FTIR Difference Spectroscopy of the Bacteriorhodopsin Mutant Tyr-185→Phe: Detection of a Stable O-like Species and Characterization of Its Photocycle at Low Temperature†

Yiwu He,‡ Mark P. Krebs,§ Wolfgang B. Fischer,‡ H. Gobind Khorana,§ and Kenneth J. Rothschild*,‡

Physics Department and Program in Cellular Biophysics, Boston University, Boston, Massachusetts 02215, and Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received July 20, 1992; Revised Manuscript Received December 18, 1992

ABSTRACT: Fourier transform infrared difference spectroscopy has been used to study the photocycle of the mutant Tyr-185→Phe expressed in native *Halobacterium halobium* and isolated as intact purple membrane fragments. We find several changes in the low-temperature bR→K, bR→L, and bR→M FTIR difference spectra of Y185F relative to wild-type bR which are not directly related to the absorption bands associated with Tyr-185. We show that these features arise from the photoreaction of a stable red-shifted species (O^{Y185F}) with a vibrational spectrum similar to the O intermediate. By using photoselection and FTIR spectroscopy, we have been able to characterize the photoproducts of this O^{Y185F} species. A K-like photoproduct is formed at 80 K which has a 13–cis structure. However, it differs from K_{630}, exhibiting an intense band at 990 cm^{-1} most likely due to a hydrogen-out-of-plane vibrational mode of the chromophore. At 170 and 250 K, photoexcitation of O^{Y185F} produces an intermediate with vibrational features similar to the N intermediate in the wild-type bR photocycle. However, no evidence for an M-like intermediate is found. Although Asp-96 undergoes a change in its environment/protonation state during the O^{Y185F} photocycle, no protonation changes involving Asp-85 and Asp-212 were detected. These results provide strong evidence that light adaptation of Y185F produces two species similar to bR_{570} and the O intermediate. Differences in their respective photocycles can be explained on the basis of differences in the protonation states of the residues Asp-85 and Asp-212 which are ionized in bR_{570} and undergo net protonation upon O^{Y185F} formation.

Bacteriorhodopsin (bR),[1] a MW 26 000 protein in the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump (Stoeckenius & Bogomolni, 1982). It contains 248 amino acid residues and has an *all-trans*-retinylidene chromophore linked through a Schiff base to Lys-216. Photon absorption initiates a photocycle which consists of a series of metastable intermediates characterized by distinct visible absorption spectra. The net result of this photocycle is proton transfer from the inside to the outside of the cell, thereby establishing an electrochemical gradient which drives ATP synthesis.

The structure of the chromophore at different stages of the photocycle has been studied extensively by resonance Raman spectroscopy [see Mathies et al., (1991) for a recent review and references cited therein]. This technique provides a means to selectively detect vibrations of retinal without interference from protein vibrational modes. Isomerization of the chromophore from an all-trans to a 13-cis structure occurs during the bR_{570}[2] →K photoreaction followed by several dark reactions which involve relaxation of the chromophore (K→L), deprotonation of the Schiff base (L→M), reprotonation of the Schiff base (M→N), and reisomerization of the chromophore back to an all-trans structure (N→O). In the last step of the photocycle (O→bR_{570}), bR returns back to its original light-adapted state.

Because of the ability of Fourier transform infrared (FTIR) difference spectroscopy to detect small conformational changes in a protein at the single amino acid residue level [for a recent review, see Rothschild (1992)], it has been used exclusively to study the structural changes occurring during the bacteriorhodopsin photocycle (Rothschild et al., 1981, 1986; Rothschild & Marrero, 1982; Bagley et al., 1982; Siebert & Mäntele, 1983; Engelhard et al., 1985; Roepe et al., 1987b) as well as in the photocycle of halorhodopsin and sensory rhodopsin I (Rothschild et al., 1988; Bousché et al., 1991b). By using isotopic labeling and site-directed mutagenesis, several infrared bands have been assigned to structurally active amino acid residues. For example, bands in the 1700–1760-cm^{-1} region were assigned to protonation and environmental changes of Asp residues 85, 96, 115, and 212 that occur during the bR→K, L, and M transitions (Braiman et al., 1988a, 1992; Gerwert et al., 1989; Rothschild et al., 1990; Maeda et al., 1992). More recently, the same approach has been used with time-resolved FTIR difference spectroscopy to assign bands in the late photocycle (Gerwert et al., 1990; Braiman et al., 1991; Bousché et al., 1991a, 1992; Chen & Braiman, 1991).

In this work, we have used low-temperature FTIR difference spectroscopy to investigate the mutant Tyr-185→Phe (Y185F)[3] expressed in native *H. halobium* and isolated as intact purple

† This research was supported by grants from the NSF (DMB-9106047), NIH (EY05499), and ONR (N00014-88-K-0464) to K.J.R. and from the NIH (GM28289 and AI11479) to H.G.K.

* Address correspondence to this author.

‡ Boston University.

§ Massachusetts Institute of Technology.

[1] Abbreviations: PM, purple membrane; bR, bacteriorhodopsin; ebR, bacteriorhodopsin produced from the expression of a synthetic wild-type gene in *Escherichia coli* using recombinant techniques and reconstituted into halobacterial lipids; FTIR, Fourier transform infrared; au, absorbance unit(s).

[2] bR_{570} denotes the normal light-adapted state of bR which in wild type has a λ_{max} near 570 nm.

[3] Designations for bR mutants make use of the standard one-letter abbreviations for amino acids. Thus, "Y185F" signifies the mutant in which tyrosine at position 185 has been replaced by phenylalanine.

0006-2960/93/0432-2282$04.00/0

membrane fragments. This extends an earlier FTIR study on a complete series of tyrosine mutants (Tyr→Phe), including Y185F, expressed in *Escherichia coli* and reconstituted into native halobacterial lipids (Braiman et al., 1988b). These measurements and studies of bR containing isotopically labeled tyrosine (Rothschild et al., 1986; Dollinger et al., 1986; Roepe et al., 1987b, 1988) led to the assignment of several bands including one at 1277 cm^{-1} to vibrations of Tyr-185. However, the changes induced in the FTIR difference spectrum of the mutant Y185F were not restricted to tyrosine vibrational bands.

In the present investigation, we demonstrate that in light-adapted Y185F most of these spectral changes arise from the existence of a stable species (O^{Y185F}) with a vibrational spectrum similar to the O intermediate along with the normal bR_{570} species. The possibility that this species is related to the all-trans chromophore component of acid-induced blue membrane BM^T is also discussed. By using different wavelengths of excitation to drive the photocycles of the two species, we demonstrate that the O^{Y185F} species undergoes a photocycle which involves an all-trans to 13-cis isomerization. At 80 K, a K-like intermediate is formed, but on the basis of the appearance of a strong HOOP mode at 990 cm^{-1}, it is deduced to have a different conformation than K_{630} of the bR photocycle. At higher temperatures (170 and 250 K), an M species is not detected. Instead, an N-like intermediate appears with a chromophore structure similar to the normal N intermediate of the bR photocycle. The difference between the bR and O^{Y185F} photocycles can be understood on the basis of the difference in protonation state of Asp-85 and Asp-212.

MATERIALS AND METHODS

Expression and Purification of Y185F. The mutant protein was expressed from an *H. halobium* strain bearing a chromosomal *bop* gene containing the Y185F mutation as described in Bousché et al. (1992). Identical spectral properties were obtained from measurements made using a plasmid-based expression system (Krebs et al., 1991). Detailed discussion of this procedure is given in the accompanying paper (Sonar et al., 1993).

FTIR Difference Spectroscopy. Difference spectra were recorded for the bR→K, bR→L, and bR→M photoreactions at 80, 170, and 250 K, respectively, using methods previously reported (Rothschild et al., 1984b; Roepe et al., 1987b; Braiman et al., 1988b). Samples were prepared by air-drying approximately 100–200 $\mu g/cm^2$ of sample on a AgCl window and then rehydrating prior to insertion into a sealed transmittance cell which was mounted in a Helitran cryostat (Air Products, Allentown, PA). The water content of the sample was checked by monitoring the 3400-cm^{-1} peak. All samples were light-adapted at room temperature prior to cooling by illuminating the sample for at least 15 min with a 505-nm long-pass filter. Spectra were recorded at 2-cm^{-1} resolution using either a Nicolet Analytical Instruments (Madison, WI) 740 or 60SX spectrometer. In order to photoselect for the red-shifted species in Y185F at 80 K, a 600-nm narrow-band interference filter was used to drive its photoreactions and a 660-nm narrow-band interference filter to photoreverse them. At 170 and 250 K, photoreactions were driven using a 660-nm narrow-band interference filter. Samples measured in D_2O were prepared by incubating a film for at least 4 h in D_2O and then removing excess D_2O by partial drying.

RESULTS

Detection of an O-like Species (O^{Y185F}). FTIR difference spectra for the bR→K, bR→L, and bR→M transitions of bR and Y185F are shown in Figure 1. The spectra of bR (WT) are almost identical to those previously reported for bR expressed in *E. coli* (ebR) (Braiman et al., 1988b). For example, all of the negative chromophore bands in the bR→M difference spectra assigned to the chromophore vibrations of bR_{570} at 1527, 1216, 1200, 1169, and 959 cm^{-1} and protein vibrations at 1762 (Asp-85), 1742 (Asp-96), 1738 (Asp-212), 1660 (amide I), 1420 (proline), 1277 (Tyr-185) and 741 cm^{-1} (Trp-86) (region not shown) match those reported in native bR (Rothschild, 1992). Positive bands characteristic of the K, L, and M intermediates also appear at the same frequencies as previously reported (Rothschild & Marrero, 1982; Roepe et al., 1987b).

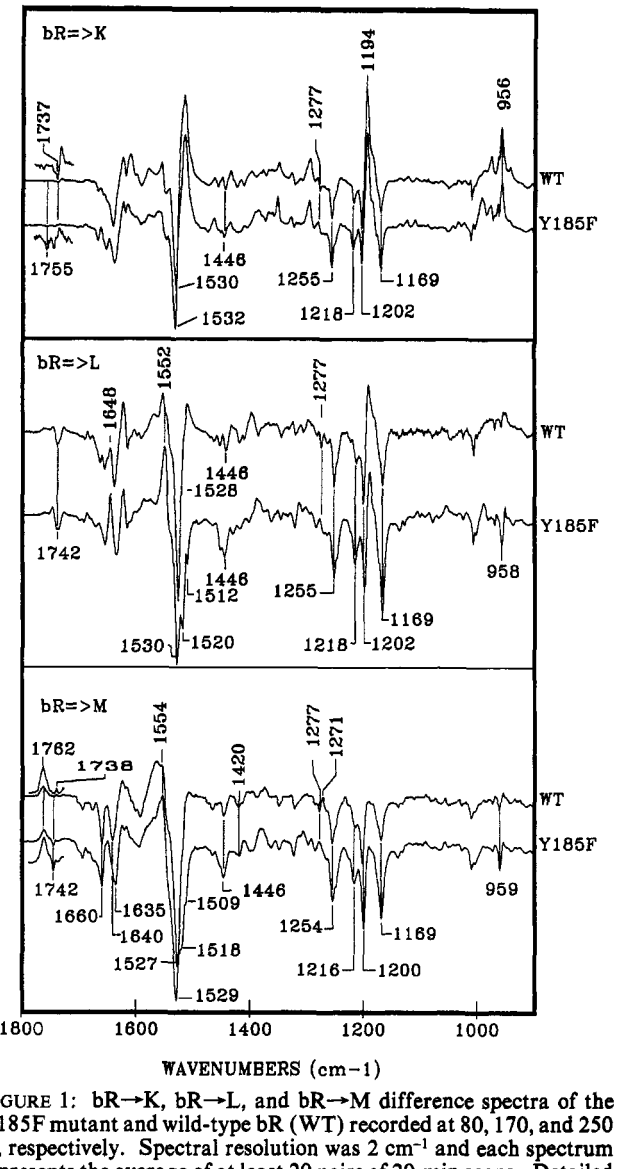

FIGURE 1: bR→K, bR→L, and bR→M difference spectra of the Y185F mutant and wild-type bR (WT) recorded at 80, 170, and 250 K, respectively. Spectral resolution was 2 cm^{-1} and each spectrum represents the average of at least 20 pairs of 20-min scans. Detailed procedures are described in Braiman et al., (1988b).

In the case of Y185F, distinct changes are found in the bR→K, bR→L, and bR→M difference spectra relative to WT (Figure 1), in agreement with an earlier study on Y185F expressed in *E. coli* (Braiman et al., 1988b). Many of these changes are due to the loss of bands assigned to the vibrations of Tyr-185, including the negative bands at 833 (not shown) and 1277 cm^{-1} and the positive band at 1271 cm^{-1} (bR→M) assigned to a CO^- stretch mode of tyrosinate (Rothschild et al., 1986; Dollinger et al., 1986; Roepe et al., 1987b). The same negative bands are also affected by isotope labeling of tyrosine (Rothschild et al., 1986; Dollinger et al., 1986).

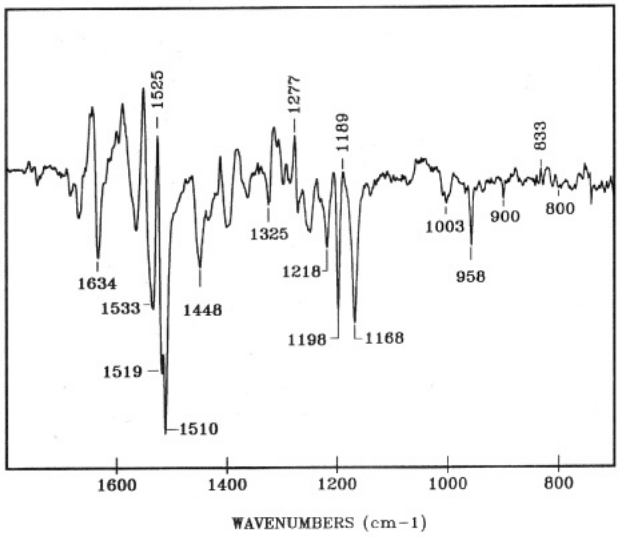

FIGURE 2: Results of interactive subtraction between bR→M difference spectra of the Y185F mutant and wild-type bR in the 1800–800-cm^{-1} region (see Results).

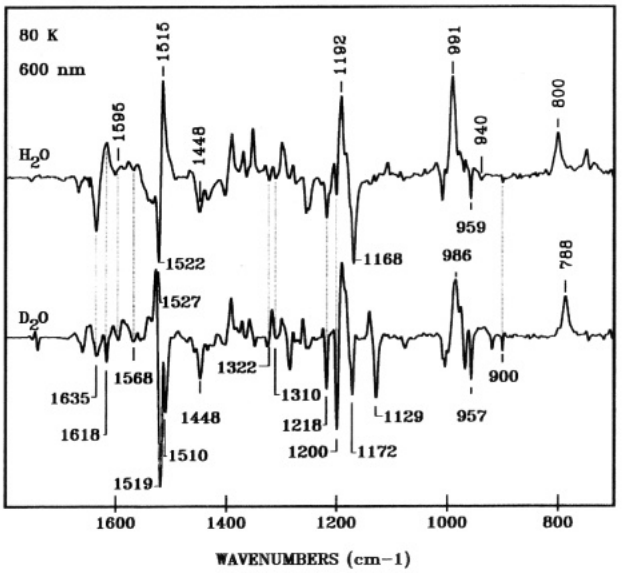

FIGURE 3: Comparison of bR→K difference spectra of Y185F in H_2O and D_2O using 600-nm illumination to drive the photoreaction.

However, there are several changes in the difference spectra of Y185F relative to WT which do not originate from the vibrations of Tyr-185. For example, in the bR→K difference spectrum, a small negative band in the carboxyl stretch region at 1755 cm^{-1} appears, the 1169-cm^{-1} band assigned to the C–C stretch mode of retinal becomes more negative, and the 956-cm^{-1} band assigned to a HOOP mode of retinal becomes less positive. In the bR→L and bR→M difference spectra of Y185F, two negative shoulders appear in the C=C stretch ethylenic region near 1510 and 1520 cm^{-1}, while the 1169-cm^{-1} C–C stretch and the 959-cm^{-1} HOOP mode increase in intensity. The negative band at 1742 cm^{-1}, assigned to the carboxyl stretch of Asp-96 (Braiman et al., 1988a; Gerwert et al., 1989; Pfefferlé et al., 1991; Bousché et al., 1991a), increases in intensity in the bR→L and bR→M difference spectra. There is also an increase in the intensity of a positive band near 1552 cm^{-1} assigned to the amide II vibrational mode (Braiman et al., 1987, 1991). In the bR→K, bR→L, and bR→M spectra, there is also an increase in a negative band near 1446 cm^{-1}.

An interactive subtraction of the WT from the Y185F difference spectrum for the bR→M transition is shown in Figure 2. These spectra were scaled so that the intensity difference of most of the common bands in the C=C ethylenic and C–C stretch regions was minimized in the subtraction. Note that all of the negative bands which increase in intensity in the difference spectra of Y185F relative to WT appear as negative bands in the subtraction corresponding to vibrational bands from the photoreacting species absent in WT. The most prominent band appears at 1510 cm^{-1} with a shoulder at 1519 cm^{-1}. Additional bands appear at 1448, 1218, 1198, 1168, 1003, 958, and 900 cm^{-1}. On the basis of an empirical linear correlation between the C=C stretch frequency of the retinylidene chromophore and its visible λ_{max} (Aton et al., 1977), the 1510-cm^{-1} band corresponds to a species with a λ_{max} near 650 nm which depletes upon illumination of the Y185F sample with 660-nm light.

Remarkably, the frequency and relative intensity of most of these bands are in agreement with bands found in the resonance Raman spectrum of the O intermediate measured at room temperature in native bR (Smith et al., 1983; Ames & Mathies, 1990). Since the vibrational spectrum of the retinylidene chromophore is strongly influenced by its structure, especially in the "fingerprint" region from 1150 to 1400 cm^{-1} (Braiman & Rothschild, 1988; Mathies et al., 1991; Ames & Mathies, 1990), this agreement is a strong indication that the chromophore structure of the red-shifted species in light-adapted Y185F (Sonar et al., 1993; Duñach et al., 1990b) is similar to the O intermediate. Similar subtractions between Y185F and wild-type bR for the bR→K and bR→L difference spectra gave similar results (data not shown). In contrast, no intensity is observed in these subtractions for the depleting red-shifted species at frequencies near 1185 and 800 cm^{-1} characteristic of the 13-cis, C=N syn chromophore structure of bR_{555} in dark-adapted bR (1183 and 800 cm^{-1}) (Smith et al., 1984) and acid-induced blue membrane (1184 and 800 cm^{-1}) (Smith & Mathies, 1985) (see Discussion). We can also exclude the N intermediate, which has a 13-cis chromophore structure, as the depleting species does not exhibit a strong band at 1187 cm^{-1} (Fodor et al., 1988; Bousché et al., 1991a; Pfefferlé et al., 1991; Rath et al., 1993). *On this basis, we conclude that a stable species O^{Y185F} with an all-trans chromophore similar to the O intermediate exists in light-adapted Y185F and undergoes a photoreaction along with the normal bR_{570} species at low temperature.* Note that the presence of the 1519-cm^{-1} band may indicate the existence of a second species which absorbs near 610 nm. Alternatively, the 1510- and 1519-cm^{-1} bands may arise from a single species with two strongly infrared-active ethylenic C=C stretch modes. We also note that some bands in this spectrum, in particular at 1277 and 833 cm^{-1}, are due to the absence of these bands in the Y185F spectrum and have previously been assigned to vibrations of Tyr-185 (Braiman et al., 1988b).

Photoreaction of the O^{Y185F} Species at 80 K. In order to enhance the photoreaction of the O^{Y185F} species at 80 K, we illuminated the sample with 600-nm light. In contrast to illumination with 500-nm light, which results in the bR→K difference spectrum shown in the top panel of Figure 1, 600-nm light is much closer to the wavelength of maximum absorption of the O^{Y185F} species. This wavelength also minimizes the bR_{570}→K_{630} photoreaction which has an isosbestic point near 600 nm. The resulting difference spectrum (Figure 3, top trace) shows weak intensity from the negative 1530-cm^{-1} band assigned to bR_{570} (Figure 1, top). Instead, negative bands characteristic of the O intermediate at 1522, 1168, and 959 cm^{-1} and even smaller features observed in the resonance Raman spectrum (Smith et al., 1983) near 1322, 1310, 940, and 900 cm^{-1} are observed.

The bR→K FTIR difference spectrum of Y185F in D_2O recorded using 600-nm illumination (Figure 3, lower trace) also showed good agreement with the corresponding resonance Raman spectrum of the O intermediate in wild-type (Smith et al., 1983). In both cases, new vibrational bands or increased intensity at 1129, 1200, and 1568 cm^{-1} is observed. These results strongly support the existence of a red-shifted species, O^{Y185F}, in Y185F with a chromophore having a similar structure to the O intermediate.

Positive bands in the O→K difference spectrum provide information about the structure of the photointermediate formed by the O^{Y185F} species (K^O) at 80 K. The overall pattern in the fingerprint region is very similar to the bR_{570}→K_{630} difference spectrum of wild-type bR, with a strong positive band appearing near 1192 cm^{-1}, close to a band at 1195 cm^{-1} in the K_{630} intermediate (Smith et al., 1984). This indicates that the primary photoreaction of O^{Y185F} involves an all-trans to 13-cis isomerization similar to the bR_{570}→K_{630} transition. However, the K^O species also exhibits several new bands including a strong band at 991 cm^{-1} (986 cm^{-1} for D_2O) in the hydrogen-out-of-plane (HOOP) mode region, different from the main HOOP mode of K_{630} at 956 cm^{-1} (Braiman, 1984). A second band at 800 cm^{-1} (788 cm^{-1} for D_2O) may correspond to a similar band in the K_{630} intermediate at 812 cm^{-1} which shifts 5 cm^{-1} in D_2O and is assigned to a HOOP mode involving a C_{14}–H wag (Braiman, 1984). Thus, while K^O is likely to have a 13-cis structure, it is not identical to K_{630}, possibly due to alterations in the torsion angles about C–C bonds which will alter the HOOP mode frequencies.

As pointed out, the frequency of the ethylenic C=C stretch mode is correlated with the visible λ_{max} of bR and its photointermediates. In the case of the O^{Y185F}→K^O photoreaction, a negative band at 1522 cm^{-1} and a positive band at 1515 cm^{-1} are observed, indicating that K^O has a red-shifted λ_{max} relative to the O^{Y185F} species. However, this region is likely to be distorted by the overlapping of positive and negative bands. This is confirmed in the O^{Y185F}→K^O difference spectrum of Y185F in D_2O (Figure 3), where the 1522-cm^{-1} band appears to downshift to 1519 cm^{-1}, revealing a strong positive band at 1527 cm^{-1} which might be due to either a chromophore (C=C stretch) or a protein (amide II) mode of K^O (or a second photoproduct). In addition, a 1510-cm^{-1} band associated with the C=C stretch of the O intermediate is now observed in D_2O. An intense negative band at 1635 cm^{-1} is also affected by D_2O, possibly shifting to 1618 cm^{-1}. This mode may be due to the C=N stretch mode of the O^{Y185F} species or alternatively a protein (amide I) vibrational mode. The latter possibility is more likely since the C=N stretch was previously assigned by resonance Raman spectroscopy to a 1627-cm^{-1} band which shifts to 1587 cm^{-1} for D_2O and may correspond to the negative 1595-cm^{-1} band seen in the corresponding FTIR difference spectrum (Figure 3). Measurements using isotopic labeling of the chromophore in Y185F will be necessary in order to make a more definitive assignment of the bands in the regions.

Small changes are also observed above 1700 cm^{-1} which might reflect a perturbation of Asp-85 during the O^{Y185F}→K^O transition. A negative band occurs at 1753 cm^{-1} close to the same frequency for the carboxyl stretch of this residue identified in the O intermediate (Bousché et al., 1992). A second small positive band at 1740 cm^{-1} is consistent with a possible perturbation of Asp-85. In addition to the appearance of these negative/positive bands in this region, the small intensity of these bands would argue against a significant protonation change of Asp-85. However, a more careful analysis will require isotope labeling and an examination of changes occurring in the 1300–1400-cm^{-1} region where the Asp carboxylate stretch modes appear (Engelhard et al., 1985).

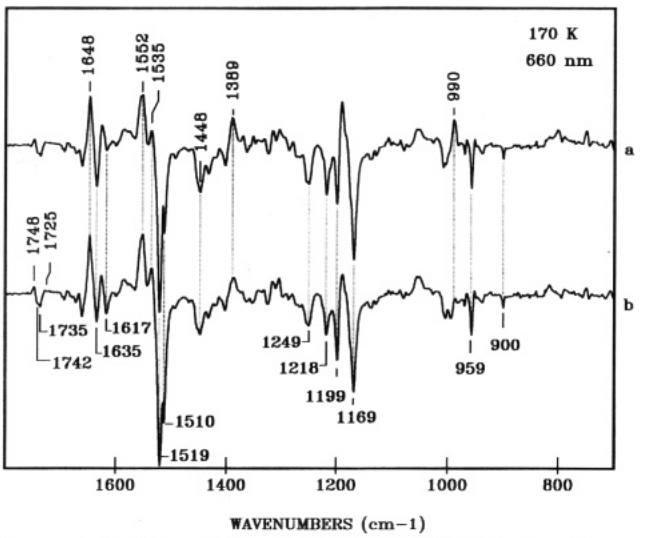

FIGURE 4: (a) bR→L difference spectrum of Y185F using 660-nm light illumination at 170 K. (b) Same spectrum corrected by subtracting the contribution from the O^{Y185F}→K^O photoreaction (Figure 3, top trace).

Photoreaction of the O^{Y185F} Species at 170 and 250 K. In order to selectively drive the photoreactions of the bR_{570} and O^{Y185F} species, different wavelengths of illumination were used. Photoselection of Y185F at 170K using 660-nm illumination resulted in a spectrum (Figure 4a) different from that obtained with 600-nm illumination (Figure 1), with an increase in intensity of negative bands appearing which are characteristic of O depletion (see also Figure 6). These include bands near 1510 and 1520 cm^{-1} due to the ethylenic C=C stretch mode(s)[4] and other characteristic O bands at 1169 and 959 cm^{-1}, confirming that the O^{Y185F} species is stable at 170 K and is selectively depleted by absorbing the 660-nm light. However, the appearance of the positive band at 990 cm^{-1} assigned to the HOOP mode of K^O indicates that this species also contributes to the difference spectrum. In order to correct for the presence of K^O, we subtracted an O^{Y185F}→K^O difference spectrum until this band disappeared (Figure 4b), which results in a relatively pure O^{Y185F}→L^O difference spectrum.

As in the case of the O^{Y185F}→K^O difference spectrum, many of the features of the O^{Y185F}→L^O difference spectrum are similar to the corresponding bR_{570}→L difference of wild-type bR. For example, pairs of bands appear in both cases in the carboxyl stretch region at 1742/1748 and 1735/1725 cm^{-1}, assigned to deionization/perturbations in the environment of Asp-96 and perturbation in the environment of Asp-115, respectively (Braiman et al., 1988a). However, large positive bands in the amide I and II regions at 1648 and 1552 cm^{-1} are not observed in the corresponding bR_{570}→L FTIR difference spectrum of wild-type bR (Figure 1) and are more characteristic of formation of the N intermediate (Bousché et al., 1991a; Pfefferlé et al., 1991). The 1535-cm^{-1} band is also characteristic of the N intermediate and assigned to an ethylenic mode (Fodor et al., 1988; Bousché et al., 1991a; Pfefferlé et al., 1991). Thus, at 170 K, L^O shares many spectral features of the N intermediate in the wild-type bR photocycle, although protonation changes between bR→L and O^{Y185F}→L^O are similar.

[4] Note that in Figure 1 these bands appear at a high frequency due to the overlaps with the major band at 1530 cm^{-1}.

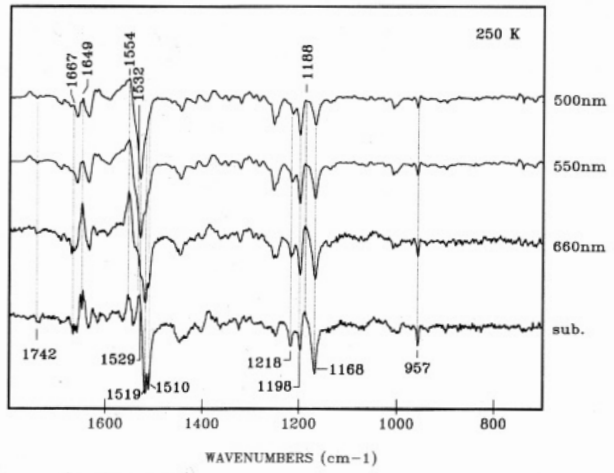

FIGURE 5: bR→M difference spectra of the Y185F mutant recorded at 250 K using different wavelengths of illumination: 500 nm (narrow-band interference filter), 550 nm (505-nm long-pass filter with a transmittance of 72% at 550 nm), and 660 nm (narrow-band interference filter). The bottom spectrum shows the subtraction between the difference spectra obtained using 550- and 500-nm illuminations.

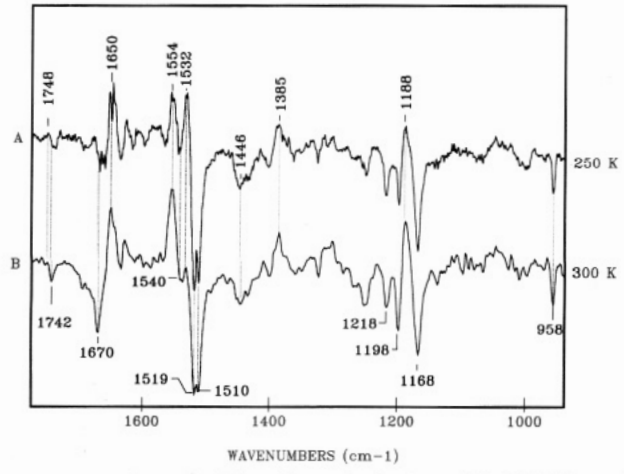

FIGURE 6: (A) Result of the subtraction between bR→M difference spectra of the Y185F mutant recorded using the illumination of 500- and 550-nm light. (B) N→O difference spectrum measured by room temperature time-resolved FTIR difference spectroscopy (the spectrum was multiplied by −1 to represent the O→N characters for the comparison).

At 250 K, a similar pattern is observed (Figure 5). Photoselection with 660-nm light produces depletion of an O-like intermediate (O^{Y185F}), whereas illumination with 550- and 500-nm light produces increasing contributions from the bR_{570}-like species present in light-adapted Y185F. We also obtained a relatively pure difference spectrum reflecting contributions only from the O^{Y185F} photocycle by interactively subtracting the difference spectrum obtained with 500-nm illumination from that obtained using 550-nm illumination until the negative 1529-cm^{-1} band disappeared (Figure 5).

Significantly, the photoexcitation difference spectrum obtained at 250 K (like that at 170 K) appears to reflect formation of an N-like intermediate. This is most clearly seen by comparing the low-temperature difference spectrum to the N→O difference spectrum of the Y185F photocycle measured by time-resolved FTIR difference spectroscopy at room temperature on a sample with high salt concentration (Figure 6) (Bousché et al., 1992). Note that this spectrum has been presented for comparison as an O→N difference spectrum. Positive peaks at 1188, 1532, 1554, and 1650 cm^{-1} are common to both spectra and highly characteristic of the vibrational modes of the N intermediate (Fodor et al., 1988; Gerwert et al., 1990; Braiman et al., 1991; Bousché et al., 1991a; Pfefferlé et al., 1991) whereas negative bands characteristic of the O species chromophore vibrations (Smith et al., 1983) appear at 1510, 1218, 1198, 1168, and 958 cm^{-1} along with the additional band at 1519 cm^{-1} in the ethylenic stretch region. A band near 1446 cm^{-1} is also found which is seen in the O^{Y185F}→K^{O} and O^{Y185F}→L^{O} difference spectra.

Bands in the FTIR difference spectrum assigned to the amide I and II modes are especially interesting since they can reflect changes in the bacteriorhodopsin secondary structure (Braiman et al., 1987, 1991; Ormos, 1991). Bands in this region in the N→O FTIR time-resolved difference spectrum (Figure 6B) at 1670 cm^{-1} (negative) and 1650 cm^{-1} (positive) assigned to the amide I mode and at 1554 cm^{-1} (positive) and 1540 cm^{-1} (negative) assigned to the amide II mode indicate that a structural refolding of the protein occurs (Bousché et al., 1992) which reverses structural changes observed earlier in the photocycle (Braiman et al., 1991; Bousché et al., 1991a; Pfefferlé et al., 1991). As seen in Figure 6A, similar spectral features are also observed in the difference spectrum obtained from Y185F with 660-nm light at 250 K; however, the intensities of the bands at both 1670 cm^{-1} (negative) and 1554 cm^{-1} (positive) are less intense in the low-temperature data. The increased intensity of the 1532-cm^{-1} band associated with the N ethylenic stretch mode could also be explained by a drop in the intensity of the negative 1540-cm^{-1} amide II mode. Thus, the structural changes which occur during the N→O transition at room temperature may be partially blocked at the lower temperature.

The protonation changes of Asp-96 which occur in the low temperature O→N photoreaction are also of interest since this residue has been found to reprotonate during the N→O transition of the Y185F photocycle (Bousché et al., 1992). The reverse reaction might then be expected to occur at low temperature when the O^{Y185F} species is converted to the N intermediate. The negative band at 1742 cm^{-1} in the O→N difference spectrum at 250 K (Figure 6A) indicates such a deprotonation; however, a small positive band at 1748 cm^{-1} is also detected. In the bR→L difference spectrum of wild-type bR, this band was interpreted as indicative of a fraction of Asp-96 undergoing a change in environment in equilibrium with deprotonated Asp-96 (Braiman et al., 1988a), whereas in a different work this band was interpreted to indicate a complete shift in the environment of Asp-96 with no deprotonation (Gerwert et al., 1989). The 1748-cm^{-1} positive band is even more evident at 170 K for the O^{Y185F} photoreaction (Figure 4), similar to the difference spectrum in this region for the bR_{570}→L reaction at 170 K (Figure 1, middle panel). Thus, while an N-like photointermediate is formed at both 170 and 250 K upon photoexcitation of the O-like species, the deprotonation of Asp-96 may be partially blocked due to the lower temperature. A clearer picture of the protonation of Asp-96 in the low-temperature O^{Y185F} photocycle might emerge by identifying the Asp-96 carboxylate band which appears near 1400 cm^{-1} in the N intermediate of wild-type bR (Bousché et al., 1992; Maeda et al., 1992). However, a strong band near 1385 cm^{-1} appears in the Y185F difference spectra at both 300 and 250 K (see Figure 6) which complicates analysis of this region of the spectrum. Further work using isotope labeling of Asp residues in Y185F will be necessary to make a more detailed analysis.

Comparison of the bR_{570} Species in Wild-Type bR and Y185F. Although we have shown that the differences between the low-temperature FTIR difference spectra of Y185F and

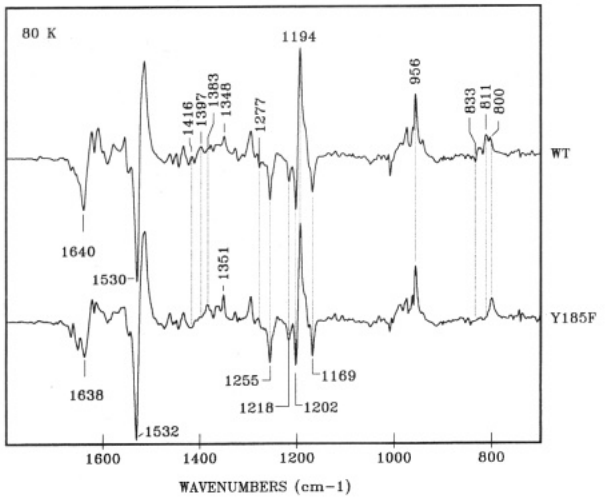

FIGURE 7: Comparison of bR→K difference spectra obtained from WT and Y185F. The later spectrum is corrected by subtracting the contribution from the O^{Y185F}→K^{O} difference spectrum (Figure 3, top trace) until the 991-cm^{-1} band disappears.

wild-type bR are mainly due to the photoreaction of an O-like species and the loss of intrinsic vibrations due to Y185F, spectral changes might still exist due to intrinsic structural alterations in the bR_{570} species present in light-adapted Y185F. In order to examine this possibility, we computed a pure bR→K spectrum of Y185F by subtracting contributions of the O^{Y185F}→K^{O} difference spectrum (Figure 3, top trace). As seen in Figure 7, these spectra are very similar, demonstrating that the structure of bR_{570} and the K_{630} intermediate in wild-type bR and Y185F are almost the same. Some changes found between the two spectra can be directly attributed to the loss of the Tyr-185 vibrations (1277 and 833 cm^{-1}) as previously shown (Braiman et al., 1988b). However, small changes are still observed which cannot be attributed directly to vibrational modes associated with Tyr-185. First, the ethylenic frequency upshifts from 1530 to 1532 cm^{-1}, corresponding to a small blue shift in the λ_{max} of Y185F to near 560 nm (Aton et al., 1980). Such a blue shift was previously detected in the low-temperature and in the room temperature bR→M visible difference absorption spectra of Y185F (Ahl et al., 1988; Duñach et al., 1990a). In addition, the C=N stretch mode of the protonated Schiff base, which normally occurs at 1640 cm^{-1}, downshifts to 1638 cm^{-1}, in agreement with time-resolved FTIR difference spectroscopy (Bousché et al., 1992). Two other positive bands at 1348 cm^{-1} (N–H in-plane bending vibration) (Maeda et al., 1992) and 811 cm^{-1} (C_{14}–C_{15} HOOP) (Smith et al., 1984) are altered, with the 1348-cm^{-1} band upshifting to 1351 cm^{-1} and the 811-cm^{-1} band disappearing or possibly shifting to 800 cm^{-1}, consistent with an alteration in the structure of the K chromophore in the vicinity of the Schiff base. Finally, a positive band located near 1397 cm^{-1} downshifts to 1383 cm^{-1}, and a positive band near 1416 cm^{-1} disappears.

We also compared the bR→M difference spectrum of Y185F obtained using 500-nm illumination (Figure 5), which minimizes O^{Y185F} photoreactions, with the corresponding bR→M difference of wild-type bR (Figure 1). The wild-type and Y185F spectra are again very similar, indicating that the dominant species in light-adapted Y185F is similar to bR_{570} and undergoes a normal photoreaction at 250 K. However, an upshift is found in the C=C ethylenic stretch mode of the chromophore from 1527 to 1529 cm^{-1}, confirming the shift of this band seen at the lower temperature. This shift accounts for the negative/positive feature at 1533/1525 cm^{-1} in the subtraction of bR→M difference spectra of light-adapted Y185F from wild-type bR in Figure 2 and in a corresponding subtraction from FT-Raman spectra (Rath et al., 1993).

DISCUSSION

We have investigated the photocycle of Y185F at low temperature using FTIR difference spectroscopy. Our results support the following conclusions.

The Altered Properties of the Mutant Y185F Are Intrinsic and Not Due to Changes in Lipid Environment. Earlier FTIR studies on bR mutants including Y185F were reported for bacteriorhodopsin expressed in *E. coli* and reconstituted into native *H. halobium* lipids (Braiman et al., 1988a,b; Rothschild et al., 1990, 1992). Since this method involves regeneration of the protein in detergent micelles followed by reconstitution into the lipids, it is possible that some of the altered spectral features of the mutants might reflect the nonnative environment and not intrinsic changes in the properties of the mutant. The present study, which examines Y185F produced in *H. halobium* and isolated as intact purple membrane fragments, resulted in FTIR difference spectra very similar to those measured for *E. coli* expressed Y185F. We therefore conclude that the photocycle and structural changes which the mutant Y185F undergoes in these two environments are very similar, in agreement with results obtained from visible absorption measurements (Sonar et al., 1993).

An O-like Species (O^{Y185F}) Exists as a Stable Form in Light-Adapted Y185F. In an accompanying paper (Sonar et al., 1993), it was concluded that many of the properties of Y185F including the formation of a long-lived red-shifted intermediate upon light adaptation (Ahl et al., 1988, 1989; Duñach et al., 1990b), photoreaction of a red-shifted species (Duñach et al., 1990a; Jang et al., 1990), and the apparent absence of an O intermediate in the Y185F photocycle could be accounted for if an O-like species existed in equilibrium with bR_{570}. *The present work confirms a key prediction of this model: the existence of a stable O-like species in the light-adapted state of Y185F.*

Our evidence is based on the direct observation of a species in light-adapted Y185F which has vibrational bands characteristic of the O chromophore. In particular, all of the major O bands measured by resonance Raman spectroscopy and characteristic of an all-trans chromophore structure (Smith et al., 1983; Ames & Mathies, 1990) are found at similar frequencies and relative intensities in the component of the FTIR difference spectrum of Y185F attributed to the red-shifted species. While an intensity correlation between resonance Raman and infrared spectra of a large molecule such as retinal is not generally expected, this effect has occurred in the bR_{570} and K chromophore of wild-type bR and may be a general property of a highly delocalized polyene molecule (Rothschild et al., 1984a).

We also considered the possibility that the O^{Y185F} reflects an acid-induced blue form of Y185F. As discussed in an accompanying paper (Sonar et al., 1993), the pK_a for acid blue formation in Y185F produced in *H. halobium* is 3.7, whereas the O^{Y185F} is detected both in hydrated films and in solution (Sonar et al., 1993; Rath et al., 1993) under conditions where the pH is much higher. Furthermore, the resonance Raman spectrum of the acid-induced blue species has also been found to be distinctly difference from the O intermediate spectrum (Smith & Mathies, 1985) with relatively weak bands at 955 cm^{-1} in the HOOP mode region and at 1167 cm^{-1} in the C–C stretch region (Smith et al. 1983). For this reason,

it was concluded that there is no significant relationship between the structure of the chromophore in acid-induced blue membrane and the O intermediate (Smith & Mathies, 1985). However, since the acid blue species contains two distinct components with an all-trans and a 13-cis chromophore, it is still possible that the structure of the all-trans form is similar to the stable O^{Y185F} detected here as well as to the O intermediate detected in the photocycle of Y185F (Bousché et al., 1992). Support for this possibility comes from the similarity of infrared bands assigned to the O intermediate in the Y185F photocycle (Bousché et al., 1992), O^{Y185F} observed here, and bands assigned to the acid blue membrane in the pH titration difference spectrum of wild-type bR (Marrero & Rothschild, 1987). Common features include the 1510- and 1520-cm^{-1} bands, assigned to the ethylenic C=C stretch modes, and bands in the 1700–1800-cm^{-1} region which reflect protonation changes of Asp groups. In a more recent study, FTIR difference spectra were obtained at 170 K for the photoreaction of light-adapted blue membrane formed by deionization (Fahmy & Siebert, 1990). These difference spectra and those recorded in D_2O are similar to the 170 K difference spectra of Y185F photoexcited with 660-nm light (Figure 4) and corresponding measurements in D_2O (data not shown). These comparisons provide evidence for an equivalence between the structure and photoreaction of the O^{Y185F} species and the all-trans component of deionized purple membrane (blue membrane). As discussed in related papers (Rath et al., 1993; Sonar et al., 1993), this equivalence might be accounted for by an increase in the pK_a of the all-trans but not the 13-cis component for acid-induced blue membrane formation in Y185F.

The O^{Y185F} Photocycle Has K and N-like Intermediates. In related work, it was proposed on the basis of static and time-resolved visible absorption measurements of Y185F that the photoexcitation of the O^{Y185F} species results in a red-shifted photoproduct (K^O) which then decays to an N-like intermediate. The O^{Y185F} species is reformed either by direct decay of N back to O or through a branched pathway to bR_{570} which then reequilibrates with O [see Figure 7 of Sonar et al. (1992)].

Our present results strongly support this model and provide the first information about the detailed structure of intermediates in the photocycle of the O^{Y185F} species. We find that photoexcitation of the O^{Y185F} species at 80 K causes an all-trans→13-cis isomerization of the chromophore and formation of a red-shifted K-like intermediate similar to the primary photoproduct in bR_{570}. However, the 13-cis structure of this species (K^O) still differs from K_{630}, with changes observed in the HOOP mode region.

We also detect protein structural changes during the O^{Y185F}→K^O transition which are not present in the bR_{570}→K_{630} photoreaction. A small band assigned to the COOH stretch mode of Asp-85 in O^{Y185F} is detected (Bousché et al., 1992), possibly due to a perturbation in the environment of this residue in the O^{Y185F}→K^O transition.[5] The K^O intermediate also appears to be more stable at higher temperatures than K_{630}, consistent with a slower decay rate observed for this intermediate at room temperature (Duñach et al., 1990a; Sonar et al., 1993).

At 250 K and to a lesser extent at 170 K, photoexcitation of the O^{Y185F} species produces a difference spectrum which is similar to the reversed N→O difference spectrum recorded by time-resolved FTIR difference spectroscopy during the Y185F photocycle (Bousché et al., 1992). The agreement is remarkable considering that the low-temperature spectrum reflects the photoexcitation of a trapped O^{Y185F} species, whereas the room temperature time-resolved spectrum represents the N→O transition which occurs during the photocycle. *Since the frequencies of positive bands in the low-temperature O→N difference spectrum (Figure 6) are very similar to those previously identified in the N intermediate of the wild-type bR photocycle at low temperature and room temperature (Fodor et al., 1988; Braiman et al., 1991; Bousché et al., 1991a; Pfefferlé et al., 1991), we conclude that the photoproduct of the O^{Y185F} species at lower temperature is very similar to the N intermediate of the bR photocycle.* The differences between the two spectra appear mainly in the amide I and II regions and may reflect a partial block in the conformational changes of the protein at lower temperature as previously observed for wild-type bR (Braiman et al., 1987). Deprotonation of Asp-96 also appears to be partially blocked at the lower temperatures, reflecting either a block in Asp-96 deprotonation or alternatively a transient deprotonation and then reprotonation.

Protonation State of Asp Residues in bR_{570} and O^{Y185F} Species. We have established that the chromophore structure of the stable red-shifted species in Y185F is similar to the O intermediate. An important question is whether this similarity also extends to the protonation states of residues Asp-85 and Asp-212, which in bR_{570} exist in an ionized state (Braiman et al., 1988a; Engelhard et al., 1989). Recent FTIR studies indicate that Asp-85 undergoes protonation and Asp-212 partial protonation during formation of the M intermediate and are retained in these protonation states until decay of the O intermediate (Bousché et al., 1991a, 1992). Since in a related work (Rath et al., 1993) we found that a similar reaction occurs during light adaptation of Y185F and formation of the O^{Y185F} species, we tentatively conclude that the protonation states of these Asp residues in the O intermediate and O^{Y185F} species are similar. It should be pointed out however, that while recent solid-state NMR studies (Metz et al., 1992; Engelhard et al., 1989) support the conclusion that Asp-85 protonates during formation of the M intermediate, a similar protonation of Asp-212 was not detected (the N and O species were not examined in this study). While further studies will be needed to explain this discrepancy, a possible explanation may stem from the fact that the NMR measurements were performed at 220 K on the mutant D96N at pH 10. It is possible that under these conditions the protonation of Asp-212, which is detected at both 250 K and room temperature in wild-type bR by FTIR, does not occur or is at a level which cannot be detected by the solid-state NMR measurements. In support of this possibility, we have previously observed (Bousché et al., 1991a) that the mutant D96A, which has a very similar phenotype to D96N, exhibits a delayed protonation of Asp-212 which in wild-type bR occurs concomitant with M formation. In addition, we have found that at lower temperature (250 K) the protonation of Asp-212 in D96A is reduced relative to wild-type bR (Bousché et al., 1991a). A decrease in the protonation of Asp-212 is also observed in wild-type bR at 220 K relative to 250 K (Roepe et al., 1987a). Thus, under the conditions of the solid-state NMR measurements, it is likely that Asp-212 has reduced protonation relative to the wild-type bR.

Assuming that the above conclusions are correct, consideration of these differences in the protonation states of Asp-85 and Asp-212 in bR_{570} and the O^{Y185F} species provides insight into understanding key differences in the bR_{570} and O

[5] As discussed, bands associated with Asp-85 in the O^{Y185F} to K^O photoreaction are mostly due to a perturbation in its environment.

photocycles despite the fact that both photocycles involve as the primary photoevent an all-trans→13-cis isomerization. These differences include: (i) shifts in the HOOP mode frequency of K^O relative to K_{630}, possibly indicating differences in the angles around C–C bonds in the polyene chain as well as a red shift in the wavelength of the absorption maximum; (ii) similarities in the photoproducts produced from the O^{Y185F} species at 250 K and to a lesser extent at 170 K and the N intermediate of the bR photocycle; (iii) absence of an M-like intermediate in the O^{Y185F} photocycle.

In the case of the K^O intermediate, the protonation of Asp-85 and Asp-212 would be expected to reduce the electrostatic interaction which normally exists with the protonated Schiff base. This would help explain both the altered configuration of the C=N portion of the chromophore relative to K_{630} and the red shift from 630 to 660 nm observed by visible absorption spectroscopy (feature i). In the case of L^O, the active site now more resembles the N intermediate of the bR_{570} photocycle (Bousché et al., 1991a). Thus, it is not surprising that some of the conformational changes involving the peptide bonds that are observed upon formation of the N intermediate have already occurred in L^O (feature ii). Finally, formation of M would not be expected (feature iii) since Asp-85 cannot function as an acceptor group for the Schiff base proton since it is already protonated.

CONCLUSIONS

The present study and related work (Bousché et al., 1992; Sonar et al., 1993; Rath et al., 1993) provide a basis for understanding the altered properties of the mutant Y185F. Low-temperature FTIR difference spectra reveal that upon cooling of light-adapted Y185F, in addition to a normal species similar to bR_{570}, a second species exists with vibrational bands very similar to the O intermediate. Furthermore, the difference spectrum of this species at 250 K is very similar to the N→O thermal reaction of Y185F measured at room temperature. We therefore conclude that the second form of Y185F which has been previously detected in a number of studies (Ahl et al., 1988, 1989; Jang et al., 1990; Duñach et al., 1990a,b) is very similar to the O intermediate.

ACKNOWLEDGMENT

We thank S. Sonar, P. Rath, T. Marti, and O. Bousché for helpful discussions during the course of this work and O. Bousché for technical assistance.

REFERENCES

Ahl, P. L., Stern, L. J., During, D., Mogi, T., Khorana, H. G., & Rothschild, K. J. (1988) *J. Biol. Chem. 263*, 13594–13601.

Ahl, P. L., Stern, L. J., Mogi, T., Khorana, H. G., & Rothschild, K. J. (1989) *Biochemistry 28*, 10028–10034.

Ames, J. B., & Mathies, R. A. (1990) *Biochemistry 29*, 7181–7190.

Aton, B., Doukas, A. G., Callender, R. H., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995–2999.

Aton, B., Doukas, A. G., Narva, D., Callender, R. H., Dinur, U., & Honig, B. (1980) *Biophys. J. 29*, 70–94.

Bagley, K., Dollinger, G., Eisenstein, L., Singh, A. K., & Zimanyi, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4972–4976.

Bousché, O., Braiman, M. S., He, Y. W., Marti, T., Khorana, H. G., & Rothschild, K. J. (1991a) *J. Biol. Chem. 266*, 11063–11067.

Bousché, O., Spudich, E. N., Spudich, J. L., & Rothschild, K. (1991b) *Biochemistry 30*, 5395–5400.

Bousché, O., Sonar, S., Krebs, M., Khorana, H. G., & Rothschild, K. J. (1992) *Photochem. Photobiol. 56*, 1085–1095.

Braiman, M. (1984) *Univ. Microfilms Int., Order No. DA8328803 44(9)*.

Braiman, M. S., & Rothschild, K. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 541–570.

Braiman, M. S., Ahl, P. L., & Rothschild, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5221–5225.

Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G., & Rothschild, K. J. (1988a) *Biochemistry 27*, 8516–8520.

Braiman, M. S., Mogi, T., Stern, L. J., Hackett, N. R., Chao, B. H., Khorana, H. G., & Rothschild, K. J. (1988b) *Proteins: Struct., Funct., Genet. 3*, 219–229.

Braiman, M. S., Bousché, O., & Rothschild, K. J. (1991) *Proc. Natl. Aca. Sci. U.S.A. 88*, 2388–2392.

Braiman, M., Klinger, A., & Doebler, R. (1992) *Biophys. J. 62*, 56–58.

Chen, W., & Braiman, M. S. (1991) *Photochem. Photobiol. 54*, 905–910.

Dollinger, G., Eisenstein, L., Lin, S., Nakanishi, K., & Termini, J. (1986) *Biochemistry 25*, 6524–6533.

Duñach, M., Berkowitz, S., Marti, T., He, Y. W., Subramaniam, S., Khorana, H. G., & Rothschild, K. J. (1990a) *J. Biol. Chem. 265*, 16978–16984.

Duñach, M., Marti, T., Khorana, H. G., & Rothschild, K. J. (1990b) *Proc. Natl. Acad. Sci., U.S.A. 87*, 9873–9877.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.

Engelhard, M., Hess, B., Emeis, D., Metz, G., Kreutz, W., & Siebert, F. (1989) *Biochemistry 28*, 3967–3975.

Fahmy, K., & Siebert, F. (1990) *Photochem. Photobiol. 51*, 459–464.

Fodor, S. P. A., Ames, J. B., Gebhard, R., van den Berg., E. M. M., Stoeckenius, W., Lugtenburg, J., & Mathies, R. A. (1988) *Biochemistry 27*, 7097–7101.

Gerwert, K., Hess, B., Soppa, J., & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943–4947.

Gerwert, K., Souvignier, G., & Hess, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9774–9778.

Jang, D. J., El-Sayed, M. A., Stern, L. J., Mogi, T., & Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4103–4107.

Krebs, M. P., Hauss, T., Heyn, M. P., RajBhandary, U. L., & Khorana, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 859–863.

Maeda, A., Sasaki, J., Shichida, Y., Yoshizawa, T., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992) *Biochemistry 31*, 4684–4690.

Marrero, H., & Rothschild, K. J. (1987) *FEBS Lett. 223*, 289–293.

Mathies, R. A., Lin, S. W., Ames, J. B., & Pollard, W. T. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 491–518.

Metz, G., Siebert, F., & Engelhard, M. (1992) *FEBS Lett. 303*, 237–241.

Ormos, P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 473–477.

Pfefferlé, J. M., Maeda, A., Sasaki, J., & Yoshizawa, T. (1991) *Biochemistry 30*, 6548–6556.

Rath, P., Krebs, M. P., He, Y., Khorana, H. G., & Rothschild, K. J. (1993) *Biochemistry* (second of three papers in this issue).

Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987a) *Biochemistry 26*, 6696–6707.

Roepe, P., Ahl, P. L., Das Gupta, S. K. Herzfeld, J., & Rothschild, K. J. (1987b) *Biochemistry 26*, 6696–6707.

Roepe, P., Ahl, P. L., Herzfeld, J., Lugtenburg, J., & Rothschild, K. J. (1988) *J. Biol. Chem. 263*, 5110–5117.

Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147–167.

Rothschild, K. J., & Marrero, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4045–4049.

Rothschild, K. J., Zagaeski, M., & Cantore, B. (1981) *Biochem. Biophys. Res. Commun. 103*, 483–489.

Rothschild, K. J., Marrero, H., Braiman, M., & Mathies, R. (1984a) *Photochem. Photobiol. 40*, 675–679.

Rothschild, K. J., Roepe, P., Lugtenburg, J., & Pardoen, J. A. (1984b) *Biochemistry 23*, 6103–6109.

Rothschild, K. J., Roepe, P., Earnest, T., & Herzfeld, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 347–351.

Rothschild, K. J., Bousché, O., Braiman, M. S., Hasselbacher, C. A., & Spudich, J. L. (1988) *Biochemistry 27*, 2420–2424.

Rothschild, K. J., Braiman, M. S., He, Y. W., Marti, T., & Khorana, H. G. (1990) *J. Biol. Chem. 265*, 16985–16991.

Rothschild, K. J., He, Y. W., Sonar, S., Marti, T., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1615–1622.

Siebert, F., & Mäntele, W. (1983) *Eur. J. Biochem. 130*, 565–573.

Smith, S. O., & Mathies, R. A. (1985) *Biophys. J. 47*, 251–254.

Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. A. (1983) *Biochemistry 22*, 6141–6148.

Smith, S. O., Myers, A. B., Pardoen, J. A., Winkel, C., Mulder, P. P., Lugtenburg, J., & Mathies, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2055–2059.

Sonar, S., Krebs, M. P., Khorana, H. G., & Rothschild, K. J. (1993) *Biochemistry* (first of three papers in this issue).

Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 51*, 587–616.