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Primary structural response in tryptophan residues of *Anabaena* sensory rhodopsin to photochromic reactions of the retinal chromophore

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ABSTRACT

Anabaena sensory rhodopsin (ASR) is a microbial rhodopsin found in eubacteria and functions as a photosensor. The photoreaction of ASR is photochromic between all-*trans*, 15-*anti* (ASR_{AT}), and 13-*cis*, 15-*syn* (ASR_{13C}) isomers. To understand primary protein dynamics in the photoreaction starting in ASR_{AT} and ASR_{13C}, picosecond time-resolved ultraviolet resonance Raman spectra were obtained. In the intermediate state appearing in the picosecond temporal region, spectral changes of Trp bands were observed. For both ASR_{AT} and ASR_{13C}, the intensities of the Trp bands were bleached within the instrumental response time and recovered with a time constant of 30 ps. This suggests that the rates of structural changes in the Trp residue in the vicinity of the chromophore do not depend on the direction of the isomerization of retinal. A comparison between spectra of the wild-type and Trp mutants indicates that the structures of Trp76 and Trp46 change upon the primary photoreaction of retinal.

1. Introduction

Protein functions are regulated by structural changes. Knowledge of protein dynamics is important for determining mechanisms of protein functions. In photoreactive proteins, light absorption induces local structural changes in the chromophore as a functional trigger. This local structural change produces sequential changes in the higher order structure, thereby facilitating function. Clarification of the local structural changes can help determine the functionally important protein's motion.

Microbial rhodopsin is a typical photoreactive protein. Four types of microbial rhodopsins exist; bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I (SRI), and sensory rhodopsin II (SRII) [1]. The two former proteins are light-driven ion pumps, whereas the latter two function as phototaxis receptors. A retinal chromophore is covalently bonded to the Lys residue *via* a protonated Schiff base linkage (see Fig. S1 in Supplementary data). Although these four microbial rhodopsins accommodate the retinal chromophore as an all-*trans*, 15-*anti* (AT) and 13-*cis*, 15-*syn* (13C) configurations in the ground state, only the AT configuration is functionally active. The photoreaction from the AT configuration is 100% cyclic. For BR, absorption of a photon by the AT configuration gives rise to isomerization to the 13-*cis*, 15-*anti* configuration. Photoisomerization of the chromophore leads to a cyclic reaction involving a series of intermediates, BR → K → L → M → N → O → BR, each of which was characterized by a distinct absorption spectrum in visible region [2].

We have investigated the primary protein response to the photoisomerization of the retinal chromophore in BR [3] and SRII [4] by ultraviolet resonance Raman (UVRR) spectroscopy. Time-resolved UVRR spectroscopy helps determine the structural dynamics at specific sites by selectively enhancing the vibrational bands attributable to aromatic amino acid side chains [5,6]. Structural changes in the Trp and Tyr residues in the vicinity of retinal were found in response to AT → 13C isomerization of retinal with a time constant of a few tens of picoseconds for both BR and SRII.

Anabaena sensory rhodopsin (ASR), which is found in a freshwater cyanobacterium [7], exhibits a unique photoreaction different from other microbial rhodopsins. Recently, it was suggested that ASR regulate the expression of a pigment protein as a gene repressor [8]. In the ground state, ASR has two stable configurations of retinal, AT (ASR_{AT}) and 13C (ASR_{13C}) [9], which exhibit photoinduced interconversion [10]. Thus, the photoreaction of ASR is not cyclic but photochromic. This ASR characteristic enables the investigation of the effect of direction of retinal isomerization on protein response. The retinal chromophore is predominantly of the AT configuration in the dark-adapted state (DA-ASR) and con-

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tains a large fraction of the 13C configuration in the light-adapted state (LA-ASR) [10–12]. In the photochromic reaction of ASR, ASR_{AT} is photoconverted to the primary photointermediate, K-ASR_{AT}, with the 13-*cis*, 15-*anti* configuration, and ASR_{13C} is converted to the K-ASR_{13C} intermediate with the all-*trans*, 15-*syn* configuration. Quantum yields of the former and latter photoisomerizations are 0.38 and 0.24, respectively [13]. Spectroscopic studies indicated that the photoreaction of both ASR_{AT} and ASR_{13C} includes several distinct intermediates [10,12,14–19]. FTIR spectroscopy revealed that the distortion of the chromophore in K-ASR_{AT} is localized in the Schiff base region, while that in K-ASR_{13C} is distributed widely along the polyene chain. In addition, although the hydrogen-bond strength between the Schiff base and the water molecule in ASR_{AT} is similar to that in ASR_{13C}, the hydrogen bond is broken in K-ASR_{AT} but not in K-ASR_{13C} [14,15]. Recently, femtosecond absorption spectroscopy demonstrated that the gross appearance of transient absorption of DA-ASR and LA-ASR is similar whereas the time constants of both intersystem crossing and buildup of K-ASR_{13C} are much faster than those of K-ASR_{AT} [19].

In the present study, the picosecond time-resolved UVRR spectra of LA- and DA-ASR was obtained and compared to the primary protein dynamics beginning with ASR_{AT} and ASR_{13C}. The Trp residue responsible for the observed UVRR spectral change was also identified based on measurements of the ASR mutants. The primary protein response of ASR was compared with those of BR and SRII.

2. Experimental section

2.1. Sample preparation

The ASR samples (wild type (WT), W76F, W176F, W183F, and W46F mutants) were prepared as described previously [7,20]. Briefly, *Escherichia coli* BL21(DE3) harboring each plasmid, which encoded the WT and mutants of the *Anabaena* opsin with a histidine tag at the C-terminus, were grown in 2 × YT medium in the presence of 50 μg/mL ampicillin after 1 mM IPTG induction with 10 μM all-*trans*-retinal. Pink-colored cells were sonicated and cell membranes were solubilized with 1% (w/v) *n*-dodecyl-β-D-maltoside (DDM). The solubilized membranes were purified with a Ni-affinity column (HisTrap HP, GE Healthcare) and an ion-exchange chromatography column (HiTrap SP HP, GE Healthcare). Then, the samples were suspended in a buffer solution containing 50 mM Tris–HCl (pH 7.0), 300 mM NaCl, and 0.1% DDM.

LA-ASR samples were prepared by yellow light illumination ($\lambda > 430$ nm) during the UVRR measurement. DA-ASR samples were prepared by incubation in the dark overnight at room temperature or for a week at 4 °C.

2.2. Steady-state UVRR measurements

The experimental apparatus for steady-state UVRR measurements has been described previously [21]. A Ti:sapphire laser pumped by a Q-switched LD-pumped Nd:YLF laser (DM laser system, Photonics Industries) operating at 1 kHz was used to generate the fourth harmonic of the laser output, 225 nm, as the probe light. The spectral width of the probe pulse was 7 cm^{-1}. Typical pulse energy at the sample point was 0.5 μJ. The probe pulse was linearly focused on a sample solution (30 μM, 1 mL) in a quartz spinning cell (a 10 mmφ NMR tube) by planoconvex and cylindrical lenses. The Raman scattering light was collected and focused onto the entrance slit of a Czerny-Turner configured Littrow prism prefilter (Bunkoukeiki) coupled to a single spectrograph with a focal length of 550 mm (iHR550, HORIBA Jobin Ybon) by two achromatic doublet lenses. The dispersed light was detected with a liquid-nitrogen-cooled CCD camera (SPEC-10:400B/LN-SN-U, Roper Scientific). The Raman shifts were calibrated with Raman bands of cyclohexane. The spectral dispersion was about 1 cm^{-1}/pixel on the CCD camera.

To accumulate the UVRR spectra, first the spectrum of DA-ASR was measured for a minute. Over 78% of the sample remained in the DA state under the present conditions. After 3 min of yellow light illumination, the spectrum of LA-ASR was measured for a minute. The DA- and LA-ASR spectra were measured 30 times. During each measurement, the damage of the sample due to the UV light irradiation was negligible.

2.3. Time-resolved UVRR measurements

The experimental setup for picosecond time-resolved UVRR measurements has been described elsewhere [3,22]. Briefly, the light source of the apparatus was a picosecond Ti:sapphire oscillator (Tsunami pumped by Millennia-Vs, Spectra-Physics) with an amplifier (Spitfire pumped by Evolution-15, Spectra-Physics) system operating at 1 kHz. The wavelength, pulse width, and energy of the laser output were 796 nm, 2.5 ps, and 800 μJ, respectively. To generate the pump and probe pulses, the second harmonic of the laser output was separated into two parts. The pump arm contained optical parametric generation (OPG) and amplification (OPA) devices [23]. The pump pulse was the output of the OPG/OPA system tuned to 549 nm. The probe arm contained a Raman shifter with compressed CH_4 gas. The probe pulse at 225 nm was the second harmonic of the first Stokes line generated from the Raman shifter and was introduced into an etalon to reduce the spectral width. The pump and probe pulses were collinearly overlapped and focused by a planoconvex lens onto a flowing thin-film of the sample solution (30 μM) with a gravity-driven wire-guided nozzle. Typical pulse energies and beam radii for pump and probe pulses at the sample point were 5 μJ, 250 μm and 0.5 μJ, 150 μm, respectively. The possibility of multiphoton excitation of the chromophore was excluded, because the linearity of the observed band intensity was kept under the present conditions of the energy density of the laser pulse. The zero-delay time was determined precisely by measuring the intensity of the difference frequency generation between the pump and probe pulses. The cross correlation time between the pump and probe pulses was 3.7 ps. The Raman scattering light was collected and focused onto the entrance slit of a Czerny-Turner configured Littrow prism prefilter [24] coupled to a 50-cm single spectrograph (500 M, SPEX) using two achromatic doublet lenses. The dispersed light was detected with a liquid-nitrogen-cooled CCD camera (SPEC-10:400B/LN, Roper Scientific). The Raman shifts were calibrated with Raman bands of cyclohexane. The spectral dispersion was 3.0–3.5 cm^{-1}/pixel on the CCD camera.

In the scan of delay time, the sequence of delay times was random. At each delay time, Raman signals were collected for three 20-s exposures with both pump and probe beams present in the sample. This was followed by equivalent exposures for pump-only, probe-only, and dark measurements. The transient Raman spectra were obtained by averaging the data for the repeated cycles. The sample bleaching was so slow that depletion of the intact component was negligible during one scan of delay time. Thus, this method enabled the avoidance of errors caused by sample bleaching as well as those caused by slowly drifting laser power.

The volume of the flowing sample solution of LA-ASR was 8 mL. During the time-resolved UVRR measurement, the sample solution in the reservoir was illuminated by yellow light before pump and probe pulse irradiation. The volume of the flowing solution of DA-ASR was 90 mL, which suppressed the accumulation of ASR_{13C} to a minimum. The data accumulation cycle took about 20 min to