at physiological pH values.[17] The lack of peptide signal intensity is also in accord with expectations; even low molecular weight amides in aqueous solution have proton exchange rates[18] which are slow in comparison with the time scale of this experiment. In bR, where most of the peptide linkages are hydrogen-bonded and/or buried in the protein interior, even the fastest exchanging amide protons are likely to be too slow to generate significant signal intensity. More surprisingly, however, the Schiff base also acquires significant polarization in the experiment. We propose that this occurs by exchange of the Schiff-base proton with solvent water during the spin-locking period, followed by direct Hartmann–Hahn magnetization transfer to the nitrogen. We have tested this hypothesis by examining the magnitude of the Schiff-base ^{15}N signal as a function of τ and t_{cp}. As a function of τ, the signal decays with a time constant similar to the T_2 of bulk water. Moreover, if we omit the refocusing π pulse, we obtain a significant ^{15}N signal only if the water (^1H) signal is on-resonance. These two facts suggest that we are indeed observing transfer from the water and not from some other putative pool of protons with an anomalously long T_2. As a function of t_{cp}, we measure essentially the same signal for times in the range 0.5–5 ms, implying that the exchange is essentially complete in 0.5 ms; thus, its time constant must be considerably less than this value. This agrees with the stopped-flow Raman measurements of Doukas et al.,[19] who showed hydrogen–deuterium exchange between this proton and bulk water to be complete within 3 ms of the start of reaction, but is shorter than the result of Ehrenberg et al.,[20] who obtained a value of 4.7 ms for the time constant of this reaction with the same technique.

It should be stressed that while the experiment shows exchange of the Schiff base proton with protons which have themselves been in a liquid phase during the τ period, it says nothing about the mechanism of this exchange. The results do not suggest or rule out proton tunnelling as a mechanism; however, they do require that, whatever the detailed exchange mechanism, it must permit *direct* exchange of the Schiff-base nitrogen with bulk water, not merely a tandem-type exchange through a chain of hydrogen bonds (this is also, of course, true of the kinetic Raman data). Recent vibrational data[21] have suggested that there may in fact be a tightly bound water near the Schiff-base nitrogen; while a tightly bound water molecule might be an intermediary in the exchange process, it would be expected itself to have a short T_2 and is thus unlikely to be the ultimate source of the slowly decaying proton signal whose exchange is detected in this experiment.

The accessibility of the Schiff-base to solvent water protons, and the role of free and bound water in the chromophore structure, is of central importance to the mechanism of the bR proton pump. The present technique may be invaluable in determining rate constants for exchange of this and other protons and in identifying residues which may be involved in proton pumping. It may also be fruitfully applied to other macromolecular systems, particularly those involved in the transport of protons across cell membranes.

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Supplementary Material Available: Graph of the measured ^{15}N signal intensity for the combined Schiff base resonances as a function of τ (1 page). Ordering information is given on any current masthead page.

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FTIR Evidence for Tryptophan Perturbations during the Bacteriorhodopsin Photocycle

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Bacteriorhodopsin (bR) functions as a light driven proton pump in the purple membrane (PM) of *Halobacterium halobium*.[1] While an atomic resolution structure of this protein is not yet available, the role of specific protein residues in the bR pump mechanism can be studied through a combination of Fourier transform infrared (FTIR) difference spectroscopy,[2,3] isotopic labeling of amino acids,[4-6,8,10] and site-specific mutagenesis.[7,10] In this paper, we utilize FTIR difference spectroscopy and tryptophan isotopic labeling to study the role of tryptophan in the bR photocycle. The location and environment of tryptophan residues has been investigated previously by using several other spectroscopic techniques.[5,11-16] In the case of low-temperature UV difference spectroscopy, two positive peaks at 290 and 294 nm appear in the BR → K difference spectrum which are consistent with an increase in hydrogen bonding of one or more tryptophan groups.[5] Additional spectral changes are detected at the M stage.[8] Recent evidence based on fluorescence energy transfer[12] and NMR[16] indicates that several tryptophan residues strongly couple with the retinal chromophore.

In this study, we have incorporated L-trp containing a perdeuteriated indole ring (L-[$^2H_5$]-trp) into bR[17] in order to identify signals in the FTIR difference spectra of the BR → K, L, and M transitions which could arise from the eight tryptophans in bR. Several peaks were found in the 760–740-cm^{-1} region which are assigned to the strongly infrared active hydrogen out-of-plane (HOOP) in phase bending mode of tryptophan. On the basis of comparison with model compound studies, a tryptophan group(s) appears to undergo an increase in hydrogen bonding at the BR → K transition which persists through M, while a second group(s), which has an anomalously high HOOP mode frequency, is perturbed during L formation.

FTIR difference measurements were made, as reported elsewhere,[5,8,9] on rehydrated films of purple membrane formed by air-drying a concentrated drop of purple membrane suspension on AgCl windows. Figure 1 shows the FTIR difference spectrum in the 1800–700-cm^{-1} region for the BR → K transition of unlabeled bR (solid) and bR-[$^2H_5$]-trp (dashed). These spectra are very similar except for the absence, in the bR-[$^2H_5$]-trp difference spectrum, of two small peaks at 742 cm^{-1} (negative) and 745 cm^{-1} (positive) as seen in the expanded view of this region (Figure 2, top). Thus, we assign the 742-cm^{-1} peak to a tryptophan residue(s) in bR whose environment changes during K formation, causing an upshift in the HOOP mode frequency. Peaks at these same frequencies are also observed in the BR → L and BR →

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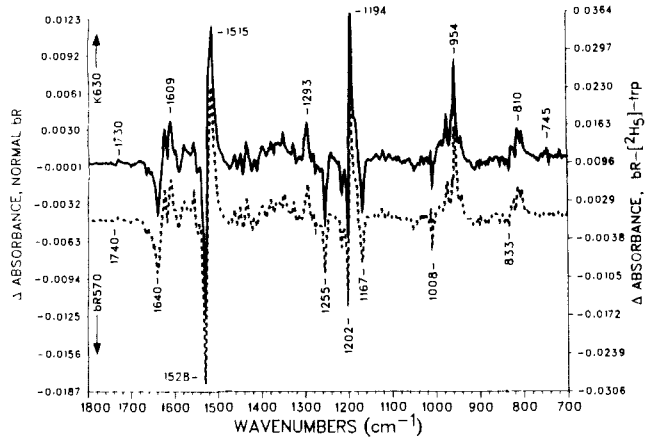

Figure 1. Comparison of $BR_{570} \rightarrow K_{630}$ FTIR difference spectra for normal bR (top, solid) and bR-[$^2H_5$]-trp (bottom, dashed). These spectra represent the average of 25 individual differences recorded with 2-cm^{-1} resolution at 81 K. One division on the absorbance axis is 0.0031 (solid), 0.0067 (dashed) au.

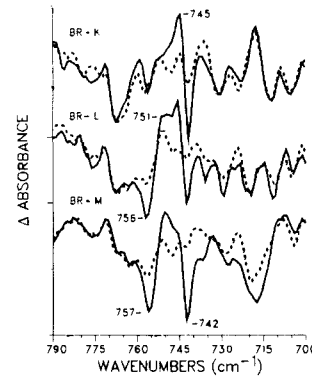

Figure 2. Comparison of the $BR_{570} \rightarrow K_{630}$ (top), $BR_{570} \rightarrow L_{550}$ (middle), and $BR_{570} \rightarrow M_{412}$ (bottom) difference spectra for bR (solid spectra) and bR-[$^2H_5$]-trp (dashed spectra). These spectra represent the average of 25 individual differences recorded with 2-cm^{-1} resolution at 81, 170, and 250 K, respectively, as previously described.[8] One division on the absorbance axis is 0.0011 (top, solid), 0.0009 (top, dashed), 0.0013 (middle, solid), 0.0012 (middle, dashed), 0.0017 (bottom, solid), 0.0010 (bottom, dashed) au.

M difference spectra (cf. Figure 2, middle, bottom), indicating that this tryptophan alteration persists through M formation. Notice, however, that the L and M difference spectra also have a new negative peak at 756 cm^{-1} accompanied by a positive peak near 751 cm^{-1}. This indicates that a second tryptophan group(s) is perturbed during L formation.

An intense tryptophan absorption near 742 cm^{-1} is found in both poly-L-tryptophan and L-tryptophan which is absent in L-[$^2H_5$]-tryptophan (Gray, D.; Roepe, P.; Rothschild, K. J.; unpublished data). Harada and co-workers[19] have assigned this peak to a CH out-of-plane bending vibration, characteristic of 1,2-disubstituted benzenes. In general, it is expected that deuterium substitutions will shift this mode down in frequency at least 100 cm^{-1}. We were unable to identify the downshifted peaks in the bR difference spectra due to high noise below 600 cm^{-1}. However, it is very likely that the tryptophan peaks we observe in the FTIR difference spectra near 740 cm^{-1} arise from the CH out-of-plane bending mode. In the case of the acetyl-tryptophan ethyl ester derivative of tryptophan, this mode displays a solvent sensitivity, shifting from 743 cm^{-1} in MeOH and benzene to 747 cm^{-1} in the strongly hydrogen bonding solvent, dimethylformamide.[19] Therefore, it is possible that the similar upshift in frequency of the 742-cm^{-1} band during the BR $\rightarrow$ K transition originates from increased hydrogen bonding of the NH indole moiety of the tryptophan. However, a different mechanism may be responsible for the abnormally high HOOP frequency (757 cm^{-1}) of the second bR tryptophan residue(s). In any case, this group appears to exist in a highly perturbed state in light-adapted BR, possibly due to direct interaction with the retinylidene chromophore.

In conclusion, our results indicate that at least two tryptophan residues undergo structural alteration during the bR photocycle. A possible role for these residues is to sterically hinder the motions of the retinylidene chromophore, thereby restricting light-induced isomerizations during the photocycle to the C_{13}–C_{14} double bond. Such a role for tryptophan residues has been recently predicted on the basis of UV–visible absorption spectroscopy of site-specific mutants of bR.[20] Through a combination of FTIR-difference spectroscopy and site-specific mutagenesis, the position of the structurally active tryptophan residues in the bR primary sequence can now be determined.[21]

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