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The Effect of S130A Mutant of *pharaonis* Halorhodopsin on Ability of Chloride Binding and Photocycle

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Bacteriorhodopsin (bR) and halorhodopsin (hR), which exist in the membrane of *Halobacterium salinarum*, are light-driven ion pumps. In spite of high similarity of primary and tertiary structures between bR and hR, these membrane proteins transport different ions, proton and chloride, in the opposite direction. From alignment of the amino acid sequences, Thr-89 of bR is homologous to Ser-115 of hR from *Halobacterium salinarum* (*sh*R). X-ray structure of *sh*R has revealed that OH group of this residue directly interacts with Cl^-. Thus, Ser-115 of *sh*R is expected to play an important role in Cl^- binding and transport. In this study, we expressed wild type hR from *Natronobacterium pharaonis* (*ph*R) and S130A, which corresponds to Ser-115 of *sh*R, in *E. coli* in order to clarify binding affinity of chloride ion and photocycle reactions. From the titration with Cl^-, affinity of S130A became quite lower than that of WT (WT 6 mM, S130A 89 mM). Furthermore, from the flash photolysis with pulse laser of λ_{max} at 532 nm, the reaction rate of S130A from O intermediate to hR ground state was found to become apparently slower than that of WT. The singular value decomposition (SVD) and global fitting analyses of the photocycles were performed to identify all photointermediates and determine the reaction rates.

Key words: *pharaonis* halorhodopsin, point mutants, photocycle, chloride binding, photointermediate

INTRODUCTION

Halorhodopsin (hR) and bacteriorhodopsin (bR) are transmembrane, seven-helix retinal proteins in the membrane of the archaeal bacterium, *Halobacterium salinarum*. These retinal proteins act as an inward-directed electrogenic light-driven chloride ion pump and an outward-directed proton pump, respectively. Although detailed knowledge on the proton transport of bR has been accumulated, much less information is available on the molecular mechanism of Cl^- transport by hR, and particularly with respect to site-specific mutagenesis.

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Recently many hRs have been identified and reported. However, only those from *Halobacterium salinarum* and *Natronobacterium pharaonis* have been extensively studied. With regard to structure, only the crystalline structure of the *salinarum* hR has been reported [1]. Since the primary structures of the *salinarum* hR and *pharaonis* hR are very highly homologous (66%), their tertiary structures would seem to be conserved. For this reason, we used the hR from *Natronobacterim pharaonis* (*ph*R) in the present paper. There are various advantages in using *ph*R. *ph*R has been reported to be more stable than *sh*R. Moreover, the retinal isomeric composition does not change by the condition of light/dark adaptation, and *ph*R transports not only halide but also nitrate at about the same rate. In addition, the expression system of *Escherichia coli* of the archaeal retinal protein was

recently reported by Shimono *et al.*[2]. And finally, the use of a histidine-tagged protein makes it possible to purify *p*hR in only one step, thereby allowing simple and large-scale preparation [3].

In this report, we expressed histidine-tagged wild-type *p*hR and Ser-130^{phR} point mutant, which corresponds to Ser-115^{shR}, in *E. coli* cells, and clarified the role of this residue in the binding of various anions and photocycle reactions. The results obtained in this report indicate that Ser-130^{phR} in helix C is one of the essential residues for the chloride binding and the transport/switch.

MATERIALS AND METHODS

The protein expression and purification procedures using *E. coli* BL21(DE3) cells harboring the plasmid were described in detail in a previous paper [3]. Wild type and Ser130^{phR} mutant substituted with Ala (S130A) of *p*hR were prepared. The anion-depleted species of the wild-type and the mutant were prepared by interchanging with buffer (10 mM MOPS (pH 7.0) and 0.1% *n*-dodecyl β-D-maltopyranoside, DM) by passing over a Sephadex-G25.

Circular dichroic (CD) spectra of the wild-type and the mutant were measured with a Jasco J-725 spectropolarimeter (Jasco, Tokyo, Japan) in the 300-750 nm region at 25°C using a scanning speed of 200 nm/min. Absorption spectra were obtained by converting a photomultiplier voltage signal of a CD apparatus into the optical density (log I_0/I) using a computer.

A computer-controlled flash-photolysis system was constructed as described in a previous paper [4]. Samples were excited at 532 nm. The output of the photomultiplier was stored in a computer equipped with an A/D converter (12-bit resolution, 0.8 μs per point). At each measuring wavelength (41 wavelengths, every 10 nm from 350 to 750 nm), the absorption change was obtained by the average of 50-100 data collection. The temperature was kept at 20°C. The bleach of the sample by actinic flash was negligible. Singular value decomposition (SVD) treatment [5] was used to decide the number of spectral components, as well as to remove the noise.

RESULTS AND DISCUSSION

Figure 1 shows absorption spectra in the range of 450-750 nm when titrated with Cl^- from 0 to 1 M at pH 7.0. In the absence of Cl^-, the replacement by Ala caused the red shifts of 12 nm. The absorption maxima of both S130A mutant and wild type was blue-shifted with an increase in the Cl^- concentration (Table 1). The S130A showed a large shift by 30 nm. The isosbestic point of the wild-type was 590 nm, but those of S130A was 604 nm. Values of K_d for the wild-type and S130A were 6 and 89 mM, respectively. The Hill coefficient for the wild-type was approximately one, but those for the Ser-130 mutant were much larger than unity. The difference of Hill coefficient between the wild-type and mutants might originate from the induced structural change.

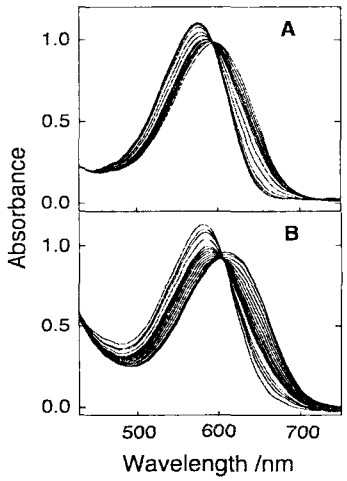

Figure 1. Change of absorption spectra of *p*hRs (A; wild type, B; S130A mutant) when titrated with Cl^- from 0 to 1 M at pH 7.0.

The CD spectra in the visible region (450-700 nm) exhibited a bilobe in the presence (1 M) and absence of Cl^- (data not shown). Since bilobes are attributed to the intermolecular interaction between neighboring retinal molecules, known as an exciton interaction, this result indicates that neither the Ser-130^{phR} mutant nor the wild-type are monomers, but rather are associated with each other [3]. In the case

of the Cl^--free condition, the exciton band of the CD spectrum of the wild-type is symmetrical with respect to the crossover point of the bilobe, but S130A mutant was slightly asymmetrical.

Table 1 Dissociation Constants, absorption maximum and apparent time constants of the photocycle of wild type and S130A mutant pf *p*hR.

	Wild type	S130A
Ground state		
K_d/ mM	5.6	89
λ_{max}/ nm (Cl^--free)	599	611
λ_{max}/ nm (1M NaCl)	577	581
Photocycle[1)]		
τ_1 /μs	237	-
τ_2 /μs	368	56
$\tau_{2'}$ /μs	-	473
τ_3 /μs	563	4356

pH 7.0 and 20°C. 1) excitation at 532 nm.

The photocycle of *p*hR has been studied extensively and the scheme was reported to be $p\text{hR}_{578} \rightarrow K_{600} \leftrightarrow L_{520} \leftrightarrow N \leftrightarrow O_{640} \leftrightarrow p\text{hR}' \rightarrow p\text{hR}_{578}$ [6], where accompanying numbers represent λ_{max} of the respective species. Kinetic analysis of the time-dependent spectral shifts from10 μs to 100 ms after a laser excitation was performed using a method of singular value decomposition [5]. Three kinds of apparent time constants of the wild type attributed to K → L (τ_1), L → O (τ_2) and O → hR (τ_3) are listed in Table 1. For the S130A mutant, the photocycle was drastically changed: The formation and decay of the K intermediate were too fast to be detected, and no equilibrium existed between K and L (or the equilibrium was completed before10 μs, the detection limit of the present experiment). The reason for this lack of equilibrium should be clarified on a molecular basis in the future. In the decay of the L intermediate, which was much faster than the decay of the other intermediates (see Table 1), a linear sequence of intermediates could not be assigned because the two positive peaks at around 520 nm had different τ values. This difference may have originated from the fact that one of the peaks corresponded to an L → O reaction (τ_2) and the other to an L → hR reaction ($\tau_{2'}$), based on the absorption maxima of the amplitude spectra.

In conclusion, the wild-type *p*hR and the Ser-130 mutant substituted by Ala showed differing levels of chloride accessibility. It was suggested that the oligomeric structure of *p*hR was slightly influenced by the substitution of Ser-130. The photocycles of the wild-type contained the typical photointermediates in a series. In contrast, the photocycle of the S130A mutant was quite different; the presence of the branched pathway from L to hR or to O was analyzed. These results indicated that the Ser residue at position 130, which contacts the chloride ion in the retinal pocket, is one of the essential residues for chloride binding and the transport/switch mechanism.

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