Ultraviolet Resonance Raman Evidence for the Opening of a Water-Permeable Channel in the M to N Transition of Bacteriorhodopsin

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Bacteriorhodopsin (bR), a 26-kDa retinal protein found in the purple membrane of *Halobacterium salinarium*, functions as a light-driven transmembrane proton pump. Upon absorption of a photon, the *all-trans*-retinal of light-adapted bR (bR_{568}) isomerizes to 13-*cis*, and the photochemically generated metastable state (J) relaxes thermally to bR_{568} through a series of intermediates (K → L → M → N → O). A proton is released to the extracellular side in the L → M transition and another proton is taken up from the cytoplasmic side in the N → O transition, concomitant with retinal reisomerization to *all-trans*.[1] A wide variety of spectroscopic methods have shown that Trp residues, particularly those located in the vicinity of retinal, are involved in the chromophore/protein conformational changes during the photocycle.[2] Here we report a drastic decrease in environmental hydrophobicity of Trp residues in the N intermediate, which was probed by the intensity of UV resonance Raman (UVRR) scattering excited at 244 nm. The decrease in hydrophobicity is mostly ascribed to a change in environment from hydrophobic to hydrophilic of Trp-182, which faces the retinal from the cytoplasmic side. This observation gives evidence for the opening of a channel for water permeation from the cytoplasmic surface to the retinal-binding pocket in the M → N transition.

Figure 1A shows the UVRR spectrum of bR_{568} excited at 244 nm. Excitation at this wavelength mainly enhances Tyr and Trp Raman bands, which are given labels Y and W, respectively, followed by their mode numbers.[3] Figure 1B shows a time-resolved UVRR difference spectrum, the photointermediate at a time of 200 μs after initiation of the photocycle *minus* the initial bR_{568} form. This delay time gives maximal concentration of the M intermediate.[4] The most prominent features in the difference spectrum are two pairs of large positive and small negative peaks at 1613/1599 and 1172/1181 cm^{-1}, which are respectively ascribed to an upshift of the Y8a band and a downshift of the Y9a band with a concomitant intensity increase. The positive peak at 1208 cm^{-1} also shows an intensity increase of the Y7a band. These changes of Tyr Raman bands are nearly identical with those observed by Ames *et al.*[5] for the M intermediate using 240-nm excitation.

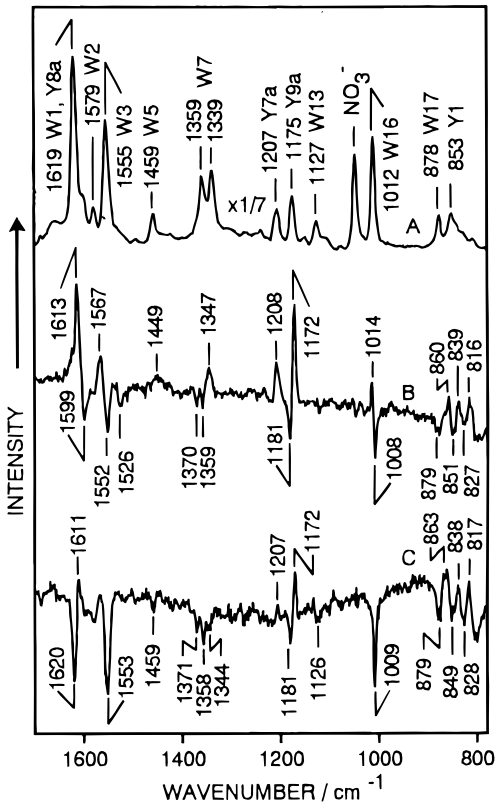

Figure 1. Time-resolved UVRR spectra of bR excited at 244 nm. (A) Probe only spectrum of light-adapted bR suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperadine-*N*′-2-ethanesulfonic acid) buffer (pH 7.0) containing 5 mM KNO_3, which was added as an internal standard of Raman scattering intensity. (B) Difference spectrum, the M intermediate *minus* light-adapted bR, recorded at a delay time of 200 μs in 10 mM HEPES buffer (pH 7.0). (C) Difference spectrum of the N intermediate recorded at a 10-ms delay time in 10 mM CHES (cyclohexylaminoethanesulfonic acid) buffer (pH 9.5) containing 200 mM KCl. The difference spectra are expanded by an intensity scale factor of 7. Excitation at 244 nm was effected by an intracavity frequency-doubled Ar^+ laser (Coherent, Innova 300 FReD). The UV laser beam (3 mW) was line-focused (height, 2 mm; width, 40 μm) on the sample by using a cylindrical lens. A 20-mL bR suspension (concentration 120 μM) was circulated through a quartz capillary tube (diameter, 1.5 mm) and Raman-scattered light was collected with a UV achromatic lens, focused onto the entrance slit of a fore-prism UV Raman spectrometer,[19] and detected on a CCD detector (Princeton Instrument, LNCCD/1152). Transient Raman spectra were obtained by irradiating the sample with a 515-nm pump beam (height, 2 mm; width, 40 μm; power, 150 mW) upstream from the probe UV beam. The delay time was determined by measuring the center-to-center distance between the pump and probe beams with a microscope. The spectra reported here are the averages of those recorded on 6−8 fresh samples with an accumulation time of 1 h.

The 244-nm difference spectrum in Figure 1B reveals additional features arising from Trp Raman bands that were not clear in the spectra reported previously.[5,6] The W7 mode of Trp usually gives a Fermi doublet at 1360/1340 cm^{-1} (e.g. see Figure 1A), whose intensity ratio serves as a marker of the environmental hydro-

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phobicity of the indole ring.[7] In the W7 frequency region of the difference spectrum (Figure 1B), two negative peaks at 1370 and 1359 cm^{-1} and a broad positive peak at 1347 cm^{-1} are seen. The 1347-cm^{-1} peak may be an overlap of peaks due to W7 and the intensified Y3 mode of Tyr.[8] A 244-nm UVRR spectrum of Trp-182 extracted from the spectrum of wild-type bR by using a mutant, W182F,[9] has shown that the W7 mode of Trp-182 gives a triplet peaking at 1370, 1357, and 1339 cm^{-1}.[10] The unusual high-frequency component at 1370 cm^{-1} is unique to Trp-182 and is attributed to a strong steric repulsion between the indole ring of Trp-182 and the methyl groups of retinal.[10] The 1370-cm^{-1} negative peak in the difference spectrum is, thus, assignable to Trp-182, and the steric repulsion must be weakened in the M intermediate. Other features in the difference spectrum may also reflect, at least partly, changes in structure and environment of Trp-182. An upshift of the W3 band, revealed by a pair of positive and negative peaks at 1567/1552 cm^{-1}, indicates an increase in the $\chi^{2,1}$ torsion angle about the $C_\beta-C_3$ bond.[11]

The N intermediate accumulates at alkaline pH and in the presence of salt.[4,12] Figure 1C shows a time-resolved (10-ms delay time) UVRR difference spectrum of the N intermediate at pH 9.5 and in the presence of 200 mM KCl. In addition to the peaks due to the Y9a, Y8a, and Y7a bands of Tyr, the difference spectrum exhibits many negative peaks assignable to the W1, W3, W5, W7, W13, and W16 bands of Trp, indicating a significant decrease of Trp Raman scattering intensity in the N intermediate. Since the W7 band characteristic of Trp-182 is also seen as a negative peak at 1371 cm^{-1} in the difference spectrum and the negative intensity in the W7 region increases in the M → N transition, the Raman intensity decrease in the N intermediate is largely attributed to Trp-182. The Raman scattering from Trp residues in bR (Figure 1A) is ~3 times stronger than that from amino acid Trp in aqueous solution, and the increased intensity has been attributed to Trp residues hydrogen bonded in hydrophobic environments.[13] Accordingly, the decrease of Trp Raman intensity in the N intermediate reflects a weakening of the hydrogen bonding and/or a change in environment from hydrophobic to hydrophilic. The frequency of the W17 band (~878 cm^{-1}) is a marker of hydrogen bonding of the Trp indole ring.[14] With 244-nm excitation, however, the W17 band is obscured by the overlap of the Tyr Y1 Fermi doublet in the M and N difference spectra (Figure 1B,C). Figure 2 shows difference spectra recorded with 229-nm excitation, which selectively enhances Trp Raman scattering. Both of the M (Figure 2A) and N (2B) difference spectra exhibit an almost identical feature composed of a negative peak at 878 cm^{-1} and a positive peak at 868 cm^{-1}. This observation indicates that the hydrogen bonding strength of Trp

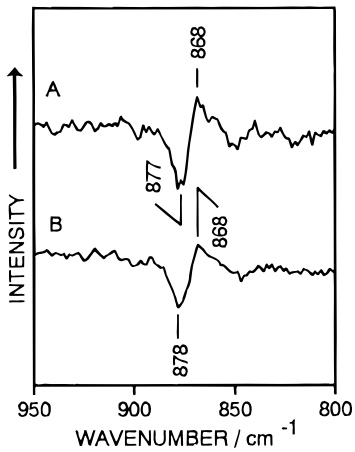

Figure 2. UVRR (229 nm) difference spectra of the (A) M and (B) N intermediates in the Trp W17 mode region. Experimental conditions are the same as in Figure 1 except for the excitation wavelength.

does not change on going from the M to N intermediate.[14] The large decrease in Trp Raman intensity observed for the N intermediate is, therefore, ascribed to a change in environment from hydrophobic to hydrophilic of one or more Trp residues including Trp-182.

In the structure of bR_{568}, the seven transmembrane α-helixes (A−G) are arranged in a circular manner to enclose the retinal chromophore bound via a protonated Schiff base to Lys-216 near the center of helix G.[1,15] Trp-182 of helix F is interacting with the retinal polyene chain from the cytoplasmic side, while the other 7 Trp residues are located on the extracellular side.[15] Asp-96, which donates a proton to the Schiff base in the M → N transition and reprotonated in the N → O transition, is located halfway between the Schiff base and the cytoplasmic surface.[15] X-ray and electron diffraction studies have suggested a tilt of the cytoplasmic half of helix F away from the main body of the protein in the M → N transition.[16,17] Such a tilt of helix F is supposed to produce an opening between the helixes on the cytoplasmic side, which are tightly packed otherwise. The opening may be large enough to allow water to have access to Asp-96[16] and subsequently to decrease the pK_a of Asp-96,[18] leading to the proton transfer from Asp-96 to the Schiff base. Here we have shown that the environment of Trp-182 changes from hydrophobic to hydrophilic in the M → N transition by using the UVRR intensity. In the N intermediate, water may be accessible to the retinal-binding pocket where Trp-182 resides. This observation gives spectroscopic evidence for the opening of a water-permeable channel extending from the cytoplasmic surface of the protein to the retinal-binding pocket in the M → N transition. A possible role of the water molecules in the channel is to mediate the proton transfer from Asp-96 to the Schiff base.

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