Articles

Detection of a Water Molecule in the Active-Site of Bacteriorhodopsin: Hydrogen Bonding Changes during the Primary Photoreaction†

Wolfgang B. Fischer,‡,§ Sanjay Sonar,‡ Thomas Marti,∥,⊥ H. Gobind Khorana,∥ and Kenneth J. Rothschild*,‡

Physics Department and Molecular Biophysics Laboratory, Boston University, Boston, Massachusetts 02215, and Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received May 5, 1994; Revised Manuscript Received August 2, 1994®

ABSTRACT: FTIR-difference spectroscopy in combination with site-directed mutagenesis has been used to investigate the role of water during the photocycle of bacteriorhodopsin. At least one water molecule is detected which undergoes an increase in H-bonding during the primary bR→K phototransition. Bands due to water appear in the OH stretch region of the bR→K FTIR-difference spectrum which downshift by approximately 12 cm^{-1} when the sample is hydrated with $H_2{}^{18}O$. In contrast to ^2H_2O, the $H_2{}^{18}O$-induced shift is not complete, even after 24 h of hydration. This indicates that even though water is still able to exchange protons with the outside medium, it is partially trapped in the interior of the protein. In the mutant Y57D, these bands are absent while a new set of bands appear at much lower frequencies which undergo $H_2{}^{18}O$-induced shifts. It is concluded that the water molecule we detect is located inside the bR active-site and may interact with Tyr-57. The change in its hydrogen-bonding strength is most likely due to the photoinduced *all-trans*→13-*cis* isomerization of the retinal chromophore and the associated movement of the positively charged Schiff base during the bR→K transition. In contrast, a second water molecule, whose infrared difference bands are not affected by the Y57D mutation, appears to undergo a decrease in hydrogen bonding during the K→L and L→M transitions.

Bacteriorhodopsin (bR),[1] the light-driven proton pump found in the purple membrane of *Halobacterium salinarium* (Oesterhelt & Stoeckenius, 1971), has been the subject of extensive investigation [for recent reviews, see Birge (1990), Krebs and Khorana (1993), and Rothschild and Sonar (1995)]. In addition to similarities with the visual receptor rhodopsin, interest in bR stems from its many unusual features including a light-triggered photocycle (Lozier et al., 1992), the ability of bR to regenerate from a denatured form (Huang et al., 1981), and the self-assembly into a two-dimensional lattice and stability at very high temperatures (Shen et al., 1993). bR is a member of a family of closely related membrane proteins which includes halorhodopsin, a light-driven chloride pump (Lanyi, 1990), and sensory rhodopsin, a light receptor for phototaxis (Spudich & Bogomolni, 1988).

The exact location and role of water molecules in bR are unknown. However, several lines of evidence suggest that water is present and may be involved in proton transport. Although the three-dimensional structure of bR determined by electron diffraction lacks sufficient resolution to locate these water molecules (Henderson et al., 1990), neutron diffraction detects several water molecules buried in the interior regions of bR (Papadopoulos et al., 1990). On the basis of spectroscopic studies, water has been postulated to be involved in the transfer of a proton from Asp-96 to the Schiff base (Cao et al., 1991; Rothschild et al., 1992). Water has also been predicted to interact with the positively charged Schiff base, accounting in part for its weak H-bonding (Hildebrandt & Stockburger, 1984; De Groot et al., 1990). Numerous studies have also predicted that water and hydroxyl-bearing side-chains of amino acid residues such as serine, threonine, tyrosine, glutamic acid, and aspartic acid could form a hydrogen-bonded network which is able to conduct protons through bacteriorhodopsin (Nagle & Mille, 1981; Merz & Zundel, 1983; Brzezinski et al., 1987; Olejnik et al., 1992).

One approach for detecting structural activity in bacteriorhodopsin is Fourier transform infrared (FTIR) difference spectroscopy [see Rothschild (1992) and Rothschild and Sonar (1994) for recent reviews and references cited therein]. Such studies in combination with site-directed mutagenesis (Braiman & Rothschild, 1988; Braiman et al., 1988b; Gerwert et al., 1989; Fahmy et al., 1992; Maeda et al., 1992c) have led to the assignment of several bands below 1800 cm^{-1} in the FTIR-difference spectra to specific amino acid residues. For example, the protonation states of the four buried aspartic acids, Asp-85, Asp-96, Asp-115, and Asp-212, have been determined for each step in the photocycle (Braiman et al., 1988a, 1991; Bousché et al., 1991, 1992; Fahmy et al., 1992; Gerwert, 1992), thus providing a framework for determining the temporal and spatial proton pathway in bR (Rothschild et al., 1992).

Recently, bands in the region above 3500 cm^{-1} in the FTIR-difference spectra of bR have been assigned on the basis of $H_2{}^{18}O$-induced shifts to the OH stretch mode of water (Maeda et al., 1992a). One or more weakly hydrogen-bonded water

† This research was supported by grants from the NSF (DMB9106017) to K.J.R. and from the NIH (GM28289 and AI11479) to H.G.K.

* Address correspondence to this author at the Department of Physics, Boston University, 590 Commonwealth Ave., Boston, MA 02215.

‡ Boston University.

§ Present address: Institute of Analytical Chemistry, Technical University of Desden, D-01062 Dresden, Germany.

∥ Massachusetts Institute of Technology.

⊥ Present address: Bernhard Nocht Institute for Tropical Medicine, D-20359 Hamburg, Germany.

® Abstract published in *Advance ACS Abstracts,* October 1, 1994.

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

0006-2960/94/0433-12757$04.50/0

molecules were detected which undergo a change in H-bonding upon formation of the L and M intermediates (Maeda et al., 1992a). However, FTIR evidence has not yet been found for the involvement of water during the primary phototransition (bR→K), even though this is the step which involves the *all-trans*→13-*cis* isomerization of the retinal chromophore.

We report here the detection of bands in the bR→K FTIR-difference spectrum of bR which are assigned to at least one water molecule which undergoes an increase in the H-bonding strength during this transition. While mutations involving several key amino acid residues including Asp-96 have no effect on these bands, the replacement of Tyr-57 by Asp-57 causes their complete disappearance. This evidence, in addition to the slow exchange rate of this water molecule with $H_2{}^{18}O$ compared to ^2H_2O, indicates that we are observing a trapped water molecule located inside the bR active-site that responds to chromophore isomerization. In addition, a second water molecule is detected which is not affected by the Y57D mutation and undergoes a decrease in H-bonding during the K→L and L→M transitions. In contrast to earlier work (Maeda et al., 1992a), we are now able to separate the contributions in the FTIR-difference spectra from these two different water molecules.

MATERIALS AND METHODS

Expression, Regeneration, and Reconstitution of Y57D. The bacteriorhodopsin mutant Y57D was prepared using an *Escherichia coli* expression system (Marti et al., 1991). FTIR spectroscopic measurements were made on samples reconstituted into vesicles with polar lipids from *H. salinarium* as described (Marti et al., 1991).

FTIR-Difference Spectroscopy. FTIR-difference spectra were recorded at 2 cm^{-1} resolution on Nicolet Analytical Instruments 740 and 60SX spectrometers (Madison, WI) using methods previously reported (Rothschild et al., 1984; Roepe et al., 1987; Braiman et al., 1988a). 100–150 μg of sample was placed on a AgCl-window and dehydrated several hours in a dry-box purged with air with a −100 °C dew-point. Before the cell was sealed, small drops of either H_2O, $H_2{}^{18}O$, or ^2H_2O were placed on the opposite AgCl-window. The sealed sample was then placed in a Helitran cryostat (Air Products, Allentown, PA) and allowed to hydrate for several hours. The amount of hydration was controlled by monitoring evaporation using the OH stretch band at 3400 cm^{-1} or the OD stretch band at 2500 cm^{-1}. After light-adaptation for 15 min at room temperature using a 150 W tungsten light source equipped with a 505 nm long-pass filter, the sample was cooled to the appropriate temperature for measuring the different transitions [bR→K (80 K); bR→L (170 K); and bR→M (250 K)]. The bR→K transition was driven with a 500 nm narrow band-pass filter and photoreversed with a 650 nm narrow band-pass filter. The bR→L transition was driven with a 600 nm narrow band-pass filter and the bR→M transition with a 505 nm long-pass filter.

RESULTS

Detection of a Water Molecule Which Is Active during the bR→K Phototransition. Figure 1A,B shows FTIR-difference spectra for the bR→K transition in the 1800–700 and 3700–3500 cm^{-1} regions, respectively, for bR hydrated in H_2O, $H_2{}^{18}O$, or ^2H_2O. In the region below 1800 cm^{-1}, $H_2{}^{18}O$ has no effect on the difference spectrum, even for bands assigned to vibrations which involve oxygen, such as the C=O stretch of Asp-115 (1741/1733 cm^{-1}, −/+) (Braiman et al., 1988a) or the C–O^- stretch of Tyr-185 (1277 cm^{-1}) (Braiman et al., 1988b). This is not surprising since a carboxylic acid group (COOH) or hydroxyl group (OH) does not normally undergo an $^{16}O/^{18}O$ exchange in the presence of $H_2{}^{18}O$. In contrast, amino acid residues containing hydroxyl, carboxylic acid, or amide groups as well as the protonated Schiff base of the retinal chromophore (C=N–H^+) are all expected to undergo a rapid H/D exchange when exposed to ^2H_2O, thus producing shifts in bands arising from vibrational modes which involve these groups. This is reflected in the bR→K FTIR-difference spectrum of bR in the presence of ^2H_2O (Figure 1A), where band shifts due to H/D exchange of chromophore and protein groups occur (Rothschild et al., 1984, 1986). For example, the Schiff base C=N–H^+ stretching mode shifts from 1640 to 1627 cm^{-1}, and the negative/positive bands assigned to a change in H-bonding of the Asp-115 C=O stretching mode shift from 1741/1733 cm^{-1} (−/+) to 1728/1722 cm^{-1} (−/+) (Figure 1A). The assignment of other bands in this region to bR and K chromophore vibrations (*e.g.*, the ethylenic C=C stretch mode of the bR_{570} and K chromophores at 1529 and 1514 cm^{-1}, respectively) as well as to specific amino acid residues such as Trp-86 (742 cm^{-1}) has been previously described [see Rothschild (1992) and Rothschild and Sonar (1994) and references cited therein].

In the region above 3500 cm^{-1} (Figure 1B), where the *Y*-scale has been expanded, several reproducible changes are observed in the bR→K difference spectrum when the sample is hydrated

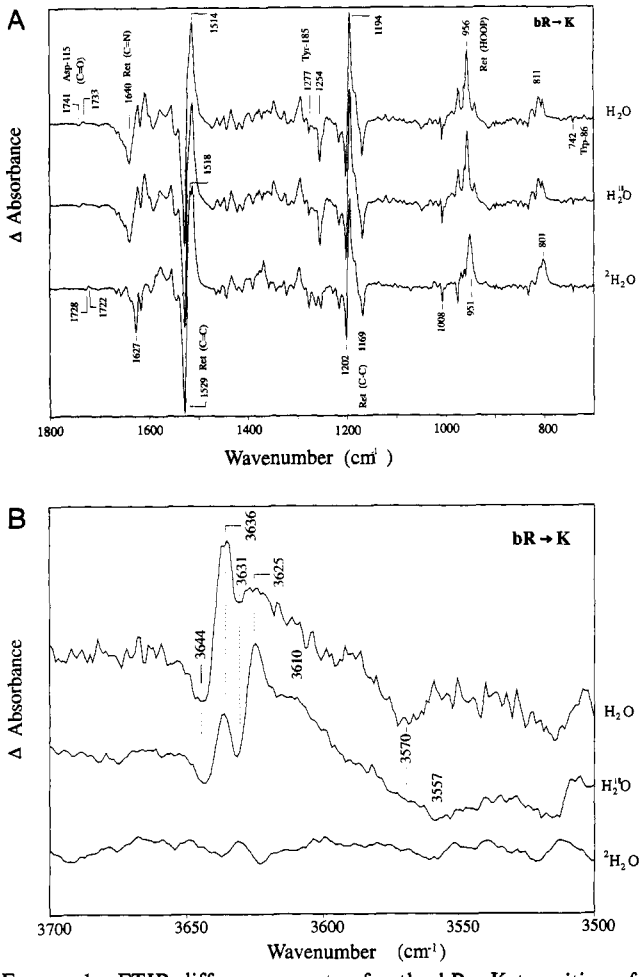

FIGURE 1: FTIR-difference spectra for the bR→K transition of bacteriorhodopsin recorded at 80 K at 2 cm^{-1} resolution. Films formed from wild-type bR (purple membrane) were humidified using H_2O (top), $H_2{}^{18}O$ (middle), and ^2H_2O (bottom) (see Materials and Methods). (A) Region from 700 to 1800 cm^{-1}. (B) Region from 3500 to 3700 cm^{-1}. Note that the *Y*-scale has been expanded (e.g., 33× for H_2O).

in $H_2{}^{18}O$. Most noticeable is an approximately 12 cm^{-1} downshift in the pair of bands at 3644/3636 cm^{-1} (−/+) to 3631/3625 cm^{-1} (−/+). Two other broader bands at 3625 cm^{-1} (+) and 3570 cm^{-1} (−) also appear to undergo a similar downshift. Such a shift is typical of the ^{18}O isotope effect on the OH stretch mode of water based on both model compound studies and normal mode analysis (Foss Smith & Overend, 1972; Fredlin et al., 1977). *Thus, these bands can all be assigned to at least one water molecule which is structurally active during the bR→K phototransition of bR.*[2] The simplest explanation for the negative/positive feature at 3644/3636 cm^{-1} is that a water molecule undergoes an increase in hydrogen-bonding strength which causes a downshift in the OH stretching frequency.

We also found that the $H_2{}^{18}O$-induced downshift of the 3644/3636 cm^{-1} (−/+) bands was incomplete, as indicated by the residual bands due to H_2O (Figure 1B, middle trace), even in the case where the film was exposed directly to bulk $H_2{}^{18}O$ for several days. In fact, the entire region from 3500–3700 cm^{-1} could be reproduced (data not shown) by downshifting the bR→K (H_2O) spectrum by 11.5 cm^{-1}, multiplying its amplitude by 1.5, and adding this shifted spectrum to the unshifted bR→K (H_2O) spectrum (Figure 1B, top trace). In contrast, hydration with ^2H_2O for only 1 h (Figure 1B, bottom trace) caused the same bands to *completely* downshift to 2689/2683 cm^{-1} (+/−) characteristic of the OD stretching mode (data not shown). *Thus, the water molecule we detect may be partially trapped in the interior of the protein since it does not freely exchange with $H_2{}^{18}O$ in the bulk medium as would be expected through simple diffusion. On the other hand, this water is still able to undergo complete H/D exchange indicating that a proton conduction pathway exists leading to the aqueous medium.* Such pathways which allow proton movement but not water diffusion are consistent with models of proton wires previously described where the positions of the water molecules in a hydrogen-bonded network are fixed (Nagle & Mille, 1981; Brzezinski et al., 1987). However, further work will be necessary to confirm the existence of such a network.

Effect of the Y57D Mutation on Bands Assigned to Water in the bR→K Difference Spectrum. In an attempt to identify the location of the water molecule(s) in bR, which respond to the primary photoreaction, we have begun to examine the effects of site-directed mutagenesis on the OH stretch mode of water (3700–3500 cm^{-1} region) in the bR→K FTIR-difference spectrum. Although most mutants thus far examined showed little effect in this region including mutations at Asp-96, Tyr-185, and Thr-46 (unpublished data of W. Fischer, K. J. Rothschild, T. Marti, S. Sonar, Y. W. He, and H. G. Khorana), we found that the bands assigned to water at 3644/3636 cm^{-1} (−/+) and 3625/3570 cm^{-1} (+/−) completely disappeared in the mutant Y57D (Figure 2).[3]

There is also an appearance of new bands at 3555/3540 cm^{-1} (+/−) in the Y57D mutant (Figure 2) which can be assigned to water, since these bands downshift by 10 cm^{-1} when the sample is hydrated with $H_2{}^{18}O$, although residual bands still remain, again indicative of incomplete exchange.

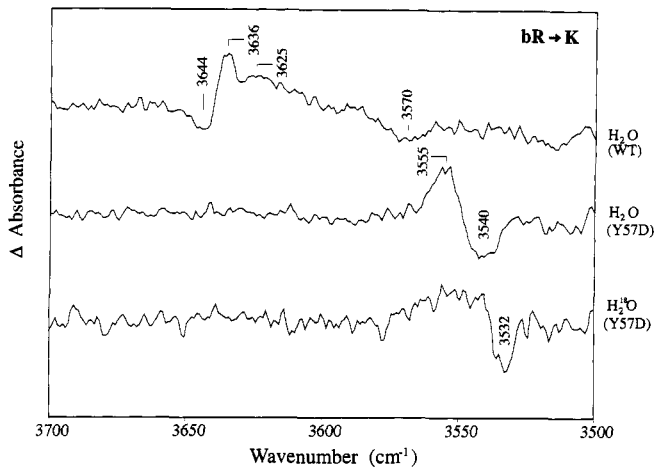

FIGURE 2: FTIR-difference spectra for the bR→K transition of the mutant Y57D recorded at 80 K at 2 cm^{-1} resolution. Top: Wild-type bR humidified in H_2O (same as in Figure 1B). Middle: Y57D humidified in H_2O. Bottom: Y57D humidified in $H_2{}^{18}O$.

The possibility that these bands arise from the OH stretch of the newly introduced Asp-57 (COOH) carboxylic acid due to an $^{16}O/^{18}O$ exchange can be excluded since a corresponding shift is not seen in the C=O stretch bands at 1716/1708 cm^{-1} (+/−) assigned to Asp-57 (Sonar et al., 1994a) or elsewhere in the region of the spectrum below 1800 cm^{-1} (data not shown). The simplest explanation for these results is that the structurally active water present in native bR has been perturbed in Y57D. Indeed, the two new bands at 3555/3540 cm^{-1} (+/−) are consistent with a water molecule with a different environment compared to native bR which now undergoes a *decrease* in H-bonding.

Evidence for a Second Water Molecule Which Is Perturbed during the K→L and L→M Transitions. In addition to a negative band at 3644 cm^{-1}, a positive band assigned to water on the basis of an $H_2{}^{18}O$-induced downshift appears near 3665 cm^{-1} in the bR→M difference spectra of wild-type bR (Maeda et al., 1992a). As seen in Figure 3, at high signal-to-noise, this band resolves into two distinct positive bands at 3668 and 3655 cm^{-1}, with the 3668 cm^{-1} band gaining intensity relative to the 3655 cm^{-1} component during the L→M transition. Furthermore, both bands undergo a partial downshift for bR hydrated in $H_2{}^{18}O$ (data not shown), confirming that they are both due to the OH stretching mode of water (Figure 3).

Maeda and co-workers (Maeda et al., 1992a) associated the 3642/3665 cm^{-1} (−/+) pair with a water molecule which undergoes a decrease in H-bonding during the bR→M transition. *However, in light of the results presented here, it is likely that at least part of the intensity of the negative band near 3644 cm^{-1} actually arises from the water molecule which we detect during the bR→K transition, whereas the positive bands near 3655 and 3668 cm^{-1} arise from at* least *one additional water molecule which undergoes changes in its H-bonding during L and M formation.* The low-frequency positive band at 3636 cm^{-1} observed in the bR→K transition appears to also be present but hidden due to an underlying negative band (see below). This would indicate that the original water which changes its hydrogen bonding during

[2] It is possible that a single water molecule is responsible for both sets of bands. This can occur if a water molecule has one strongly and one weakly hydrogen-bonded hydrogen. In this case, band splitting is typically 100 cm^{-1} with the lower-frequency band, arising from the stronger hydrogen-bonded oscillator. This band should also have the greatest half-width of the two components, in agreement with our results (Mohr et al., 1965; Glew & Roth, 1971). We also note that the possibility exists that a water molecule undergoes a change in its hydrogen bonding in a fraction of the bacteriorhodopsin molecules photocycling.

[3] The bR→K difference spectrum of Y57D displays bands characteristic of both the *all-trans* and 13-*cis*/C=N syn components of bR. However, this effect cannot be attributed to the increased content of dark-adapted bR in Y57D (Sonar et al., 1994), since the water bands from the *all-trans*-retinal component of Y57D (in its dark-adapted form) in the bR→K difference spectrum should still appear [see Sonar et al. (1994) for further information].

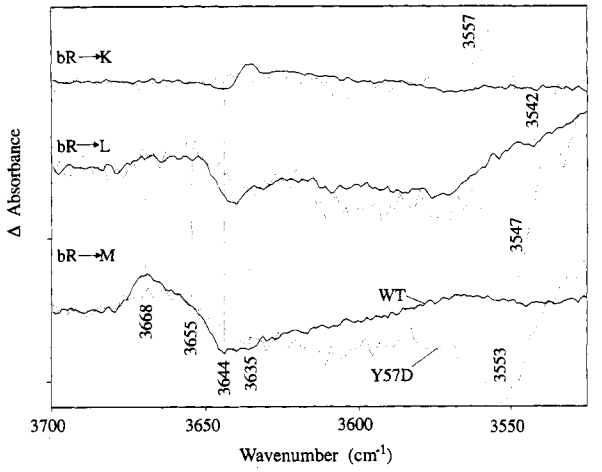

FIGURE 3: FTIR-difference spectra for the bR→K, bR→L, and bR→M transitions of wild-type bR (solid line) and Y57D (dotted line). Each spectrum was recorded at 2 cm^{-1} resolution under conditions described in the text. Spectra were scaled using bands in the region below 1800 cm^{-1}.

the bR→K transition may not change its hydrogen bonding in the K→L or L→M transitions.

This picture is supported by the effects of the Y57D mutation on the OH stretch region of the bR→L difference spectrum recorded at 170 K (Figure 3). In this case, the negative band at 3644 cm^{-1} disappears, as in the bR→K difference spectrum, and is now replaced by a much weaker negative band at 3635 cm^{-1}, whereas the bands near 3660 cm^{-1} still are present. A similar pattern is obtained at 250 K, where it has been found that formation of the M intermediate is blocked for the case of Y57D (Sonar et al., 1994a). *Thus, the negative band at 3644 cm^{-1} in the bR→L and bR→M difference spectra of native bR appears to arise from the perturbation of a water molecule during the bR→K transition, whereas the positive bands at 3655 and 3668 cm^{-1} are due to a second water molecule which is perturbed during the K→L and L→M transitions, respectively.* It is likely that this water molecule undergoes a decrease in hydrogen bonding since a negative band appears at 3635 cm^{-1}. We also found that the bR→L and bR→M difference spectra of Y57D exhibited a new negative band near 3550 cm^{-1} (Figure 3).

DISCUSSION

We have investigated the involvement of water in the bacteriorhodopsin photocycle by using a combination of FTIR-difference spectroscopy and site-directed mutagenesis. Any water which is structurally active, *i.e.*, undergoes a change in H-bonding, orientation, or protonation state, should give rise to bands in the FTIR-difference spectra in the region above 3500 cm^{-1} where the OH stretch mode of water is found. Indeed, earlier work led to the assignment of several bands in the bR→L and bR→M difference spectra to water as shown by the isotope-induced shift of these bands when the bR sample is hydrated in $H_2{}^{18}O$ (Maeda et al., 1992b).

In this paper, we have extended this work by investigating possible changes in water during the bR→K primary phototransition. Since the chromophore undergoes an all-trans to 13-*cis* isomerization during this transition, it is likely that any water molecule positioned in the immediate vicinity of the chromophore will undergo a change in its environment. This is especially true of water in the vicinity of the Schiff base (Harbison et al., 1988; De Groot et al., 1990), since movement of the Schiff base positive charge is likely to alter the electrostatic environment sensed by a nearby water.[4] The

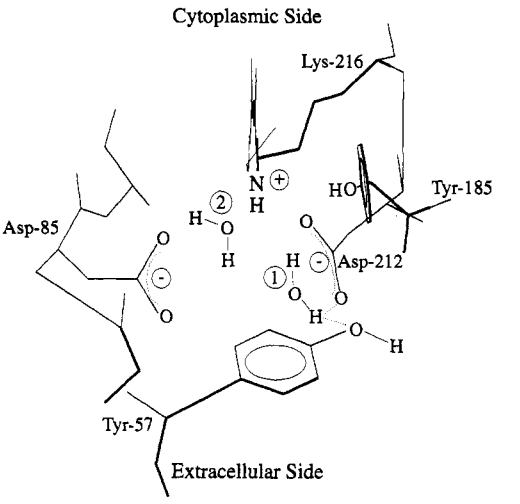

FIGURE 4: Schematic model of the bR active-site based on the 3-D structure coordinates determined by cryoelectron microscopy (Henderson et al., 1990). The protonation states of Asp-85 and Asp-212 were derived from FTIR-difference spectroscopy (Braiman et al., 1988a). The water molecule was positioned so that it can form hydrogen bonds with Tyr-57 and Asp-212 (position 1) or with Asp-85 and the Schiff base (position 2).

OH stretch mode of water is expected to undergo a shift in frequency in this case if the hydrogen-bonding pattern is altered. Water might also react more indirectly to chromophore isomerization through a change in its interaction with nearby residues such as Asp-85, which serves as the Schiff base proton acceptor (Braiman et al., 1988a; Rothschild et al., 1989a; Fahmy et al., 1992), or Asp-212, which is also located nearby (Rothschild, 1992; Rothschild & Sonar, 1994).

In this work, we have also obtained extremely high signal-to-noise data that enabled us to resolve bands that were previously not observable due to their weak intensity and/or overlap with other bands. In addition, the use of site-directed mutagenesis has allowed us to separate the contributions from different water molecules as well as to localize to some extent their position in the bR structure. The major conclusions that can be reached from this work are the following:[5]

(1) At least one water molecule undergoes an increase in H-bonding during the bR→K primary phototransition.

(2) This water molecule may be partially trapped in bR, since it exchanges slowly with other water molecules in the bulk medium.

(3) This water is located inside the bR active-site.

(4) An additional water molecule may undergo a decrease in H-bonding during the K→L and L→M transitions which is not affected by substitution of Tyr-57 with Asp.

A model showing two possible locations of the water molecule which is active during the bR→K transition is shown in Figure 4. Based on our present results and the electron diffraction derived structure of bR (Henderson et al., 1990), one probable location (1) is in the immediate vicinity of Tyr-57. We positioned the water molecule to form hydrogen bonds with both Tyr-57 and negatively charged Asp-212 in the native bR structure. In this position, movement of the nearby Schiff base is likely to alter the H-bonding of the water either due to direct electrostatic influence or because of changes in the

[4] In fact, a large downshift in the Schiff base C=N stretch frequency is observed at this stage of the photocycle (Rothschild et al., 1984), indicative of such a change in environment.

[5] Similar results have been recently obtained from the Y57D mutant expressed in the native *Halobacterium halobium* (A. Nillsson, M. Coleman, and K. J. Rothschild, unpublished results).

positioning of Asp-212 and/or Tyr-57. In support of this model, two other residues which are also located close to Asp-212, Tyr-185, and Trp-86 (not shown) have also been found to respond to chromophore isomerization (Braiman et al., 1988b; Rothschild et al., 1989b) during the bR→K transition. This model would also account for the large shift in frequency of the water bands we detect when Tyr-57 is replaced by Asp-57. In this case, the substitution of a hydroxyl group by a carboxyl group is expected to result in a repositioning of the water to again optimize H-bonding (Rath et al., 1993).

A second possible position for the structurally active water molecule is between Asp-85 and the protonated Schiff base (position 2). This would help explain why Y57D does not form the M intermediate (Sonar et al., 1994a), since disruption of this water might interfere with proton transfer between the Schiff base and Asp-85 (Braiman et al., 1988a). However, in this case it is necessary to postulate that substitution of Tyr-57 disrupts a water molecule near Asp-85. This is possible if this substitution altered the overall structure of the active site. On the other hand, the C=N stretch frequency of Y57D as well as the frequency of other chromophore bands is unaltered compared to native bR (Sonar et al., 1994), a strong indication that the environment around the Schiff base is very similar to native bR. In addition, substitution of an asparagine for Asp-85 does not substantially alter the hydrogen bonding of the Schiff base, consistent with a more direct interaction between Asp-85 and the Schiff base (Rath et al., 1993). An additional possible position of a water molecule is between Arg-82, Asp-212, and the Schiff base [see Figure 6 of Rath et al. (1993)]. In this case, a water molecule may still interact with the Schiff base by forming a weak hydrogen bond in a bifurcated interaction.

Recently, it was found that the mutation Asp-85→Asn (D85N) causes the 3644 cm^{-1} band to disappear in the bR→L FTIR-difference spectrum (Maeda et al., 1994). It was concluded from this study that a water molecule interacts directly with Asp-85 and the protonated Schiff base in the L intermediate similar to the water shown at position 2 in Figure 4. However, in view of our similar results on Y57D, a unique position of the water molecule cannot yet be specified. In particular, the replacement of the Asp-85 residue with a neutral residue could also alter the structure of the active-site sufficiently to affect the H-bonding of a water molecule located in position 1.

Our results also indicate that the water molecule which is altered during the primary phototransition is still altered at the L and M stages of the bR photocycle, since the 3644 cm^{-1} negative band is detected at these stages. However, since this band appears to gain intensity, we cannot exclude the possibility that this water molecule undergoes a partial deprotonation at these stages of the photocycle. In the case of the mutant Y57D, the 3644 cm^{-1} band is absent in all of the difference spectra, replaced by new bands in the 3550 cm^{-1} region. This most likely reflects the response of a water molecule which is still located in the active site, but is now sensing the different environment caused by replacement of Tyr-57 with an Asp residue. In a related paper (Sonar et al., 1994a), we show that Asp-57 *deprotonates* at the L stage of the photocycle. An interesting possibility is that the water molecule we detect in Y57D also deprotonates, thereby accounting for the strong negative band near 3550 cm^{-1} in the bR→L difference spectrum.

In conclusion, we have established by this work that a water molecule is structurally active during the primary photoreaction of bacteriorhodopsin and is most likely located inside the active-site. Furthermore, at least one additional water molecule is perturbed later during the K→L and L→M steps of the photocycle undergoing a decrease in its H-bonding, although we do not as yet have information about the location of this (these) water(s). It is likely that future studies employing FTIR spectroscopy and site-directed mutagenesis, as well as the recently introduced technique of site-directed isotope labeling (SDIL) (Sonar et al., 1994b), will be able to provide further information about the role of these waters in the bR proton pump mechanism.

ACKNOWLEDGMENT

We thank Drs. P. Rath, J. Olejnik, and O. Bousché for help during the course of this work and Xiao-Mei Liu for assistance in some of the measurements.

REFERENCES

Birge, R. R. (1990) *Biochim. Biophys. Acta 1016*, 293–327.
Bousché, O., Braiman, M., He, Y. W., Marti, T., Khorana, H. G., & Rothschild, K. J. (1991) *J. Biol. Chem. 266*, 11063–11067.
Bousché, O., Sonar, S., Krebs, M. P., Khorana, H. G., & Rothschild, K. J. (1992) *Photochem. Photobiol. 56*, 1085–1096.
Braiman, M. S., & Rothschild, K. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 541–570.
Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G., & Rothschild, K. J. (1988a) *Biochemistry 27*, 8516–8520.
Braiman, M. S., Mogi, T., Stern, L. J., Hackett, N. R., Chao, B. H., Khorana, H. G., & Rothschild, K. J. (1988b) *Proteins: Struct., Funct., Genet. 3*, 219–229.
Braiman, M. S., Bousché, O., & Rothschild, K. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2388–2392.
Brzezinski, B., Zundel, G., & Kraemer, R. (1987) *J. Phys. Chem. 91*, 3077–3080.
Cao, Y., Varo, G., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1991) *Biochemistry 30*, 10972–10979.
De Groot, H. J. M., Smith, S. O., Courtin, J., Van den Berg, E., Winkel, C., Lugtenburg, J., Griffin, R. G., & Herzfeld, J. (1990) *Biochemistry 29*, 6873–6883.
Fahmy, K., Weidlich, O., Engelhard, M., Tittor, J., Oesterhelt, D., & Siebert, F. (1992) *Photochem. Photobiol. 56*, 1073–1083.
Foss Smith, D., & Overend, J. (1972) *Spectrochim. Acta 28A*, 471–483.
Fredlin, L., Nelaniter, B., & Ribbegard, G. (1977) *J. Chem. Phys. 66*, 4065–4072.
Gerwert, K. (1992) *Biochim. Biophys. Acta 1101*, 147–153.
Gerwert, K., Hess, B., Soppa, J., & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943–4947.
Harbison, G. S., Roberts, J. E., Herzfeld, J., & Griffin, R. G. (1988) *J. Am. Chem. Soc. 110*, 7221–7223.
Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899–929.
Hildebrandt, P., & Stockburger, M. (1984) *Biochemistry 23*, 5539–5548.
Huang, K.-S., Bayley, H., Liao, M.-J., London, E., & Khorana, H. G. (1981) *J. Biol. Chem. 256*, 3802–3809.
Krebs, M. P., & Khorana, H. G. (1993) *J. Bacteriol. 175*, 1555–1558.
Lanyi, J. (1990) *Physiol. Rev. 70*, 319–329.
Lozier, R. H., Xie, A., Hofrichter, J., & Clore, G. M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3610–3614.
Maeda, A., Sasaki, J., Ohkita, Y. J., Simpson, M., & Herzfeld, J. (1992a) *Biochemistry 31*, 12543–12545.
Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992b) *Biochemistry 31*, 462–467.

Maeda, A., Sasaki, J., Shichida, Y., Yoshizawa, T., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992c) *Biochemistry 31*, 4684–4690.
Maeda, A., Sasaki, J., Yamazaki, Y., Needleman, R., & Lanyi, J. K. (1994) *Biochemistry 33*, 1713–1717.
Marti, T., Otto, H., Mogi, T., Rosselet, S. J., Heyn, M. P., & Khorana, H. G. (1991) *J. Biol. Chem. 266*, 6919–6927.
Merz, H., & Zundel, G. (1983) *Chem. Phys. Lett. 95*, 529–532.
Mohr, S. C., Wilk, W. D., & Barrow, G. M. (1965) *J. Am. Chem. Soc. 87*, 3048–3052.
Nagle, J. F., & Mille, M. (1981) *J. Chem. Phys. 74(2)*, 1367–1372.
Oesterhelt, D., & Stoeckenius, W. (1971) *Nature (London), New Biol. 233*, 149–152.
Olejnik, J., Brzezinski, B., & Zundel, G. (1992) *J. Mol. Struct. 271*, 157–173.
Papadopoulos, G., Dencher, N. A., Zaccai, G., & Bualdt, G. (1990) *J. Mol. Biol. 214*, 15–19.
Rath, P., Marti, T., Sonar, S., Khorana, H. G., & Rothschild, K. J. (1993) *J. Biol. Chem. 268*, 17742–17749.
Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987) *Biochemistry 26*, 6696–6707.
Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147–167.
Rothschild, K. J., & Sonar, S. (1995) in *CRC handbook of organic photochemistry and photobiology* (Song, P.-S., Ed.) CRC Press, Inc., London (in press).
Rothschild, K. J., Roepe, P., Lugtenburg, J., & Pardoen, J. A. (1984) *Biochemistry 23*, 6103–6109.
Rothschild, K. J., Roepe, P., Ahl, P. L., Earnest, T. N., Bogomolni, R. A., Das Gupta, S. K., Mulliken, C. M., & Herzfeld, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 347–351.
Rothschild, K. J., Braiman, M. S., Mogi, T., Stern, L. J., & Khorana, H. G. (1989a) *FEBS Lett. 250*, 448–452.
Rothschild, K. J., Gray, D., Mogi, T., Marti, T., Braiman, M. S., Stern, L. J., & Khorana, H. G. (1989b) *Biochemistry 28*, 7052–7059.
Rothschild, K. J., He, Y. W., Sonar, S., Marti, T., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 1615–1622.
Shen, Y., Safinya, C. R., Liang, K. S., Ruppert, A. F., & Rothschild, K. J. (1993) *Nature 366*, 48–50.
Sonar, S., Marti, T., Rath, P., Fischer, W., He, Y.-W., Khorana, H. G., & Rothschild, K. J. (1994a) *J. Biol. Chem.* (in press).
Sonar S., Lee, C.-P., Coleman, M., Patel, N., Liu, X., Marti, T., Khorana, H. G., RajBhandary, U. L., & Rothschild, K. J. (1994b) *Nature Struct. Biol. 1*, 512–516.
Spudich, J. L., & Bogomolni, R. A. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 193–215.