Conformational Changes in the Core Structure of Bacteriorhodopsin†

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Received January 30, 1998; Revised Manuscript Received May 18, 1998

ABSTRACT: Bacteriorhodopsin (bR) is the light-driven proton pump found in the purple membrane of *Halobacterium salinarium*. In this work, structural changes occurring during the bR photocycle in the core structure of bR, which is normally inaccessible to hydrogen/deuterium (H/D) exchange, have been probed. FTIR difference bands due to vibrations of peptide groups in the core region of bR have been assigned by reconstituting and regenerating delipidated bR in the presence of D_2O. Exposure of bR to D_2O even after long periods causes only a partial shift of the amide II band due to peptide NH → ND exchange only of peripheral peptide structure. However, the amide II band completely downshifts when reconstitution/regeneration of bR is performed in the presence of D_2O, indicating that almost the entire core backbone structure of bR undergoes H/D exchange. Peripheral regions can then be reexchanged in H_2O, leaving the core backbone region deuterated. Low-temperature FTIR difference spectra on these core-deuterated samples reveal that peptide groups in the core region respond to retinal isomerization as early as the K intermediate. By formation of the M intermediate, infrared differences in the amide I region are dominated by much larger structural changes occurring in the core structure. In the amide II region, difference bands appear upon K formation and increase upon M formation which are similar to those observed upon the cooling of bacteriorhodopsin. This work shows that retinal isomerization induces conformational changes in the bacteriorhodopsin core structure during the early photocycle which may involve an increase in the strength of intramolecular α-helical hydrogen bonds.

Bacteriorhodopsin (bR)[1] is the light-driven proton pump found in the purple membrane of *Halobacteria salinarium* (*3*, *4*). It consists of a 26 000 Da integral membrane protein and a retinylidene chromophore covalently linked through a Schiff base to Lys-216 (*5*). Upon light absorption, bR undergoes a photocycle consisting of a series of intermediates with different visible absorption:

$$bR_{568} \rightarrow K_{630} \rightarrow L_{550} \rightarrow M_{412} \rightarrow N_{550} \rightarrow O_{640} \rightarrow bR_{568}$$

Vectorial proton transport through the membrane results from the ejection of a proton into the extracellular medium during M_{412} formation and uptake of a proton from the cytoplasmic medium during O_{640} formation. Other major steps in the photocycle include the all-trans to 13-cis retinal isomerization during K_{630} formation and retinal reisomerization back to an all-trans configuration during O_{640} formation (*6*−*8*).

Although the three-dimensional structure of bR has recently been solved with a resolution of 2.5 Å (*9*), important questions still remain about the molecular basis for proton transport. Perhaps the most important is how light-driven retinal isomerization couples to protein structural changes and subsequent proton transport. In this regard, FTIR difference spectroscopy has been shown to be an effective method to monitor conformational changes in receptors and other membrane proteins (*10*−*14*). In the case of bR, structural changes of the retinylidene chromophore and protonation and/or hydrogen-bonding changes of specific Asp, Tyr, Trp, Pro, Lys, and Thr residues have been detected at different stages of the photocycle (*1*, *2*, *15*−*17*). These studies along with other spectroscopic approaches have led to an increasingly detailed picture of how specific residues are involved in proton translocation during the photocycle (*8*).

Much less is known about structural changes occurring in the peptide backbone of bR. BR consists of a peripheral region where H/D exchange of backbone amide peptide groups occurs rapidly and a core region where the backbone amide peptide groups are largely inaccessible to H/D exchange (*18*, *19*). Polarized FTIR spectroscopy combined with H/D exchange has shown that this core region consists primarily of α-helical structure oriented predominantly perpendicular to the membrane plane and buried largely within the lipid bilayer (*19*, *20*). However, until now, detailed information has not been available from FTIR about the conformational changes of this core region during specific steps in the photocycle. Part of the difficulty is that amide I and amide II difference bands which reflect conformational changes in the backbone region of bR overlap with bands due to other protein (*21*) and chromophore groups (*22*) and thus have been difficult to assign. In addition, thus far, there

† This work was supported by a grant from the NSF (MCB9419059) to K.J.R.

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[1] Abbreviations: PM, purple membrane; bR, bacteriorhodopsin; IR, infrared; FTIR, Fourier transform infrared; OD, optical density; H/D, hydrogen/deuterium; HH-bR, HD-bR, DH-bR, DD-bR, H/D exchange state of peptide groups in the core and peripheral structure of the reconstituted sample, where the first letter stands for the state of the core region and the second letter for the state of the peripheral region.

S0006-2960(98)00246-3 CCC: $15.00
Published on Web 06/25/1998

has been no systematic method to separate vibrational contributions of peptide groups in the peripheral and core backbone regions of bR.

In this work, we have selectively probed backbone structural changes in the core and peripheral regions of bR during its photocycle.[2] This has been accomplished by removing lipids and retinal from bR, partially denaturing it in detergent, and then reconstituting/regenerating bR in native *H. salinarium* lipids. This approach has previously been shown to result in reconstituted bR with properties similar to bR in the native purple membrane (*23*, *24*). As discussed below, when reconstitution/regeneration is performed in the presence of D_2O, the entire peptide backbone of bacteriorhodopsin undergoes H/D exchange. Peripheral regions can then be reexchanged in H_2O, leaving the core region fully deuterated. FTIR difference spectra on such samples reveal that the core region of bacteriorhodopsin responds to retinal isomerization as early as during the bR → K transition with the amide I and II bands undergoing frequency shifts similar to those observed upon lowering the temperature of purple membrane. Even larger changes in the core region occur by formation of the M intermediate, as indicated by the appearance of a more intense set of bands which undergo frequency shifts in the amide I and amide II region.

MATERIALS AND METHODS

Delipidation, Regeneration, and Reconstitution of bR. All chemicals were obtained from Sigma (St. Louis, MO). Purple membrane was isolated from cultures of *H. salinarium* (strain S9) and purified as described previously (*25*). Total lipids from whole cells were extracted as described (*26*). Delipidation/denaturation of purple membrane was carried out by a solvent extraction procedure as described by Braiman et al. (*27*). Refolding and regeneration was performed according to Popot (*26*). For refolding in a deuterated medium, solutions of delipidated bacterioopsin (bO) in SDS were repetitively freeze-dried from D_2O. All buffers used were deuterated by repetitive evaporation with D_2O. Finally, refolded bR was washed several times with D_2O.

Low-Temperature FTIR Difference Spectroscopy. bR → K and bR → M FTIR difference spectra were recorded using previously reported methods (*28*−*30*). Samples were prepared by air-drying approximately 100−200 μg/cm^2 of sample on a AgCl window and then rehydrating prior to insertion into a sealed transmittance cell which was mounted in a Helitran cryostat (Air Products, Allentown, PA). For the purpose of peripheral deuteration, the sample was exposed to a drop of D_2O inside a drybox for approximately 1 h, which allows H/D exchange of most peptide groups directly exposed to the external medium (*31*). The H_2O (D_2O) content of the sample was checked by monitoring the intensity of the 3400 cm^{-1} (2600 cm^{-1}) band relative to the amide I and amide II bands. All samples, except where noted, were light-adapted at room temperature prior to cooling by illuminating the sample for at least 15 min with a 150 W tungsten light source (Dolan-Jenner Industries, Inc., Lawrence, MA) equipped with a 505 nm long-pass filter.

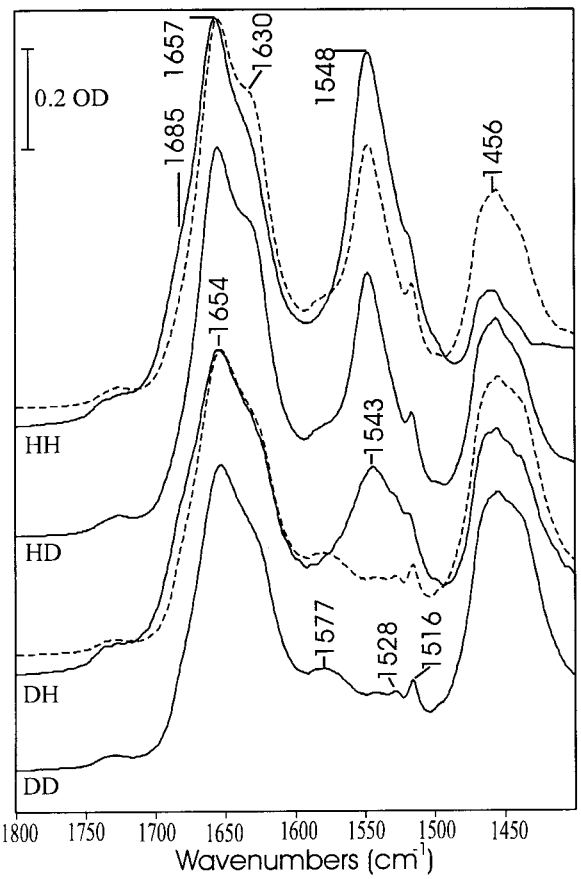

FIGURE 1: FTIR absorption spectra of bR reconstituted in native *H. salinarium* lipids. Samples were refolded and regenerated according to the procedure of Popot (*26*) in either H_2O or D_2O buffer and prior to measurement exposed to either H_2O or D_2O for the purpose of hydration (see Materials and Methods). The resulting samples, HH-bR, HD-bR, DH-bR, and DD-bR, where the first letter specifies the refolding/regeneration medium and the second the hydration/deuteration medium, were measured at 250 K with 2 cm^{-1} resolution (solid traces). Dashed traces reproduce the HD-bR spectrum which is superimposed on the HH-bR spectrum for comparison (top) or DD-bR spectrum which is superimposed on the DH-bR spectrum (bottom). The scale bar refers to the *y*-scale of HH-bR.

The samples were cooled to 250 or 80 K in order to measure the bR → M or bR → K FTIR difference spectra, respectively. Spectra were recorded at 2 cm^{-1} resolution using a Nicolet Analytical Instruments 740 spectrometer (Madison, WI).

RESULTS

Hydrogen/Deuterium Exchange in the Core Region of Bacteriorhodopsin. Figure 1 compares the FTIR absorption spectra of the four reconstituted samples HH-bR, HD-bR, DH-bR, and DD-bR recorded at 250 K. The infrared spectrum of bR reconstituted in *H. salinarium* lipids and measured at 250 K in the presence of H_2O (HH-bR) is similar to previously published spectra of native purple membrane measured under similar conditions (*31*). For example, an amide I band appears near 1657 cm^{-1} and an amide II band near 1548 cm^{-1} in both cases.[3] However, HH-bR also displays a more prominent shoulder near 1630 cm^{-1} which

[2] Peripheral and core are used here to describe bR structure which is available for H/D exchange during exposure to the external medium (peripheral) or when exposed during reconstitution/regeneration (core).

[3] The amide II band shifts in frequency from 1545 to 1548 cm^{-1} upon cooling from RT to 250 K.

is normally associated with β-structure (*31*−*33*). This most likely arises from incomplete refolding of bR in the membrane during reconstitution. For example, a similar effect is observed for samples of bR expressed in *E. coli* and reconstituted using a similar procedure (*27*). One interesting possibility is that this effect is associated with the G-helix, which forms a β-sheet structure when it is chemically synthesized as a separate helix fragment and reconstituted into phospholipid vesicles (*34*). The amide II/amide I ratio is also lower in the reconstituted sample (HH-bR) compared to bR in native purple membrane. This effect most likely arises from a lower degree of orientation of the reconstituted membranes (e.g., higher mosaic spread) with respect to the sample plane (*20*, *35*).

A major effect of H/D exchange on the infrared absorption of native bacteriorhodopsin is to lower the absorption of the amide II band near 1548 cm^{-1} and increase the absorption in the region near 1456 cm^{-1} (amide II′ mode) (*31*). This effect occurs due to the NH → ND exchange of peptide backbone groups which causes a downshift in the vibrational frequency of the amide II vibration of approximately 100 cm^{-1} (*36*). In bacteriorhodopsin, the effect is relatively small because of the existence of a core region which is inaccessible to H/D exchange. The total extent of H/D exchange in bR peptide groups was estimated on the basis of infrared measurements as 20% after 2 h of exposure to D_2O and only 27% after 48 h at room temperature (*31*).

As seen in Figure 1, a similar amide II downshift occurs in delipidated bR, which is refolded/reconstituted in H_2O and deuterated in D_2O (HD-bR). However, as in the case of purple membrane, the exchange is still incomplete due to the inaccessibility of the core region of bR. In contrast, delipidated bR refolded/reconstituted in the presence of D_2O and subsequently exposed to D_2O in the external medium (DD-bR) shows almost complete disappearance of the amide II band with the appearance of a new band corresponding to the amide II′ mode at 1456 cm^{-1}. This demonstrates that the delipidated/denatured bR undergoes complete H/D exchange of all peptide groups in the core region during the refolding/reconstitution procedure. The reexposure of this core-deuterated sample to H_2O (DH-bR) then causes a reappearance of the amide II band and decrease of the amide II′ band intensities, reflecting the D/H exchange of the peripheral structure of bR.

Several other conclusions can be drawn from the absolute absorption of core-deuterated bacteriorhodopsin (DH-bR and DD-bR). First, core deuteration causes only a small isotope downshift in the amide I band from 1657 to 1654 cm^{-1} consistent with the assignment of this band to α-helical structure (*37*, *38*). In contrast, as previously noted (*31*), the major change induced by peripheral H/D exchange (HH-bR to HD-bR) is a drop in absorption near 1685 cm^{-1}, reflecting a higher frequency subcomponent of the amide I band assigned to β-structure or reverse turns (*31*, *39*−*41*). This indicates that non-α-helical structure is present mainly in regions of the bR backbone accessible to H/D exchange, most likely corresponding to surface loop regions. The amide II frequency for this structure, which is reflected in the DH-bR spectrum, is also downshifted 5 cm^{-1} to 1543 cm^{-1}, again indicating the presence of non-α-helical structure. We also note that almost complete disappearance of the amide II band in DD-bR unmasks much smaller bands such as at

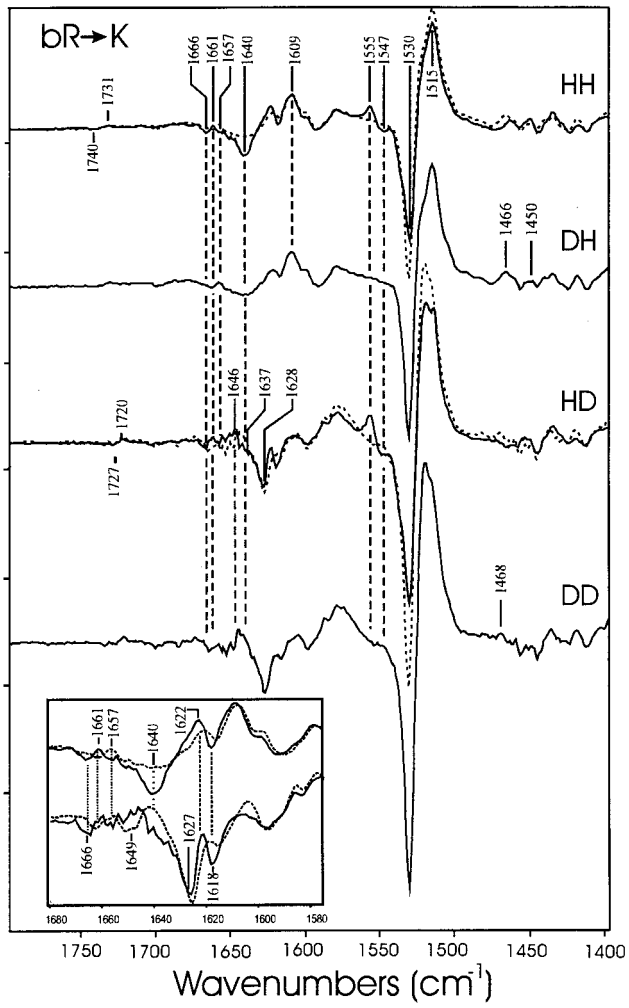

FIGURE 2: bR → K FTIR difference spectra of bR reconstituted in native *H. salinarium* lipids. Difference spectra were recorded at 80 K at 2 cm^{-1} as described (Materials and Methods) on the four different reconstituted samples HH-bR, DH-bR, HD-bR, and DD-bR (solid traces). Dashed traces reproduce the DH-bR spectrum which is superimposed on the HH-bR spectrum for comparison (top) or DD-bR spectrum which is superimposed on the HD-bR spectrum for comparison (bottom). The tick marks on the *y*-axis are 0.008 OD spacing for the HH-bR spectrum. Inset: Expansion in the region 1575−1680 cm^{-1} with top traces the superposition of HH-bR (solid line) and DH-bR (dashed line) and bottom traces the superposition of HD-bR (solid line) and DD-bR (dashed line).

1528 cm^{-1} from the C═C stretch mode of the all-trans retinal chromophore (*42*), 1516 cm^{-1} due to the aromatic ring mode of tyrosines (*43*), and 1577 cm^{-1} due to the asymmetric $C-O^-$ stretch mode of carboxylate groups present in Asp and Glu groups (*21*).

Structural Changes in the K Intermediate. Figure 2 compares the bR → K FTIR difference spectra in the region from 1400 to 1800 cm^{-1} for the four reconstituted samples. The HH-bR spectrum is very similar to the corresponding bR → K spectrum of native bR (e.g., bR in purple membrane) in H_2O in this region and other regions of the spectrum below 1400 cm^{-1} (not shown). For example, a weak pair of bands appears at 1740/1731 cm^{-1} (−/+), assigned to the carbonyl (C═O) stretch mode of Asp-115 (*2*). As expected, negative bands due to the all-trans retinylidene chromophore of light-adapted bR (bR_{570}) appear at 1640 cm^{-1} (C═N stretch of protonated Schiff base) and 1530 cm^{-1} [ethylenic C═C stretch (*44*)], while positive bands characteristic of the 13-

cis retinylidene chromophore in the K intermediate are found at 1609 cm^{-1} (C═N stretch of protonated Schiff base) (*29*) and 1515 cm^{-1} (ethylenic C═C stretch). A similar agreement is also found between the HD-bR and native bR measured in D_2O, including a downshift of the Asp-115 carbonyl stretch bands to 1727/1720 cm^{-1} (−/+) and a downshift of the C═N vibration to 1628 cm^{-1} due to deuteration of the Asp-115 carboxylic acid group and the Schiff base, respectively. A splitting of the positive band near 1515 cm^{-1} also occurs as previously noted (*29*).

To assign bands in the bR → K difference spectra, spectra recorded with and without core deuteration were compared, e.g., HH-bR and DH-bR (samples exposed to H_2O) and HD-bR and DD-bR (samples exposed to D_2O). Previous studies on the effect of H/D exchange on the bR → K difference spectrum of native bR revealed changes mainly due to the effects of Schiff base deuteration (*29*, *44*). In contrast, the major effect of core deuteration is to cause a shift in the frequency of several bands in the amide I and II regions which arise from the vibrations of protein groups, leaving bands assigned to the chromophore vibrations unaltered (Figure 2 and inset).

In the amide I region, a pair of weak negative/positive bands at 1666/1661 cm^{-1} is downshifted ∼5 cm^{-1} to 1661/1657 cm^{-1} upon core deuteration, indicating that this pair is assignable to the amide I mode of one or more peptide groups which are located in the core region of bR. A second effect of core deuteration is the apparent reduction in the intensity of the negative band at 1640 cm^{-1} assigned to the C═N stretch of the protonated Schiff base. However, this is most likely due to a frequency shift of one or more amide I bands into the region of the 1640 cm^{-1} band, thereby partially canceling its intensity. For example, the pair of negative/positive peaks in DD-bR at 1649/1640 cm^{-1} would act to partially cancel the intensity of the 1640 cm^{-1} band in DH-bR. The absence of a similar reduction in intensity upon core deuteration in the case of the 1628 cm^{-1} band assigned to the C═N stretch of the deuterated Schiff base (*29*) is a further indication that this effect is not due to an intrinsic change in the C═N mode. Spectral changes are also observed at lower frequency upon core deuteration with positive/negative bands at 1622/1618 cm^{-1} downshifting 4−5 cm^{-1}. Although this is in the range of a tyrosine ring mode, the assignment to tyrosine is unlikely since isotopic labeling of tyrosines has little effect on the bR → K difference spectrum in this region (*45*).

One of the clearest effects of core deuteration is the disappearance of a pair of positive/negative bands at 1555/1547 cm^{-1} in DH-bR and DD-bR. This change is accompanied by the appearance of a positive band near 1466−1468 cm^{-1} in both spectra, although the expected appearance of a negative band at lower frequency is not clearly seen. Overall, these changes are consistent with the assignment of the 1555/1547 cm^{-1} difference bands to the amide II mode of backbone peptide groups with at least the positive component downshifting approximately 90 cm^{-1} due to core deuteration. As described below, a similar but much larger effect is observed in the bR → M difference spectrum.

Structural Changes in the M Intermediate. Figure 3 compares bR → M FTIR difference spectra of the four reconstituted samples. Overall, spectra of the non-core-deuterated samples (HH-bR and HD-bR) compare well to

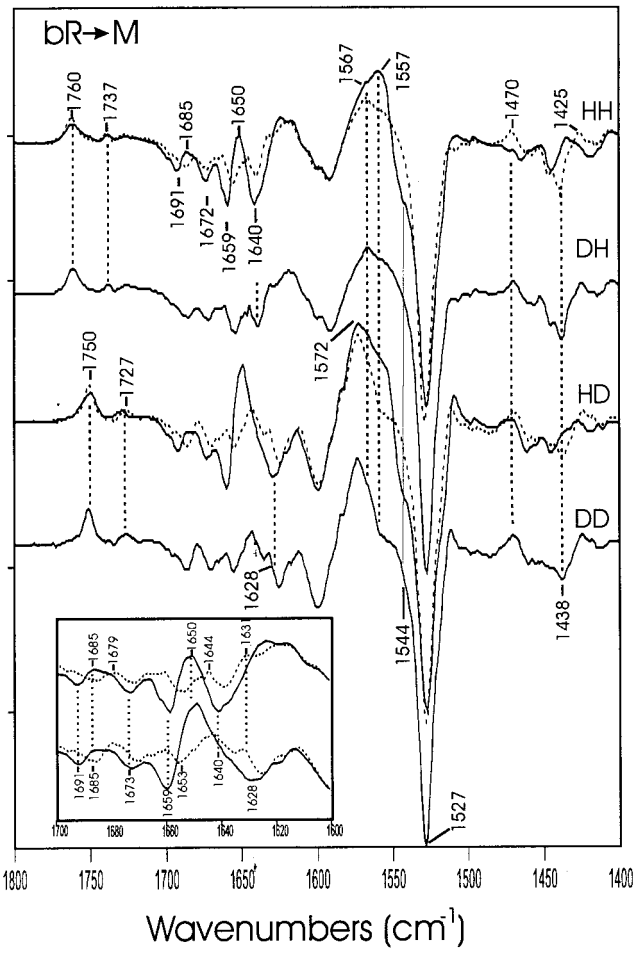

FIGURE 3: bR → M FTIR difference spectra of bR reconstituted in native *H. salinarium* lipids. Difference spectra were recorded at 250 K at 2 cm^{-1} as described (Materials and Methods) on four different reconstituted samples HH-bR, DH-bR, HD-bR, and DD-bR (solid traces). Dashed traces reproduce the DH-bR spectrum which is superimposed on the HH-bR spectrum for comparison (top) or DD-bR spectrum which is superimposed on the HD-bR spectrum for comparison (bottom). The tick marks on the *y*-axis are 0.01 OD spacing for the HH-bR spectrum. (Inset) Expansion in the region 1700−1600 cm^{-1} with top traces the superposition of HH-bR (solid line) and DH-bR (dashed line) and bottom traces the superposition of HD-bR (solid line) and DD-bR (dashed line).

the corresponding bR → M difference spectra of native purple membrane recorded in H_2O and D_2O, respectively (*30*).[4] For example, several major bands appear above 1700 cm^{-1} which are assigned to the C═O stretch mode of Asp/Glu carboxylic acid groups including the Asp-85 C═O stretch at 1760 and 1750 cm^{-1} for COOH and COOD, respectively (*1*, *2*, *14*). Major bands assigned to vibrations of the bR_{570} retinylidene chromophore include the negative bands at 1640 cm^{-1} (1628 cm^{-1} in the case of HD) assigned to the C═N stretch mode of the Schiff base and at 1527 cm^{-1} due to the ethylenic C═C stretching mode.

In general, core deuteration induces significant changes in both the amide I and amide II regions, which are much more pronounced compared to changes in the bR → K

[4] Small differences are found with native bR in purple membrane including a less intense pair of negative/positive bands at 1742/1748 cm^{-1} corresponding to a change of hydrogen bonding in Asp-96 at this stage of the photocycle (*1*). These differences are also found in bR expressed in *E. coli* (*2*) and refolded using a similar procedure.

difference spectrum (Figure 2). The major effect in the amide I region is the downshift of a set of negative/positive bands of approximately 2−10 cm^{-1}, consistent with their assignment to the carbonyl stretch of peptide groups (i.e., amide I mode). For example, in both DH-bR and DD-bR, negative/positive bands at 1691/1685 cm^{-1} downshift approximately 6 cm^{-1}. A small negative band at 1673 cm^{-1} also downshifts 2−3 cm^{-1}. The largest effect is the 3−6 cm^{-1} downshift of the negative/positive bands from 1659/1650 to 1653/1644 cm^{-1}. As in the case of bR → K, the apparent reduction in intensity of the negative C═N band at 1640 cm^{-1} is most likely due to the downshift of the positive band at 1650 to 1644 cm^{-1} in DH-bR. A new positive band also appears at 1631 cm^{-1} in core-deuterated samples.

Since these downshifts are consistent with those expected for amide I bands upon peptide deuteration (e.g., CONH → COND), the results indicate that most of the bands in the region between 1640 and 1700 cm^{-1} arise from structural rearrangements in peptide groups located in the core region of bR, which are normally inaccessible to H/D exchange. The 1691/1685 cm^{-1} bands deserve special mention, since H/D exchange could also cause a downshift of the C═O stretch of carboxylic acid groups. However, this is unlikely since ^{13}C labeling of the carboxylic acid group of Asp or Glu residues does not produce significant frequency shifts in this region (*46*). We cannot exclude, however, the possibility that this band arises from the C═O stretch mode of the amide group on the side chain of Asn. However, such an assignment would require that these side-chain residues are buried in the core region of bR and not accessible for H/D exchange.

Large changes are also observed in the amide II region upon core deuteration. Most dramatic is the drop in intensity of a positive band near 1557 cm^{-1} along with a negative shoulder located near 1544 cm^{-1}. The loss of the former unmasks the band at 1567 cm^{-1} due to the ethylenic stretch mode of the M intermediate (1572 cm^{-1} in the case of HD-bR). Deuteration of peptide groups should cause the amide II bands to downshift as is observed in the absolute absorption of bR (Figure 1). In agreement, a pair of positive/negative bands appears in the DH-bR and DD-bR spectra at 1470 and 1438 cm^{-1}. Although, the $\Delta\nu$ splitting between the positive and negative components in the amide II′ region is larger than that observed in the amide II region (32 cm^{-1} vs 13 cm^{-1}), the H/D exchange induced frequency downshift may depend on the degree of coupling with the amide III mode which in the case of α-helices is in the 1275−1300 cm^{-1} region (*47*). Note also that a positive band appears near 1425 cm^{-1} due to core deuteration, although its assignment is not clear.

DISCUSSION

A number of studies have shown that bacteriorhodopsin contains a core structure which is resistant to H/D exchange (*18*, *19*, *48*). It was concluded on the basis of FTIR dichroism measurements on oriented samples of purple membrane (*31*) that this core structure corresponds to the oriented α-helical structure buried in the interior of the purple membrane (*49*). For example, although H/D exchange produces shifts in the amide I and amide II bands similar to those observed here (Figure 1), almost no changes occur in the dichroism difference spectrum reflecting mainly oriented α-helices (*19*). The small frequency shift we observe of the amide I band (∼3 cm^{-1}) upon core deuteration is also characteristic of α-helical structure (*38*) and, hence, agrees with this conclusion. In contrast, the higher frequency shoulder on the amide I band near 1685 cm^{-1}, which drops in intensity when bR is exposed to D_2O (*31*), is not sensitive to core-deuteration and, hence, most likely reflects structure which is present in peripheral regions of the protein.

In this paper, we have examined whether the H/D resistant core structure in bR undergoes structural changes during the bR photocycle. This is an important question since a complete elucidation of the bR proton pump mechanism will most likely require an understanding of the role of the bR peptide backbone. Earlier studies on bR were not able to address this question directly since bands in the region from 1500 to 1700 cm^{-1} had not been definitively assigned to amide I and II modes. This requires either NH → ND exchange of core peptide groups, which are normally resistant to H/D exchange, or isotope labeling of specific peptide groups in the core region (*50*, *51*).

Our results clearly establish that many of the prominent bands detected in the amide I and amide II region of the bR → K and bR → M difference spectra are directly attributable to structural changes in the core of bR. Furthermore, since these changes occur as early as the bR → K transition, they must reflect the response of the core to light-driven isomerization of the chromophore. In the amide I region, weak bands are found in the bR → K difference spectrum (1666/1661 cm^{-1}) which increase in magnitude upon formation of M (1659/1650 cm^{-1}). The frequency and small downshift (∼5 cm^{-1}) of these bands is within the range expected of α-helical structure in bR (*20*, *37*). On the other hand, changes are also seen in the amide I region which are not typical of α-helices, including negative/positive features at 1649/1640 cm^{-1} and 1622/1618 cm^{-1} in the bR → K difference spectrum and at 1691/1685 cm^{-1} in bR → M. These frequencies are more typical of *β*-structure and might represent several core residues, which do not participate in α-helical hydrogen bonding (*33*).

One of the most important results of this study is the assignment of positive/negative bands near 1557/1544 cm^{-1} to the amide II mode of the core peptide backbone structure which appear upon formation of the K intermediate and increase in magnitude up to at least formation of the M intermediate. This is a highly congested region of the difference spectrum which can contain contributions from both protein and chromophore group modes. The assignment of these positive/negative bands to the amide II mode is consistent with a frequency upshift of this mode during the early bR → M portion of the bR photocycle. Recent time-resolved FTIR measurements of core-deuterated bR show that these amide II changes continue to increase in magnitude upon formation of the N intermediate (Kluge, T., Smilowitz, L., Olejnik, J. and Rothschild, K. J., unpublished data) and correspond to the appearance of an intense positive band at 1556 cm^{-1}, which was previously identified as a characteristic feature of the N intermediate (*52*−*55*). Since this band does not shift in the bR → N difference spectrum upon prolonged exposure of bR to D_2O, it was concluded that it reflects peptide structural changes in the core structure of bR (*56*).

Interestingly, a similar frequency upshift of the amide II band also occurs when bacteriorhodopsin is cooled, with bands appearing at 1558 (+) and 1543 (−) cm^{-1} in the temperature-induced difference spectrum of bR (135 → 80 K) (*57*). A cooling-induced downshift in the amide I frequency from 1665 to 1654 cm^{-1} also occurs in bR (*57*), similar to the amide I band shifts we observe during the bR → K and bR → M difference spectra (1666/1661 cm^{-1} and 1659/1650 cm^{-1}, respectively). One possibility is that both phenomena reflect an increase in the strength of intramolecular hydrogen bonding in some regions of bR α-helices. In particular, an increase in hydrogen-bonding strength is generally correlated with a decrease in frequency of the amide I mode and increase in the amide II mode (*58*). Other effects should also be considered including alterations in the dipole−dipole coupling between neighboring α-helical segments (*59*) and tilting of transmembrane α-helical segments in bR which can lead to dichroism effects (*56*). Spectroscopic evidence for tilting of polypeptide segments between bR_{568} and formation of the M intermediate has also been reported on the basis of CD studies (*60*). It is interesting to note that similar temperature-induced shifts in the amide I and II band have been observed in polypeptides such as poly(L-alanine) (*61*) as well as individual fragments of bR reconstituted into membranes (Hunt, J., Engelman, D. M. and Rothschild K. J., unpublished data). Clearly, additional studies will be necessary to further investigate the origin of the amide I and II shifts during the bR photocycle including assignments of these bands to specific regions of the bR peptide backbone.

The approach used in this work can in principle be applied to other protein systems provided a means of deuterating the core structure exists. Bacteriorhodopsin is special in this regard, since it can be folded and reconstituted into membranes without the need for chaperone proteins. In general, most integral membrane proteins are expected to have significant core structure. For example, structure inaccessible to H/D exchange even after prolonged exposure to D_2O has been found in rhodopsin (*18*, *40*, *62*, *63*), acetylcholine receptor (*64*), and a truncated form of phospholamban (*65*). The assignment of amide I and II bands in FTIR difference spectra to core and peripheral regions of membrane proteins can be especially useful in order to correlate structure and function. For example, FTIR spectroscopy and H/D exchange have revealed that photoactivation of rhodopsin causes a portion of the core structure to become exposed to the external medium (*66*). FTIR spectroscopy and H/D exchange of acetylcholine receptor have also led to the conclusion that peripheral regions undergo structural changes during receptor desensitization (*67*). Future studies which utilize this approach can also take advantage of the ability of FTIR difference spectroscopy to measure nanosecond changes in membrane protein conformation (*68*).

ACKNOWLEDGMENT

We wish to thank Xiao-Mei Liu for technical assistance and Dr. Maarten Heyn for assistance with Tilmann Kluge's arrangements to conduct research at Boston University.

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BI9802465