Photo-induced Regulation of the Chromatic Adaptive Gene Expression by Anabaena Sensory Rhodopsin

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Background: ASR is categorized as a microbial sensory rhodopsin.

Results: ASR represses the transcription of the chromatic adaptive gene crpB through its C-terminal region.

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

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

Rhodopsin molecules are photochemically reactive membrane-embedded proteins, with seven transmembrane α-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. Anabaena sensory rhodopsin (ASR), found in 2003 in the cyanobacterium 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 Escherichia coli cells with the reporter gene 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 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 the 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 α-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); whereas type-2 rhodopsins, such as visual pigments, are, in general, G-protein-coupled receptors 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, or the cation channel channelrhodopsin. 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).

Because the discovery of BR (5), rhodopsins had for nearly 3 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 an evolutionary relationship to the well studied archaean 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, tentatively named the ASR transducer (ASRT) (10), which is different from the members of the membrane-embedded taxis transducer family proteins, haloarchaeal phototaxis transducers I for SRI and haloarchaeal phototaxis transducers II 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 of both ASR and ASRT in Anabaena is still poorly understood, although isothermal titration calorim-
etry measurements revealed that ASRT binds to ASR with a dissociation constant of 8 \mu M (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 of its photochemical reactions are also photochromic (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 co-workers 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 Escherichia coli because of its lack of photo-induced behaviors. Furthermore, the promoter sequence of phycocyanin, which is an orange light-inducible blue pigment (subunits \(\beta\) and \(\alpha\) encoded by \(\text{cpc}B\) and \(\text{cpc}A\)) in cyanobacteria involved in the chromatic adaptation (22), was also introduced into the cells with a reporter gene \(\text{crp}\). Using this system, the functions of ASR and ASRT were investigated.

**EXPERIMENTAL PROCEDURES**

**Gene Preparations**—As shown in supplemental Fig. S1A, the HindIII-KpnI fragment, including the putative promoter sequence of phycocyanin (P\(_{\text{cpc}B}\)) amplified by PCR from the Anabaena PCC7120 genome, was inserted into the upstream region of the \(\text{crp}\) gene on the plasmid pHA7MK, a derivative of pBR322. The HindIII-EcoRI fragment, including P\(_{\text{cpc}B}\) and \(\text{crp}\) from pHA7MK, was inserted into the multicloning site of the vector plasmid pSU21. As shown in supplemental Fig. S1B, the putative promoter sequence of the \(\alpha\) subunit of allophycocyanin (P\(_{\text{apc}A}\)) (23) was amplified by PCR from the Anabaena PCC7120 genome, and it was digested by PsiI and inserted into the upstream region of the \(\text{crp}\) gene on the plasmid pHA7MK. 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-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 Prism 3100, Applied Biosystems) to confirm the expected nucleotide sequences.

Sample Preparations—LB medium supplemented with 50 μg/ml ampicillin and 25 μg/ml 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 mid logarithmic phase. For the expression of ASR, all-trans-retinal (10 μM) and isopropyl-1-thio-β-D-galactopyranoside (1 mM) 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 ImageJ software. For the Northern blotting analysis, the total RNA content was isolated from cells grown in LB medium to their mid logarithmic phase as described previously (25) and resolved by 1.5% agarose-gel electrophoresis followed by Northern blotting with 620 bp of the digoxigenin(DIG)-labeled DNA probes, containing the crp-coding region using the following primers: FW, GCAAACCGCAA-CAG; 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 His6 tag at the C or N terminus were expressed in E. coli cells, solubilized by 2% (w/v) n-dodecyl-β-D-maltoside, and purified with a Ni2+ affinity column. The samples were concentrated and exchanged using an Amicon Ultra Filter (Millipore).

RESULTS

Fig. 1 shows the strategy of this study. The 502-bp fragment of the Anabaena PCC7120 genome, which sufficiently includes F_{pcpB} was amplified by PCR and inserted into the upstream region of the crp gene on a plasmid vector DNA (Fig. 1A). It should be noted that the Shine-Dalgarno sequence, AGGAGA, exists on the upstream region of the ATG initiation codon of cpcB (supplemental Fig. 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 (Fig. 1B). The cells were illuminated with light of various wavelengths (Fig. 1C). The amount of CRP expressed depending on the wavelength of the light illumination was analyzed by the Western blotting.

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, pH 7.0) containing 1 M NaCl and 0.05% DDM. Fig. 2A shows the absorption spectra of the purified fraction from the cells without ASR (w/o), with N-terminal His-tagged ASR (His_{6}-ASR), or with C-terminal His-tagged ASR (ASR-His_{6}) 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 ImageJ software 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 S.D. D, Northern blotting analysis of the E. coli cells with and without ASR in the dark is shown.
both the His<sub>6</sub>-ASR and ASR-His<sub>6</sub> 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 Fig. 2A. However, the expression level of ASR-His<sub>6</sub> was ~5 times greater than that of His<sub>6</sub>-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 approximately 15,000 molecules of the His<sub>6</sub>-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 (Fig. 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 staining. Without ASR, the protein expression of CRP was confirmed as an intense band both in the dark and under illumination, suggesting that P<sub>cpcB</sub> 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 His<sub>6</sub>-ASR in the dark (Fig. 2B, lane 3), whereas under illumination (Fig. 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 His<sub>6</sub>-ASR (Fig. 2C). In contrast, the addition of ASR-His<sub>6</sub> 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 (Fig. 2D). As shown, compared with the other conditions, the amount of mRNA of crp extracted from cells expressing His<sub>6</sub>-ASR was significantly decreased (Fig. 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 nonspecific against the promoter sequence, we performed further experiments by using PapcA 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 (Fig. 3A). Although the total amount of CRP was increased 3–4-fold in Papc as compared with PcpcB, no significant change upon illumination was observed (Fig. 3B), indicating that the effect of ASR is specific for PcpcB. The increase of CRP by PapcA could be related to the functionality of the promoter sequence in E. coli. In other words, PapcA seems more effective for the transcription in E. coli cells than PcpcB. It should be also noted that the ASR protein was similarly expressed in cells containing PcpcB or PapcA, as judged by the absorption at 550 nm of the purified protein (Fig. 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 (Fig. 4). For this, the sample was illuminated with light of different wavelengths (see Fig. 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 Fig. 4, the amount of CRP depends on the wavelength of the light. This dependence was compared with 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%), whereas upon illumination with orange light this ratio is rapidly shifted to the 13-cis form (λmax of 537 nm). As shown in Fig. 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 \( \alpha \)-helical structure (Fig. 5, A and B) (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 Fig. 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 (Fig. 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. Fig. 5C also shows the amount of CRP in the presence of an Arg-lacking mutant 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 important 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 Fig. S2, A and B). 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 Fig. S2, A and B), 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 (Fig. 6A) and ASRT alone (Fig. 6B). For the fusion construct, ASRT was linked to ASR through a flexible linker of nine residues (29) connecting the cytoplasmic C terminus of ASR and N terminus of ASRT (Fig. 6A). The fusion construct was used because the protein expression of both 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 Fig. S2C) or in the repression of CRP compared with His6-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 Fig. 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 et al. 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 (Fig. 6B), regulated by the addition of arabinose. 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 or indirect interaction with P_{cpcB} through its C-terminal Arg residues in the absence of light. 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. ASRT may affect the expression via interaction with both ASR or P_{cpcB}.

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 an 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 the future, it would be interesting to study the structure and dynamics of the C terminus of ASR while interacting with its partners. This would give an idea about the 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 the future.

Fig. 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 bacteriorhodopsin promoter (37–40). In the cyanobacterium *Synechocystis* PCC6803, the cpcBACD promoter is recognized by the σ factors SigA, SigD, and SigE (41). SigA, a functional homologue of *E. coli* σ70 (rpoD), is responsible for the basal transcription, while SigD and SigE, functional homologues of *E. coli* σ38 (rpoS), contribute to the light-induced transcription. The putative P_cpcB sequence (−35 and −10 elements) of *Anabaena* PCC7120 is based on that of *Synechocystis* PCC6803 (supplemental Fig. S1A), which has a σ70-like promoter sequence and could be recognized by the endogenous σ70 and σ38 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, they 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 knock-out 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 the 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 could function as a transcriptional factor.

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