Hydrogen bonding changes of internal water molecules in rhodopsin during metarhodopsin I and metarhodopsin II formation

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Rhodopsin is a 7-helix, integral membrane protein found in the rod outer segments, which serves as the light receptor in vision. Light absorption by the retinylidene chromophore of rhodopsin triggers an 11-*cis* → all-*trans* isomerization, followed by a series of protein conformational changes, which culminate in the binding and activation of the G-protein transducin by the metarhodopsin II (Meta II) intermediate. Fourier transform IR difference spectroscopy has been used to investigate the structural changes that water, as well as other OH- and NH-containing groups, undergo during the formation of the metarhodopsin I (Meta I) and Meta II intermediates. Bands associated with the OH stretch modes of water are identified by characteristic downshifts upon substitution of $H_2{}^{18}O$ for H_2O. Compared with earlier work, several negative bands associated with water molecules in unphotolysed rhodopsin were detected, which shift to lower frequencies upon formation of the Meta I and Meta II intermediates. These data indicate that at least one water molecule undergoes an increase in hydrogen bonding upon formation of the Meta I intermediate, while at least one other increases its hydrogen bonding during Meta II formation. Amino acid residue Asp-83, which undergoes a change in its hydrogen bonding during Meta II formation, does not appear to interact with any of the structurally active water molecules. Several NH and/or OH groups, which are inaccessible to hydrogen/deuterium exchange, also undergo alterations during Meta I and Meta II formation.

INTRODUCTION

Rhodopsin is a 7-helix, integral membrane protein found in the disc photoreceptor membranes of rod outer segments, and is responsible for scotopic vision in vertebrates [1,2]. Light causes a rapid 11-*cis* to all-*trans* isomerization of the rhodopsin retinylidene chromophore [3,4], followed by a series of spectral changes which correspond to the different bleaching intermediates [bathorhodopsin (Batho) → lumirhodopsin (Lumi) → metarhodopsin I (Meta I) → metarhodopsin II (Meta II)] in the photoactivation cascade [5,6]. Signal transduction occurs upon formation of the Meta II intermediate, the only intermediate known to bind and activate the G-protein transducin [7,8]. In a much slower process, Meta II decays to metarhodopsin III, or to opsin and all-*trans* retinal [9]. An important goal in vision research is to understand the key molecular events that underlie rhodopsin activation. On a broader level, research on rhodopsin may hold significance for understanding the molecular mechanism for activation of a variety of other G-protein-coupled receptors.

Fourier transform (FT)IR difference spectroscopy has been used extensively to study conformational changes in membrane proteins [10–12]. In the case of rhodopsin, conformational changes of the retinylidene chromophore, protonation and/or hydrogen bonding changes of Asp, Glu and Cys residues, structural changes of the peptide backbone, and structural changes of the membrane lipid matrix have been detected at different stages of the photoactivation cascade [13–23]. Assignment of vibrational bands to individual protein groups has been facilitated by site-directed mutagenesis [19,20] and recently by uniform isotope labelling (F. DeLange, P. Rath, C. Klaassen, J. Raap, K. J. Rothschild, W. J. DeGrip and J. Lugtenburg, unpublished work).

FTIR difference spectroscopy can also be used to study changes in the environment of individual water molecules in rhodopsin during the different steps in the photoactivation cascade. Bands due to the OH stretching mode of water can be identified by a characteristic approx. 10 cm^{-1} shift upon substitution of H_2O with $H_2{}^{18}O$. Accessibility of these water molecules to hydrogen/deuterium exchange (H/D exchange) can also be probed by using ^2H_2O, which causes much larger band shifts of the order of 1000 cm^{-1}. Negative bands, which are shifted by isotope substitution of water, can be assigned to the unphotolysed rhodopsin, whereas positive bands that are shifted correspond to the state of water in the bleaching intermediate (e.g. Meta I or Meta II). Only by detecting bands from both states (e.g. negative/positive bands) can information be obtained about changes in the structure of water molecules during bleaching. In previous FTIR studies of the Batho, Lumi, Meta I and Meta II intermediates, positive bands were assigned to water molecules whose OH stretching modes undergo an alteration in hydrogen bonding strength during photoactivation [24,25]. However, in the case of the formation of the Meta I and Meta II intermediates, low signal/noise prevented the detection of clear negative bands which could be assigned to water in unphotolysed rhodopsin [24]. Thus an important goal is to obtain increased signal/noise difference spectra sufficient to assign negative bands in the FTIR difference spectrum. Probing the involvement of internal water molecules during formation of the Meta II intermediate is especially important due to its role in G-protein activation.

In this work, several negative bands associated with water

Abbreviations used: FT, Fourier transform; Rho, rhodopsin; Batho, bathorhodopsin; Lumi, lumirhodopsin; Meta I, metarhodopsin I; Meta II, metarhodopsin II; H/D exchange, hydrogen/deuterium exchange.

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molecules in unphotolysed rhodopsin are detected that shift to lower frequency, indicative of increased hydrogen bonding, upon formation of the Meta I and Meta II intermediates [19,20]. At least one water molecule is altered during Meta I formation, whereas at least one other is altered during Meta II formation. Site-directed mutagenesis indicates that none of these water molecules interacts with Asp-83, which was previously found to undergo hydrogen bonding changes during Meta II formation. Several other bands are found that are associated with NH and/or OH groups that are unavailable for H/D exchange in unphotolysed rhodopsin. However, one NH group, possibly associated with a tryptophan residue, becomes exposed to the external medium during Meta II formation.

EXPERIMENTAL

Rhodopsin membranes were prepared from bovine rod outer segments according to methods described previously [26]. The A_{280}/A_{500} ratio of the resulting washed photoreceptor membrane was typically 2.0 ± 0.1. Membrane suspensions at a concentration of 55 nmol/ml of rhodopsin were stored at -20 °C until further use. Sample films for transmission spectroscopy were prepared by isopotential spin-drying [27,28] of an aqueous suspension of photoreceptor membranes in H_2O, containing approx. 1–3 nmol of rhodopsin, on to an AgCl window. The film is then rehydrated before insertion into a sealed transmittance cell as described previously [19]. For $H_2{}^{18}O$ measurements, the rhodopsin film was first dried for more than 12 h in a dry-air box in order to remove residual H_2O. The dried film was then exposed to bulk $H_2{}^{18}O$ for more than 24 h by putting a 10 μl drop directly on to the film and then sealing it with a second AgCl window. It was then redried in a dry-air box and assembled into a sealed IR cell using a second AgCl window containing small drops (approx. 3 μl) of $H_2{}^{18}O$ placed outside of the IR beam path. The D83N mutant was produced using recombinant baculovirus and the *Spodoptera frugiperda* cell line, IPLB-Sf9, and the FTIR difference spectrum obtained as reported previously [19].

Transmission FTIR difference spectra of hydrated rhodopsin films were recorded by using methods similar to those reported previously [17,29]. The H_2O, ^2H_2O or $H_2{}^{18}O$ content of the sample was monitored by measuring the intensity ratio of the 3400 cm^{-1} band (O–H stretch mode) or 2600 cm^{-1} band (O–^{2}H stretch mode) to the methyl and methylene C–H stretch bands of the protein and lipids in the 2800–3000 cm^{-1} region. The Rho → Meta I and Rho → Meta II difference spectra were recorded at -15 °C and 10 °C respectively. The sample was photobleached for 3 min using light from a 150 W tungsten illuminator (Model 180, Dolan–Jenner Industries, Lawrence, MA, U.S.A.) filtered by a 500 nm long-pass filter (Corion, Holliston, MA, U.S.A.) and several heat filters, and transmitted to the sample with an annular optical fibre. At -15 °C, only a small amount of Meta II is formed under these conditions, based on the appearance of bands at 1766/1750 cm^{-1} associated with the formation of Meta II. Spectra were recorded at 8 cm^{-1} resolution and 11 min intervals for several hours before and after illumination (3000 scans for each spectrum) on a Bio-Rad FTS-60A spectrometer (Bio-Rad, Digilab Division, Cambridge, MA, U.S.A.) equipped with a HgMnTe detector. Each difference spectrum shown represents a subtraction of the spectrum recorded immediately before the light is turned on, from the spectrum recorded immediately after, which includes the 3-min period of light illumination. Note that both the H_2O and $H_2{}^{18}O$ difference spectra exhibited some broad baseline distortion in the region 3200–3400 cm^{-1}, most probably due to slow water evaporation from the IR cell.

RESULTS

FTIR difference spectra of rhodopsin in the region below 1800 cm^{-1} have been reported previously for the Batho, Lumi, Meta I and Meta II intermediates [16]. These spectra mainly reflect changes in the C–C, C–N, C–O, C=O, C=N stretching modes and C–H, N–H and OH bending modes of both the retinylidene chromophore and the protein. In contrast, the region above 3200 cm^{-1} reflects OH and NH stretching vibrations, including modes from peptide groups (amide A, NH stretch), amino acid side chains with NH-containing groups, such as tryptophan residue (indole NH stretch), water (OH stretch) and other hydroxy-containing residues, such as serine and threonine (OH stretch). (Alterations in the SH stretch mode of cysteine appear in the 2500–2600 cm^{-1} region [21].) In principle, the band assigned to the NH stretching mode of the protonated Schiff base could also be found in this region. Generally, the intensity and frequency of these modes are sensitive to changes in hydrogen bonding. For example, the OH stretch of water, as well as the NH stretch of peptides and tryptophan, downshift in frequency upon an increase in hydrogen bonding [30,31]. However, since there are both symmetric (low frequency) and asymmetric (high frequency) stretch modes for water, it is sometimes not possible to assign the absolute strength of a hydrogen bond but only the changes in the relative hydrogen bonding strength [32].

Water molecules that change upon formation of Meta I

Figure 1 shows the Rho → Meta I difference spectrum in the region above 3200 cm^{-1} for rhodopsin hydrated with H_2O, $H_2{}^{18}O$ and ^2H_2O. Several highly reproducible bands appear in this region, including two intense pairs of negative/positive bands at 3286/3304 and 3478/3500 cm^{-1}. These bands, as well as all other bands below 3525 cm^{-1}, are not significantly affected by ^2H_2O, indicating that the corresponding groups are inaccessible to the outer medium and do not undergo H/D exchange. In contrast, almost all of the bands above 3525 cm^{-1} disappear when rhodopsin is exposed to ^2H_2O. This indicates that these groups are freely accessible for H/D exchange even in the unphotolysed rhodopsin.

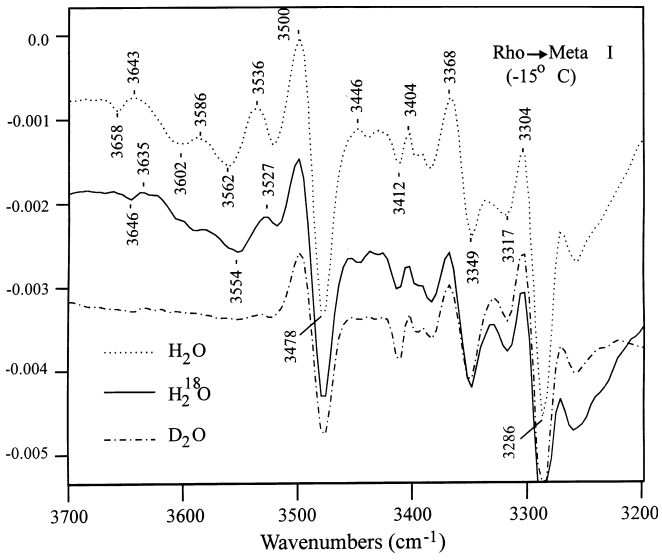

Figure 1 FTIR difference spectra of the Rho → Meta I transition recorded at -15 °C for rhodopsin films hydrated with H_2O, ^2H_2O (D_2O) and $H_2{}^{18}O$

The *y*-axis shown is for the spectrum in H_2O.

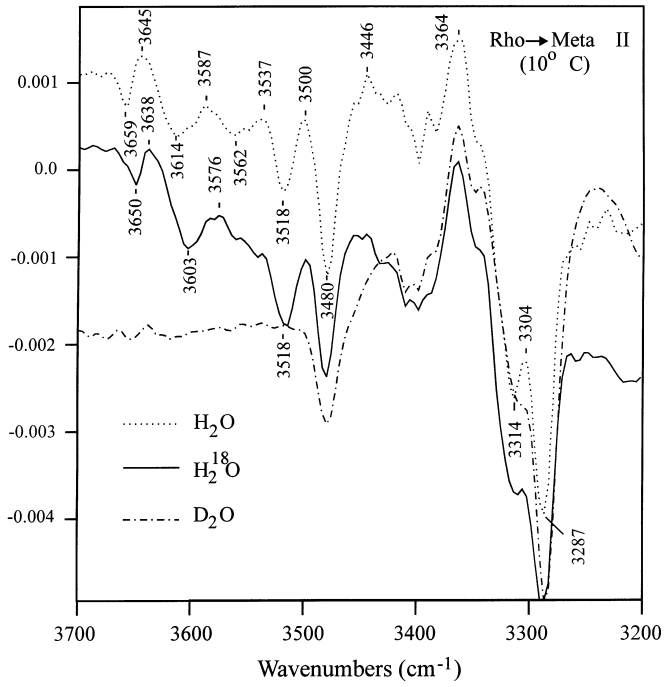

Figure 2 FTIR difference spectra of the Rho → Meta II transition recorded at 10 °C for rhodopsin films hydrated with H_2O, ^2H_2O (D_2O) and $H_2{}^{18}O$

The *y*-axis shown is for the spectrum in H_2O.

In order to assign bands due to the OH stretch mode of water, rhodopsin films were humidified with $H_2{}^{18}O$. As seen in Figure 1, only ^2H_2O-sensitive bands are altered, exhibiting a downshift in the range 7–10 cm^{-1}, characteristic of the OH stretch of water molecules. Three individual sets of negative/positive bands can be distinguished at 3658/3643, 3602/3586 and 3562/3536 cm^{-1}. However, the pair at higher frequency (3658/3643 cm^{-1}) is likely to be associated with the Meta II formation (see below). We therefore conclude on this basis that one and possibly two water molecules are present in rhodopsin, which undergo an increase in hydrogen bonding strength during Meta I formation. In an earlier study [24], only the positive band near 3536 cm^{-1} was detected in both the Lumi and the Meta I intermediates, and was found to be $H_2{}^{18}O$-sensitive.

Water molecules that change upon formation of Meta II

Rho → Meta II difference spectra for rhodopsin exposed to H_2O, $H_2{}^{18}O$ and ^2H_2O are shown in Figure 2. This spectrum is considerably different from the Rho → Meta I difference spectrum and reveals several new bands. In the region above 3525 cm^{-1}, all of the bands can be assigned to the OH stretch of water on the basis of an approx. 10 cm^{-1} downshift for the spectrum in $H_2{}^{18}O$. Furthermore, as in the Rho → Meta I case, all of the bands in this region disappear due to H/D exchange. Compared with the Rho → Meta I difference spectrum, the 3659/3645 cm^{-1} pair of bands significantly increases in intensity and is therefore assigned to a water molecule that is altered during formation of the Meta II intermediate. The less intense bands observed at the same frequency in the Rho → Meta I difference spectrum most probably arise from residual amounts of Meta II formed at −15 °C. A second set of bands also appears at 3614/3587 cm^{-1}, which are distinct from the less intense set at 3602/3586 cm^{-1} in the Rho → Meta I difference spectrum (the latter may still be present but hidden under the more intense bands). The bands at 3562/3536 cm^{-1} found in the Rho → Meta I spectrum are also still present but are less intense. In an earlier report, the positive $H_2{}^{18}O$-sensitive component at 3645 cm^{-1} was identified but the other bands were not clearly seen [24]. Thus we conclude that the Meta I → Meta II transition involves at least one (3659/3645 cm^{-1}) and possibly a second water molecule (3614/3587 cm^{-1}) that undergo an increase in hydrogen bonding upon formation of the Meta II intermediate.

Several bands are also observed in the Rho → Meta I and Rho → Meta II difference spectra below 3525 cm^{-1}, which appear to be insensitive to both $H_2{}^{18}O$ and ^2H_2O. Most notable is the band near 3480 cm^{-1} (negative), which has been previously assigned, by analogy to bacteriorhodopsin, to the NH stretch mode of a tryptophan indole group [24]. A positive band at 3500 cm^{-1} may also be associated with this NH group in the Meta I intermediate. However, this band becomes less prominent in the Rho → Meta II difference spectrum, possibly due to overlap with a new negative band at 3518 cm^{-1}. This new negative band does not shift in $H_2{}^{18}O$ but is absent in ^2H_2O, and thus is associated with an NH or OH group that can undergo H/D exchange in unphotolysed rhodopsin. We could not confirm the disappearance of the band at 3500 cm^{-1} in the Rho → Meta II spectrum for the case of ^2H_2O as reported previously [24] due to its overlap with the negative band at 3518 cm^{-1}. However, a second positive, broad band near 3446 cm^{-1} is also present in the Rho → Meta I and Meta II difference spectra and absent only in the case of Meta II in ^2H_2O. Hence this band may represent an additional NH group, possibly from a tryptophan residue, or an OH group, which becomes accessible for H/D exchange only upon formation of Meta II.

The largest change in this region is the appearance of a previously unreported, intense negative band at 3287 cm^{-1} that is present in Meta I and intensifies upon Meta II formation. This band falls in the region of the amide A mode (NH stretch vibration) of peptide groups [31]. For example, the major band in the absolute absorption spectrum of rhodopsin in this region appears at 3290 cm^{-1} at −15 °C and 3305 cm^{-1} at 10 °C. In the Rho → Meta I difference spectrum this band appears to be associated with a positive component at 3304 cm^{-1}. A second pair is also found in the Rho → Meta I difference spectrum at 3349/3368 cm^{-1} (−/+). Hence these pairs of bands may arise from two peptide groups that undergo a decrease in hydrogen bonding strength upon Meta I formation. The same two pairs of bands are also observed in the Rho → Meta II difference spectrum, but the positive components are masked by the increase in intensity of a negative band near 3287 cm^{-1}.

Asp-83 does not interact with structurally active water molecules during the Meta I → Meta II transition

In an earlier FTIR study of rhodopsin bleaching, two bands at 1767 cm^{-1} (negative) and 1748 cm^{-1} (positive) were assigned on the basis of site-directed mutagenesis to the C=O stretch mode of the Asp-83 carboxylic acid group [19,20]. It was concluded that Asp-83 undergoes an increase in hydrogen bonding during the Meta I → Meta II transition. In order to determine if this change involved alterations in the interaction of Asp-83 with water molecules, the region above 3200 cm^{-1} was examined for a mutant of rhodopsin, where Asp-83 is replaced by an Asn residue (D83N). As seen in Figure 3, all of the bands assigned to water molecules in the Rho → Meta II difference spectrum of wild-type rhodopsin are still observed in the corresponding spectrum of D83N. Since substitution of an Asn for an Asp

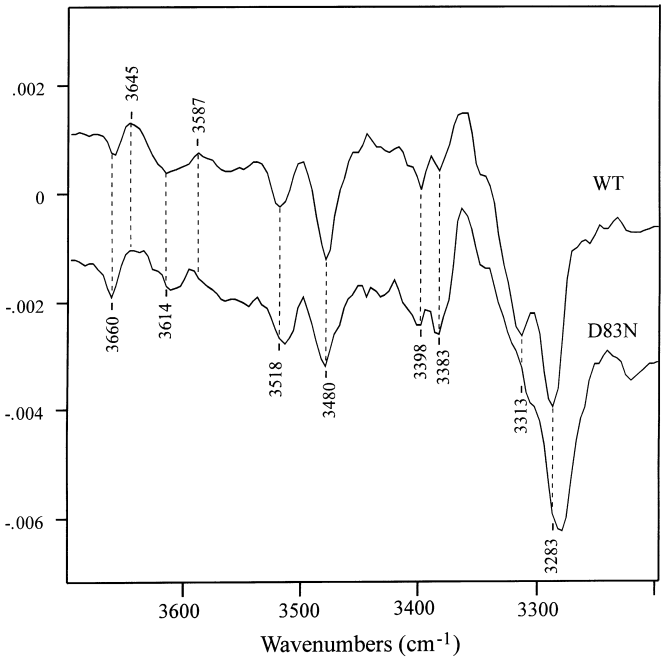

Figure 3 Comparison of the FTIR difference spectra for the Rho → Meta II transition for wild-type rhodopsin (WT) and the mutant Asp-83 → Asn (D83N)

The D83N mutant was produced and FTIR difference spectrum obtained as reported previously [19]. The *y*-axis shown is for the WT spectrum.

residue results in the replacement of a hydroxy group with an amino group, we would expect the hydrogen bonding to nearby water molecules to be significantly altered by this substitution. Since we do not detect significant shifts in the vibrational frequency of the water molecules detected, we conclude that it is unlikely that Asp-83 hydrogen bonds with any of these water molecules. A downshift is, however, observed in the intense negative peak at 3283 cm^{-1}, which may indicate that the mutation alters a structurally active peptide group(s).

DISCUSSION

In this work we have focused on water molecules which are structurally active during the formation of the Meta I and Meta II intermediates of the rhodopsin photocascade by using a combination of site-directed mutagenesis, FTIR difference spectroscopy, H/D exchange and water replacement with $H_2{}^{18}O$. Any water that is structurally active, i.e. undergoes a change in hydrogen bonding, orientation or protonation state, should give rise to bands in the FTIR difference spectra in the region above 3200 cm^{-1}, where the OH stretch mode of water is found. ^2H_2O and $H_2{}^{18}O$ substitutions allow the assignment of bands first to all groups accessible for H/D exchange and secondly to specific water molecules. Earlier work using this approach led to the detection of positive bands associated with water molecules that change their hydrogen bonding during formation of the Batho, Lumi, Meta I and Meta II intermediates [24,25]. However, the low signal/noise ratio prevented the assignment of negative bands to water molecules, thereby making it difficult to determine how water is altered in rhodopsin in response to photoactivation.

The increased signal/noise obtained in the present work made possible the assignment of both negative and positive bands to water molecules that are structurally active in rhodopsin. Our findings show that one water molecule is altered during the formation of Meta I and a second during the Meta I → Meta II transition. At least one extra water molecule may also be involved in each of these steps, but could not be unambiguously assigned because of the possible detection of multiple bands from a single water molecule. This can occur for example if a water molecule has one strongly and one weakly bonded hydrogen atom. 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 and therefore may be more difficult to detect [30,32]. In either case of one or two water molecules involved at each step, the pattern of bands (e.g. negative/positive pairs with the negative being the highest frequency component) is consistent with an increase in the hydrogen bonding of the water molecules.

It can also be concluded that the weakest hydrogen bonded water molecule, which we associated with the 3658/3643 cm^{-1} pair of bands, becomes altered during formation of the Meta II intermediate. This is similar to the pattern observed in bacteriorhodopsin, where at least one very weakly hydrogen bonded water molecule is altered during the formation of the M intermediate in the bacteriorhodopsin photocycle [33]. However, the pattern in this case shows a reversal in hydrogen bonding changes, with the band shifting up in frequency being indicative of a decrease in hydrogen bonding upon M formation.

The location of the structurally active water molecules in rhodopsin will be an important element in ascertaining their role in the photoactivation. In an initial step towards this goal, we examined a possible interaction of water with Asp-83, which has previously been found to undergo a change in hydrogen bonding during Meta II formation. Following earlier work on bacteriorhodopsin [33,34], this is possible by examining whether substitutions of specific residues, which in this case involved Asp-83 → Asn, induced changes in the assigned water OH bands. Our negative result demonstrates that Asp-83, located on helix-II towards the centre of the membrane, does not interact with any of the assigned water molecules. Instead, Asp-83 may form a direct interaction with some other nearby protein groups or with the chromophore during the Meta I → Meta II transition. A second possible location for the interaction of a water molecule is Glu-113, the putative proton acceptor and counterion of the Schiff base [35–37]. In the case of bacteriorhodopsin, FTIR evidence indicates that a water molecule interacts with the analogous residue Asp-85 [34]. In support of such an interaction in rhodopsin, the removal of water blocks the Meta I → Meta II step, and under ultrahigh vacuum a shift is induced in the rhodopsin absorption from 500 → 390 nm [38].

In addition to structurally active water, our results demonstrate that a variety of other NH or OH groups, including peptide NH, undergo distinct changes in hydrogen bonding during the Meta I and Meta II steps of photoactivation. Almost all of these groups were inaccessible for H/D exchange in both the unphotolysed rhodopsin and the Meta I and Meta II intermediates. This indicates that a significantly large core of rhodopsin remains buried in the membrane interior during the photoactivation cascade. However, we also detect the existence of an NH or OH group(s), possibly from the indole ring of a tryptophan residue, as suggested earlier [24], which becomes available for H/D exchange only upon formation of the Meta II intermediate. This is consistent with recent evidence from FTIR that photoactivation also causes buried portions of the rhodopsin backbone structure to become more accessible during Meta II formation [38a].

Further assignments in this region can be made through a combination of site-directed mutagenesis and isotope labelling.

For example, the possibility that one of these bands arises from the NH stretch of the protonated Schiff base can be probed through uniform labelling of lysines in rhodopsin with ^{15}N at the ϵ-amino group. The possible assignment to a threonine hydroxy group can be probed with an ^{18}O label located at the hydroxy group. In this regard, recent progress has been made in this direction by introducing isotope labels into rhodopsin expressed in an insect culture system (C. H. W. Klaassen, F. DeLange, J. Raap, P. Rath, K. J. Rothschild, J. Lugtenburg and W. J. DeGrip, unpublished work). More specific assignments can be obtained in the future, either by substitution of specific amino acid residues (site-directed mutagenesis) or through isotope labelling of specific amino acid residues (site-directed isotope labelling) [39].

This research was supported by grants from the NIH-NEI (EY05499) to K.J.R. and from the Netherlands Organization for Scientific Research, Chemical Division (NWO-SON, WGM 330-011 and 328-050) to W.J.D. We thank Dr. S. Williams for many valuable suggestions for improving the manuscript.

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Received 3 June 1997/5 September 1997; accepted 17 September 1997