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Identification of the key determinant of the transport promiscuity in Na^+-translocating rhodopsins

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ABSTRACT

Bacterial Na^+-transporting rhodopsins convert solar energy into transmembrane ion potential difference. Typically, they are strictly specific for Na^+, but some can additionally transport H^+. To determine the structural basis of cation promiscuity in Na^+-rhodopsins, we compared their primary structures and found a single position that harbors a cysteine in strictly specific Na^+-rhodopsins and a serine in the promiscuous *Krokinobacter eikastus* Na^+-rhodopsin (Kr2). A Cys253Ser variant of the strictly specific *Dokdonia sp.* PRO95 Na^+-rhodopsin (NaR) was indeed found to transport both Na^+ and H^+ in a light-dependent manner when expressed in retinal-producing *Escherichia coli* cells. The dual specificity of the NaR variant was confirmed by analysis of its photocycle, which revealed an acceleration of the cation-capture step by comparison with the wild-type NaR in a Na^+-deficient medium. The structural basis for the dependence of the Na^+/H^+ specificity in Na^+-rhodopsin on residue 253 remains to be determined.

1. Introduction

Rhodopsins are retinal-containing proteins that convert solar energy into transmembrane ion potential difference. These simplest ion pumps are formed by a single 27-kDa polypeptide and contain a retinal molecule, which forms an aldimine with a lysine side chain, as a single prosthetic group. Retinal isomerization from all-*trans* to 13-*cis* form upon photon absorption triggers a cascade of conformational changes in rhodopsins resulting in ion translocation across the membrane [1]. The first-discovered rhodopsins employ H^+ and Cl^- as the pumped ions, but more recent studies of marine flavobacteria have identified the rhodopsins that catalyze energy-dependent Na^+ transport from the cytoplasm to the external medium [2].

The first-discovered and best-known Na^+-transporting rhodopsin is that of the flavobacterium *Krokinobacter eikastus* NBRC 100814T (Kr2) [3]. The 3D-structure of Kr2 has been reported by two groups [4,5] (Fig. 1), and its photocycle and the ion translocation mechanism have been described in detail [3]. In the absence of Na^+ (Li^+), Kr2 is additionally capable of catalyzing light-dependent H^+ transport [3] at a rate even exceeding that of the Na^+ transport at equal cation concentrations [6]. The dual Na^+/H^+ transport specificity was also reported for Na^+-rhodopsin from *Indibacter alkaliphilus* LW1 [7].

The other characterized Na^+-rhodopsins, such as those from *Dokdonia sp.* PRO95, *Nonlabens marinus* S1-08T, *Gillisia limnaea* R-8282T, *Flagellimonas* sp_DIK and *Nonlabens* sp_YIK_SED-11 [8–11], are similar to Kr2 but are more specific transporters. None of these rhodopsins exhibit a significant H^+-transport activity, even in the absence of Na^+. The structural basis for the difference in the cation specificity between Kr2 and the other Na^+-rhodopsins has not yet been determined. *Dokdonia sp.* PRO95 Na^+-rhodopsin (NaR) provides a convenient model to address this problem because its amino acid sequence differs from Kr2 in only five positions (Fig. 1); the structures of other Na^+-rhodopsins are more divergent (Fig. S1). The aim of the present study was, therefore, to determine which of the five positions carries a residue whose identity determines the difference in the cation specificity between Kr2 and NaR.

Abbreviations: CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; DDM, *n*-dodecyl β-D-maltoside; Kr2, Na^+-transporting rhodopsin from *Krokinobacter eikastus* NBRC 100814T; NaR, Na^+-transporting rhodopsin from *Dokdonia sp.* PRO95; RMSD, root-mean-square deviation.

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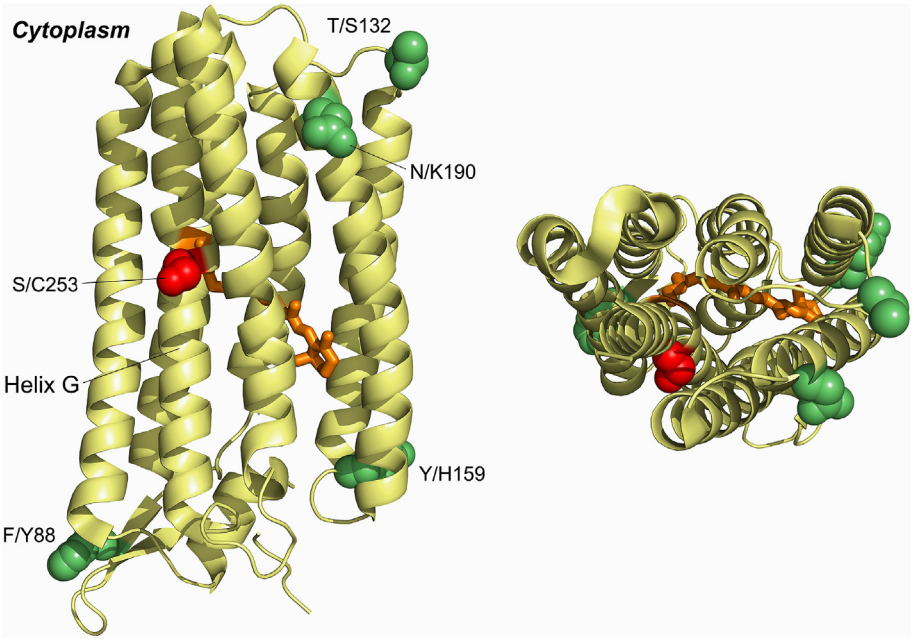

Fig. 1. The structure of *K. eikastus* Na^+-rhodopsin (Kr2; PDB ID: 4XTL) [5]. The right panel shows the top view. The bound retinal is depicted as orange sticks. Side chains of the residues that are different in NaR are shown in a spherical representation, and residue identities are indicated in a Kr2/NaR format using a single-letter notation. The figure was created with PyMOL (PyMOL Molecular Graphics System, version 1.5.0.4; Schrödinger, LLC). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

2.1. Construction of the plasmid encoding the enzymes of the retinal biosynthetic pathway

The operon containing *crt*E, *crt*I, *crt*B, *crt*Y and *blh* genes encoding geranylgeranyl diphosphate synthase, phytoene desaturase, phytoene synthase, lycopene cyclase and 15,15′-β-carotene dioxygenase, respectively, which form a retinal biosynthetic pathway in *Vibrio campbellii* CAIM 519 [12], was amplified from genomic DNA of this bacterium by PCR with long-reading Encyclo polymerase (Evrogen, Russia) and the forward/reverse primers 5′-GCTTTGCTCCCTGGTCCTCA/5′- GGGCCCAATAACCAACCGACTAA-GATGC. The amplified 5479-bp fragment was cloned into the pBAD-TOPO vector (Invitrogen). The *Nru*I-*Apa*I fragment of this plasmid was subcloned into the p15BCM7 vector [13] treated with the *Nru*I and *Apa*I restriction enzymes, resulting in the plasmid p15Ret, which contained operon for retinal biosynthesis under control of an *ara*BAD promoter and a *p15A* origin of replication and confers chloramphenicol resistance. The p15Ret plasmid was transformed into the *Escherichia coli* BL21 strain, and the ability of the transformed cells to produce retinal was determined in a two-phase culture system as described by Jang et al. [14].

2.2. Site-directed mutagenesis of the dokdonia sp. PRO95 NaR gene

The forward mutagenic primer used to substitute a Ser codon for that of Cys253 in the wild-type NaR gene (AEX55013) was 5′-TACACAATTGCAGATGTATcTTCTAAAGTCATCTATGGTG (the substituted nucleotide is indicated by lower case letter). Mutagenesis was carried out using the QuikChange II kit (Stratagene) and the plasmid pRhod_10.1 [15] as the template. The NaR-encoding region of the final expression construct (pRhod_C253S) was checked by DNA sequencing. The pRhod_C253S and the intact pRhod_10.1 plasmids harboring the wild-type NaR gene were transformed into *E. coli* BL21/p15Ret cells.

2.3. Expression of the dokdonia sp. PRO95 NaR gene and its Ser253/Cys variant

For induction of synthesis of NaR variants, *E. coli* BL21/p15Ret/pRhod_10.1 or *E. coli* BL21/p15Ret/pRhod_C253S cells were grown at 37 °C in the TB medium containing 100 μg/mL ampicillin and 30 μg/mL chloramphenicol up to late-exponential phase ($A_{600} \approx 1.5$), after which the growth medium was supplemented with 0.15% (w/v) L-arabinose. The cells were further grown for 16 h at 15 °C and used for measurements of light-driven H^+ transport or for NaR isolation.

2.4. Purification of wild-type and variant NaR

The recombinant 6 × His-tagged NaR and its Cys253Ser variant were isolated from *E. coli* cells by a metal-chelating chromatography as described previously [15].

2.5. Measurements of light-driven proton transport in E. coli cells

Prior to measurements, *E. coli* cells were depleted of endogenous substrates and loaded with a required cation (Na^+ or K^+) as described previously [10]. For the H^+ transport assay, the Na^+- or K^+-loaded cells (2 mg of protein per mL) were placed into a 1.4-mL chamber and the pH of the suspension was adjusted to ~7.5 with NaOH or KOH solution. The cells were incubated in darkness and illuminated through a 470–610 nm bandpass filter with a 100-W halogen lamp equipped with an infrared cut-off filter at light irradiance ~300 W/m^2. Light-induced pH changes were monitored with a pH electrode and calibrated with an argon-saturated 20 mM H_2SO_4 solution.

2.6. Flash photolysis measurements of the NaR photocycle

Light-induced absorption changes were monitored with a single-beam differential spectrophotometer with a resolution time

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