Structure of an Atypical Orphan Response Regulator Protein Supports a New Phosphorylation-independent Regulatory Mechanism*

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Two-component signal transduction systems, commonly found in prokaryotes, typically regulate cellular functions in response to environmental conditions through a phosphorylation-dependent process. A new type of response regulator, hp1043 (HP-RR) from Helicobacter pylori, has been recently identified. HP-RR is essential for cell growth and does not require the well-known phosphorelay scheme. Unphosphorylated HP-RR binds specifically to its own promoter (P1043) and autoregulates the promoter of the gene tlpB from H. pylori, has been recently identified. HP-RR is essential for cell growth and does not require the well-known phosphorelay scheme. Unphosphorylated HP-RR binds specifically to its own promoter (P1043) and autoregulates the promoter of the gene tlpB from H. pylori.

Two-component systems are the predominant signal transduction systems used by prokaryotes and are frequently involved in the regulation of cellular functions in response to variable environmental conditions. These systems exhibit a phosphorylation process, a sensor histidine kinase to an intracellular response regulator protein (RR), which typically acts as a transcription regulator. The environmental stimulus triggers the autophosphorylation of the transmitter domain of histidine kinase and then the phosphate group is transferred to an aspartate residue in the N-terminal regulatory domain of RR. Phosphorylation induces a conformational change resulting in dimerization of RR and binding to the promoter of target genes. RR is a conserved N-terminal regulatory domain and a variable C-terminal transactivation domain. The transactivation domains can be divided into three major subfamilies based on the homology of their DNA-binding region: the OmpR/PhoB winged-helix domain (2–4), the NarL/FixJ four-helix domain (5, 6), and the NtrC ATPase-coupled transcription factors (7). Other response regulator proteins, which are classified as a fourth subfamily, contain different effector domains, such as enzymes.

In most RR, phosphorylation creates conformational changes in a conserved region, the β3-α4 loop and the α4 helix. In CheY, the phosphate provides hydrogen bonds to the Thr106 O-γ and the Ala108 amide nitrogen, which then provide space for Tyr106 to access the interior, resulting in a stabilizing hydrogen bond between the Tyr106 hydroxyl and the Glu89 carbonyl oxygen in the loop. This conformational change drives the conformational change of the α4-β5-α5 face of the regulatory domain that in turn promotes dimerization. A conserved tyrosine (corresponding to Tyr106 of CheY) is the key residue indicating the status of an RR: the inward position, in which the hydroxyl group points toward the N terminus of helix α4; is found in the active state, whereas the outward position is found in the inactive state. It is well known that dimerization allows or increases DNA binding to the promoter recognition element (8).

Recent analysis of the H. pylori genome sequence revealed the presence of four two-component systems and two orphan proteins. Two-component systems are the predominant signal transduction systems used by prokaryotes and are frequently involved in the regulation of cellular functions in response to variable environmental conditions. These systems exhibit a phosphorylation process, a sensor histidine kinase to an intracellular response regulator protein (RR), which typically acts as a transcription regulator. The environmental stimulus triggers the autophosphorylation of the transmitter domain of histidine kinase and then the phosphate group is transferred to an aspartate residue in the N-terminal regulatory domain of RR. Phosphorylation induces a conformational change resulting in dimerization of RR and binding to the promoter of target genes. RR is a conserved N-terminal regulatory domain and a variable C-terminal transactivation domain. The transactivation domains can be divided into three major subfamilies based on the homology of their DNA-binding region: the OmpR/PhoB winged-helix domain (2–4), the NarL/FixJ four-helix domain (5, 6), and the NtrC ATPase-coupled transcription factors (7). Other response regulator proteins, which are classified as a fourth subfamily, contain different effector domains, such as enzymes.

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response regulators (9). Based on structural and functional homologies, one of these pairs (analogs of the CheA-CheY proteins) may play a role in regulating random tumbling motions and flagellar rotation in response to signals from chemotaxis receptors (10, 11). The other three pairs of histidine kinase and RR proteins (HP0165-HP0166, HP1364-HP1365, and HP0244-HP0703) and two RR proteins (HP1043 and HP1021) are predicted to be involved in transcriptional regulation (12). The hp1043 and hp1021 genes encode orphan RRs, for which no histidine kinases have been identified (12, 13). The orphan response regulator HP1043 (HP-RR) has been shown to be essential for the growth of H. pylori (14).

HP-RR binds to its own promoter and performs an autoregulatory function (13, 15). Interestingly, a recent report revealed that the regulatory domain (HP-RR') is not phosphorylated in vitro and phosphorylation is not necessary for its function, indicating that HP-RR differs from the well known two-component response regulators. HP-RR has thus been classified as belonging to a new response regulator family (14, 15). According to structural studies of the ArcA regulatory domain, it is suggested that the OmpR/PhoB family has a common dimerization mechanism mediated by the α4-β5-α5 interface with the participation of well conserved residues (16). However, HP-RR has 5 residue substitutions of 11 conserved residues, which could affect the interactions of the dimer interface. A recent report showed that PhoB adopts two different dimerization modes: the inactive α1-α5 interface dimer and the active α4-β5-α5 interface dimer (17). These reports raise questions about the structure-function relationships of HP-RR related to dimerization and activation. Previous studies proposed that HP-RR forms a dimer in vivo (13). We have also reported preliminary NMR data showing that HP-RR is a symmetric dimer with two functional domains, an N-terminal regulatory domain (~14 kDa) and C-terminal DNA-binding/transactivation domain (~11 kDa) (18). Additionally, ultracentrifugation data also support that HP-RR is a stable dimer in solution. In this report, we present a detailed three-dimensional structure of HP-RR, a new class of phosphorylation-independent response regulator, using NMR spectroscopy, x-ray crystallography, and site-directed mutagenesis. The structural information presented here will promote understanding of the molecular function of this new type of response regulator.

### TABLE 1

| Structural statistics of HP-RR | HP-RR | HP-RR' | HP-RR'' |
|-------------------------------|-------|--------|---------|
| **NOE distance restraints**    |       |        |         |
| All                           | 3299  | 2622   | 1817    |
| Intraresidue                  | 778   | 673    | 458     |
| Sequential (i–j = 1)          | 932   | 622    | 433     |
| Medium range (2 ≤ i–j ≤ 5)    | 717   | 491    | 416     |
| Long range (i–j > 5)          | 872   | 782    | 510     |
| Intermolecular                | 52    | 54     |         |
| Hydrogen bonds distance restraints* (No.) | 93 | 56 | 38 |

**Ramachandran plot (%)**
- Most favored regions: 84.2%
- Additional allowed regions: 12.0%
- Generously allowed regions: 2.7%
- Disallowed regions: 1.1%

**X-ray (HP-RR)**
- Space group: P2₁2₁2₁
- Unit cell parameters: *a* = 89.0, *b* = 41.5, *c* = 36.7 Å
- Resolution: 1.5 Å
- R_{free} (%) = 9.4 (39.5)
- R-factor (%) = 20.6 (25.5)
- Root mean square deviation bond length (Å): 0.0052
- Root mean square deviation bond angle (%): 1.1673

**Mean root mean square deviations from the average coordinate**
- Backbone atoms (N, Cα, C, O): 1.199 Å, 0.31 Å, 0.24 Å
- Heavy atoms: 1.838 Å, 0.78 Å

**Additional restraints**
- Hydrogen bonds distance: Intermolecular 52, Long range 54, Medium range 54

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**TABLE 2**

**Structural statistics of HP-RR**

**A**

**Promoter DNA sequence (5' → 3')**

| DNA Sequence | Relative Binding (%) |
|-------------|----------------------|
| D1          | 100                  |
| D2          | 100                  |
| D3          | 100                  |
| D4          | 100                  |
| D5          | 100                  |
| D6          | 100                  |
| D7          | 100                  |
| D8          | 100                  |
| D9          | 100                  |

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**B**

**Relative Binding (%)**

**FIGURE 1**

**Binding affinities of HP-RR for different DNA sequences.** A. DNA sequences (D1–D8) are displayed together with that of the consensus sequence. The DNA sequence with a blue box showed higher affinity to HP-RR than its own promoter sequence. B. Electrophoretic mobility shift assay experiments were performed with HP-RR and labeled target DNA. Binding activities were determined by the signal intensity relative to that of the wild-type DNA.
EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—Plasmid construction, protein expression, and purification of full-length HP-RR was performed as described (18). DNA fragments encoding the N-terminal regulatory domain, HP-RRr (residues 1–119), and the C-terminal effector domain, HP-RRe (residues 120–223), were inserted into pET-21a(+/H11001) with a C-terminal (His)₆ tag and into pET-21b3-2 (GE Healthcare) with an N-terminal (His)₆ tag. These vectors were used to transform the Escherichia coli strain BL21(DE3) (Invitrogen) for fusion protein expression. HP-RRr and HP-RRe were overexpressed and purified according to the methods used for full-length HP-RR (18). For x-ray crystallography, selenomethionine-substituted HP-RRr was prepared by transforming E. coli B834(DE3) methionine auxotroph cells (Novagen) with the vector containing HP-RRr and growing the cells in selenomethionine-containing minimal medium.

Site-directed Mutagenesis—Mutations were introduced into both HP-RR and HP-RRe using an overlapping PCR strategy. Two PCRs were performed using pET21b3-2 as templates. The PCR products were purified with QIAquick (Qiagen), mixed together, and used as the templates in the final stage. All mutant proteins were expressed and purified as described above.
Structure of a New Response Regulator hp1043

FIGURE 3. NMR structures of the DNA-binding domain and chemical shift mapping of the residues important for DNA binding. A, stereoview of the backbone traces from the final ensemble of 20 solution structures. The α-helices are displayed in orange and β-sheets in blue. B, electrostatic potential surface of the transactivation domain is displayed. Red, blue, and white colors represent negative, positive, and neutral electrostatic potential, respectively. The backside view of the molecule rotated by 180° around the vertical axis is also shown. Residues important for DNA binding are labeled. C, chemical shift change of the effector domain upon DNA binding. The chemical shift changes are calculated by using the equation: \( \Delta \delta_{\text{tot}} = (\langle \Delta \delta_{\text{prot}} W_{\text{prot}} \rangle)^2 + (\langle \Delta \delta_{\text{res}} W_{\text{res}} \rangle)^2 \), where \( \delta_{\text{i}} \) is the chemical shift of nucleus i, and W denotes its weight factor (\( WN_{\text{H}} N_{\text{W}} = 1 \), \( WN_{\text{H}} N_{\text{C}} = 0.2 \)). D, residues that exhibit significant chemical shift perturbation upon DNA binding. Magenta indicates \( \Delta \delta_{\text{tot}} > 0.2 \), and yellow indicates 0.1 < \( \Delta \delta_{\text{tot}} < 0.2 \).

Electrophoretic Mobility Shift Assay—A double-stranded phosphorothioate oligonucleotide was synthesized with the following sequence: 5'-ATTATATTTTTCTAATTTAAAAT-3'. DNA was end-labeled with 20 units of T4 polynucleotide kinase and 250 μCi of \([γ-^{32}P]ATP\). Labeled DNA was purified with a Qiagen nucleotide removal kit. To measure DNA binding, purified HP-RR and HP-RR' were incubated with radiolabeled DNA at room temperature for 30 min in the presence of 0.2 μg of poly(dI-dC). DNA complexes were separated on a 5% native polyacrylamide gel in 1× Tris glycine (10% Tris glycine) at 140 V for 4 h and visualized by autoradiography.

NMR Spectroscopy—All NMR experiments were recorded at 303 K on a Bruker DRX500 or a DRX600 equipped with an xy,z-shielded gradient triple resonance probe or a z-shielded gradient triple resonance cryoprobe. NMR spectra were processed with the NMRPipe/nmrDraw software package (19) and analyzed using SPARKY (20). Backbone resonance data of HP-RR were collected by using deuterium-decoupled TROSY-based triple resonance pulse sequences (21). Data for HP-RR' and HP-RR'' were collected by conventional triple resonance pulse sequences. Three-dimensional experiments used for sequential assignments included HNCO, HNCACB, CBCA(CO)NH, HBHA(CO)NH, C(CCCO)NH, H(CCCO)NH, and HCH-TOCSY. For structural information, \( ^{15}N \)-edited and \( ^{13}C \)-edited NOESY-HSQC spectra were used. Dihedral backbone restraints were derived from \( ^{1}H_{\text{a}}, ^{13}C_{\text{a}}, ^{13}C_{\text{β}}, \) and \( ^{13}C_{\text{β}} \) chemical shifts using the program TALOS (22). The hydrogen bond restraints were determined from hydrogen exchange experiments on amide protons. Residual dipolar couplings were measured by taking the difference in the corresponding /splittings in oriented and isotropic media and \( ^{1}H_{\text{a}} \) dipolar coupling constants were obtained using a two-dimensional in-phase or anti-phase \( ^{1}H_{\text{a}}{^15}N \) HSQC spectra in a liquid crystalline medium (23).

NMR Structure Calculation—Initial structure calculations were performed using CYANA 2.0 (24, 25). The structures were refined with NIH-XPLOR (version 2.9.7) software (26) by a combination of torsion angle and Cartesian dynamics. A total of 100 structures were calculated, and 20 structures with the lowest target function values were selected for structural analysis. A summary of NMR-derived restraints and structures of HP-RR, HP-RR', and HP-RR'' is given in Table 1. The final structures with the lowest NOE energies were validated by the program PROCHECK (27). Solution structures were analyzed and visualized using the programs VMD-XPLOR (28), PyMOL (29), and MOLMOL (30). The electrostatic surface potential was calculated with the program APBS (31).

Crystallization and Structure Determination—The purification and crystallization of HP-RR' were carried out as described previously (32). Selenomethionine-substituted HP-RR' crystals were also obtained under the same crystallization conditions with the addition of 5 mM dithiothreitol. Before data collection, the crystals were immersed briefly in a cryoprotectant solution, which was the reservoir solution plus 15% ethylene glycol. A three-wavelength MAD data set of the selenomethionine-labeled crystal based on 200 images (1° rotation) was collected at 0.9794 Å (edge wavelength), 0.97929 Å (peak wavelength), and 0.97162 Å (remote wavelength). All data were collected at beam line 6B of the Pohang Accelerator Laboratory (PAL) in Pohang,
Korea using a Bruker AXS proteum300 CCD detector (Bruker, Madison, WI). The data were then indexed, integrated, and scaled using the HKL2000 suite (33). Four selenium sites in the asymmetric unit were located and used for phase determination at 2.2 Å with the program SOLVE (34). The phases were subsequently improved by density modification with the program RESOLVE (35). The program O (36) was used for the electron density model building. The 1.8-Å peak wavelength anomalous dispersion data were re-averaged and used for the refinement of the model with the CNS program package (37). Statistics for crystallographic data are summarized in Table 1.

**RESULTS**

Characterization of DNA Binding of HP-RR—A recent report proposed that unphosphorylated HP-RR specifically binds to a 29-mer target DNA containing its own promoter sequence (13), which is dependent upon the growth phase at the post-transcriptional level. We have determined the promoter DNA sequences (ATTAATTTTCTTAACTAATTAAAAT) important in binding to HP-RR based on data from electro-photometric mobility shift assays (Fig. 1, A and B). HP-RR has well conserved residues in its transactivation domain, just like OmpR (2), PhoB (4, 39), and the DrrD. As expected, unphosphorylated HP-RR successfully bound to its target DNA sequences (ATTAATATTTTCTTAAACTAATTTAAAAT) with a central five-stranded β-sheet surrounded by five α-helices in each monomer unit (Fig. 2, A and B). HP-RR has well conserved residues in its transactivation domain, just like OmpR (2), PhoB (4, 39), and the DrrD. As expected, unphosphorylated HP-RR successfully bound to its target DNA sequences (Fig. 1B). Interestingly, the protein had increased affinity for binding DNA mutated at the 11th position (D4) (Fig. 1B).

Structure of HP-RR Dimer—Based on gel filtration chromatography and cross-linking experiments, HP-RR is a symmetric dimer. A superposition of the final 20 NMR structures over the energy minimized average structure shows that it consists of 12 β-strands and 8 α-helices in each monomer unit (Fig. 2A). The molecular topology of the HP-RR resembles that of the OmpR/PhoB subfamily with a 2-fold symmetry in the absence of phosphorylation. The N-terminal regulatory domain forms a compact dimer and the transactivation domain is connected to the regulatory domain by a short flexible linker (Fig. 2, B and C). A total of 51 intermolecular NOEs for the dimer interface are observed between two subunits (e.g. Thr274(A)/Arg100(B), Ser80(A)/Ser101(B), Ser80(A)/Ala104(B), Val84(A)/Ala104(B), and Arg108(A)/Arg108(B)). However, nonintermolecular interactions between transactivation domains have been observed. The molecular topology of the regulatory domain is an α/β-fold with a central five-stranded β-sheet surrounded by five α-helices. The electrostatic surface of HP-RR mainly consists of two oppositely charged regions, which differs slightly from PhoB.
An ensemble of the 20 lowest energy NMR structures of the transactivation domain is displayed in Fig. 3A. The transactivation domain (HP-RR) consists of an N-terminal four-stranded anti-parallel β sheet (strands β6-β9) and a helix bundle with three α-helices (α6, α7, and α8). The β-hairpin between α6 and α7 enables close interactions with β10. The two helices comprised of α7 and α8 form a helix-turn-helix DNA binding motif. The recognition helix (α8) is also very well defined. The electrostatic surface showed that the transactivation domain consists of two distinct regions with opposite charge distribution (Fig. 3B). The loop between α7 and α8, referred to as a transcription loop for both transcription activation and interaction with the σ20 subunit of the RNA polymerase, is identified. Similar to PhoB/OmpR family proteins, the DNA-binding domain is also stabilized by a conserved hydrophobic core. Comparing the structure of HP-RR with OmpR and PhoB, the root mean square deviations of the Cα atoms of the secondary structural regions are 3.638 Å (OmpR) and 2.146 Å (PhoB, data not shown). Most of the differences originate from the transactivation and winged loop regions (Fig. 3B). To map the DNA-binding site and residues critical for DNA binding, chemical shift perturbations were measured by NMR titration with its consensus DNA. The most significant chemical shift changes were observed for the residues of α8, a recognition helix as shown in Fig. 3, C and D.

Dimeric Interface of HP-RR—The crystal structure clearly shows that the 2-fold symmetric dimeric interface is stabilized by the α4-β5-α5 interface that buries 1610 Å² of surface area (800 Å² per monomer) (Fig. 4A). The dimeric interface of the HP-RR regulatory domain is similar to that of the active, phosphorylated protein of ArcA (16) and PhoB (17); however, the electrostatic interactions and the hydrophobic patch are different from that of active PhoB, which is composed of four salt bridges and three hydrophobic residues. This might be due to amino acid substitutions in the consensus sequence of the dimeric interface. Residues Arg91, Glu111, Lys117, Arg122, and Leu95 in PhoB are replaced by Glu83, Ala104, Glu110, Phe115, and Phe87 in HP-RR, respectively. In addition, solution structure of the regulatory domain based on NMR data confirmed that HP-RR forms a stable dimer through both electrostatic and hydrophobic interactions. An electrostatic charge network among charged side chains forms three salt bridges: Asp93–Arg108, Glu83–Arg108, and Arg100–Glu83 (Fig. 4B). The interaction is consolidated by inter-subunit hydrogen bonds of the Arg112 side chain with the carbonyl oxygen of Asp92 and the side chain of Arg108 with the carbonyl oxygen of Tyr94. In addition, the hydrophobic interactions among residues Val84, Phe87, Ala104, Ala107, and Ala111 serve as a major stabilizing force for the HP-RR dimer (Fig. 4B). Therefore, it is conceivable that the ionic interactions could serve as a driving force for the initial dimerization process of HP-RR, whereas the hydrophobic interactions contribute to dimer stability.

Protein Dynamics of HP-RR—NMR relaxation data were analyzed within the model-free formalism of protein dynamics and indicate an extensive reduction in backbone motion for the residues in the interface region. These effects are reflected by an increase in the generalized order parameter, S², of the residues in the interface region. These results imply that the dynamics of the dimer illuminates the relative contributions of charged and hydrophobic interactions between these residues. Hydrophobic interactions between the residues in the interface region, such as Val84, Phe87, Ala104, and Ala107 resulted in high order parameters for these residues (Val84, S² = 0.84; Phe87, S² = 0.92; Ala104, S² = 1; Ala107, S² = 0.99; Ala111, S² = 0.99) (Fig. 4C). Also, interactions between the charged residues such as Glu83/Arg108 and Asp93/Arg112 resulted in high order parameters for these residues (Glu83, S² = 0.94; Asp93, S² = 0.94; Arg108, S² = 0.98; Arg112, S² = 0.96). The R₂/R₁ ratio is 1.54 ± 0.498 for the regulatory domain, and 5.34 ± 0.527 for the transactivation domain, indicating that the regulatory domain forms a rigid dimer, whereas the transactivation domain acts as a monomer. The S² from backbone data of the transactivation domain averages 0.8882 ± 0.013 for all residues (data not shown). The NH exchange rates of the residues in the solvent exposed loops are higher than that of those in regions of secondary structure. However, Ile105, Ala96, and Val86 show relative high exchange rates indicating that the dimeric interface is mainly comprised of side chain-side chain interactions. In addition, the relaxation rates are relatively uniform across the whole transactivation domain except some residues.

Site-directed Mutagenesis Supports Structural Data of HP-RR—Based on structural and sequence information, mutational analysis for both the regulatory and transactivation domains was performed (Fig. 5A). Seventeen mutant proteins were prepared to study both structural stability and DNA binding, although only 10 mutants are correctly folded and purified. Because phenylalanine 87 in the dimeric interface is a leucine or alanine residue in all other response regulator proteins, we constructed F87L and F87A mutants to examine a correlation—The crystal structure clearly shows that the 2-fold symmetric dimeric interface is stabilized by the α4-β5-α5 interface that buries 1610 Å² of surface area (800 Å² per monomer) (Fig. 4A). The dimeric interface of the HP-RR regulatory domain is similar to that of the active, phosphorylated protein of ArcA (16) and PhoB (17); however, the electrostatic interactions and the hydrophobic patch are different from that of active PhoB, which is composed of four salt bridges and three hydrophobic residues. This might be due to amino acid substitutions in the consensus sequence of the dimeric interface. Residues Arg91, Glu111, Lys117, Arg122, and Leu95 in PhoB are replaced by Glu83, Ala104, Glu110, Phe115, and Phe87 in HP-RR, respectively. In addition, solution structure of the regulatory domain based on NMR data confirmed that HP-RR forms a stable dimer through both electrostatic and hydrophobic interactions. An electrostatic charge network among charged side chains forms three salt bridges: Asp93–Arg108, Glu83–Arg108, and Arg100–Glu83 (Fig. 4B). The interaction is consolidated by inter-subunit hydrogen bonds of the Arg112 side chain with the carbonyl oxygen of Asp92 and the side chain of Arg108 with the carbonyl oxygen of Tyr94. In addition, the hydrophobic interactions among residues Val84, Phe87, Ala104, Ala107, and Ala111 serve as a major stabilizing force for the HP-RR dimer (Fig. 4B). Therefore, it is conceivable that the ionic interactions could serve as a driving force for the initial dimerization process of HP-RR, whereas the hydrophobic interactions contribute to dimer stability.

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gel filtration high pressure liquid chromatography, and our structure predicts it would disturb the dimeric interface. However, the F87A mutant expresses as an inclusion body, suggesting that the size of the hydrophobic residues is of importance for hydrophobic interactions, which might be critical for protein folding. It is interesting to see that even though F87L is a monomer, it retains DNA binding ability like wild-type, whereas a dimerization-induced conformational change allows DNA binding in the case of the PhoB and NarL response regulator proteins (17, 40). In addition, mutation of the DNA-binding domain does not affect dimerization of the regulatory domain. Taken together, these data suggest that unlike apo-PhoB, the HP-RR regulatory domain does not inhibit DNA binding activity of the transactivation domain. The two domains of HP-RR could in fact act independently.

The charged mutants, D93N and R108E, express as inclusion bodies, indicating that these charged residues are also critical for protein folding. However, R100A folded successfully, implying that Arg100 might be not critical for protein folding. None of the mutations of the dimeric interface in the regulatory domain affect DNA binding affinity. Mutations at Val183, Asn189, Gln190, and Gln193, located in the putative DNA recognition helix (α8), reduced DNA binding affinity compared with that of the wild type. Interestingly, mutations at Thr206 and Thr208 completely destroy the DNA binding ability, indicating that the two residues are critical for DNA binding (Fig. 5B).

DISCUSSION

Recent studies have already proposed that the HP-RR is capable of specific binding to its target genes without phosphorylation (14, 15). Other RRIs, such as PhoP or BvgV, can bind to DNA without phosphorylation (41–43); however, the affinity of DNA binding is increased by phosphorylation. Surprisingly, HP-RR does not have the conserved phosphorylation site corresponding to Asp57 in E. coli CheY and avidly binds its own promoter sequence without phosphorylation. Previously, Schar et al. (14) modified a putative phosphorylation site and showed that it was not required for HP-RR function in vivo. In addition, they showed that HP-RR could not be phosphorylated in vitro.

For DrrB and CheB, the regulatory and DNA-binding domains are located very close in space such that the α4-β3-α5 face of the regulatory domain packs extensively against the DNA-binding domain providing steric hindrance in the non-phosphorylated state. However, in the case of the OmpA/PhoB family protein DrrD, the end of helix α5 of the regulatory domain contacts only with the antiparallel β-sheet of the DNA-binding domain. Although there are subtle differences in the interdomain interface of the known RR proteins, the DNA-binding domains of RR proteins are generally inhibited by the regulatory domain. The inhibition of DNA binding is relieved through a conformational change induced by phosphorylation of the regulatory domain that triggers dimerization of RR proteins. In contrast, we propose that the two domains of HP-RR act independently because no interdomain interaction was observed from NMR data. This finding is supported by mutagenesis, showing that the monomorphic F87L mutant successfully binds to DNA. This implies that dimerization of the regulatory domain in HP-RR does not affect the activity of the DNA-binding domain.

Recent studies of activated regulatory domains have provided significant structural insights into their function as inducible switches. The unphosphorylated NarL protein could not bind to DNA because the regulatory domain is in close contact with the recognition helix of the effector domain, resulting in the inhibition of DNA binding (40). The inhibitory role of the regulatory domain was also observed for the CheB protein from Salmonella typhimurium (44). In both cases, phosphorylation drives a structural transition of the response regulator protein to permit DNA binding. The effect of phosphorylation has been also studied for several response regulator proteins including NtrC (45), Spo0A (46), and FixJ (47) and in the phosphorylation mimic state of phosphono-CheY (48) and BeF3-activated CheY (49). All studies reported that modification-induced conformational changes. For NtrC, the α4 helix of the phosphorylated state provides a hydrophobic surface allowing interactions with the other domain of the protein.

From primary sequence comparison of HP-RR with the OmpR/PhoB subfamily, it is also found that HP-RR does not have the consensus sequence at the acidic pocket that is required for the phosphotransfer reaction (Fig. 5C). However, the molecular topology of HP-RR resembles that of the OmpR/PhoB subfamily and the dimeric interface formed by the α4-β3-α5 face is also similar to the active, phosphorylated form of ArcA and PhoB proteins (Fig. 4A). In HP-RR, a major structural difference occurs in the β3-α3 loop due to a 4-residue deletion. By detailed comparison with the phosphorylated form of ArcA and PhoB, the deviations of the Cα atoms of the regulatory domain are 1.97 Å (ArcA) and 3.35 Å (PhoB) (Fig. 4A). In addition, the orientation of key residues responsible for the conformational change is also similar to that of the activated PhoB protein (data not shown). Tyr49 (equivalent to Tyr1052 in PhoB) in particular adopts an inward position, indicating that HP-RR is in an active conformation. This data supports the idea that the conserved canonical phosphorylation site is unnecessary in HP-RR (14) because it is already in an active conformation without phosphorylation. The chemical shift perturbation of the effector domain upon DNA binding is relatively small, implying that a conformational change does not occur upon DNA binding except in binding loops (Fig. 3D). This is supported by the fact that the NMR spectra of the HP-RRs are nearly identical to those of the full-length protein (data not shown). 15N relaxation showed that the mobility of the putative binding sites is enhanced from the microsecond to millisecond time scale. Based on significant R2 values of HP-RR, both regulatory and transactivation domains might not experience cross-talk or a dynamic interaction between two domains in solution. Localized collective motions within backbone residues also support that the transactivation domain might bind like a monomer upon DNA binding. Based on our findings, we built a protein-DNA complex model by molecular modeling (Fig. 5D). Our structure showed that HP-RR has a smaller flexible linker connecting the two domains than PhoB (39). Considering that the regulatory domains form a symmetric dimer mediated by the α4-β5-α5 face with a very short 2-residue linker, it is conceivable that HP-RR might bind to DNA in a
head-to-head orientation, which is quite consistent with the fact that the DNA sequence of the binding site is an inverted repeat (Fig. 5D).

From our data, we have a clearer understanding about how this atypical bacterial response regulator protein could function as a cell growth-associated regulator without a phosphorylation event. A recent report showed that expression of HP-RR is regulated both on the post-transcriptional and post-translational levels (15). However, the question as to how the regulation of the protein can be achieved still remains. We conclude that HP-RR could possess its activity without phosphorylation because the structure of the regulatory domain resembles that of the active, phosphorylated form of ArcA and PhoB. Our study suggests how HP-RR differing from the well known two-component systems possibly functions in the absence of post-translational modification.

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