significant for ASR. In this regard, I found the absence of strongly hydrogen-bonded water molecules in ASR (4).
Figure 4-8
The structural changes of ASR in L-intermediate. (left) The ASR<sub>L</sub> minus ASR infrared spectra in protonated carboxylic acid region. (Right) The structure of ASR in cytoplasmic surface. Yellow broken lines indicates hydrogen bond, green spheres are water molecules.
Since there is a positive correlation between the strongly hydrogen-bonded water molecules and the proton pumping activity, weakly hydrogen-bonded water molecules in the Schiff base region may be the key element. Interestingly, the replacement of Pro206 to Asp was not sufficient for ASR to function as a BR-like proton pump (14). Since the Schiff base proton is transferred to the cytoplasmic side, ASR is a very good model system to study the general mechanism of proton pumps in archaeal-type rhodopsins.
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6557–6561.


Chapter 5

Engineering an inward proton pump from a bacterial sensor rhodopsin

Introduction

ATP is synthesized by an enzyme that utilizes proton motive force and thus nature creates various proton pumps such as an outward-directed light-driven proton pump bacteriorhodopsin (BR). In contrast, an inward proton pump has been neither created naturally nor artificially, presumably because proton pumps must have a specific mechanism to exclude transport in the reverse direction to maintain a proton gradient.

In BR, a retinal chromophore is located at the center of the membrane, and the hydrophobicity is different between the cytoplasmic and extracellular domains (1). The cytoplasmic domain is highly hydrophobic, whereas the extracellular domain is composed of charged and polar amino acids that form a hydrogen-bonding network. Figure 5-1a shows the presence of 7-8 water molecules in the extracellular domain, but only 2 water molecules in the cytoplasmic domain (2). Such an asymmetric hydrogen-bonded network could be the reason of unidirectional proton transport in BR, where the proton transfer to the extracellular side occurs in 10^-5 seconds, followed by reprotonation through a transiently formed proton pathway in the cytoplasmic domain on a slower timescale (10^-4-10^-3 seconds) (1). Many other light-driven proton pumps seem to possess BR-like asymmetric architecture and a similar mechanism for proton transport. Sasaki et al. previously converted BR into an inward chloride-ion pump (3), but an inward proton pump has never been created. It may be difficult to design an inward proton pump from “normal” outward proton pumps.

Anabaena sensory rhodopsin (ASR) is an archaeal-type rhodopsin found in Anabaena (Nostoc) sp. PCC7120, a freshwater cyanobacterium. ASR does not
Figure 5-1
X-ray crystallographic structures of BR (6) (a) and ASR (7) (b). Top and bottom panels represent views from the membrane plane and the cytoplasmic side, respectively. In the top panel, top and bottom regions correspond to the cytoplasmic and extracellular sides, respectively. The retinal chromophore is colored yellow, and green spheres represent internal water molecules. BR (a) is a light-driven outward proton pump, where Asp85 accepts a proton from the Schiff base and Asp96 donates a proton to the Schiff base (1). No proton pump activity has been reported for ASR (3). ASR is a sensor protein that activates a soluble protein at the cytoplasmic surface, and it was reported that the Schiff base proton is transferred to Asp217 in the cytoplasmic region (8) (b). Polar amino acids as well as five water molecules in the cytoplasmic region, characteristic for ASR, which must be advantageous for primary proton transfer that may be important for activation of the soluble transducer protein. This is an experimental basis of designing an inward proton pump in the present study.
show proton pump activity, and as it forms a single operon with a soluble protein of 14 kDa (4), it has been suggested that ASR is a photochromic sensor activating the 14 kDa transducer protein at the cytoplasmic surface (5, 6). The X-ray crystallographic structure of ASR has a similar α-helical arrangement to that of BR (7), but a very different hydrogen-bonded network. Figure 5-1b shows that in ASR both extracellular and cytoplasmic domains contain 5 water molecules, and form hydrogen-bonded networks. Consistent with the hydrogen-bonded network in the cytoplasmic domain, an unusual proton transfer has been found in this protein. Shi et al. reported reverse proton transfer, from the Schiff base to Asp217 in the cytoplasmic domain (8), whereas Sineshchekov et al. reported that the direction of this proton transfer is dependent on C-terminus truncation (9). Therefore, proton conduction in ASR remains unclear at present, although proton conductivity toward the cytoplasmic domain appears possible. This suggests the potential to design an inward proton pump out of ASR, and indeed I have achieved this. A single amino acid replacement of Asp217 to Glu confers inward proton pump activity to ASR.

Materials and Methods

Sample Preparation. In the present study, I prepared C-terminally truncated and full-length ASR according to a method described previously (4, 6, 10). The D217E mutant was designed based on the wild-type ASR, which was produced by a two-step megaprimer PCR method (11), with an oligonucleotide (Hokkaido System Science, Japan): 5′-ACG TAA GCC GTG TAA TTC CAG AAA ACT AAA TCC-3′. The final PCR products were cloned into plasmid pKJ606 (12), derived from pMS107, by replacing the original insert with XbaI/NotI digestion. After ligation, the plasmids were transformed in E. coli strain JM109. All of the mutations were confirmed by DNA sequencing (Hokkaido System Science, Japan).

E. coli strain BL21 (Stratagene) was transformed by introducing pMS107-derivative plasmid, which encodes the wild-type, D217E and D217N ASR, and the wild-type GR, and was grown in 2x YT medium in the presence of ampicillin.
(50 µg/ml) at 38 °C. Three hours after IPTG induction with the addition of 10 µM all-trans retinal, pink-colored cells were harvested by centrifugation at 3,600 × g for 15 min at 4 °C and suspended into 10 mL of 30 mM Tris-HCl, pH 8.0, and 20 % sucrose. Then, sphaeroplast sample was prepared as follows. 100 µg of lysozyme was added and stirred gently at room temperature for 15 min. Sphaeroplasts were spun down by 3,600 × g for 15 min at room temperature and resuspended in 100 mM potassium phosphate (KPi), pH 7.0, 20 mM MgSO₄·7H₂O, 20 % sucrose, and 4 mg of DNase (400 µL) and injected slowly using 1-mL syringe (18 gauge needle) into 200 mL of rapid stirring (200 rpm) solution of 50 mM KPi, pH 7.0 at 37 °C. After 15-min of gentle stirring, Na-EDTA was added to a final concentration of 10 mM and stirred for another 15 min. The right side-out of sphaeroplast vesicles were collected at 3,600 × g for 15 min at 4 °C (Hitachi CF16RX centrifuge, Japan), washed with 10 mL unbuffered solution (150 mM NaCl, 50 mM MgSO₄·7H₂O), and spun down by 3,600 × g for 15 min at 4 °C. The sphaeroplast suspension (pH between 6.3-6.7) was used for the measurements of proton pump activity.

For spectroscopic and HPLC analysis, the harvested samples after IPTG induction were sonicated, solubilized by 1 % dodecyl maltoside (DM), and purified by a Ni²⁺-NTA column as described previously (13, 14). The purified ASR samples in 0.1 % DM solution (300 mM NaCl, 50 mM Tris-HCl, 150 mM imidazole at pH 7.0) were used for UV-visible spectroscopy and HPLC analysis (14). For FTIR spectroscopy, the purified ASR was then reconstituted into phosphatidylcholine (PC) liposomes by removing the detergent with Bio-beads, where the molar ratio of the added PC to ASR was 30:1 (13, 14). The liposomes were washed three times with a buffer (2 mM sodium borate (pH 9.0)).

**Light-Induced pH Changes.** Proton pump activity of each protein was measured by monitoring pH changes in sphaeroplast suspension by a glass electrode (15). Sphaeroplasts containing ASR or GR protein in unbuffered solution (150 mM NaCl, 150 mM MgSO₄) were illuminated at >500 nm through a glass filter (AGC Techno
Glass Y-52, Japan) and the pH value changes were monitored (Horiba pH meter F-55, Japan). The light source was a 1 kW tungsten-halogen projector lamp (Rikagaku Master HILUX-HR, Japan). The samples were then illuminated after addition of CCCP to a final concentration of 10 µM. Proton pumping activities were calculated by adding 10 µl of 0.01 N HCl to the suspension. The amount of proteins in the sphaeroplast suspension was estimated by measuring absorption spectra after solubilizing proteins with 1 % DM. 3-6 independent measurements were averaged to obtain the initial rate of proton transport.

For the light intensity dependence of the initial rate of proton transport, neutral density filters of 50, 25, 10, 1 % were used. The action spectrum for proton pump activity was measured with monochromatic light (half width: 10 nm) from interference filters (Melles Griot, 03FIV107, 006, 109, 008, 117, 018 and 119, USA), where wavelength-dependent light intensity of the tungsten-halogen lamp was calibrated by using a CCD linear detector (Hamamatsu Photonics PMA-12, Japan).

**UV-Visible Spectroscopy.** Absorption spectra of the wild-type and D217E ASR were measured in 0.1 % DM solution (300 mM NaCl, 50 mM Tris-HCl, 150 mM imidazole at pH 7.0) at 20 ºC by use of a SHIMADZU UV-2400PC UV-visible spectrometer. The samples were illuminated with a >500 nm light for 4 sec at 277 K, and decay kinetics of the M intermediate were monitored at 388 nm.

**HPLC Analysis.** HPLC analysis was performed as described previously (14). Dark-adapted ASR was prepared by keeping the samples in the dark overnight at 4 ºC. Extraction of retinal oxime from the sample was carried out by hexane after denaturation by methanol and 500 mM hydroxylamine at 4 ºC.

**FTIR Spectroscopy.** FTIR spectroscopy was performed as described previously (13, 14). A 40 µl aliquot of the PC liposomes was deposited on a BaF₂ window of 18 mm diameter and dried in a glass vessel that was evacuated by an aspirator. The ASR film sample was hydrated with 1 µL of H₂O or D₂O before the measurements. Then, the sample was placed in a cryostat (Oxford DN-1704, UK) mounted in the FTIR spectrometer (Bio-Rad FTS-40, USA). The cryostat was equipped with a
temperature controller (Oxford ITC-4, UK), and the temperature was regulated with 0.1 K precision. All experimental procedures until setting the samples were performed in the dark or under dim red light (>670 nm).

Illumination with >500 nm light at 230 K for 4 sec converted ASR to ASR_{im}. Each difference spectrum was calculated from two spectra constructed from 64 interferograms taken before and after the illumination. Three difference spectra obtained in this way were averaged to produce the ASR_{im} minus ASR spectrum.
Results and Discussions

The wild-type (WT) and mutant proteins of ASR were expressed in E. coli as described previously (10, 13). I used full-length ASR here, but C-terminally truncated ASR was also prepared for WT. For proton pump measurement, I prepared sphaeroplast vesicles by removing the cell wall by lysozyme treatment. Illumination caused a net inward transport of protons for D217E ASR, resulting in alkaline pH of the medium (Figure 5-2, top panel). The observation of no transport after addition of CCCP (Figure 5-2, top panel) and in the absence of retinal (Figure 5-2, second panel) clearly demonstrates that the D217E mutant functions as a light-driven inward proton pump. The inward proton-pump activity was also observed for the wild-type (WT) ASR (Figure 5-2, third panel), but the pump activity was much less than D217E ASR (initial slope 5-times less). The proton-pump activity was negligible for D217N ASR (Figure 5-2, fourth panel). I also measured an outward-directed proton pump as a control. Figure 5-2 bottom panel shows the result with Gloeobacter rhodopsin (GR) found in a primitive cyanobacterium. In sphaeroplast vesicles containing GR, light caused outward proton transport, like BR.

It should be noted that light-driven inward proton transport associated with two-photon reactions was reported for the D85N mutant of BR in films attached to planar lipid bilayers (16), where the molecular mechanism has not been well established. It appears that absorption of a second photon by the deprotonated Schiff base is necessary for the inward proton pump. To test such a possibility for the present case, I measured proton pump activity with different light intensities.

Figure 5-3a clearly shows a linear relationship, indicating that the inward proton transport in D217E ASR is driven by a single-photon reaction. The action spectrum for proton pump activity, measured with monochromatic light, resembles the absorption spectrum of D217E ASR (Figure 5-3b). The initial slope for the inward proton pump in D217E ASR was $15.1 \pm 4.0 \text{ H}^+$/protein/min, which is about half of that in GR (Figure 5-2, bottom panel) and 15-times smaller than that in BR (17).
Figure 5-2
Light-driven proton pump activity in sphaeroplast vesicles containing ASR or GR (50 mM MgCl₂, 150 mM NaCl, initial pH ~6.5). “On” and “Off” indicate the onset and offset of illumination (with yellow light, > 500 nm), respectively, and positive signal corresponds to a decrease in pH (inward proton pump). Signal amplitude of each protein was normalized to its absorption after the pump activity measurement, indicating that pump activity can be numerically compared among different samples. Dotted lines represent identical measurements in the presence of 10 µM CCCP. One division of the y-axis corresponds to a 0.1 pH unit.
Figure 5-3c shows that the inward proton pump by D217E ASR is pH-independent between pH 6.5 and 8.0.

What determines the inward proton-pump activity of D217E ASR? Aspartate and glutamate have similar properties, but the proton pump activity was much higher for glutamate. The absorption spectra of WT and D217E are similar (Figure 5-4a) and HPLC analysis showed that the all-trans form of retinal is dominant in both dark-adapted samples (Figure 5-4b) (14). The similarity of D217E to WT in the unphotolyzed state is reasonable because Asp217 is located at about 15 Å from the retinal chromophore (Figure 5-1b). The M intermediate state is similarly formed for D217E, whose decay was also similar to the case for WT (Figure 5-4c).

Light-induced M-minus-ASR difference FTIR spectra are also similar between WT and D217E (Figure 5-5a), but a remarkable difference is seen at 1760-1700 cm⁻¹, the characteristic frequency region of protonated carboxylic acids. The spectrum of WT (Figure 5-5b, top panel) exhibits a broad positive band at 1740-1700 cm⁻¹, and Shi et al. interpreted this feature as evidence for protonation of Asp217 in the M intermediate (8). A stronger positive peak is observed at 1713 cm⁻¹ for D217E, which is down-shifted in D₂O (Figure 5-5b, bottom panel). These observations suggest that the Schiff base proton is transferred to Glu217 in the M intermediate. It should be noted that the spectra of WT and D217E are normalized by the retinal bands at 1250-1200 cm⁻¹ (Figure 5-5a), indicating that the same amount of ASR is converted to the M state for WT and D217E. Nevertheless, protonation of Glu217 is about 10-times larger than that of Asp217 in WT. This is completely coincident with the proton-pump activity (Figure 5-2). It is thus likely that proton affinity at position 217 is correlated with the inward proton pump activity.

On the basis of the present FTIR data, the mechanism of the inward proton pump in D217E ASR can be explained as follows. M formation accompanies deprotonation of the Schiff base, and Glu217 acts as a proton acceptor in D217E ASR. Figure 5-3c suggests that pKa of Glu217 is lower than 6.5 in the unphotolyzed state, while being higher than 8.0 in M. In BR, the proton acceptor is
Figure 5-3
(a) Dependence of the initial rate of proton transport of D217E ASR on light intensity. Inset expands the region of light intensity between zero and 15 %. (b) Spectral coincidence of the proton pump activity and absorption of D217E ASR. Open circles represent proton pump activity upon illumination with monochromatic light by use of an interference filter. Solid line shows the absorption spectrum of the dark-adapted form of D217E ASR. (c) pH dependence of the proton pump activity of D217E ASR.
Figure 5-4
Molecular properties of WT and D217E ASR, which were measured in a DM solution at pH 7.0. (a) Absorption spectra of the dark-adapted form of WT (dotted line) and D217E (solid line) ASR. (b) Isomeric composition of the retinal chromophore in the dark-adapted form. WT and D217E ASR possess 97 and 98 % all-trans forms, respectively. (c) Decay kinetics of the M intermediate at 388 nm, which were measured at 277 K. Time constants of WT and D217E ASR were 162 and 188 ms, respectively.
Figure 5-5
(a) Light-minus-dark difference infrared spectra of WT and D217E ASR in the 1800-800 cm\(^{-1}\) region. The spectra were measured for PC liposomes at pH 9.0 and 230 K. One division of the y-axis corresponds to 0.02 absorbance units. (b) The spectra in a are highlighted at the frequency region (1780-1690 cm\(^{-1}\)) of protonated carboxylic acids. Solid and dotted lines correspond to the measurements in H\(_2\)O and D\(_2\)O, respectively. One division of the y-axis corresponds to 0.004 absorbance units.
Asp85 in the extracellular side (1), and the Schiff base nitrogen interacts with the side-chain oxygen (distance 4.4 Å (2)) through a strongly hydrogen-bonded water in the unphotolyzed state (Figure 5-1a) (18). Interestingly, ASR also has a negatively charged Asp75 at 3.5 Å (7), but the Schiff base proton is transferred to Glu217 that is far distant (~ 15 Å) (Figure 5-1b) (8). This suggests that the accessibility of the Schiff base just before releasing the proton is toward the cytoplasmic side in ASR, while being toward the extracellular side in BR. This may suggest the important role of Asp212 in BR, the second negative charge in the Schiff base region, which is replaced by Pro in ASR (Figure 5-1).

For BR-like proton pumps, outward vectoriality is particularly important to create a proton gradient. It has been inferred that the ancestral rhodopsin functioned as a light-driven outward proton pump (19). In fact, the archaeal-type photosensors, SRI and SRII, pump protons outwardly in the absence of transmembrane transducer protein (20, 21). During evolution, conversion into inward proton pumps must be strongly prohibited, because it is dangerous for survival. Then, why can ASR be easily converted into an inward proton pump? I infer that ASR became a light sensor that activates a soluble transducer protein, and the hydrogen-bonding network in the cytoplasmic domain and its changes must be important for the activation. It is the symmetrical hydrogen-bonded network from the Schiff base (Figure 5-1b) that allowed creation of an inward proton pump by a single amino acid replacement.

The newly designed inward proton pump may be useful as an application tool in cell biology. The recently found channelrhodopsin, with a light-activated cation channel, allowed numerous applications in neurobiology, because transport of cations can be triggered by light (22). Currently, channelrhodopsin and halorhodopsin, a light-driven inward chloride pump, are used as neuroengineering tools to investigate neural circuit function (23). The newly designed inward proton pump (D217E ASR) could provide another kind of active control of electrochemical potential in cells by light in contrast to channelrhodopsin of a purely passive nature.
Another application of this protein may be in the field of acidosis-induced cell death. Intracellular pH is precisely regulated around 7.2 by various transporters, which may be changed by acidification around tumor cells (6.9-7.0). Thus, tumor metabolism and pH-control systems have been targets for novel anticancer therapies (24). Acidification of cells by light using an inward proton pump will be useful in the research field. For these applications, a more efficient proton pump may be required, because the current efficiency of D217E ASR is 20-times lower than that of BR, and an additional mutation study is in progress.

In conclusion, the present study created a light-driven inward proton pump from ASR, a bacterial photochromic sensor protein, by a single amino acid replacement. The native ASR has only small proton pump activity, but a mutation of Asp217 to Glu creates an inward proton pump. FTIR spectroscopy clearly detects the protonation signal of Glu217, but little for Asp217 in WT ASR. The strong proton affinity of the acceptor in the cytoplasmic side appears to force proton uptake from the extracellular side after the Schiff base deprotonation, even though the carboxylate is 15 Å distant from the retinal Schiff base.
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Chapter 6

Conclusion and Perspectives

In this dissertation, I showed photoreaction behavior of *Anabaena* sensory rhodopsin (ASR). In addition, I created a new function, an inward proton pump, to ASR. The results in each chapter are summarized as follows.

In chapter 2-1, I applied low-temperature FTIR spectroscopy to the all-trans form of ASR, and compared the difference spectra at 77 K with those of BR. The K intermediate minus ASR difference spectra show that the retinal isomerizes from the all-trans to the distorted 13-cis form like BR. The N-D stretching of the Schiff base was observed at 2163 (-) and 2125 (-) cm\(^{-1}\), while the O-D stretchings of water molecules were observed in the >2500 cm\(^{-1}\) region. These results indicate that the protonated Schiff base forms a strong hydrogen bond with a water molecule, which is connected to Asp75 with a weak hydrogen bond. This result with ASR supports the working hypothesis by Kandori group about the strong correlation between the proton pump activity and the existence of strongly hydrogen bonded water molecules in archaeal rhodopsins. Also I discuss the structural reason why the bridged water molecule does not form a strong hydrogen bond in ASR.

I extended the low-temperature spectroscopic study at 77 K to the 13-cis, 15-syn form of ASR (13C-ASR) (Chapter 2-2). HPLC analysis revealed that light-adapted ASR with light >560 nm at 4 °C possesses 78% 13C-ASR, while dark-adapted ASR has AT-ASR predominantly (97%). Then, I established the illumination conditions to measure the difference spectra between 13C-ASR and its K state without subtracting the difference between AT-ASR and its K state. Spectral comparison between 13C-ASR and AT-ASR provided useful information on structure and structural changes upon retinal photoisomerization in ASR. In particular, previous X-ray crystallographic study of ASR reported the same protein structure for 13C-ASR and
AT-ASR (1), whereas the present FTIR study revealed that protein structural changes upon retinal photoisomerization were significantly different between 13C-ASR and AT-ASR. The differences were seen for HOOP modes of the retinal chromophore, amide I, cysteine S-H stretch, the Schiff base N-D stretch, and water O-D stretch modes. These must trigger different global protein structural changes in each photoreaction cycle leading to the observed photochromic behavior.

ASR has been believed to function as a photoreceptor for chromatic adaptation (2). In this case, branching reactions, from ASR$_{AT}$ to ASR$_{13C}$ and from ASR$_{13C}$ to ASR$_{AT}$ (Figure 3-1c), are favorable for ARS, but they are in striking contrast to what is known for microbial rhodopsins. Ideally, the conversion ratios should be unity for photochromic reactions ($x = y = 1$ in Figure 3-1c), but this is exactly the opposite of the properties of pump rhodopsins, such as BR. X-ray crystal structures reported similar chromophore structures and protein environments for ASR$_{AT}$ (4) and BR$_{AT}$ (3). Do photochromic reactions indeed take place for ASR$_{AT}$ and ASR$_{13C}$? In Chapter 3, I determined the branching ratios ($x$ and $y$ values) for ASR$_{AT}$ and ASR$_{13C}$ by means of low-temperature UV-visible spectroscopy. Surprisingly, the obtained $x$ and $y$ values were unity, indicating that the photoreactions of ASR$_{AT}$ and ASR$_{13C}$ are completely photochromic. The complete photochromic reactions are highly advantageous for the chromatic sensor function of ASR.

In chapter 4, I applied low-temperature FTIR spectroscopy at 170 K to the dark-adapted ASR that has predominantly all-trans retinal (97%). The obtained ASR$_{L}$ minus ASR spectra were similar between the full-length and C-terminally truncated ASR, implying similar protein structural changes for the L state. The ASR$_{L}$ minus ASR spectra were essentially similar to those of BR, but a unique spectral feature was observed in the carboxylic C=O stretching region. The bands at 1722 (+) and 1703 (-) cm$^{-1}$ were observed at pH 5, which was reduced at pH 7 and disappeared at pH 9. The mutation study successfully assigned the bands to the C=O stretch of Glu36. Interestingly, Glu36 is located at the cytoplasmic side, and the distance from the retinal Schiff base is about 20 Å (Figure 4-1). I also observed
pH-dependent frequency change of a water stretching vibration, which is located near Glu36. Unique hydrogen-bonding network in the cytoplasmic domain of ASR will be discussed.

In chapter 5, I created a light-driven inward proton pump from ASR, a bacterial photochromic sensor protein, by a single amino acid replacement. The native ASR has only small proton pump activity, but a mutation of Asp217 to Glu creates an inward proton pump. FTIR spectroscopy clearly detects the protonation signal of Glu217, but little for Asp217 in WT ASR. The strong proton affinity of the acceptor in the cytoplasmic side appears to force proton uptake from the extracellular side after the Schiff base deprotonation, even though the carboxylate is 15 Å distant from the retinal Schiff base.

I have the following research plan in near future. The 100 % photochromic reaction in ASR is unique and unusual among archaeal-type rhodopsins. I like to reveal the origin of the photochromism in ASR. I have already published the FTIR spectra of the K states of the all-trans and 13-cis forms, and the L state of the all-trans form. I also measured the spectra of the M state. Proton conduction into the cytoplasmic domain could be correlated to the photochromism, but it is not conclusive. The key issue is that thermal isomerization takes place at the C15=N bond. Since the states cannot be well separated for late intermediates, the analysis will not be easy. I hope that some mutants help understanding of the unique photochromism, and I like to use other archaeal-type rhodopsins.

Almost nothing has been known about the interaction between ASR and 14kDa-protein, and I like to elucidate the molecular mechanism of protein-protein interaction for signal transduction. One of the difficulties in the study is that the binding affinity appears to be very low. Therefore, I will use a fusion protein of ASR and the 14kDa-protein. ATR-FTIR spectroscopy and other techniques will reveal when and how the light signal is transferred into the soluble protein.

Engineering ASR for different function is of particular interest. I have been able
to design an inward proton pump from ASR. I like to design the BR-like outward proton pump. In addition, an inward chloride ion pump, Na+/K+ ion pump and SRI and SRII-like sensory function could be gained for ASR.

The newly designed inward proton pump may be useful as an application tool in cell biology. The recently found channelrhodopsin, with a light-activated cation channel, allowed numerous applications in neurobiology, because transport of cations can be triggered by light (4). Currently, channelrhodopsin and halorhodopsin, a light-driven inward chloride pump, are used as neuroengineering tools to investigate neural circuit function (5). The newly designed inward proton pump (D217E ASR) could provide another kind of active control of electrochemical potential in cells by light in contrast to channelrhodopsin of a purely passive nature. Another application of this protein may be in the field of acidosis-induced cell death. Intracellular pH is precisely regulated around 7.2 by various transporters, which may be changed by acidification around tumor cells (6.9-7.0). Thus, tumor metabolism and pH-control systems have been targets for novel anticancer therapies (6). Acidification of cells by light using an inward proton pump will be useful in the research field. For these applications, a more efficient proton pump may be required, because the current efficiency of D217E ASR is 20-times lower than that of BR, and an additional mutation study is in progress.

In far future, I like to continue studying photoreactive proteins such as rhodopsins in view of fundamental and application research fields. The fundamental study could be performed by spectroscopic techniques as has been so far done, or I would try some other techniques. In any case, I like to know the reason why they can pump ions or sense environmental lights by use of only about 300 amino acids. It is surely difficult question, but I like to challenge. The application study could be focused on developing analytical tools for physiology. Advantage of rhodopsins is that the reaction can be controlled by light, so that the analysis can be not only qualitative but also quantitative. I like to develop such analytical tool based on rhodopsins, as has been the case for GFP. In the application field, energy problem
has to be taken into account particularly in Japan. Solar cells from plant photosynthesis have been proposed by many researchers, while rhodopsins are the potential source of solar cells, I believe. I would like to keep thinking about such application.
REFERENCES


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