Structural Basis for the Transcriptional Regulation of Heme Homeostasis in Lactococcus lactis*

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Background: A transcriptional regulator HrtR regulates the expression of the heme efflux transporter.

Results: The crystal structures of HrtR in apo, holo, and DNA-bound forms were solved.

Conclusion: Heme sensing by HrtR induces a coil-to-helix transition to regulate DNA binding activity.

Significance: Elucidating the structure and function of HrtR is fundamental to understand the molecular mechanism of heme homeostasis.

Although heme is a crucial element for many biological processes including respiration, heme homeostasis should be regulated strictly due to the cytotoxicity of free heme molecules. Numerous lactic acid bacteria, including Lactococcus lactis, acquire heme molecules exogenously to establish an aerobic respiratory chain. A heme efflux system plays an important role for heme homeostasis to avoid cytotoxicity of acquired free heme, but its regulatory mechanism is not clear. Here, we report that the transcriptional regulator heme-regulated transporter regulator (HrtR) senses and binds a heme molecule as its physiological effector to regulate the expression of the heme-efflux system responsible for heme homeostasis in L. lactis. To elucidate the molecular mechanisms of how HrtR senses a heme molecule and regulates gene expression for the heme efflux system, we determined the crystal structures of the apo-HrtR-DNA complex, apo-HrtR, and holo-HrtR at a resolution of 2.0, 3.1, and 1.9 Å, respectively. These structures revealed that HrtR is a member of the TetR family of transcriptional regulators. The residue pair Arg-46 and Tyr-50 plays a crucial role for specific DNA binding through hydrogen bonding and a CH–π interaction with the DNA bases. HrtR adopts a unique mechanism for its functional regulation upon heme sensing. Heme binding to HrtR causes a coil-to-helix transition of the α4 helix in the heme-sensing domain, which triggers a structural change of HrtR, causing it to dissociate from the target DNA for derepression of the genes encoding the heme efflux system. HrtR uses a unique heme-sensing motif with bis-His (His-72 and His-149) ligation to the heme, which is essential for the coil-to-helix transition of the α4 helix upon heme sensing.

Iron is an essential element for nearly all organisms. Although iron ions are used as an essential cofactor constituting heme, iron-sulfur clusters, and nonheme iron complexes that participate in many biologically critical reactions, excess iron ions are toxic. Therefore, cellular iron homeostasis is tightly regulated to avoid the toxic effects of iron, for which nature has evolved sophisticated regulatory systems (1, 2). Uptake and efflux of iron ions and/or iron complexes are important processes to be regulated for iron homeostasis. In most of these regulatory systems, the cellular iron level is monitored by sensing iron ions (3, 4) or by sensing free heme molecules (5, 6).

The widely studied biological function of heme is to act as a prosthetic group in hemeproteins that show a variety of functions, including oxygen storage and transport, electron transfer, redox catalysis, and sensing of gas molecules. Besides acting as a prosthetic group in a protein matrix, it has become apparent that free heme molecules can act as physiological effectors of several proteins, including transcriptional regulators (6, 7), heme-regulated eIF2α kinase (8), and sensor kinases in two-component signal transduction systems (5, 9). Reversible heme binding regulates the physiological function of these proteins. Although research on these proteins has shown new physiological functions of heme as a signaling molecule, the detailed molecular mechanisms by which heme regulates the functions of these proteins remain to be elucidated, mainly because the three-dimensional structures of these regulatory proteins have not yet been solved.

Pedersen et al. (10) have reported a new heme-responsive regulatory system in the lactic acid bacterium Lactococcus lactis that is widely used in dairy and other food fermentations. L. lactis undergoes a metabolic shift from fermentation to respiration in an aerobic environment when heme is supplied (11, 12). Acquired heme is used as a source of heme, rather than of iron, to activate cytochrome oxidase (cytochrome bd) for oxygen respiration because this bacterium cannot biosynthesize heme (10,
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13, 14). As free heme molecules are cytotoxic, some regulatory system is required to avoid this toxicity. Pedersen et al. (10) have reported that the ygfCBA operon is involved in heme tolerance and homeostasis and proposed that ygfA and ygfB encode an ATP-binding cassette transporter ATP-binding protein and an ATP-binding cassette transporter permease, respectively, responsible for heme efflux to maintain heme homeostasis. Recently, Lechardeur et al. (15) have reported that YgfC is a heme-responsive repressor that regulates the expression of the hrtRBA (formerly ygfCBA) operon in response to free heme molecules as physiological effectors and renamed YgfC as HtrR (heme-regulated transporter regulator). HtrR acts as a heme molecules as physiological effector and renamed YgfC as HtrR (heme-regulated transporter regulator). HtrR acts as a heme-sensing repressor for the regulation of the hrtRBA operon. Although Lechardeur et al. (15) report that the DNA binding activity of HtrR is regulated by heme binding, it is not clear how heme binding regulates the biological function of HtrR. Here, we report the crystal structures of HtrR in heme-free (apo-), heme-bound (holo-), and DNA-bound forms with biochemical and spectroscopic characterization, based on which we propose a heme-responsive regulatory mechanism of HtrR responsible for transcriptional regulation of heme homeostasis.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of HtrR**—The htrR gene synthesized by GenScript was inserted between the NdeI and BamHI sites of pET3a to construct an expression vector for HtrR, by sized by GenScript was inserted between the NdeI and BamHI sites of pET3a to construct an expression vector for HtrR. HtrR was induced with 0.5 mM isopropyl-β-D-thiogalactoside, and then cultivation was allowed to continue for another 15 h at 20 °C with shaking at 80 rpm.

**Fluorescence Polarization Analysis**—Heme titrations into apo-HtrR were carried out by the addition of 1 μl of 1 mM hemin (Sigma-Aldrich) solution to 1 ml of 5 μM apo-HtrR dimer in 50 mM Tris-HCl (pH 7.4). Hemin was initially dissolved in dimethyl sulfoxide (DMSO) was added into the sample solution after 20 min of the incubation and then incubated at room temperature for another 20 min before electrophoresis. After electrophoresis, gels were observed with a BioDoc-It-Plus system (UV).

**Fluorescence Polarization Analysis**—A 19-bp DNA fragment (sequence of the sense strand: 5′-TAGAATTTAATAAATGACACAGTGTCATAAAATTT-3′ and 5′-TAGAATTATAAAATGATGTCACACTGCAAATT-3′, respectively. The 5′ end of the antisense strand of the target DNA was labeled with 6-carboxyfluorescein. The sense and antisense strands in equimolar amounts (25 μM in final) were mixed in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (pH 8.0) and 100 mM NaCl and then boiled at 100 °C for 10 min followed by cooling to room temperature to obtain double-stranded DNA.

The DNA fragment and apo-HtrR were mixed in a 10-μl reaction mixture containing 20 mM Tris-HCl (pH 7.4), 50 mM KCl, and 5% glycerol and then incubated for 20 min at room temperature. If necessary, hemin dissolved in dimethyl sulfoxide (DMSO) was added into the sample solution after 20 min of the incubation and then incubated at room temperature for another 20 min before electrophoresis. After electrophoresis, gels were observed with a BioDoc-It-Plus system (UV).

**Electrophoretic Mobility Shift Assays**—The EMSAs were performed with 33 bp of double-stranded DNA on a native polyacrylamide gel (8%) using Tris borate-EDTA buffer as a running buffer. The sequences of the sense strand for the target and nontarget DNA used in EMSAs are 5′-TAGAATTTAATAAATGACACAGTGTCATAAAATTT-3′ and 5′-TAGAATTATAAAATGATGTCACACTGCAAATT-3′, respectively. The 5′ end of the antisense strand of the target DNA was labeled with 6-carboxyfluorescein. The sense and antisense strands in equimolar amounts (25 μM in final) were mixed in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (pH 8.0) and 100 mM NaCl and then boiled at 100 °C for 10 min followed by cooling to room temperature to obtain double-stranded DNA.

The DNA fragment and apo-HtrR were mixed in a 10-μl reaction mixture containing 20 mM Tris-HCl (pH 7.4), 50 mM KCl, and 5% glycerol and then incubated for 20 min at room temperature. If necessary, hemin dissolved in dimethyl sulfoxide (DMSO) was added into the sample solution after 20 min of the incubation and then incubated at room temperature for another 20 min before electrophoresis. After electrophoresis, gels were observed with a BioDoc-It-Plus system (UV).
sense strand: 5′-ATGACACAGTGTCAAT-3′) were mixed with a 1:1 molecular ratio and then incubated at 20 °C for 10 min before crystallization of the apo-HrtR-DNA complex. The sense and antisense strands of the 15-bp DNA in 20 mM Trizma/HCl (pH 7.4) were incubated at 100 °C for 2 min and then cooled to room temperature to prepare the blunt end 15-bp DNA duplex at a final concentration of 2 mM.

Initial crystallization screening was performed using commercially available sparse matrix screens, in which 1 μl of protein solution was mixed with an equal volume of reservoir solution and equilibrated against 100 μl of reservoir solution by the sitting-drop vapor diffusion method at 20 °C. For the optimization of crystallization conditions, 3 μl of protein solution was mixed with an equal volume of reservoir solution and equilibrated against 1 ml of reservoir solution by the hanging-drop vapor diffusion method at 20 °C. The crystals of holo-HrtR were obtained using reservoir solution containing 19% (w/v) polyethylene glycol 4000, 0.1M sodium cacodylate (pH 6.0), and 0.2 M magnesium chloride. The crystal of holo-HrtR belongs to space group C2221 and contains two dimers in an asymmetric unit.

Crystals of apo-HrtR were obtained using 3.0M sodium formate, 0.4M sodium chloride, and 0.1M Trizma/HCl (pH 8.6) as a reservoir solution. The crystal of apo-HrtR belongs to space group P2221, with one protomer in asymmetric unit. Crystals of apo-HrtR were obtained using 3.0 M sodium formate, 0.4 M sodium chloride, and 0.1 M Trizma/HCl (pH 8.6) as a reservoir solution. The crystal of apo-HrtR belongs to space group P2221, contains two dimers in an asymmetric unit. The heavy-atom derivative of apo-HrtR crystal was prepared by soaking into the mother liquor containing ~4.5 mM ammonium tetrachloroplatinate (II) for 1 h. The crystals of apo-HrtR-DNA complex were obtained using 1.2 M sodium citrate and 0.1 M PIPES/NaOH (pH 7.3) as a reservoir solution. The crystal of apo-HrtR-DNA complex belongs to space group P6522.

**Data Collection, Structure Determination, and Refinement**—Crystals were cryo-protected for x-ray diffraction data collection by soaking crystals into mixed oil (66.5% (v/v) Paratone-N, 28.5% (v/v) paraffin oil, 5.0% (v/v) glycerol) for 30 s (16). The crystal was then picked up in the cryoloop and flash-cooled in a stream of cold nitrogen gas at 100 K. X-ray diffraction data were collected on BL26B1, BL26B2, and BL41XU at the SPring-8 facility. The data were integrated and scaled using the program HKL2000 (17) and further processed using the CCP4 package (18). Phases of holo-HrtR crystals were calculated by SOLVE/RESOLVE (19) from single wavelength anomalous dispersion (SAD) data measured at the absorption peak of the heme iron. Most parts of the atomic model were built automatically using the ARP/warp (20) program. The heme and remaining residues were manually built in Coot (21). Phases of apo-HrtR crystal were obtained from SAD data of platinum derivatives. Four platinum atoms were located, and phases were calculated by autoSHARP (22). The structure of apo-HrtR-DNA complex was solved by molecular replacement using an apo-HrtR dimer and 15 bp of double-stranded DNA in TetR-DNA complex (Protein Data Bank (PDB) accession code 1QPI) as the search models in Phaser (23). The models were manually rebuilt in Coot (21) and refined using PHENIX (24). The heme molecule in holo-HrtR was refined in full occupancy. The model geometry was checked using the program WHAT IF (25). The figures were generated by PyMol (26). The statistics of data collection and refinement are summarized in Table 1.

**RESULTS**

**Heme-responsive Regulation for DNA Binding of HrtR**—To avoid structural distortions that could be produced as the consequence of the presence of tags, intact HrtR without any tag sequence was expressed and purified in this work. Titration of apo-HrtR with heme revealed that apo-HrtR takes up 1 mol eq of heme with respect to each HrtR protomer to form a 1:1 complex that shows a Soret peak and α and β bands, at 413, 562, and 536 nm, respectively, in the ferric form (Fig. 1). Reduction of holo-HrtR changed the Soret, α, and β peaks to 425, 560, and 530 nm, respectively. These spectra are similar to those of heme proteins with bis-His axial ligands, suggesting that the heme in holo-HrtR is coordinated by two histidines. The crystal structure of holo-HrtR described below showed that this is the case.

Gel filtration analyses showed that apo-HrtR and holo-HrtR eluted as a single peak with an estimated molecular weight of 45,500 and 48,000, respectively, indicating that both apo-HrtR and holo-HrtR exist as a homodimer in solution. Thus, heme binding to apo-HrtR did not change its quaternary homodimer structure.

Electrophoretic mobility shift assays (EMSAs) revealed that apo-HrtR can directly interact with the putative operator-promoter region of the hrtRBA operon (Fig. 2). The HrtR-binding sequence (5′-ATGACACAGTGTCAAT-3′) is located at the position of −6 to 20 relative to the putative transcriptional start site of the hrtRBA operon, which is overlapped with the putative −10 region of the hrtRBA promoter. Apo-HrtR bound to

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FIGURE 1. A, spectral change upon titration of hemin. Inset, titration curve of hemin binding to apo-HrtR measured at 413 nm. B, electronic absorption spectra of HrtR (3 μM in dimer) in the ferric (black) and ferrous (red) forms.

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AUGUST 31, 2012•VOLUME 287•NUMBER 36

JOURNAL OF BIOLOGICAL CHEMISTRY 30757

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the target DNA, which gave a band shift due to the formation of the apo-HrtR-DNA complex (Fig. 2). The addition of a 10–50 mol eq excess of unlabeled target DNA relative to labeled target DNA to the reaction mixture resulted in the disappearance of the retarded band concomitantly with the appearance of a band due to free DNA, whereas 50 mol eq excess of unlabeled non-target DNA did not show any competition (Fig. 2A). These competition assays revealed that the binding of HrtR to target DNA is specific. Fluorescence polarization assays also supported this notion. The equilibrium dissociation constant ($K_d$) for the apo-HrtR-DNA complex was determined to be 0.2 nM by fluorescence polarization assays (Fig. 2B), which shows a high DNA binding affinity of apo-HrtR.

Next we determined the regulatory role of heme for DNA binding using EMSAs and fluorescence polarization assays. The addition of 1 mol eq of heme relative to HrtR protomer was sufficient to fully dissociate HrtR from DNA (Fig. 2C). Consistent with these results, holo-HrtR showed neither a band shift on EMSAs nor a change in fluorescence polarization when reacting with the target DNA. These results indicate that heme acts as an effector molecule by which the DNA binding activity of HrtR is regulated to derepress the hrtRBA operon upon sensing free heme molecules in L. lactis.

**Global Fold of HrtR**—To obtain structural insights into the heme-responsive regulatory mechanisms of HrtR function, we determined the crystal structures of apo-HrtR, holo-HrtR, and the apo-HrtR-DNA complex. The structures were determined at resolutions of 3.1, 1.9, and 2.0 Å for apo-HrtR, holo-HrtR, and the apo-HrtR-DNA complex, respectively (Fig. 3 and Table 1). We crystallized apo-HrtR in complex with DNA duplexes of 15, 17, 18, or 19 bp in length and found that co-crystals with the 15-bp duplex DNA gave diffractions with the highest resolution. The sequence of the sense strand of 15-bp duplex DNA used in this study was 5’-ATGACACAGTGTCA-3’, which has a perfect inverted repeat as shown with underlines.

Although the crystallographic asymmetric unit consists of one protomer in the case of holo-HrtR and the apo-HrtR-DNA complex, applying crystallographic symmetry generated a homodimer. Two homodimers of apo-HrtR exist in the crystallographic asymmetric unit. Gel filtration experiments revealed a dimeric form for both apo-HrtR and holo-HrtR in solution. Based on these results, we propose that this crystallographic dimer of holo-HrtR and apo-HrtR-DNA complex is the physiologically relevant form.

The global fold of HrtR is similar to that of TetR family transcriptional regulators (TFRs) that possess nine conserved helices, within which helices 1–3 comprise the DNA-binding domain and helices 4–9 comprise the ligand-binding domain (27, 28). In addition to the nine conserved helices, HrtR possesses extra short helices ($\alpha_N$ and $\alpha_C$ shown in Fig. 3) at the N and C termini, although it is not clear due to disorder whether there is an $\alpha_C$ helix in holo-HrtR. The DNA-binding domain and the heme-sensing (heme-binding) domain consist of $\alpha_N$, $\alpha_1$, $\alpha_2$, and $\alpha_3$ helices and $\alpha_4$, $\alpha_5$, $\alpha_6$, $\alpha_7$, $\alpha_8$, $\alpha_9$, and $\alpha_C$ helices, respectively. The interface between the DNA-binding and heme-binding domains is formed through interactions among $\alpha_1$, $\alpha_4$, and $\alpha_6$ helices (see below). The $\alpha_8$ and $\alpha_9$ helices in each subunit were found to form a four-helix bundle that constitutes a dimer interface. Ser-146 in one subunit forms a hydrogen bond with Asn-150 in the other subunit in this interface (Fig. 4). Hydrophobic interactions are also present at this interface, in which Val-143, Val-147, Leu-175, and Val-176 in one subunit form hydrophobic interactions with those in the other one (Fig. 4). These interactions are conserved among the apo-HrtR-DNA complex, apo-HrtR, and holo-HrtR and are responsible for stabilization of the dimeric form of HrtR.
The structure of apo-HrtR in the absence of DNA is very similar to that of the DNA-bound HrtR. Superimposition of these structures (protomer of the DNA-bound HrtR in the crystallographic asymmetric unit versus A-D chains of apo-HrtR in the absence of DNA) gives an overall root mean square deviation (r.m.s.d.) of 0.5–0.7 Å calculated over the Cα atoms (Fig. 3D). In the following section, therefore, the structures of the DNA-bound HrtR and holo-HrtR are used to discuss the structural difference between apo- and holo-forms of HrtR unless otherwise noted.

Pairwise Interactions of Arg-46 and Tyr-50 for the Specific DNA Binding—The apo-HrtR/DNA complex structure reveals how the apo-HrtR dimer binds target DNA. The helix-turn-helix motif of each protomer contacts two consecutive DNA major grooves (Fig. 3A), and α2 and α3 helices in the helix-turn-helix motifs interact with the target DNA (Fig. 5). Arg-46 (εNH) forms a hydrogen bond with a base (N7 of G11 (guanine 11)), which is the sole hydrogen bond involving the bases of DNA. The guanidium group of Arg-46 also forms hydrogen bonds with the phosphate backbone of T10 (thymine 10) and Tyr-50. There are also hydrogen bonds between the imidazole group of His-37 and the phosphate group of G9 (guanine 9), between the NH group of Ile-35 and the phosphate group of T10, between the backbone NH group of Met-36 and the phosphate group of T10, and between the OH group of Tyr-50 and the phosphate group of G11. In addition to these hydrogen bonds, a CH–π interaction should be present between the methyl group of T12 (thymine 12) and Tyr-50. The methyl group of T12 is oriented perpendicularly to the phenyl ring of Tyr-50, and the distance is 3.8 Å between the carbon atom of the methyl group of T12 and the Cγ of Tyr-50 (Fig. 5B), which are reasonable conditions for a CH–π interaction (29).

The structure of the apo-HrtR/DNA complex suggests that Arg-46 and Tyr-50 are responsible for the recognition of the DNA sequence specific for HrtR binding. To confirm this hypothesis, we carried out EMSA with R46A and Y50A variants. With a 1:1 ratio of HrtR dimer to DNA (0.5 μM), no band shift was observed for the R46A variant, indicating the loss of DNA binding activity of R46A variant. Under the same conditions, a smear band shift was observed for the Y50A variant (Fig. 6). Under these conditions, wild-type HrtR completely binds to the DNA to form the HrtR/DNA complex. These results show that Arg-46 and Tyr-50 play a crucial role for sequence-specific DNA binding using a hydrogen bond and a CH–π interaction with the bases G11 and T12 of the target DNA, respectively.

The Y50F variant also showed a smear band shift, as did the Y50A variant (Fig. 6). If both the CH–π interaction and the critical hydrogen bond between Arg-46 and G11 are still present...
ent in the Y50F variant, it should exhibit DNA binding comparable with the wild-type protein. However, this was not the case. The OH group of Tyr-50 forms hydrogen bonds with Arg-46 and the phosphate group of G11 (Fig. 5). These hydrogen bonds fix the orientation of the side chains of Arg-46 and Tyr-50 in pairs suitable for specific DNA binding. Thus, Arg-46 and Tyr-50, whose orientations are fixed in pairs by the hydrogen bond between them, are required for high affinity specific DNA binding of HrtR. HrtR is the first example of the TetR family of repressors that uses a pairwise residue to recognize the target DNA sequence.

Conformational Change of HrtR Induced by Heme Sensing—
The crystal structure of holo-HrtR revealed that the heme is accommodated in a large cavity that is open to solvent (Fig. 7, A and B), with His-72 and His-149 as the axial ligands to form a 6-coordinated heme. The α4, α5, α8, and α9 helices compose a hydrophobic heme pocket in the cavity, where the heme is surrounded by hydrophobic residues Ile-71, Phe-75, Phe-76, Leu-...
These hydrophobic interactions along with the axial ligation of His-72 and His-149 will be responsible for a high heme binding affinity of HrtR. Although a loop from Pro-125 to Glu-135 is located over the edge of the opening of the heme binding cavity on the protein surface in holo-HrtR, the electron density of this loop was not observed in apo-HrtR, probably due to disorder, suggesting flexibility of the loop from Pro-125 to Glu-135. This flexible loop may change its conformation over the entrance of the heme-binding cavity, by which the heme binding affinity would be regulated.

A comparison of the apo-HrtR-DNA complex (expressed as apo-HrtR hereafter unless otherwise noted) and holo-HrtR...
structures revealed that heme binding triggers a coil-to-helix transition at the α4a-α4b region (Fig. 8). In apo-HrtR, a loop (residues 68–71) intervenes between α4a and α4b helices. Glu-70 in the middle of this intervening loop forms a hydrogen bond with Trp-123 in apo-HrtR. Upon heme binding, a coil-to-helix transition occurs in this intervening loop, which results in the formation of a long α4 helix in holo-HrtR. As the location of Glu-70 is largely altered by this coil-to-helix transition, the hydrogen bond between Glu-70 and Trp-123 is lost in holo-HrtR as shown in Fig. 8.

**Hydrogen-bonding Networks at the Interface between the DNA-binding and Heme-binding Domains**—Heme binding caused not only the coil-to-helix transition, but also reconstruction of the hydrogen-bonding networks. The coil-to-helix transition at the α4a-α4b region resulted in the loss of the hydrogen bond between Glu-70 and Trp-123. The helical axis of the α1 helix was inclined by 22° upon heme binding (Fig. 9), which resulted in the formation of a hydrogen-bonding network among Tyr-18, Gln-26, Glu-67, and Gln-104 at an interface between the DNA-binding and heme-binding domains (Fig. 10). The hydrogen-bonding network among these residues may be responsible for the stabilization of the orientation of the DNA-binding domain in holo-HrtR. To test these hypotheses, we carried out *in vitro* DNA binding experiments with E70A, Y18F, and Y18F/Q26A/E67A/Q104A variants.

EMSAs revealed that these variants retained DNA binding ability in the apo-form, and the addition of 1 mol eq of heme was sufficient for these variants to dissociate from DNA, as is the case for wild-type HrtR (data not shown). Thus, the response to heme was not changed in these variants. Fluorescence polarization assays revealed that the DNA binding affinity was also not changed by these mutations. The $K_d$ value for the apo-HrtR-DNA complex was found to be 0.3, 0.3, and 0.6 nM for E70A, Y18F, and Y18F/Q26A/E67A/Q104A variants, respectively (data not shown). These results indicate that these hydrogen-bonding interactions do not play a crucial role for the regulation of heme-responsive DNA binding of HrtR. In other words, the formation of the hydrogen-bonding interactions among Tyr-18, Gln-26, Glu-67, and Gln-104 is not a driving force for the conformational change induced by heme binding.
Heme-responsive Change in CD Spectra of HrtR—Fig. 11A shows the CD spectra of DNA-free apo-HrtR and holo-HrtR. The development of the negative band at 208 nm was observed upon heme binding. Elongation of the length of helical segments causes an increase in the intensity of $\theta_{208}$ (30, 31). In the case of HrtR, the length of the $\alpha_4$ helix increased by the coil-to-helix transition upon heme binding. No increase of helical contents was observed except for the coil-to-helix transition at the $\alpha_4$ region. Thus, the change in CD spectra shown in Fig. 11A corresponds to the elongation of the $\alpha_4$ helix upon heme binding. These results indicate that the coil-to-helix transition at the $\alpha_4a-\alpha_4b$ region proceeds physiologically in solution and is not an artifact caused by crystal packing in the protein crystal.

CD spectra of H72A and H149A variants are shown in Fig. 11, B and C, respectively. Although a slight increase in the intensity of $\theta_{208}$ was observed for the H149A variant upon heme binding, the H72A variant showed no change in its CD spectra regardless of the presence or absence of heme. Given that the coil-to-helix transition at the $\alpha_4a-\alpha_4b$ region mainly contributes to the change in CD spectra upon heme binding,
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CD spectroscopy reveals that the coil-to-helix transition at the α4a-α4b region does not occur in H72A and H149A variants. These results indicate that the ligation of His-72 and His-149 in pairs to the heme is essential for the coil-to-helix transition at the α4a-α4b region, which plays a crucial role for the heme-responsive regulation of DNA binding activity of HrtR as shown below.

**Functional Regulation of HrtR with a Unique Heme-sensing Motif**—To elucidate the functional roles of the axial ligation to the heme, EMSAs were carried out with H72A and H149A variants. Although the apo-forms of the H72A and H149A variants retained the DNA binding activity, substitution of His-72 or His-149 with Ala rendered the variant HrtR DNA complexes more resistant to dissociation by added heme. In H72A and H149A variants, 100 mol eq of heme relative to HrtR caused little dissociation of the HrtR-DNA complexes (Fig. 12), whereas 1 mol eq of heme was sufficient for the dissociation of the wild-type HrtR-DNA complex. H72A and H149A variants bound heme with a molecular ratio of 1:1 for HrtR protomer to heme, as did wild-type HrtR (data not shown). Although both variants retained specific heme binding ability, their response to heme was lost for the dissociation of the HrtR-DNA complex. Thus, it is essential for the function of HrtR as a heme-responsive repressor that both His-72 and His-149 are coordinated to the heme iron to form a 6-coordinated heme.

**DISCUSSION**

Heme is one of the widely studied classes of the metal-containing prosthetic groups, which show a variety of functions in biological systems. As free heme molecules show cytotoxicity, heme homeostasis should be regulated strictly. Although numerous lactic acid bacteria, including *L. lactis*, acquire heme molecules exogenously to establish an aerobic respiratory chain, they cannot biosynthesize heme molecules due to the lack of the enzymes for heme biosynthesis. Although the regulation of efflux of excess heme molecules out of an *L. lactis* cell is reported to be the key process to maintain heme homeostasis in *L. lactis*, the detailed regulatory mechanism remains to be elucidated (10, 15). In this study, we have elucidated the molecular mechanisms of heme-responsive transcriptional regulation for the heme-efflux system in *L. lactis* based on the crystal structures of the heme-sensing transcriptional regulator HrtR.

We found that apo-HrtR binds to the target DNA in vitro. Assuming that an *L. lactis* cell is a sphere with a 0.8-μm diameter (32), the cell volume is estimated to be ~3 × 10^{-16} liters. A single copy of a gene or a protein in the same volume corresponds to a concentration of 6 nM. As the *K_d* of the apo-HrtR-DNA complex is 0.2 nM, the operator site of the *hrtRBA* operon will be occupied at all times with apo-HrtR if apo-HrtR is present at any level in an *L. lactis* cell. Under these conditions, therefore, the most physiologically important process for the functional regulation of HrtR is the dissociation of HrtR from DNA upon heme sensing. Once derepression of the *hrtRBA* operon occurs upon heme sensing, the concentration of DNA-free apo-HrtR increases along with the expression of the heme-efflux system, HrtBA. DNA-free apo-HrtR also binds heme molecules to form holo-HrtR, which would contribute to avoiding the toxic effect of free heme molecules.

HrtR adopts a unique heme-sensing motif. The heme regulatory motif (HRM or CP motif) that consists of a Cys-Pro sequence is known to be a heme-sensing and heme-binding motif in heme-responsive signal transducers and regulators (33, 34). HrtR adopts bis-His ligation to the heme as the heme-sensing and heme-binding motif instead of the CP motif.
Although bis-His ligation to the heme is widely found in many cytochromes acting as electron transfer proteins, this has not been the case for proteins whose function is regulated by heme sensing. HrtR is the first example of a heme-responsive regulatory protein that does not adopt the CP motif for heme sensing and heme binding.

The crystal structures of HrtR determined in this study reveal that heme binding to apo-HrtR triggers a conformational change that regulates the DNA binding activity of HrtR. The change in relative orientation of the DNA-binding domain is induced upon heme binding with a rigid-body motion of the DNA-binding domain, which is not driven by formation of a hydrogen bond network among Tyr-18, Gln-26, Glu-67, and Gln-104. The coil-to-helix transition of the α4 helix upon heme binding causes relocation of the α3 helix and the rigid-body motion of the DNA-binding domain. Interactions in the hydrophobic core consisting of Leu-9, Val-17, Phe-25, Ile-35, Ile-38, Val-39, Leu-42, Ile-44, Phe-49, Phe-53, and Leu-56 are responsible for maintaining the integrity of the structure of the DNA-binding domain (Fig. 13).

The change in relative orientation of the DNA-binding domain upon heme binding resulted in a change in the center-to-center distance between the recognition helices in the helix-turn-helix DNA-binding motif of each subunit. The distance between the Cα atoms of Tyr-50 in the α3 helix of each subunit increased upon heme binding from 35 to 47 Å. As the distance between two consecutive major grooves in B-DNA is 34 Å, the interhelical distance between the recognition helices in holo-HrtR (47 Å between the Cα atom of Tyr-50 in the α3 helix) is too wide for the recognition helices to fit into two consecutive major grooves and to bind to the target DNA. Thus, the coil-to-helix transition induced by the coordination of His-72 and His-149 to the heme shifts HrtR from a DNA binding-competent conformation to one that is not.

The helical axis of the α4b helix was inclined by about 15° by the coil-to-helix transition upon heme binding (Fig. 9), by which the distance between the Ne atoms of His-72 and His-149 decreased from 9 to 4 Å. As His-72 is located in the N terminus of the α4b helix in apo-HrtR, the ligation of His-72 to the heme may cause a rigid-body movement of the α4b helix to trigger the formation of a helix in the intervening loop between the α4a and α4b helices. As shown in Fig. 8, the location of His-72 is significantly altered by the coil-to-helix transition upon heme binding, although this is not the case for His-149. These structural features suggest functional roles of His-149 and His-72 as an “anchor” to fix the heme at the proper position, and as a “switch” to trigger the coil-to-helix transition, respectively.

Although two histidines, His-72 and His-149, play an important role for heme sensing as described above, H72A and H149A variants retain the DNA binding activity. Lechardeur et al. (15) reported that H72A and H149L variants lost their ability to bind DNA, which is inconsistent with our results. They used a tagged HrtR in which maltose-binding protein (MBP) was fused at the N terminus of HrtR. As the N terminus of HrtR is located near the DNA recognition helix (α3) in the helix-turn-helix motif, as shown in Fig. 3, MBP fused to the N terminus may interfere with the interaction between

![FIGURE 13. Hydrophobic interactions in the core of the DNA-binding domain for DNA-bound apo-HrtR (A) and holo-HrtR (B) (stereo view).](image)
the α3 helix and the target DNA by steric hindrance and/or perturbation of the conformation of the DNA-binding domain. As wild-type MBP-HrtR, however, shows formation of the MBP-HrtR-DNA complex (15), the reasons for this discrepancy remain unclear for the DNA binding ability of H72A and H149L variants.

The crystal structures of HrtR reveal that HrtR is a member of the TFR family. The closest structural homologues of apo-HrtR and holo-HrtR were identified using the DALI server (35). The top three hits were a TFR from Chloroflexus aurantiacus (PDB code 3NRG, Z-score of 11.0, and an r.m.s.d. of 4.2 Å), E. coli AcrR (PDB code 3BCG, Z-score of 11.0, and an r.m.s.d. of 3.9 Å), and Pseudomonas aeruginosa DesT (PDB code 3LSR, Z-score of 11.0, and an r.m.s.d. of 3.3 Å) for apo-HrtR and a TFR from Streptomyces coelicolor (PDB code 2QIB, Z-score of 12.9, and an r.m.s.d. of 3.3 Å), a transcriptional regulator from Bacillus subtilis (PDB code 1V10, Z-score of 12.7, and an r.m.s.d. of 3.3 Å), and Staphylococcus aureus QacR (PDB code 3BTL, Z-score of 12.5, and an r.m.s.d. of 2.9 Å) for holo-HrtR. Among these homologues, detailed structure and function relationships are reported only for AcrR (36–38), DesT (39, 40) and QacR (41, 42).

Although these TFRs bind different ligands (many structurally unrelated compounds such as ethidium, proflavin, and rhodamine 6G for AcrR (36), saturated fatty acid-CoA and unsaturated fatty acid-CoA for DesT (39, 40), and numerous structurally dissimilar monovalent and bivalent cationic and lipophilic compounds for QacR (41, 42)), their ligand-binding cavities are similarly positioned in the ligand-binding domain (28). Ligand binding results in a conformational change of the DNA-binding domain, for which the relocation of α6 helix upon ligand binding induces a pendulum-like motion of the DNA-binding domain in TFRs including TetR (43), QacR (41), and DesT (39).

FIGURE 14. A and B, ribbon model of the dimeric structure for E. coli apo-AcrR (PDB code 3BCG) (A) and DNA-bound apo-HrtR (8) (DNA is omitted for clarity). The α4a-loop–α4b region is shown in green and yellow for AcrR and HrtR, respectively. C, overlay of the protomer for apo-AcrR (green and light green) and apo-HrtR (yellow and orange) shown in a cylinder model.
Although the position of the ligand-binding cavity in HrtR is similar to that of other TFRs, HrtR adopts a unique mechanism by which ligand binding causes a conformational change for HrtR derepression. In the case of HrtR, the coil-to-helix transition at the α4a-loop-α4b upon ligand binding induces the relocation of the DNA-binding domain, which results in HrtR derepression. HrtR is the first example of TFR in which the coil-to-helix transition of α4 helix triggers a conformational change for derepression.

Ligand-free AcrR, a member of TFR from E. coli, has an α4a-loop-α4b structural motif similar to that in apo-HrtR (37, 38) (Fig. 14). As no crystal structure of ligand-bound AcrR has been solved, it is not clear whether or not ligand binding causes a helix-to-coil transition at the α4a-loop-α4b in AcrR as it is the case with HrtR. However, the structure of ligand-free AcrR suggests that a similar mechanism to that of HrtR is adopted in some subgroups of TFR, in which the coil-to-helix transition at the α4a-loop-α4b triggered by ligand binding plays a crucial role for the regulation of their activities.

Acknowledgments—We thank Dr. Tetsunari Kimura of the Institute for Molecular Science for the discussion on CD spectra, Drs. Koki Makabe and Takashi Nakamura and Prof. Kunihiro Kuwajima of Okayama Institute for Integrative Bioscience for the help in measuring CD spectra, and Prof. Hiroshi Nakajima and Yoshikito Watanabe of Nagoya University for the help in doing fluorescence polarization assays at the initial stage.

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