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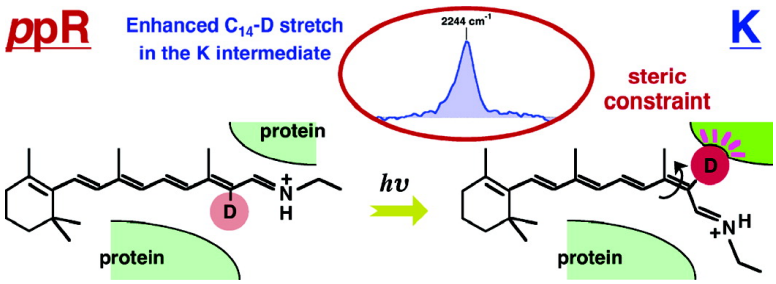
J. Am. Chem. Soc., **2005**, 127 (46), 16036-16037• DOI: 10.1021/ja056203a • Publication Date (Web): 01 November 2005



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Published on Web 11/01/2005

Steric Constraint in the Primary Photoproduct of an Archaeal Rhodopsin from Regiospecific Perturbation of C−D Stretching Vibration of the Retinyl Chromophore

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Rhodopsins convert light into either signal or energy. Visual rhodopsins possess an 11-cis retinal as the chromophore, and the 11-cis to all-trans photoisomerization initiates protein structural changes for visual transduction.[1,2] On the other hand, archaeal rhodopsins possess an all-trans retinal, and the all-trans to 13-cis photoisomerization initiates protein structural changes for phototaxis or ion pumping.[3,4] Photocycle intermediates are designated as the K, L, M, N, and O states. It is well known that the isomerization in protein is a selective and efficient reaction according to the potential surface of the excited state in rhodopsins.[1] One of the characteristics in the isomerization is that the reaction occurs even at low temperatures. Since the protein motion is restricted at low temperatures, the structure of the retinal binding site is hardly altered. X-ray crystallography indeed showed little change of the retinal pocket for the primary K intermediates of bacteriorhodopsin (BR), a light-driven proton-pump,[5−7] and *pharaonis* phoborhodopsin (*p*pR), a light-sensor protein for negative phototaxis,[8] where the measurements were conducted at 100 K. This suggests that the isomerized chromophore has a remarkably distorted structure, by which light energy is stored.

How is such structural constraint observed experimentally? Current X-ray crystallographic structures of photointermediates with >2 Å resolution are not sufficient for constructing the detailed structure of the retinal chromophore. In fact, the retinal structure is considerably different among three X-ray structures for the K intermediate of BR.[5−7] The distorted retinal chromophore has been best investigated through vibrational analysis particularly on the hydrogen out-of-plane (HOOP) wagging vibrations in the 800−1000 cm^{-1} region.[9] Previous resonance Raman spectroscopy of rhodopsins successfully observed the enhanced HOOP vibrations for the primary intermediates of rhodopsins, and they were interpreted in terms of the origin of the chromophore distortion.[9] Similar enhanced HOOP modes were also observed for infrared bands.[10] Thus, it is generally accepted that HOOP vibrations are a good probe of chromophore distortion in rhodopsins.

In contrast to the HOOP vibrations, the C−H stretching vibration has not been used for probing structural information of the chromophore, probably due to the many overlapping signals from the protein as well as the chromophore in the narrow 3000−3100 cm^{-1} region.[11] However, it should be clear that selective deuteration readily shifts such stretching frequencies to the 2200−2300 cm^{-1} region, being devoid of any signals from protein as well as the chromophore. Hence, we have initiated a program to prepare selectively deuteratured archaeal rhodopsin analogues and examined the C−D vibrations at 2200−2300 cm^{-1}. In this study, all seven monodeuterated all-trans retinal analogues (Figure 1) were synthesized according to the method described previously.[12] Deuterated retinal was incorporated into *p*pR, and the photochemical property was examined by use of the light-sensor protein.[3] The *p*pR protein with histidine tag at the C-terminus was expressed in *Escherichia coli*, solubilized with 1.0% *n*-dodecyl-β-D-maltoside (DM), and purified by a Ni-column.[13] Deuterated all-trans retinal (1 mg) at position 7, 8, 10, 11, 12, 14, or 15 was added into the *E. coli* culture (1 L), while unlabeled all-trans retinal was used as a control sample.[14] The purified *p*pR sample was then reconstituted into L-α-phosphatidylcholine (PC) liposomes by the removal of the detergent with Bio-beads, where the molar ratio of the added PC to *p*pR was 50:1. Hydrated films of the *p*pR sample were used for low-temperature FTIR spectroscopy.

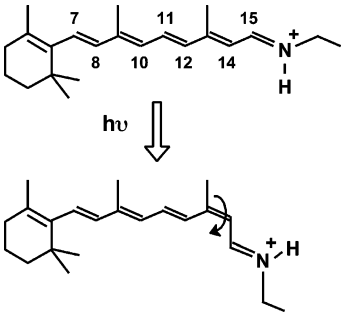

Figure 1. All-trans retinal is photoisomerized into 13-cis in archaeal rhodopsins. Retinal deuterated at position 7, 8, 10, 11, 12, 14, or 15 is used in the present study.

The ppR$_K$ minus *p*pR difference spectra were measured at 77 K according to the method described previously.[13] The retinal vibrations in the ppR$_K$ minus *p*pR difference spectra differ significantly among samples, whereas protein signals were identical (data not shown). Therefore, we normalized each spectrum by use of the C=O stretches of Asn105 at 1704 (−)/1700 (+) cm^{-1}.[15] The dotted line in Figure 2 shows the ppR$_K$ minus *p*pR difference spectrum for the unlabeled retinal at 2160−2330 cm^{-1}, where only a broad spectral feature was observed. The observation of no peaks is expected of a native system as noted above. In contrast, a C−D group is introduced for each deuterated retinal, whose stretch should exist in this region. However, Figure 2a−e,g shows almost identical spectra between unlabeled and deuterated retinal at positions 7, 8, 10, 11, 12, and 15. No peaks originate from either (i) a small C−D stretch that is comparable with baseline or (ii) no frequency change of the C−D stretch between *p*pR ad ppR$_K$. On the other hand, a

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10.1021/ja056203a CCC: $30.25

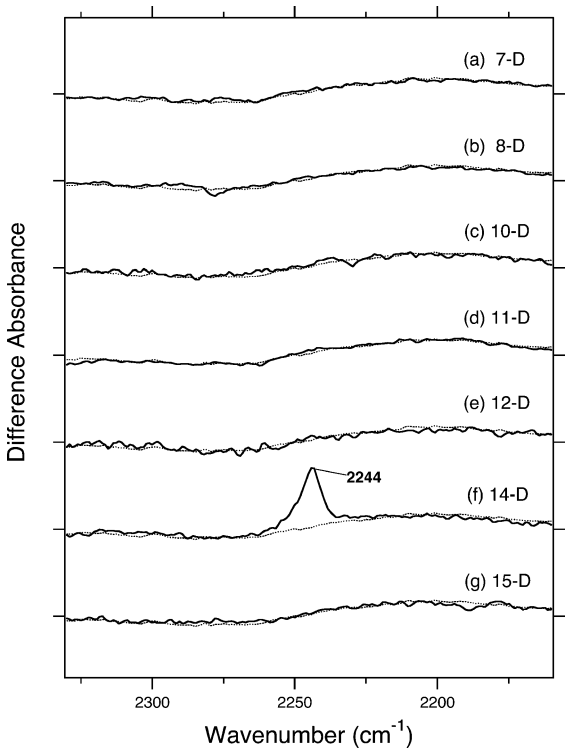

Figure 2. *p*pR$_K$ minus *p*pR difference spectra in the 2160−2330 cm^{-1} region. The solid line represents the spectrum for the deuterated retinal at positions 7 (a), 8 (b), 10 (c), 11 (d), 12 (e), 14 (f), and 15 (g), while the dotted line corresponds to that for the unlabeled retinal. Vertical amplitudes of all spectra are normalized by the bands of Asn105 at 1704 (−)/1700 (+) cm^{-1}. One division of the *y*-axis corresponds to 0.0003 absorbance unit.

highly contrasting spectral feature was obtained for C_{14}−D (Figure 2f), where a strong positive peak appeared at 2244 cm^{-1}. Therefore, we assigned the band as the C_{14}−D stretch in *p*pR$_K$.

The corresponding C−H stretching vibration of the 2244-cm^{-1} band is calculated to be about 3060 cm^{-1}, which coincides well as the vinylic C−H stretch.[11] We then expected that the positive peak at about 3060 cm^{-1} is also observable in the C−H stretch region for the unlabeled retinal. However, baseline distortion due to strong absorption of other vibrations including water O−H stretches made it difficult to identify the C−H stretch. In other words, by shifting the specific C−H stretch of retinal by deuteration, we could find the stretching band of the C_{14} atom for the first time. Interestingly, no clear negative peak was observed in Figure 2f. This could be due to (i) a small band in another frequency or (ii) the same frequency for *p*pR. In both cases, the C_{14}−D stretch for *p*pR is much smaller in amplitude than for *p*pR$_K$. We thus concluded that only the C_{14}−D stretching vibration is much enhanced upon retinal photoisomerization.

A similar enhanced stretching vibration was observed for the N−H stretch of indole of Trp182 in the L intermediate of BR,[16] where the H−D unexchangeable band appears at 3486 cm^{-1}, whose molar extinction coefficient was estimated to be 4 times greater than that of the normal N−H stretch of indole. We inferred that the steric constraint near the indole ring yields this enhancement of absorption. Presumably, similar steric constraint occurs at the C_{14}−D group in *p*pR$_K$. Electrostatic field effects may also play a role in such enhancement. Formation of *p*pR$_K$ accompanies rotation of the C_{13}=C_{14} double bond, leading to the movement of the C_{14}−H (C_{14}−D) group. It is noted that *p*pR$_K$ exhibits various HOOP vibrations, suggesting that the chromophore is widely distorted along the polyene chain.[13] In contrast, the C−D stretching vibration was only specific for the C_{14} group. This fact strongly suggests that the C−D stretch provides structural information different from that of HOOP modes, namely, information on the direct contact of deuterium (hydrogen) with it surroundings.

The counterpart of the C_{14}−D group in *p*pR$_K$ is thus interesting. According to the X-ray structure of *p*pR,[17] the distance from the C_{14} atom is 3.8 Å to the phenol oxygen of Tyr174, 3.4 Å to the carboxylic oxygen of Asp201, 4.1 Å to the hydroxyl oxygen of Thr204, and 4.4 Å to the hydroxyl oxygen of Thr79. The structure of *p*pR$_K$ shows that only the distance to Thr204 is significantly reduced from 4.1 to 3.3 Å.[8] Therefore, Thr204 may be the counterpart of the C_{14}−D group. Thr204 plays an important role for the signal relay to the transducer protein,[18,19] and the counterpart of the C_{14}−D group will be studied in the future.

In conclusion, this paper reports a novel technique to monitor steric constraint of the retinal chromophore with the binding pocket. In an archaeal light-sensor *p*pR, only the C_{14}−D stretching vibration is significantly enhanced upon retinal photoisomerization. The local steric constraint at the C_{14}−D group probably contributes to the high energy state of the primary intermediate, and the relaxation leading to the function of the rhodopsin. Comparative investigations of other rhodopsins such as BR and visual rhodopsin will lead to a better understanding of the specific isomerization mechanism of retinal in protein.

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JA056203A