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Regulation of gene expression by a heme as a signaling molecule in *Lactococcus lactis*

Shigetoshi AONO

National Institutes of Natural Sciences, Okazaki Institute for Integrative Bioscience

Research aims

Lactococcus lactis undergoes a metabolic shift from fermentation to respiration in an aerobic environment when heme is supplied[1]. 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. As free heme molecules are cytotoxic, some regulatory system is required to avoid this toxicity. In *L. lactis*, a heme-efflux system is reported to be responsible for heme tolerance and hemeostasis. Though a transcriptional regulator HrtR will regulate the expression of the heme-efflux system, the detailed mechanisms remain to be elucidated. In this work, I have determined the crystal structures of HrtR in heme-free (apo-), heme-bound (holo-), and DNA-bound forms with biochemical and spectroscopic characterization, based on which I propose a heme-responsive regulatory mechanism of HrtR responsible for transcriptional regulation of heme homeostasis.

Methods

The *hrtR* gene synthesized by GenScript was inserted between the *Nde*I and *Bam*HI sites of pET3a to construct an expression vector for HrtR, by which an intact HrtR is expressed without any tag sequences. The codons in the synthesized *hrtR* gene were optimized for protein expression in *Escherichia coli*. X-ray diffraction data were collected on BL26B1, BL26B2, and BL41XU at the SPring-8.

Results & Discussion

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 equivalent 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[2]. 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.

Electrophoretic mobility shift assays (EMSAs) revealed that apo-HrtR can directly interact with the putative operator-promoter region of the *hrtRBA* operon (Fig. 2)[2]. The HrtR-binding sequence (5′-ATGACACAGTGTCAT-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 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 equivalent 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, while 50 mol equivalent excess of unlabeled non-target DNA did not show any competition. These competition assays revealed that the binding of HrtR to tar-

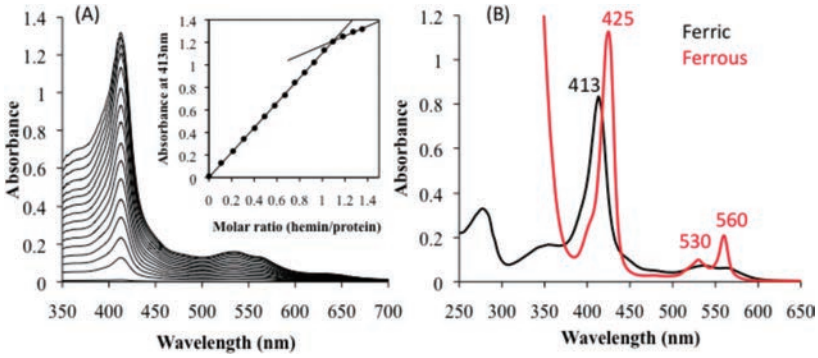

Figure 1 (A) Heme titration into apo-HrtR (B) Electronic absorption spectra of holo-HrtR

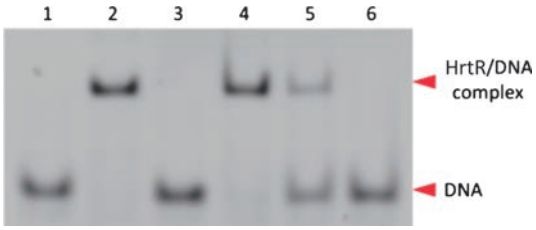

Figure 2 EMSA of HrtR
Lane 1: free DNA
Lane 2: apo-HrtR, Lane3: holo-HrtR
Lane 4: apo-HrtR + 0.2 eq. hemin
Lane 5: apo-HrtR + 0.5 eq. hemin
Lane 6: apo-HrtR + 1.0 eq. hemin

get DNA is specific.

Next the regulatory role of heme for DNA-binding was studied by EMSAs and fluorescence polarization assays. The addition of 1 mol equivalent of heme relative to HrtR protomer was sufficient to fully dissociate HrtR from DNA (Fig. 2). 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*.

To obtain structural insights into the heme-responsive regulatory mechanisms of HrtR function, the crystal structures of apo-HrtR, holo-HrtR, and the apo-HrtR/DNA complex have been determined at resolutions of 3.1, 1.9, and 2.0 Å for apo-HrtR, holo-HrtR, and the apo-HrtR/DNA complex, respectively[2]. The global fold of HrtR is similar to that of TetR family transcriptional regulators that posses nine conserved helices, within which helices 1 to 3 comprise the DNA-binding domain, and helices 4 to 9 comprise the ligand-binding domain. In addition to the nine conserved helices, HrtR possesses extra short helices at the N- and C-termini, though it is not clear due to disorder if there is an α_C helix in holo-HrtR. The DNA-binding domain and the heme-sensing (heme-binding) domain consist of α_N, α1, α2, and α3 helices, and α4, α5, α6, α7, α8, α9, and α_C helices, respectively. The interface between the DNA-binding and heme-binding domains is formed through interactions among α1, α4, and α6 helices (see below). The α8 and α9 helices in each subunit were found to form a four-helix bundle that constitutes a dimer interface.

The crystal structure of holo-HrtR revealed that the heme is accommodated in a large cavity that is open to solvent, with His72 and His149 as the axial ligands to form a 6-coordinated heme. 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. 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 C^{α} atoms of Tyr50 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 inter-helical distance between the recognition helices in holo-HrtR (47 Å between the C^{α} atom of Tyr50 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 His72 and His149 to the heme shifts HrtR from a DNA-binding competent conformation to one that is not.

Conclusion

In this work, it has been reported that the transcriptional 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, the crystal structures of the apo-HrtR/DNA complex, apo-HrtR, and holo-HrtR have been determined.

References

1) Pedersen, M. B. *et al.* (2008) Impact of aeration and heme-activated respiration on *Lactococcus lactis* gene expression: identification of a heme-responsive operon. *J. Bacteriol.* **190**: 4903–4911.
2) Sawai, H., Yamanaka, M., Sugimoto, H., Shiro, Y., and Aono, S. (2012) Structural basis for the transcriptional regulation of heme homeostasis in *Lactococcus lactis*. *J. Biol. Chem.* **287**: 30755-30768.