# The Photocycle and Proton Translocation Pathway in a Cyanobacterial Ion-Pumping Rhodopsin

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ABSTRACT The genome of thylakoidless cyanobacterium *Gloeobacter violaceus* encodes a fast-cycling rhodopsin capable of light-driven proton transport. We characterize the dark state, the photocycle, and the proton translocation pathway of GR spectroscopically. The dark state of GR contains predominantly all-*trans*-retinal and, similar to proteorhodopsin, does not show the light/dark adaptation. We found an unusually strong coupling between the conformation of the retinal and the site of $Glu^{132}$, the homolog of $Asp^{96}$ of BR. Although the photocycle of GR is similar to that of proteorhodopsin in general, it differs in accumulating two intermediates typical for BR, the L-like and the N-like states. The latter state has a deprotonated cytoplasmic proton donor and is spectrally distinct from the strongly red-shifted N intermediate known for proteorhodopsin. The proton uptake precedes the release and occurs during the transition to the O intermediate. The proton translocation pathway of GR is similar to those of other proton-pumping rhodopsins, involving homologs of BR Schiff base proton acceptor and donor $Asp^{85}$ and $Asp^{96}$ ($Asp^{121}$ and $Glu^{132}$). We assigned a pair of FTIR bands (positive at 1749 $cm^{-1}$ and negative at 1734 $cm^{-1}$) to the protonation and deprotonation, respectively, of these carboxylic acids.

## INTRODUCTION

Microbial rhodopsins are ubiquitous retinal-binding membrane proteins with ion-transporting and photosensory functions. The archetypal well-studied microbial rhodopsins are of haloarchaeal origin and are best represented by the light-driven proton pump BR. PR is a eubacterial homolog of BR originally found in uncultured marine γ-proteobacteria (1). Similar to BR, it binds all-*trans*-retinal covalently and performs transmembrane light-driven proton translocation, implying that it can be used as a supplementary source of energy (1–3). The exact contribution of the PR-dependent bioenergetic pathway is still controversial and may vary with the taxonomy of the host (4–7). In the past few years it was realized that there are thousands of different PR species, with great taxonomic, ecological, structural, spectral, and, possibly, functional diversity (8–14). The last is suggested not only by the analysis of the primary structures (11) but also by the presence of both fast (typical for ion pumps) and slow (typical for photosensors) photocycles and corresponding photoelectric signals (15,16). Genes encoding PR-like proteins are found not only in α-, β-, and γ-proteobacteria but also in *Bacteroidetes*, *Actinobacteria*, *Planctomycetes*, *Firmicutes,* and *Cyanobacteria* (8,12,17,18) as well as in marine archaea and some dinoflagellates, which probably obtained them by lateral gene transfer (19,20).

Most of the biophysical studies done on the original green-absorbing PR (1) expressed in *Escherichia coli* imply the BR-like nature of the light-driven proton translocation. Under physiological conditions (mildly alkaline pH), PR transports protons light-dependently in the extracellular direction in liposomes, oocytes, and *E. coli* cells (16,21,22). Accordingly, it shows light-induced spectral changes in the infrared region of strongly bound water characteristic for transport rhodopsins (23). The photocycle of PR is relatively fast, and it showed most of the photointermediates known for BR, with the apparent exception of the L intermediate (21,24,25). Finally, a combination of site-directed mutagenesis and time-resolved spectroscopy showed that the homologs of the primary proton acceptor ($Asp^{85}$, present as $Asp^{97}$ in PR) and donor ($Asp^{96}$, present as $Glu^{108}$ in PR) of the Schiff base of BR are involved in proton translocation in PR as well (24). On the other hand, there are many notable differences between PR and BR, the most interesting one being the unusually high $pK_a$ of $Asp^{97}$ (7.1–7.6), which may be related to the alkaline character of the marine environment (21,24). This can originate from the structural differences in the extracellular half of PR, such as the absence of the pair of proton-releasing glutamates ($Glu^{194}$ and $Glu^{204}$ of BR), weaker coupling of the Schiff base counterion to $Arg^{94}$ ($Arg^{82}$ of BR), its unique interaction with $Asn^{230}$ and $His^{75}$, and a relatively strong hydration of $Asp^{227}$ ($Asp^{212}$ of BR) (26–29).

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*Abbreviations used*: BR, bacteriorhodopsin; PR, green-absorbing proteorhodopsin; GR, *Gloeobacter* rhodopsin; ASR, sensory rhodopsin from *Anabaena*; XR, xanthorhodopsin from *Salinibacter ruber*; DM, *n*-dodecyl-β-D-*maltoside*; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; FTIR, Fourier-transform infrared.

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Among approximately a dozen sequenced cyanobacterial genomes, only two rhodopsin species have been found. The first one is a unique photosensory rhodopsin from *Anabaena* (*Nostoc*) sp. PCC7120 (ASR) (30), possibly serving as a receptor for phycobilin biosynthesis. The second rhodopsin was found in a thylakoidless unicellular cyanobacterium *Gloeobacter violaceus* PCC 7421 (31). Its primary structure resembles that of PR, and because it has a glutamate residue at the position of the Schiff base proton donor, it was suggested to have a proton-pumping function as well (32). Indeed, when expressed in *E. coli*, GR demonstrates light-driven proton transport and correspondingly fast photocycle (A. R. Choi, L. Shi, L. S. Brown, and K.-H. Jung, unpublished). It should be noted that the exact physiological role of such proton-pumping rhodopsin in the presence of the proton gradient-generating chlorophyll-based photosynthetic apparatus is puzzling. Even though the amino acid sequence of GR is closer to that of PR than of BR, it forms a separate cluster together with XR, the carotenoid-associated proton pump from *Salinibacter ruber* (33,34), and a few other sequences from proteobacteria, actinobacteria, and *Chloroflexii* (8,12). The recently obtained crystal structure of XR (35) may give more hints toward general structural features of this group of rhodopsins, in particular, participation of a His side chain in the extracellular hydrogen-bonded network and unusual connectivity of the cytoplasmic Glu (homolog of $Asp^{96}$ of BR) to the Schiff base-forming Lys.

In this work, we tried to define the pathway of proton translocation in the photocycle of GR using the combination of site-directed mutagenesis, heterologous expression, and three kinds of spectroscopy, including Raman, time-resolved FTIR, and time-resolved visible. We compared characteristics of the photocycle and the dark state of GR with those of PR and BR and concluded that overall path of proton transport involves the same carboxylic residues (homologs of BR's $Asp^{85}$ and $Asp^{96}$). At the same time, we found that the photocycle and the dark state of GR are different from those of both PR and BR in several respects. In addition to substantially lower $pK_a$ of the Schiff base counterion $Asp^{121}$ (A. R. Choi, L. Shi, L. S. Brown, and K.-H. Jung, unpublished), the most interesting features of GR are an apparent accumulation of the BR-like N intermediate with the deprotonated cytoplasmic proton donor $Glu^{132}$ and unusually strong coupling between this proton donor and the retinal.

## MATERIALS AND METHODS

### Heterologous expression of the wild-type and mutant GR

The heterologous expression of wild-type GR in *E. coli* as well as production of GR mutants are described in detail elsewhere (A. R. Choi, L. Shi, L. S. Brown, and K.-H. Jung, unpublished). Briefly, genomic DNA of *Gloeobacter violaceous* was used to amplify the gene encoding GR by standard PCR using nondegenerate primers 5′ATGTTGATGACC GTATTTTCTTCTGC3′ and 5′CTAGGAGATAAGACTGCC-TCCCCG3′. *E. coli* strain DH5$\alpha$ was used for the cloning, and transformants were grown in LB medium in the presence of ampicillin (50 $\mu$g/mL) at 35°C. The amplification product was inserted under the *lacUV5* promoter into the pKJ900 plasmid, which also carried a gene for $\beta$-carotene dioxygenase under pBAD promoter (36). GR was expressed in *E. coli* strain $\beta$/UT, which was constructed by transforming plasmid pORANGE into UT5600, resulting in the ability to synthesize $\beta$-carotene (36). $\beta$/UT cells transformed with pKJ900 were able to produce retinal and GR endogenously after being induced with 1 mM IPTG and 0.2% L-(+)-arabinose for 24 h at 30°C. The pKJ900 plasmid encoding wild-type gloeoopsin was used as a template for site-directed mutagenesis. Site-directed mutagenesis was carried out with the two-step megaprimer PCR method with *Pfu* polymerase (36).

### Isolation, purification, and reconstitution of GR

The typical protein yields were in the range of 2–5 mg of GR per liter of culture, which produces *E. coli* membranes with an intense color and negligible cytochrome bands (around 410 nm). Isolation of the GR-containing *E. coli* membranes, their treatment with DM and incorporation into polyacrylamide gels was performed as described for ASR (37). For vibrational spectroscopy and proton kinetics, further purification was performed via solubilization in DM, $Ni^{2+}$-NTA resin binding, and reconstitution into DMPC/DMPA liposomes as described earlier (37). The notable difference between GR and other His-tagged microbial rhodopsins is its relatively fast rate of hydrolysis of the C-terminus, so that His-tag purification had to be performed using freshly isolated membranes.

### Spectroscopy in the visible and infrared ranges

Static visible spectra were collected using a Cary 50 spectrophotometer (Varian, Palo Alto, CA). Time-resolved laser difference spectroscopy in the visible range and global multiexponential fitting of the data were performed as before (37,38). Static Raman spectra were collected using either a Renishaw (Toronto, Ontario, Canada) Raman Imaging Microscope, System 2000, with excitation at 785 nm, or Bruker (Billerica, MA) FRA 106/s accessory to the IFS66vs spectrometer, with excitation at 1024 nm, at 2 $cm^{-1}$ resolution, using concentrated suspensions of GR liposomes. Time-resolved rapid-scan FTIR difference spectra were measured in transmission mode using Bruker IFS66vs spectrometer as described elsewhere (37,38), using fast return and 4 $cm^{-1}$ spectral resolution. The interferogram acquisition time was 12 ms.

## RESULTS AND DISCUSSION

### Characterization of the dark state of GR by Raman spectroscopy

Similar to many other microbial rhodopsins, GR exists in two spectral forms in a pH-dependent equilibrium, involving the bluish acidic ($\lambda_{max} \approx 550$ nm) and reddish alkaline ($\lambda_{max} \approx 543$ nm) pigments. The transition between the two forms was found to be rather broad, with the midpoint at pH $\approx$ 5 (A. R. Choi, L. Shi, L. S. Brown, and K.-H. Jung, unpublished), which is higher than that for BR but lower than for PR and XR (24,39). For this reason, we chose to study the alkaline (proton-pumping) form of GR at pH 9 to avoid contamination by the acidic form. First, we characterized the chromophore of GR by preresonance Raman spectroscopy (Fig. 1 *A*), using GR reconstituted into DMPC/DMPA liposomes to increase the protein concentration and purity. The basic features of the spectra in the fingerprint