Backbone Dynamics of an Atypical Orphan Response Regulator Protein, *Helicobacter pylori* 1043

Ki-Woong Jeong, Hyunsook Ko, Sung-Ah Lee, Eunmi Hong¹, Sunggeon Ko¹, Hyun-Soo Cho²,
Weontae Lee¹, and Yangmee Kim*

An atypical orphan response regulator protein, HP1043 (HP-RR) in *Helicobacter pylori*, is proven to be essential for cell growth and does not require the well known phosphorelay scheme. HP-RR was identified as a symmetric dimer with two functional domains, an N-terminal regulatory domain (HP-RR^r) and a C-terminal effector domain (HP-RR^e). HP-RR is a new class of response regulator, as a phosphorylation-independent regulator. Previously, we have presented a detailed three-dimensional structure of HP-RR using NMR spectroscopy and X-ray crystallography. In this study, in order to understand the functional importance of flexibilities in HP-RR^r and HP-RR^e, T₁, T₂, heteronuclear NOE experiments have been performed and backbone dynamics of HP-RR^r and HP-RR^e were investigated. HP-RR^r is a symmetric dimer and the interface region, α 4- β 5 of dimer, showed high rigidity (high S^2 values). Site of rearrangements associated with phosphorylation of HP-RR^r (Ser⁷⁵: $R_{ex} = 3.382$, Ile⁹⁵: $R_{ex} = 5.228$) showed slow chemical exchanges. HP-RR^e is composed of three α -helices flanked on two sides by anti-parallel β -sheets. Low order parameters as well as conformational exchanges in the centers of loop regions known as the DNA binding site and transcription site of HP-RR^e suggested that flexibility of HP-RR^e is essential for interaction with DNA. In conclusion, backbone dynamics information for HP-RR implies that structural flexibilities in HP-RR^r are necessary for the phosphorylation site and the dynamic nature of HP-RR^e is essential for the regulation of interaction between protein and DNA.

INTRODUCTION

Analysis of *Helicobacter pylori* genome sequence revealed that the *H. pylori* genome encodes four two-component systems and two orphan response regulators (Tomb et al., 1997). Two-component systems are widespread prokaryotic signal transduction devices which allow the regulation of cellular functions in response to changing environmental conditions (Parkinson and Kofoid, 1992). These signaling pathways, referred to as

'two-component' systems, are structured around two conserved proteins: a histidine protein kinase (HK) and a response regulator protein (RR) these are phosphorylated at His and Asp residues, respectively. Phosphotransfer from the HK to the RR results in activation of the RR and generation of the output response of the signaling pathway (West and Stock, 2001). Usually, two-component systems are composed of a sensor protein perceiving environmental stimuli via its N-terminal input domain and a cognate response regulator. In the presence of the appropriate stimulus, the sensor protein autophosphorylates highly conserved histidine residues in the transmitter domain. Subsequently, the phosphate group is transferred to an aspartic acid residue in the N-terminal receiver domain of the response regulator, resulting in a conformational change and the activation of its C-terminal output domain, which frequently has DNA binding capacity (Beier and Frank, 2000).

The *H. pylori* genome encodes analogues of the CheA and CheY proteins, whose roles on regulating the random tumbling motion due to flagellar rotation in response to signals from chemotaxis receptors has been well documented in other systems (Armitage, 1999; Eisenbach, 1996). Three other pairs of HK 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 (Beier and Frank, 2000). One of these proteins, HP1043 (HP-RR), encodes orphan RRs for which no HKs have been identified. RRs are typically found at the ends of autophosphotransfer pathways where they regulate transcription functioning as phosphorylation-activated switches that regulate output responses. The majority of RRs are transcription factors with effector domains that can be divided into three major subfamilies based on their DNA-binding domains: the OmpR/PhoB winged-helix domains (Kondo et al., 1997; Martinez-Hackert and Stock, 1997; Okamura et al., 2000), the NarL/FixJ four-helix domains (Baikalov et al., 1996; 1998), and NtrC ATPase-coupled transcription factors (Pelton et al., 1999). The HP-RR is grouped into the OmpR/PhoB subfamily of response regulators, which shares structural similarities on its C-terminal effector domain with these proteins.

The orphan response regulator HP-RR was shown to be es-

Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Institute of SMART Biotechnology, Konkuk University, Seoul 143-701, Korea, ¹Department of Biochemistry, ²Department of Biology, Yonsei University, Seoul 120-749, Korea
*Correspondence: ymkim@konkuk.ac.kr

Received November 23, 2012; revised December 13, 2012; accepted December 17, 2012; published online February 21, 2013

Keywords: backbone dynamics, C-terminal effector domain, HP1043 (HP-RR), NMR spectroscopy, N-terminal regulatory domain, spin relaxation

essential for the growth of *H. pylori* and did not require the well known phosphorelay scheme (Schär et al., 2005). HP-RR is a DNA-binding protein, and two targets have been reported for this RR protein.

HP-RR binds specifically to its own promoter, suggesting an autoregulatory role, and also to the promoter of the HP0103 methyl-accepting chemotaxis protein *tlpB* gene (*PtlpB*) (Delany et al., 2002). Recent studies have already proposed that the HP-RR is capable of specific binding to its target genes without phosphorylation (Müller et al., 2007; Schär et al., 2005). Other RRs, such as PhoP or BvgV, are able to bind to DNA without phosphorylation (Boucher et al., 1997; Liu and Hulett, 1997; Zu et al., 1996); however, the affinity of DNA binding is increased by phosphorylation. Surprisingly, HP-RR lacks the conserved phosphorylation site which corresponds to Asp⁵⁷ in *Escherichia coli* CheY and, instead, avidly binds its own promoter sequence without phosphorylation. Previously, Schär group modified the putative phosphorylation site and showed that it was not required for HP-RR function *in vivo* (Schär et al., 2005). In addition, these investigators found that HP-RR could not be phosphorylated *in vitro*.

HP-RR was identified as a symmetric dimer with two functional domains, an N-terminal regulatory domain (HP-RR^r: residues 1-119) and a C-terminal DNA-binding/transactivation effector domain (HP-RR^e: residues 120-223). In our previous study, the three-dimensional structures of HP-RR^r and HP-RR^e were determined by NMR spectroscopy, X-ray crystallography, and site-directed mutagenesis studies (Hong et al., 2004; 2007). HP-RR^r has an $\alpha/\beta/\alpha$ sandwich structure, with a central five-stranded parallel sheet surrounded by two α -helices on one face and three on the other face. HP-RR^e is composed of three α -helices flanked on two sides by anti-parallel β -sheets. Structure and mutagenesis studies have supported a new phosphorylation-independent regulatory mechanism (Hong et al., 2004; 2007). In our previous paper, the dynamic properties of the charged and hydrophobic interactions at dimer interface of the N-terminal regulatory domain were explained briefly and the dimeric state of the regulatory domain was confirmed by R₁ and R₂ spin relaxation data.

Flexibilities and dynamics in protein conformation are essential for its function, playing key roles in molecular recognition, rate-limiting conformational transitions, and catalysis (Cole et al., 2002; Eisenmesser et al., 2002). The time scales of these protein conformational motions vary widely, from picoseconds to seconds, making solution NMR a suitable technique for analyzing such motions. Here, the functional importance of motional properties of HP-RR protein was investigated using NMR spin-relaxation experiments. We analyzed the details about the dynamics properties of residues at dimer interface of regulatory domain. The dynamic properties of the residues near the phosphorylation site were investigated to prove the phosphorylation-independent regulation mechanism of regulatory domain, too. Also, for the first time, the dynamic properties of the effector domain were analyzed to understand the significance of dynamic features of the HP-RR protein in its function as a bacterial response regulator

MATERIALS AND METHODS

Protein expression, isotopic enrichment, and purification

DNA fragments of HP-RR^r (residues 1-119) and HP-RR^e (residues 120-223) were previously cloned into pET-21b3-2 and pET-21a(+) vectors (GE Healthcare, Sweden) containing an IPTG inducible promoter and resistance to ampicillin (Hong et

al., 2007). These vectors were used to transform the *E. coli* strain BL21 (DE3) (Invitrogen, USA) for fusion protein expression. ¹⁵N-labeled HP-RR^r and HP-RR^e were produced in 250 ml of M9 minimal media supplemented with 100 μ g/ml ampicillin and 1 g/L ¹⁵NH₄Cl (Cambridge Isotope Laboratories, Andover, MA, USA). The same protocol for full length HP-RR was used to purify the overexpressed HP-RR^r and HP-RR^e (Hong et al., 2004). His-tags of HP-RR^r and HP-RR^e were cleaved by bovine thrombin (GE Healthcare, Sweden) for 24 h and 12 h in batch, respectively. Further purification was carried out by gel filtration column chromatography with HiLoad Superdex 75 prep grade column (GE Healthcare, Sweden). The final buffer for gel filtration purification consisted of 20 mM HEPES, 100 mM KCl for HP-RR^r and 50 mM potassium phosphate, 2 mM dithiothreitol (pH 7.23) for HP-RR^e. All protein NMR experiments were performed on 1.2 mM ¹⁵N-labeled HP-RR^r and 1.3 mM ¹⁵N-labeled HP-RR^e samples.

NMR experiments for backbone dynamics

All NMR experiments were recorded at 303K on a Bruker Avance 500 MHz with cryogenic probehead located in the Korea Basic Science Institute (Korea). NMR spin-relaxation experiments were performed using gradient-selected sensitivity-enhanced pulse sequences, as previously published (Kördel et al., 1992; Meiboom and Gill, 1958; Noggle and Shimmer, 1971; Vold et al., 1968). The longitudinal (R₁) spin-relaxation rates of HP-RR^r were measured with relaxation delays of 0.002 ($\times 2$), 0.15, 0.3 ($\times 2$), 0.6, 0.78, 1.0, and 1.2 s. The transverse (R₂) relaxation rates of HP-RR^r were obtained with total relaxation delays of 0.0 ($\times 2$), 0.0158, 0.0316, 0.0475 ($\times 2$), 0.0633, 0.0791, and 0.0949 seconds. The longitudinal (R₁) spin-relaxation rates of HP-RR^e were measured with relaxation delays of 0.002 ($\times 2$), 0.065, 0.145, 0.246, 0.366 ($\times 2$), 0.527, and 0.759 seconds. The transverse (R₂) relaxation rates of HP-RR^e were obtained with total relaxation delays of 0.0 ($\times 2$), 0.0172, 0.0345, 0.0517 ($\times 2$), 0.0690, 0.0862, and 0.103 seconds. All relaxation spectra of HP-RR^r were acquired with the ¹H carrier set coincident with the water resonance and ¹⁵N frequency set to 116.5 ppm; spectral widths were 6,012.821 Hz and 1,470.588 Hz in the t₂ and t₁ dimension, with 2,048 and 200 complex points in each dimension, respectively. All relaxation spectra of HP-RR^e were also acquired with the ¹H carrier set coincident with the water resonance and ¹⁵N frequency set to 117 ppm; spectral widths were 6,012.821 Hz and 1,621.863 Hz in the t₂ and t₁ dimension, with 2,048 and 256 complex points in each dimension, respectively. The recycle delay in all R₁ and R₂ relaxation measurements was 2.5 s. The heteronuclear cross-relaxation rate was obtained from heteronuclear NOE (hNOE) experiments by interleaving pulse sequences with and without proton saturation. The recycle delay and proton saturation time in the hNOE measurements were 4.5 s and 3.0 s, respectively. All spectra were processed with NMRPipe (Delaglio et al., 1995) and visualized with Sparky (Goddard and Kneller, 2004). The hNOE was determined from the ratio of peak heights for experiments with and without ¹H-saturation pulses. R₁ and R₂ rates were determined by fitting the peak heights using the program Curvfit available from Arthur Palmer at Columbia University (USA) (<http://biochemistry.hs.columbia.edu/labs/palmer/software/curvfit.html>).

Model-free analysis

Protein amide backbone dynamics were characterized by fitting ¹⁵N NMR relaxation rates to one of five semiempirical forms of the spectral density function with model-free formalism (Lipari

and Szabo, 1982a; 1982b). The spin-relaxation data were described using five models: (1) S^2 only; (2) S^2 and τ_e ; (3) S^2 and R_{ex} ; (4) model 2 plus R_{ex} ; and (5) S^2 , S^2_h , S^2_e , and τ_e , the extended two-time scale model (Cole and Loria, 2003; Lipari and Szabo, 1982b). Herein, S^2 is the generalized order parameter, τ_e is the internal correlation time, S^2_h is the fast motion order parameter with a typical correlation time of <10 ps; and R_{ex} is the additional line broadening owing to assumed two-site conformational exchange, which depends on the equilibrium site population, the rate of exchange between conformers, and chemical shift differences (Mandel et al., 1995). Motion parameters were fitted to the spin-relaxation data using the program FAST-Model-free with Model-Free 4.2 (Cole and Loria, 2003; Palmer et al., 1991). Model selection was based on the statistical testing protocol described previously by Mandel group (Mandel et al., 1995). The rotational diffusion tensor was estimated from R_2/R_1 ratios and the program 'R2R1_diffusion' using the solution coordinates, and the program 'pbdinertia' (Fairbrother et al., 1998; Mandel et al., 1995; Palmer et al., 1991). The criteria for the inclusion of residues in the diffusion tensor estimate relied on the method of Tjandra et al. (1995). Because R_2 values are loaded with R_{ex} contribution, the data containing R_{ex} were excluded from the calculation of the diffusion tensor and correlation time. During the model free analysis, an axially symmetric rotational diffusion tensor was used, the N-H bond lengths were assumed to be 1.02 Å and the ^{15}N chemical shift anisotropy was assumed to be -160 ppm (Fairbrother et al., 1998; Hiyama et al., 1988). For each model, 300 randomly distributed data sets were generated. Models were selected by comparing the sum-squared error of the optimal fit with the 0.05 critical value of the distribution. In cases where F-statistics were applicable, comparisons were made with the 0.20 critical value of the distribution.

RESULTS

Solution NMR experiments of HP-RR'

The results of the spin-relaxation experiments, R_1 , R_2 and hNOE of HP-RR' are shown in Fig. 1. The average (10% trimmed) R_1 , R_2 , and hNOE values of HP-RR' in 20 mM HEPES buffer with 100 mM KCl were $1.41 \pm 0.04 \text{ s}^{-1}$, $16.51 \pm 0.35 \text{ s}^{-1}$, and $0.79 \pm 0.02 \text{ s}^{-1}$, respectively. HP-RR' has an $\alpha/\beta/\alpha$ sandwiched structure, with a central five-stranded parallel β -sheet surrounded by two α -helices on one face and three on the other face. Large R_2 values ($> 20 \text{ s}^{-1}$) were observed for the backbone N-H of Val³, Ile⁴², Met⁴⁹, Ile⁹⁵, and Arg¹¹⁴ in HP-RR' spin-relaxation rate data. These residues are distributed in the terminal region or loop region between the short helix and the β -sheet, and regions near the phosphorylation site. These large R_2 values could be due to large contributions from conformational exchange (R_{ex}) of the protein on the micro- to millisecond time scale. The residues at both termini in HP-RR' usually have low hNOE values due to intrinsic flexibility. The significantly reduced hNOEs were found for the C-terminus as well as the loop region including Ser³², Ser⁶⁷, Ile⁶⁹, Asp⁹³, and Trp¹¹⁶. Even though Asp⁹³ is in the $\beta 5$ region, it has a low hNOE value. In most RRs, phosphorylation induces conformational changes of the conserved region including the $\beta 4$ - $\alpha 4$ loop and the $\alpha 4$ helix. The signature "switch" residues involved in rearrangements associated with phosphorylation are Ser⁷⁵ and Tyr⁹⁴, located in the $\beta 4$ - $\alpha 4$ loop and $\beta 5$ region, respectively (Hong et al., 2007). Therefore, this may be the reason why Asp⁹³ and Ile⁹⁵, residues located in the vicinity of Tyr⁹⁴, are associated with a low hNOE value and a high R_2 value with conformational exchange, re-

Fig. 1. Summary of NMR spin-relaxation results for HP-RR' in 20 mM HEPES buffer with 100 mM KCl. NMR data are displayed as a function of HP-RR' amino acid residues. Breaks in the lines indicate regions of missing data as a result of data peak overlap or data of inadequate S/N. Secondary structure is shown above the data plot with rectangles indicating α -helical or β -sheet regions.

spectively.

NMR-derived dynamics parameters of HP-RR'

Model free analysis was performed as described to obtain global and site-specific dynamics information (Cole and Loria, 2003; Mandel et al., 1995). Using an axially symmetric diffusion tensor, the rotational correlation time, τ_m , of HP-RR' was found to be $12.67 \pm 0.28 \text{ ns}$ and the determined $D_{\text{par}}/D_{\text{per}}$ ratio was 1.30. The order parameter, S^2 , of HP-RR' describing the degree of spatial restriction of the backbone NH bond vector, was determined by model free analysis and is presented in Fig. 2. The backbone dynamics for the majority of amino acid residues are best described by model 1 [equation (1)]. The 72 residues of

Fig. 2. Histograms illustrating the order parameters, S^2 , and internal mobility parameters, R_{ex} , calculated for residues of NMR HP-RR'. Breaks in the lines indicate regions of missing data as a result of data peak overlap, inadequate S/N, or residues which could not fit a motion model.

Fig. 3. (A) The S^2 values are colored onto the ribbon structure of HP-RR'. The color scheme is red ($S^2 < 0.70$) and yellow ($S^2 > 0.90$). For values $0.70 < S^2 < 0.90$, residues are colored from orange to yellow, linearly. (B) For the residues with $R_{ex} > 0.5 \text{ s}^{-1}$, the ribbon diameter of the cyan color also increased linearly. Ser⁷⁵ and Ile⁹⁵ are marked in pink. The figures were prepared with MolMol (Koradi et al. 1996). (C) Dimeric interface of HP-RR' showing the interactions between the charged residues, marked with a black dotted line. (D) Hydrophobic interactions between the residues in the dimeric interface of HP-RR' are in orange. (E) Hydrogen bonding interactions in the dimeric interface of HP-RR' are represented with blue dotted lines. The figure was prepared with PyMol (<http://www.pymol.org>).

HP-RR' are best fit using model 1, 7 residues are fit with model 2, 10 with model 3, 6 with model 4, and 7 with model 5. The average S^2 value for the HP-RR' is 0.94 ± 0.01 (102 quantifiable residues). As shown Fig. 2A, most residues participating in secondary structure exhibited high S^2 values while significantly reduced S^2 values were observed for the loop region, results consistent with the hNOE values obtained in solution-NMR experiments. Particularly noteworthy, residue Ala²⁷ in the end of

α 1- β 2 loop, Asp⁵² in the β 3- α 3 loop, and Asp⁹³ near the phosphorylation site showed very low S^2 values.

The slow time scale (μ s-ms) motions were also captured from the model-free analysis. The values of R_{ex} greater than 0.5 s^{-1} are shown in Fig. 2B. Chemical exchange appeared at the loop regions; these regions were also found to have low order parameters. Residues such as Ile⁴², Met⁴⁹, Ser⁶⁸, Ser⁷⁵, Ala⁸⁶, and Ile⁹⁵ showed slow conformational motions (R_{ex} greater than

3.0 s^{-1}). R_{ex} values appeared on the opposite side to HP-RR^e connected to HP-RR^e. Particularly, Ser⁷⁵ in the $\beta 4$ region and Ile⁹⁵ in the $\beta 5$ region, displayed a large R_{ex} values. In the HP-RR structure, Ser⁷⁵ is oriented toward the active site and Tyr⁹⁴ adopts an inward position forming a hydrogen bond with the main chain nitrogen atom of Thr⁷⁹ located at the $\beta 4$ - $\alpha 4$ loop. Namely, “inward” orientation of Tyr⁹⁴ side chain adjacent to that of the conserved residue Ser⁷⁵ on $\beta 4$ is shown. According to the model-free result, site rearrangements, associated with phosphorylation of HP-RR^e, showed significant conformational exchanges (Ser⁷⁵: $R_{ex} = 3.382 \text{ s}^{-1}$, Ile⁹⁵: $R_{ex} = 5.228 \text{ s}^{-1}$).

HP-RR^e is a symmetric dimer (Hong et al., 2004; 2007) and Fig. 3A shows the ribbon structure of HP-RR^e with order parameters, S^2 , colored onto the structure. Loop regions as well as the end of helices have low S^2 values. In Fig. 3B, the ribbon structure of HP-RR^e, showing the residue with $R_{ex} > 0.5 \text{ s}^{-1}$, increases its diameter in a linear fashion. Ser⁷⁵ and Ile⁹⁵, residues that gave large R_{ex} values and also situated near the phosphorylation site, are marked in pink in Fig. 3B. The detailed interactions in the $\alpha 4$ - $\beta 5$ - $\alpha 5$ interface dimer region are shown in Figs. 3C, 3D, and 3E. There are strong electrostatic interactions between the charged residues such as Glu⁸³/Arg¹⁰⁸ and Glu⁸³/Arg¹⁰⁰ and these interactions resulted in rigid structure with high order parameters (Glu⁸³: $S^2 = 0.97$, Arg¹⁰⁸: $S^2 = 0.98$, Arg¹⁰⁰: $S^2 = 1.00$). The residues with hydrophobic interactions, Phe⁸⁷, Ala¹⁰⁴, Ala¹⁰⁷, and Ala¹¹¹ in the interface as shown in Fig. 3D, displayed high S^2 values (Phe⁸⁷: $S^2 = 0.97$, Ala¹⁰⁴: $S^2 = 1.00$, Ala¹⁰⁷: $S^2 = 1.00$, Ala¹¹¹: $S^2 = 0.99$). Residue interactions at the dimer interface are also consolidated by inter-subunit hydrogen bonds between the Arg¹¹² side chain and the backbone carbonyl oxygen of Asp⁹², as are those between the side chain of Arg¹⁰⁸ and the carbonyl oxygen of Tyr⁹⁴ as shown in Fig. 3E. The order parameters for these residues are high (Asp⁹²: $S^2 = 0.94$, Tyr⁹⁴: $S^2 = 0.91$, Arg¹⁰⁸: $S^2 = 0.98$, Arg¹¹²: $S^2 = 0.96$) (Fig. 3E). Notably, the key residue, Asp⁹³ in the middle of the dimeric interface, which by forming two salt bridges shows very low order parameter ($S^2 = 0.65$).

Solution NMR experiments of HP-RR^e

The results of the spin-relaxation experiments, R_1 , R_2 , and hNOE of N-terminal regulatory domain HP-RR^e are shown in Fig. 4. The average (10% trimmed) R_1 , R_2 , and NOE values of HP-RR^e are $1.97 \pm 0.02 \text{ s}^{-1}$, $10.50 \pm 0.19 \text{ s}^{-1}$, and $0.74 \pm 0.01 \text{ s}^{-1}$, respectively (Fig. 4). The relaxation rates R_2 in HP-RR^e indicated relatively uniform behavior across the protein sequence with the exception of the loop regions, including Glu¹⁷⁵ and Phe²¹⁴ and residues in the β -sheet region at the N-terminus. Significantly reduced hNOE values were determined for the $\beta 9$ - $\alpha 6$ loop region including Val¹⁴⁴ and Lys¹⁴⁵ and the $\alpha 7$ - $\alpha 8$ loop region including Glu¹⁷⁴, Glu¹⁷⁵, Val¹⁷⁹, and Thr¹⁸⁰.

NMR-derived dynamics parameters of HP-RR^e

Global and site-specific dynamics information for HP-RR^e were obtained by model-free analysis. Using an axially symmetric diffusion tensor, we found that the τ_m for HP-RR^e was $7.94 \pm 0.26 \text{ ns}$, with corresponding $D_{\text{par}}/D_{\text{per}}$ ratio of 1.09. The S^2 value of HP-RR^e was determined by model free analysis and is shown in Fig. 5. The 27 residues are best described by model 1, 15 by model 2, 4 by model 3, 19 by model 4, and 14 by model 5. Figure 5 shows S^2 and R_{ex} values calculated for the residues of HP-RR^e. The average S^2 values for the entire HP-RR^e are 0.88 ± 0.01 (80 quantifiable residues) and is drawn in Fig. 6A. Flexibility of HP-RR^e is essential for its function to interact with DNA. Helices $\alpha 7$ and $\alpha 8$ form a helix-turn-helix DNA binding motif, in

Fig. 4. NMR spin-relaxation results for HP-RR^e in 50 mM potassium phosphate buffer. NMR data are displayed as a function of HP-RR amino acid residue. Breaks in the lines indicate regions of missing data as a result of data peak overlap or data of inadequate S/N. Secondary structure is shown above the data plot with rectangles indicating α -helical or β -sheet regions.

which the $\alpha 8$ is the major groove DNA recognition helix and the turn between $\alpha 7$ and $\alpha 8$ is a transcription loop as shown in Fig. 6B. The values of R_{ex} for the residues greater than 0.5 s^{-1} are shown with larger ribbon diameter (Fig. 6B) and the transcription loop marked with circle is essential for transcription activation for interacting with the σ^{80} subunit of the RNA polymerase (Hong et al., 2004; 2007). The β -hairpin wing between two β -sheets ($\beta 11/\beta 12$) of the C-terminus shown in Fig. 7A with pink arrows involves the minor groove DNA recognition (Hong et al., 2007). According to the chemical exchange data from the model free analysis, the centers of loop regions known as the transcription loop (Glu¹⁷⁵: $R_{ex} = 6.501$) and the residues at the

Fig. 5. Histograms illustrating the S^2 and R_{ex} calculated for residues of NMR HP-RR^e in 50 mM potassium phosphate buffer. Breaks in the lines indicate regions of missing data as a result of data peak overlap, inadequate S/N, or residues which did not fit to a motion model.

Fig. 6. Spatial locations of NMR-derived dynamic parameters of HP-RR^e. (A) The S^2 values are colored onto the ribbon structure of HP-RR^e in 50 mM potassium phosphate buffer. The color scheme is red ($S^2 < 0.70$) and yellow ($S^2 > 0.90$). For values $0.70 < S^2 < 0.90$, residues are colored from orange to yellow, linearly. (B) The R_{ex} values are colored onto the ribbon structure of HP-RR^e. For the residues with $R_{ex} > 0.5 \text{ s}^{-1}$, the ribbon diameter and the depth of the pink color also increase linearly.

Fig. 7. (A) The DNA recognition sites and (B) hydrophobic packing site of HP-RR^e. The major groove DNA recognition site of HP-RR^e has an important core hydrophobic packing region.

DNA recognition sites (Met¹⁹⁵: $R_{ex} = 1.386$, Asp¹⁹⁶: $R_{ex} = 1.16$, Lys¹⁹⁷: $R_{ex} = 0.826$, Val²⁰⁷: $R_{ex} = 1.302$, Arg²¹⁰: $R_{ex} = 0.619$, and Gly²¹¹: $R_{ex} = 0.991$) show chemical exchanges (Figs. 5 and 6B). Ala¹⁵⁶ and Phe²¹⁴ at the core hydrophobic packing region showed large conformational exchange, too (Fig. 7B).

DISCUSSION

HP-RR in *Helicobacter pylori* is proven to be essential for cell

growth (Schär et al., 2005). HP-RR, a member of the OmpR subfamily of response regulators (RRs), also binds to its consensus DNA sequences. HP-RR recognizes specifically to its own promoter, suggesting an autoregulatory role, and also to the promoter of the *tlpB* gene (PtlpB) (Delany et al., 2002). HP-RR was determined to be a symmetric dimer with two functional domains, an N-terminal domain and a C-terminal DNA-binding/transactivation domain (Hong et al., 2004; 2007). There are no significant inter-domain interactions in HP-RR and two domains

have independent functions. Furthermore, it is impossible to perform the spin relaxation experiment on HP-RR intact protein because of the large molecular size and line broadening in protein. Therefore, we performed the spin relaxation experiments for the individual domains and investigate the relationship between structure, dynamics, and function of individual domains.

The R_2/R_1 ratio was determined as 11.70 ± 0.39 for the regulatory domain (HP-RR'), whereas that for the trans-activation domain (HP-RR^e) was 5.28 ± 0.21 , indicating that the regulatory domain forms a rigid dimer, whereas trans-activation domain acts as a monomeric structure. The average S^2 value for the HP-RR' as a dimer is higher than the average S^2 value for the HP-RR^e monomeric form (HP-RR': $S^2 = 0.94 \pm 0.01$, HP-RR^e: $S^2 = 0.88 \pm 0.01$). The interface region, $\alpha 4$ - $\beta 5$ - $\alpha 5$ of the HP-RR' dimer, shows high rigidity (high S^2 values) except at Asp⁹³ which is the key residue forming two salt bridges in the middle.

Recent studies have already proposed that the HP-RR is capable of specific binding to its target genes without phosphorylation (Müller et al., 2007; Schär et al., 2005). Other RRs, such as PhoP or BvgV, could bind to DNA without unphosphorylation events (Boucher et al., 1997; Liu and Hulet, 1997; Zu et al., 1996); however, the affinity of DNA binding was increased by phosphorylation. The signature "switch" residues involved in rearrangements associated with phosphorylation are Ser⁷⁵ and Tyr⁹⁴ in HP-RR, located in the $\beta 4$ - $\alpha 4$ loop and $\beta 5$ region, respectively. In the HP-RR structure, Ser⁷⁵ is oriented toward the active site and Tyr⁹⁴ adopts an inward position forming a hydrogen bond with the main chain nitrogen atom of Thr⁷⁹ located at the $\beta 4$ - $\alpha 4$ loop (Hong et al., 2007). According to the model-free result, the site of rearrangements associated with phosphorylation of HP-RR' showed the great conformational exchanges (Ser⁷⁵: $R_{ex} = 3.382$, Ile⁹⁵: $R_{ex} = 5.228$). Furthermore, Asp⁹³, near the phosphorylation site which forms strong electrostatic network to maintain the dimeric interface, as shown in Fig. 5C showed very low S^2 values. Therefore, these structural flexibilities are necessary for the phosphorylation site even though HP-RR is already in active form without phosphorylation. All the other residues in the dimeric interface showed high rigidity compared with the other regions.

The detailed interactions in the $\alpha 4$ - $\beta 5$ - $\alpha 5$ interface region of dimer are shown in Figs. 3C, 3D, and 3E. As shown in Fig. 3C, there are interactions between the charged residues such as Glu⁸³/Arg¹⁰⁸ and Glu⁸³/Arg¹⁰⁰ and these interactions resulted in rigid structure with high order parameters (Glu⁸³: $S^2 = 0.97$, Arg¹⁰⁸: $S^2 = 0.98$, Arg¹⁰⁰: $S^2 = 1.00$). The residues with hydrophobic interaction, Phe⁸⁷, Ala¹⁰⁴, Ala¹⁰⁷ and Ala¹¹¹ as shown in Fig. 3D, also showed high S^2 values (Phe⁸⁷: $S^2 = 0.97$, Ala¹⁰⁴: $S^2 = 1.00$, Ala¹⁰⁷: $S^2 = 0.99$, Ala¹¹¹: $S^2 = 0.99$). The interactions between residues at the dimeric interface are also consolidated by inter-subunit hydrogen bonds between the Arg¹¹² side chain and the backbone carbonyl oxygen of Asp⁹², and those between the side chain of Arg¹⁰⁸ and the carbonyl oxygen of Tyr⁹⁴ as shown in Fig. 3E. The order parameters for these residues are high (Asp⁹²: $S^2 = 0.94$, Arg¹⁰⁸: $S^2 = 0.98$, Arg¹¹²: $S^2 = 0.96$).

The model-free results of HP-RR^e indicate that there are slow exchanges in DNA binding region (Met¹⁹⁵, Asp¹⁹⁶, Lys¹⁹⁷, Val²⁰⁷, Arg²¹⁰, Gly²¹¹) which may be essential for its function to interact with DNA. The centers of loop regions known as the DNA binding site and transcription site displayed large flexibilities (low S^2 values and high R_{ex} values). According to model free analysis shown in Fig. 5, Ala¹⁵⁶ and Phe²¹⁴, at the hydrophobic core and residues located near the major groove DNA binding site (Met¹⁹⁵, Asp¹⁹⁶) have large R_{ex} values. These results implied

that the major groove DNA recognition on HP-RR^e may result in changes of conformational exchange of hydrophobic packing region. As a consequence, these dynamic behaviors in the proteins are probably related to the regulation of binding affinity between protein and DNA by making transient interaction.

In conclusion, backbone dynamics information for HP-RR implies that structural flexibilities in HP-RR' are necessary for the phosphorylation site and the dynamic nature of HP-RR^e is essential for the regulation of interaction between protein and DNA.

ACKNOWLEDGMENTS

This work was supported by grants from the Basic Science Research Program (2011-0022873, Y. Kim), Priority Research Centers Program (2012-0006686, Y. Kim) through the National Research Foundation of Korea, funded by the Ministry of Education, Science, and Technology, and World Class University (WCU) program (R33-2009-000-10123-0, W. Lee).

REFERENCES

- Armitage, J.P. (1999). Bacterial tactic responses. *Adv. Microb. Physiol.* 41, 229-289.
- Baikalov, I., Schroder, I., Kaczor-Grzeskowiak, M., Grzeskowiak, K., Gunsalus, R.P., and Dickerson, R.E. (1996). The structure of the *Escherichia coli* response regulator, NarL. *Biochemistry* 35, 11053-11061.
- Baikalov, I., Schroder, I., Kaczor-Grzeskowiak, M., Cascio, D., Gunsalus, R.P., and Dickerson, R.E. (1998). NarL dimerization? Suggestive evidence from a new crystal form. *Biochemistry* 37, 3665-3676.
- Beier, D., and Frank, R. (2000). Molecular characterization of two-component systems of *Helicobacter pylori*. *J. Bacteriol.* 182, 2068-2076.
- Boucher, P.E., Murakami, K., Ishihama, A., and Stibitz, S. (1997). The nature of DNA-binding and RNA polymerase interaction of the *Bordetella pertussis* BvgA transcriptional activator at the *fla* promoter. *J. Bacteriol.* 179, 1755-1763.
- Cole, R., and Loria, J.P. (2002). Evidence for flexibility in the function of ribonuclease A. *Biochemistry* 41, 6072-6081.
- Cole, R., and Loria, J.P. (2003). FAST-Modelfree: a program for rapid automated analysis of solution NMR spin-relaxation data. *J. Biomol. NMR* 26, 203-213.
- Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* 6, 277-293.
- Delany, I., Spohn, G., Rappuoli, R., and Scarlato, V. (2002). Growth phase-dependent regulation of target gene promoters for binding of the essential orphan response regulator HP1043 of *Helicobacter pylori*. *J. Bacteriol.* 184, 4800-4810.
- Eisenbach, M. (1996). Control of bacterial chemotaxis. *Mol. Microbiol.* 20, 903-910.
- Eisenmesser, E.Z., Bosco, D.A., Akke, M., and Kern, D. (2002). Enzyme dynamics during catalysis. *Science* 295, 1520-1523.
- Fairbrother, W.J., Liu, J., Pisacane, P.I., Sliwowski, M.X., and Palmer, A.G.III. (1998). Backbone dynamics of the EGF-like domain of heregulin- α . *J. Mol. Biol.* 279, 1149-1161.
- Goddard, T.D., and Kneller, D.G. (2004). SPARKY 3. In., 3.110 ed., (San Francisco, University of California).
- Hiyama, Y., Niu, C.-H., Silverton, J.V., Bavoso, A., and Torchia, D.A. (1988). Determination of ¹⁵N nuclear shift tensor via ¹⁵N-¹H dipolar coupling in Boc-glycylglycyl[¹⁵N]glycine benzyl ester. *J. Am. Chem. Soc.* 110, 2378-2383.
- Hong, E., Jung, J.W., Shin, J., Kim, J.H., Jeon, Y.H., Yamazaki, T., and Lee, W. (2004). Backbone ¹H, ¹³C and ¹⁵N resonance assignments of the response regulator HP1043 from *Helicobacter pylori*. *J. Biomol. NMR* 28, 85-86.
- Hong, E., Lee, H.M., Ko, H., Kim, D.U., Jeon, B.Y., Jung, J.W., Shin, J., Lee, S.A., Kim, Y., Jeon, Y.H., et al. (2007). Structure of an atypical orphan response regulator protein supports a new phosphorylation-independent regulatory mechanism. *J. Biol. Chem.* 282, 20667-20675.

- Kondo, H., Nakagawa, A., Nishihira, J., Nishimura, Y., Mizuno, T., and Tanaka, I. (1997). *Escherichia coli* positive regulator OmpR has a large loop structure at the putative RNA polymerase interaction site. *Nat. Struct. Biol.* **4**, 28-31.
- Koradi, R., Billeter, M., and Wuthrich, K. (1996). MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14**, 51-55.
- Kördel, J., Skelton, N.J., Akke, M., Palmer, A.G.III., and Chazin, W.J. (1992). Backbone dynamics of calcium-loaded calbindin D9k studied by two-dimensional proton-detected ¹⁵N NMR spectroscopy. *Biochemistry* **31**, 4856-4866.
- Lipari, G., and Szabo, A. (1982a). Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range validation. *J. Am. Chem. Soc.* **104**, 4546-4559.
- Lipari, G., and Szabo, A. (1982b). Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. Analysis of experimental results. *J. Am. Chem. Soc.* **104**, 4559-4570.
- Liu, W., and Hulett, F.M. (1997). *Bacillus subtilis* PhoP binds to the phoB tandem promoter exclusively within the phosphate starvation-inducible promoter. *J. Bacteriol.* **179**, 6302-6310.
- Mandel, A.M., Akke, M., and Palmer, A.G.III. (1995). Backbone dynamics of *Escherichia coli* ribonuclease HI: correlations with structure and function in an active enzyme. *J. Mol. Biol.* **246**, 144-163.
- Martinez-Hackert, E., and Stock, A.M. (1997). The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure* **5**, 109-124.
- Meiboom, S., and Gill, D. (1958). Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instrum.* **29**, 688-691.
- Müller, S., Pflock, M., Schär, J., Kennard, S., and Beier, D. (2007). Regulation of expression of atypical orphan response regulators of *Helicobacter pylori*. *Microbiol. Res.* **162**, 1-14.
- Noggle, J.H., and Shirmer, R.E. (1971). The nuclear overhauser effect: chemical applications. (New York: Academic Press), pp. 1-259.
- Okamura, H., Hanaoka, S., Nagadoi, A., Makino, K., and Nishimura, Y. (2000). Structural comparison of the PhoB and OmpR DNA-binding/transactivation domains and the arrangement of PhoB molecules on the phosphate box. *J. Mol. Biol.* **295**, 1225-1236.
- Palmer, A.G.III., Rance, M., and Wright, P.E. (1991). Intramolecular motions of a zinc finger DNA-binding domain from Xfin characterized by proton-detected natural abundance ¹³C heteronuclear NMR spectroscopy. *J. Am. Chem. Soc.* **113**, 4371-4380.
- Parkinson, J.S., and Kofoed, E.C. (1992). Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26**, 71-112.
- Pelton, J.G., Kustu, S., and Wemmer, D.E. (1999). Solution structure of the DNA-binding domain of NtrC with three alanine substitutions. *J. Mol. Biol.* **292**, 1095-1110.
- Schär, J., Sickmann, A., and Beier, D. (2005). Phosphorylation-independent activity of atypical response regulators of *Helicobacter pylori*. *J. Bacteriol.* **187**, 3100-3109.
- Tjandra, N., Feller, S.E., Pastor, R.W., and Bax, A. (1995). Rotational diffusion anisotropy of human ubiquitin from ¹⁵N NMR relaxation. *J. Am. Chem. Soc.* **117**, 12562-12566.
- Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A., et al. (1997). The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539-547.
- Vold, R.L., Waugh, J.S., Klein, M.P., and Phelps, D.E. (1968). Measurements of spin relaxation in complex systems. *J. Chem. Phys.* **48**, 3831-3832.
- West, A.H., and Stock, A.M. (2001). Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem. Sci.* **26**, 369-376.
- Zu, T., Manetti, R., Rappuoli, R., and Scarlato, V. (1996). Differential binding of BvgA to two classes of virulence genes of *Bordetella pertussis* directs promoter selectivity by RNA polymerase. *Mol. Microbiol.* **21**, 557-565.