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Vibrational Spectroscopy of Bacteriorhodopsin Mutants: Chromophore Isomerization Perturbs Tryptophan-86[†,‡]

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ABSTRACT: Fourier transform infrared difference spectra have been obtained for the bR → K and bR → M photoreactions of bacteriorhodopsin mutants with Phe replacements for Trp residues 10, 12, 80, 86, 138, 182, and 189 and Cys replacements for Trp residues 137 and 138. None of the tryptophan mutations caused a significant shift in the retinylidene C=C or C—C stretching frequencies of the light-adapted bR_{570} state. Since these frequencies are known to be strongly correlated with the visible absorption maximum of the chromophore, it is concluded that none of the tryptophan residues are essential for forming a normal bR_{570} chromophore. However, a 742-cm^{-1} negative peak attributed previously to the perturbation of a tryptophan residue during the bR → K photoreaction was found to be absent in the bR → K and bR → M difference spectra of the Trp-86 mutant. On this basis, we conclude that the structure or environment of Trp-86 is altered during the bR → K photoreaction. All of the other Trp → Phe mutants exhibited this band, although its frequency was altered in the Trp-189 → Phe mutant. In addition, the Trp-182 → Phe mutant exhibited much reduced formation of normal photoproducts relative to the other mutants, as well as peaks indicative of the presence of additional chromophore conformations. A model of bR is discussed in which Trp-86, Trp-182, and Trp-189 form part of a retinal binding pocket. One likely function of these tryptophan groups is to provide the structural constraints needed to prevent chromophore photoisomerization other than at the C_{13}=C_{14} double bond.

Understanding light-driven proton transport in bacteriorhodopsin (bR)[1] remains an important problem in biology and biophysics. This 26 000-dalton protein found in the purple membrane (PM) of *Halobacterium halobium* contains a retinylidene chromophore. Due to bR's relative simplicity, it has been extensively investigated by using a wide variety of biochemical and biophysical techniques (Stoeckenius & Bogomolni, 1982). Recent success in the isolation of site-directed mutant forms of bR (Nassal et al., 1987; Braiman et al., 1987) has made it possible to study the role of individual residues in the bR proton pump mechanism (Hackett et al., 1987; Mogi et al., 1987, 1988, 1989; Khorana, 1988; Ahl et al., 1988).

Several studies have focused on the possible involvement of tryptophan residues in bR proton pumping and color regulation [e.g., Maggiora and Schowen (1977)]. Fluorescence and NMR techniques have detected tryptophan residues interacting

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[1] Abbreviations: PM, purple membrane; bR, bacteriorhodopsin; bO, bacterioopsin; hR, halorhodopsin; FTIR, Fourier transform infrared; au, absorbance units; HOOP, hydrogen out-of-plane.

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closely with the retinylidene chromophore (Sherman, 1982; Kalisky et al., 1981; Acuna et al., 1984; Polland et al., 1986; Arseniev et al., 1987). Time-resolved fluorescence spectroscopy has revealed that some of these tryptophans may undergo a change in environment during the lifetime of the L and M intermediates (Fukumoto et al., 1981). Low-temperature UV–visible absorption difference spectroscopy has detected tryptophan alterations at an earlier stage, during the formation of the K intermediate (Ahl et al., 1988). However, it is unclear from these studies which tryptophan residues interact directly with the retinal chromophore and undergo a change in environment during the bR photocycle.

In a recent study utilizing site-directed mutagenesis, it was found that of all eight Trp residues in the bR sequence only replacement of Trp-86, Trp-137, Trp-182, and Trp-189 by Phe resulted in significant alterations in the λ_{max} of the chromophore (Hackett et al., 1987; Mogi et al., 1989).[2] In addition, low levels of chromophore regeneration and proton pumping were observed for the W137F mutant, while W86F also showed a significant reduction in proton pumping (Mogi et al., 1989). The above-mentioned work as well as a recent low-temperature UV–visible absorption study of Trp-182 → Phe and Trp-189 → Phe substitutions (Ahl et al., 1988) suggests that Trp-86, Trp-182, and Trp-189 all interact directly with the retinal chromophore.

It has been demonstrated by several groups that Fourier transform infrared (FTIR) difference spectroscopy can be used to probe structural alterations in bacteriorhodopsin (Rothschild et al., 1981; Rothschild & Marrero, 1982; Bagley et al., 1982; Siebert & Mäntele, 1983; Braiman & Rothschild, 1988). In combination with isotopic labeling, specific amino acids involved in the photocycle can be identified such as tyrosine (Rothschild et al., 1986; Dollinger et al., 1986a) and aspartic and glutamic acids (Engelhard et al., 1985; Dollinger et al., 1986b; Eisenstein et al., 1987). We report here on the use of FTIR difference spectroscopy in combination with site-directed mutagenesis to probe structural changes in individual tryptophan residues in the bR primary sequence. This approach has also been recently applied by us to investigate tyrosine (Braiman et al., 1988b) and aspartic acid (Braiman et al., 1988a) residues in bR. The method requires the assignment of peaks in the FTIR difference spectra to particular amino acids on the basis of isotope labeling. In the case of tryptophan, this has been recently accomplished by incorporation of L-[$^2H_5$]tryptophan into bR (Roepe et al., 1988a). FTIR difference spectroscopy of this isotopically labeled bR permitted assignment of several bands in the difference spectra of the bR → K, bR → L, and bR → M photoreactions to the in-phase, out-of-plane rocking vibration of indolyl hydrogens in tryptophan.

In particular, the presence of such a band in the bR → K difference spectrum reflects a tryptophan residue undergoing a change in its local environment during the primary phototransition (bR → K). Our present study has enabled us to identify this residue as Trp-86. Trp-86 also appears to interact (directly or indirectly) with Asp-85 and Tyr-185, whose vibrations were recently detected in two related studies (Braiman et al., 1988a,b). Substitutions at Trp-182 and Trp-189 also caused distinct effects. The Trp-182 → Phe substitution led to a substantial decrease in photocycling, while FTIR spectra of the Trp-189 → Phe mutant indicated that Trp-189 interacts with Trp-86.

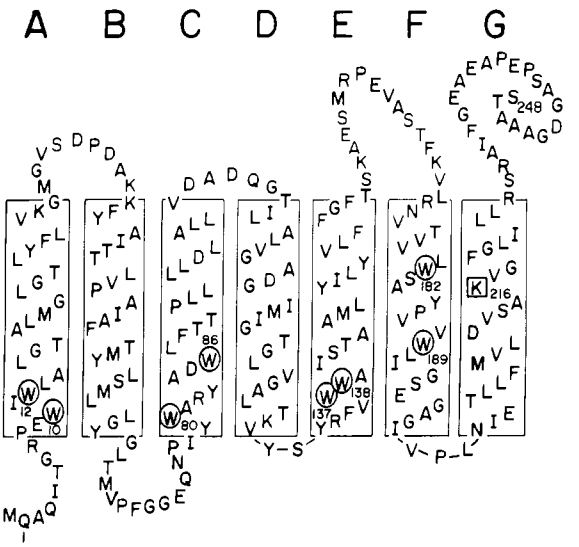

FIGURE 1: Folding model of bR showing mutated Trp residues (circled) and Lys-216, site of attachment of retinal chromophore (boxed).

This evidence, along with additional information about the effects of substitutions at Trp-182 and Trp-189 on the low-temperature UV–visible absorption (Ahl et al., 1988), supports a 3-dimensional structural model for a bR retinal binding pocket that includes the residues Trp-86, -182, and -189. However, none of the Trp residues in bR appear to be responsible for the bathochromic shift of the bR retinylidene chromophore.

Methods

Preparation of bR Mutant Samples. The construction of Trp mutants (Figure 1) has been described elsewhere (Mogi et al., 1989). The mutants were purified from crude membranes of *Escherichia coli* as described previously (Braiman et al., 1987). PM-like vesicles were reconstituted from bacterioopsin and *all-trans*-retinal with polar lipids from *H. halobium* by using a protein:lipid ratio of 1:1 by weight (Popot et al., 1987). This procedure generally resulted in a species with a bR-like chromophore, with the exception that in the case of W137F[3] the preparation appears to contain principally a denatured form as indicated by a λ_{max} at 380 nm, close to that of free retinal. The W137C mutant was therefore used to investigate the role of Trp-137. Wild-type bacteriorhodopsin expressed in *E. coli* (e-bR) prepared in the manner described was found to give FTIR difference spectra nearly identical with those of native bR in purple membrane (Braiman et al., 1988a,b).

FTIR Difference Spectroscopy. Difference spectra were recorded for the bR → K and bR → M photoreactions at 77 and 250 K, respectively, by using methods previously reported (Roepe et al., 1987; 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 in a humid atmosphere. The sample was inserted into a sealed transmittance cell that was then 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

[2] These shifts in λ_{max} were dependent on the use of lipid/detergent micelles for reconstitution. Less significant shifts are observed for these mutants incorporated into protein/lipid vesicles (see Discussion).

[3] Designations for bR mutants make use of the standard one-letter abbreviations for amino acids. Thus, "W138F" signifies the mutant in which the tryptophan at residue 138 has been replaced by phenylalanine, while "W138C" signifies replacement of the same residue by cysteine.

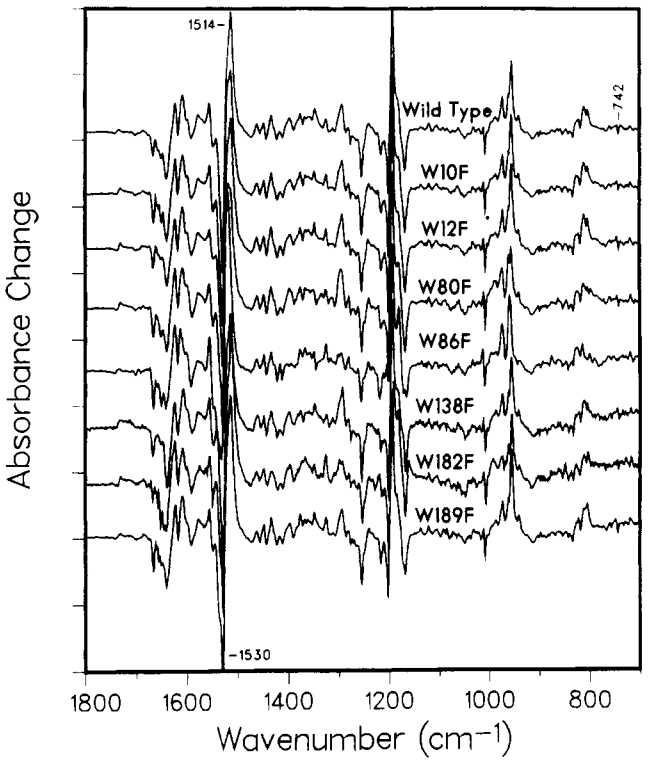

FIGURE 2: FTIR difference spectra of bR → K photoreaction at 77 K for wild-type bR and seven Trp → Phe mutants. All spectra were recorded by using procedures previously reported (Roepe et al., 1987a; Braiman et al., 1988b). Spectral resolution was 2 cm^{-1}, and each spectrum represents the average from at least 15 pairs of 20-min scans.

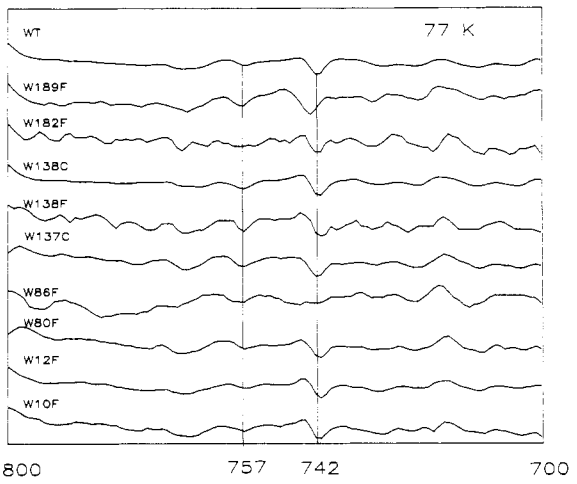

FIGURE 3: Expanded view of spectra from Figure 2 in the 700–800-cm^{-1} region, with the addition of the Trp → Cys mutants W137C and W138C.

(Madison, WI) 60SX spectrometer.

Results

FTIR difference spectra of the bR → K and bR → M photoreactions for wild-type bR and bR tryptophan mutants are shown in Figures 2–5 (the mutants W137C and W138C are shown only in Figures 3 and 5). All of the mutants, with the exception of W86F, produced difference spectra generally similar to those of wild type. In the case of W86F, substantial changes were observed, which appear to arise in part from the presence of a species similar to (although not identical with) bR_{548}, the 13-cis component of dark-adapted bR (Figure 6). W182F was unusual because of reduced peak intensities in the bR → K and bR → M difference spectra relative to the other mutants, indicative of a lowered photoalteration for this

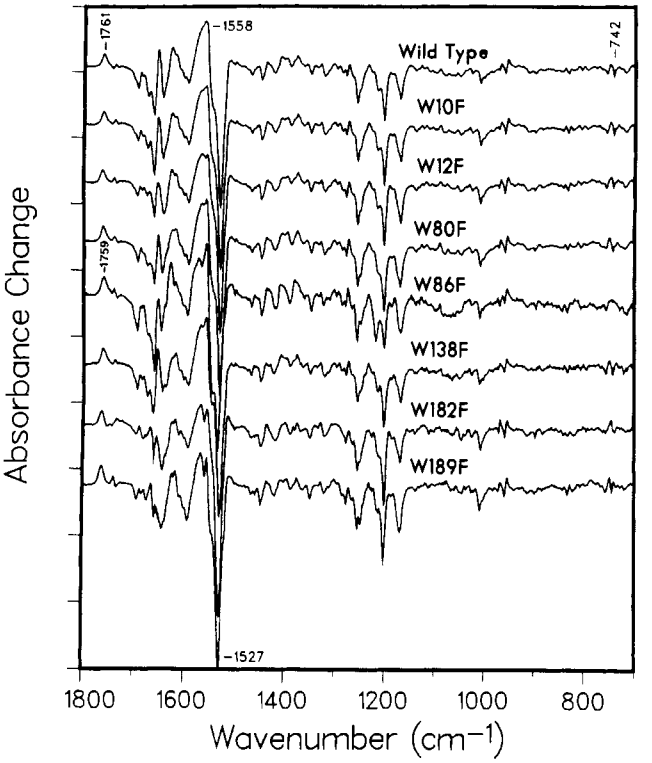

FIGURE 4: FTIR difference spectra of bR → M photoreaction at 250 K for wild-type bR and seven Trp → Phe mutants. Spectral resolution was 2 cm^{-1}, and each spectrum represents the average of at least 15 scans of 20-min duration.

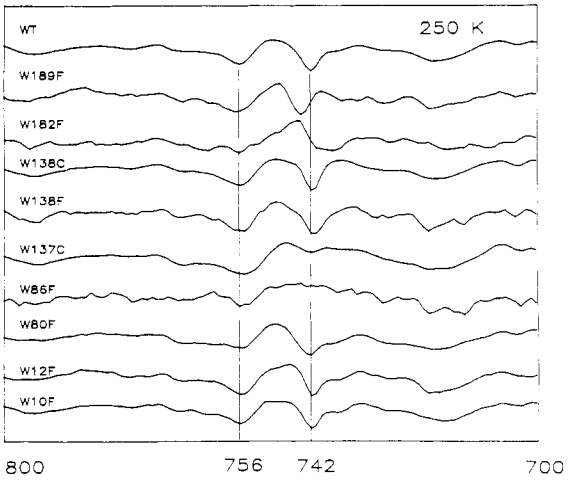

FIGURE 5: Expanded view of spectra from Figure 4 in the 700–800-cm^{-1} region with the addition of mutants W137C and W138C.

mutant under the conditions used. Some nonnative configurations of the retinal chromophore were also detected with this mutant and to a lesser degree with the mutants W80F, W137C, and W189F. However, these differences appear to be due in part to an increased level of the dark-adapted species, bR_{548}, and its photoproducts.

bR → K Photoreaction

As seen in Table I, the frequencies of bands assigned to the C—C and C=C stretching modes of the retinylidene chromophore of light-adapted bR (bR_{570}) in the mutants are very close to those found in wild-type bR. However, W86F and W182F exhibited small but significant shifts. Since the frequencies of these C—C and C=C stretching bands are dependent on the chromophore configuration and are correlated closely with the visible absorption maximum of bR (Smith et al., 1987a,b), all of the Trp mutants must be capable of

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
W010F	1639.2	1529.7	1276.6	1254.9	1215.9	1202.4	1168.6	741.9
W012F	1639.2	1529.7	1277.1	1254.9	1216.3	1201.9	1168.6	741.5
W080F	1639.7	1529.7	1276.6	1254.9	1216.3	1202.4	1169.1	741.5
W086F	1641.1	1532.1	n.o.[c]	1255.9	1218.8	1201.9	1166.2	n.o.[c]
W137C	1639.7	1530.2	1276.6	1254.4	1215.4	1201.9	1168.1	742
W138C	1639.7	1529.7	1276.6	1254.4	1216.3	1201.9	1167.7	742
W138F	1639.2	1528.8	1276.6	1254.4	1216.8	1201.9	1167.7	742
W182F	1640.2	1528.3	1276.6	1254.4	1215.9	1201.4	1168.1	742
W189F	1640.1	1529.7	1276.6	1254.9	1216.8	1202.8	1168.2	743.4

[a] C—O⁻ stretching mode of tyrosinate-185. [b] In-phase HOOP mode of Trp-86 at 742 cm^{-1}. [c] n.o., not observed.

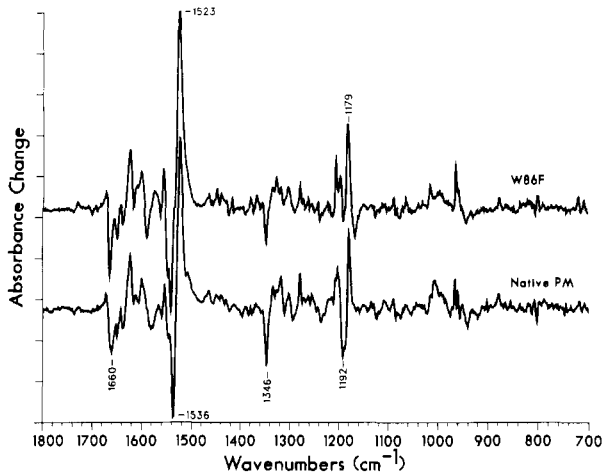

FIGURE 6: Comparison of the $bR_{548} \rightarrow K_{610}$ difference spectrum for native purple membrane (bottom) [see Roepe et al. (1988b) for details] and the nonnative component of the W86F difference spectrum (top). Both spectra were recorded at 77 K. The top spectrum was produced by interactively subtracting a difference spectrum of W86F derived by using the low-temperature light-adaptation procedure, which favored production of the normal light-adapted species, from a difference spectrum of W86F obtained without this procedure, which favored the dark-adapted bR_{548} like species. The subtraction was performed so that the 1530-cm^{-1} negative band in the difference, which reflects the normal light-adapted bR species, disappeared.

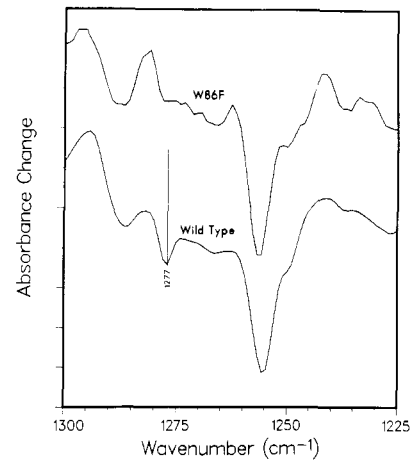

FIGURE 7: Expanded view of the bR → K difference spectrum of W86F and wild-type bR in the 1200–1300-cm^{-1} region.

forming a pigment very similar to bR_{570}.

W86F exhibits the largest spectral deviations from e-bR. The normal procedure of cooling the sample to 77 K immediately after light adaptation produced a difference spectrum which resembled that observed for the dark-adapted bR primary photoreaction (Roepe et al., 1988b) rather than the bR → K difference spectra usually obtained (Figure 6). This observation may be related to the presence of major shifts in the λ_{max} of this mutant in lipid/detergent micelles at room temperature (Mogi et al., 1989; see also footnote 2). However, a more normal (light-adapted) difference spectrum could be obtained if the sample was initially cooled to 250 K and cycled through the M intermediate by using alternate 20-min periods of yellow illumination followed by no light prior to cooling to 77 K. This "low-temperature light adaptation" procedure apparently traps the W86F mutant in a form similar to bR_{570}.

Spectra of the mutants W80F, W137C, W138C, W138F, W182F, and W189F also exhibited evidence of some increase in the level of bR_{548}. This was most pronounced in the case of W182F. This is visually evident in resolution-enhanced difference spectra of these mutants (data not shown) that reveal negative peaks characteristic of bR_{548}, e.g., bands at 1536 cm^{-1} (C=C stretch), 1346 cm^{-1} (NH bend), and 800 cm^{-1} (NH HOOP) (Smith et al., 1987b). Positive bands characteristic of the primary photoproduct of bR_{548} (Roepe et al., 1988b) are also present at 1522 cm^{-1} (C=C stretch) and 1183 cm^{-1} (C—C stretch). As can be seen from Table I, W182F also exhibited small shifts in the C=C (1528 cm^{-1}) and C—C (1201 cm^{-1}) stretching modes relative to the wild type.

The Trp-182 → Phe substitution was also distinguished by the unusually low photoalteration under conditions used to observe the bR → K phototransition. The peak intensities were only 10% of that observed for wild type. This may be due to the presence of altered forms of this mutant that do not photocycle. A similar conclusion has been reached recently on the basis of a photokinetic study of this mutant (Ahl et al., 1989).

Assignment of Peaks to Trp-86. Wild-type bR and all of the mutants except W86F displayed a pair of peaks at 742 cm^{-1} (negative) and 745 cm^{-1} (positive) (Figure 3). These peaks have previously been assigned on the basis of isotopic labeling of PM to a tryptophan residue that is perturbed during the bR → K primary photoreaction (Roepe et al., 1988a). Thus, the absence of these peaks *only* in the W86F bR → K difference spectrum indicates that the residue being perturbed is Trp-86. It is important to note that the absence of this peak cannot be attributed to the presence of a form of W86F similar in behavior to bR_{548}, since even in the samples where this form was minimized by repeated cycling at 250 K the 745-cm^{-1} band was absent.

Indirect Effects Involving Tyr-185 and Trp-189. A negative peak at 1277 cm^{-1} is present in wild-type bR and all of the Trp mutants except W86F (Figures 2 and 7 and Table I). This band is sensitive to isotopic substitutions of tyrosine (Rothschild et al., 1986) but not tryptophan (Roepe et al., 1988a). In a previous study of tyrosine mutants (Braiman et al., 1988b), this peak was assigned to the C–O⁻ stretching vibration of Tyr-185, which exists in an ionized state in bR and undergoes a protonation when the K intermediate is formed (Rothschild

et al., 1986). This band is still absent in the W86F mutant under conditions where the bR_{548}-like species is minimized (Figure 6). We cannot, however, exclude the possibility that the band is shifted to another region. A second band at 833 cm^{-1} that is assigned to a Fermi resonance of tyrosine (Rothschild et al., 1986) is also diminished but not absent in the W86F difference spectrum. Thus, replacement of Trp-86 with Phe appears to affect indirectly the alterations that Tyr-185 undergoes during the bR → K reaction.

Small frequency upshifts of 2–3 cm^{-1} in the 742-cm^{-1} band were observed for the W189F mutant (Figure 3). In view of our assignment of these peaks to Trp-86, this suggests some form of interaction between Trp-189 and Trp-86. A similar influence of the Trp-189 substitution on the peaks assigned to Trp-86 was observed for the bR → M transition (see below).

bR → M Photoreaction

All Tryptophan Mutants Form an M Intermediate. As seen in Figure 4, the bR → M difference spectra of all of the mutants except W86F were generally similar to that of wild-type bR. Only the W86F mutant exhibited significantly altered spectral features; however, these altered features were observed to diminish after repeated photocycling at 250 K. In particular, a negative band appears near 1540 cm^{-1}, which most likely arises from the ethylenic C=C stretch mode of the same bR_{548}-like species observed in the bR → K photoreaction. Along with this peak, a second band appears near 1532 cm^{-1}, closer to the normal C=C stretching frequency for bR_{570}. Under conditions where the 1540-cm^{-1} band is reduced in size, the difference spectra obtained at 250 K resembled normal bR → M difference spectra obtained from native bR. Thus, all of the Trp mutants can form an M intermediate with a conformation similar to that of wild-type M.

Assignment of Bands in the Tryptophan Region. Wild-type bR and all of the mutants except W86F and W137C exhibited a clear negative band at 742 cm^{-1} in the bR → M difference spectrum. In the case of W137C, the band still may be present at a reduced intensity (Figure 5). Since only W86F exhibited a loss of this band in *both* the bR → K and bR → M difference spectra, the simplest explanation is that the band arises directly from vibrations of Trp-86 and that its weakening in the bR → M spectrum of W137C is due to a secondary effect.

A second negative peak at 755 cm^{-1} was also assigned on the basis of isotope labeling (Roepe et al., 1988a) to a tryptophan residue. While this peak appears to be weaker in many of the mutants, especially W182F (cf. Figure 5), it does not disappear completely. However, a careful examination of the effects of Trp isotope labeling on this region in the bR → M difference spectrum shows that there exists a label-insensitive component to this 755-cm^{-1} peak (K. J. Rothschild and Yi-Wu He, unpublished results). Thus, it is likely that the 755-cm^{-1} peak does not disappear completely in any of the mutants because a component of the peak arises from a non-tryptophan vibration in the bR → M transition. It was not possible, however, to conclusively assign the label-sensitive component of the 755-cm^{-1} peak to a specific tryptophan residue since this band is weak to begin with and diminishes in intensity in several of the mutants. Several other smaller bands in this region, particularly near 736 cm^{-1}, also appear to be altered in some of the mutants, as well as in bR containing isotope-labeled tryptophan. Thus, it is likely that this region of the spectrum contains contributions from tryptophan residues in addition to Trp-86 and that these other Trp groups therefore undergo some structural perturbations during the bR photocycle. On the basis of our data, the most likely candidates are Trp-137, Trp-138, Trp-182, and Trp-189.

Secondary Effects. In contrast to the case of the bR → K photoreaction, a negative peak at 1277 cm^{-1} is present in the bR → M difference spectrum of W86F along with a second peak at 1272 cm^{-1}. These peaks have previously been assigned to protonation changes in Tyr-185 during the bR → K and L → M steps of the bR photocycle (Braiman et al., 1988b). Thus, the Tyr-185 protonation change that was altered at 77 K in W86F is no longer altered at 250 K. We also found that W86F was the only mutant that produced a small (2 cm^{-1}) downshift in the 1760-cm^{-1} vibration assigned previously to the protonation of Asp-85 (Braiman et al., 1988a). This evidence, which is discussed further below, suggests that some interaction exists between Trp-86 and Asp-85, as might be expected because of their proximity in the bR primary protein sequence. Finally, the W189F mutant produced an upshift in the frequency of the 742-cm^{-1} band, which again suggests an interaction between Trp-189 and Trp-86.

Discussion

In the present study, we were able to probe the effect of tryptophan substitutions on the structure of the chromophore, on the ability of these mutants to produce K and M intermediates, and on other specific residues involved in the bR mechanism whose vibrations have been previously assigned in the FTIR difference spectra.

Formation of Light-Adapted bR with a Normal Chromophore Structure Was Possible for All Trp Mutants. All of the Trp mutants displayed a negative ethylenic (C=C stretch) band near 1529 cm^{-1} in the bR → K and bR → M difference spectra. This is the same frequency observed in the wild-type spectrum. The largest deviation was found for W86F and W182F, which exhibited a 2-cm^{-1} upshift and 1.5-cm^{-1} downshift, respectively (cf. Table I). Since an empirical linear correlation exists between the $\nu_{C=C}$ and λ_{max} of bR and its photointermediates (Doukas et al., 1978), all of the mutants appear to form a light-adapted species with a visible absorption λ_{max} near 570 nm. In the case of W182F and W189F, visible absorbance measurements at low temperature have confirmed that these mutants have λ_{max} values very close to that of wild-type bR (Ahl et al., 1988).

We also found that the frequencies of the major bands previously assigned to vibrations of the retinylidene chromophore were not significantly affected by the nine Trp substitutions examined (cf. Table I). The largest shifts, less than 3 cm^{-1}, were found for W86F and W182F. Resonance Raman studies indicate that the frequencies of chromophore vibrational modes, particularly those in the 1100–1300-cm^{-1} fingerprint region, are very sensitive to retinal configuration (Smith et al., 1987a,b). *Thus, the configuration of retinal in the photochemically active fraction of these mutants under low-temperature conditions must be the same as in native bR_{570}, i.e., all trans.*

Trp-86 Undergoes a Structural Alteration during the bR → K Phototransition. The assignment of the 742-cm^{-1} peak in the bR → K difference spectrum to Trp-86 indicates that this residue undergoes a change in its local environment or structure during the bR → K primary photoreaction. Previously, FTIR difference spectroscopy combined with site-directed mutagenesis led us to infer a protonation change of Tyr-185 (Braiman et al., 1988b) and a perturbation of Asp-115 (Braiman et al., 1988a) during this early step of the photocycle.

Our results also suggest that Trp-86 interacts with residues Asp-85, Tyr-185, and Trp-189. The W189F mutation caused detectable shifts in the frequency of the 742-cm^{-1} negative peaks in both the bR → K and bR → M difference spectra.

This presumably reflects an alteration in the state of the local environment of the Trp-86 residue in bR_{570} due to the replacement of Trp-189 by Phe. This could arise, for example, if a change occurred in hydrogen bonding of the indolyl nitrogen of Trp-86. The disappearance of the 1277-cm^{-1} tyrosinate band in W86F in the bR → K (but not bR → M) difference spectrum suggests an influence of Trp-86 on Tyr-185. Similarly, the shift in the band at 1760 cm^{-1} in the W86F bR → M difference spectrum assigned recently to Asp-85 suggests some interaction exists between residues 85 and 86. However, it is not possible to ascertain on the basis of the present data if these are effects due to direct interactions between residues or secondary effects resulting from long-range interactions involving, for example, altered secondary structure or side-chain packing.

Evidence for Interaction of Trp-182 and Trp-189 with the Retinal Chromophore. A recent study revealed that the substitution of Trp-182 and Trp-189 with Phe resulted in changes in the properties of bR (Mogi et al., 1989). An earlier study of bR F-helix mutants in lipid/detergent micelles also revealed large blue shifts for Phe replacements of Trp-182 and Trp-189 (Hackett et al., 1987). In contrast, recent low-temperature UV–visible absorption measurements on humidified membrane films of Trp-182 and Trp-189 mutants with *H. halobium* polar lipids (Ahl et al., 1988) revealed much smaller shifts of λ_{max}. However, alterations in the band shape of the visible bR → K and bR → M depletions and positive UV peaks indicated that a subpopulation of the protein and/or retinal becomes trapped in altered configurations, producing pigments with different absorption maxima. This led to the suggestion that Trp-182 and -189 residues are not directly responsible for the bathochromic shift of retinal but instead help restrict the allowed conformations of retinal in its binding pocket. In view of the larger λ_{max} shifts observed for these mutants in lipid/detergent micelles, it is plausible that replacement of these two Trp residues produces a conformational flexibility of the retinal in the binding pocket that is greatest when the constraints associated with the 2-dimensional lattice are also removed.

The present FTIR results support the idea that Trp-182 and Trp-189 are located inside a retinal binding pocket (see discussion below). In the case of Trp-189, peaks in the W189F spectrum indicate the presence of a bR_{548}-like (13-cis) species under light-adapted conditions where normally only the bR_{570} (all-trans) species is observed. As discussed, the W189F mutation also affects the 742-cm^{-1} peak assigned to Trp-86. Both of these observations can be explained if Trp-189 is located close to Trp-86 and also influences (directly or indirectly) the configuration of retinal. In the case of W182F, the great reduction in photocycling after prolonged illumination and the appearance of new peaks in the difference spectrum are both consistent with a trapping of a nonnative species. In addition, small shifts were observed in the frequency of the bR_{570} chromophore. Again, direct interaction of Trp-182 with retinal could account for these findings.

Model for Tryptophan/Retinal Interactions. A 3-dimensional model (Figure 8) for the interaction of retinal with the putative C, F, and G helices in bR provides a basis to explain our present results. In a previous paper (Braiman et al., 1988a), we used this same structural model as a framework for a proton transport mechanism involving the protonation changes deduced by FTIR spectroscopy of Asp-85, -96, and -212 as well as Tyr-185 (Braiman et al., 1988b). Additional support for the arrangement of the F and G helix has been found (Rothschild et al., 1989). In the context of our present

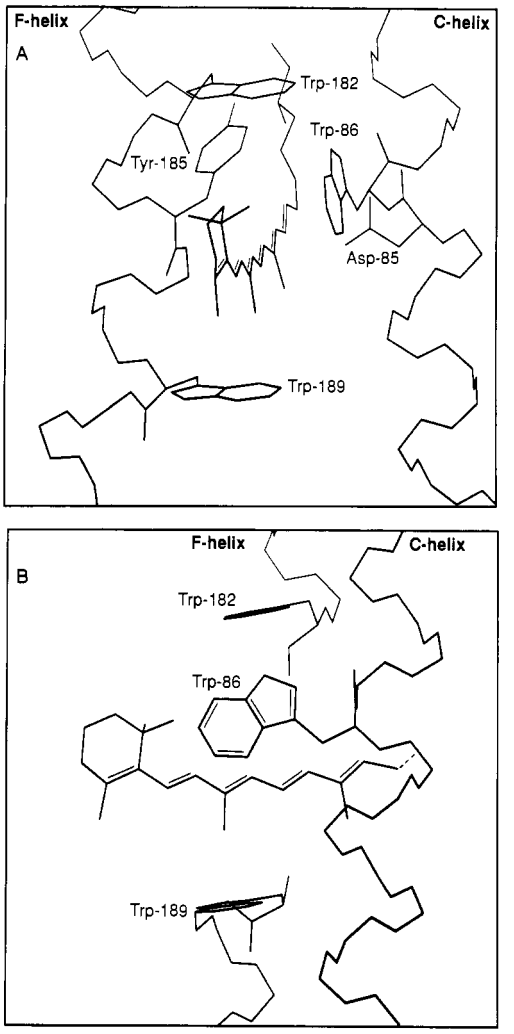

FIGURE 8: Structural model for the interaction of Trp-86, -182, and -189 with the retinal chromophore. (A) Projection with a perspective looking down the axis of the retinylidene polyene chain with β-ionone ring out of the page. (B) Perspective viewing sideways onto the polyene plane. The positions of the C, F, and G helix residues are the same as in a proposed 3-dimensional model of the proton-pumping mechanism site (Braiman et al., 1988a). To simplify the image, helix G and parts of helix F are not shown. The retinal chromophore can also be incorporated into the proposed pocket with the polyene plane rotated 180° so that the methyl groups on C_9 and C_{13} are pointed up toward the cytoplasmic membrane surface (Rothschild et al., 1989).

results, it can be seen that the G and F helices form a retinal binding pocket. In this pocket, Trp-182 is above and Trp-189 below the polyene chain. Trp-86 on the C helix is in contact with the polyene chain close to the C_{13}=C_{14} double bond. With this model, the relative positions of the C, F, and G helices as projected onto the membrane plane are close to those proposed recently (Mogi et al., 1988) on the basis of the effects of site-directed mutations on proton pumping and chromophore formation. The in-plane positioning is also consistent with the results of neutron diffraction studies (Heyn et al., 1988). A similar model can also be constructed with the orientation of the retinal polyene plane reversed so that the methyl groups linked to C_9 and C_{12} of retinal are pointed toward the cytoplasmic side of the membrane (Rothschild et al., 1989) in agreement with recent studies (Grzesiek et al., 1989; Lin & Mathies, 1989).

The exact positioning of the G and F helices was chosen on the basis of the constraint that Tyr-185 should be able to interact directly with the Schiff base as predicted previously (Braiman et al., 1988b). The position of the C helix relative to the F and G helices was adjusted so that Asp-85 could act

as a proton acceptor from the Schiff base during the bR → M transition (Braiman et al., 1988a).[4] In addition, reprotonation of the Schiff base can be accomplished from Asp-212 as suggested (Braiman et al., 1988a). This model is consistent with the chromophore–protein changes as suggested by Fodor et al. (1988).

The proposed structural model of the retinal–protein interaction can account for many of our current results. The bR → K primary photoreaction involves a photoisomerization about the C_{13}=C_{14} bond of retinal (Braiman & Mathies, 1982) as well as a change in the environment of the Schiff base (Rothschild et al., 1984, 1985). Chromophore isomerization in the bR → K step would change the Trp-86 local environment, due to an alteration in electrostatic interaction with the nearby positively charged Schiff base. Alternatively, Trp-86 might be forced sterically to undergo a small displacement in response to the C_{13}=C_{14} retinal bond isomerization, giving rise to the perturbations we observe in the bR → K difference spectrum. The proximity of Trp-189 to Trp-86 in this model could also account for the observed effect of the W189F substitution on the Trp-86 vibrational mode.

The positioning of Trp-86, Asp-85, Tyr-185, and the retinal Schiff base all within 5 Å of each other is another key feature of this model. It has already been discussed above how the placement of Trp-86 and Tyr-185 near the structurally active Schiff base could explain the alterations observed for these groups during the primary phototransition [see also Braiman et al. (1988a)]. The proximity of these groups could also account for the observed effects of substitution of each one on the others. For example, as discussed above, the substitution of Trp-86 by Phe appears to affect the state of Asp-85 and Tyr-185 at several different stages of the photocycle. This might occur because the Phe residue is much smaller than Trp and could therefore alter the relative positions of groups in the active site.

An important aspect of this model is the positioning of the residues Trp-86, Trp-182, and Trp-189 so as to constrain the motions of the retinal during photoisomerization. The model is consistent with the present work and with a previous study (Ahl et al., 1988), both of which indicate that replacement of any of these residues by Phe relaxes such constraints and allows new chromophore isomers to form. The conservation of all three Trp residues in the primary sequence of hR (Blanck & Oesterhelt, 1987), a protein that also undergoes a light-driven all-trans → 13-cis isomerization, supports an important role for these residues. A more complete model for a retinal binding pocket in bR that involves Trp-182, Trp-189, Pro-186, and Tyr-185 in the F and G helices has been proposed (Rothschild et al., 1989).

Conclusions

FTIR difference spectroscopy combined with protein engineering can provide an effective method for probing the role of specific residues in a protein. While an atomic resolution structural model for bR will be required for a complete description of the bR proton pump mechanism, this work and two related studies (Braiman et al., 1988a,b) have now provided information about the structure and local environment of specific Trp, Tyr, and Asp residues in bR and its photointermediates. This information provides important constraints for the construction of any model describing proton transport in bR.

[4] In the construction of this model, it was assumed that the C, G, and F helices had a normal α-helical conformation, except for the φ and ψ angles of the peptide bonds between Pro-186 and immediately adjacent residues. For these two peptide groups, we used the set of φ and ψ angles recently determined (G. Stubbs, private communication) for the peptide linkages to Pro-78 in the right radial α-helical region of the tobacco mosaic virus (TMV) coat protein (Namba & Stubbs, 1986).

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^{1}H NMR Studies of the Solution Conformations of an Analogue of the C-Peptide of Ribonuclease A†

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ABSTRACT: Two-dimensional NMR experiments have been performed on a peptide, succinyl-AETAAAKFLRAHA-NH_2, related to the amino-terminal sequence of ribonuclease A. This peptide contains 50–60% helix in 0.1 M NaCl solution, pH 5.2, 3 °C, as measured by circular dichroism. NOESY spectra of the peptide in aqueous solution at low temperatures show a number of NOE connectivities that are used to determine the highly populated conformations of the peptide in solution. Short-range $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ connectivities and medium-range $d_{\alpha\beta}(i, i + 3)$ and $d_{\alpha N}(i, i + 3)$ connectivities are detected. The pattern of NOE connectivities unambiguously establishes the presence of helix in this peptide. The magnitudes of the $^3J_{HN\alpha}$ coupling constants and the intensities of the $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ NOEs allow the evaluation of the position of the helix along the peptide backbone. These data indicate that the amino terminus of the peptide is less helical than the remainder of the peptide. The observation of several long-range NOEs that are atypical of helices indicates the presence of a high population of peptide molecules in which the first three residues are distorted out of the helical conformation. The absence of these NOEs in a related peptide, RN-31, in which Arg 10 has been changed to Ala, suggests that this distortion at the amino-terminal end of the peptide arises from the formation of a salt bridge between Glu 2 and Arg 10. We propose that the conformational ensemble of RN-24 in solution includes three principal conformations: a set of extended conformations, a set of largely helical conformations, and a set of conformations that contain a salt bridge between the side chains of Glu 2 and Arg 10.

According to the framework model of protein folding, elements of secondary structure form early in the folding process. These interact and become mutually stabilizing, and the tertiary structure of the protein is built up from the sum of these interactions (Kim & Baldwin, 1982). The factors that affect the formation and localization of secondary structure are clearly important in directing the early stages of protein folding. Short- and medium-range interactions (along the peptide chain and within a given element of secondary structure) should predominate over long-range or tertiary interactions (between different elements of secondary structure) in these early stages. This prediction has made linear

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