Role of the semi-conserved histidine residue in the light-sensing domain of LitR, a MerR-type photosensory transcriptional regulator

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The LitR/CarH protein family transcriptional regulator is a new type of photoreceptor based on the function of adenosyl B$_{12}$ (AdoB$_{12}$) as a light-sensitive ligand. Here, we studied a semi-conserved histidine residue (His$^{132}$) in the light-sensing (AdoB$_{12}$-binding) domain at the C-terminus of LitR from a thermophilic Gram-negative bacterium, Thermus thermophilus HB27. The in vivo mutation of His$^{132}$ within LitR caused a reduction in the rate of carotenoid production in response to illumination. BIACore analysis revealed that the illuminated-LitR$_{H132A}$ possesses high DNA-binding activity compared to the wild-type protein. The subunit structure analysis showed that LitR$_{H132A}$ performed an incomplete subunit dissociation. The ability of LitR$_{H132A}$ to associate with AdoB$_{12}$ was reduced compared with that of the wild-type protein in an equilibration dialysis experiment. Overall, these results suggest that His$^{132}$ of LitR is involved in the association with AdoB$_{12}$ as well as the light-sensitive DNA-binding activity based on oligomer dissociation.

INTRODUCTION

The light-induced transcription regulator LitR/CarH family of proteins belongs to the MerR family of transcriptional regulators and utilizes coenzyme B$_{12}$ (5’-deoxyadenosylcobalamin; AdoB$_{12}$; AdoCbl) as a light-sensitive ligand (Diez et al., 2013; Jost et al., 2015; Ortiz-Guerrero et al., 2011; Pérez-Marín et al., 2008; Takano, 2016; Takano et al., 2005, 2006a, b, 2011, 2015a, 2016). The LitR/CarH family of proteins consists of an N-terminal winged-helix DNA-binding domain with MerR-type helix-turn-helix (HTH) motif and a C-terminal light-sensing domain containing a four-helix bundle and a Rossmann-fold cobalamin binding domain. An intriguing feature of the LitR/CarH family is that homologous proteins are encoded in the phylogenetically divergent non-phototrophic bacteria, including Gram-negative and Gram-positive bacteria (Ortiz-Guerrero et al., 2011; Takano et al., 2006b). Previous studies have revealed that the LitR/CarH family of proteins commonly plays a central regulatory role in controlling the light-inducible expression of the genes responsible for carotenoid biosynthesis. This has been demonstrated in a number of bacteria, including Myxococcus xanthus (Pérez-Marín et al., 2008), Thermus thermophilus (Diez et al., 2013; Takano et al., 2011, 2014), Bacillus megaterium (Takano et al., 2015b), and Streptomyces coelicolor (Takano et al., 2005). These findings suggest the possibility that the regulatory mechanism based on the function of the AdoB$_{12}$—LitR/CarH family is essential for the control of light-inducible carotenoid production in bacteria.

Recently, a three-dimensional structure for CarH of T. thermophilus HB8 was determined in three relevant states: dark conditions, free form and bound to the operator DNA, and light conditions (Jost et al., 2015). The structural analysis showed that: (i) the tetramer of CarH consists of dimer-of-dimers type architecture, (ii) CarH binds to the −35 element within carH promoter, and (iii) CarH undergoes a large-scale conformational change upon illumination...
from tetramer to monomer in response to illumination. In this large conformational change, light triggers the movement of a helical bundle located in the light-sensing domain, which drastically alters the orientation of the helical bundle relative to the AdoB12-binding domain. The 5′-deoxyadenosyl (5′-dAdo) group of AdoB12 blocked the movement of the helical bundle under dark conditions, whereas under light conditions, loss of the 5′-dAdo group by photolysis caused the movement of the helical bundle, which led to disassembly of the tetramer.

Structural and biochemical analyses of CarH also revealed that His132 coordinates with AdoB12 to form a bis-His Cbl ligation in the illuminated CarH, which is involved in the retention of AdoB12 in CarH. Bis-His Cbl ligation has been reported in heme-binding proteins and, recently, it was also proposed in AerR, a Cbl-binding transcriptional regulator (Cheng et al., 2014). Thus, the involvement of His132 of CarH in bis-His Cbl ligation is known, but the role of His132 in carotenoid production by T. thermophilus cells and DNA-binding activity of this type of regulator, has not yet been studied in detail. Therefore, we focused on His132 of T. thermophilus HB27 LitR and performed a functional in vivo and in vitro characterization of a LitR(H132A) mutant. The evidence obtained in this study indicates that the His132 residue plays a critical role in the fine-tuning of photosensing by LitR.

**METHODS**

**Bacterial strains, plasmids and culture media.** T. thermophilus HB27 TH104 (proC) (pTT8) (Hoshino et al., 1994) was used as the wild-type strain in this study. Escherichia coli HST08 and Rosetta2 (DE3) pLysS (Takara Bio Shiga, Japan) were used as hosts for DNA manipulation and protein expression, respectively. pUC118 and pMD19 (Takara Bio and New England Biolabs (Ipswich, MA, USA). The conditions for the culture and genetic manipulation of E. coli and T. thermophilus are described by Maniatis et al. (1982) and Koyama et al. (1986), respectively. T. thermophilus was grown at 60°C in TM medium (Takano et al., 2011). To enable the selection of transformants of E. coli and T. thermophilus, ampicillin, kanamycin and hygromycin B were added at 50 µg l⁻¹.

**Plasmids for genetic complementation.** The pTEV carrying litR162A, litR164A and litR172A was constructed as follows. A two-stage PCR procedure using complementary mutagenic primers was applied. In the first PCR, the upstream and downstream region from the mutation point was amplified by the primer set R-F/132-MR and 132-MF/R-R for litR164A, R-F/142-MR and 142-MF/R-R for litR162A and R-F/177-MR and 177-MF/R-R for litR172A (Table 1). The second PCR was performed with the primer pair R-F/R-R by using the two amplified DNA fragments as a template. The PCR amplicons were cloned into pUC118, and the nucleotide sequences were verified by Eurofins Genomics K.K. (Tokyo, Japan). The DNA fragments were then cloned between the NdeI and SphI sites of pTEV. pTEV-litR containing an intact litR was constructed in our previous study (Takano et al., 2011). The litR genes were under control of the sfp promoter (Faraldo et al., 1992), which directed the constitutive expression of each coding sequence. The resulting plasmids were introduced into the pTT8 of the wild-type strain in this study. E. coli HB27, the method of extracting carotenoids, and the measurement of UV-V is absorbance for carotenoid was performed as described previously (Takano et al., 2011). An illuminating incubator (BR-180L; Tai Tech; Saitama, Japan) equipped with white light fluorescent lamps (20 W; Toshiba; Tokyo, Japan) was used for the liquid shaking culture under light conditions. White light was illuminated at approximately 2.4 µmol·m⁻²·s⁻¹ onto the solid culture. Light irradiation of the recombinant proteins was carried out by using light-emitting diode lamps, blue (λmax=450 nm), or white lights.

**Light irradiation and carotenoid production.** The culture conditions for carotenoid production by T. thermophilus HB27, the method of extracting carotenoids, and the measurement of UV-V is absorbance for carotenoid was performed as described previously (Takano et al., 2011). An illuminating incubator (BR-180L; Tai Tech; Saitama, Japan) equipped with white light fluorescent lamps (20 W; Toshiba; Tokyo, Japan) was used for the liquid shaking culture under light conditions. White light was illuminated at approximately 2.4 µmol·m⁻²·s⁻¹ onto the solid culture. Light irradiation of the recombinant proteins was carried out by using light-emitting diode lamps, blue (λmax=450 nm), or white light.

**Table 1. Oligonucleotide primers used in this study**

*Restriction sites are underlined. The mutated nucleotides are shown by bold letters. †Dashes indicate the absence of restriction site. ‡Corresponding positions in the genome sequence database of Thermus thermophilus HB27 (http://www.genome.jp/kegg/kegg2.html) are shown.

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light (Optocoe Tokyo, Japan). The measurement of light strength was recorded by a Model LI-250 light meter (LI-COR; Tokyo, Japan). An absorption spectrum of the carotenoid fraction was recorded by using a UV spectrometer U-2800A (Hitachi High-Tech Science Tokyo, Japan) and a NanoDrop 2000 (Thermo Fisher Scientific Waltham, MA, USA).

**Protein overexpression and purification of LitR recombinant proteins.** The expression vector for the wild-type LitR in *E. coli* was constructed as described in the previous study (Takano et al., 2011). The LitR derivatives were amplified by PCR with primer set RexF/RexR using pTEV-litR, pTEV-litRΔH132, pTEV-litRΔH142 and pTEV-litRΔH177 as a template. The amplicons were cloned between the BamHI and EcoRI site of pGEX-6P-2. The nucleotide sequence was verified by sequencing using a BigDye Terminator v3.1 cycle sequencing kit on an ABI3100 automated DNA sequencer (Thermo Fisher Scientific). *E. coli* Rosetta 2 (DE3)pLysS were used as the host for protein expression. The expression and purification of LitR proteins for biochemical analyses was performed as described previously (Takano et al., 2011).

![Fig. 1. Structure-based amino acid sequence alignment of the AdoB12-binding domain of *T. thermophilus* LitR-CTD (LitRth) with that of representative homologous proteins. The sequences are from the phylum Deinococcus-Thermus, including *T. thermophilus* HB8 (LitRtt, E-value; 8 × 10⁻¹³), *Thermus oshimai* (LitRtos, 5 × 10⁻⁷), *Meiothermus ruber* DSM 1279 (LitRmrb, 3 × 10⁻⁴), *Deinococcus maricopensis* (LitRdmr, 2 × 10⁻¹⁰), and *Truepera radiovictrix* (LitRtra, 3 × 10⁻⁵–23); the phylum *Chloroflexus*, including *Roseiflexus castenholzii* (LitRrca, 4 × 10⁻⁵–19) and *Chloroflexus aurantiacus* (LitRcau, 4 × 10⁻⁷–17); the phylum *Proteobacteria*, including *Stigmatella aurantiaca* (LitRsur, 2 × 10⁻⁶–10) and *Sorangium cellulosum* (LitRscl, 5 × 10⁻⁶–6); and the phylum *Firmicutes*, including *Bacillus megaterium* QM B1551 (LitRbmq, 2 × 10⁻⁶–11). White letters on a red background represent strictly conserved residues, and boxed letters show similar residues. Alignment of the primary structure was performed using the program Clustal W2 (Larkin et al., 2007), and the figures were generated with ESPript 3.0 (http://esprit.ibcp.fr) (Robert & Gouet, 2014). The two B12-binding domains are shown by divergent arrows. The conserved motifs of each domain are shown below the alignment (X indicates any amino acid). The positions of the two conserved and one semi-conserved His residues of the B12-binding domain are indicated by arrows.](http://www.microbiologyresearch.org)
**DNase I footprint analysis.** The condition of DNase I footprint analysis used for the determination of LitR binding site was carried out as described previously (Takano et al., 2011). A Fuji imaging plate (Fuji Film; Tokyo, Japan) was used to detect the radioactive signals of dried gels. The images were scanned with a Typhoon 9410 or Typhoon FLA 9500 image analyser (GE Healthcare). For a reference, Maxam–Gilbert sequencing ladders (G+A reaction) were prepared from the sample DNA containing the LitR-binding sites, and then the radioactive signals of the dried gel were recorded. To estimate the bulk refractive index background, AdoB (Sigma-Aldrich; St Louis, MO, USA) was dialysed against 1× PBS buffer at 4°C overnight to remove the free AdoB. The absorbance spectra of proteins collected from the inner fraction were measured with a UV spectrometer U-2800A and a NanoDrop 2000. White light was used to irradiate AdoB–LitR complexes at 587 µmol s⁻¹ m⁻² for 10 min. 100µM AdoB was used as a reference compound.

**BlAcore analysis.** The DNA-binding activity of LitR proteins to its binding site was analysed by surface plasmon resonance spectroscopy using a BlAcore 1000 biosensor (GE Healthcare). 5′-Biotin-conjugated oligonucleotide, BlA-F (containing a sense strand for the LitR-binding site [−52 to −101]) was immobilized on the streptavidin surface of a Sensor Chip SA (GE Healthcare), and then a 5′-unlabelled oligonucleotide, BlA-R (containing an antisense strand of the LitR-binding site), was annealed to the sense-strand oligonucleotide on the Sensor Chip. The oligonucleotides used in this process were of HPLC purification grade and prepared by Eurofins Genomics (Table 1). HBS-EP (GE Healthcare) was used as basal running buffer at a flow rate of 30 µl min⁻¹ at 28°C. AdoB-bound LitR proteins were injected at 100–320 pmol over the flow cell of the Sensor Chip fixed with the dsDNA standards used were those included in the gel-filtration calibration kit (GE Healthcare): ferritin, albumin, carbonic anhydrase, ribonuclease, and blue dextran, indicating 440, 67, 29, 13.7, and 2 kDa, respectively.

**Gel-filtration column chromatography.** Wild-type LitR (1.2 µg µl⁻¹) and LitR₃₁₃₂₃ (1.5 µg µl⁻¹) proteins in complex with AdoB₁₂ in a 200 µl volume were loaded onto a Superdex 200 HR 10/30 column equipped with an ÄKTA FPLC system (GE Healthcare). The absorbance was monitored at 280 nm. The column was equilibrated with 1× PBS (containing 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, and 1.8 mM KH₂PO₄) at a flow rate of 0.3 ml min⁻¹. The molecular size standards used were those included in the gel-filtration calibration kit (GE Healthcare): ferritin, albumin, carbonic anhydrase, ribonuclease, and blue dextran, indicating 440, 67, 29, 13.7, and 2 kDa, respectively.

**Fig. 2.** Carotenoid production of the litR mutants. UV-visible absorption spectrum of carotenoids extracted from cell culture under dark conditions (solid line) and light conditions (dashed line) at 60°C for 19-h culture in TM liquid medium. The strains used were: wild-type (WT) strain, the litR mutant (ΔH1R) harbouring the pTT8 integrated by pTEV (empty plasmid), litR (pTT8 carrying an intact litR), ΔH1R/H1₃₂₃ (pTT8 carrying ΔH1₃₂₃), ΔH1R/H42₄ (pTT8 carrying ΔH4₂₄) and ΔH1R/H1₇₇₇ (pTT8 carrying ΔH1₇₇₇)
For the light condition, white light was used to illuminate the proteins at approximately 5.7 µmol s⁻¹ m⁻² for 10 min.

**Chemical cross-linking.** The oligomer formation of full-length native LitR recombinant proteins was analysed by chemical crosslinking as described previously (Komatsu et al., 2006). Aliquots of 250 pmol of purified wild-type LitR and LitR<sub>H132A</sub> in complex with AdoB<sub>12</sub> were illuminated at approximately 5.5 µmol s⁻¹ m⁻² for 1 to 9 min at 28 °C. The chemical crosslinking was carried out by the addition of 20 µl of 25 mM DSP (dithiobis succinimidyl propionate) to each reaction for 1 h at 28 °C in the dark. After the reaction was stopped by 15-min incubation at 28 °C in the presence of 20 µl of 1 M Tris-HCl (pH 7.5), the samples were analysed by SDS-PAGE, and proteins were detected by silver staining. A low-molecular-weight and high-molecular-weight marker (GE Healthcare) were used as standards to estimate the protein sizes in SDS-PAGE.

**RESULTS**

**The conserved histidine residues in the AdoB<sub>12</sub>–binding domain of LitR**

The litR gene of *T. thermophilus* HB27 encodes 285 amino acid residues (calculated molecular weight is 31.1 kDa), and contains two conserved domains. A conserved domain database (Marchler-Bauer et al., 2002) search indicates that the
the B$_{12}$-binding domain (Pfam02607). His$^{142}$ was strictly conserved in the LitR/CarH family derived from Gram-positive as well as Gram-negative bacteria (Fig. 1), but not in B$_{12}$-dependent enzymes (data not shown). In contrast, the His$^{132}$ residue was conserved in LitR homologues derived from bacteria belonging to the phyla Deinococcus–Thermus and Chloroflexus, and a portion of the Proteobacteria, but not Firmicutes. This suggests that His$^{142}$ and His$^{177}$ are crucial for the function of all types of LitR, while His$^{132}$ may contribute to the function of a subtype distributed to extremophiles. Recently, it was reported that His$^{132}$ of CarH binds to cobalt to form bis-His ligation with His$^{177}$ in a light-exposed form, and is involved in the retention of Cbl after photolysis (Jost et al., 2015).

### Substitution of His$^{132}$ to Ala

In order to examine whether the semi-conserved His residue (His$^{132}$) is involved in the LitR function, we constructed an Ala-substitution mutant cassette (litR$_{H132A}$) and introduced it into the litR mutant to observe its effect on carotenoid production (Fig. 2). We also constructed the litR mutants (litR$_{H142A}$ and litR$_{H177A}$) as a control. The litR

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Fig. 4. BIAcore analysis. The response curves are shown for the dark-incubated and illuminated LitR proteins. The LitR-binding site (–52 to –101) was immobilized on the surface of sensor chip SA via 5'-biotin. The AdoB$_{12}$-bound LitR proteins were injected over the DNA surface at concentrations ranging from 10 to 320 nM (lower to upper curves) in HBS buffer.

![BIAcore Analysis](image-url)

N-terminal domain (corresponding to residues Thr$^8$–Tyr$^{45}$) of LitR exhibits distinct similarity to the HTH motif of a MerR family regulatory protein (e-value = 1.6 x 10$^{-18}$). The C-terminal region of LitR (designated as LitR-CTD) corresponding to residues Gly$^{132}$–Leu$^{150}$ and Val$^{167}$–Glu$^{169}$ exhibits a distinct similarity to the B$_{12}$-binding domain (Pfam02607) with e-value = 1.6 x 10$^{-18}$, and B$_{12}$-binding domain (Pfam02310) with e-value = 1 x 10$^{-5}$, respectively (Fig. 1). LitR-CTD has an amino acid sequence similar to the conserved motifs, ‘MxxVG’ in the B$_{12}$-binding domain and ‘DXHxxGx(41)SxVx(26)GG’ in the B$_{12}$-binding domain (Fig. 1).

Fig. 1 shows the amino acid sequence alignment of proteins similar to LitR–CTD, derived from the bacteria belonging to the phyla Deinococcus–Thermus, Chloroflexus, Proteobacteria, and Firmicutes. The alignment showed the presence of the three conserved His residues. The His residue at position 177 of *T. thermophilus* LitR–CTD (designated His$^{177}$) corresponds to that included in the conserved ‘DXHxxGx(41)SxVx(26)GG’ motif of the B$_{12}$-binding domain (Pfam02310). The His residue within this motif is involved in the binding of the cobalt atom (Kräutler, 2005; Ludwig & Matthews, 1997; Reitzer et al., 1999). Consistent with this fact, the His$^{177}$ residue of *T. thermophilus* LitR is essential for the association with AdoB$_{12}$ (Ortiz-Guerrero et al., 2011). Two other His residues (His$^{132}$ and His$^{142}$) exist in the B$_{12}$-binding_2 domain (Pfam02607). His$^{142}$ was strictly conserved in the LitR/CarH family derived from Gram-positive as well as Gram-negative bacteria (Fig. 1), but not in B$_{12}$-dependent enzymes (data not shown). In contrast, the His$^{132}$ residue was conserved in LitR homologues derived from bacteria belonging to the phyla Deinococcus–Thermus and Chloroflexus, and a portion of the Proteobacteria, but not Firmicutes. This suggests that His$^{142}$ and His$^{177}$ are crucial for the function of all types of LitR, while His$^{132}$ may contribute to the function of a subtype distributed to extremophiles. Recently, it was reported that His$^{132}$ of CarH binds to cobalt to form bis-His ligation with His$^{177}$ in a light-exposed form, and is involved in the retention of Cbl after photolysis (Jost et al., 2015).

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Fig. 5. Spectral characterization of AdoB$_{12}$-treated LitR proteins. The absorbance spectra of 100 μM AdoB$_{12}$ (a) as a reference compound, 10 μM AdoB$_{12}$–LitR$_{H142A}$ (b) and 10 μM AdoB$_{12}$–LitR$_{H177A}$ (c). The dark-incubated and illuminated samples are shown by solid and dashed lines, respectively.
Fig. 6. Gel-filtration chromatography (a) and chemical cross-linking (b) for the analysis of light-induced subunit dissociation of LitR proteins. (a) The recombinant protein of 240 μg AdoB_{12}–LitR_{WT} (upper) and 300 μg AdoB_{12}–LitR_{H132A} (lower) were loaded onto a Superdex 200 HR 10/30 column, and the absorbance spectra were monitored at 280 nm. The dark-incubated and illuminated proteins are shown by solid and dashed lines, respectively. The molecular size standards used were Fe: ferritin, Al: conalbumin, Ca: carbonic anhydrase, and Rn: ribonuclease, indicating 440, 75, 29 and 13.7 kDa, respectively. (b) Aliquots of 250 pmol of purified LitR_{WT} and LitR_{H132A} proteins were incubated under dark or light conditions for 1 to 16 min in the presence of DSP, a chemical crosslinker. The crosslinked proteins were separated by SDS-PAGE and detected by silver staining. Apo-LitR shows the non-associated proteins with AdoB_{12}. The lane on the right edge represents LitR proteins untreated with crosslinker or illumination. Closed and open triangles indicate oligomers (tetramer and more) and dimers, respectively. The arrowhead denotes monomeric LitR.
mutant, harbouring the plasmid-borne intact litR gene (ΔlitR/litR) performed light-dependent carotenoid production similarly to the wild-type. Meanwhile, the litR mutant introduced with litRΔH177A (ΔlitR/litRΔH177A) or litRΔH142A (ΔlitR/litRΔH142A) produced marked amounts of carotenoid both in light and dark conditions as did the mutant with empty vector (ΔlitR). These results indicate that His132 and His142 are essential for LitR-dependent inhibition of carotenoid production under dark conditions, as shown by the previous study on CarH from T. thermophilus HB8 (Ortiz-Guerrero et al., 2011). Carotenoid production by the mutant retaining litRΔH132A (ΔlitR/litRΔH132A) was significantly inhibited in the dark (Fig. 2). However, this transformant cultured under light produced carotenoid to a moderate level. This indicates that His132 is crucial for the full activation of carotenoid biosynthesis by LitR. Based on this result, we focused on the biochemical property of LitRHis132.

**Association of LitR with AdoB12**

The association of LitR with AdoB12 is fundamental to its DNA-binding activity, and the photolysis of AdoB12 to OHB12 causes the inactivation of LitR (Díez et al., 2013; Ortiz-Guerrero et al., 2011). Therefore, the ability of LitR to associate with AdoB12 was evaluated by equilibration dialysis (Fig. 5) (see Methods). The wild-type LitR protein was associated with AdoB12, and the spectrum was changed to the OHB12 profile by illumination as reported previously (Fig. 5b). In contrast, the absorption level of LitRΔH132A treated with AdoB12 was about one-half that of LitRWT (Fig. 5c). However, the light-induced spectral change was still observed. This result suggests that His132 is involved in the association of LitR with AdoB12.

**Light-induced subunit dissociation of AdoB12–LitR complex**

CarH of T. thermophilus undergoes light-inducible dissociation from tetramers to monomers (Ortiz-Guerrero et al., 2011). Thus, we investigated the subunit structure of LitRWT and LitRΔH132A in complex with AdoB12 by gel-filtration column chromatography. The result showed that both types of LitR eluted at the position corresponding to a tetramer (Mr 112 000 or 117 000) if not illuminated (Fig. 6a). On the other hand, illuminated LitRWT eluted as a monomer (Mr 37 000), and illuminated LitRΔH132A as a dimer (Mr 72 000) and monomer (Mr 42 000). Based on the detection of dimerized LitRΔH132A under illuminated conditions, the self-interaction of AdoB12–LitR was also studied by chemical crosslinking analysis (Fig. 6b) (see Methods). Both AdoB12–LitRWT and AdoB12–LitRΔH132A formed oligomers under dark conditions. Exposure to light caused complete subunit dissociation of AdoB12–LitRWT in 3–4 min. Meanwhile, AdoB12–LitRΔH132A formed an oligomer even if it was subjected to 16-min illumination. These results indicate that His132 is crucial for the photosensitivity of subunit dissociation of LitR.

**DISCUSSION**

LitR/CarH family proteins consisting of light-sensitive transcriptional regulators are widely distributed among divergent bacterial genera. His132 is the semi-conserved amino acid residue in thermophilic bacteria harbouring LitR/CarH. Here, we investigated the role and function of the His132 residue of T. thermophilus HB27 LitR. Our results from *in vivo* and *in vitro* analyses indicated that His132 is crucial for the photosensitivity of LitR. We also confirmed that His177 is essential for the repressor function under dark conditions. The mutation of this residue causes constitutive carotenoid production (Fig. 2); LitRΔH177A did not bind the LitR recognition site irrespective of illumination (Fig. 4). These two His residues are known for their significant role in the light-dependent conformational change of CarH, the
LitR equivalent of *T. thermophilus* HB8 (Jost et al., 2015). Our results not only support the view from the detailed structural analysis, but also suggest an additional role for His$^{132}$. H132A mutation caused a decrease in carotenoid production under light conditions (Fig. 2). This result was supported by data from BIAcore analysis, which indicated that the LitR$^{H132A}$ mutant protein retained moderate DNA-binding activity under light conditions (Fig. 4). Probably, the moderate DNA-binding causes partial repression of the transcription of *crt* even under the light conditions; hence, the level of carotenoid production is reduced (Fig. 2). Thus, we speculate that His$^{132}$ is critical for the light-sensitivity of LitR. The residue may affect the efficiency of light-dependent subunit dissociation from tetramer to monomer, which is fundamental to the inactivation of the repressor function of LitR.

LitR$^{H132A}$ exhibited incomplete subunit dissociation from tetramers to monomers in a gel-filtration chromatography analysis (Fig. 6a). The result demonstrated the occurrence of a putative dimer structure. This result supports the view that His$^{132}$ is involved in light-dependent subunit dissociation. Jost et al. (2015) reported that CarH$^{H132A}$ dissociates into monomers as easily as the CarH wild-type protein. Probably the inconsistency is due to the difference in experimental conditions with regard to the time of light exposure: Jost et al. (2015) observed dissociation after 1 h of white-light treatment, longer than ours. We assume that our short and weak illumination conditions gave a clue to discover the occurrence of the putative dimer state of LitR$^{H132A}$.

The result of gel-filtration chromatography was supported by that of the chemical cross-linking experiment (Fig. 6b). LitR$^{H132A}$ formed a tetramer or dimer despite the long illumination period. It suggests that the conformational change of LitR$^{H132A}$ occurs slowly in comparison to that of the wild-type LitR. Another possibility is that the conformation of the LitR$^{H132A}$ mutant protein is locked in the dark state or tends to form an intermediate state between the dark and light ones. Since LitR$^{H132A}$ retains the AdoB$_{12}$-binding activity (Fig. 5c), the most serious influence of His$^{132}$ mutation is not on the defect of AdoB$_{12}$ binding but the increased affinity of protein–protein interaction, leading to the stabilization of the active form of repressor. We assume that His$^{132}$ is a crucial residue for the sensitivity of the conformational change of LitR in the transition between the dark and light states.

Jost et al. (2015) suggested that His$^{132}$ is involved in the stabilization of CarH–Cbl complex after light-induced dissociation via bis-His ligation. The H132A mutant of CarH easily loses Cbl due to the inability to perform the ligation. We propose that the amino acid at this position has an additional role, modulating the sensitivity of the photosensor to light. The organisms retaining His at this position could be sensitive to photo-induced oxidative damage; hence they produce a high level of carotenoid in response to even a weak level of illumination in order to fully protect the cells. On the other hand, organisms with a different amino acid at this position could be relatively tolerant to the damage. The insensitive type of LitR may contribute to energy saving in such organisms by adjusting the carotenoid production to a moderate level. Understanding the relationship between structure and function of such sensing molecules will become fundamental knowledge in terms of microbial physiology and ecology.

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