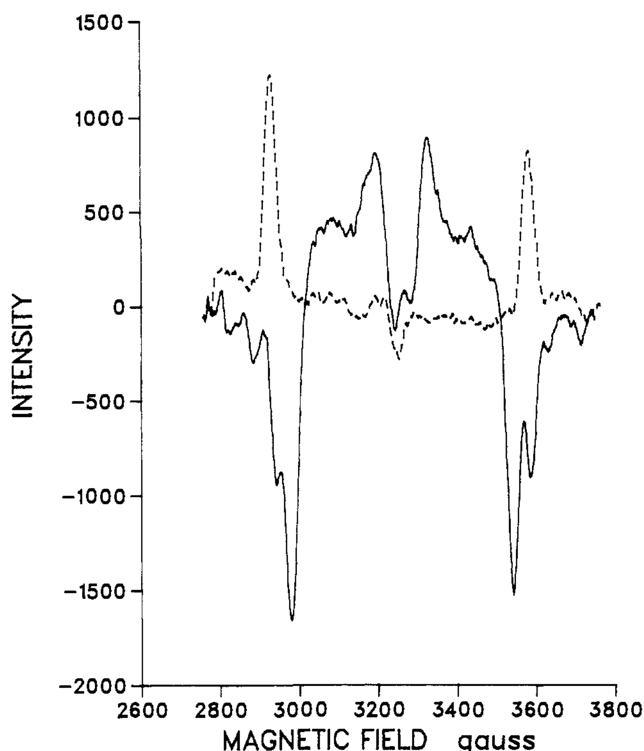

Figure 2. EPR powder spectra of the lowest excited triplet states of ZnTPP (--) and **1** (—) at 10 K in MTHF. Positive signals are in absorption, and negative signals are in emission. The zero-field splitting parameters for ZnTPP and **1** are, respectively, $|D| = 0.0305$ and 0.0300 cm^{-1}, $|E| = 0.0085$ and 0.0080 cm^{-1}.

spin-orbit induced intersystem crossing primarily to the *z* sublevel of the triplet state. This is typical of Zn containing porphyrins and chlorophylls.[13] The *z*-direction in the principal magnetic axis system for porphyrin π–π^* triplet states is normal to the plane of the macrocycle. In strong contrast to ZnTPP, **1** shows an intense triplet EPR signal possessing a spin polarization pattern (eea eaa) which shows that an in-plane sublevel of the triplet state is overpopulated.[12] Thus, the triplet state of **1** is not formed via the usual intersystem crossing mechanism for Zn porphyrins. Singlet radical ion pairs in covalently linked donor–acceptor molecules can undergo subnanosecond intersystem crossing leading to triplet formation.[14] Intersystem crossing rates are enhanced by a spin-orbit interaction when the symmetry axes of the donor HOMO and the acceptor LUMO lie between 45° and 90° relative to one another. The π systems of the donor and acceptor in **1** and **2** lie about 60° relative to one another.[15] Since the electron acceptor lies approximately on the inplane symmetry axis of the porphyrin, rapid intersystem crossing preceding ion pair recombination produces a triplet state in which an in-plane sublevel of the porphyrin is overpopulated. On the other hand, *no triplet EPR signal is observed for **2***. This result agrees with the optical transient absorption data which show that excitation of **2** yields little or no triplet Zn-porphyrin.

The optical and EPR results at 10 K suggest that the radical ion pair state of **1** is above the triplet state of its Zn-porphyrin, while that of **2** is below the triplet state of its Zn-porphyrin. Thus, we can estimate the change in the radical ion pair energy that occurs when the solvent is frozen to a solid at low temperature. Since the energies of the lowest excited triplet states of the porphyrins in **1** and **2** are about 1.58 and 1.55 eV, respectively, and since the energies of the radical ion pairs for **1** and **2** at 294 K in fluid solution are about 0.75 and 0.56 eV, respectively, the increase in radical ion pair energy is 0.99 eV > ΔG > 0.83 eV or about 0.9 eV. Thus, the fact that most porphyrin-based donor–acceptor molecules exhibit very low quantum yields for charge separation in low-temperature solids results in a large part from destabilization of the ion pair state in low-temperature solids relative to polar liquids. These experiments permit us to begin to study more closely the role of the medium in electron-transfer reactions and may allow us to understand the behavior of photosynthetic reaction centers at low temperature.

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Supplementary Material Available: Synthesis information for **1** and **2** (2 pages). Ordering information is given on any current masthead page.

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Solid-State NMR Detection of Proton Exchange between the Bacteriorhodopsin Schiff Base and Bulk Water

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Liquid-phase NMR has been employed extensively to investigate the rates and mechanisms of chemical reactions, particularly exchange reactions at equilibrium.[1,2] By monitoring the dynamics of either longitudinal[3] or transverse magnetization,[4] in one- and/or two-dimensional NMR[5] experiments, one can usefully measure

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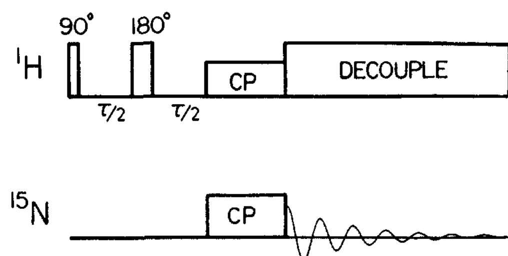

Figure 1. Pulse sequence employed to detect proton exchange between liquid and immobile phases in a hydrated bacteriorhodopsin sample.

rate constants for exchange processes in biological molecules as large as 10–20 kD. The principal limitation to liquid-phase NMR studies is the decreased rate of molecular tumbling with increasing molecular size. This leads to line-broadening of solution NMR spectra due to incomplete averaging of anisotropic molecular interactions.[6] As a result, larger proteins, and particularly membrane proteins (for which isotropic reorientation is effectively forbidden), are not amenable to kinetic analysis by such conventional techniques. However, high resolution solid-state NMR has recently been applied with great success by us[7,8] and by others[9] to rotationally restricted proteins. We have been able to resolve single ^{15}N[7] and ^{13}C[8] resonances in the high-resolution solid-state NMR spectra of the membrane protein bacteriorhodopsin (bR) and to interpret these results in terms of the molecular structure in the resting state and in photocycle intermediates. In this communication we introduce a new experimental approach which permits us to monitor the *kinetic* transfer of magnetization from solvent water to a single membrane protein site, specifically the Schiff-base nitrogen of bR. This magnetization transfer, to a site which in most models is buried within the protein,[10] apparently occurs via chemical exchange of the Schiff-base proton with solvent water.

The NMR pulse sequence employed in this experiment (Figure 1) resembles the standard cross-polarization sequence[11] but includes an additional delay τ between the initial high frequency $\pi/2$ pulse and the start of Hartmann–Hahn spin-locking.[12] A π pulse is applied in the middle of the τ period in order to refocus inhomogeneous interactions; this pulse may be omitted in some circumstances. During this delay, the proton magnetization dephases with a time constant T_2, which in a solid is typically of the order of microseconds, rather than the milliseconds or seconds usually observed in solution. Thus, a τ period of the order of milliseconds effectively selects for liquid-state proton magnetization. It is well-established that the anisotropic interactions in bR are large[8,13]: more specifically, the Schiff-base nitrogen itself[14] and residues in its vicinity[8] show full chemical shielding anisotropies, essentially unaveraged by motion. In NMR terms this means that the interior of the protein more resembles a solid than a liquid. Therefore, the proton T_2 in the interior of the protein

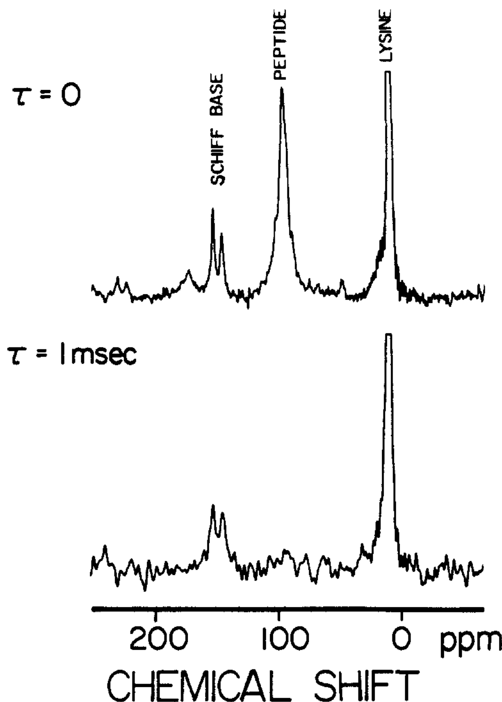

Figure 2. (Top) Standard cross-polarization/magic-angle sample spinning ^{15}N spectrum of bacteriorhodopsin obtained by using the pulse sequence shown in Figure 1 with a τ of 0.0 μs, and with the π pulse, superfluous here, omitted. (Bottom) Spectrum of the same sample with the same pulse sequence but with a π of 1 ms. Note the absence of the nonexchanging peptide resonances. In both spectra the lysine ϵ-amino resonances are truncated in order to allow the weaker Schiff-base signals to be seen. Other experimental parameters: $\nu_{^{15}N}$ = 32.2 MHz, $\nu_{^1H}$ = 317.6 MHz, number of acquisitions = 20000, t_{cp} = 1 ms. The ^{1}H and ^{15}N pulses were 3.0 and 8.5 μs, respectively.

in general, and in the vicinity of the chromophore in particular, will be short. In contrast, we measure the T_2 of the solvent water (which makes up approximately 70% of the weight of our hydrated bR samples) to be approximately 50 ms. Therefore, after a τ period of the order of 1 ms, only the solvent water possesses significant transverse magnetization; this residue is preserved by the proton spin-lock. A matched Hartmann–Hahn radio-frequency field on a rare spin species allows transfer of the proton magnetization to the rare spin system, if there exists a significant dipolar coupling between the two spin systems. Thus, this experiment selects for rare-spin nuclei which are in a relatively rigid environment but are bonded to or close to protons which a short time previously have been in a site with a long T_2.

Figure 2 shows the results of applying this pulse sequence to ϵ-(^{15}*N*-amino)lysyl-bR.[18] The top spectrum, obtained via conventional cross-polarization (i.e., with τ = 0) shows the characteristic magic-angle spectrum of this protein[7]—the intense line from the six free ϵ-amino groups of lysine, the natural-abundance peptide-backbone signal at about 100 ppm, and the Schiff-base resonances from bR_{568} and bR_{548} at 145 and 152 ppm, respectively. The bottom spectrum was obtained with τ = 1 ms, and while it is apparent that we still detect appreciable signals from the surface lysines and from the Schiff-base, the peptide-nitrogen signals are almost totally suppressed. That the free-lysine signals persist is not unexpected, since they are likely on the membrane surface,[15,16] and free amino groups exchange protons rapidly with solvent water

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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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Registry No. Water, 7732-18-5.

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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