Vibrational Spectroscopy of Bacteriorhodopsin Mutants

EVIDENCE THAT THR-46 AND THR-89 FORM PART OF A TRANSIENT NETWORK OF HYDROGEN BONDS

(Received for publication, July 22, 1991)

Kenneth J. Rothschild‡, Yi-Wu He‡, Sanjay Sonar‡, Thomas Marti¶, and H. Gobind Khorana¶
From the ‡Department of Physics and Program in Cellular Biophysics, Boston University, Boston, Massachusetts 02215 and the ¶Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The role of Thr-46 and Thr-89 in bacteriorhodopsin photocycle has been investigated by Fourier transform infrared difference spectroscopy and time-resolved visible absorption spectroscopy of site-directed mutants. Substitutions of Thr-46 and Thr-89 reveal alterations in the chromophore and protein structure during the photocycle, relative to wild-type bacteriorhodopsin. The mutants T89D and to a lesser extent T89A display red shifts in the visible λmax of the light-adapted states compared with wild type. During the photocycle, T89A exhibits an increased decay rate of the K intermediate, while a K intermediate is not detected in the photocycle of T89D at room temperature. In the carboxyl stretch region of the Fourier transform infrared difference spectra of T89D, a new band appears as early as K formation which is attributed to the deprotonation of Asp-96. Along with this band, an intensity increase occurs in the band assigned to the protonation of Asp-212. In the mutant T46V, a perturbation in the environment of Asp-96 is detected in the L and M intermediates which corresponds to a drop in its pKa. These data indicate that Thr-89 is located close to the chromophore, exerts steric constraints on it during all-trans to 13-cis isomerization, and is likely to participate in a hydrogen-bonding network that extends to Asp-212. In addition, a transient interaction between Thr-46 and Asp-96 occurs early in the photocycle. In order to explain these results, a previously proposed model of proton transport is extended to include the existence of a transient network of hydrogen-bonded residues. This model can account for the protonation changes of key amino acid residues during the photocycle of bacteriorhodopsin.

Bacteriorhodopsin (bR) is the light-driven proton pump

* This research was supported by National Science Foundation Grant DMB-8806007, National Institutes of Health Grant EY05499, and ONR Grant N00014-88-K-0464 (to K. J. R.) and by National Institutes of Health Grant GM28289 and AI111479 and ONR Grant N00014-82-K-0189 (to H. G. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed.
‡ Recipient of a fellowship from the Swiss National Science Foundation.
§ The abbreviations used are: bR, bacteriorhodopsin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; FTIR, Fourier transform infrared; WT, wild-type; bR mutants are designated by the wild-type amino acid residue (standard one-letter code) and its position number followed by the substituted amino acid residue. Thus, T46V signifies the mutant in which threonine at position 46 has been replaced by valine.

1992 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 267, No. 3, Issue of January 25, pp. 1615-1622, 1992
Printed in U.S.A.
work now shows that substitutions of Thr-46 and Thr-89 do not alter the structure of the all-trans chromophore in the light-adapted ground state. However, the light-adapted Thr-89 mutants exhibit a red-shifted visible $\lambda_{max}$ and altered structure and stability of the K intermediate. Replacement of Thr-89 by Asp results in deprotonation of Asp-89 early in the photocycle and increased protonation of Asp-212. This suggests the existence of a proton pathway between the 2 Asp residues in T89D. In the case of T46V, the stretching frequency of the Asp-96 carboxyl group is perturbed in the L and M intermediates, indicating the formation of a Thr-46/Asp-96 hydrogen bond at this stage of the photocycle. In order to account for these findings, a model for proton transport is described which extends an earlier spectroscopically derived model. It includes as a key feature the existence of a transient network of hydrogen bonds which acts to transport a proton from Asp-96 to the Schiff base during the M to N transition.

MATERIALS AND METHODS

FTIR Measurements—Low temperature static FTIR difference measurements were made as previously reported on rehydrated films formed by air-drying an aqueous suspension of a sample on an AgCl window (27, 28). Each sample was sealed in a specially designed cell, light-adapted, and cooled to 80 K (bR→K), 170 K (bR→L), or 250 K (bR→M) (28, 29). All spectra were recorded at 2 cm$^{-1}$ resolution using a Nicolet 740 spectrometer equipped with an MCT detector. As described in Ref. 55, photoreversal from K to bR of T89D was accomplished using light filtered by a 660-nm interference filter in contrast to the other samples including wild-type bR where a 628-nm narrow band interference filter was used.

Time-resolved Absorption Spectroscopy—Time-resolved spectra were obtained with a 1420 UV-enhanced optical multichannel analyzer and model 1460 controller (Princeton Applied Research, Princeton, NJ), interfaced with a 340S monochromator (Spex Industries, Edison, NJ) using procedures which have been previously reported (25).

Sample Preparation—The construction, expression, and purification of the bacterioopsin mutants T46V, T89A, and T89D has been previously reported (26). Apoproteins were regenerated with retinal window (27, 28). Each sample was sealed in a specially designed cell, and reconstituted in vesicles with polar lipids from ton, NJ), interfaced with a 340s monochromator (Spex Industries, Edison, NJ) using procedures which have been previously reported (25).

RESULTS

Substitutions of Thr-89 Perturb the K Chromophore—Both low temperature FTIR difference spectroscopy and room temperature time-resolved visible absorption spectroscopy indicate that the substitutions T89A and T89D perturb the structure of the K intermediate.

Fig. 1 shows the bR→K FTIR difference spectra of wild-type bR (WT) and the mutants T46V, T89A, and T89D recorded at 80 K. Bands in the wild-type bR difference spectrum have been previously assigned to vibrational modes of the chromophore of bR$_{30}$ (negative bands) and the K$_{30}$ intermediate (positive bands) by comparison with the corresponding resonance Raman spectra (21-23, 31, 32). The negative chromophore bands in the T46V, T89A, and T89D difference spectra appear at very similar frequencies to the wild-type spectrum, particularly in the conformationally sensitive C-C stretching region from 1150 to 1300 cm$^{-1}$. These results indicate that the chromophore structure in the light-adapted state of the T46V, T89D, and T89A mutants is largely the same as in wild-type bR.

In contrast, shifts in the positive bands assigned to the K chromophore of both T89A and T89D indicate that alterations occur in the K chromophore structure relative to wild-type bR. For example, the frequency of the hydrogen-out-of-plane mode is shifted from 957 cm$^{-1}$ in wild-type bR to 950 cm$^{-1}$ in both T89D and T89A. The intensity of the 1184 cm$^{-1}$ shoulder is increased in T89D relative to wild-type bR. Both these features are characteristic of the L intermediate chromophore, suggesting that the Thr-89 substitutions remove some of the constraints which normally prevent the K$_{30}$ chromophore from adopting a more relaxed 13-cis configuration (33).

In order to further investigate the properties of the K intermediates of T89D and T89A, we measured the time-resolved visible absorption difference spectra of these mutants at room temperature. Surprisingly, the positive band near 600 nm, which appears in the difference spectrum of wild-type bR (Fig. 2A) recorded during the first 200 ns, is not observed in T89D (Fig. 2C). In contrast, a K intermediate was detected in T89A, although it had a faster decay time of 280 ns compared to 500 ns for wild-type bR (Fig. 2, A and B).

The most likely explanation for the above results is that Thr-89 does not play a major role in determining the configuration of the all-trans chromophore in light-adapted bR but comes into direct contact with the chromophore during all-trans→13-cis isomerization. The proximity of Thr-89 to retinal (12) supports this picture. It is also interesting to note that the negative-positive bands at 1423/1429 cm$^{-1}$ assigned to structural changes involving the Xaa-Pro C–N peptide bond (34) are absent in the T89D bR→K, bR→L, and bR→M difference spectra. Thus, it is possible that an interaction occurs between the chromophore and Thr-89 during chromophore isomerization, which acts as a trigger for a protein conformational change involving 1 or more proline residues. Several other residues in helix C appear to also be structurally active or play a critical role during the photocycle including Asp-85 (5), Trp-86 (35), Asp-96 (5, 6, 17, 18), and possibly Leu-93 (36). We also found that the T46V mutation had no significant effects on the structure of the K intermediate at either low temperature, as determined by the similarity of the T46V bR→K difference spectrum with wild-type bR (Fig. 1), or at room temperature, based on the normal time-resolved visible absorption difference spectra (data not shown).

T89A and T89D Have Red-shifted Chromophores—The
ethylenic stretch frequency, \( \nu_{\text{C=C}} \), of the retinylidene chromophore, which appears at 1527 \( \text{cm}^{-1} \) in light-adapted bR, is downshifted in the bR\( \rightarrow \)K, bR\( \rightarrow \)L, and bR\( \rightarrow \)M low temperature difference spectra of the T89A and T89D mutants by 2 and 5 \( \text{cm}^{-1} \), respectively (Figs. 1, 3, and 5). Based on an empirical linear correlation which exists between the \( \nu_{\text{C=C}} \) and \( \lambda_{\text{max}} \) of bR and its photointermediates (32, 37), the \( \lambda_{\text{max}} \) of T89A and T89D should be red-shifted by approximately 8 and 20 \( \text{nm} \), respectively, relative to wild-type bR. The time-resolved visible bR\( \rightarrow \)M difference spectrum of T89D confirms that there exists a red-shifted \( \lambda_{\text{max}} \) for this mutant, but only by 10 \( \text{nm} \) relative to wild-type bR (Fig. 2D). No red shift could be detected in the T89A visible difference spectrum, however, this may be due to a reduced production of N\(^{5} \) which would tend to shift this band to lower wavelength. We therefore conclude that T89D and to a lesser extent T89A have red-shifted \( \lambda_{\text{max}} \) values and that the degree of the red shift may increase at lower temperature.

A red-shifted \( \lambda_{\text{max}} \) of T89D could also account for the dramatic reduction in amplitude we observed for the K\( \rightarrow \)bR difference spectrum (data not shown). Apparently, the 628-nm light normally used for photoreversal of the K intermediate back to bR is also absorbed strongly by the red-shifted bR state of T89D. In contrast, photoreversal using 660-nm light (Fig. 1) produced a more normal amplitude bR\( \rightarrow \)K difference spectrum relative to wild-type bR.

In a previous study, in which these mutants were characterized in L-a-dimyristoylphosphatidylcholine/CHAPS/sodium dodecyl sulfate micelles (26), a 6-nm red shift was observed for the dark-adapted state of T89D, whereas no red shift was noted for the light-adapted form. Furthermore, the \( \lambda_{\text{max}} \) of the light-adapted state of T89A was blue-shifted by 28 nm relative to the wild type. However, extractions of the light-adapted chromophores of these mutants in micelles indicate the presence of significant fractions of inactive cis isomers (26), which are blue-shifted, whereas in reconstituted membranes their proportion is likely to be significantly reduced. This has previously been observed for example in the tryptophan mutants W182F and W189F (38). In addition, difference spectroscopy reveals solely the \( \lambda_{\text{max}} \) of the fraction of sample which is photoactive. Thus, it is possible that the \( \lambda_{\text{max}} \) of the visible absorption spectrum of a mutant can differ from that determined by difference spectroscopy, as was observed here.

Asp-89 Deprotonates Early in the Photocycle—The bR\( \rightarrow \)L and bR\( \rightarrow \)M difference spectra of T89D exhibited alterations in the 1700-1800-\( \text{cm}^{-1} \) carboxyl stretch region relative to the corresponding wild-type difference spectra (Figs. 3–6). Most apparent is the negative band near 1753 \( \text{cm}^{-1} \) which most likely originates from the carboxyl group of Asp-89. Resolution enhancement shows that this band is superimposed on the normal set of bands previously assigned in the wild-type difference spectra to the 4 membrane-embedded Asp residues in bR (5). For example, in the T89D bR\( \rightarrow \)L difference spectrum (Figs. 3 and 4), the 1753-\( \text{cm}^{-1} \) band is superimposed on top of the pair of negative/positive bands at 1742/1748 \( \text{cm}^{-1} \) due to Asp-96 and a second set of bands at 1735/1728 \( \text{cm}^{-1} \) assigned to Asp-115 (5). The bR\( \rightarrow \)M difference spectrum of T89D (Figs. 5 and 6) displays a positive band near 1761 \( \text{cm}^{-1} \) assigned to the protonation of Asp-85. However, this band is upshifted in frequency and diminished in intensity due to partial cancellation with the strongly negative 1753-\( \text{cm}^{-1} \) band.

A second effect of the T89D mutant in the bR\( \rightarrow \)M difference spectrum (Figs. 5 and 6) is the intensification of a band at 1738 \( \text{cm}^{-1} \), near the frequency assigned to the carboxyl stretch mode of Asp-212 (5, 14). A similar effect may occur also in the bR\( \rightarrow \)L difference spectrum of T89D (Figs. 3 and 4), accounting for the reduction in intensity of the negative

---

**FIG. 2.** Time-resolved visible difference spectra of _Escherichia coli_ expressed wild-type bacteriorhodopsin and the T89A and T89D mutants. All spectra were recorded at pH 6.0. Panels A–C show difference spectra recorded during the life-time of the K intermediate of wild-type bR. Difference spectra are shown in the sequence of decreasing absorbance at 630 nm for the time points 0.1, 0.26, 0.56, 0.96, 1.55, 2.47, and 3.84 \( \mu \text{s} \) using a 100-ns data acquisition window. Panel D compares the difference spectra of wild-type bR (dotted line) and T89D (solid line) at 150 \( \mu \text{s} \) after the laser flash, using a 10-\( \mu \text{s} \) data acquisition window.

---

**FIG. 3.** Steady-state bR\( \rightarrow \)L FTIR difference spectra of wild-type bR and the T46V, T89A, and T89D mutants recorded at 170 K. The difference spectrum consisted of the difference of two spectra recorded before and after illumination with 550-nm light at 170 K. The film was then warmed to 250 K in the dark where the L intermediate was allowed to thermally decay for 20 min. The sample was then cooled back to 170 K and the process repeated. Spectral resolution was 2 \( \text{cm}^{-1} \), and each spectrum represents the average of at least 10 pairs of 20-min scans. For a more complete description see Ref. 27.
FTIR Spectroscopy of Threonine Mutants of bR

The most likely explanation for this data is that Asp-89 undergoes a partial deprotonation during the br→L and M stages of the photocycle, while Asp-212 undergoes an increased protonation. As discussed later, transfer of a proton from Asp-89 to Asp-212 can account for several of the other properties of T89D, and suggests the existence of a proton transport pathway between the 2 residues.

T46V Perturbs the Environment of Asp-96 in the L and M Intermediates—The positive/negative bands at 1748 and 1742 cm⁻¹ in the wild-type br→L and br→M difference spectra reflect a change in the environment of the Asp-96 carboxyl group (5, 6, 17). As seen in Figs. 4 and 6, the T46V mutation causes a shift in the positive component from 1748 to 1754 cm⁻¹ in both the br→L and br→M difference spectra. The negative band at 1742 cm⁻¹ also appears to be broadened and slightly upshifted in frequency. However, spectral deconvolution (data not shown) reveals that this shift is mainly due to the upshift in frequency of the overlapping positive component at 1742 cm⁻¹. Thus, the T46V substitution mainly affects the environment of the Asp-96 carboxyl group at the L and M stages of the photocycle. As discussed below, these results suggest that Thr-46 forms a hydrogen bond with Asp-96 during the early part of the br photocycle.

DISCUSSION

In this study, we have combined FTIR difference spectroscopy (20, 24, 39) and site-directed mutagenesis (3) in order to investigate the effects of the substitutions T46V, T89A, and T89D on structural changes which occur during the photocycle of bR. These mutants were previously studied by kinetic visible absorption spectroscopy in L-α-dimyristoylphosphatidylcholine/CHAPS/sodium dodecyl sulfate mixed micelles (26). T46V exhibited an increased decay rate of the M intermediate while the Thr-89 mutants had abnormal dark-light adaptation reactions and greater than 10-fold increased regeneration rates with 13-cis retinal compared with all-trans retinal.

The present study provides additional information about the roles of Thr-89 and Thr-46 in the br photocycle as well as several other key residues in bR, including Asp-96, Tyr-185, and Asp-212. These findings are discussed below.

Interaction between Thr-46 and Asp-96 during the Early Photocycle—The T46V substitution perturbed the environment of Asp-96 at the L and M stages of the photocycle, shifting the carboxyl stretching vibration of Asp-96 from 1748 to 1753 cm⁻¹. The direction of this shift is consistent with a drop in the pKₐ of Asp-96 (20, 40), as previously postulated in order to explain the increase in the rate of the M→N transition as well as a slow down in the proton uptake observed for the T46V mutant (26).

These results, along with the proximity of Thr-46 and Asp-96 in the electron diffraction-derived br structure (12), point to the occurrence of an interaction between Asp-96 and Thr-46 during the early photocycle. At low temperature, Asp-96 appears to undergo a change in its environment during L formation (5, 6). However, recent room temperature time-resolved FTIR measurements (16) show that Asp-96 undergoes a transient deprotonation during the life-time of the L intermediate. Such a transient deprotonation was also suggested on the basis of earlier FTIR studies (5, 41).

Based on the above information and other recent FTIR studies (17)³ the following picture emerges of the protonation changes of Asp-96 during the photocycle as illustrated in Fig.

---

³ O. Boussé, S. Sonar, M. Krebs, H. G. Khorana, and K. J. Rothschild, unpublished results.
FIG. 7. Model of hydrogen-bonded proton transport network and protonation changes that occur during the bR photocycle. The approximate position of the residues shown is based on the coordinate map of the electron diffraction derived bR structure (12). Single bond rotations were allowed in the side-chains of residues Asp-96, Thr-46, and Arg-82 for positional adjustments. The positions of the Schiff base nitrogen and Lys-216 side chain are approximate except in the case of the bR<sub>570</sub> state. Shaded arrows denote polarizable hydrogen bonds which allow delocalization and collective movement of protons in a hydrogen-bonded network of such bonds (44). For example, in the bR<sub>570</sub> state, the network allows a net movement of a proton toward ionized Asp-212. This is equivalent to a partial delocalization of the negative charge of the Asp-212 carboxylate group into the network. In cases where a negative charge is shared among several high pK<sub>a</sub> residues and water molecules, such as in the M intermediate, a δ(-) is shown at only 1 of the residues which shares the charge (i.e. Tyr-185). A black arrow is used to indicate when a net proton transfer will occur at the next step of the photocycle. Further details of the model are given in the text.

7. 1) bR<sub>570</sub>: Asp-96 exists in a predominantly protonated state at pH 7 (5, 6), even though the pK<sub>a</sub> of an Asp residue is normally close to 3.5. 2) L<sub>550</sub>: bR undergoes a conformational change (42) which alters the local environment of Asp-96 (5, 6). At physiological temperatures, this results in a partial deprotonation of Asp-96 (5, 16). 3) M<sub>412</sub>: during M formation, Asp-96 reprotonates and establishes a hydrogen-bonding interaction with Thr-46. 4) N<sub>550</sub>: during the M→N transition, Asp-96 undergoes a net deprotonation (15-18). 5) O<sub>640</sub>: during O formation, which involves a 13-cis→all-trans isomerization of the chromophore (2), Asp-96 undergoes a reprotonation from the cytoplasmic side of the membrane. 3) This protonation would account for the block of the N decay at high pH (43).

Possible Existence of a Proton Wire Extending from Thr-89 to Asp-212—Both the T89D and T89A mutants exhibited altered properties relative to wild-type bR, as summarized here. 1) In T89D, Asp-89 deprotonates early in the photocycle while Asp-212 undergoes an increased protonation. 2) The λ<sub>max</sub> of the light-adapted states of T89D and to a lesser extent T89A were red-shifted. 3) The K intermediates of both mutants were destabilized with the largest effect observed for T89D. 4) The rate of dark adaptation of T89D was increased severalfold relative to wild-type bR. 5) The rate of chromophore regeneration with 13-cis retinal in T89D is increased relative to wild-type bR (26). 6) The O decay is slowed down in T89D relative to wild-type bR (26).

The above effects could be explained if a network of polarizable hydrogen bonds existed in light-adapted bR which extends from Thr-89 to Asp-212. A direct interaction between these 2 residues is unlikely based on the electron diffraction-derived structure of bR (12). However, as shown in Fig. 7 and discussed in the next section, such a network could be formed if one or more water molecules were located between Thr-89 and Tyr-185. Evidence for the existence of a polarizable hydrogen bond between Tyr-185 and Asp-212 has been previously presented (14). Alternatively, a pathway might exist between Thr-89 and Asp-212 which does not involve Tyr-185.

One of the major consequences of such a hydrogen-bonding network would be the existence of a pathway for proton transfer from Thr-89 to Asp-212. Normally, protons in such a network are expected to be localized predominantly on the
hydroxyl groups of the high pK$_s$ residues Thr-89 and Tyr-185 (44). However, substitution of Thr-89 by Asp, which has a much lower pK$_s$ is expected to result in an increased protonation of Asp-212. A similar effect, although less pronounced, could occur for the mutant T89A, if an additional water molecule substituted for the hydroxyl group of Thr-89 in the network. If Thr-89 were involved in a transient proton-transporting network of hydrogen bonds (see next section), the presence of such a water molecule could further explain why the mutant T89A is able to pump protons with a steady-state activity which is approximately two-thirds of that of the wild type (26). In addition to the apparent deprotonation of Asp-89 and increased protonation of Asp-212 in T89D, the br $\rightarrow$ M difference spectrum of T89D (Fig. 5) shows a reduction in the 1271-cm$^{-1}$ band assigned to Tyr-185 deprotonation during formation of the M intermediate (19). Such a decrease would be expected since Asp-89 can now function as the major proton donor for Asp-212, rather than the higher pK$_s$ residues which form a network of polarizable hydrogen bonds.

Several other features of the Thr-89 mutants can be explained by an increase in the protonation state of Asp-212 as discussed below.

**Intrinsic Red Shift in the $\lambda_{max}$ of Thr-89 Mutants**—Since Asp-212 is located close to the positively charged Schiff base and is in a position to serve as a partial counterion, increased protonation of Asp-212 relative to wild-type br would be expected to cause a red shift of the $\lambda_{max}$ (45). Such a red shift is observed for T89D and to a lesser extent for T89A. Furthermore, this red shift has been shown to reflect an intrinsic property of the light-adapted chromophore and is not due to an increased amount of acid-blue membrane present in these mutants, as has been found in the case of the mutants R82A, R82Q, D85E, and Y185F (25, 46, 47).

***Increased Isomerization Rate in Thr-89 Mutants***—Based on the concepts previously proposed by Seltzer (48), Asp-212 is in an ideal position to play an important role in determining the energy barriers for retinal isomerization during the photocycle and light-dark adaptation. In the case of the Thr-89 mutants, partial neutralization of Asp-212 could explain the above listed properties 3–5, all of which involve the ability of the chromophore to undergo isomerization. For example, the rapid decay of the K intermediate in T89D is likely to reflect an enhanced ability of the chromophore to relax into a more planar 13-cis configuration and/or a facilitation of single bond isomerization around the C$_{14}$-C$_{15}$ bond as is postulated in the model below, this deprotonation occurs by a small delocalization of the negative charge on Asp-212 into the network. This is equivalent to a movement of a proton in this network. The movement of a proton in this network does not occur until M formation (49).

**Slow O Decay Rate of T89D**—Recent time-resolved FTIR measurements demonstrate that the O decay involves deprotonation of Asp-212. The slower decay rate of the O intermediate in T89D relative to wild-type br (26) could thus be caused by an inhibition of Asp-212 deprotonation. As described in the model below, this deprotonation occurs by movement of a positive charge back into the network of hydrogen-bonded high pK$_s$ residues, including Thr-89. The T89D mutation would be expected to slow down the deprotonation of Asp-212 due to the introduction of a low proton affinity residue (Asp-89).

**A Model for Proton Transport**—An extended network of hydrogen bonds formed between hydrophilic residues such as Asp, Glu, Tyr, Thr, and Ser as well as water molecules could provide the basis for proton transport within the interior of membrane proteins (44, 50, 51). In support of such a proton transport mechanism, Zundel and co-workers (44, 52) have demonstrated that the polarizable hydrogen bonds formed in such networks allow collective proton motions. Thus far, however, there is no direct evidence for the existence of an extended hydrogen-bonded network in br.

Some of the “channel lining residues” recently identified by Henderson and co-workers (12), which are located between the Schiff base and the cytoplasmic side of br, are in good positions to form a hydrogen-bonded network. In an attempt to account for the protonation changes of specific residues deduced from FTIR as well as a variety of other data, we have constructed a model of such a network that is based on the electron diffraction-derived br structure (12) and an earlier proposed mechanism for proton transport (5).

Specific steps in the proton transfer process are shown in Fig. 7 and described below.

**bR$_{650}$**—In the bR$_{650}$ state (light-adapted br), Asp-212 and Asp-85 exist in a predominantly ionized form which is stabilized by the protonated Schiff base and Arg-82 as originally proposed (5). As indicated in Fig. 7 by a series of shaded arrows (see figure legend), a network of hydrogen-bonded residues which includes Asp-212, Tyr-185, and Thr-89 allows a small delocalization of the negative charge on Asp-212 into the network. This is equivalent to a movement of a proton toward Asp-212. This delocalization could account for the partial deprotonation observed in Tyr-185 by FTIR (19). However, the extent of ionization of any of the high pK$_s$ residues in the chain is still expected to be small, consistent with recent NMR and UV resonance Raman studies which reveal a predominantly protonated state for Tyr-185 (53, 54).

**K$_{iso}$**—Upon K formation, the movement of the positively charged Schiff base causes a disruption in the network of hydrogen bonds including the Tyr-185/Asp-212 hydrogen bond. As a consequence the protonation of Tyr-185 increases (19, 55, 56). The red-shifted $\lambda_{max}$ of the K chromophore is most likely due to the movement of the positively charged Schiff base away from its counterions Asp-85 and Asp-212. This charge separation could also account for the downshift in the C=N stretch frequency (32) and the energy (15 kcal/mol) stored in the K intermediate (57).

**L$_{650}$**—During formation of the L intermediate, Asp-96 undergoes a partial deprotonation due to a protein structural change which lowers its effective pK$_s$. At low temperature, where the decay of the L intermediate is blocked, Asp-96 rapidly reprotonates, hence the ionized form is not directly observed. However, at room temperature, this reprotonation does not occur until M formation (16).

**M$_{412}$**—As illustrated by the shaded arrows, upon M formation, a transient network of polarizable hydrogen bonds is established which includes Asp-96, Thr-46, Thr-89, Tyr-185, Asp-212, and several water molecules. This network allows a partial protonation of Asp-212 to occur. While none of the high pK$_s$ residues in this network are expected to exist in a predominantly ionized form, as a consequence of this partial protonation the polarizable hydrogen bonds allow some of the negative charge of Asp-212 to be delocalized throughout the network.

This is denoted by placing a $\delta$ near Tyr-185. Note, that in a complete description, several other conjugate forms would be shown with the $\delta$ placed at the other high pK$_s$ residues in the network. The movement of a proton in this network towards Asp-212 (i.e. partial protonation of this residue) can be understood as due to the transfer of the Schiff base proton to Asp-85 (5). Prior to formation of the M intermediate, the positively charged Schiff base forms ionic interactions with Asp-212, Asp-85, and Arg-82 (5, 11). The disruption of these ionic interactions due to Schiff base deprotonation, and the

---

$^4$ P. Rath, T. Marti, H. G. Khorana, and K. J. Rothschild, unpublished results.
subsequent release of Arg-82 from the active site act as a trigger for diffusion of a proton to the extracellular medium (5) as indicated in Fig. 7.

$N_{max}$—The $M \rightarrow N$ transition involves a net proton transfer from Asp-96 to the Schiff base (8–10). The deprotonation of Asp-96 along with a protein secondary structural change at this stage of the photocycle was recently detected by time-resolved FTIR difference spectroscopy (16, 17). As illustrated in Fig. 7, this proton transfer involves the entire transient network of hydrogen-bonded residues including Asp-212, which acts as the proximal proton donor to the Schiff base. The overall process involves the transfer of a proton from Asp-212 to the Schiff base, reprotonation of Asp-212 and simultaneous donation of a proton from Asp-96 into the hydrogen-bonded network. In effect, this amounts to a net movement of a proton from Asp-96 to the Schiff base. Asp-96 is now deprotonated and no longer interacts with Thr-46, accepting a proton from the cytoplasmic medium during the next photocycle step. Similar to the M intermediate, a partial negative charge is delocalized among the high $p_K_a$ residues in the chain and Asp-212 is partially protonated.

$O_{max}$—The $N \rightarrow O$ transition involves the reprotonation of Asp-96, thus accounting for the observed slow $O$ decay at high pH (9, 43). This transition also involves a reisomerization of the chromophore from 13-cis to all-trans. As discussed in the previous section, the partial neutralization of Asp-96 could act to facilitate this isomerization. It is not yet clear, however, how Asp-96 reprotonation influences this process. One possibility is that conformational changes of the protein associated with the $N \rightarrow O$ transition is triggered by Asp-96 reprotonation. The red-shifted $\lambda_{max}$ of the O chromophore is most likely due to neutralization of Asp-85 and partial neutralization of Asp-212, which serve as the counterions of the Schiff base when the chromophore is in the all-trans configuration.

$O$ Decay—The entire system resets itself during the $O \rightarrow bR$ transition. This involves a movement of the proton localized on Asp-212 back toward the high $p_K_a$ residues including Tyr-185 and Thr-89. A block in the deprotonation of Asp-212 is expected to slow the $O$ decay rate, as is observed for the mutant Y185F.6 The ionic interactions between Asp-85, Asp-212, Arg-82, and the Schiff base are also reestablished during this step of the photocycle.7

Many of the features of this model remain to be established. For example, bands in the FTIR difference spectrum due to protonation changes of Thr-46 and Thr-89 have not yet been identified. In addition, the postulated role of Asp-212 as the proximal proton donor to the Schiff base is based on indirect evidence (5, 14). Alternative candidates for a proximal proton donor include Thr-89 (58, 59). However, such a model is consistent with Asp-85 serving as the proximal proton acceptor for the Schiff base. In this case as seen in Fig. 7, a small movement of the Schiff base would allow it to switch from a position where it donates a proton to Asp-85 ($L \rightarrow M$ transition) to a position where it accepts a proton from Asp-212 ($M \rightarrow N$ transition). In support of the role for Asp-212 as the proximal proton donor, recent time-resolved FTIR measurements reveal that a slowed M decay, as observed in the mutant D96A, is also accompanied by a delay in the protonation of Asp-212 (17).

CONCLUSIONS

We have investigated by FTIR difference spectroscopy the effects of the substitutions, T89A, T89D, and T46V on structural changes occurring in the bR photocycle. The information obtained here and in earlier studies utilizing a combination of FTIR, isotope labeling and site-directed mutagenesis (5, 13, 14, 16, 17, 19) has led us to propose a tentative model for proton transport in bR which can account for a variety of data on bR and its mutants. Several structural aspects of this model, first proposed in 1988, have recently been confirmed by electron diffraction (12). Higher resolution analyses may help determine the possible existence of a hydrogen-bonding network in a position to transport protons from Asp-96 to the Schiff base. However, the movement of protons through such a hydrogen-bonding network cannot easily be observed directly by diffraction techniques. Thus, further progress and tests of this and other models will most likely continue to rely on the application of spectroscopic probes including static and time-resolved FTIR difference spectroscopy.

Acknowledgments—We thank M. Heyn and S. Subramaniam for helpful discussions during the course of this work and S. Berkowitz and O. Bousc̈h for technical assistance.

REFERENCES

1. Stoeckenius, W. & Bogomolni, R. A. (1982) Annu. Rev. Biochem. 51, 587–616
2. Smith, S. O., Lugtenburg, J. & Mathies, R. A. (1985) J. Membr. Biol. 85, 95–109
3. Khorana, H. G. (1988) J. Biol. Chem. 263, 7439–7442
4. Mogi, T., Stern, L. J., Marti, T., Chao, B. H. & Khorana, H. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4148–4152
5. Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G. & Rothschild, K. J. (1988) Biochemistry 27, 8516–8520
6. Gerwert, K., Hess, B., Soppa, J. & Oesterhelt, D. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4943–4947
7. Marinetti, T., Subramaniam, S., Mogi, T., Marti, T. & Khorana, H. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 529–533
8. Holz, M., Drachev, L. A., Mogi, T., Otto, H., Kaulen, A. D., Heyn, M. P., Skulachev, V. P. & Khorana, H. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2167–2171
9. Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H. G. & Heyn, M. P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9229–9232
10. Butt, H. J., Fendler, K., Bamberg, E., Tittor, J. & Oesterhelt, D. (1989) EMBO J. 8, 1657–1663
11. Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G. & Heyn, M. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1018–1022
12. Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E. & Downing, K. H. (1990) J. Mol. Biol. 213, 899–929
13. Rothschild, K. J., Braiman, M. S., Mogi, T., Stern, L. J. & Khorana, H. G. (1989) FEBS Lett. 250, 448–452
14. Rothschild, K. J., Braiman, M. S., Hey, Y. W., Marti, T. & Khorana, H. G. (1990) J. Biol. Chem. 265, 16885–16891
15. Gerwert, K., Souvignier, G. & Hess, B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9774–9778
16. Braiman, M. S., Rouschë, O. & Rothschild, K. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2388–2392
17. Bousc̃h, O., Braiman, M. S., Hey, Y. W., Marti, T., Khorana, H. G. & Rothschild, K. J. (1991) J. Biol. Chem. 266, 11063–11067
18. Pfefferle, J. M., Maeda, A., Sasaki, J. & Yoshizawa, T. (1991) Biochemistry 30, 8548–8556
19. Braiman, M. S., Mogi, T., Stern, L. J., Hackett, N. R., Chao, B. H., Khorana, H. G. & Rothschild, K. J. (1988) Proteins Struct. Funct. Genet. 3, 219–229
20. Rothschild, K. J., Zagaeski, M. & Cantore, W. A. (1981) Biochem. Biophys. Res. Commun. 103, 482–489
21. Rothschild, K. J. & Marrero, H. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4045–4049
22. Bagley, K., Dollinger, G., Eisenstein, L., Singh, A. K. & Zimanyi, L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4972–4976
23. Siebert, F. & Mantele, W. (1983) Eur. J. Biochem. 130, 565–573
24. Braiman, M. S. & Rothschild, K. J. (1988) Annu. Rev. Biophys. Biophys. Chem. 17, 541–570
25. Duñach, M., Berkowitz, S., Marti, T., Hey, Y. W., Subramaniam, S. Sonar, M., Krebs, H. G., Khorana, and K. J. Rothschild, unpublished observation.
FTIR Spectroscopy of Threonine Mutants of bR

S., Khorana, H. G. & Rothschild, K. J. (1990) J. Biol. Chem. 265, 16978-16984

26. Marti, T., Otto, H., Mogi, T., Rösselet, S. J., Heyn, M. P. & Khorana, H. G. (1991) J. Biol. Chem. 266, 6919-6927

27. Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J. & Rothschild, K. J. (1987) Biochemistry 26, 6686-6707

28. Roepe, P., Scherrer, P., Ahl, P. L., Das Gupta, S. K., Bogomolni, R. A., Herzfeld, J. & Rothschild, K. J. (1987) Biochemistry 26, 6708-6717

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

30. Popot, J. L., Gerchman, S. E. & Engelman, D. M. (1987) J. Mol. Biol. 198, 655-676

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

32. Rothschild, K. J., Roepe, P., Lugtenburg, J. & Pardoen, J. A. (1984) Biochemistry 23, 6105-6109

33. Braiman, M. S. & Mathies, R. A. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 403-407

34. Rothschild, K. J., He, Y. W., Gray, D., Roepe, P. D., Pelletier, S. L., Brown, R. S. & Herzfeld, J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9832-9835

35. Rothschild, K. J., Gray, D., Mogi, T., Marti, T., Braiman, M. S., Stern, L. J. & Khorana, H. G. (1989) Biochemistry 28, 7052-7059

36. Subramaniam, S., Greenhalgh, D. A., Rath, P., Rothschild, K. & Khorana, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6873-6877

37. Aton, B., Doukas, A. G., Callender, R. H., Becher, B. & Ebrey, T. G. (1977) Biochemistry 16, 6775-6778

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

39. Rothschild, K. J. (1988) Photochem. Photobiol. 47, 883-887

40. Bellamy, L. J. (1968) Vol. 2, The Infrared Spectra of Complex Molecules, Chapman and Hall, London

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

42. Subramaniam, S., Marti, T., Rösselet, S. J., Rothschild, K. J. & Khorana, H. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2583-2587

43. Kouyama, T., Nasuda-Kouyama, A., Ikegami, A., Mathew, M. K. & Stoeckenius, W. (1988) Biochemistry 27, 5855-5863

44. Zundel, G. (1988) J. Mol. Struct. 177, 43-68

45. Honig, B., Dinur, V., Nakanishi, K., Bogh-Nair, V., Gawinowicz, M. A., Arnaboldi, M. & Motto, M. G. (1979) J. Am. Chem. Soc. 101, 2503-2507

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

47. Subramaniam, S., Marti, T. & Khorana, H. G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1013-1017

48. Seltzer, S. (1987) J. Am. Chem. Soc. 109, 1627-1631

49. Fahmy, K., Siebert, F., Grossjean, M. F. & Tavan, P. (1989) J. Mol. Struct. 214, 257-288

50. Eigen, M. & DeMayer, L. (1958) Proc. R. Soc. Lond. Ser. A 247, 505-533

51. Nagle, J. F. & Tristram-Nagle, S. (1983) J. Membr. Biol. 79, 655-676

52. Brzezinski, B., Zundel, G. & Krämer, R. (1987) J. Phys. Chem. 91, 3077-3080

53. Ames, J. B., Bolton, S. R., Netto, M. N. & Mathies, R. A. (1990) J. Am. Chem. Soc. 112, 9007-9009

54. Smith, S. O., Courin, J., Van den Berg, E., Winkel, C., Lugtenburg, J., Herzfeld, J. & Griffin, R. G. (1989) Biochemistry 28, 237-243

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

56. Dollinger, G., Eisenstein, L., Lin, S., Nakanishi, K. & Termnii, J. (1986) Biochemistry 25, 6524-6533

57. Birge, R. R. & Cooper, T. M. (1985) Biophys. J. 42, 61-69

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

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