Vibrational Spectroscopy of Bacteriorhodopsin Mutants: Evidence for the Interaction of Proline-186 with the Retinylidene Chromophore†

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ABSTRACT: Fourier-transform infrared difference spectroscopy has been used to study the role of the three membrane-embedded proline residues, Pro-50, Pro-91, and Pro-186, in the structure and function of bacteriorhodopsin. All three prolines were replaced by alanine and glycine; in addition, Pro-186 was changed to valine. Difference spectra were recorded for the bR → K and bR → M photoreactions of each of these mutants and compared to those of wild-type bacteriorhodopsin. Only substitutions of Pro-186 caused significant perturbations in the frequency of the C=C and C—C stretching modes of the retinylidene chromophore. In addition, these substitutions reduced bands in the amide I and II region associated with secondary structural changes and altered signals assigned to the adjacent Tyr-185. Pro-186 → Val caused the largest alterations, producing a second species similar to bR_{548} and nearly blocking chromophore isomerization at 78 K but not at 250 K. These results are consistent with a model of the retinal binding site in which Pro-186 and Tyr-185 are located in direct proximity to the chromophore and may be involved in linking chromophore isomerization to protein structural changes. Evidence is also found that Pro-50 may be structurally active during the bR → K transition and that substitution of this residue by glycine preserves the normal protein structural changes during the photocycle.

Prolines are often found in the hydrophobic transmembrane regions of integral membrane proteins involved in ion transport and receptor functions (Brandl & Deber, 1986). In the case of bacteriorhodopsin (bR),[1] the light-driven proton pump from *Halobacterium halobium* (Stoeckenius & Bogomolni, 1982), three buried prolines, Pro-50, Pro-91, and Pro-186, are membrane embedded and located in the putative helices B, C, and F, respectively (Engelman et al., 1981) (cf. Figure 1). Recent studies using site-directed mutagenesis have shown that none of these residues are essential for proton transport (Hackett et al., 1987; Mogi et al., 1989). However, the substitution of Pro-186 by valine or leucine can significantly lower proton pumping activity (Mogi et al., 1989), cause a large blue shift in the λ_{max} of the visible absorption (Ahl et al., 1988; Mogi et al., 1989), and alter the rate of M decay (Ahl et al., 1989). Substitutions of Pro-50 and Pro-91 caused significant alterations in the chromophore regeneration rate (Mogi et al., 1989). Thus, while it is unlikely that buried prolines in bR are directly involved in proton-transfer reactions, they may still be essential in stabilizing the native structure of bR as well as being involved in conformational changes associated with the bR photocycle.

In this study, we have combined FTIR difference spectroscopy (Braiman & Rothschild, 1988) with site-directed mutagenesis to further investigate the role of prolines in bR. This work complements our previous FTIR based investigation of tyrosine, aspartic acid, and tryptophan residues in bR (Braiman et al., 1988a,b; Rothschild et al., 1989b). Those studies led to the identification of specific residues in bR that undergo protonation changes or structural rearrangements during the bR → K and bR → M photoreactions. One residue, Tyr-185, located adjacent to Pro-186, appears to undergo protonation changes during the primary phototransition to K and again upon M formation (Braiman et al., 1988b). Three other residues, Trp-86, Trp-182, and Trp-189, are believed to help limit the possible conformations of the retinal chromophore (Rothschild et al., 1989b). A sequence of protonation changes and a possible proton pathway involving Asp-85, Asp-96, and Asp-212 have been proposed on the basis of FTIR difference measurements of site-directed mutants of the membrane-embedded Asp residues (Braiman et al., 1988a). Asp-85 is predicted to serve as the proton acceptor group of the Schiff base during M formation, whereas Asp-96 is believed to be part of the Schiff base reprotonation pathway (Braiman et al., 1988a; Gerwert et al., 1989). Recent spectroscopic studies support several aspects of this model [cf., for example, Butt et al. (1989), Otto et al. (1989), Holz et al. (1989), and Otto et al. (1990)].

The present work is based on a recent FTIR study of bacteriorhodopsin containing isotopically labeled prolines (Rothschild et al., 1989c). Specific bands in the 1420–1440-cm^{-1} region of the bR → K and bR → M photoreactions were assigned to proline residues. This indicates that one or more prolines are structurally active during the bR photocycle and that chromophore isomerization is closely coupled to these changes.

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[1] Abbreviations: PM, purple membrane; bR, bacteriorhodopsin; FTIR, Fourier-transform infrared; au, absorbance units; DMPC, dimyristoylphosphatidylcholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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Table I: Frequency (Peak Position) of Selected Chromophore and Protein Vibrations of bR_{570} Identified in the bR → K Difference Spectrum

mutant	C=N	C=C	Tyr-185[a]	C—C	C—C	C—C	C—C	Trp-86[b]
WT	1640.6	1529.7	1277.1	1254.1	1216.8	1202.4	1169.1	741.9
P50A	1640.1	1529.2	1276.6	1254.4	1215.9	1202.4	1168.6	742.5
P50G	1641.1	1530.2	1276.6	1255.4	1216.8	1202.4	1168.6	742.5
P91A	1639.2	1529.2	1276.6	1254.4	1216.8	1202.4	1168.6	741.5
P91G	1641.1	1530.2	1276.6	1254.4	1215.9	1202.4	1168.6	741.5
P186A	1639.2	1528.3	c	1255.4	1217.8	1202.4	1170.5	739.6
P186G	1641.1	1530.2	1272.8	1255.4	1216.8	1202.3	1168.6	742.5
P186V	c	1528.3	c	1254.4	c	c	1165.7	c

[a] C—O stretching mode of tyrosinate-185. [b] In-phase HOOP mode of Trp-86. [c] Band not detected.

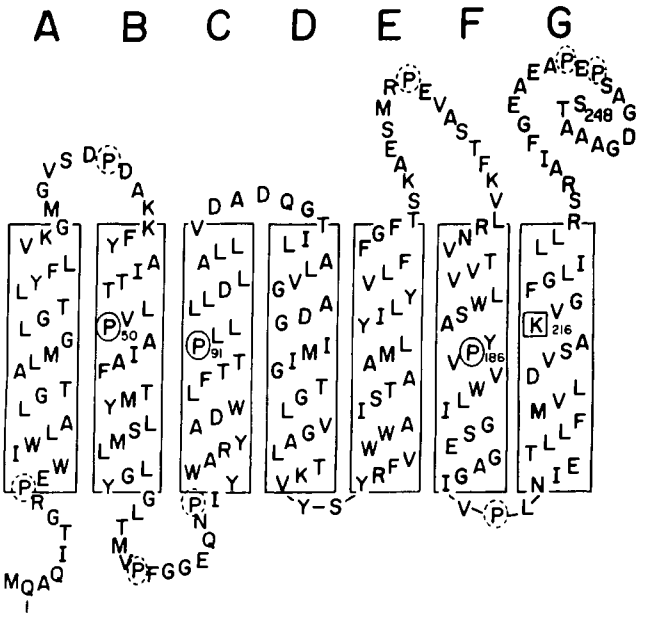

FIGURE 1: Amino acid sequence and two-dimensional folding model of bacteriorhodopsin showing the location of membrane-embedded proline residues (continuous circles) and those prolines in loop regions (dashed circles). Lys-216, which forms the Schiff base with retinal, is shown in a box.

Our results establish that none of the three buried prolines are essential for maintaining the normal bR chromophore structure or for the all-trans to 13-cis isomerization. However, the effect of substitutions at Pro-186 on the vibrational spectra of the retinylidene chromophore and on the overall photoconversion during the bR → K and bR → M transitions strongly suggests that this residue is located close to the chromophore and along with Tyr-185, Trp-86, Trp-182, and Trp-189 helps form a retinal binding pocket. In addition, alterations in Tyr-185 and structural changes involving the protein backbone appear to be affected by substitutions of Pro-186. Evidence is also presented that Pro-50 may undergo a structural change during the bR → K photoreaction.

Experimental Procedures

Preparation of bR Mutant Samples. The construction, expression, and purification of bR mutants have been reported (Karnik et al., 1987; Mogi et al., 1988). Mutant bR was regenerated with retinal and reconstituted in vesicles (Popot et al., 1987) with polar lipids from *H. halobium* using a 1:1 weight ratio. This procedure resulted in a species with a bR-like chromophore, except in the case of P186V,[2] which exhibited a blue-shifted λ_{max}.

[2] Designations for bR mutants make use of the standard one-letter abbreviations for amino acids. Thus "P186V" signifies the mutant in which the proline at position 186 has been replaced by valine, while "P186G" signifies replacement of the same residue by glycine.

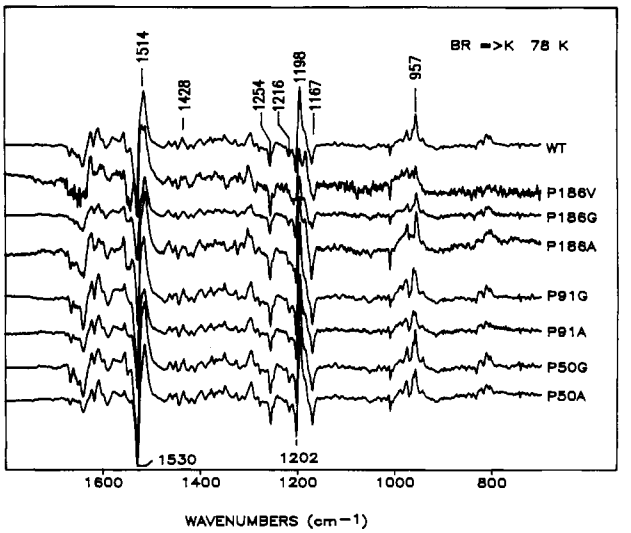

FIGURE 2: FTIR difference spectra of the bR → K photoreaction at 78 K for wild-type bR and mutants of proline residues Pro-50, Pro-91, and Pro-186. All spectra were recorded by using procedures previously reported (Roepe et al., 1987a; Braiman et al., 1988b). Spectral resolution was 2 cm^{-1}, and each spectrum represents the average from at least 15 pairs of 20-min scans.

FTIR Difference Spectroscopy. Difference spectra were recorded for the bR → K and M photoreactions at 78 and 250 K, respectively, by using methods previously reported (Rothschild et al., 1984; Roepe et al., 1987a; 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 that was mounted in a Helitran cryostat (Air Products, Allentown, PA). Water content of the sample was checked by monitoring the 3400-cm^{-1} peak. All samples were light-adapted prior to cooling. Spectra were recorded at 2-cm^{-1} resolution by using a Nicolet Analytical Instruments (Madison, WI) 60SX spectrometer.

Results

FTIR difference spectra of the bR → K and bR → M photoreactions for wild-type bR and the membrane-embedded proline mutants of bR are shown in Figures 2 and 3, respectively. With the exception of P186V these mutants produced difference spectra that were similar to, although not identical with, those of wild-type bR. The results are discussed in detail below.

Substitutions of Pro-50 and Pro-91

Effects on Chromophore. Chromophore vibrational modes in bR, K, and M appear at similar frequencies in the difference spectra of the Pro-50 and Pro-91 mutants and wild-type bR (Figures 2 and 3 and Table I). This includes the ethylenic C=C stretching mode ($\nu_{C=C}$) at 1530 cm^{-1} and the C—C stretching modes (ν_{C-C}) near 1169, 1202, 1216, and 1254 cm^{-1}

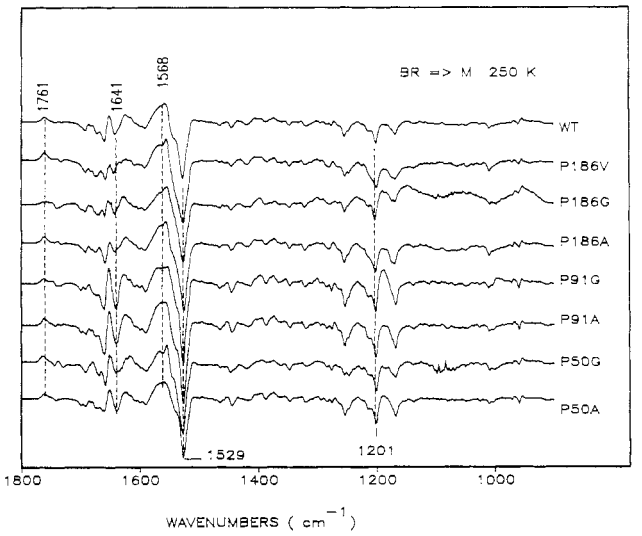

FIGURE 3: FTIR difference spectra of bR → M photoreaction at 250 K for wild-type bR and mutants of proline residues Pro-50, Pro-91, and Pro-186. All spectra were recorded by using the same procedures as those of Figure 2.

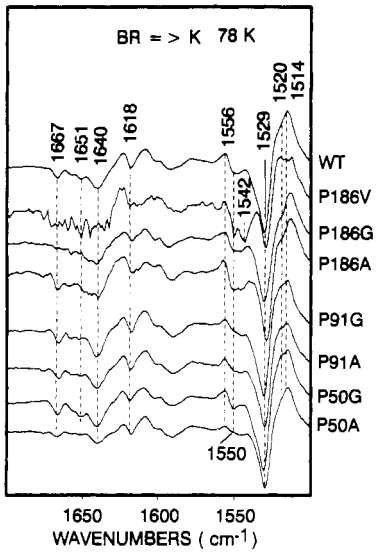

FIGURE 4: Expanded view of Figure 2 in the 1500–1700-cm^{-1} region.

(Figures 4 and 5). Since a close correlation exists between $\nu_{C=C}$ and the wavelength of the visible absorption (λ_{max}) (Doukas et al., 1978), we conclude that the *species which photocycles in these mutants absorbs near 570 nm.*

However, the hydrogen out-of-plane (HOOP) modes of the chromophore in the K intermediate of P50A, P91A, and P91G are altered relative to those of wild-type bR. The 956-cm^{-1} band is reduced in intensity and an increase in intensity occurs at 962 cm^{-1} and near 974 cm^{-1} (Figure 6). In contrast, no significant change was found in the HOOP mode region for P50G. This indicates that Gly allows a more normal chromophore structure to exist in the K intermediate than the Ala substitution.

Effects on Protein. Most of the bands assigned previously to specific bR residues in the bR → K difference spectrum are still present in the difference spectra of P50A, P50G, P91A, and P91G as seen in Table I. This includes bands at 1277 cm^{-1} (Tyr-185) (Braiman et al., 1988b) and 742 cm^{-1} (Trp-86) (Rothschild et al., 1989b) (data not shown). However, small features in the amide I (1660–1680 cm^{-1}) and amide II region (1545–1560 cm^{-1}) (Figure 4) are altered in the spectra of all of these mutants except P50G. For example, the negative/

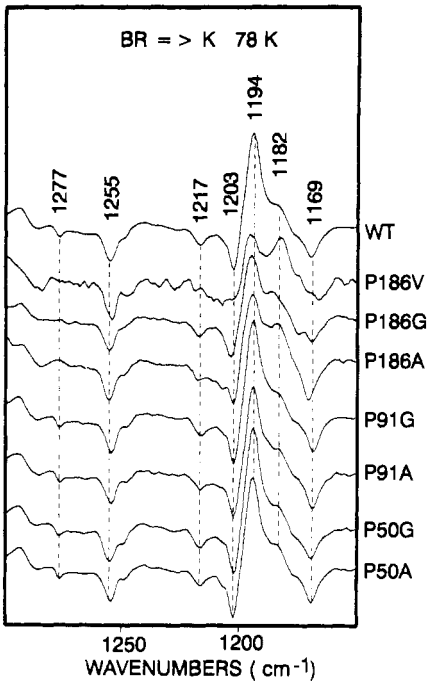

FIGURE 5: Expanded view of Figure 2 in the 1150–1300-cm^{-1} region.

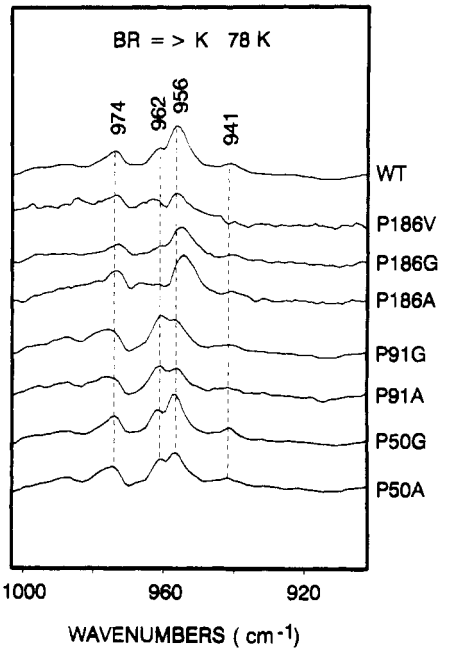

FIGURE 6: Expanded view of Figure 2 in the 900–1000-cm^{-1} region.

positive feature at 1550/1560 cm^{-1} is reduced in intensity except in the case of the P50G bR → K difference spectrum. In addition, the small negative band in wild-type bR at 1651 cm^{-1} is reduced in P91G, P91A, and P50A mutants but not in P50G. Thus, we again conclude that the most normal photocycle behavior at 78 K occurs for P50G.

In contrast, P50G exhibited the most perturbed bR → K difference spectrum in the 1420–1440-cm^{-1} region, which has previously been assigned to a mode involving the X-Pro C–N band (Rothschild et al., 1989c). As seen in Figure 7, the 1428-cm^{-1} positive shoulder is almost completely absent while P50A, P91A, and P91G exhibited only a partial reduction in intensity in this band. The band shape and intensity in the amide I region near 1618 cm^{-1} is also altered in P50G, similar to the effect of isotope labeling of proline in this region (K. J. Rothschild and Y.-W. He, unpublished results). Thus, while

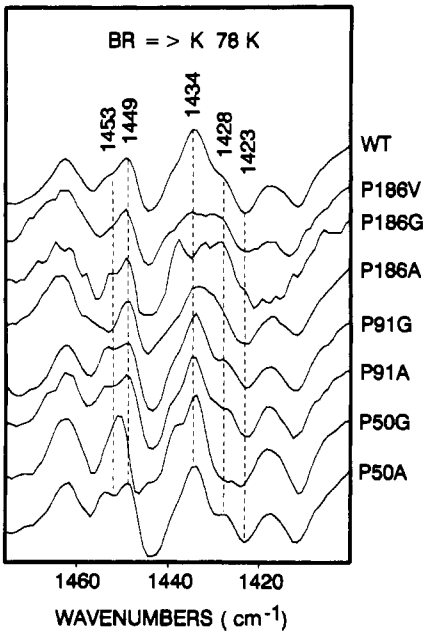

FIGURE 7: Expanded view of Figure 2 in the 1400–1480-cm^{-1} region.

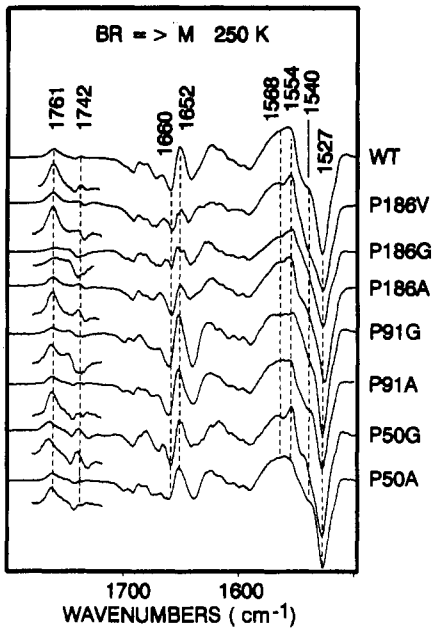

FIGURE 8: Expanded view of Figure 3 in the 1500–1800-cm^{-1} region. Insets below each spectrum in the 1720–1780-cm^{-1} region are included with expansion of the y scale.

the P50G bR → K difference spectrum is very similar to the wild-type spectrum in most regions, bands assigned to proline vibrations are absent or altered in P50G. As discussed later, this may indicate that Pro-50 is structurally active during the bR → K transition in normal bacteriorhodopsin.

In the case of the bR → M difference spectra, we observe changes in protein bands relative to wild-type bands for all of the mutants including P50G. For example, peaks are altered in the aspartic acid carbonyl stretch region (1700–1760 cm^{-1}), assigned on the basis of site-directed mutagenesis to Asp-85, Asp-96, Asp-115, and Asp-212 (Braiman et al., 1988a) (Figure 8). In the case of P91G and P91A, the bands at 1742 cm^{-1} (negative) and 1748 cm^{-1} (positive) that are associated with changes in Asp-96 (Braiman et al., 1988a; Gerwert et al., 1989) appear reduced or absent. In addition, the 1760-cm^{-1} band assigned to Asp-85 protonation (Braiman et al., 1988a) is shifted to higher frequency in P50G. However, in no case

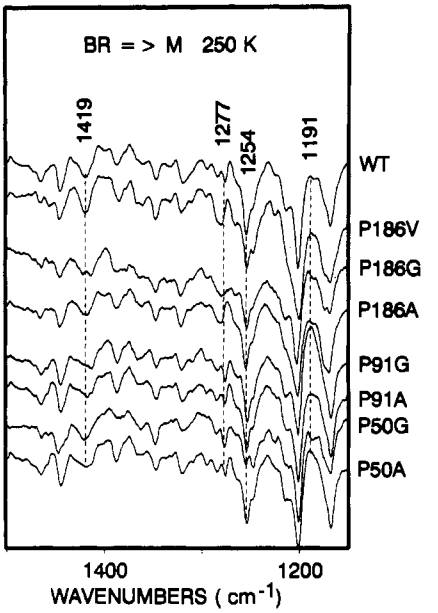

FIGURE 9: Expanded view of Figure 3 in the 1150–1500-cm^{-1} region.

is the 1760-cm^{-1} band absent.

Substitutions of Pro-186

Effects on Chromophore. The Pro-186 → Val substitution and to a lesser extent the Ala and Gly substitutions produced changes in bands assigned to chromophore vibrations in both the bR → K and bR → M difference spectra. For example, two negative bands at 1530 and 1542 cm^{-1} and two positive bands at 1510 and 1515 cm^{-1} (Figure 4) are observed in the bR → K difference spectrum of P186V. This indicates the presence of two different species, one that has a normal λ_{max} and one that is blue-shifted relative to the normal species. The appearance of a positive band near 1184 cm^{-1} in the C—C stretch region of the bR → K difference spectra of P186V and to a lesser extent in P186A and P186G (Figure 5) also indicates the presence of an altered K photoproduct. Similar bands are also observed in the bR → K photoreaction of the dark-adapted purple membrane (bR_{548}) (Roepe et al., 1988), which contains a chromophore in the 13-cis, C=N anti configuration (Harbison et al., 1984).

The 956-cm^{-1} HOOP band (Figure 6) is still present in P186V, although it is reduced in intensity relative to the 962- and 974-cm^{-1} bands. In the case of P186G and P186A, smaller changes are observed with a possible loss of intensity at 962 cm^{-1}. This indicates that substitutions of Pro-186 affect the K intermediate retinal structure but in a qualitatively different way than the Pro-50 and Pro-91 mutants.

The yield of the bR → K photoreaction was drastically reduced for the P186V mutant and to a lesser extent for the P186A mutant. In the case of P186V, the reaction was almost 10% of that of the wild type and required extensive averaging in order to obtain the difference spectrum shown in Figure 2. In contrast, the yield of the bR → M transition of P186V was reduced by only 25%.

Effects on the Protein Structure. The negative band at 1277 cm^{-1}, previously associated with the protonation of Tyr-185 during the bR → K photoreaction (Rothschild et al., 1986; Braiman et al., 1988b) is absent in the bR → K difference spectrum for all of the Pro-186 substitutions (Figure 5 and Table I). This might be due to an upshift of this band to near 1280 cm^{-1}, since a positive band at this frequency (Roepe et al., 1987a) is also not present. The band near 833 cm^{-1}, assigned to a Fermi resonance of tyrosine (Rothschild et al.,

1986), appears to also be upshifted in P186V (data not shown). In the case of P186G and P186A, this region has insufficient signal/noise to make a reliable identification of the bands. In the bR → M difference spectrum (Figure 9), the region between 1270 and 1280 cm^{-1} assigned to tyrosine-185 is again altered, with an apparent upshift of the negative band from 1277 to 1280 cm^{-1}. In the Fermi resonance region, the negative band appears to again be upshifted to near 835 cm^{-1} in the case of all three mutants. On this basis, we conclude that changes that occur in Tyr-185 and possibly a second tyrosine residue (Roepe et al., 1987b) are influenced by substitution of the proline in the neighboring 186 position.

A second common effect of the Pro-186 substitutions is the reduction of bands in the amide I and II region (Figure 8). This is most evident in the bR → M difference spectrum, where a reduction occurs in the negative 1660-cm^{-1} and positive 1652-cm^{-1} amide I bands. In addition, the negative/positive pair at 1540/1554 cm^{-1} assigned tentatively to the amide II vibration (Braiman et al., 1987) is also altered. In the case of P186A and P186V, these alterations cannot be attributed to incomplete formation of M or rapid M decay, since the bands at 1568 and 1760 cm^{-1} associated with M formation are clearly present. However, in the case of P186G, a reduction in these bands is apparent as well as an increase near 1191 cm^{-1} associated with the L intermediate (Roepe et al., 1987a), indicating that at 250 K, M formation is partially blocked.

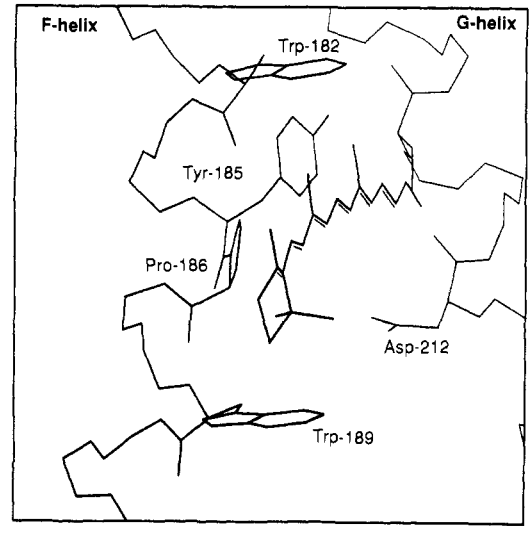

FIGURE 10: Proposed structural model of the retinal binding pocket in bR showing the relative position of Pro-186 [from Rothschild et al. (1989b)]. The proposed orientation is in agreement with polarized visible (Heyn et al., 1977; Clark et al., 1980), FTIR (Earnest et al., 1986), and neutron diffraction measurements (Heyn et al., 1988) and second Harmonic interference (Huang & Lewis, 1989). Recent evidence indicates that the plane of the polyene chain is oriented so that the C_9- and C_{12}-methyl groups of retinal are pointed toward the cytoplasmic side of the membrane and the NH bond is toward the extracellular medium (Lin & Mathies, 1989; Hauss et al., 1990).

DISCUSSION

Proline residues that are embedded in the transmembrane α-helical regions of bR are potential sites for structural activity. In particular, it has been found that one or more prolines undergo structural alteration as early as the bR → K phototransition (Rothschild et al., 1989c). An interesting possibility is that strains in the retinal binding pocket due to retinal isomerization might be relieved to some extent by movements around the X-Pro C–N bonds. In other proteins, it has been demonstrated that cis–trans isomerization of X-Pro bonds occurs during protein renaturation (Brandts et al., 1975; Lin & Brandts, 1978). In the case of RNase A, this process involves a Tyr-Pro C–N bond (Lin & Brandts, 1983).

In a previous study that examined the effects of mutations of the membrane-embedded proline-50, -91, and -186 (Mogi et al., 1989),[3] it was established that none of these residues are obligatory for proton pumping. However, the mutant Pro-186 → Val had a significantly lower proton pumping rate (10–20% of normal) and a chromophore blue-shifted to near 470 nm in DMPC/CHAPS mixed micelles. Pro-50 → Ala, Pro-91 → Ala, and Pro-91 → Gly had approximately 30-fold slower regeneration rates with all-trans retinal, whereas Pro-50 → Gly regenerated the chromophore 10 times faster than wild type.

In this study, we have utilized FTIR difference spectroscopy to examine the structural changes that proline mutants undergo during the photocycle relative to wild-type bR. We find that none of the three buried proline residues are essential for the normal all-trans to 13-cis isomerization of the chromophore that occurs during the BR → K phototransition. In addition, all of the mutants appear capable of forming a light-adapted pigment that absorbs near 570 nm. This includes P186V and P186G, which also exhibit a blue-shifted form of the light-adapted protein. These mutants were reported to have a λ_{max} near 470 nm in DMPC/CHAPS mixed micelles (Mogi et al., 1989). However, in native lipids the ethylenic stretching frequency near 1530 cm^{-1} indicates the existence of a normal λ_{max} closer to 570 nm. *Thus, membrane-embedded prolines are not essential for the normal chromophore/protein interactions that give rise to the bathochromic absorption shift in bacteriorhodopsin.*

Despite the above conclusions, on a finer level FTIR difference spectroscopy reveals that the proline mutants exhibit several defects in the protein and chromophore structural alterations that normally occur during the bR photocycle. For example, changes in the HOOP vibrations indicate that the configuration of the chromophore in the K intermediate is altered in all of the proline mutants except P50G. Structural changes involving specific residues as well as peptide groups in the protein backbone are also altered in many of these mutants. As discussed below, this information provides clues to the overall mechanism of proton transport in bR and the role of prolines in this mechanism.

Pro-186 Is Located in the Retinal Binding Pocket and May Be Structurally Active during the bR Photocycle. In contrast to substitutions of Pro-50 and Pro-91, the Pro-186 → Val mutation and to a lesser extent the Pro-186 → Gly and Pro-186 → Ala mutations produced nonnative chromophore structures in light-adapted bR and its K and M photoproducts. Such changes are likely to reflect alterations in the protein/chromophore interaction inside the retinal binding pocket. For example, when bR is regenerated with 13-demethylretinal, the 13-cis isomer dominates, even in the light-adapted state (Gartner et al., 1983). Our result is also consistent with a recently proposed model for the retinal binding pocket which envisions that Pro-186, along with the residues Tyr-185, Trp-182, and Trp-189 from the helix F, Asp-85 and Trp-86 from the helix C, and Asp-212 from the helix G, is located close to the chromophore (Braiman et al., 1988a; Rothschild et al., 1989a,b) (cf. Figure 10). Since Pro-186 is located in this model directly adjacent to the polyene chain of the chromophore, replacement with compact residues such as Ala and Gly is expected to have much less effect on the chromo-

[3] These studies were made on bR mutants incorporated into lipid–detergent micelles.

phore structure and isomerization, as compared to a bulkier residue like valine.

However, even in the case of the Pro-186 → Val substitution, we found that a subpopulation of this mutant has a normal chromophore structure in the bR, K, and M states. This might occur if the valine side chain could exist in different conformations, some of which did not interfere with the normal structure of retinal in the protein. The fact that the level of bR → K photoalteration for P186V was much lower at 78 K than 250 K might reflect a greater flexibility of the valine side chain at the higher temperature, thereby allowing the chromophore more opportunity to find the proper configuration necessary for photoisomerization. This explanation is also supported by recent low-temperature and flash kinetic absorption measurements of Pro-186 mutants (Ahl et al., 1988, 1989).

Substitutions at Pro-186 also produced distinct effects on the normal protein structural changes that occur during the photocycle. First, the extent of secondary structural changes, as reflected by the amide I and II bands, is reduced for all three mutants. These bands have also been shown by time-resolved FTIR to be sensitive to temperature and relative humidity (Braiman et al., 1987). This may indicate that Pro-186 is important for linking chromophore isomerization to rearrangement of protein structure. The Pro-186 substitutions also appear to block or alter tyrosine changes previously attributed to Tyr-185 (Braiman et al., 1988b) during the bR → K transition and possibly alter the deprotonation of this group during M formation. Bands in the carboxyl C=O stretching region associated with a change in the environment of Asp-96 between bR and M (Braiman et al., 1988a; Gerwert et al., 1989) are also absent in P186V and P186A. Thus, while we are unable to determine if Pro-186 is itself undergoing a structural alteration during the photocycle, it is clear that its presence is required for other protein structural changes that occur as early as the formation of the K intermediate.

Are Pro-50 and Pro-91 Structurally Active during the bR Photocycle? The substitution Pro-50 → Gly produced substantial changes in the bR →K difference spectrum only in the regions assigned to proline vibrations. This indicates that Pro-50 may be structurally active during this step of the photocycle. However, in this case we would expect similar changes to occur in this region for the Pro-50 → Ala substitution. In fact, changes do occur, although not to the same extent as in P50G. In addition, changes are observed in this region for the other proline mutants. Thus, an unambiguous determination could not be made if Pro-50 is structurally active or if the changes observed are due to indirect effects of this substitution on the behavior of another proline group (see below). However, the minimal changes that the Pro-50 → Gly substitution produced in other regions of the bR → K difference spectrum indicate that Pro-50 is not absolutely necessary for normal functioning of bR. This finding is also in agreement with the near normal proton pumping and increased regeneration rate observed for this mutant (Mogi et al., 1989).

In contrast to P50G, both P91A and P91G produced changes in chromophore and protein bands including those in the proline vibrational region 1420–1440 cm^{-1}. However, it is again not possible on the basis of these data to determine if the changes we observe in the proline vibrational region are due to a direct contribution from Pro-91 or due to more indirect effects on other proline groups.

Do Buried Prolines Function Cooperatively in Response to Chromophore Isomerization? The present study indicates that all three membrane-embedded prolines play a role in the functioning of bR, although they are not essential for proton pumping. In particular, all of the mutants, except Pro-50 → Gly, produced chromophore and protein structural alterations that occur during the bR photocycle. However, we were unable to assign specific peaks in the difference spectrum to individual proline residues in contrast to earlier studies, which focused on Tyr, Asp, and Trp residues (Braiman et al., 1988a,b; Rothschild et al., 1989b). This may indicate that the proline-assigned bands in the difference spectrum arise from one of the eight prolines located in the loop regions of the protein as shown in Figure 1.

Alternatively, our results could be explained without postulating structural activity of the loop prolines if the three membrane-embedded prolines acted cooperatively. In particular, then substitution of one proline might induce a second proline to alter its normal behavior during the photocycle. Such an effect could explain why the P50A bR → K difference spectrum does not show a complete loss of proline-assigned bands, even in the case where Pro-50 is normally the only proline that is structurally active during the bR → K transition.

Cooperative behavior of prolines during the bR photocycle would be understandable if protein structural changes are driven by a change in shape of the chromophore in the binding pocket upon light-driven isomerization. This strain that chromophore isomerization imposes on the protein could be relieved gradually during the photocycle by the rotation of the different X-Pro C–N bonds of buried prolines. If any one of these prolines is replaced, then the response of the buried prolines to the chromophore isomerization could be altered. Models that show how protein structural changes can be driven by shape changes of the isomerized chromophore have been previously presented (Braiman et al., 1988a; Fodor et al., 1988).

Conclusions

FTIR difference spectroscopy and site-directed mutagenesis have been used to examine the role of specific prolines in the functioning of bR. Our results indicate that of the three membrane-embedded prolines, only Pro-186 appears to influence the structure of the chromophore and its ability to undergo a normal all-trans to 13-cis isomerization. This result is consistent with previous evidence presented that indicates Pro-186 is located in the retinal binding pocket, which also includes Tyr-185, Trp-86, Trp-182, and Trp-189 (Braiman et al., 1988a; Rothschild et al., 1989a,b). A similar model has recently been derived on the basis of high-resolution electron cryomicroscopy (Henderson et al., 1990). However, this model shows that the flat ring of Pro-186 is located closer to the β-ionone ring of retinal than shown in Figure 10.

While we have not yet been able to identify which buried prolines are structurally active, our evidence points to the involvement of Pro-50 in helix B along with Pro-186, which appears to be linked to conformational changes of the protein. This later result would be consistent with the Tyr-185, Pro-186 region of the F helix acting as a hinge that links chromophore isomerization with protein structural changes. It is also possible that all three buried prolines are structurally active and act cooperatively. FTIR studies on site-directed mutants of prolines in the loop regions of bR along with more selective incorporation of isotope labels in the prolines of bR will be necessary to further explore these possibilities.

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