Engineering retinal-based phototrophy via a complementary photosystem in Synechocystis sp. PCC6803
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Chapter 4

Functional expression of *Gloeobacter* rhodopsin in *Synechocystis* sp. PCC6803

Que Chen, Jos Arents, Srividya Ganapathy, Willem J. de Grip, Klaas J. Hellingwerf

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Abstract:

Proteorhodopsins are retinal-based, light-driven proton pumps that occur widespread in Nature. They predominantly appear to function in environments with high incident irradiance for phototrophy (and photosynthesis) of their host, by boosting the magnitude of the proton motive force across the organism’s cytoplasmic membrane. Their maximal absorbance is usually in the blue to the orange range but can be extended into the (far)red. Since they are also easily heterologously expressed, these pumps can play an important role in further attempts to increase the efficiency of oxygenic photosynthesis. Here we report on an extension of our initial studies towards this goal, by comparison of the effect of expressing two different bacterial rhodopsins (*i.e.* Proteorhodopsin and *Gloeobacter* rhodopsin) in the model cyanobacterium *Synechocystis* sp. PCC6803, with special emphasis on the pigments bound by the respective apo-opsins, and the oligomeric state of the corresponding holo-rhodopsins, in *Escherichia coli* and in the cyanobacterial membranes.

From these studies, we tentatively conclude that the two proton-pumping rhodopsins are predominantly present as hexamers and trimers, respectively. Furthermore, *Gloeobacter* rhodopsin is able to bind (an antenna) carotenoid, in addition to retinal, and also has a higher turnover rate of proton pumping at a given light intensity than Proteorhodopsin. The smaller amount of GR expressed, however, will decrease its effectiveness. It still remains to be established which of these two bacterial rhodopsins can give the highest stimulatory effect on the growth rate of their cyanobacterial host.

Key words:

bacterial rhodopsins, growth-phase dependence, oligomeric state, carotenoids, echinenone, canthaxanthin, 3-hydroxy-echinenone, pumping rate
Introduction

Rhodopsins are light-sensitive seven-helix transmembrane proteins that bind a retinal molecule as their chromophore. This family has members with either a sensory- (48), or a chemiosmotic function in free-energy transduction. Most members of this group utilize light energy to translocate protons over a membrane against an electrochemical proton gradient (77). More recently, however, bacterial rhodopsins have been discovered that pump ions other than protons, like sodium\(^+\) (50-53), lithium\(^-\) (52), or chloride ions (54-56). Site-directed engineering can broaden this range even further to even include Cs\(^+\) and K\(^+\) (57-59). The gene encoding the first discovered member of the Bacterial group of the proteorhodopsins was detected in the genome sequence of an uncultured \(\gamma\)-proteobacterium from oceanic waters (67). Since then, PRs have turned out to be highly abundant in the oceans (68-72) and organisms containing them, including cyanobacteria (56, 73) can be found in many other habitats as well (70, 74-76). Surprisingly, Proteorhodopsin turned out to function as a hexameric assembly (85), rather than the hexagonal array of trimers characteristic for bacteriorhodopsin from *Halobacterium salinarum* (62).

In vivo experiments with organisms that express a Proteorhodopsin have shown that its pumping activity can lead to an increase in growth rate under nutrient-limited conditions (68, 69, 86), production of ATP (87), increased fixation of CO\(_2\) (68, 88), and/or survival under starvation or stress conditions (87, 89-92). Such enhancements, however, generally require nutrient-limitation or stress conditions before they exceed the limit of detection. Nevertheless, the occurrence of Proteorhodopsin-mediated light-driven ATP synthesis implies that next to chlorophyll-based phototrophy and photosynthesis, there is a second type of phototrophy: retinal-based phototrophy (226, 227) and presumably even retinal-based photosynthesis (227). This type of photosynthesis is characterized by a very limited use of antenna pigments and is based on light-driven E/Z isomerization, rather than on light-driven charge-separation and electron transfer, as in anoxygenic- and oxygenic phototrophy and photosynthesis.

Which of the two types of photosynthesis (i.e. the retinal-based or the chlorophyll-based), is most efficient, is very difficult to decide (4, 228), and may even depend on the abiotic environment of the cell in which this is tested (see also above). This issue has gained special importance within the context of the bio-based economy and the worries about global warming. These concerns have
brought the need for society to develop alternative, non-fossil based, sustainable, methods for fuel- and commodities production. Cyanobacteria play a crucial role in this development because they combine the ability to use water-derived electrons to reduce CO\textsubscript{2} and combine this with the highest oxygenic photosynthetic efficiency (9) and high growth rates. Genetic engineering of such organisms then allows one to produce a range of valuable carbon-based products via ‘direct conversion’ (11-13).

Because of the importance of oxygenic photosynthesis for a variety of sustainability applications, multiple proposals have been made to increase the efficiency of oxygenic photosynthesis beyond its current biological and thermodynamic limits (27, 43, 123). The most recent of these (43) proposes to transfer electrons from water to NADPH by PSII only, and combine this activity with a far-red light-driven proton pump that then would provide the complementary energy-rich intermediate for the ‘dark reactions of photosynthesis’ (i.e. ATP). Although in the latter publication it is proposed to use an (infra)red-light-absorbing and bacterio-chlorophyll-based cyclic electron transfer chain as this proton pump, a far-red absorbing proteorhodopsin (229) would equally qualify, particularly because heterologous expression of the latter is much more straightforward. For this reason, we have expressed holo-proteorhodopsin (the green proteorhodopsin from Monterey Bay (67) in Synechocystis and observed that this retinal-based proton pump can slightly but measurably increase the rate of photoautotrophic growth of the cyanobacterium Synechocystis sp. PCC6803 (46).

Here we report on the comparison of the functional expression of (green) Proteorhodopsin with Gloeobacter rhodopsin from Gloeobacter violacea in Synechocystis sp. PCC6803. The latter is of interest because of: (i) the higher turnover rate of its photocycle (i.e. 230 ms vs 140 ms of the largest decay time constant for the alkaline forms of Proteorhodopsin and Gloeobacter rhodopsin, respectively (73, 79, 82) (ii) its ability to bind carotenoids like salinixanthin and echinenone (100-102), which function as an antenna for the retinal-based proton pumping and in this way increase the absorption cross section of the pump for visible light about 4-fold (97).

As this beneficial effect of PR expression in Synechocystis was only very small it is important to find out whether more efficient or faster pumping, proton pumps can increase it. One of the possible candidates for this is Gloeobacter rhodopsin, i.e. because of its faster pumping rate. Here we characterize this Gloeobacter rhodopsin with respect to the holo-protein expression level in
Synechocystis, carotenoid binding and oligomeric state. The latter aspect was included because of the difficulty to functionally express bacteriorhodopsin in \textit{i.e.} Escherichia coli, partly because it forms hexagonal arrays of protein trimers (62).

**Materials and Methods**

**Strains and growth conditions**

Strains of \textit{Escherichia coli} were routinely grown in LB-Lennox (LB) liquid medium at 37°C with shaking at 200 rpm, or on solid LB plates containing 1.5% (w/v) agar. Where appropriate, antibiotics were added to a final concentration as follows: ampicillin (100 μg/ml) or kanamycin (25 to 50 μg/ml), either separately or in combination.

*Synechocystis* sp. PCC6803 (a glucose tolerant strain, obtained from D. Bhaya, Stanford University, USA) was routinely grown at 30°C with continuous illumination with white light at the moderate intensity of approximately 45 μmol • m$^{-2}$ • s$^{-1}$ (= μmol photons • m$^{-2}$ • s$^{-1}$). Liquid cultures were grown in BG-11 medium (Sigma-Aldrich), supplemented with 50 mM sodium bicarbonate, 25 mM TES-KOH (pH = 8) and appropriate antibiotics, and with shaking at 120 rpm (Innova 43, New Brunswick Scientific). The BG-11 agar plates were supplemented with 10 mM TES-KOH (pH= 8), 5 mM glucose, 0.3% (w/v) sodium thiosulfate, and 1.5% (w/v) agar. Where appropriate, antibiotics were added to a final concentration of: ampicillin (100 μg/ml) or kanamycin (25 to 50 μg/ml), either separately or in combination.

To exert salt stress, cells were grown in the presence of increasing concentrations of NaCl (up to 1 M). Cells were grown in BG-11 with 50 mM NaHCO3, 25 mM TES, pH = 8, at 30°C with shaking at 120 rpm, and illuminated with RGB light (red, green, blue) at a total light intensity of 47 μmol • m$^{-2}$ • s$^{-1}$ (containing 21 μmol • m$^{-2}$ • s$^{-1}$ red, 23 μmol • m$^{-2}$ • s$^{-1}$ green, and 3 μmol • m$^{-2}$ • s$^{-1}$ blue light). The red, green, and blue LEDs emitted maximally at 635 nm, 527 nm, and 459 nm, respectively. Growth was monitored via cell density by measuring the OD$_{750}$ of a small volume (≤ 150 μl) sample from each culture in a Multiskan FC Microplate Photometer (Thermo Scientific, Finland).
Strain construction

The gene coding for *Gloeobacter* rhodopsin (Genebank accession number NP_923144, (102)) with a C-terminal 6×histidine tag (GR-His) was amplified with the proof-reading Pwo DNA Polymerase (Roche Diagnostics) with primers JBS308: 5′, *tac* gaaattcgccgccgcttctag ATGTTGATGACCGTATTTTCTTC 3′, and JBS312: 5′, *tac* ctgcagcggccgctactagta CTA GTGATGGTGGGTTGATG GGAGATAAGACTGCCTCCC 3′, from plasmid pKJ900 (73, 178), which was a kind gift of Dr. K.H. Jung from the University of Seoul, South Korea. The obtained fragment was digested with XbaI (Thermo Scientific) and then ligated by T4 DNA Ligase (Thermo Scientific) into AvrII-digested pJBS1312, which is a derivative of the broad-host-range conjugation vector pVZ321, with the relevant structure: *PpsbA2-RBS-AvrII-BBa_B0014* (46). This newly constructed plasmid was named pQC012, with the relevant structure: *PpsbA2-RBS-GR-His-BBa_B0014*. *E. coli XL1-Blue* (Agilent Technologies) was used as cloning host. Constructed plasmids were verified with primers JBS308 and JBS315: 5′, GATGTATGCTCTTCTGCTC 3′, using colony PCRs by 2 × MyTaq Polymerase (Bioline), followed by additional verification via sequencing.

This plasmid was conjugated into wild-type *Synechocystis* sp. PCC6803 via tri-parental mating, following the description in (46). *E. coli J53/RP4* (180, 181) was used as a helper strain and *E. coli XL1-Blue* carrying pQC012 was used as the donor strain.

The construction of plasmid pQC006 (with the structure: *PpsbA2-RBS-PR-His-BBa_B0014*), pQC011 (with a structure: *PpsbA2-RBS-PROPS -BBa_B0014*), as well as the generation of the *E. coli* and *Synechocystis* strains containing these plasmids, has been described in (46).

Isolation of His-tagged *Gloeobacter* rhodopsin from *E. coli* and *Synechocystis* cells

The GR-His expressing *E. coli* strain (*i.e.* *E. coli* with pQC012) was grown in the presence of all-trans retinal at a final concentration of 10 µM. Cells were harvested by centrifugation (11,000 g, 10 min, 4 °C) and re-suspended into binding buffer A (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1.5% (w/v) n-dodecyl-β-D-maltopyranoside (DDM; GAMPRO Scientific), pH = 8), and then disrupted by sonication. The obtained lysate was stirred at 4 °C overnight, followed by centrifugation (10,000g, 30 min at 4 °C) to get rid of cell debris. The obtained cell-free lysate was loaded on a His-Trap column (His-
expression of holo-Gr in synechocystis

Trap FF Crude 5ml), connected to an ÄKTA FPLC system (GE Healthcare). Histidine-tagged protein was eluted with elution buffer B (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 0.1% (w/v) DDM, pH 8). The UV/Vis spectrum of the main eluted fractions has been recorded on an Agilent 8453 UV–vis spectrophotometer (Agilent Technologies, Germany). The fractions containing protein were collected and dialyzed overnight against buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) DDM, pH = 8.0) at 4°C. The purity of the protein was verified by the ratio of absorption at 280 nm over 541 nm. For fractions with a high purity, the protein concentration was determined via the absorption at 541 nm with an extinction coefficient of 50,000 L mol$^{-1}$ cm$^{-1}$ (102). The isolation and purification of GR-His from *Synechocystis* cells (*Synechocystis* with pQC012) followed the same protocol except that all-trans retinal was not added to the culture.

**Reconstitution of GR-His with carotenoids in vitro**

*Holo-Gloeobacter* rhodopsin isolated from *E. coli* (see above) was dialyzed and dissolved in a buffer (25 mM MOPS, 100 mM NaCl, 0.02% DDM, pH = 7.2) (101) and then aliquoted for storage at – 20 oC. Echinonene, 3-hydroxy-echinenone (both were a kind gift from Dr. J. Lugtenburg, Leiden University, The Netherlands), and canthaxanthin (Sigma-Aldrich, >95% pure) were used as the carotenoids in the carotenoid-binding test. All of the above carotenoids were dissolved in acetone (AR grade) and added into a GR-His sample at a 1:1 molar ratio, at the final concentration of 4 µM. Absorption spectra were measured at various points in time with an Agilent 8453UV–vis spectrophotometer (Agilent Technologies, Germany).

For calculations, an extinction coefficient of 50,000 L mol$^{-1}$ cm$^{-1}$ at 541 nm (102) was used for *Gloeobacter* rhodopsin in the DDM-containing buffer (see above), while 119,000 L mol-1cm-1 was used for all above-mentioned carotenoids in acetone (101).

**Measurement of the Proton-Pumping Activity of GR-His**

Light-dependent rates of proton extrusion of *Escherichia coli* cells was measured in a darkened temperature-controlled vessel (230) of 1.9 ml at 25°C with a pH electrode (Mettler Toledo type no. 51343164) and a white light source (Schott KL 1500 LCD) connected via an optical fiber. Stirring was performed by an overhead constant-stirring device, which also allows solute addition.
E. coli XL1 cells expressing PR-His or GR-His (i.e. containing plasmid pQC006 (46) or pQC012) were grown in LB-Lennox medium with kanamycin (25 µg/ml) overnight at 37°C, and 200 rpm. The culture was then diluted 100-fold and grown again to an OD600 of 0.35. Retinal was then added at a final concentration of 10 µM and growth was continued overnight in darkness. To stop protein synthesis, chloramphenicol was added to the cells at a final concentration of 50 µg/ml and incubated for 2 hours. Next, carotenoids (echinenone, 3-hydroxy echinenone, or canthaxanthin) dissolved in acetone, were added to the culture at a final concentration of 10 µM. After overnight incubation with carotenoids, cells were harvested by centrifugation and washed three times. Finally the cells were re-suspended in starvation buffer (10 mM NaCl, 10 mM MgSO4, 0.1 mM CaCl2, 10 mM Tris-HCl, pH = 7.0). The cells were starved in darkness at room temperature for 4 - 5 days at 60 rpm. 1 ml of this cell suspension was centrifuged in the dark (i.e. in a black tube), washed 3 times with measurement solution (10 mM NaCl, 10 mM MgSO4, 0.1 mM CaCl2, and 250 mM KCl) and diluted to an OD600 of 1.5 with measurement solution. The initial rate of proton extrusion elicited by illumination was measured at different light intensities (from 250 to 6,000 μmol • m⁻² • s⁻¹) by measuring the extracellular pH for about 30 sec in the light, and during re-equilibration during about 20 minutes in the dark. The light intensity was calibrated with a LI-COR Radiation Sensor. The pH response was calibrated with the addition of 5 µl 0.01 N oxalic acid. The proton extrusion activity of E. coli cells without addition of all-trans retinal was used as a control.

To determine the expression level of PR or GR in E. coli production strains, a small amount of cells (approximately 100 OD600 cells, equal to 100 ml culture with OD600 of 1.0) were disrupted with 100-µm glass beads (Sigma) using a Precellys®24 bead beater (Bertin Technologies) in 1 ml buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1.5% (w/v) DDM, pH = 8) and then stirred overnight at 4°C. The obtained cell extract was centrifuged to discard cell debris at 10,000 g and 4°C for 15 minutes. The resulting supernatant was considered as the GR or PR sample, and its protein concentration was determined by spectrometry at 525 nm for PR and 541 nm for GR, on the basis of the molar extinction coefficient of 44,000 M⁻¹ cm⁻¹ for PR (231) and 50,000 M⁻¹ cm⁻¹ for GR.

**Dependency of GR-His expression and retinal production on growth phase**

To investigate the dependency of the retinal production and the level of GR-
Expression of holo-Gr in Synechocystis containing pQC012 was harvested at different growth phases, for quantification of the GR-His expression level (by quantitative Western blot) and retinal production (by HPLC analysis).

Quantitative Western blotting

Cells were harvested and disrupted with 100-μm glass beads (Sigma) using a Precellys®24 bead beater (Bertin Technologies) in a buffer containing 20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, 1.5% (w/v) DDM, pH = 8, and then stirred overnight at 4°C. The obtained cell extract was centrifuged to discard cell debris at 10,000 g and 4°C for 15 minutes. The supernatant was used as the GR sample; its protein concentration was determined with the Pierce BCA Protein Assay (Thermo Scientific) according to the manufacturer’s protocol.

Proteins in the cell-free lysates were separated via SDS-PAGE in ‘Any kD Mini-PROTEAN TGX Precast Gels’ (Bio-Rad) or ExpressPlus Page gels 8-16% (GenScript) using the same procedures as described before (46). Purified GR-His from E. coli was quantified by spectrophotometry, based on its absorption maximum at 541 nm and a molar extinction coefficient of 50,000 L mol⁻¹ cm⁻¹ (102); then loaded in different volumes on the same gel as the standard. For quantification, band intensities of GR-His samples were determined using ImageJ v1.49a (W. S. Rasband, U.S. National Institutes of Health, http://imagej.nih.gov/ij/) and compared with a series of standards. To estimate the number of GR molecules per cell, we assumed that the efficiency of cell disruption was 100%. The total protein content (as measured with the Pierce BCA Protein Assay) loaded on a gel was converted into the corresponding number of cells, on the basis of the OD₇₅₀ of a sample, using the conversion factor that 1 ml culture of wild-type Synechocystis with an OD₇₅₀ = 1 contains 10⁸ cells, as determined with a Casy 1 TTC cell counter (Schärfe System GmbH, Reutlingen, Germany)(232).

Retinal identification and quantification

Retinal was identified and quantified in the same batches of samples as those used for quantification of GR-His expression. Cell pellets were re-suspended in 1 M hydroxylamine at pH 8.0 in 50% (v/v) methanol and disrupted via vortexing for 1 min, prior to incubation at 30°C for 10 min. The resulting reaction mixtures were subsequently extracted at least three times with petroleum ether (boiling point 40 to 60°C) and finally dissolved in n-heptane (HPLC...
grade). The extracted pigments were separated on an HPLC system with an EC 150/4.6 NUCLEOSIL 100-5 C18 column (MACHEREY-NAGEL), and n-heptane (HPLC grade) at 1 ml • min⁻¹ as the mobile phase. Spectra of the eluting fractions were recorded using a SPD-M30A diode array detector (Shimadzu Nexera X2, Japan).

All-trans retinal purchased from Sigma-Aldrich was used as a standard compound for pigment identification and quantification. After reacting with hydroxylamine, all-trans retinal was converted into the more stable compound retinal oxime (233). Elution of retinal oxime was monitored at 354 nm in our system. The obtained peaks were integrated using the software Labsolution (Shimadzu, Japan). To determine the retinal content in a sample, the peak area of retinal oxime in the sample was compared with that of a series of known amounts of retinal (oxime). To present retinal production as the number of retinal molecules per cell, the number of cells was estimated as described for the Western blotting procedure above.

Size-exclusion chromatography (SEC)

A Superdex 200 Increase column 10/300GL (GE Healthcare) was used with an AKTA FPLC system to perform size-exclusion chromatography. GR-His isolated from E. coli and Synechocystis was first concentrated with a 10 kD spin filter (Corning) and then 0.1 ml of each sample was (separately) loaded on the column. The protein was eluted with filtered elution buffer (25 mM MOPS, 100 mM NaCl, 0.02% DDM, pH = 7.2) with a flowrate of 0.4 ml/min and collected in fractions. The GR-His protein isolated from E. coli was reconstituted with echinenone, as described above, to investigate the effect of carotenoid binding on the oligomeric state of GR.

Results and Discussion

Stimulation of growth of Synechocystis under salt stress by Proteorhodopsin

We previously have demonstrated that, in regular BG-11 medium, PR provides a small but measurable enhancement of photoautotrophic growth of Synechocystis, as compared to the corresponding strain expressing PROPS (the D97N mutant form of PR (46, 202). With the aim of further exploring this effect, we also compared the growth rates of these three strains under some stress conditions that dissipate (most of) the proton motive force (PMF)
EXPRESSION OF HOLO-GR IN SYNECHOCYSTIS

generated by the chlorophyll-based photosynthesis machinery of the cells, like: growth with only green light, with and without low concentrations of glucose; growth in the presence of an inhibitor of photosynthetic electron transfer (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU); growth in the presence of a protonophorous uncoupler of the PMF ([(3-chlorophenyl) hydrazono] malononitrile, CCCP); and growth in a high-salinity medium.

Under most conditions described above, we observed that Synechocystis containing an empty plasmid (pJBS1312) always had a higher growth rate (as deduced from the OD$_{730}$ of the cultures) and cell count than Synechocystis expressing PR (pQC006) or expressing its non-proton-pumping derivative PROPS; the same trend as shown under standard condition in BG-11 medium (46). The best situation encountered was that pQC006 could enhance the growth rate to the level of wild-type cells (with pJB1312) but never overtake the wild type without the self-replicative empty plasmid. This is probably because expressing PR/PROPS in Synechocystis causes an energy burden or space limitation in the thylakoid membrane, thereby decreasing photosynthetic efficiency in the relevant mutants.

Figure 1: PR-stimulated growth in high-salt conditions. Synechocystis with the empty plasmid pJBS1312 (triangles), plasmid pQC006 that leads to expression of PR-His (squares), or plasmid pQC011, expressing the non-proton-pumping PROPS (202) homologue of PR (circles). Cells were grown in standard medium with 1 M additional NaCl added. Data shown are from a representative experiment from a series of five independent experiments. Error bars represent the standard deviation of biological replicates within the representative experiment (n = 3) and are only visible when they exceed the size of the symbols. Time point zero indicates the inoculation of the cultures from a pre-culture growing linearly in the same medium.
When the three strains were exposed to different levels of salinity (0.8 M (data not shown) and 1 M NaCl), the *Synechocystis* strain expressing PR (pQC006) consistently displayed a higher growth rate and cell density than the *Syn-echocystis* strain expressing PROPS (pQC011), or expressing no opsin at all (empty plasmid pJBS1312). A representative result of these experiments is shown in Fig. 1. In this experiment, we observe a slightly larger stimulatory effect of PR on growth in *Synechocystis* under high salinity stress. It is known from literature that salt stress strongly induces cyclic electron transfer around PSI (234), which is the endogenous alternative light-driven proton pump for generating additional amounts of ATP (without simultaneous generation of NADPH, since that would bring redox stress to the cells), presumably to secrete sodium ions that leak into the cells under these stress conditions (234). The additional PMF (or ATP) generated by PR could further accelerate the process of sodium expulsion, thereby better-restoring growth.

However, the stimulatory effect of PR on growth so far has been rather small (a few percent at most), but could be further increased via various approaches. The most straightforward one is to use a proteorhodopsin variant that pumps faster than PR, like *Gloeobacter* rhodopsin (GR), which has both a higher photo-cycle rate (73) than PR (82) and additionally binds one molecule of carotenoid as an antenna pigment, thereby increasing GR’s effective absorption cross section 3.5 fold (97).

**Isolation of His-tagged *Gloeobacter* rhodopsin from *E. coli***

The poly-histidine (6× His) tag engineered at the C-terminus of GR (GR-H) allows the isolation and purification of GR from *E. coli* by using a His-Trap column. The results shown in Fig. 2, i.e. the color (magenta-red) and the spectra (with maximum absorption at 541 nm) of the main fractions (9 - 11) suggest an efficient isolation procedure of GR-His with the His-trap chromatography. Beyond that, we obtained one fraction (Frac 5) containing cytochrome. Furthermore, to determine the purity of GR-His in the main fractions, we estimated the ratio of absorption at 541 nm over 280 nm (A541/A280) of each fraction (see Table 1). Assuming that the fraction with the highest A541/A280 ratio only contains GR-His protein, the ratio of A541/A280 of this particular fraction should be equal to the ratio of the molar extinction coefficient (ε) of GR at 541 nm and 280 nm. The estimation of ε280 is based on the convention that the molar extinction coefficient at 280 nm of a particular protein is related to its tryptophan (W), tyrosine (Y) and cysteine (C) amino acid content, and can be calculated from the formula: \( \varepsilon_{280} = (nW \times 5500) + (nY \times 1490) + (nC \times 125) \).
In this formula, n refers to the number of the corresponding amino acid. On the above basis, $\varepsilon_{280}$ of GR-His is equal to 55,350 M$^{-1}$ cm$^{-1}$, while $\varepsilon_{541}$ is 50,000 M$^{-1}$ cm$^{-1}$(102). Therefore, a ratio of 0.9 of A541/A280 would indicate an absolutely pure fraction of GR-His. The three main fractions show a constant value of ~0.7 of this ratio, which indicates a high purity. Although this numerical value is not identical to the theoretical number, one should realize that the theoretical number is an approximation.

Figure 2: The spectra of the main fractions of a purification run of his-tagged *Gloeobacter* rhodopsin from *E. coli* with a His-Trap column.

<table>
<thead>
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<th>Wavelength (nm)</th>
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<th>F11</th>
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<td>0.522788</td>
</tr>
<tr>
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<td>0.465747</td>
<td>0.370724</td>
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</table>

Reconstitution of *Gloeobacter* rhodopsin with carotenoids in *vitro*

Protein homology analysis (GR has 50% identical amino acid residues with xanthorhodopsin (100)) and successful reconstitution of *Gloeobacter* rhodopsin with salinixanthin (Imasheva et al., 2009) and echinenone (Balashov et al., 2010) have proven that *Gloeobacter* rhodopsin is a light-driven proton pump with a dual chromophore system (i.e. a carotenoid and all-trans retinal). The presence of a carotenoid in a rhodopsin is advantageous, as the carotenoid appears to act as an antenna system to increase the effective absorp-
tion cross section of the protein. The selectivity of *Gloeobacter* rhodopsin with respect to carotenoid binding indicates that the 4-keto group of the carotenoid plays a key role in the successful binding and (resonance) energy transfer between the carotenoid and the retinal chromophore (102).

Intriguingly, *Synechocystis* naturally produces a considerable variety and amount of carotenoids, including the 4-keto derivatives: echinenone; 3′-hydroxy echinenone and canthaxanthin (235, 236). Therefore, prior to exploring the issue in *vivo*, *i.e.* whether or not, and if so which, carotenoids will bind to GR in *Synechocystis*, we have characterized the binding of carotenoids to GR in *vitro*.

![Figure 3: Time course of the reconstitution of *Gloeobacter* rhodopsin (4 µM) with A): echinenone (4 µM); B): 3-hydroxy-echinenone (4 µM) and C): canthaxanthin (4 µM). The spectra were recorded in buffer containing 20 mM Tris-HCl, 80 mM NaCl, 0.08% (w/v) DDM, pH = 8.0.](image)

Echinenone is the most promising candidate for such binding as it is considered as one of the main carotenoids (18%) produced by *Synechocystis* (235), and the association between echinenone and GR has been reported in the literature (101). Another two interesting candidates would be 3′-hydroxyechinenone, and canthaxanthin, as their presence has been confirmed in *Synechocystis* by HPLC (236), although they are not the main carotenoid products. However, due to the fact that 3′-hydroxyechinenone is not commercially
available, it has not been included in this experiment; instead, 3-hydroxyechinenone has been tested.

Fig. 3 presents the spectra of the reconstitution of GR-His with the three carotenoids as a function of time. The addition of echinenone to *Gloeobacter* rhodopsin at a molar ratio of 1:1 produced a shifted and broad absorption peak in 10 minutes, of which the maximum was blue-shifted from 540 nm to 530 nm. Moreover, extended incubation (21 h at room temperature) resulted in a further shift in the absorption maximum to 491 nm. This latter shift was accompanied by the appearance of fine structure in the spectrum with peaks at 428, 458 nm and 541 nm, typical for a carotenoid molecule embedded in a hydrophobic environment. Comparatively, reconstitution of *Gloeobacter* rhodopsin with 3-OH echinenone proceeded faster (in 3 hours) and showed a sharper absorbance maximum at 458 nm, but with a less-explicit shoulder at 541 nm. In contrast, reconstitution with canthaxanthin generated less clear carotenoid fine-structure, but with 8 nm blue shift of the maximum of the peak (from 541 nm to 533 nm).

In accordance with the previous observation that salinixanthin, with the additional 2'-hydroxy group and glycoside moiety, bound to *Gloeobacter* rhodopsin much faster than echinenone (101), our results also clearly show that an additional hydroxy group accelerated the binding of 3-hydroxy echinenone to *Gloeobacter* rhodopsin, while an extra 4'-keto group (*i.e.* in canthaxanthin) prevented this and perhaps even impairs functional binding to GR. This illustrates that the hydroxy group definitely plays a role in the binding process, although the further detail of the mechanism is still unknown. However, considering that an extra 4'-keto group (canthaxanthin) impaired the binding, presumably the polarity of the group at the 4' position profoundly influences the binding.

The binding between carotenoids and GR-His was not very stable in aqueous solutions of DDM (showing *i.e.* a high off-rate), because the carotenoids disassociated from GR-His during incubation in buffer containing 0.1% DDM.

**Effect of carotenoid-binding on the molecular turnover rate of proton-pumping of *Gloeobacter* rhodopsin**

Comparing the photocycle turnover rates of proteorhodopsin and *Gloeobacter* rhodopsin shows that the former has a slower photocycle (~230 ms of the largest decay time constant for the alkaline form) (73, 79, 82), which implies a
lower rate of proton pumping. To directly compare the proton pumping activity of proteorhodopsin and *Gloeobacter* rhodopsin, we measured the proton pumping rate of GR and PR in intact *E. coli* cells under various intensities of white light (from 250 μmol • m⁻² • s⁻¹ to 6000 μmol • m⁻² • s⁻¹, see Fig. 4). The proton pumping rate initially increased linearly with the light intensity (≤ 1500 μmol • m⁻² • s⁻¹), and then gradually saturated with higher light intensities. Fig. 4 shows that under the conditions selected GR displays a two-fold higher molecular proton-pumping rate than PR, with a maximum rate of 4.8 protons per GR molecule per second.

An important question is whether or not association with carotenoids can improve the proton pumping activity of GR-His. In order to investigate this, we measured the proton pumping rate of *holo*-GR-His (*all-trans* retinal bound GR-His) in vivo under conditions with and without the addition of echinenone; 3-OH echinenone or canthaxanthin. However, our attempts to reconstitute *holo*-GR-His with carotenoids in vivo in *E. coli* were not successful. Spectra of intact *E. coli* cells expressing *holo*-GR showed no differences with and without the addition of carotenoids. Presumably, the outer membrane of *E. coli* prohibits the uptake of carotenoids from the medium into the cells, or the carotenoids may only bind GR in *E. coli* very weakly.

**Dependency of GR-His expression and retinal production on growth phase**

To explore the possibility of functional expression of GR holoprotein in *Synechocystis* and its effect on the photo-autotrophic growth rate of this organ-
ism, the Synechocystis strain containing pQC012 (for GR-H expression) was cultivated in batch culture at a moderate light intensity (~ 45 μmol • m\(^{-2}\) • s\(^{-1}\)). Cells were collected at different growth phases for quantification of the GR-expression level and the level of retinal production, respectively.

The results summarized in Fig. 5 show that GR-His had the highest expression level (~9.5×10\(^4\) molecules per cell) in the linear growth phase, which is shifted to an earlier phase as compared to PR-His in Synechocystis, the expression of which reached a peak at the transition between linear growth and the stationary phase (46). Moreover, retinal production increased during the subsequent growth phases and reached a peak (at ~8.4 ×10\(^4\) molecules per cell) at the early stationary phase, and was then followed by a decrease. In contrast, the PR-expressing strain (Synechocystis with pQC006) showed an overall increasing trend in retinal production and displayed a sharp increase in the late stationary phase (data not shown). In addition, the changes in retinal production level did not correlate with those of GR-His expression. Significantly, the number of functional GR-His molecules clearly depends on the growth phase of the cells. Therefore, in i.e. the linear growth phase of Synechocystis, in which a large fraction of GR-His is present as apo-GR-His, exogenous ad-

Figure 5: Retinal production- and expression level of His-tagged apo-GR in a batch culture of Synechocystis in various growth phases. Cells were grown in the BG-11 medium at a moderate light intensity. Solid line: Growth curve monitored via the OD\(_{730}\). Bars: Amount of apo-PR-His per cell, quantified via Western blots and amount of retinal per cell, quantified via HPLC. The number of cells per sample was determined using the conversion factor that 1 ml culture of wild-type Synechocystis with an OD\(_{730}\) 1 contains 10\(^8\) cells, as determined with a Casy 1 TTC cell counter (Schärfe System GmbH, Reutlingen, Germany) (184). The data shown are from three biological replicates. Error bars represent the standard error of the mean, and are only visible when they exceed the size of the symbols.

The results summarized in Fig. 5 show that GR-His had the highest expression level (~9.5×10\(^4\) molecules per cell) in the linear growth phase, which is shifted to an earlier phase as compared to PR-His in Synechocystis, the expression of which reached a peak at the transition between linear growth and the stationary phase (46). Moreover, retinal production increased during the subsequent growth phases and reached a peak (at ~8.4 ×10\(^4\) molecules per cell) at the early stationary phase, and was then followed by a decrease. In contrast, the PR-expressing strain (Synechocystis with pQC006) showed an overall increasing trend in retinal production and displayed a sharp increase in the late stationary phase (data not shown). In addition, the changes in retinal production level did not correlate with those of GR-His expression. Significant-ly, the number of functional GR-His molecules clearly depends on the growth phase of the cells. Therefore, in i.e. the linear growth phase of Synechocystis, in which a large fraction of GR-His is present as apo-GR-His, exogenous ad-
dition of all-trans retinal could well increase the amount of functional GR so as to further increase the extent of the beneficial effect of this retinal-based proton pump on light-energy conversion in the cyanobacterium.

Isolation of His-tagged Gloeobacter rhodopsin from Synechocystis

To explore the possibility that carotenoids would bind to holo-GR-His in vivo in Synechocystis, GR-His was isolated from the Synechocystis strain containing pQC012 (for GR-H expression) by using a His-Trap column (see Materials and Methods). The relevant elution fractions showed a yellow-brown color with a spectrum containing a broad absorption peak in the range of 350 - 550 nm. In addition, a peak with a maximum absorption at 678 nm in the same fraction strongly suggests the presence of contaminating (i.e. Chl a-binding) proteins. The co-elution with a small Chl a-binding protein has been consistently observed in fractionations of His-tagged proteins from Synechocystis. A corresponding fraction from a retinal-deficient Synechocystis strain (JBS14003; unpublished results) containing pQC006 (for apo-PR-His expression) was used as a reference to correct for the presence of this contaminant. The spectrum corrected with this reference spectrum should represent the spectrum of His-tagged GR from Synechocystis. Fig. 6 clearly shows that the corrected spectrum contained an absorption peak in the range of 400 to 600 nm, with a maximum at 486 nm rather than 540 nm. A closer look at the spectra shows that two shoulders are present at 458 nm and 540 nm. These latter characteristics strongly suggest the binding of carotenoids to GR-His. HPLC analysis of pigment extracts of this purified GR-His protein fraction further confirmed this finding as we detected a peak with a close retention time (0.3 minutes later) as echinenone, but with a different UV/Vis spectrum, i.e. a blue-shift of its maximum absorption of a few nm as compared to echinenone (data not shown). However, not enough information so far is available to propose a tentative structure of this/these compound(s). We consider it likely, however, that it is a derivative of echinenone, as Synechocystis displays a very complex carotenoid metabolism, and conversion of echinenone is certainly possible. Moreover, we also detected a peak of retinal oxime from these extracts of the GR-His fraction, which confirms that all-trans retinal had been bound by GR-His. Therefore, based upon the analysis of the spectrum and the results of chromophore extraction, we conclude that GR-His expressed in vivo in Synechocystis has bound all-trans retinal well as (a) carotenoid(s). Although the structure of the carotenoid(s) is still unknown, we were able to isolate (a) carotenoid(s) bound to holo-GR from Synechocystis in a buffer with 0.1% DDM, which implies that the binding of the carotenoid(s) to GR-His is
rather strong. Therefore, these carotenoids would fit better into the carotenoid-binding pocket of GR than echinenone. Revealing their structure will help to delineate the carotenoid-binding pocket of GR.

Figure 6: Spectra eluted from a Ni$^{2+}$-affinity column for purification of His-tagged GR from *Synechocystis*. A strain deficient in retinal synthesis (i.e. JBS14003, obtained via deletion of *slr1541* and *slr1648*), and conjugated with plasmid pQC006 (for the expression of PR-His), was cultivated in BG-11 medium and served as the control. Wild-type *Synechocystis* conjugated with plasmid pQC012 (for the expression of GR-His) was investigated for the formation of holo-PR.

The oligomeric state of *Gloeobacter* rhodopsin isolated from *E. coli* and from *Synechocystis*

Transmembrane proteins often form functional oligomers, as oligomer formation can provide increased structural- and proteolytic stability may facilitate membrane insertion, and allows functional cooperativity (237). Proteorhodopsin and Bacteriorhodopsin both function in the form of oligomers (i.e. hexamers (85) and a two-dimensional array of trimers (62), respectively). The quaternary structure of *Gloeobacter* rhodopsin, in particular when isolated from *Synechocystis* has so far remained unexplored.

The oligomeric state of GR-His isolated from *E. coli* and from *Synechocystis*, both purified by His-Trap affinity chromatography was analyzed by size-exclusion chromatography on a Superdex 200 column.

As shown in Fig. 7, three peaks (labeled a, b, and c) stood out in the elution pattern, which indicates the co-existence of three oligomeric structures for GR-His isolated from *E. coli*, which is comparable to results published elsewhere (238). Therefore, we assigned these three peaks (a, b, c) according to size as representing oligomers, trimers and monomers of GR-His, respective-
ly. Apparently, of these, the trimer is the dominant species. CD-spectroscopy of equivalent samples has confirmed this assignment (229). In addition, Tsukamoto et al. showed that the quaternary structure of GR-His is pH dependent: The monomer state dominates at acidic pH (pH <4), while the ratio of trimers over monomers increased with increasing pH, and the oligomers have a considerable population only when pH >7.5 (238).

Binding a carotenoid could stimulate the formation of oligomers of a rhodopsin, as such a hydrophobic pigment molecule may bind at the interface between monomers so as to connect and assemble them into oligomers (239). As GR-His also can be present in either of three oligomeric states at different ratios in a pH-dependent manner, it is interesting to know whether binding of a carotenoid has a significant influence on oligomer formation of GR-His. To explore this, GR-His was reconstituted with echinenone in vitro as described in Materials and Methods and analyzed by size-exclusion chromatography. The change in the ratio of the three structural isomers was estimated in the eluted samples by comparing the results obtained with and without the addition of echinenone. No significant changes in the relative composition of three structural isomers were observed, which strongly indicates that echinenone does not play a crucial role in the formation of the quaternary structure of GR-His (data not shown). The SEC chromatography of GR-His isolated from Synechocystis (Fig. 7) showed a different monomer/oligomer distribution than GR-His isolated from E. coli, although they both presumably do form the same type of oligomers. Besides this, for GR, in addition, a large fraction showed up in front of the eluting oligomers. Overall these results indicate that the

Figure 7: SEC analysis of GR-His dissolved in buffer with 20mM Tris-HCl, 0.1 M NaCl, 0.1%DDM, pH 8. Three peaks, named a, b, and c, were identified as an oligomer, trimer and monomer, respectively.
same oligomeric states are present in GR-His isolated from *E. coli* and from *Synechocystis*. Further spectral analysis showed that the material eluting at 7 – 9 ml mainly contained contaminating proteins (*i.e.* Chl a-binding protein), while between 10 and 13 ml, GR-His eluted from the column with two chromophores bound (*i.e.* retinal and a carotenoid; data not shown).

**Conclusions and Perspectives**

Our previous study demonstrated that functional expression of Proteorhodopsin in *Synechocystis* stimulates the growth rate of this organism, only with a few percent (46). In an attempt to further increase the energy contribution from retinal based phototrophy, *Gloeobacter* rhodopsin was heterologously expressed in *Synechocystis*. Although this latter rhodopsin shows a higher pumping rate (two-fold higher than PR) and has a unique ability to bind (an antenna) carotenoid (which can further increase its pumping rate), its lower expression level, however, will decrease its effectiveness and may offset its advantage in pumping rate, so that GR contributes approximately to the same extent to light-energy conversion of *Synechocystis* as Proteorhodopsin.

To be able to better quantitate the bio-energetic contribution of the two rhodopsins to the energy metabolism of *Synechocystis*, and compare which of the two is more effective, expression of these two rhodopsins in (a) mutants of *Synechocystis* may be a promising approach. The results of physiological studies strongly suggest that PR activity in its endogenous host is most beneficial when cells are grown under harsh (stress) conditions. However, the growth data collected from those conditions on *Synechocystis* are of lesser technical reproducibility than those obtained with growth in standard conditions. The use of a mutant, which is subject to severe energy limitation because of a genetic lesion, could solve this problem.

Among such mutants, we consider a PSI deficient *Synechocystis* strain as the most promising one. Cyclic electron flow around PSI essentially also functions as a light-driven proton pump, and therefore works to generate extra proton motive force and/or ATP synthesis, without the coupled formation of NADPH (145). Considering this, one may expect that functional expression of proteorhodopsin in a PSI deletion strain could partially restore the impaired production of ATP and therefore growth of such a mutant to the corresponding wild-type levels. This idea even makes more sense if linear electron flow in a PSI deletion strain could be adjusted to occur via PSII plus NDH-1 only, for water-driven reduction of NADPH, as proposed in (43).
As compared to *Gloeobacter* rhodopsin, bacteriorhodopsin even pumps much faster (~ 100 protons/BR/s; (63). However, the poor expression level of bacteriorhodopsin in many heterologous hosts limits its usefulness. A recent report showed improved overexpression of bacteriorhodopsin in *E. coli*, based on constructing chimeric proteins of bacteriorhodopsin and a sensory rhodopsin, and/or optimization of the absence of tertiary structure in the 5’ region of its mRNA (65). This opens up the possibility to try expression of bacteriorhodopsin derivatives also in *Synechocystis*, although there is no guarantee that functional holo protein will be formed from such chimera’s. More exciting is the possibility to functionally express a far-red-shifted proteorhodopsin which can absorb and function with the light of a wavelength beyond 700 nm (*i.e.* a proteorhodopsin with maximum absorption at 750 nm; (229)). This will allow us to construct a *Synechocystis* strain which can (also) utilize infrared light. Replacement of PSI by such an infrared-absorbing proton pump is a promising strategy to increase the maximum areal energetic efficiency of oxygenic photosynthesis.

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**Authors’ contributions**

QC and KJH designed experiments; QC and JCA performed experiments; QC, JCA and KJH wrote the paper; and SG and WJdG contributed to the writing of the paper and the overall experimental design.

**Conflict of interest**

The authors declare that they have no conflict of interest. KJH is a scientific advisor to the start-up company Photanol BV. This does not create a conflict of interest nor does it alter the authors’ adherence to accepted policies on sharing data and materials.