FTIR Spectroscopy of the M Photointermediate in *pharaonis* Phoborhodopsin

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ABSTRACT *pharaonis* phoborhodopsin (*p*pR; also called *pharaonis* sensory rhodopsin II, *p*sR-II) is a photoreceptor for negative phototaxis in *Natronobacterium pharaonis*. During the photocycle of *p*pR, the Schiff base of the retinal chromophore is deprotonated upon formation of the M intermediate (ppR_M). The present FTIR spectroscopy of ppR_M revealed that the Schiff base proton is transferred to Asp-75, which corresponds to Asp-85 in a light-driven proton-pump bacteriorhodopsin (BR). In addition, the C=O stretching vibrations of Asn-105 were assigned for *p*pR and ppR_M. The common hydrogen-bonding alterations in Asn-105 of *p*pR and Asp-115 of BR were found in the process from photoisomerization (K intermediate) to the primary proton transfer (M intermediate). These results implicate similar protein structural changes between *p*pR and BR. However, BR_M decays to BR_N accompanying a proton transfer from Asp-96 to the Schiff base and largely changed protein structure. In the D96N mutant protein of BR that lacks a proton donor to the Schiff base, the N-like protein structure was observed with the deprotonated Schiff base (called M_N) at alkaline pH. In *p*pR, such an N-like (M_N-like) structure was not observed at alkaline pH, suggesting that the protein structure of the M state activates its transducer protein.

INTRODUCTION

pharaonis phoborhodopsin (*p*pR) from *Natronobacterium pharaonis* is a member of the archaeal rhodopsins (Kamo et al., 2001; Sasaki and Spudich, 2000). *p*pR activates a cognate transducer protein upon light absorption, leading to negative phototaxis. It possesses a retinal chromophore that is embedded within seven-transmembrane helices, like the well-studied proton-pump protein bacteriorhodopsin (BR) (Kamo et al., 2001; Sasaki and Spudich, 2000; Spudich and Lanyi, 1996). In *p*pR or BR, the retinal forms a Schiff base linkage with Lys-205 or Lys-216, respectively, and the protonated Schiff base is stabilized by a negatively charged counterion, Asp-75 or Asp-85, respectively. Light absorption of *p*pR triggers *trans-cis* photoisomerization of the retinal chromophore in its electronically excited state (Kandori et al., 2002b), followed by rapid formation of the ground-state species such as the K intermediate (Lutz et al., 2001). This process is also the case in BR. Relaxation of the primary intermediates eventually leads to functional processes during their photocycles (Kamo et al., 2001; Sasaki and Spudich, 2000; Spudich and Lanyi, 1996).

Comparative investigation of *p*pR and BR is a powerful method to understand their molecular mechanisms. We started such comparative studies by means of low-temperature FTIR spectroscopy. The results on the primary K intermediate revealed the structural similarity between *p*pR and BR on the polyene chain of the chromophore (Kandori et al., 2001b), and hydrogen bonds of internal water molecules (Kandori et al., 2001a). These observations were consistent with the similar crystallographic structures of *p*pR (Luecke et al., 2001; Royant et al., 2001) and BR (Belrhali et al., 1999; Luecke et al., 1999). In contrast, the structure of the K state after photoisomerization was more extended in *p*pR than in BR (Kandori et al., 2001b), which was probably correlated with the high thermal stability of ppR_K (Hirayama et al., 1992). In fact, ppR_K was stable even at 170 K, where the L intermediate was formed in BR (Kandori et al., 2001b).

Accompanying the relaxation of ppR_K and BR_L, the M intermediates appear by deprotonation of the Schiff base. The M intermediates of *p*pR and BR are functionally important in transducer activation and proton pumping, respectively (Kamo et al., 2001; Sasaki and Spudich, 2000; Spudich and Lanyi, 1996). ppR_M is formed in tens of microseconds, like BR_M, and decays in 1–2 s (Imamoto et al., 1992), which is two orders of magnitude longer than the lifetime of BR_M. The long-lived M-state in *p*pR must be advantageous in interaction with the transducer. The longer lifetime of ppR_M than BR_M predominantly originates from the lack of the proton-donating groups (Asp-96–Thr-46 in BR) to the Schiff base, because the M intermediate of the F86D/L40T (Phe-86 and Leu-40 in *p*pR correspond to Asp-96 and Thr-46 in BR, respectively) mutant of *p*pR decays as fast as BR_M (Iwamoto et al., 1999). By means of spin-labeling, Wegener et al. observed the opening of the F-helix at the cytoplasmic side during M formation (Wegener et al., 2000, 2001), as was the case for BR. These facts suggested similar structural changes at the M states in *p*pR and BR.

Despite functional importance, molecular understanding of ppR_M has been much less than that of BR_M. Engelhard et al. reported the difference infrared spectra between ppR_M

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and *p*pR, whereas vibrational bands have not been assigned (Engelhard et al., 1996). A positive band appeared in the carboxylic C═O stretching region upon M formation (Engelhard et al., 1996), suggesting the proton transfer from the Schiff base to a carboxylate. Because the structure of *p*pR is similar to that of BR (Belrhali et al., 1999; Luecke et al., 1999, 2001; Royant et al., 2001), the proton acceptor is likely to be Asp-75, a corresponding amino acid of Asp-85 in BR. Indeed, the D75N mutant experiment strongly suggests that Asp-75 is the proton acceptor (Schmies et al., 1998). Nevertheless, it has not been assigned to date. Protein structural changes are much less known for $p\mathrm{pR_M}$, while extensive studies have been reported for $\mathrm{BR_M}$ (Lanyi et al., 2000).

One of the notable aspects in *p*pR is that the N state has not been well identified in its photocycle. It is reasonable because the corresponding amino acid residue of Asp-96 in BR is Phe-86 in *p*pR (Iwamoto et al., 1999). In BR, the M intermediate decays accompanying a proton transfer from Asp-96 to the Schiff base, and the formed N intermediate possesses the protonated Schiff base and deprotonated Asp-96 with largely changed protein structure. The N-specific protein structure can be described by the highly dichroic strong amide-I vibrations at 1671 (−), 1663 (+), and 1649 (+) $\mathrm{cm^{-1}}$ in the $\mathrm{BR_N}$ minus BR difference infrared spectrum (Kandori, 1998). The frequency of the C═O stretch of Asp-85 is shifted from 1762 in $\mathrm{BR_M}$ to 1754 $\mathrm{cm^{-1}}$ in $\mathrm{BR_N}$ (Braiman et al., 1991; Hessling et al., 1993; Kandori, 1998; Ormos et al., 1992; Pfefferlé et al., 1991). Great changes in amide-I vibrations presumably correspond to the opening of the F-helix at the cytoplasmic side in $\mathrm{BR_N}$, which was probed by diffraction (Dencher et al., 1989; Kamikubo et al., 1996; Subramaniam et al., 1999; Vonck, 1996) and spin-labeling (Rink et al., 2000; Thorgeirsson et al., 1997) experiments. When Asp-96 is replaced to Asn in BR, the N state is not observed and the M state is highly stabilized at alkaline pH. Previous FTIR spectroscopy of the D96N protein of BR revealed the appearance of the $\mathrm{M_N}$ state after the M state, where the chromophore was M-like (deprotonated) but the protein structure was N-like (largely changed) (Sasaki et al., 1992). This fact suggested that protonation of the chromophore was not a prerequisite for formation of the N-like protein structure in BR. Observation of the $\mathrm{M_N}$ state in D96N of BR then raised a question on *p*pR; does the $\mathrm{M_N}$-like state appear during the photocycle of *p*pR?

In this paper we report the structural changes occurring upon formation of $p\mathrm{pR_M}$ by means of FTIR spectroscopy. By use of mutant proteins, we were able to assign the C═O stretch of Asp-75 and the C═O stretch of Asn-105 in the $p\mathrm{pR_M}$ minus *p*pR spectrum. These data provided similar protein structural changes between *p*pR and BR at the M state. In contrast, the present study revealed that the photocycle of *p*pR lacked the N-like protein structure, which was in clear contrast to that of BR. Lack of the N-like ($\mathrm{M_N}$-like) structure in the photocycle of *p*pR may be substantial to its functional processes. Protein structural changes in the M intermediates are discussed on the basis of the present FTIR data.

MATERIALS AND METHODS

Preparation of the *p*pR sample

The wild-type and mutant proteins of *p*pR were prepared as described previously (Kandori et al., 2001a, b; Shimono et al., 1997). Briefly, the *p*pR proteins possessing histidine tag at the C-terminus were expressed in *Escherichia coli*, solubilized with 1.5% *n*-dodecyl-β-D-maltoside (DM), and purified by Ni-column. The purified *p*pR sample was then reconstituted into L-α-phosphatidylcholine (PC) liposome by dialysis, where the molar ratio of the added PC was 50 times that of *p*pR.

FTIR spectroscopy

FTIR spectroscopy was applied as described previously (Kandori et al., 2001a, b). The *p*pR sample in the PC liposome was washed twice by buffers at pH 7 (2 mM phosphate) or 9 (2 mM borate). Five mM NaCl was added to test the chloride effect. A 90 μl sample of the *p*pR was dried on a $\mathrm{BaF_2}$ window with a diameter of 18 mm. After hydration by either $\mathrm{H_2O}$ or $\mathrm{D_2O}$, the sample was placed in a cell, which was mounted in an Oxford DN-1704 cryostat (Oxon, England) equipped in the Bio-Rad FTS-40 spectrometer (Cambridge, MA).

Illumination with >480 nm light (VY-50, Toshiba, Shizuoka, Japan) provided by a 1 kW halogen-tungsten lamp at 250 K for 90 s converted *p*pR to $p\mathrm{pR_M}$. Because the $p\mathrm{pR_M}$ completely reverted to *p*pR upon illumination with a UV light (UG-5, Melles Griot, Irvine, CA) for 90 s, as evidenced by the same but inverted spectral shape, the cycles of alternative illuminations with >480 nm light and UV light were repeated a number of times. The difference spectrum was calculated from the spectra constructed with 64 interferograms after minus before the illumination. Twenty-four spectra obtained in this way were averaged for the $p\mathrm{pR_M}$ minus *p*pR spectrum.

Because photointermediates decay rapidly at 290 K, a slightly different experimental setup was applied to study the $\mathrm{M_N}$ state in *p*pR, as described previously (Chon et al., 1999). In this case, the sample film was tilted 45° relative to the probe light, and the >480 nm light from a 150 W xenon lamp was focused on the sample at an angle of 90° with respect to the probe light. During illumination infrared spectra were obtained, and the difference spectrum was calculated from the spectra constructed with 128 interferograms during minus before the illumination. Ten spectra obtained in this way were averaged at 250, 270, and 290 K.

RESULTS

Infrared spectral changes of *p*pR upon formation of the M photointermediate

We tested various conditions such as pH, temperature, and illumination wavelength to accumulate $p\mathrm{pR_M}$. It was found that *p*pR and $p\mathrm{pR_M}$ can be photoconverted with each other at 250 K, as shown by the mirror images of the difference IR spectra. Photoreversion of $p\mathrm{pR_M}$ to *p*pR was previously reported by Balashov et al. (2000). The dotted line in Fig. 1 *a* shows the typical $p\mathrm{pR_M}$ minus *p*pR spectrum measured at 250 K for the hydrated film at pH 9. There were no spectral differences between pH 9 (*dotted line*) and 7 (*solid line*),

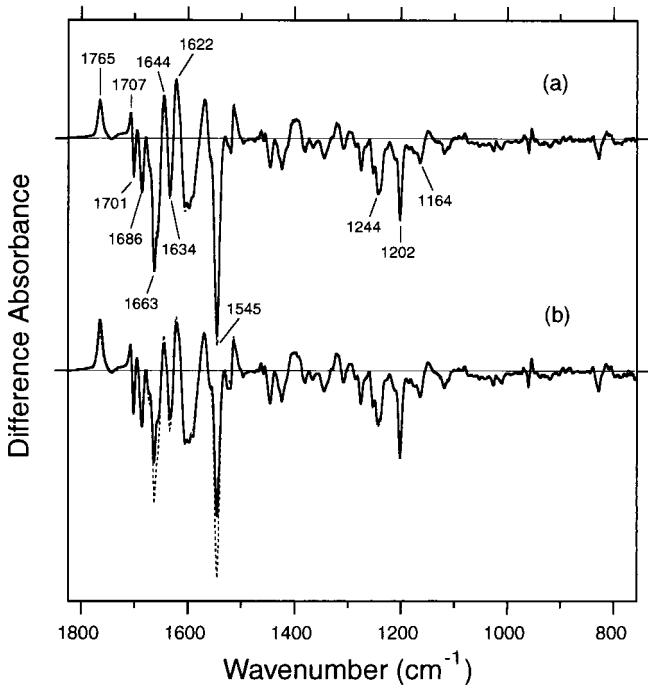

FIGURE 1 (*a*) *p*pR$_M$ minus *p*pR spectra measured at pH 7 (*solid line*) and 9 (*dotted line*) in the 1820–760 cm^{-1} region. The spectra are measured at 250 K for the hydrated film with H_2O. *p*pR is converted to *p*pR$_M$ with >480 nm light, while being reverted with a UV-pass filter. (*b*) *p*pR$_M$ minus *p*pR spectra measured in the absence (*dotted line*) and the presence (*solid line*) of NaCl at 250 K and pH 9. The dotted line is reproduced from the dotted line in *a*. One division of the *y* axis corresponds to 0.01 absorbance unit.

which was consistent with the previous report (Engelhard et al., 1996). In contrast, the O intermediate appeared at higher temperature and neutral pH, whereas only *p*pR$_M$ was observed at pH 9 even at higher temperatures (data not shown). Thus, alkaline pH is favorable to accumulate *p*pR$_M$, as was reported by the flash photolysis (Miyazaki et al., 1992). Such pH dependence is also the case for BR. FTIR studies on *p*pR$_O$ will be reported elsewhere.

Fig. 1 *b* exhibits a spectral comparison of *p*pR$_M$ between the absence (*dotted line*) and presence (*solid line*) of NaCl. Recent structural determination of *p*pR showed the presence of a chloride ion in the extracellular side of the chromophore (Royant et al., 2001). It is known that chloride does not affect the absorption of *p*pR (Shimono et al., 2000), while it is intriguing to determine whether the structure of *p*pR$_M$ is influenced by chloride ions. Fig. 1 *b* shows that both spectra are identical in the 1500–800 cm^{-1} region. Frequencies in the 1800–1500 cm^{-1} were also the same between the absence and presence of chloride ions, though amplitudes of some bands were different. Thus, we concluded that chloride ions do not affect the protein structural changes between *p*pR and *p*pR$_M$.

The *p*pR$_M$ minus *p*pR spectrum (Fig. 1 *a*) exhibits a negative peak at 1545 cm^{-1} in the ethylenic C═C stretching region. The value is identical to that in the native *p*pR (Engelhard et al., 1996) and also in good agreement with the previous resonance Raman spectroscopy of the native *p*pR in DM solution (1548 cm^{-1}) (Gellini et al., 2000). Negative bands at 1244, 1202, and 1164 cm^{-1} are attributable to the C—C stretching vibrations of the retinal chromophore. The 1244 and 1202 cm^{-1} bands were also observed in the *p*pR$_K$ minus *p*pR spectrum, and tentatively assigned as a mixture of C12—C13 stretch and N—H in-plane bending, and C14—C15 stretch in *p*pR, respectively, from the analogy to BR (Kandori et al., 2001b). The 1164-cm^{-1} band was not observed in the *p*pR$_K$ minus *p*pR spectrum (Kandori et al., 2001b), and newly appeared in the *p*pR$_M$ minus *p*pR spectrum. It is likely that the 1164-cm^{-1} band originates from C10—C11 stretch, because the corresponding band of BR appears at 1170 cm^{-1} (Smith et al., 1985). This suggests that there are no structural changes at the C10—C11 moiety between *p*pR$_K$ and *p*pR, and some changes in *p*pR$_M$. It may also be possible that the chromophore structure is not altered at position C10—C11 between *p*pR and *p*pR$_M$, whereas deprotonation in *p*pR$_M$ weakens the absorbance of the IR band so that it appeared as a negative band. Another possibility is that the appearance of a different band at 1164 cm^{-1} cancels out the negative band at the same frequency in the *p*pR$_K$ minus *p*pR spectrum, possibly because of the extended structural changes upon K formation (Kandori et al., 2001b).

Because the band corresponds to the C10—C11 stretching vibration of the retinal chromophore, these results can be interpreted in terms of no structural changes between *p*pR$_K$ and *p*pR, and some changes in *p*pR$_M$. It may also be possible that the chromophore structure is not altered at position C10—C11 between *p*pR and *p*pR$_M$, whereas deprotonation in *p*pR$_M$ weakens the absorbance of the IR band so that it appeared as a negative band.

The appearance of the positive 1765-cm^{-1} band is characteristic of the M intermediate (Engelhard et al., 1996; Maeda, 1995; Rath et al., 1996). In addition, various peaks were observed in the >1600 cm^{-1} region; 1707 (+), 1701 (−), 1686 (−), 1663 (−), 1644 (+), 1634 (−), and 1622 (+) cm^{-1}. These bands originate from protein vibrations. The C═N stretching vibration of the protonated Schiff base at 1650 cm^{-1} is present as the shoulder of the strong negative band at 1663 cm^{-1}. The negative 1663-cm^{-1} band is located at the typical frequency region of amide-I vibration in the αII-helix.

Assignment of the C═O stretching vibrations of Asp-75 in *p*pR$_M$

The appearance of a positive band at 1770–1760 cm^{-1} upon formation of the M intermediate is characteristic among BR, *p*pR, and phoborhodopsin of *Halobacterium salinarum* (pR), and interpreted in terms of protonation of a carboxylate by the proton transfer from the Schiff base. By means

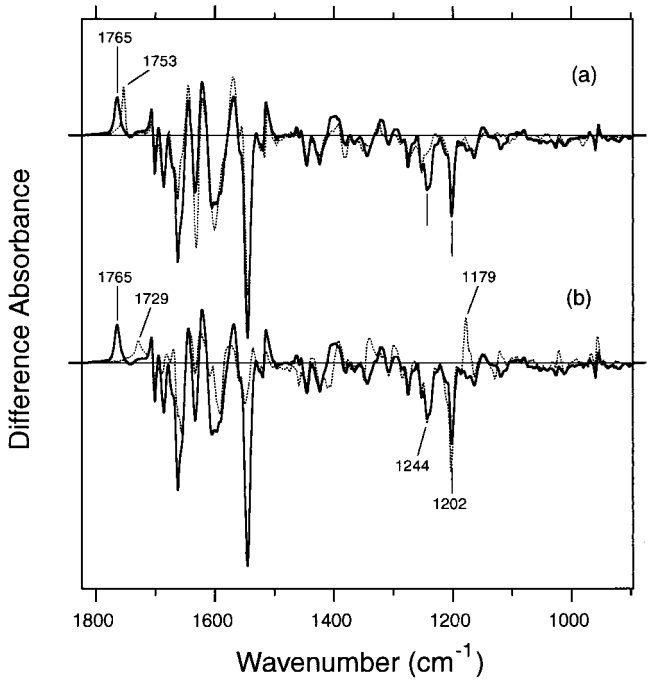

FIGURE 2 (*a*) *p*pR$_M$ minus *p*pR spectra of the wild-type protein in H_2O (*solid line*) and D_2O (*dotted line*) in the 1820–900 cm^{-1} region. The spectra are measured at 250 K and pH 9. (*b*) Difference IR spectra of the wild-type (*solid line*) and the D75E mutant (*dotted line*) proteins in H_2O. The latter spectrum is measured at 220 K and pH 9. One division of the *y* axis corresponds to 0.01 absorbance unit.

of FTIR spectroscopy, the proton acceptors were identified as Asp-85 (Maeda, 1995) and Asp-73 (Bergo et al., 2000) in BR and pR, respectively, whereas the acceptor has not been assigned for *p*pR. Fig. 2 *a* shows the positive bands at 1765 and 1753 cm^{-1} in H_2O and D_2O, respectively, in the carboxylic C═O stretching region.

To assign the positive 1765-cm^{-1} band in the *p*pR$_M$ minus *p*pR spectrum, we replaced Asp-75 to Glu. HPLC analysis revealed that the D75E mutant protein possesses 40% all-*trans* and 60% 13-*cis* forms in the dark, like BR, whereas the wild-type *p*pR possesses only the all-*trans* form (Hirayama et al., 1995; Shimono et al., 2001). We found that, unlike BR, D75E does not show light-adaptation, possibly because of the slow photocycle of the all-*trans* form. Therefore, we searched the suitable illumination conditions to accumulate the M intermediate of the D75E protein. Absorption spectra in the UV and visible region showed that the M intermediate is formed by illumination with >480 nm light at 220 K, being a lower temperature than for the wild type (not shown). Thus, we measured the difference IR spectrum for the D75E protein. The obtained spectrum exhibited negative bands at 1244 and 1202 cm^{-1} and a positive band at 1179 cm^{-1} in the fingerprint region (Fig. 2 *b*). The negative bands imply that the photocycle of the all-*trans* form was predominantly involved in the spectrum, whereas the appearance of the positive band at 1179 cm^{-1} may originate from formation of another product possessing a protonated Schiff base.

In the carboxylic C═O stretching region, a positive band was observed at 1729 cm^{-1} (Fig. 2 *b*). In BR, the C═O stretching vibrations of Asp-85 and Glu-85 appeared at 1760 and 1724 cm^{-1}, respectively (Braiman et al., 1988). These facts strongly suggest that the positive 1765-cm^{-1} band originates from the C═O stretching vibration of Asp-75, which down-shifts to 1729 cm^{-1} in the D75E mutant protein of *p*pR. The C═O stretching vibrations of the aspartic acids at 1760 and 1765 cm^{-1} indicate that Asp-85 in BR and Asp-75 in *p*pR, respectively, exist in the highly hydrophobic environment (Dioumaev and Braiman, 1995). In addition, it is likely that the environment of the position 75 in *p*pR$_M$ is more hydrophobic than that of the corresponding position 85 in BR$_M$, because higher frequency represents a more hydrophobic environment for carboxylates.

Assignment of the C═O stretching vibrations of Asn-105 in *p*pR$_M$

The *p*pR$_M$ minus *p*pR spectrum has a characteristic peak pair at 1707 (+)/1701 (−) cm^{-1} (Fig. 1 *a*). In the *p*pR$_K$ minus *p*pR spectrum, there is a peak pair at 1704 (−)/1700 (+) cm^{-1}, which is not shifted in D_2O (Fig. 3 *a*) (Kandori et al., 2001b). We recently assigned the bands as the C═O stretching vibration of Asn-105 in D-helix (Kandori et al., 2002a). In addition, from the analysis of the amplitude of the C═O stretches, we provided experimental evidence that photoisomerization yields more extended protein structural changes in *p*pR than in BR (Kandori et al., 2002a). Fig. 3 *b* shows the *p*pR$_M$ minus *p*pR spectra measured in H_2O (*solid line*) and D_2O (*dotted line*). The bands at 1707 (+) and 1701 (−) cm^{-1} are not shifted in D_2O, while the positive 1695-cm^{-1} band disappeared in D_2O.

The bands at 1707 (+) and 1701 (−) cm^{-1} completely disappeared in the N105D mutant protein, where the D_2O-sensitive 1695-cm^{-1} band remained (Fig. 3 *c*). Instead, a negative peak newly appeared at 1739 cm^{-1}, which shifts to 1728 cm^{-1} in D_2O. This band is close in frequency to the negative 1744-cm^{-1} band in the *p*pR$_K$ minus *p*pR spectrum (Kandori et al., 2002a). Thus, we concluded that the bands at 1707 (+) and 1701 (−) cm^{-1} originate from the C═O stretch of Asn-105. In the difference spectrum of N105D, it seems that there is a positive peak at 1729 cm^{-1} in addition to the positive one at 1746 cm^{-1}, which may suggest the structural heterogeneity in the M state of the mutant.

In *p*pR, the C═O stretching frequency of Asn-105 is down-shifted in *p*pR$_K$ and up-shifted in *p*pR$_M$, indicating that the hydrogen bond is strengthened upon photoisomerization, and weakened upon primary proton transfer. In BR, it is known that the C═O stretching frequency of Asp-115 is down-shifted in BR$_K$ and BR$_L$, and up-shifted in BR$_M$ (Maeda, 1995; Sasaki et al., 1994). Thus, the structural

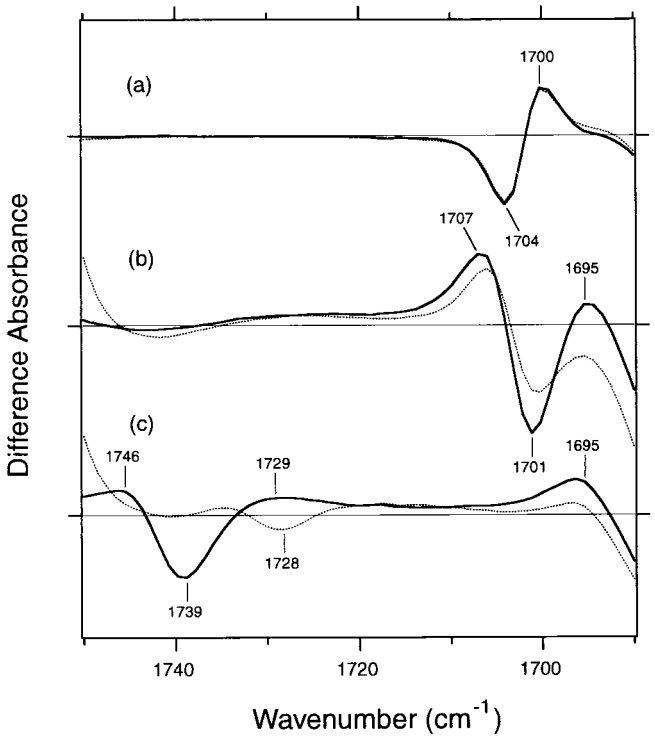

FIGURE 3 (*a*) *p*pR$_K$ minus *p*pR spectra of the wild-type protein in H_2O (*solid line*) and D_2O (*dotted line*). The spectra measured at 77 K are reproduced from Kandori et al., 2001b. (*b*) *p*pR$_M$ minus *p*pR spectra of the wild-type protein in H_2O (*solid line*) and D_2O (*dotted line*) measured at 250 K and pH 9. The solid line is reproduced from the dotted line in Fig. 1 *a*. (*c*) *p*pR$_M$ minus *p*pR spectra of the N105D mutant protein in H_2O (*solid line*) and D_2O (*dotted line*). One division of the *y* axis corresponds to 0.003 absorbance unit.

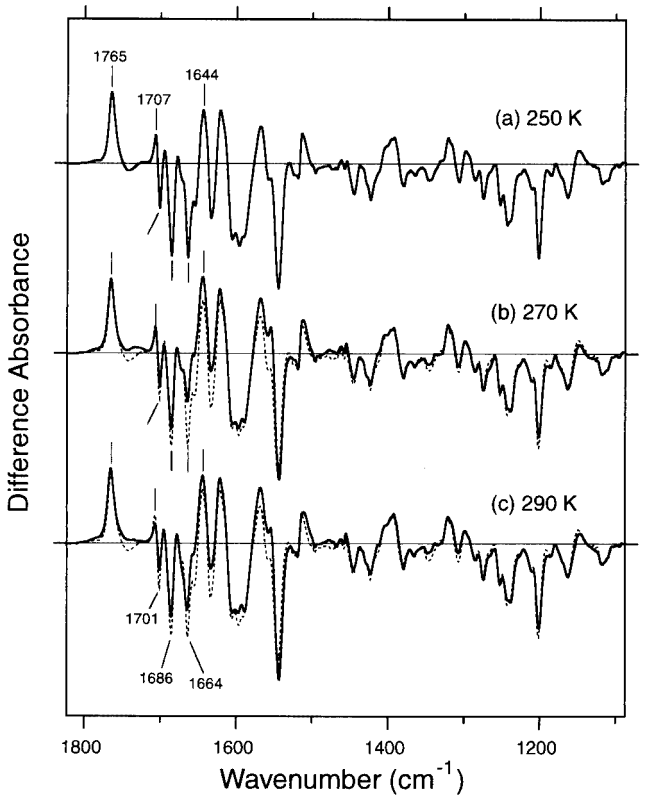

FIGURE 4 *p*pR$_M$ minus *p*pR spectra measured in the 1820–1090 cm^{-1} region at 250 (*a*), 270 (*b*), and 290 (*c*) K. Dotted lines in *b* and *c* reproduce the spectrum of *a* for comparison. The spectra are measured for the hydrated film at pH 9. One division of the *y* axis corresponds to 0.008 absorbance unit.

changes at position 105 in *p*pR are similar to those at the corresponding position 115 in BR through light absorption and M formation.

Lack of the N-like (M_N-like) structure in *p*pR

In BR, the M intermediate decays accompanying a proton transfer from Asp-96 to the Schiff base, and the formed N intermediate possesses 1) a 13-*cis* chromophore; 2) a protonated Schiff base; 3) deprotonated Asp-96; and 4) largely changed protein structure. The N-specific protein structure can be described in the BR$_N$ minus BR difference infrared spectrum by the highly dichroic strong amide-I vibrations at 1671 (−), 1663 (+), and 1649 (+) cm^{-1} (Kandori, 1998), and the frequency shift of the C═O stretch of Asp-85 from 1762 (BR$_M$) to 1754 (BR$_N$) cm^{-1} (Braiman et al., 1991; Hessling et al., 1993; Kandori, 1998; Ormos et al., 1992; Pfefferlé et al., 1991). As described in the Introduction, protonation of the chromophore is not prerequisite for the formation of an N-like structure in BR. When Asp-96, an internal proton donor to the chromophore, is replaced to Asn, the M state is highly stabilized at alkaline pH. Previous FTIR spectroscopy of the D96N protein of BR revealed the appearance of the M_N state after M, where the chromophore is M-like (deprotonated) but the protein structure is N-like (largely changed) (Sasaki et al., 1992).

Observation of the M_N state in BR provides an implication for *p*pR. In *p*pR, the N state has not been well identified in its photocycle. It is reasonable because the corresponding amino acid residue of Asp-96 in BR is Phe-86 in *p*pR. Therefore, it is an intriguing question whether the M_N-like state is present during the photocycle of *p*pR. To answer this question, we measured infrared spectral changes of the alkaline film of *p*pR at various temperatures. BR$_N$ is normally trapped at 273 K (Kandori, 1998). In the present study, we also examined higher temperatures (290 K). Consequently, we had to modify the experimental setup as described in Materials and Methods because photointermediates decayed rapidly at room temperature. Fig. 4 *a* shows the *p*pR$_M$ minus *p*pR spectrum measured at 250 K. It is noted that the amplitudes of some bands were different in Fig. 4 *a* from those in Fig. 1 *a*, though frequencies were identical. Such difference presumably originates from the fact that the *p*pR molecules were partially oriented in the film. In Fig. 4 the film sample was tilted by 45°, so that

vibrations whose dipole moments were parallel to the membrane normal were enhanced in amplitude. In fact, the C═O stretch of Asp-75 and various amide-I vibrations whose dipole moments tend to be parallel to the membrane normal were likely to be enhanced in Fig. 4, while C═C and C—C stretches of the chromophore whose dipole moments are parallel to the membrane were reduced in intensity from Fig. 1.

Fig. 4 shows that spectral shape and amplitude do not change among 250, 270, and 290 K. This is clear contrast to the D96N mutant of BR, where the M state was observed at lower temperature, and the M_N state was observable at higher temperature (Sasaki et al., 1992). Formation of the M_N state accompanied 1) appearance of the prominent amide-I bands at 1669 (−) and 1649 (+) cm^{-1}; and 2) frequency shift of the C═O stretch of Asp-85 from 1762 to 1755 cm^{-1} (Sasaki et al., 1992). In *p*pR, the positive 1765-cm^{-1} band of Asp-75 did not change its frequency at 250–290 K. In addition, there are no significant amplitude changes in the amide-I vibrations (1686 (−), 1664 (−), and 1644 (+) bands), which can be observed in N and M_N (Sasaki et al., 1992; Kandori, 1998). Thus, we concluded that the N-like (M_N-like) structure was not involved in the photocycle of *p*pR at alkaline pH.

DISCUSSION

In this paper we studied the structure of *p*pR_M by means of FTIR spectroscopy. Balashov et al. reported that *p*pR_M was formed at ~220 K in their low-temperature visible spectroscopy (Balashov et al., 2000). However, we observed spectral contamination of *p*pR_K at 220–240 K in the present FTIR measurement (data not shown). The difference between the two experiments probably originates from the sample condition; the *p*pR molecule was solubilized by DM in the previous visible spectroscopy (Balashov et al., 2000), while the protein was reconstituted into the PC liposome in this work. Thus, it is likely that *p*pR_K is more stabilized in membrane than in the DM solution. A similar observation was reported for visual rhodopsin, where metarhodopsin II, having the deprotonated Schiff base like *p*pR_M, was highly stable in the DM solution (Hofmann et al., 1995).

The present study assigned the positive 1765-cm^{-1} band as the C═O stretch of the protonated Asp-75 in *p*pR_M (Fig. 2). Upon formation of *p*pR_M, Asp-75 receives a proton from the Schiff base, while BR_M formation accompanies a proton transfer from the Schiff base to Asp-85. Thus, such mechanism is common between *p*pR and BR. In addition, this study assigned the bands at 1707 (+)/1701 (−) cm^{-1} as the C═O stretches of Asn-105 (Fig. 3). Together with the previous report on Asn-105 in *p*pR_K (Kandori et al., 2002a), it was revealed that hydrogen-bonding alterations at position 105 in *p*pR (115 in BR) are common between *p*pR and BR, first strengthened upon photoisomerization, followed by weakened upon primary proton transfer from the Schiff base to Asp-75 in *p*pR (Asp-85 in BR). These observations provided the experimental evidence for the similar structural changes. However, there is a certain difference between *p*pR and BR. In the absence of transducer, *p*pR can pump protons, whereas the pumping efficiency is much lower in *p*pR than in BR (Schmies et al., 2001; Sudo et al., 2001a). This has to be explained in terms of structural factors.

The present study revealed that the photocycle of *p*pR lacked the N-like structure at alkaline pH. This observation was in clear contrast to that of BR. In the D96N mutant protein of BR, the N-like protein structure is formed with the deprotonated Schiff base (called M_N), even though the protein does not have an internal proton donor to the Schiff base (Sasaki et al., 1992). Thus, lack of the N-like (M_N-like) structure implies different structural changes between *p*pR and BR. They may be substantial to the low efficiency in the proton pumping of *p*pR (Schmies et al., 2001). It is, however, noted that the recent spin-labeling experiment of *p*pR reported the opening of F-helix in *p*pR and in BR (Wegener et al., 2000, 2001). This fact may suggest that the N-specific amide vibrations in BR_N are not directly correlated with the outward motion of F-helix. An alternative explanation is that the mechanism of the F-helix opening is different between *p*pR and BR.

The lack of the N-like (M_N-like) structure in *p*pR also suggests the mechanism of the interaction with its transducer protein. The protein structure characteristic of *p*pR_M is likely to activate its transducer protein. It is, however, noted that the association between *p*pR and its transducer is weakened upon formation of *p*pR_M (Sudo et al., 2001b, 2002). This may suggest that the structure of *p*pR_M is not important in the complex formation with the transducer. Rather, a *p*pR-transducer complex forces the transducer in the non-active state, and light-induced dissociation of the complex may be an essence of the transducer activation. Further experiments will lead to better understanding of the structural changes in the transducer activation.

The present study also provided implications for the chloride binding site. Royant et al. showed the presence of the chloride binding site inside *p*pR according to their x-ray structure (Royant et al., 2001), which was not visible in the x-ray structure of Luecke et al. (2001). The present results clearly showed little chloride effect on the *p*pR_M minus *p*pR spectrum (Fig. 1 *b*), suggesting that there are no structural alterations at the chloride binding site present in the extracellular side. In contrast, chloride ions influence the structure of *p*pR_O according to our FTIR spectroscopy (Furutani et al., manuscript in preparation). Thus, chloride ions are likely to influence the last step in the photocycle of *p*pR, which is our next focus.

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