Conformational Changes in the Photocycle of *Anabaena* Sensory Rhodopsin

**ABSENCE OF THE SCHIFF BASE COUNTERION PROTONATION SIGNAL**

Vladislav B. Bergo, Maria Ntefidou, Vishwa D. Trivedi, Jason J. Amsden, Joel M. Kralj, Kenneth J. Rothshchild, and John L. Spudich

From the Center for Membrane Biology, Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas 77030 and the Department of Physics, Molecular Biophysics Laboratory, Boston University, Boston, Massachusetts 02215

*A* *Anabaena* sensory rhodopsin (ASR) is a novel microbial rhodopsin recently discovered in the freshwater cyanobacterium *Anabaena* sp. PCC7120. This protein most likely functions as a photosensory receptor as do the related haloarchaeal sensory rhodopsins. However, unlike the archaeal pigments, which are tightly bound to their cognate membrane-embedded transducers, ASR interacts with a soluble cytoplasmic protein analogous to transducers of animal vertebrate rhodopsins. In this study, infrared spectroscopy was used to examine the molecular mechanism of photoactivation in ASR. Light adaptation of the pigment leads to a phototransformation of an all-trans/15-anti to 13-cis/15-syn retinylidene-containing species very similar in chromophore structural changes to those caused by dark adaptation in bacteriorhodopsin. Following 532 nm laser-pulsed excitation, the protein exhibits pre-

Microbial rhodopsins form a large family of photoactive membrane proteins that mediate ion transport and photosensory reception in Archaea, eubacteria, and lower eukaryotes (1–5). These pigments all have seven transmembrane α-helices and a retinylidene chromophore covalently bound to a lysine residue on the C-terminal helix G via a protonated Schiff base. Photon absorption causes an isomerization of the chromophore and induces subsequent changes within the protein structure, giving rise to a sequence of intermediate states with distinct absorption maxima. The kinetics of the overall photocycle, which culminates in the recovery of the ground state, is related to the protein function. Proton pumps such as bacteriorhodopsin (BR)3 and proteorhodopsin (PR) return to the initial state within tens of milliseconds, whereas the photocycles of archaeal photosensory receptors SRI and SRII are slower by at least an order of magnitude. In the latter case, the longer photocycles reflect their long lived M and O intermediates, which are signaling states for activation of their cognate transducer proteins (6).

Several new members of the microbial rhodopsin family have been discovered recently through genome sequencing studies (3, 7, 8). Of special interest is *Anabaena* sensory rhodopsin (ASR) from the freshwater cyanobacterium *Anabaena* sp. PCC7120. This protein has a slow photocycle, typical of sensory proteins, and does not exhibit light-induced proton pumping, consistent with a signaling rather than transport function (7). In contrast to archaeal sensory rhodopsins, which transmit the light-induced signals through interaction with integral membrane transducer proteins (HtrI and HtrII), a small (14 kDa) soluble protein appears to be a transducer for ASR (7). The gene encoding the 14-kDa putative transducer occurs in a dicistronic operon with the coding sequence of ASR.

In this study, the kinetics and absorbance changes of the ASR photocycle were studied in vitro and in lipid bilayers. The photocycle is characterized by a slow cis-trans isomerization of the retinylidene chromophore with the dark-adapted state containing predominantly all-

The absorbance changes during the ASR photocycle were monitored by infrared (FTIR) spectroscopy. The results show that the primary effect of light is a phototransformation of the chromophore from an all-trans-retinal to a 13-cis-retinal form. This transformation is followed by a slower cis-trans isomerization to a 15-syn-retinal form, which is then protonated by the Schiff base counterion Asp-75, which remains unprotonated throughout the photocycle.

To further investigate the conformational changes of ASR during its photocycle, we have applied Fourier transform infrared (FTIR) difference spectroscopy. Previously, this approach has been successfully applied to a number of microbial rhodopsins including BR (11–14), sensory rhodopsins I and II (15–19), the more recently discovered *Neurosorpa* rhodopsin (NR) (20, 21), and proteorhodopsin (22, 23). An FTIR study of spectral transitions during the early ASR photocycle has been recently reported (24). Here we used millisecond rapid scan time-re-
solved FTIR combined with FT-Raman spectroscopy to examine the late photocycle and the light-dark adaptation process in this pigment. Although structural changes of the retinal chromophore during the all-trans photocycle are very similar to those of other microbial rhodopsins, many of the light-induced protein changes, especially proton transfer reactions, appear to be significantly different.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Mutations at codons encoding cysteine at the protein residue positions 134, 137, and 203 were introduced into asr by the QuikChange site-directed mutagenesis method (Stratagene Inc., La Jolla, CA) using oligonucleotide primers coding for serine at each of the three cysteine-coding positions. The mutagenesis was conducted on the pMS710 plasmid (7) containing full-length asr with a C-terminal hexahistidine tag, and mutations were confirmed by DNA sequencing. Expression and purification of the three mutant proteins were performed as described previously (9) in BL21 E. coli cells. Similar procedures were used to prepare the purified ASR mutant protein D75E.

Proteoliposome Reconstitution—Purified C-terminally His-tagged Anabaena sensory rhodopsin (10) was reconstituted in E. coli polar lipids (Avanti, Alabaster, AL) at a 1:10 protein-to-lipid (w/w) ratio. Lipids initially dissolved in chloroform were dried under argon and resuspended in the dialysis buffer (50 mM potassium phosphate, 300 mM NaCl, pH 7.0) to which octyl glucoside was added to the final concentration of 1%. The lipid solution was incubated with the octyl glucoside-solubilized protein for 1 h on ice and dialyzed against the dialysis buffer with three buffer changes every 24 h. The reconstituted protein was centrifuged for 15 min and resuspended in the sample buffer (50 mM Tris–HCl, 150 mM NaCl, pH 8.0).

Rapid Scan Time-resolved FTIR Difference Spectroscopy—Protein films were prepared by depositing 5–10 µl of the proteoliposome suspension onto a polished 2-mm thick, 25-mm diameter CaF2 window (Wilmad, Buena, NJ) and drying the sample under a gentle stream of argon. Films were rehydrated via the vapor phase and then sealed in a temperature-controlled IR cell (Model TFC, Harrick Scientific Corp., Ossining, NY) using a second CaF2 window. Spectra were recorded with a BRUKER IFS 66 v/s FTIR spectrometer (Bruker Optics, Germany) at 5 °C as described previously (21) at 4 cm⁻¹ spectral resolution and 240-kHz scanner velocity corresponding to the data acquisition window of 18 ms. Each time-resolved spectrum represents an average of four individual time slices collected in the 25–85 ms time range after initiation of the photocycle. The difference spectra were produced by subtracting the reference (dark) spectrum recorded 100 ms before the laser photoexcitation from the spectra recorded after excitation. The negative bands in the difference spectra arise from the vibrations of molecular groups in the protein dark state, and the positive bands arise from the photointermediates.

FT-Raman Spectroscopy—Protein samples were prepared similar to those used for FTIR measurements. Spectra were collected on a Bruker RFS 100/S instrument using 180° scattering geometry. The excitation wavelength of the Nd:YAG laser was 1064 nm, and the excitation energy was 100 milliwatts. For each spectrum, 1000 scans were averaged at 4 cm⁻¹ spectral resolution.

RESULTS

Structural Changes of the ASR Retinylidene Chromophore during Dark to Light Adaptation Are Reversed from Those of Bacteriorhodopsin—Previous data from x-ray crystallography, UV-visible spectroscopy, and retinal extraction studies have demonstrated that unphotolyzed ASR can exist in a combination of two distinct states depending on preillumination conditions (9, 10). In the dark, the ASR chromophore exists predominantly in an all-trans configuration, whereas illumination with orange light (580 nm) leads to a predominantly 13-cis form. We recorded the FTIR difference spectra of ASR under conditions that favored the transition from the dark- to light-adapted state (Fig 1, top trace) and compared it to that of the light to dark transition in BR (bottom trace) (25, 26). The two spectra are remarkably similar. In particular, bands in the retinal fingerprint region found at 1168, 1200, 1215, and 1253 (cm⁻¹) in light-adapted BR, which contain contributions from various C–C stretching modes of the all-trans/15-anti chromophore (27) are also present in dark-adapted ASR at 1167, 1196, 1212, and 1245 cm⁻¹. Positive bands found at 1182 and 1230 cm⁻¹ in the dark-adapted BR are assigned to the 13-cis, 15-syn chromophore (28) and also appear in the light-adapted ASR spectrum at 1179 and 1232 cm⁻¹. A strong peak near 1342 cm⁻¹ in BR, which is indicative of the 15-syn chromophore configuration (26), is found at 1338 cm⁻¹ in ASR.

In the ethylenic stretching region peaks are detected at 1543/1532 cm⁻¹ (+/−) in ASR and at 1536/1524 cm⁻¹ (+/−) in BR. The upshift in frequency in both proteins reflects a blue-shift in the chromophore wavelength of maximum absorption. In particular, an empirical inverse correlation is observed for almost all microbial rhodopsins between the retinylidene chromophore C=C stretching frequency (1500–1600 cm⁻¹) and its wavelength of maximum visible absorbance (λmax) (29).

These results demonstrate that the dark-adapted form of ASR, which contains predominantly an all-trans/15-anti-retinal species, is converted by light adaptation to a blue-shifted form with an increased amount of a 13-cis/15-syn species. Strikingly, this is opposite to BR in which light adaptation produces a red-shifted pigment with all-trans/15-anti-retinal species. This conclusion strongly supports earlier studies based on visible UV absorption and retinal extraction (9, 10). In addition, it provides information about the state of isomerization around the C=N Schiff base bond.

In contrast to the intense chromophore peaks, few bands appear in the ASR and BR difference spectra that can be ascribed to the protein
vibrations. In fact, the only strong band in the amide I region (1600–1700 cm$^{-1}$), which is characteristic of protein backbone changes, appears at 1642 cm$^{-1}$. However, a band at this frequency is assigned to the protonated Schiff base C=O stretching vibration on the basis of FT-Raman and isotope-labeling experiments (see below), in agreement with its position in ASR spectra obtained at low temperature (24). Therefore these data indicated that the structural transitions during light adaptation involve primarily the retinylidene chromophore and do not produce large conformational changes in the protein structure between the two states.

The Photocycle of the All-trans-retinal Form of ASR Is Detected by Time-resolved FTIR Spectroscopy—The photochemical reaction cycle of ASR was measured by time-resolved FTIR spectroscopy in the millisecond time range using pulsed photoexcitation provided by a 532 nm Nd:YAG laser. A major negative band, which arises from the depleted initial state(s) of ASR, is evident at 1537 cm$^{-1}$ (Fig. 2, top). This band is assigned to the all-trans/15-anti-retinal species based on several factors. (i) A band at a similar frequency (1534 cm$^{-1}$) is observed in the FT-Raman spectrum of ASR measured in the dark (Fig. 2, inset), which contains predominantly the all-trans/15-anti species. (ii) The 1537 cm$^{-1}$ peak falls midway between the 1532 cm$^{-1}$ peak assigned in the dark–light difference spectrum to all-trans/15-anti form and 1543 cm$^{-1}$ peak assigned to the 13-cis/15-syn form. However, if spectral overlap is taken into account the peaks at 1532 and 1537 cm$^{-1}$ would be much closer. (iii) Because of the short duration of the laser pulse (~6 ns), no contributions from secondary photoexcitations of ASR photointermediates are expected in the spectra.

The predominance of the all-trans/15-anti form of retinal in the photocycle of ASR recorded by time-resolved FTIR is also indicated by bands appearing in the C–C stretch fingerprint region (1150–1400 cm$^{-1}$). Negative bands at 1164, 1196, 1214, and 1245 cm$^{-1}$ (Fig. 3, top) are all indicative of the all-trans-retinal configuration. For example, similar bands also appear in the all-trans photocycles of NR (1166, 1200, 1211, and 1244 cm$^{-1}$) (21) and SRII (1163, 1200, and 1242 cm$^{-1}$) (19).

These frequencies also closely match peaks observed in the FT-Raman spectrum of ASR (Fig. 3, bottom), which primarily reflects retinal vibrations because of the preresonance enhancement phenomena in analogy to BR (30). However, on the basis of bands appearing in the ethylenic and fingerprint regions we cannot eliminate smaller contributions from the 13-cis/15-syn photocycle. For example, a negative shoulder at 1548 cm$^{-1}$ and a small negative band at 1184 cm$^{-1}$ present in both FTIR and FT-Raman spectra may be because of photoisomerization of the 13-cis/15-syn form (28).

The All-trans Photocycle of ASR Contains M and O Photointermediates—As seen in Fig. 2, along with the negative 1537 cm$^{-1}$ band, positive bands appear near 1567 and 1523 cm$^{-1}$. The 1567 cm$^{-1}$ peak is characteristic of the M-intermediate of BR ($\lambda_{\text{max}}$ = 412 nm) as well as most other microbial rhodopsins. The weak intensity of this band is typical of a deprotonated Schiff base retinylidene chromophore (31). The second band at 1523 cm$^{-1}$ reflects the late-photocycle red-shifted O intermediate ($\lambda_{\text{max}}$ near 600 nm), which is also observed in many microbial rhodopsins including BR, SRII, and NR. In the case of ASR, the assignment of the 1537 (−), 1567, and 1523 (+) cm$^{-1}$ bands to the chromophore ethylenic modes is confirmed by their 5–10 cm$^{-1}$ down-shift when the normal retinal is replaced by a C15D isotope-labeled retinal (Fig. 2, bottom) as previously observed in BR (27). No significant changes in the relative intensity of these bands were observed between the early and late time points (data not shown).

The positive 1176 cm$^{-1}$ peak found in this region most likely reflects the all-trans chromophore of an O-like intermediate similar to BR (32, 33). Such a band is also found in the late photocycle spectra of NR and SRII at 1176 and 1179 cm$^{-1}$, respectively (21, 34). However, unlike these pigments, no strong positive band is detected near 1187 cm$^{-1}$, that would be characteristic of the N-like photocointermediate with a 13-cis chromophore (28, 35). One possible explanation is that the positive intensity in this region is canceled by an overlapping negative band near 1184 cm$^{-1}$.

Assignment of the Schiff Base C=O Stretch Vibration Indicates Hydrogen Bonding Similar to BR—Using a retinal isotope label along with FTIR difference and FT-Raman spectroscopy we were able to conclusively assign the C=O stretch mode of the protonated Schiff base in ASR. The FT-Raman spectrum exhibits a major peak at 1655 cm$^{-1}$ along with a smaller shoulder at 1642 cm$^{-1}$ (Fig. 2, inset). Substituting
deuterium for hydrogen at the C-15 position of the retinal affects only the 1642 cm\(^{-1}\) component by causing its disappearance. The remaining 1655 cm\(^{-1}\) band most likely reflects the strong Amide I vibration of the protein, which is found at the same frequency in the absolute absorption FTIR spectrum (data not shown). A downshift of the negative band at 1642 cm\(^{-1}\) to near 1632 cm\(^{-1}\) is also observed in the FTIR difference spectrum of C15D retinal-containing ASR (Fig. 2) confirming its assignment to the Schiff base vibration. The 1642 cm\(^{-1}\) peak also disappears in the spectrum of ASR hydrated with D\(_2\)O; however analysis in this case is complicated by the shift of other bands most likely arising from the protein Amide I backbone difference vibrations, which are affected by the H/D substitution (data not shown). We therefore assign the 1642 cm\(^{-1}\) band to the Schiff base C=NH\(^{+}\) stretching mode in the ASR dark state in agreement with an earlier study (24). The similar frequency of the ASR Schiff base mode to that of BR (26) along with the similar downshift of this mode upon NH to ND substitution in both room temperature (data not shown) and low temperature studies (36) indicates that the hydrogen bond strength of the Schiff base is similar in BR and ASR.

The Schiff Base Counterion Asp-75 Is Unprotonated during the Late Photocycle—The C=O stretching vibration of protonated carboxylic acid side chains in proteins is found in the 1700–1780 cm\(^{-1}\) region (37, 38). For example, in the FTIR difference spectrum of BR, a strong positive band appears near 1760 cm\(^{-1}\) in the M-intermediate (11) reflecting a transfer of the proton from the Schiff base to its counterion Asp-85 (39). This band downshifts slightly (∼5 cm\(^{-1}\)) in the N and O intermediates reflecting environmental changes of Asp-85, which deprotonates during the O decay (14, 33). Similar bands near 1760 cm\(^{-1}\) are also observed in the M, N, and O intermediates of other microbial rhodopsins including SRII, NR, and PR (18, 21, 22) and attributed in each case to protonation of the counterion homologous to ASR Asp-75. In contrast, the light-driven chloride pump halorhodopsin does not exhibit such a band because the homologous counterion is replaced by the chloride anion (40), and the band is absent also in SRI in which the homologous aspartyl residue is protonated in the dark state and does not function as a Schiff base counterion (41).

Sequence alignment and x-ray crystallographic structure analysis (10) indicates that Asp-75 in ASR is in a position to act as the Schiff base counterion and Schiff base proton acceptor. In support of its role as a counterion, like other microbial rhodopsins the visible absorption of this pigment undergoes a red shift at lower pH as seen in Fig. 4. The pH\(_{\mathrm{pK}_{a}}\) of this transition appears to be near pH 8. Similar to other microbial rhodopsins this transition is likely to arise from the neutralization of the unprotonated Asp-75. The replacement of Asp-75 with Glu-75 also produces a ∼14 nm blue shift (Fig. 4, inset), which resembles the effect of the same substitution in BR and SRII (42) and suggests similar electrostatic interactions between this group and the chromophore.

Despite the above evidence, there is a conspicuous absence of an Asp-75 protonation signal in the FTIR difference spectrum. Specifically, no positive bands appear near 1760 cm\(^{-1}\), although peaks are present at a lower frequency near 1742 cm\(^{-1}\) (∼) and 1715 cm\(^{-1}\) (+) (Fig. 5, dotted trace). This absence of bands arising from Asp-75 protonation occurs even under the measurement conditions where an ASR all-trans-retinal photocycle predominates and the M and O photocycle intermediates are present.

To confirm the absence of signal from protonation of Asp-75, spectra of the D75E mutant were measured (Fig. 5, solid trace). Because of the lower C=O stretching frequency of the glutamic acid side chain (43), this substitution is expected to downshift any positive band(s) arising from the protonated carboxyl group at position 75. However, no significant alterations are observed in the 1700–1800 cm\(^{-1}\) region. On the other hand, spectral changes are found near 1600 and 1400 cm\(^{-1}\) where anti-symmetric and symmetric stretching vibrations, respectively, of COO\(^{-}\) groups appear (43). In particular, bands at 1399 (−) and 1422 (+) cm\(^{-1}\) in the wild-type spectrum are shifted in the mutant spectrum (Fig. 5, right inset). Thus, these vibrations most likely arise from the unprotonated Asp-75 in the wild-type pigment, which undergoes an environmental change between the initial and light-activated states.

The Asp-75 → Glu replacement also alters the protein environment...
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near the Schiff base causing an alteration of the assigned 1642 cm\(^{-1}\) C=N stretching peak, which is expected because of the close proximity of these groups.

Evidence for Lipid Conformational Changes Involving Ester Carbonyl Groups during the Late Photocycle of ASR—Vibrations observed in the C=O stretching region may also arise from perturbations of lipid head groups as was previously discovered for bovine rhodopsin (44, 45). In support of this explanation, the 1742 cm\(^{-1}\) (–) band is absent in the difference spectra of ASR reconstituted in halobacterial polar lipids, which lack ester carbonyl groups (Fig. 5, left inset). This peak is therefore assigned tentatively to the lipid molecule(s) whose head groups are disturbed in the late photocycle of ASR. A more definitive assignment would require the use of isotope-labeled lipids. The remaining peak near 1715 cm\(^{-1}\) appears to contain several subcomponents, which are not affected by lipid substitution. It could therefore reflect endogenous E. coli lipids, which are tightly associated with the protein, or alternatively arise from Asn/Gln or Asp/Glu residues.

The SH Groups from Cys-137 and Cys-203 Undergo Hydrogen Bonding Changes during the Photocycle—Difference bands are detected in the 2500–2600 cm\(^{-1}\) region, which arise from S-H stretching vibrations of cysteine sulphydryl groups (46, 47). Small reproducible peaks appear at 2544 (–), 2554 (+) and 2564 (–) cm\(^{-1}\) (Fig. 6, left panel, top trace) in the spectra of wild-type ASR recorded in H\(_2\)O but not in D\(_2\)O (data not shown), in agreement with an expected ~700 cm\(^{-1}\) downshift of the S-D stretching mode. These bands were assigned to specific cysteine residues using site-directed mutagenesis. The C134S mutant difference spectrum (Fig. 6, left, second trace from top) is very similar to that of wild type indicating that Cys-134 does not change significantly between the initial and light-activated states. In contrast, the C137S mutant spectrum (third trace) shows a disappearance of the 2564 cm\(^{-1}\) (–) band and a slight decrease in intensity of the 2554 cm\(^{-1}\) (+) band. The C203S mutation (bottom trace) results in disappearance of the 2544 (–) band and a weaker 2554 (+) cm\(^{-1}\) peak. We therefore assign the 2554/2564 (+/–) cm\(^{-1}\) pair of bands to Cys-137 and 2544/2554 (+/–) cm\(^{-1}\) pair of bands to Cys-203. We also note that the C203S mutation significantly perturbs secondary structural changes during the photocycle as indicated by the disappearance of several bands in the amide I region near 1650 cm\(^{-1}\) (Fig. 6, right panel).

All three cysteine residues in ASR are located in the transmembrane region with Cys-134 and Cys-137 in helix E facing the lipids and Cys-203 in helix G pointed toward helix A (10) (Fig. 7). None of these residues appear to interact directly with the retinol inside the chromophore pocket. Thus, the observed perturbation of the two cysteine groups indicates that helices E and G undergo at least local changes in the vicinity of these cysteine groups during the light activation in ASR.

**DISCUSSION**

This paper reports the first analysis using time-resolved FTIR difference spectroscopy of the late photochemical reaction cycle of Anabaena sensory rhodopsin, a novel microbial rhodopsin from the freshwater cyanobacterium Anabaena sp. 7120. An earlier low temperature (77 K) FTIR difference study of ASR focused on the all-trans form of the protein and its transition to the K-intermediate (24). That study revealed the similarity of the ASR and BR chromophore isomerization during the primary step of their respective photocycles. In addition, it showed that the hydrogen bonding of a water molecule, which bridges the Schiff base and the Asp-75 counterion in ASR, differs significantly from BR.

The present study focused on the structural changes occurring in the late photocycle of ASR (e.g. the M intermediate and later). The fact that 532 nm pulsed excitation of ASR drives mainly the photocycle of the all-trans/15-anti species enabled direct comparison between the photocycle of ASR and other previously studied microbial rhodopsins including BR, SRII, NR, and PR. A recent UV-visible kinetic spectroscopy study of the late ASR photocycle using 532 nm pulsed excitation showed accumulation of the M intermediate, which decays with a time constant at 18 °C of ~100 ms, and also significant contributions from the O intermediate with a slower decay time (7). Our detection of the ethylenic vibrations of the M and O intermediates by time-resolved FTIR confirms this conclusion. However, direct correspondence of the decay times is not expected because of the significantly different measurement conditions including temperature, amount of water in the samples and the buffer composition.

Several major conclusions can be drawn about the ASR photocycle...
from the FTIR time-resolved measurements. (i) The retinal isomerization between the light- and dark-adapted forms of ASR is very similar to BR but occurs in the opposite direction. In particular, on the basis of comparison to the light-dark BR difference spectrum and previous band assignments, the all-trans/15-anti retinylidene configuration predominates in the dark whereas the 13-cis/15-syn configuration forms under light-adapting conditions in agreement with earlier retinal extraction studies (9). However, our study extends these earlier findings by providing evidence of the configuration at the C–N bond. This interconversion between two different forms of ASR may have functional significance, allowing ASR to sense color (10). (ii) The overall retinal structure of ASR in the all-trans dark adapted conformation (also photocycling conformation analyzed) is similar to that of other all-trans microbial rhodopsin chromophores as evident from the similar frequency of the Schiff base C–N stretching mode and C–C fingerprint region. These results agree with the crystallographic analysis of the ASR dark state (10). (iii) Despite the similar structure of the ASR chromophore with BR and other microbial rhodopsins, a band in the 1700–1800 cm⁻¹ region corresponding to the protonation of the Asp-75 counterion was not observed. In fact, unlike other microbial rhodopsins, no bands were assigned to protonation changes of carboxylic acid groups. This later observation can be explained partially by the replacement of two residues in BR, which undergo ionization and hydrogen bonding changes (Asp-96 and Asp-115, respectively), with non-ionizable residues in ASR. However, the evidence that the Schiff base counterion Asp-75 is unprotonated in the late photocycle including the M intermediate suggests that ASR has a fundamentally different proton transfer mechanism underlying its function.

One possibility is that another residue functions as the proton acceptor for the Schiff base. From the ASR crystal structure, there is no obvious candidate group other than Asp-75 to serve as the proton acceptor. The structure therefore favors the alternative that the proton is released from the Schiff base into an extensive network of hydrogen-bonded groups. In fact, several residues and internal water molecules form such a hydrogen-bonded chain in the protein dark state, which extends from the Schiff base-forming residue Lys-210 to the cytoplasmic surface (10) (Fig. 7). This mechanism would imply that the proton transfer occurs in the cytoplasmic direction, opposite to all other microbial rhodopsins thus far studied. In support of this possibility, inwardly directed proton transfer has been suggested from measurements of light-induced charge movements in intact E. coli cells containing ASR (48). Such a change in the direction of proton transfer is intriguing because the signaling mechanism of ASR involves interaction with a soluble cytoplasmic protein.

It is also worth noting that a basic change in the conventional transfer of a proton from the Schiff base to the counterion might be caused by a difference in the charge distribution in the active site, sometimes referred to as the complex counterion. In particular, Asp-212, which is located near the protonated Schiff base in the BR active site, is replaced by a neutral Pro-206 residue in ASR. This alteration in charge could lead to a fundamentally different proton release mechanism from the Schiff base.

The possibility that Asp-75 undergoes a transient protonation, which is not detected during the time scale of our measurements (milliseconds), should also be considered. However, the formation of the M intermediate of ASR requires several milliseconds (9). Thus, protonation of Asp-75 during the M formation appears unlikely. We also do not detect peaks corresponding to the Asp-75 protonation in the low temperature difference spectra of ASR where the M intermediate is stable.

(iv) The time-resolved FTIR measurements provide information about protein and lipid conformational changes in ASR. (a) Conformational changes occur near the center of transmembrane helices E and G as indicated by perturbations of cysteine groups located in these regions. These spectral changes may reflect a local distortion in the polypeptide chain, for example in response to the chromophore isomerization, or a more global event, such as a rigid body displacement of the whole helix from its initial position. It remains to be determined whether the outward tilting motion of helix F, which was previously detected in archaeal rhodopsins (49, 50), also occurs in ASR. We note that the proline residue in helix F, which is believed to be involved in the helix tilt in BR (49) is conserved in ASR, as well as in most other microbial rhodopsins, arguing that the helix F tilt is a general feature of the family. (b) Spectral changes were detected that arise from perturbations of the lipid polar head groups suggesting that conformational changes also occur near the surface of the membrane. Strong lipid perturbations are not always observed in other microbial rhodopsin spectra. For example, no difference bands because of lipid carboxyl groups have been thus far identified in FTIR spectra of the eubacterial proton pump PR (22) nor the fungal NR (20, 21). The different behavior of ASR may be because of the unusual lipid-protein interaction first observed by the x-ray crystallographic analysis (10). On the other hand, it may also reflect considerably different structural changes that potentially occur in this protein. Interestingly, very similar lipid changes were found in the spectra of bovine rhodopsin during formation of the signaling Meta-II state (44, 45).

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Addendum—During the review process, an independent analysis of the late photocycle of ASR was reported (Shi, L., Yoon, S. R., Bezerra, A. G., Jr., Jung, K. H., and Brown, L. S. (2006) J. Mol. Biol., in press) using UV-visible and FTIR spectroscopy. In agreement with our conclusions, no light-induced protonation changes of the counterion were detected. In addition a band was assigned to the protonation of the cytoplasmic group Asp-217. The amplitude of the band assigned to Asp-217 is significantly smaller in our spectra and does not shift with H/D exchange making it unlikely that it reflects a protonation change under our conditions. This may be explained by the different temperatures used in the two studies resulting in accumulation of different intermediates (e.g. “early” versus “late” M intermediate) or possibly the C-terminally shortened form of the protein (L. Brown, personal communication) used in the Shi et al. study.

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