Tryptophan Interactions in Bacteriorhodopsin: A Heteronuclear Solid-State NMR Study†

Aneta T. Petkova,§ Minoru Hatanaka,§ Christopher P. Jaroniec,§ Jingui G. Hu,§ Marina Belenky,‡ Michel Verhoeven,|| Johan Lugtenburg,|| Robert G. Griffin,§ and Judith Herzfeld*‡,§

Department of Chemistry and Keck Institute for Cellular Visualization, MS #015, Brandeis University, Waltham, Massachusetts 02454-9110, Francis Bitter Magnet Laboratory and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Rijksuniversiteit te Leiden, 2300 RA Leiden, The Netherlands

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ABSTRACT: The bulky and amphiphilic nature of tryptophan residues makes them particularly interesting components of proteins. In bacteriorhodopsin, four of the eight tryptophan residues are in the active site, forming parts of the retinal binding pocket. In this work, we use solid-state NMR to study the interactions of the tryptophan residues in wild-type bacteriorhodopsin, in the resting state, and in critical intermediates of the proton-motive photocycle. The range of the chemical shifts of the indole nitrogens suggests that all eight of them are hydrogen bonded. Using difference spectroscopy, we isolate several changes in these hydrogen bonds in the early and late M states. As found earlier for the peptide backbone, some perturbations found in the early M state relax in the transition to the late M state while new perturbations arise. Interestingly, Rotational Echo DOuble Resonance (REDOR) difference spectroscopy of [20-13C]retinal,-[indole-15N]Trp-bR shows that indole of Trp182 is not involved in the significant hydrogen bond perturbations. We also use REDOR to measure dipolar interactions in [20-13C]retinal,[indole-15N]Trp-bR, and thereby determine the distance between the C20 of retinal and the indole nitrogen of Trp182. The internuclear distance changes only slightly from the light-adapted state (3.36 ± 0.2 Å) to the early M state (3.16 ± 0.4 Å).

Bacteriorhodopsin (bR) is a retinal pigment that functions as a light-driven proton pump in the cell membranes of Halobacterium salinarum. Intermediates in the proton-motive photocycle with distinct absorption spectra are labeled sequentially as bR568 (or LA), J, K, L, M, N, and O. The photocycle begins with photoinduced isomerization of the retinal to Lys216 (in the L state). The SB is reprotonated; MAS, magic angle spinning; NMR, nuclear magnetic resonance; REDOR, rotational echo double resonance; SB, Schiff base; SSNMR, solid-state NMR; TMS, tetramethylsilane; TPPM, two-pulse phase modulation; WT, wild type.

from a complex of residues at the extracellular surface. On the other hand, proton uptake occurs at the cytoplasmic side of the membrane, after Asp96 has donated its proton to the SB (in the M→N transition). The reprotoxinated chromophore then reisomerizes from 13-cis,15-anti to all-trans,15-anti, and Asp85 deprotonates to recover the initial state [see references (1–3)].

Although considerable progress has been made toward elucidating the detailed proton transfer mechanism in bR, many key issues remain unresolved. Primary among them is the means by which backflow is prevented. Thus, much attention has focused on differences between the early M state in which the SB proton has just been released to the extracellular side of the protein and the late M state in which the SB is about to be reprotonated from the intracellular side of the protein. Multiple M intermediates have been predicted by molecular dynamics simulations (4–6) and by different sequential kinetic schemes to which time-resolved visible absorption profiles were fitted (7, 8). It was also found that in various bR preparations, two successive M’s could be distinguished by a 4–7 nm blue shift in the absorption (7, 9). However, FTIR studies have so far found only very small variations in the M intermediates in native bR (10).

Recently we (11) succeeded in distinguishing early M (M0) and late M (M5) photocycle intermediates in solid-state nuclear magnetic resonance (SSNMR) magic angle spinning (MAS) spectra. The M0 and M5 states were accumulated at different temperatures and shown to comply with the scheme...
Both M intermediates have a 13-cis,15-anti conformation as determined from the chemical shifts of C13 and C14 in the retinal (for nomenclature, see Figure 1) and C\textsubscript{9} in Lys216. However, the \textsuperscript{15}N chemical shift of the SB moves upfield by about 8 ppm in the M\textsubscript{0}→M\textsubscript{a} transition. This shift indicates an increase in the pK\textsubscript{a} and/or hydrogen bonding of the SB, and therefore greater readiness to reprotonate. Furthermore, the contrast with the blue shift in the visible spectrum indicates that the relaxation of chromophore distortion between the L and N states can be localized to the M\textsubscript{0}→M\textsubscript{a} transition. In addition, the M\textsubscript{0}→M\textsubscript{a} transition is associated with significant changes in the protein backbone (11). Given the observed changes in the SB, the chromophore, and the peptide backbone, it seems likely that the M\textsubscript{0}→M\textsubscript{a} transition serves as the “reprotonation switch” of the bR photocycle.

Tryptophan has the bulkiest side chain of all the amino acids, and shows both hydrophobic properties (through the aromatic system) and hydrophilic properties (through the indole nitrogen). The replacement of tryptophan with other aromatic systems (through the C9 methyl group of the retinal). The replacement of tryptophan with other aromatic systems (through the indole nitrogen). The replacement of tryptophan with other aromatic systems (through the indole nitrogen). The replacement of tryptophan with other aromatic systems (through the indole nitrogen).

Figure 1: Representation of the tryptophan residues surrounding the retinal based on X-ray diffraction results (58).
FIGURE 3: Rotational echo double resonance (REDOR) pulse sequence (31). The S-spins are cross-polarized from the \(^1\)H reservoir, and the signal is observed as a spin–echo. The rotor-synchronized \(\pi\) pulses (one pulse every \(1/2\tau_r\)) applied to the I nuclei reintroduce the I–S dipolar couplings. The I-spin pulses were phased according to the xy-8 scheme (33) to minimize pulse imperfections. To account for relaxation effects, a reference (\(S_0\)) experiment was recorded for each REDOR (S) experiment, by acquiring the spectrum in the absence of I-spin \(\pi\) pulses.

**Photocycle Intermediates.** The bR photocycle intermediates were accumulated in situ. The light-adapted (LA) state was obtained from the dark-adapted state via white light illumination (1000 W Xe lamp, Oriel Instruments, Stratford, CT) for 4 h at 2 °C, or upon thermal relaxation of LA photoproducts at 0 °C for 1 h. The \(M_n\) and \(M_o\) intermediates were accumulated by illumination of LA at \(-60\) and \(-20\) °C, respectively, with \(\lambda > 530\) nm (long-pass filter from Oriel Instruments) for 2 h. Once accumulated, all intermediates were cryo-trapped at \(-90\) °C, where NMR data were acquired in the dark.

**Solid-State NMR Spectroscopy.** The NMR experiments were performed on custom-designed spectrometers (courtesy of Dr. D. J. Ruben) operating at \(^1\)H Larmor frequencies of 317 and 400 MHz, with quadruple- and triple-resonance transmission line MAS probes (designed by Dr. C. M. Rienstra), equipped with 5 and 4 mm Chemagnetics spinner modules, respectively. The \(^13\)C chemical shifts are referenced to TMS with adamantane as a secondary standard [resonances at 38.56 and 29.50 ppm (28)]. The \(^15\)N chemical shifts are referenced to virtual liquid NH\(_3\) by using the \(^15\)N/\(^13\)C frequency ratios (29) and the chemical shift of TMS relative to DSS [\(+1.7\) ppm (30)]. All shifts have an uncertainty of 0.4 ppm.

The REDOR (31) pulse sequence employed to recouple heteronuclear dipolar interactions is shown in Figure 3. The initial S-spin magnetization was created via ramped cross-polarization (32) from \(^1\)H. The S-spin was observed as a spin–echo, where the echo intensity was modulated due to I–S dipolar interactions reintroduced under MAS by the rotor-synchronized \(\pi\) pulses applied to the I spins. To account for relaxation effects, reference spin–echo spectra were acquired in the absence of I-spin pulses. The I and S radio frequency fields during REDOR were \(-40\) kHz, and the pulses on the I channel were phased according to the xy-8 scheme (33) to compensate for pulse imperfections. CW \(^1\)H decoupling (~100 kHz) was applied during the REDOR period, and TPPM decoupling (34) (~95 kHz) was used during the acquisition of the free induction decay. For each bR photocycle intermediate studied, REDOR experiments were performed for three mixing times (9.6, 12.8, and 16.0 ms). The data were acquired in blocks of 320 scans, and approximately 14 000 transients were accumulated for each time point. The recycle delay was 2 s.

**Internal Distances.** Experimental \(^13\)C REDOR data were analyzed by calculating dephasing ratios for each mixing time, using difference spectroscopy to eliminate the natural-abundance \(^13\)C signals from each spectrum. \(S\) and \(S_0\) intensities were obtained from the difference spectra by least-squares fits of the retinal 20-\(^13\)C peak to Gaussian line shapes. The variation of the experimental (\(S_0 - S/S_0 = \Delta S/S_0\)) with mixing time was fit to the analytical expression describing the evolution of the observable S spin magnetization (\(^13\)C in this case) under the REDOR pulse sequence (31):

\[
\Delta S/S_0(t) = \lambda \left( 1 - \cos(\omega_{IS} t) \right)
\]

(1)

where \((\cdots)\) represents averaging over all possible orientations of the I–S dipole vector in the powder sample. The scaling factor \(\lambda\) accounts for the contribution to the \(S_0\) curve from \(^13\)C nuclei not dephased by \(^15\)N (\(^15\)N labeling of Trp residues in the sample used for the REDOR experiments was 63%, i.e., \(\lambda = 0.63\)). \(\omega_{IS}\) is the orientation-dependent dipolar coupling, which is a function of the dipolar coupling constant, \(b_{IS}\) (35):

\[
b_{IS} = -\left( \frac{\mu_0}{4\pi} \right) \gamma_I \gamma_S \frac{h^3}{I_{IS}}
\]

(2)

Here, \(r_{IS}\) is the I–S internuclear distance and \(\gamma_I\) and \(\gamma_S\) are the gyromagnetic ratios characteristic of the I and S spins, respectively.

**RESULTS**

**\(^15\)N Chemical Shifts of Trp Model Compounds.** To help interpret the \(^15\)N spectra of tryptophan residues in bR, we have measured the indole \(^15\)N chemical shifts in five natural-abundance tryptophan-containing crystals. The crystallographic studies indicate that the indole nitrogen (\(N_1\)) is hydrogen bonded to an oxygen in Trp-Gly-Gly-HBr (24), Ac-Trp-OMe (21), and L-tryptophan picrate (22), and is not hydrogen bonded in N-methyl-L-tryptophan (23) and Trp-HBr (24). We find a \(^15\)N chemical shift range of 133.5–125.6 ppm for the H-bonded \(N_1\), and 127.5–122.3 ppm for the non-H-bonded \(N_1\). The relatively deshielded nitrogen (127.5 ppm) in Trp-HBr could perhaps be due to very short contacts (3.42–3.80 Å) between \(N_1\) and four carbons of adjacent indole rings. Figure 4 shows the correlation between the \(N_1\)-O distance for the H-bonded indoles and the measured \(^15\)N chemical shift. We have tentatively assigned the two points for Trp picrate to conform with the overall trend. Consistent with Shoji et al. (26), we find that stronger H-bonding correlates with a less shielded \(^15\)N nucleus. This correlation suggests the possibility of interpreting tryptophan \(^15\)N chemical shifts in terms of H-bond strengths. A linear regression analysis of the data gives the relationship:

\[
r_{NO} = -0.0424 \text{ (Å/ppm)} \cdot \sigma_N + 8.46 \text{ (Å)}
\]

(3)

shown by the dashed line in Figure 4.

**\(^15\)N CPMAS Spectra of the Trp Side Chains in bR Photocycle Intermediates.** We proceed by considering the \(^15\)N indole resonances of the eight tryptophan residues in bR. The \(^15\)N CPMAS spectra of \([\epsilon_1, ^{15}\text{N}]\text{Trp-bR}\) for the LA,
M₀, and Mₙ states are shown in Figure 5. In the LA state (Figure 5, top), we observe an unresolved broad envelope of ¹⁵N signals ranging from 117 to 137 ppm. The larger peak centered at 130.6 ppm, with a downfield shoulder at 133.3 ppm and an upfield shoulder at 127.2 ppm, corresponds to the ¹⁵N signals of the eight labeled indole rings. The low intensity peak centered at 120.8 ppm represents the amide backbone signals due to the ¹⁵N natural abundance (and perhaps some scrambling of the original label). Changes in the indoles are apparent in the Mo state (Figure 5, middle), where the maximum intensity is shifted from 130.6 to 132.4 ppm, and a pronounced upfield shoulder is observed at 130.1 ppm. Yet a different ¹⁵N profile is acquired for the Mₙ state (Figure 5, bottom). We identify the most intense peak at 129.5 ppm, another peak at 132.6 ppm, and a shoulder on the downfield side at 134.1 ppm.

Since fitting the signals of the eight tryptophans is a nontrivial task, we try to identify the changes between the photocycle intermediates from the CP difference spectra (Figure 6). A careful examination of these spectra suggests that at least three residues are involved in the chemical shift changes between the states. The LA minus Mo spectrum (Figure 6, top) shows positive peaks at 130.5 and 127.4 ppm (LA), and a doubly intense negative peak at 133.1 ppm (Mo). Thus, at least two resonances shift downfield (suggesting stronger H-bonding) in the LA→M₀ transition. The LA minus Mₙ spectrum (Figure 6, middle) shows a doubly intense positive peak at 130.8 ppm (LA) and negative peaks both upfield and downfield at 135.0 and 128.6 ppm (Mₙ).

¹⁵N Chemical Shifts of the Trp182 Side Chain in bR Photocycle Intermediates. One might expect that the perturbations that we see in the ¹⁵N CP difference spectra (Figure 6) are most likely to be due to the tryptophans closest to the retinal, i.e., Trp86, Trp138, Trp182, and Trp189. In particular, Trp182 can be considered a likely candidate, since it has been suggested to interact with the C₉ and C₁₃ methyl groups of the retinal, and affect different steps of the photocycle (17–19, 37). Therefore, we direct our interest to Trp182, and will select the indole nitrogen signal of Trp182 (among the eight indole nitrogens) via its dipolar interaction with the ¹³C in the C₁₃ methyl group, i.e., the C₂₀ of the retinal. Since Trp182 is the only tryptophan residue near the C₁₃ and C₉ methyl groups of the chromophore, we expect only the ¹⁵N of Trp182 and the ²⁰-¹³C of retinal to be recoupled by REDOR in our sample. As a result, the ¹⁵N resonance of the Trp182 indole nitrogen should be isolated by subtracting the ¹⁵N REDOR signals from the ¹⁵N echo signals of [20-¹³C]ret,[ε₁-¹⁵N]Trp-bR (Figure 7). As expected, a single resonance is detected in each photocycle intermediate. The Trp182 N₁₁ signal appears at 130.4 ppm in the LA state (Figure 7, top), and shifts to 131.7 ppm in M₀ (Figure 7, middle) and to 131.2 ppm in Mₙ (Figure 7, bottom). As these
results show, the $N_{11}$ chemical shift of Trp182 in $M_0$ and $M_n$ is shifted slightly downfield compared to the LA state. Reference to Figure 4 suggests that in all three states, the indole of Trp182 is moderately strongly hydrogen bonded, perhaps a bit more so in $M_0$ and $M_n$ than in LA. However, it appears that Trp182 is not responsible for the peaks observed in the $^{15}N$ CP difference spectra (Figure 6).

$^{13}C$ Chemical Shift of the Retinal $C_{20}$ in bR Photocycle Intermediates. Since the retinal isomerization during the LA to $M_0$ and $M_n$ transition does not influence the $N_{11}$ chemical shift of Trp182 substantially, and because the efficiency of the $^{13}C$ channel of our NMR probe is much higher than the $^{15}N$ channel, we look for a better marker of the bR state in the $C_{20}$ resonance of the retinal. Figure 8 shows the retinal $^{13}C$ signals obtained for each photocycle intermediate when the $^{13}C$ spectrum of natural-abundance bR is subtracted from the $^{13}C$ spectrum of [20-$^{13}C$]ret,[1-$^{15}N$]Trp-bR. As expected two $C_{20}$ signals are present in the dark-adapted state of bR (Figure 8a) and only one in the light-adapted state (Figure 8b). Signals at $\sim 13.1$ ppm for the all-trans,15-anti-retinal of bR$_{568}$ and at 22.4 ppm for the 13-cis,15-syn-retinal of bR$_{555}$ are in agreement with the results obtained by Smith et al. ($38$). The upfield shift in bR$_{568}$ relative to bR$_{555}$ is attributed to the sterical interaction between the protons of $C_{20}$ and the proton of $C_{15}$ in the all-trans species that is absent in the 13-cis species. The $C_{20}$ signal shifts to 19.6 ppm in $M_0$ (Figure 8c) and to 17.8 ppm in $M_n$ (Figure 8d). Thus, the shift of the $C_{20}$ signal is a convenient marker of bR states. The similarity of the shifts in the two M species suggests that sterical interactions of the $C_{20}$ group are not much affected by the relaxation from $M_0$ to $M_n$, although the interactions seem to be somewhat stronger than in the 13-cis-bR$_{555}$.

**Intermolecular Distances in bR Photocycle Intermediates.** We have employed the REDOR experiment to determine the $C_{20}$-retinal to $N_{11}$-Trp182 distances for the LA and early M ($M_0$) photocycle intermediates. Figure 9 shows the $^{13}C$ spin–echo reference ($S_0$) (Figure 9a), REDOR dipolar dephasing ($S$) (Figure 9b), and difference ($S_0 - S$) (Figure 9c) spectra of [20-$^{13}C$]retinal,[1-$^{15}N$]Trp-bR in the LA state for the mixing time of 12.8 ms. The 13.2 ppm resonance which corresponds to the retinal $C_{20}$ is prominent in the reference spectrum (Figure 9a), and its intensity is substantially reduced in the REDOR spectrum (Figure 9b), due to dipolar dephasing by the $^{15}N_{11}$ of Trp182.

Similar spectra (not shown) have been obtained for various mixing times for both the LA and M$_0$ states. The resulting
The 15N chemical shift of the SB indicates that its channel. The connection of the SB to the extracellular intermediate which is gone in the N intermediate, where the spectroscopy find distortion of the chromophore in the L state but stronger than in the LA state. The electrostatic counterion interaction in the N state is weaker than in the L state. However, by 15N REDOR difference spectroscopy (Figure 6), hydrogen bonding of Trp182 is moderately hydrogen bonded in LA (ε15N resonance at 130.4 ppm), and remains so in the M state (ε15N resonance at 131.7 ppm) and the M state (ε15N resonance at 131.2 ppm). Thus, if the hydrogen bonding of Trp182 is perturbed by bending of the chromophore, this perturbation has largely dissipated by the early M state, and the transition from the early M state to the late M state occurs with essentially no effect on the hydrogen bonding of Trp182. These results are consistent with FTIR, UV, and visible Resonance Raman studies (17, 19) which show moderately strong H-bonding between N14 of Trp182 and a water molecule in the LA state which is absent in the L state and recovered in the M state. In any case, none of the large perturbations identified by 15N NMR difference spectroscopy (Figure 6) are due to Trp182.

Using the linear regression obtained from the model compound data (Figure 4), hydrogen bond lengths for the indole nitrogens can be estimated from their 15N chemical shifts and compared with the hydrogen bond lengths found chromophore to relax and the SB to be reoriented toward the intracellular transport channel. This timing is ideal for a protonation switch, the spring-loaded chromophore unwinding immediately after deprotonation, so that reprotonation must occur from a different direction.

The unwinding of the deprotonated chromophore is not entirely unimpeded, however. Two different M states are found when bR is illuminated and relaxed at different temperatures, indicating a significant kinetic barrier from one to the other (11, 45–47). In this step, from early M to late M, the distortions in the chromophore relax (12), and the SB nitrogen becomes more shielded, indicating greater readiness to reprotonate (11). In addition, some perturbations in the peptide backbone of the protein relax, while new perturbations appear elsewhere (11). Such adjustments may be responsible for the substantial kinetic barrier of the transition.

In the present work, we have found a similar pattern for the tryptophan residues. Again, perturbations are found in the early M (M0) state, some of which relax in the transition to late M (Mn) while new perturbations arise. As seen in Figure 6, the activity seems to involve two or three indoles that have moderate hydrogen bonding strengths (ε15N resonances at 130.5–130.8 ppm, close to the most common value of 130.6 ppm) in the LA state and one indole that is relatively weakly hydrogen bonded (ε15N resonance at 127.4 ppm) in the LA state. The indole that is relatively weakly hydrogen bonded in LA (ε15N resonance at 127.4 ppm) is quite tightly hydrogen bonded in M0 (ε15N resonance at 133.1 ppm), but no longer perturbed in Mn. On the other hand, whereas only one of the indoles that is moderately hydrogen bonded in LA (ε15N resonances at 130.5–130.8 ppm) is perturbed in Mn (ε15N resonance at 133.1 ppm), two such indoles are perturbed in Mn (ε15N resonances at 135.0 and 128.6 ppm). In Mn, both of the perturbed indoles have become more strongly hydrogen bonded. In Mn, one indole hydrogen bond becomes stronger (in fact exceptionally so), and one becomes weaker.

With its proximity to the C13 methyl group of the retinal, Trp182 seemed likely to be perturbed by the bending of the chromophore when it isomerizes around the C13=CH14 bond. However, by 15N REDOR difference spectroscopy (Figure 7), we have shown that the indole nitrogen of Trp182 is moderately hydrogen bonded in the LA state (ε15N resonance at 130.4 ppm), and remains so in the M state (ε15N resonance at 131.7 ppm) and the Mn state (ε15N resonance at 131.2 ppm). Thus, if the hydrogen bonding of Trp182 is perturbed by bending of the chromophore, this perturbation has largely dissipated by the early M state, and the transition from the early M state to the late M state occurs with essentially no effect on the hydrogen bonding of Trp182. These results are consistent with FTIR, UV, and visible Resonance Raman studies (17, 19) which show moderately strong H-bonding between N14 of Trp182 and a water molecule in the LA state which is absent in the L state and recovered in the M state. In any case, none of the large perturbations identified by 15N NMR difference spectroscopy (Figure 6) are due to Trp182.

Using the linear regression obtained from the model compound data (Figure 4), hydrogen bond lengths for the indole nitrogens can be estimated from their 15N chemical shifts and compared with the hydrogen bond lengths found.
by diffraction (listed in Table 1 for two relatively high-resolution structures). For Trp182, at 130.4 ± 0.4 ppm in the LA state (top panel of Figure 7), we estimate a hydrogen bond length of 2.928 ± 0.04 Å. This compares well with 2.81 Å in the 1C3W diffraction structure and 3.00 Å in the IQHJ diffraction structure for the distance between the Trp182 indole nitrogen and the oxygen of the hydrogen bonded water. For the downfield shoulder at 133.3 ppm in the 15N spectrum of the LA state (Figure 5), we estimate a hydrogen bond distance of 2.805 ± 0.04 Å. This also compares well with the shortest indole hydrogen bond distance found in recent diffraction structures—that of Trp137 with water at 2.78 Å in the 1C3W structure and 2.75 Å in the IQHJ structure. These congruences are encouraging considering the limited resolution of the diffraction and the approximate nature of the interpretation of the 15N chemical shifts. However, divergence occurs for the other end of the spectrum. For the upfield shoulder at 127.2 ppm, we estimate a maximum hydrogen bond distance of 3.064 (0.04 Å. This compares well with the shortest indole hydrogen bond between Trp189 and the OH of Tyr83 is unperturbed in Mo but is perturbed in the Mo→Mn transition, and the hydrogen bond between Trp189 and the OH of Tyr83 is unperturbed in Mn but is perturbed in the Mn→Mn transition. This is satisfying in that the active residues are the ones that are part of the retinal binding pocket. Unfortunately, however, although the chemical shift variation for these three indoles (133.3–130.5 ppm) is outside the error bars of the NMR experiment, the variation in the hydrogen bond lengths (2.75–2.97 Å) is probably within the error bars of the diffraction experiments and therefore not suitable for making convincing distinctions and assignments.

More exact distances can be obtained from measurements of the dipolar interactions between magnetic nuclei. By analyzing the REDOR data for [20-13C]retinal,[indole-15N]W182 in bR-Trp-bR (Figure 10), we have obtained distances from the C20 of retinal to the Nδ1 of Trp182 of 3.36 ± 0.2 Å in the LA state and 3.16 ± 0.4 Å in the Mo state. These distances are compared in Table 2 with the results of diffraction and simulation studies. The 3.36 Å distance that we obtain for the LA state is short compared to the distances in the early diffraction structures (and simulations based on those structures), but agrees closely with the distances from more recent studies at improved resolution. Of greater interest is the change in the transition from the LA state to the Mn state. In this transition, isomerization of the retinal from all-trans to 13-cis causes the backbone of the chromophore to become bent. Indeed, an increased tilt (−11°) of the C5 to C13 part of the polyene chain out of the plane of the membrane has been detected in the Mn state by linear dichroism and neutron-diffraction (48, 49). The result could be to push the C20 of retinal toward Trp182. In fact, the one diffraction structure

<table>
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<th>Trp nearest oxygen</th>
<th>1C3W (Å)</th>
<th>1QHJ (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 CO of Thr5 (1C3W) or Arg7 (1QHJ)</td>
<td>3.52</td>
<td>4.25</td>
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</tr>
<tr>
<td>138 CO of Pro186</td>
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<tr>
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<tr>
<td>189 OH of Tyr83</td>
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<td>2.83</td>
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Table 2: Distance from [20-13C]Retinal to [indole-15N]W182 in bR

<table>
<thead>
<tr>
<th>Trp nearest oxygen</th>
<th>resolution (Å)</th>
<th>distance in LA (Å)</th>
<th>distance in photocyte intermediates (Å)</th>
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<td>NMR, WT*</td>
<td>3.36 ± 0.2</td>
<td>3.16 ± 0.4 (Mn)</td>
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<td>Scharmagl et al.</td>
<td>4.02</td>
<td>3.57 (L)−3.42</td>
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<td>Schulten et al.</td>
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<td>3.73</td>
<td>3.47 (L)</td>
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<td>1CSS (60)</td>
<td>2.0</td>
<td>3.55 (M)</td>
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* Derived from the present REDOR recoupling experiments. * Coordinates kindly supplied by the principal investigators. * Structures are from the Protein Data Bank. * Sass et al., to be published. * Takeda et al., to be published. * Takeda et al., to be published.
of the K intermediate suggests that such compression does occur, with a [20-13C]retinal-[indole-15N]Trp182 distance of just 2.79 Å. However, other diffraction structures suggest less compression in subsequent intermediates. In the present NMR study, the REDOR results suggest that slight compression remains in the M₀ state. Although our 95% confidence limits for the M₀ state cover the entire range of the diffraction results for photocycle intermediates as well as the range for our LA distance, direct comparison of the most critical data points (see Figure 10) indicates that it is likely that the [20-13C]retinal-[indole-15N]Trp182 distance is a bit shorter in the M₀ state than in the LA state. Still, the difference is small.

CONCLUSIONS

Using solid-state 15N NMR, we have studied the interactions of the side chains of the eight tryptophan residues in bR and have observed changes in these interactions in the LA→M₀ and M₅→M₆ transitions of the proton-motive photocycle.

The chemical shifts of the indole nitrogens indicate that they are probably all hydrogen bonded. The indole nitrogen that resonates furthest downfield (and therefore is probably the most strongly hydrogen bonded) in the LA state is unperturbed in the M₀ and M₅ states. The indole nitrogen that resonates the furthest upfield (and therefore is probably the least strongly hydrogen bonded) in the LA state moves far downfield in the M₆ state (and therefore has probably become tightly hydrogen bonded), but relaxes completely in the M₅→M₆ transition. At least two other residues are perturbed in the M₅ state, one of which is unperturbed in the M₆ state and the other of which is differently perturbed in the M₆ state. None of these perturbations in indole hydrogen bonding are attributable to Trp182. But other residues in the retinal binding pocket appear to be involved.

We have also measured the internuclear distance between C₂₀ of the retinal and N₁ of Trp182 in the LA and M₅ states. The results suggest a slight compression, but the change in distance, from 3.36 ± 0.2 to 3.16 ± 0.4 Å, is too small to be reliably distinguished. From the M₅ state to the M₆ state, the 1.8 ppm upfield shift in the [20-13C]retinal resonance suggests a slight increase in compression, but again it is very small.

ACKNOWLEDGMENT

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REFERENCES

Tryptophan Interactions in Bacteriorhodopsin