Photo-induced regulation of the chromatic adaptive gene expression by \textit{Anabaena} sensory rhodopsin

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Running Title: Transcriptional regulation by ASR

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**Background:** ASR is categorized as a microbial sensory rhodopsin.

**Results:** ASR represses the transcription of the chromatic adaptive gene \textit{cpcB} through its C-terminal region.

**Conclusion:** We demonstrate a novel function of a retinal containing protein, and suggest that a membrane spanning protein can be able to function as a transcriptional factor.

**Significance:** The knowledge gained in this study will help to understand the functional diversity of microbial rhodopsins.

**SUMMARY**

Rhodopsin molecules are photochemically reactive membrane-embedded proteins, with seven transmembrane alpha-helices which bind the chromophore retinal (vitamin A aldehyde). They are roughly divided into two groups according to their basic functions i) ion transporters such as proton pumps, chloride pumps and cation channels, and ii) photo-sensors such as sensory rhodopsin from microbes and visual pigments from animals. \textit{Anabaena} sensory rhodopsin (ASR), found in 2003 in the cyanobacterium \textit{Anabaena} PCC7120, is categorized as a microbial sensory rhodopsin. To investigate the function of ASR in vivo, ASR and the promoter sequence of the pigment protein phycocyanin were co-introduced into \textit{E. coli} cells with the reporter gene \textit{crp}. The result clearly showed that ASR functions as a repressor of the CRP protein expression, and that this is fully inhibited by the light activation of ASR, suggesting that ASR would directly regulate the transcription of \textit{crp}. The repression is also clearly inhibited by the truncation of the C-terminal region of ASR, or mutations on the C-terminal Arg residues, indicating the functional importance of the C-terminal region. Thus, our results demonstrate a novel function of rhodopsin molecules, and raise the possibility that the membrane spanning protein ASR could work as a transcriptional factor. In future, the ASR activity could be utilized as a tool for arbitrary protein expression in vivo regulated by visible light.

Photoactive proteins with their cognate chromophores are widespread in organisms, and function as light-energy converters or receptors for light-signal transduction. Rhodopsins have retinal (vitamin A aldehyde) as their chromophore within seven transmembrane alpha-helices, and they have been classified into two groups, microbial (type-1) and animal (type-2) rhodopsins (1).
Type-1 rhodopsins are widespread in the microbial world, in prokaryotes (bacteria and archaea) and in eukaryotes (fungi and algae), while type-2 rhodopsins, such as visual pigments, are, in general, G-Protein Coupled Receptors (GPCRs) that are widespread in vertebrates and in invertebrates (1). Although rhodopsins show seemingly dissimilar functions, they can be roughly divided into two groups based on their basic functions. Some are light-driven transporters, such as the proton pump bacteriorhodopsin (BR), the chloride pump halorhodopsin (HR) or the cation channel channelrhodopsin (ChR). Others are light sensors, such as the visual pigments from animals or the phototaxis receptors sensory rhodopsins I and II (SRI and SRII) from microbes (1-4).

Since the discovery of BR (5), ‘rhodopsins’ had for nearly three decades been thought to be unique proteins, found only in extremely halophilic archaea, and in animal photoreceptors. New discoveries started at the end of the last century, when the techniques in mass genome sequencing and bioinformatics started to advance dramatically (3,4). Today, the number of rhodopsin genes identified is up to several thousand, and keeps growing (6,7). Most of them have been found in eubacteria and fungi. They have evolutionary relationship to the well-studied archaeal rhodopsins (6,8).

Anabaena sensory rhodopsin (ASR), isolated in 2003 from the freshwater nitrogen-fixing cyanobacterium Anabaena PCC7120, is a member of the type-1 (microbial) rhodopsins (9). ASR is encoded in an operon, along with a second gene that encodes a small soluble cytoplasmic protein, named the ASR Transducer (ASRT) tentatively (10), which is different from the members of the membrane embedded taxis transducer family proteins, haloarchaeal phototaxis transducers Htr1 for SRI and HtrII for SRII (11,12). The photochemical properties of ASR, and the interaction between ASR and ASRT have already been studied (9,10,13-19), but the physiological function both of ASR and ASRT in Anabaena is still poorly understood, though isothermal titration calorimetry (ITC) measurements revealed that ASRT binds to ASR with a dissociation constant of 8 µM (10). The crystallographic analysis of ASR has revealed its photochromic nature, and the existence of a water molecule network in its cytoplasmic half, where it possibly interacts with ASRT (20). ASR accommodates both all-trans and 13-cis retinal in the original state, which can be interconverted by the illumination with blue (480 nm) or orange (590 nm) light (13,18). Furthermore, it has been shown that all its photochemical reactions are also photocromic (18), and that there is no photocycle similar to those observed in all other microbial rhodopsins.

What are the functional roles of ASR and ASRT? Dr. John Spudich and coworkers have proposed that one possibility would be the regulation of the gene expression in response to the light intensity and/or quality (4,20). This control could either occur via the direct transcriptional regulation by ASRT (making ASRT a transcription factor), or via transcription factors further downstream in an uncharacterized signaling cascade. Because Anabaena shows various light-dependent phenomena, including chromatic adaptation and photosynthesis (21), it is complicated to study the functions of ASR and ASRT in native Anabaena cells. Therefore, in this study ASR and ASRT were heterologously introduced into the eubacterium E. coli because of its lack of photo-induced behaviors. Furthermore, the promoter sequence of phycocyanin, which is an orange light-inducible blue-pigment (subunits β and α encoded by cpcB and A) in cyanobacteria involved in the chromatic adaptation (22), was also introduced into the cells with a reporter gene crp. Using this system, the functions of ASR and ASRT were investigated.

Experimental Procedures

Gene preparations

As shown in the Supplemental Figure S1a, the Hind III-Kpn I fragment, including the putative promoter sequence of phycocyanin (PcpcB) amplified by PCR from the Anabaena
PCC7120 genome, was inserted into the upstream region of the crp gene on the plasmid pH7MK, a derivative of pBR322. The Hind III-EcoRI fragment, including P_{pcpB} and crp from pH7MK, was inserted into the multicloning site of the vector plasmid pSU21. As shown in the Supplemental Figure S1b, the putative promoter sequence of the α subunit of allophycocyanin (P_{apcA}) (23) was amplified by PCR from the Anabaena PCC7120 genome and it was digested by Psi I and inserted into the upstream region of the crp gene on the plasmid pH7MK. The BamHI-EcoRI fragment was inserted into the multicloning site of pSU21. The ASR- and ASR-ASRT fusion-encoding plasmids were derived from pMS107 as described previously (8). For the expression plasmid of ASRT alone, the gene of ASRT, amplified by PCR from the Anabaena PCC7120 genome, was inserted into the arabinose (Ara)-inducible plasmid pBAD33. The mutant gene of ASR was constructed by PCR using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) as described previously (24). All constructed plasmids were analyzed using an automated sequencer (ABI 3100) to confirm the expected nucleotide sequences.

**Sample preparations**

LB medium supplemented with 50 µg/ml of ampicillin and 25 µg/ml of chloramphenicol was inoculated at 1:30 dilution with a fresh overnight culture of TA341 cells carrying corresponding plasmids. Cells grown under shaking at 30°C were harvested at their midlogarithmic phase. For the expression of ASR, all-trans retinal (10µM) and IPTG (1mM) were added to the cell culture. When necessary, the cells were for 30 min at 30°C illuminated with light of various wavelengths (MAX-302, ASAHI SPECTRA) before harvesting. The samples were subjected to an SDS-PAGE (12% polyacrylamide) followed by immunoblotting with anti-CRP antibodies. To quantify the intensity of the CRP band, the immunoblots were scanned, and the resulting images were analyzed using the software ImageJ (rsb.info.nih.gov/ij/). For the Northern blotting analysis, the total RNA content was isolated from cells grown in LB medium to their midlogarithmic phase as described previously (25), and resolved by 1.5% agarose-gel electrophoresis followed by Northern blotting with 620bp of the DIG-labeled DNA probes, containing the crp coding region using the following primers; FW: GCAAACCGCAAACAG, RV: ACGAGTGCCGTAAAC.

The preparation of crude membranes and the purification of ASR proteins were performed using essentially the same method as described previously (26). In short, ASR proteins with a six-histidine tag at the C- or N-terminus were expressed in E. coli cells, solubilized by 2%(w/v) DDM and purified with a Ni²⁺ affinity column. The samples were concentrated and exchanged using an Amicon Ultra filter (Millipore, Bedford, MA, USA).

**RESULTS**

Figure 1 shows the strategy of this study. The 502bp fragment of the Anabaena PCC7120 genome which sufficiently includes the promoter sequence of the β subunit of phycocyanin (P_{pcpB}) was amplified by PCR, and inserted into the upstream region of the crp gene on a plasmid vector DNA (Figure 1a). It should be noted that the Shine-Dalgarno (SD) sequence, AGGAGA, exists on the upstream region of the ATG initiation codon of cpcB (Supplementary Figure S1a). Then, we introduced the engineered plasmid into the E. coli TA341 strain which does not have CRP (27). Into these cells, another plasmid for the expression of ASR, the ASR-ASRT fusion protein or ASRT was co-introduced (Figure 1b). The cells were illuminated with light of various wavelengths (Figure 1c). The amount of CRP expressed depending on the wavelength of the light illumination was analysed by the Western blotting analysis.

**Photo-induced protein expression in cells**
containing ASR alone

Cells having the expression plasmid of ASR showed a pink coloring, indicating the successful expression of ASR. To confirm this quantitatively, the cells from 100 mL cultures were disrupted by sonication, and ASR was purified by affinity chromatography from the membrane fraction. The purified protein was suspended into 1 mL of a buffer (50 mM Tris-Cl pH7.0) containing 1 M NaCl and 0.05 % DDM.

Figure 2a shows the absorption spectra of the purified fraction from the cells without ASR (w/o), with the purified N-terminal His-tagged ASR (His6-ASR) or with the purified C-terminal His-tagged ASR (ASR-His6). The cells were grown in the dark (D) or under illumination (L) for 30 min with light of ~550 nm obtained through a band pass filter (see Figure 1c). As can be seen, the absorption maxima of both the His6-ASR and ASR- His6 were located at 548 nm, which is almost identical to the value reported previously (549 nm) (20), indicating the successful expression of the recombinant ASR in the E. coli cells. It should be noted that there was no significant effect of light on the amount of the proteins expressed, as shown by the dotted lines in Figure 2a. However, the expression level of ASR-His6 was approximately 5-times greater than that of His6-ASR (the expression of which was similar to the expression level of WT ASR), suggesting the importance of the C-terminus for the protein expression. From the calculation using the molecular coefficient of ASR, it was estimated that about 15,000 molecules of the His6-ASR were expressed in one E. coli cell.

Under the same conditions, the amount of the reporter protein CRP was investigated by using the western blotting analysis (Figure 2b). It should be noted that, under all conditions, no significant change of the amount of the total proteins was observed, as judged by Coomassie Brilliant Blue (CBB) staining. Without ASR (w/o), the protein expression of CRP was confirmed as an intense band both in the dark (D) and under illumination (L), suggesting that P_{crpB} from Anabaena was also functional in the E. coli cells. On the other hand, the amount of CRP was markedly decreased in the cells having His6-ASR in the dark (Figure 2B, lane 3), while under illumination (Figure 2b, lane 4), the amount of CRP returned to the level in the absence of ASR (lanes 1 and 2). Quantitative analysis revealed that the CRP expression was repressed 3.6-fold in the dark condition upon addition of His6-ASR (Figure 2c). In contrast, the addition of ASR-His6 did not affect the amount of CRP significantly, both in the dark and under illumination, suggesting the functional importance of the C-terminal region of ASR.

To confirm whether the amount of protein was related to the transcriptional process, the amount of mRNA was investigated using the northern blotting analysis (Figure 2d). As shown, compared with the other conditions, the amount of mRNA of crp extracted from cells expressing His6-ASR was significantly decreased (Figure 2d, middle lane), implying the transcriptional inhibition of crp by ASR. The results also indicate that this repression is prevented by the addition of a His-tag at the C-terminus (ASR-His6), or by the light activation of ASR. The results were qualitatively reproducible in our experimental conditions. Thus, our results suggest that a light-induced regulation of CRP by ASR occurred in the transcriptional level.

To confirm whether the effect of ASR is specific or non-specific against the promoter sequence, we performed further experiments by using P_{apcA}, which is the promoter sequence of the a subunit of allophycocyanin (apcA) from Anabaena PCC7120. It is well-known that apc is a component of phycobilisome as well as cpc. The amount of the reporter protein CRP was investigated by using the western blotting analysis (Figure 3a). Though the total amount of CRP was increased 3~4-fold in P_{apc} as compared with P_{cpcB}, no significant change upon illumination was observed (Figure 3b), indicating that the effect of ASR is specific for P_{cpcB}. The increase of CRP by P_{apcA} could be related to the functionality of the promoter sequence in E. coli. In other word, P_{apcA}
seems more effective for the transcription in E. coli cells than p_cpcB. It should be also noted that the ASR protein was similarly expressed in cells containing p_cpcB or p_arpC, as judged by the absorption at 550 nm of the purified protein (Figure 3c), indicating no significant effect of the promoter sequence on the expression of ASR.

**The action spectrum and the role of the C-terminal region of ASR**

To confirm whether ASR (His6-ASR) functions as a photoreceptor for the CRP expression, the action spectrum was obtained (Figure 4). For this, the sample was illuminated with light of different wavelengths (see Figure 1c). To ensure equal conditions, the light intensity at different wavelengths was corrected by using an illuminometer (optical power meter and sensor, 9742 and 3664, Hioki, Japan). As can be seen in Figure 4, the amount of CRP depends on the wavelength of the light. This dependency was compared to the absorption spectrum of purified ASR. As already mentioned, ASR has a photochromic nature, binding both all-trans and 13-cis retinal. It was reported that ASR exhibits a light-induced interconversion between its two retinal forms (18, 20). In the dark-adapted state, the all-trans form (absorption maximum of 549 nm in detergent) is predominant (almost 100 %), while upon illumination with orange light this ratio is rapidly shifted to the 13-cis form (λ_max of 537 nm). As shown in Figure 4, the obtained action spectrum overlays well with the absorption spectrum of purified ASR in the dark (red line) compared with that under illumination (blue line), indicating that dark-adapted ASR (all-trans form) is likely to be the photoreceptor in the E. coli cells. Thus, unexpectedly, ASR itself has the potential to regulate the transcription of the chromatic adaptive gene cpcB via the direct or indirect interaction with p_cpcB.

Unlike other microbial rhodopsins such as BR and SRII, ASR has an extended C-terminal region at its cytoplasmic end, which is predicted to be of α-helical structure (Figures 5a and 5b) (28). As described above, the addition of a His-tag at the C-terminus enhances the ASR expression, but also inhibits the repression of the CRP expression by ASR (see Figure 2), suggesting the functional importance of the C-terminal region. This was also supported by our result that upon truncation of the C-terminal region of ASR no significant effect on the CRP expression could be observed, both in the dark and under illumination (Figure 5c). We speculated that five Arg residues at the C-terminus of ASR were involved in the DNA binding through electrostatic interaction with the negative charge of the DNA, leading to the repression of the CRP expression by ASR in the dark. Figure 5c also shows the amount of CRP in the presence of an Arg-lacking mutant (R→A) of ASR, in which the five Arg residues (closed circles in panel-A) were replaced by Ala. As can be seen, the repression of the CRP expression by ASR in the dark was also not observed in the case of this mutation, suggesting the importance of these Arg residues for the ASR function. It should be noted that the ASR mutants were normally expressed in the E. coli cells as judged by their absorption spectra (Supplemental Figures S2a and S2b). This was also the case for the C-terminal His-tagged mutants, in the presence of which also no effect of light was observed (Supplemental Figures S2a and S2b), suggesting no significant change of the structure of ASR by the mutations.

**Role of ASRT**

To investigate the role of the putative transducer protein ASRT, we performed similar experiments for the ASR-ASRT fusion complex (Figure 6A) and ASRT alone (Figure 6B). For the fusion construct, ASRT was linked to ASR through a flexible linker of 9 residues (29) connecting the cytoplasmic C terminus of ASR and N terminus of ASRT (Figure 6A). The fusion construct was used, because the protein expression both of ASR and ASRT in E. coli cells with ASR-ASRT operon gene was of low yield. To ensure that the observed properties are not associated with the addition of the linker to ASR, a mutant in which only the linker was added
(ASR-linker) was also investigated. As expected, no significant change in protein expression (Supplemental Figure S2c) or in the repression of CRP compared to Hisc-ASR was observed, indicating that the linker does not influence the function. The protein expression was also not significantly affected by the fusion with ASRT (Supplemental Figures S2d). As shown, the absorption maximum of the fusion protein was slightly shifted to 552 nm compared with that of the ASR-linker (549 nm). This confirms that the linker does not inhibit the interaction between ASR and ASRT, as a similar red-shift in the absorption spectrum of ASR upon interaction with ASRT (~5 nm) had also been reported in the study by Sineshchekov and coworkers in which ASR and ASRT were expressed separately (13). Interestingly, the repression of the CRP expression by ASR was partially (~60%) inhibited by the fusion with ASRT, suggesting that ASRT functions as an inhibitor of ASR through the interaction with ASR. Furthermore, no increase of the expression was observed upon illumination, which provides a possibility that ASRT might also work as an inhibitor for the CRP expression. To investigate the role of ASRT alone, similar experiments were performed with cells containing only ASRT (Figure 6b), regulated by the addition of Arabinose (Ara). As can be seen, the amount of CRP was slightly decreased in the ASRT-expressing cells as judged by the Western blotting analysis using anti-His-tag antibodies and anti-CRP antibodies, suggesting the potential role of ASRT as a transcriptional factor through the interaction with DNA.

**DISCUSSION**

Based on the results obtained in this study combined with other findings, we propose that the all-trans form of ASR regulates the protein expression through cationic residues in the C-terminal region of ASR via the direct interaction with the promoter sequence of the β-subunit of phycocyanin. Thus, we raise the possibility that the membrane spanning protein ASR is able to act as a transcriptional factor. It is well-known that phycocyanin forms the phycobilisomes, together with the other pigments, allophycocyanin and phycoerythrin (phycoerythrocyanin), which functions as a blue pigment in cyanobacteria, and is responsible for the complementary chromatic adaptation (CCA) (30-32). CCA is a light-dependent acclimation process used by cyanobacteria that results in optimal growth and development, in response to changes in the ambient light environment (30-32). This process has been most readily identified by observing changes in the cell color between brick red and blue-green due to variations in the prevalence of green and red wavelengths in the ambient light. These pigmentation changes have been shown to result from the reconfiguration of the light-harvesting complexes, and allow cyanobacteria to finely tune light absorption to the predominant wavelengths of the ambient light, and thereby, maximize the photosynthesis. Although it is unclear, whether ASR regulates the transcription in the native cells, the correlation between the irradiation with orange light and the increment of the expression of CRP controlled by P_cpeB is consistent with the synthetic pattern of the color-sensitive pigment phycocyanin in cyanobacteria (30-32). This supports the function of ASR demonstrated here. As already mentioned, so far, rhodopsins molecules were roughly divided into two groups based on their basic functions, ion transport and signal transduction. Here, the existence of a third function, the transcriptional regulation, is proposed. It should be noted that it has been already previously reported that pigmentation changes could be controlled by the phytochrome-related photoreceptor RcaE, containing tetrapyrrole as a chromophore, in the cyanobacterium *Fremyella diplosiphon* (also called *Calothrix* sp. strain PCC 7601) (33).

How important is the structural change(s) upon illumination for the function of ASR? The crystal structure of ASR has revealed its photochromic nature, as well as the existence of a water molecule network in
its cytoplasmic half. Although the structure of the extended C-terminal region at its cytoplasmic end is still unknown, it is predicted to be of α-helical structure. We presented here the functional importance of this region. It is well known that in the case of other microbial rhodopsins such as BR and SRII, the cytoplasmic region of the F-helix is opened in outward direction upon illumination, and this movement is thought to be important for the proton pumping activity of BR and photosignal transfer of SRII (34, 35). If this structural change is conserved in ASR, it might cause a structural perturbation against the extended C-terminal region of ASR through the hydrogen bonding network in the cytoplasmic region (36). This might be essential for the function of ASR. Extending our investigation in future, it would be interesting to study the structure and dynamics of the C-terminal end of ASR while interacting with its partners. This would give an idea about details of the binding mechanisms. Some information could be gained from spectroscopic techniques such as NMR, EPR and fluorescence spectroscopy. Further studies are planned to investigate these questions in future.

Figure 7 shows a hypothetical model of the function of ASR and ASRT on the CRP expression based on our results and other findings. i) In the dark state, ASR having all-trans retinal as its chromophore inhibits the transcription of cpcB by the interaction with the promoter sequence through the Arg residues in the C-terminus. It should be noted that the transcriptional system of E. coli is, in general, similar to that of cyanobacteria. Both in vivo and in vitro, it had already been demonstrated that the RNA polymerase of E. coli could cross-react with the cyanobacterial promoter (37-40). In the cyanobacterium *Cynechocystis* PCC6803, the cpcBACD promoter is recognized by the σ factors SigA, SigD and SigE (41). SigA, a functional homologue of E. coli σ^70 (rpoD), is responsible for the basal transcription, while SigD and SigE, functional homologues of E. coli σ^38 (rpoS), contribute to the light-induced transcription. The putative P_{cpcB} sequence (-35 and -10 elements) of *Anabaena* PCC7120 is based on that of *Cynechocystis* PCC6803 (Supplementary Figure S1a), which has a σ^70-like promoter sequence, and could be recognized by the endogenous σ^70 and σ^38 in E. coli (41). ii) ASR is activated by the illumination with orange light. As already mentioned, ASR exhibits a light-induced interconversion of its retinal chromophore between its all-trans and 13-cis form (18,20). Upon illumination with orange light, the retinal composition shifts rapidly to its 13-cis form (λ_{max} of 537 nm). A conformational change occurs in ASR simultaneously, leading to the cancellation of the transcriptional repression of cpcB by ASR. A hydrogen bonding network and the structural changes in the cytoplasmic region are characteristic for ASR in contrast to other microbial rhodopsins, and might be related to the repression of cpcB by ASR and/or the cancellation of that repression by light. iii) We demonstrated here that ASRT inhibits the activity of ASR in the dark, presumably through the interaction between ASR and ASRT. Thus ASRT seems to be a modulator of ASR.

These engineered E. coli cells could also be used as a tool for controlling the arbitrary protein expression by visible light in vivo. As proteins, that are correctly transcribed and translated as gene products from the genome, are responsible for various physiological events in the cells, such an artificial protein expression/repression in vivo could be of great use. In fact, gene knockout and overexpression techniques utilizing stimuli such as reagents, heat and so on, have already been applied to investigate the protein function. Our ASR-cpcB system provides the advantage in controlling the amount of protein at temporal/spatial resolution and in providing control of the timing, compared with other techniques. In future, ASR might be a novel tool for optogenetics, next to the ion transporting rhodopsins. Further analysis is, however, indispensable to increase the understanding of the function of ASR, and to establish more
efficient light-inducible protein expression systems.

In conclusion, the orange light absorbing microbial rhodopsin ASR was shown to regulate, as a gene repressor, the expression of the pigment protein phycocyanin forming phycobilisomes. The extended C-terminal region of ASR including its positively charged residues could be related to the regulation. Thus, we demonstrate a novel function of a retinal containing protein, and suggest that a membrane spanning protein can be able to function as a transcriptional factor.

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REFERENCES


FOOTNOTES

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†The abbreviations used are: ASR, *Anabaena* sensory rhodopsin; *P* _pcpB_, promoter sequence of the β subunit of phycocyanin; *P* _apcA_, promoter sequence of the α subunit of allophycocyanin; CRP, cyclic AMP receptor protein.

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FIGURE LEGENDS

Figure 1 : Schematic illustration of the experimental strategy
A) The fragment including P_{pcpB} from the *Anabaena* PCC7120 genome was inserted into the upstream region of the *crp* gene on a plasmid. B) The constructed plasmids were introduced into the TA341 strain, which lacks CRP, together with the expression plasmid of ASR, the ASR-ASRT fusion protein or ASRT. C) The sample is grown in the dark and in under light of various wavelengths. The light of a selective wavelength was obtained by filtering a light source of high brightness with one of eight band pass filters (for 450, 480, 502, 520, 550, 570, 600 and 638nm; %T indicates percent transmittance). The amount of CRP in the cells depending on light condition was monitored by immunoblotting with anti-CRP antibodies.

**Figure 2: Effect of ASR on the CRP expression**

A) Absorption spectra of purified fraction taken from the cells without ASR (w/o), with N-terminal His-tagged ASR (His<sub>6</sub>-ASR), or with C-terminal His-tagged ASR (ASR-His<sub>6</sub>) grown in the dark (D) or under illumination (L: ~550 nm). B) CRP expression levels in the absence/presence of ASR in the dark or under illumination (550 nm) were monitored by immunoblotting with anti-CRP antibodies. C) Quantitative analysis by estimating the amount of CRP using the software ImageJ with correction of the light intensity obtained by using an illuminometer (optical power meter and sensor, 9742 and 3664, Hioki, Japan). Ten independent experiments were performed, and the results were averaged. Error bars represent the standard deviation. D) Northern blotting analysis of the *E. coli* cells with and without ASR in the dark.

**Figure 3 : Specificity of the promoter sequence of P_{pcpB}**

A) CRP expression levels of P_{pcpB} and P_{apcA} in the dark or under illumination (550 nm) were monitored by immunoblotting with anti-CRP antibodies. B) Quantitative analysis of the amount of CRP. Five independent experiments were performed, and the results were averaged. Error bars represent the standard deviation. C) Absorption spectra of purified fraction taken from the cells with His<sub>6</sub>-ASR grown in the dark (D) or under illumination (L: ~550 nm). Five independent experiments were performed, and the results were averaged. Error bars represent the standard deviation.

**Figure 4 : Action spectrum of the ASR-induced repression of the CRP expression prevented by light.**

Western blotting analysis of the *E. coli* cells expressing CRP after illumination with various wavelengths of light (see Figure 1c) (upper panel). The band intensity was estimated by using the software ImageJ, and subtracting the signal in the dark. Five independent experiments were performed, and the results were averaged. Error bars represent the standard deviation. The visible absorption spectra of the purified His<sub>6</sub>-ASR were measured in a buffer containing 1 M NaCl and 50 mM Tris-Cl, pH 7.0. The dark-adapted sample (dashed line) was illuminated for 5 min with light of lambda > 560 nm (O-58 cutoff filter; Toshiba), filtered from a high power light source (MAX-302, ASAHI SPECTRA), to produce the light-adapted form (solid line).

**Figure 5: Role of the extended C-terminal region in ASR**

A) Representation of the full-length ASR. The transmembrane helices (pink), the retinal (yellow), and the extended C-terminal region are shown according to the crystal structure of the truncated protein (19). The structure of the 35 cytoplasmic residues beyond residue 226 is unknown, and has been predicted to be an alpha-helical extension. The Arg residues marked by a closed circle in the C-terminus region are the sites of attention in this study. B) The estimated secondary structure using the software PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). Conf, Pred and AA indicate the confidence, the predicted secondary structure (H=helix, E=strand, C=coil) and the amino acid sequence, respectively. C) Western blotting and its quantitative analysis of the amount of CRP expressed in *E. coli* cells in the presence of either His<sub>6</sub>-ASR, the truncated
mutant which consist of the 226 amino acid residues at the N-terminal of ASR (Truncated form), or the Arg mutant (R→A) with (L) or without (D) light of 550 nm. All mutants have a His-tag at the N-terminus. Six independent experiments were performed, and the results were averaged. Error bars represent the standard deviation.

**Figure 6: Role of ASRT**
A) Western blotting analysis of the *E. coli* cells expressing His<sub>6</sub>-ASR-linker (ASR-linker) or His<sub>6</sub>-ASR-linker-ASRT (ASR-linker-ASRT) with and without light of 550 nm. Four independent experiments were performed, and the results were averaged. Error bars represent the standard deviation. B) The effect of ASRT on the CRP expression. ASRT and CRP were detected by anti-His and anti-CRP antibodies, respectively. Three independent experiments were performed, and the results were averaged. Error bars represent the standard deviation. In all cases, the signal in the absence of protein was used as a baseline.

**Figure 7: Hypothetical model of the transcriptional regulation by ASR and ASRT**
i) ASR having an all-trans retinal chromophore in the dark represses the transcription of the chromatic adaptive gene *cpcB* via the direct or indirect interaction with P<sub>cpcB</sub> through its C-terminal Arg residues in the absence of light. ii) Upon illumination, a structural change(s) of ASR is induced by the conversion of the retinal chromophore from all-trans to 13-cis, leading to the inhibition of the repression. iii) ASRT may affect the expression *via* interaction with both ASR or P<sub>cpcB</sub>. 
FIGURE 1

A) Anabaena genome

B) ASR

C) Wavelength [nm]
FIGURE 2

A) Absorbance [OD]

B) CRP

C) p<0.001

D) crp
FIGURE 3

A) 

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<th>$P_{apcB}$</th>
<th>$P_{apcA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light (550 nm)</td>
<td>D</td>
<td>L</td>
</tr>
<tr>
<td>CRP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B) 

B) Dark, Light

C) 

Absorbance at 550 nm [OD]

Light (550 nm) | D | L | D | L

$P_{apcB}$ | $P_{apc}$
FIGURE 4
FIGURE 7
Photo-induced regulation of the chromatic adaptive gene expression by Anabaena sensory rhodopsin
Hiroki Irieda, Teppei Morita, Kimika Maki, Michio Homma, Hiroji Aiba and Yuki Sudo

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