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Interhelical interactions between D92 and C218 in the cytoplasmic domain regulate proton uptake upon N-decay in the proton transport of *Acetabularia* rhodopsin II

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ABSTRACT

Acetabularia rhodopsin II (ARII or Ace2), an outward light-driven algal proton pump found in the giant unicellular marine alga *Acetabularia acetabulum*, has a unique property in the cytoplasmic (CP) side of its channel. The X-ray crystal structure of ARII in a dark state suggested the formation of an interhelical hydrogen bond between $C218^{ARII}$ and $D92^{ARII}$, an internal proton donor to the Schiff base (Wada et al., 2011). In this report, we investigated the photocycles of two mutants at position $C218^{ARII}$: $C218A^{ARII}$ which disrupts the interaction with $D92^{ARII}$, and $C218S^{ARII}$ which potentially forms a stronger hydrogen bond. Both mutants exhibited slower photocycles compared to the wild-type pump. Together with several kinetic changes of the photoproducts in the first half of the photocycle, these replacements led to specific retardation of the N-to-O transition in the second half of the photocycle. In addition, measurements of the flash-induced proton uptake and release using a pH-sensitive indium-tin oxide electrode revealed a concomitant delay in the proton uptake. These observations strongly suggest the importance of a native weak hydrogen bond between $C218^{ARII}$ and $D92^{ARII}$ for proper proton translocation in the CP channel during N-decay. A putative role for the $D92^{ARII}$-$C218^{ARII}$ interhelical hydrogen bond in the function of ARII is discussed.

1. Introduction

Microbial rhodopsins are a family of photoreceptive membrane proteins broadly distributed in various microorganisms, such as archaea, eubacteria, and lower eukaryotes [1–4]. Notwithstanding their diverse functions, microbial rhodopsins possess common properties, which are often described as structural and photochemical similarities. These proteins are commonly composed of seven transmembrane α-helical bundles (helices A-G) and enclose an all-*trans* retinal (ATR) as a chromophore. ATR covalently attaches to a conserved lysine residue on helix G of the opsin, which forms a protonated Schiff base (PSB) linkage [5]. Thus, these proteins can be photoactivated by the absorption of visible light. Light irradiation induces the isomerization of the retinal from all-*trans* to 13-*cis*, initiating a stepwise cyclic photochemical reaction via several photointermediates called the photocycle. During the photocycle, microbial rhodopsins perform their respective functions, including ion pumping, channeling, and photosensing [3,6].

Among many microbial rhodopsins, bacteriorhodopsin (BR), which acts as a light-driven outward proton pump, is the most well-studied [7–10]. The proton transport of BR is essentially accomplished via three

Abbreviations: AR, *Acetabularia* rhodopsin; BR, bacteriorhodopsin; ChR2, channelrhodopsin-2; PR, proteorhodopsin; ATR, all-*trans* retinal; PSB, protonated Schiff base; SB, deprotonated Schiff base; CP, cytoplasmic; EC, extracellular; H-bond, hydrogen bond; λ_{max}, absorbance maximum wavelength; P_i, *i*th photochemically-defined state determined by global fitting; DDM, n-dodecyl-β-D-maltoside; PC, L-α-phosphatidylcholine; ITO, indium-tin oxide; MES, MOPS, HEPES, CHES, CAPS, all abbreviations of Good's buffers

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main groups associated with proton transfer events: a protonated/deprotonated Schiff base (PSB/SB), its proton acceptor $D85^{BR}$, and donor $D96^{BR}$, which are located at the center of the protein, the extracellular (EC), and the cytoplasmic (CP) transmembrane region of helix C, respectively. In addition, two groups on the EC and CP side modulate the unidirectional proton movement in BR. A proton-releasing complex, which is established by several amino-acid residues and water molecules in the EC channel, causes the ejection of a proton towards the external bulk medium during the first half of the photocycle and subsequently receives a proton from $D85^{BR}$ during the second half of the photocycle. Another crucial residue is $T46^{BR}$ on helix B, the side chain of which forms a hydrogen bond with protonated $D96^{BR}$ in the CP domain in the unphotolyzed state. This interhelical interaction contributes to the maintenance of the high pK_a of $D96^{BR}$ (> ~11 [11]) at the initial state together with the hydrophobic residues surrounding it [12]. Through the entry of water via the outward tilt of helix F into the CP region at the site of the photoproduct, $D96^{BR}$ forms a new interaction with nearby water chains concurrently with the disruption of the H-bond with $T46^{BR}$ [13]. This H-bonding rearrangement leads to the lowering of the pK_a in $D96^{BR}$ from greater than 11 to approximately 7 [14,15], with subsequent proton donation of $D96^{BR}$ to SB. $T46^{BR}$, via this interhelical interaction, thereby regulates the function of $D96^{BR}$ in the CP region and facilitates efficient proton translocation in BR, although this residue itself does not directly participate in the proton transfer events.

Acetabularia rhodopsin (AR) is a light-driven proton pump found in a marine alga, *Acetabularia acetabulum*, whose characterization was first performed by Hegemann and colleagues [16]. Two AR homologue genes differing from the initially identified AR were subsequently cloned by Jung and colleagues: *Acetabularia* rhodopsin I and II (ARI and ARII, also abbreviated as Ace1 and Ace2, respectively) [17,18]. Previous electrophysiological studies revealed that these two AR proteins function as an outward proton pump, like BR [17,18]. However, their detailed characterization has not progressed due to their relatively low expression in a general host, such as *E. coli*, in the absence of certain modifications [19]. Recently, a unique cell-free protein expression method developed by Shimono et al. [20] enabled the large-scale synthesis of the two ARs, allowing accelerated investigation of their photochemical and structural properties [18,21,22].

We previously determined the X-ray crystal structures of ARI and ARII [18,21]. In both, the residues corresponding to $T46^{BR}$ were substituted by asparagine ($N48^{ARI}$ and $N45^{ARII}$, respectively). The oxygen and nitrogen atoms on the side chains of these asparagine residues interact with two carboxyl oxygens of putative proton donor residues ($D100^{ARI}$ or $D92^{ARII}$) through two H-bonds (see Fig. 1B and ref. [21]). Although the role of $N48^{ARI}$ or $N45^{ARII}$ has not been yet clarified, these interactions might provide stabilization of the protonation state of proton donors in the resting state, instead of the T46-D96 interaction in BR. In addition, a unique feature was found in the CP region of ARII. In the structure of the unphotolyzed ARII, at 3.2-Å resolution [18], $C218^{ARII}$ on helix G was located in the neighborhood of $D92^{ARII}$ on helix C (Fig. 1B), although the distance (approximately 3.9 Å) is somewhat far compared to the typical H-bond cutoff distance of 3.2 Å [23]. The residue corresponding to $C218^{ARII}$ is usually a hydrophobic one in many microbial rhodopsins, thus, this is specific for ARII. If $C218^{ARII}$ directly interacts with $D92^{ARII}$ in the dark state, it is likely that a sulfhydryl group of $C218^{ARII}$ forms a weak H-bond with a carbonyl oxygen ($O_{\delta 2}$) on the side chain of protonated $D92^{ARII}$, which serves as a H-bond donor (Fig. 1C). From FTIR spectroscopic studies by Nack et al., a similar Asp-Cys interaction was identified in channelrhodopsin-2 (ChR2), a light-gated cation channel [24]. They proposed that this interhelical interaction on helices C and D acted as a molecular switch (called the DC gate) to control the lifetime of the conductive state in ChR2 [24]. However, the existence of a functional Asp-Cys interhelical interaction in the proton-pumping rhodopsins has not been yet reported. Since it is unclear whether the Asp-Cys interaction implied by the crystal structure of ARII in the dark state is formed even in the photolyzed state and functional, we herein investigated the effects of replacement of $C218^{ARII}$ on the photoreactivity of ARII. The present study is the first report in which the effects of the Asp-Cys interaction on the photochemistry of a proton-pumping rhodopsin are described, and provides implications for its functional role.

2. Materials and Methods

2.1. Sample Preparation

Plasmids for the expression of $D92N^{ARII}$ and two $C218^{ARII}$ mutants ($C218A^{ARII}$ and $C218S^{ARII}$) were prepared by PCR using the QuikChange site-directed mutagenesis kit (Stratagene). In this mutagenesis procedure, a previously constructed expression plasmid for the cell-free protein synthesis of the wild-type ARII [22] was employed as a template. The sequences of the PCR product were confirmed using an automated DNA sequencer (Applied Biosystems). The expression and purification of ARII mutant proteins were performed as described previously [18].

2.2. Flash Photolysis

Flash photolysis experiments were conducted using the same apparatus and procedure as previously described [25]. A laser pulse (Nd:YAG 532 nm, 7 ns, 5 mJ/pulse) was employed as a pump light. The temperature was maintained at 20 °C with a thermostat (NCB-1200; Eyela). The solution for measurements contained 400 mM NaCl, 0.05% n-dodecyl-β-D-maltoside (DDM), and 6 mixed buffers (citrate, MES, HEPES, MOPS, CHES, CAPS, at 10 mM each). The medium pH was adjusted to be desired values by the addition of diluted HCl or NaOH solutions. The concentration of ARII mutant proteins in the sample solution was ~4–5 μM.

Global fitting analysis, assuming an irreversible sequential scheme including quasi-equilibria [26], was performed for the obtained data by flash photolysis at pH 7.0. The details of this analysis were described in several previous publications [27–29]. Briefly, through simultaneous fitting using a multiexponential function for the dataset at wavelengths from 340 to 700 nm with a 10-nm interval, the optimal number of exponents in the fitting function was determined from the reductions in the standard deviations of the weighted residuals. From this analysis, the time constant (τ_i) of the P_i-state, which represents the *i*th-appearing photochemically-defined state in the determined scheme, and the absorbance difference ($\Delta\varepsilon_i$) between P_i and P_0 (the dark state) were calculated. Regression analysis of the data was performed using Microcal Origin software (Microcal Software) and Igor Pro software (WaveMetrics).

The flash photolysis data at the pH values other than 7.0 were collected at three characteristic wavelengths for ARII, 400, 520, and 610 nm, which primarily monitor the M-state, resting state, and red-shifted K- and O-states, respectively.

2.3. Measurement of Absorption Spectra at the Dark State and Determination of Photointermediate Spectra during the Photocycle

The absorbance spectra in the dark state were measured using a spectrophotometer (V-560; Jasco). The obtained spectra contained the effects of the background scattering. Thus, this effect, which is represented as $A + B/\lambda^4$ (λ in nanometers), was removed from each spectrum. To calculate the spectra of the P_i-states determined by the global fitting analysis, the scattering-removed spectra in the dark (P_0) were added to $\Delta\varepsilon_i$.

To estimate the absorbance spectra of the photoproducts, the obtained P_i-spectra were further analyzed using the following equation, represented by the sum of the skewed Gaussian functions [26,30]: