Structural Basis of a Physical Blockage Mechanism for the Interaction of Response Regulator PmrA with Connector Protein PmrD from "Klebsiella pneumoniae"*

Received for publication, May 1, 2013, and in revised form, July 13, 2013. Published, JBC Papers in Press, July 16, 2013, DOI 10.1074/jbc.M113.481978

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Significance:

Background: PmrD binds to phospho-PmrA and sustains its phosphorylation state.

Results: Phospho-PmrA interacts with PmrD via several specific intermolecular interactions.

Conclusion: A steric inhibition mechanism was proposed for protecting phospho-PmrA against dephosphorylation.

For all organisms, sensing and responding to environmental cues is essential to their survival. Compared with eukaryotic signaling cascades, which are mainly involved in mediating signals through specific Ser-, Thr-, and Tyr-phosphorylated residues among the related sub-tracts, in prokaryotic signaling systems, a distinct phosphorylation scheme predominates, the two-component system (TCS)\(^2\) (1–3). Hundreds of TCSs have been found in eubacteria, archaea, and a few eukaryotic organisms (4), and the TCS is the most prevalent system in bacteria for transducing external information into the cell and coping with environmental stresses (2, 5). A PmrA-PmrB TCS, dealing with cationic antimicrobial peptides and polymyxin resistance in pathogens (6), is responsible for sensing the external stimuli caused directly or indirectly by excess Fe\(^{3+}\), low Mg\(^{2+}\), and mild acidic environments (7–9). Under this TCS, pathogens can alter the composition of their cell walls to resist neutralization of polymyxin for drug resistance and allow bacterial survival within macrophages by reducing the affinity with cationic antimicrobial peptides (10, 11).

The increasing antibiotic resistance to "Klebsiella pneumoniae" bacteria, a common cause of nosocomial bacterial infections causing pneumonia and urinary tract infections, especially in immunocompromised patients (12), led us to investigate how the virulence and drug resistance persist via the associated TCS. Similar to most response regulators, the PmrA of "K. pneumoniae" belonging to the OmpR-PhoB family is composed of an N-terminal receiver domain (Fig. 1) and a C-terminal effector/DNA-binding domain. Interestingly, at low Mg\(^{2+}\) concentration, the PhoP-PhoQ system promotes the expression of a small basic protein, PmrD (7), which can prevent the intrinsic dephosphorylation of phospho-PmrA and enhance the expression of PmrA-activated downstream genes (13). Therefore, PmrD is termed a “connector” because it connects the PmrA-PmrB and PhoP-PhoQ systems (14). Besides PmrD, other small-sized proteins that connect TCSs for "Escherichia coli" include SafA, which connects the PhoP-PhoQ and EvgS-EvgA systems (15); IraM, which connects PhoP-PhoQ and binds to response regulator RssB, thereby preventing the proteins from recruiting targets for degradation (16); and MrzA, which connects CpxR-Cpxa and OmpR-EnvZ systems (17). To date, the complicated mechanisms of cross-regulation among these TCSs are still not clear (14), and no detailed structural information is available about how the phosphorylation site of the PmrA; HDX, hydrogen/deuterium exchange; r.m.s.d, root mean square deviation; MTSL, (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl) methanethiosulfonate).
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FIGURE 1. Primary sequence alignment of N-terminal receiver domains of PmrA and five other response regulators. The secondary structural elements of PmrAN are at the top of the figure. Identical residues are shown in white with a red background, and similar residues are shown in red. The alignment was generated by use of CLUSTAL-W (60) and ESPript (61).

response regulator is protected by the connector protein in the interconnecting signal transduction pathways such as PmrD and phospho-PmrA.

Previously, we determined the solution structure for K. pneumoniae PmrD and mapped its binding sites when it bound to PmrAN (18). In this study, we present the structure of the BeF₃⁻-activated N-terminal receiver domain of PmrA (PmrAN) solved by x-ray crystallography. The backbone assignment of PmrAN involved transverse relaxation-optimized spectroscopy-type three-dimensional NMR experiments with ²H,¹³C,¹⁵N-labeled proteins was verified by SDS-PAGE and mass spectrometry.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of the Recombinant Proteins—The coding regions of pmr and PmrAN (N-terminal 123 residues) were PCR-amplified from the genomic DNA of K. pneumoniae. The amplified gene products were cloned as an Ndel/Xhol fragment into the pET29b vector (Novagen). The resulting plasmids (pET-PmrD and pET-PmrAN) allowed for the in-frame fusion of each coding region containing an additional LEHHHHHH sequence at the C terminus to facilitate the in-frame fusion of each coding region containing an additional LEHHHHHH sequence at the C terminus to facilitate protein purification. The recombinant proteins were overexpressed in the host E. coli strain BL21(DE3) (Novagen) induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C (for PmrD) or 30 °C (PmrAN). After lysis with use of an M-110S microfluidizer (Microfluidics) and subsequent centrifugation, the overproduced proteins were purified from the soluble fraction by affinity chromatography on His-Bind resin (Novagen) and size-exclusion chromatography with a Superdex75 10/300 GL column. The eluted samples were dialyzed against 50 mM Tris (pH 8.0), 100 mM NaCl, and concentrated by use of Amicon (molecular weight of 5000; Millipore). For isotopically enriched samples, cells were grown in M9 minimal medium (20) containing 1 g/liter ¹⁵NH₄Cl and 2 g/liter [¹³C]glucose. PmrD and PmrAN mutants were generated according to QuikChange™ protocol (Stratagene). The authenticity of the recombinant proteins was verified by SDS-PAGE and mass spectrometry.

Crystalization of BeF₃⁻-activated PmrAN—For crystallization trials, the BeF₃⁻-activated PmrAN was first concentrated to 5 mg/ml in buffer containing 20 mM sodium phosphate, pH 6.0, and 30 mM NaCl with use of a Centricon concentrator (Millipore). The BeF₃⁻-activated PmrAN was obtained by adding 5.3 mM BeCl₂ (Fluka), 35 mM NaF, and 7 mM MgCl₂. The hanging drop vapor diffusion method was then performed at 298 K by mixing 1 μl BeF₃⁻-activated PmrAN with an equal volume of crystal screening solutions. Hexagonal crystals appeared after 3 days under the condition of 100 mM imidazole and 1 mM sodium acetate at pH 6.5. The crystals belong to space group P4₁ and contain one PmrAN₄ dimer per asymmetric unit. Collection of the cryogenic multiwavelength anomalous dispersion data involved use of an Area Detector Systems Quantum-315 charge-coupled device detector with a synchrotron radiation x-ray source at beamline BL13B1 at the National Synchrotron Radiation Research Center in Taiwan. X-ray diffraction data integration and scaling involved use of the HKL2000 package (21). The extension of initial phases to 1.99 Å and the preliminary auto-model building involved use of RESOLVE (22). XtalView (23) was used to examine electron density maps and for manual model building. Further refinement involved use of CNS (24) and PHENIX (25). After completion, the Rfree value of the final model for all reflections above 1σ between 25.38 and 1.70 Å resolution was refined to 16.5%, and an Rfree value of 17.5% was obtained with 5.2% randomly distributed reflections (26). The structures were visualized and plotted with use of PyMOL (27).

NMR Backbone Resonance Assignments of Free and Bound Forms of BeF₃⁻-activated PmrAN—NMR experiments with 0.3 mM ²H,¹³C,¹⁵N-labeled PmrAN in complex with PmrD (with
a molar ratio of 1:2) in a Shigemi NMR tube (Allison Park, PA) were performed at 310 K with the use of Bruker AVANCE 600 and 800 NMR spectrometers (Bruker, Karlsruhe, Germany) equipped with triple (1H, 13C, 15N) resonance cryoprobe including a shielded z-gradient. All heteronuclear NMR experiments were performed as described (28). Sequence-specific assignment of the backbone atoms was achieved by independent connective analysis of transverse relaxation-optimized spectroscopy-type three-dimensional HNCA, HN(CO)CA, CBCA(CO)NH, HNCACB, HNCO, and HN(CA)CO experiments. The chemical shifts of individual spin systems (HN, N, Ca, Cβ, Cc, and Ha) were collected manually, and the backbone resonances were assigned by visual inspection. Transverse relaxation-optimized spectroscopy-based NOESY and HNCO spectra were acquired on the triple-labeled PmrAN in complex with PmrD. From the resonance assignments for free PmrAN, most of the resonances in the bound PmrAN could be easily identified because their cross-peaks were well superimposed. For the residues with significant shift perturbations, their backbones were assigned by comparing NOE connectivities and carbonyl carbon chemical shifts between the free and bound forms. All NMR spectra were processed and analyzed by use of Topspin (Bruker Biospin), NMRPipe (29), and NMRView (30) software packages.

**HDX Experiments**—HDX experiments were initiated by adding D2O (99.9%) to lyophilized protein, which had been prepared at the required pH and buffer conditions. The concentration of 15N,13C,2H-labeled PmrAN was ~100 μM, and the unlabeled PmrD was ~200 μM. The 15N–1H selective optimized flip-angle short-transient heteronuclear multiple quantum coherence procedure (31) was used to obtain the 15N–1H correlation spectra with 1024 (t2) and 64 (t1) complex points and 4 scans at 310 K.

**Chemical Shift Perturbation Experiments**—To map the binding sites on PmrAN with PmrD, we collected 1H–15N HSQC spectra for 1H,15N-labeled PmrAN with unlabeled PmrD at a molar ratio of 1:2. We investigated and ruled out the possibility that the shift changes of PmrD were due to constituents of BeF3. All spectra processing were analyzed by use of XWIN-NMR (Bruker Biospin) and SPARKY (32). Normalized chemical shift changes were calculated as follows.

\[
\Delta \delta = [\Delta H^2 + (0.17 \Delta N)^2]^{1/2}
\]

(Eq. 1)

The cut-off (0.135 ppm) was set as the S.D. for all chemical shift changes. All residues with values above the cut-off were considered affected by interaction with PmrD.

**Complex Structure Determination using HADDOCK**—The information-driven docking program HADDOCK (version 2.0) (19) was used to generate a PmrD–PmrAN complex model. The starting structures for docking were an x-ray crystal structure of PmrAN and the 10 lowest energy structures of PmrD, which were refined with the constraints measured from the chemical shift perturbations (18). From NMR titration data, different sets of ambiguous interaction restraints were used to generate the complex model. PmrD contained sets that included the active residues 8, 9, 53, 59, 99, 104, 105, 138, 139, 183, 189, 229, 234, and 236. The active residues were chosen on the basis of the chemical shift perturbation data and high solvent accessibility (>50%). The passive residues are the solvent-accessible surfaces neighboring active residues. During the rigid body energy minimization, 10,000 structures were calculated, and the 199 best solutions based on the intermolecular energy were used for the semi-flexible, simulated annealing, followed by explicit water refinement. The best 199 docked models were clustered by use of a cut-off r.m.s.d. of 3.5 Å. The clusters were ranked by the averaged HADDOCK score of their top 10 complex models (Table 1).

**Site-directed Spin Labeling and Paramagnetic Relaxation Enhancement (PRE) Experiments**—PmrDC54 constructs containing a single cysteine residue were generated by use of site-directed mutagenesis to convert existing extra cysteine residues to serine. Mutations were confirmed by DNA sequencing over the entire open reading frame and were found to be properly expressed. Directed mutagenesis to convert existing extra cysteine residues to ascorbic acid and DTT (2–5 μL from a concentrated stock; dilution < 1.25%) to NMR samples. Samples were placed at 25 °C for at least 1 h after the addition of reducing agent. The intensity ratios of paramagnetic versus diamagnetic spectra were obtained from the peak heights and further normalized before distance constraints were converted.

| Docking statistics | Ensemble (199 structures) | Best 10 structures |
|--------------------|---------------------------|-------------------|
| Haddock score      | -144.61 ± 19.86           | -181.35 ± 4.61    |
| E_elec (kcal/mol)  | -82.18 ± 8.16             | -89.55 ± 8.22     |
| E_nuc (kcal/mol)   | -550.71 ± 79.78           | -694.77 ± 52.86   |
| E_tors (kcal/mol)  | -583.84 ± 80.92           | -738.89 ± 51.84   |
| E_elec (kcal/mol)  | 48.34 ± 11.51             | 43.83 ± 3.37      |
| BSA (Å)            | 3143.53 ± 151.53          | 3738.55 ± 157.648 |
| r.m.s.d. from lowest energy structure (Å) | 0.984 | 0.847 ± 0.33 |
| No. of AIR violations > 0.3 Å | 1.23 ± 0.47 | 1.1 ± 0.3 |

| Deviations from idealized geometry | r.m.s.d. for bond angles | r.m.s.d. for bond lengths (Å) |
|-----------------------------------|-------------------------|-------------------------------|
|                                   | 0.5°                    | 0.004                         |
| Most favored regions (%)          | 82.2                    | 82.9                          |
| Additionally allowed regions (%)  | 15.8                    | 14.9                          |
| Generously allowed regions (%)    | 1.4                     | 1.4                           |
| Disallowed regions (%)            | 0.6                     | 0.8                           |
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RESULTS

Structure of PmrAN and Dimer Interface—Beryllium fluoride, a phosphoryl analog (33), was used to produce an activated form of PmrAN for structural studies. The structure of PmrAN was solved with experimental phases from molecular replacement data at 1.70 Å. The initial phases of PmrAN were solved with use of the molecular replacement software MOLREP from the CCP4 suite (34). The overall structure of PmrAN adopts a signature CheY-like α/β-fold architecture of receiver domains shared by all response regulators (35). Alternating β-strands and α-helices in the primary structure fold into a central five-stranded parallel β-sheet surrounded by two α-helices on one side and three on the other side (Fig. 2A). The asymmetric unit has only one molecule that forms a dimer with another crystallographic symmetry-related molecule using the face formed by helix α4, strand β5, and helix α5 (Fig. 2A). The presence of a large number of intra- and intermolecular interactions involving hydrophobic and polar residues was revealed by analysis of the interface. The α4 helix of one monomer packs against the α5 helix of the other monomer through a hydrophobic patch formed between Leu-84 (α4), Ile-88 (α4), and Leu-91 (α5) and the aliphatic portions of Ala-104 (α4), Ala-110 (α5), and Ala-114 (α5) side chains (Fig. 2B). The interface is further stabilized by a number of side chains involved in attributive salt bridges, especially toward the center region, which creates an extensive network that surrounds the hydrophobic packing between the α4 and α5 helices. Intermolecular salt bridges are formed between Arg-87(α4)–Glu-107(α5), Asp-92(α4)–Arg-113(α5), and Asp-97(β5)–Arg-111(α5) at the outer sides (Fig. 2C). The x-ray structures of other members of the activated form of receiver domains in the OmpR-PhoB subfamily were reported previously, including PhoB and PhoP from E. coli and DrrB and DrrD from Thermotoga maritima (36–38). In all cases, the receiver domains form a dimer with an interface at α4-β5-α5, which reveals a common dimerization mechanism on activation with members of the OmpR-PhoB subfamily.

Coordination of the Active Site in PmrAN—PmrA is phosphorylated at Asp5, the residue that corresponds to the conserved phosphorylation site shared by the receiver domains of response regulators (39). In the crystal structure, Mg2+ binds to F1 of BeF3, the side chain carboxyl oxygens of Asp-8 and Asp-51; the main chain carbonyl oxygen of Gly-53, a water molecule hydrogen-bonded to Asp-8 and another water molecule hydrogen-bonded to Asp-9, thus satisfying the octahedral coordination of Mg2+ (Fig. 2D). The BeF3 moiety contacts with the side chain oxygen atom of Thr-79, the Nε atom of Lys-101 and the backbone nitrogen atoms of Gly-53, Leu-52, and Ala-80. Carbon, oxygen, and nitrogen atoms are shown in green, red, and blue, respectively. E, active site electron density map (Fo–Fc) of the BeF3-activated PmrAN, calculated at 2σ is shown in gray within 1.7 Å of the residues depicted in sticks.

FIGURE 2. X-ray crystal structure of PmrAN. A, ribbon structure of PmrAN shows the formation of a dimer with the interface at α4-β5-α5. The phosphate analog, BeF3, is shown as a ball and stick figure in magenta, and Mg2+ is represented by a green sphere. B, hydrophobic interactions identified at the dimer interface. A hydrophobic patch (spheres) brings helices α4 (Leu-84, Leu-91, and Ile-88 colored in orange) and α5 (Ala-104, Ala-110, and Ala-114 colored in purple) together. C, the dimer interface is stabilized by an extensive network of salt bridges, Arg-87(α4)–Glu-107(α5), Asp-92(α4)–Arg-113(α5), and Asp-97(β5)–Arg-111(α5). D, an extended view of the active site. The Mg2+ is octahedrally coordinated to Asp-51, Gly-53, Asp-8, BeF3, and two water molecules. The BeF3 moiety contacts with the side chain oxygen atom of Thr-79, the Nε atom of Lys-101 and the backbone nitrogen atoms of Gly-53, Leu-52, and Ala-80. Carbon, oxygen, and nitrogen atoms are shown in green, red, and blue, respectively. E, active site electron density map (Fo–Fc) of the BeF3-activated PmrAN, calculated at 2σ is shown in gray within 1.7 Å of the residues depicted in sticks.
middle of the β4-α4 loop. Crystalllographic data are summarized in Table 2, and electron density for the active site of the refined model is shown in Fig. 2E.

{1H, 13C, and 15N Assignments and Secondary Structure Comparisons between Solution and X-ray PmrAN Structures—We could not grow good crystals of PmrAN in complex with PmrD and therefore used NMR techniques to obtain detailed structural information and gain insight into the protein-protein interactions (41). Two-dimensional 15N-1H HSQC spectrum of PmrAN is well dispersed, which indicates a well folded structure. Following a standard sequential assignment procedure, all of the observed cross-peaks in the HSQC spectrum were completely assigned, which correspond to 97% of 1H, 13C, and 15N pairs (115 of 119). The four unassigned residues were His-44, Leu-105, Gln-122, and Gly-123. Their cross-peaks disappeared, presumably because of solvent exposure. For the other backbone atoms, 98% of 1H resonances (116 of 119) and 98% of 13C, 15N, and 13Cβ resonances (351 of 360) were assigned. Hα of Arg-87 was shifted up-field at 3.54 ppm, which is likely due to the shielding effect by Tyr-98 as seen in the crystal structure. The chemical shift index (29) based on Hα, Cα, Cβ, and C atoms, 98% of 1H, 13C, and 15N resonances (115 of 119) for free PmrAN. Most of the water-inaccessible residues were similar to those in the free form of PmrAN (Fig. 4B), so these residues are not involved in binding with PmrD. However, three residues, Leu-11, Phe-103, and Arg-81, showed a lower water-accessible rate in the presence of PmrD (Fig. 4B), presumably because of the steric occlusion effect of PmrD. Interestingly, Leu-11, Arg-81, and Phe-103 are all close to the phosphorylation site and are consistent with the binding interface determined by chemical shift perturbation (Fig. 4C).

{Modeling of the PmrD-PmrAN Complex—With formation of the PmrD-PmrAN complex, we observed significant broadening of NMR signals for both PmrD and PmrAN, which may be due to the slower tumbling of the complex and/or a contribution from intermediate timescale chemical exchange broadening. These significantly broadened NMR resonances limited the acquisition of intermolecular NOEs critical for determining the structure of multiprotein complexes, although this effect aided in identifying interfacial residues. Because of a lack of intermolecular NOEs, we generated the complex model of a PmrD-PmrAN heterotetramer using the experimental data-driven docking method of HADDOCK (19). The generated complex

### Table 2

| Data collection and refinement statistics | Data collection |
|------------------------------------------|-----------------|
| Space group                              | P4_1            |
| Cell dimensions (Å)                       | a = b = 49.99 and c = 145.86 Å |
| Wavelength (Å)                            | 0.97315         |
| Resolution (Å)                            | 1.7             |
| Redundancy                                | 12.2 (11.9)     |
| %I/(σI)                                   | 40.1 (6.7)%     |
| Completeness (%)                          | 97.2 (94.5)%    |
| Rmerge (%)                                | 6.6 (41.7)      |

*Numbers in parentheses refer to the highest resolution shell.*
model is dominated by intermolecular interactions between residues located on the active site pockets of PmrAN and the solvent-exposed face of the β-barrel of PmrD (Fig. 5A). Seven intermolecular hydrogen bonds were identified: Ser-23(HG)–Asp-9(OD1), Ser-23(HG)–Asp-9(OD2), Ala-49(O)–Leu-105(HN), Asn-67(OD1)–Thr-83(HN), His-70(HD1)–Gly-53(O), His-70(HE2)–Asp-9(OD1), and Arg-75(HH12)–Glu-85(OE2) (Fig. 5B). In addition, two intermolecular salt bridges were found between Lys-64–Glu-31 with 2-fold symmetry (Fig. 5B). Therefore, the hydrogen bonds and salt bridges for each partner interact with appropriately charged residues on the corresponding interface, providing a specificity of structural arrangement for the interaction between PmrD and PmrAN.

PRE Effect of Site-directed Spin Labeling on PmrDC54 and PmrAN D56C—To double-confirm the orientations of the PmrD-PmrAN complex model, we used site-directed spin labeling with a nitroxide spin-label compound, MTSL, covalently attached to a Cys residue (42) for PRE experiments, which
can be used as an independent biophysical method to obtain intermolecular long distance (<30 Å) information. PmrD contains three Cys residues (Cys-17, Cys-35, and Cys-54), and PmrAN contains only one Cys residue (Cys-27). To determine which Cys residues could be labeled under the conditions described under “Experimental Procedures,” we initially covalently linked the stable mutant 15N-labeled PmrD C35S and wild-type 15N-labeled PmrAN using MTSL. The backbone HN of Cys-54 in PmrD C35S was significantly shifted as compared with PmrD, so Cys-54 was able to bind with MTSL. However, Cys-27 of PmrAN did not show any shift perturbation on adding MTSL, so Cys-27 is unlikely to bind to MTSL. We therefore produced two mutants, C17S/C35S on PmrD (PmrDC54) and D56C on PmrAN (PmrAN D56C), for PRE experiments. Spin labeling did not change the protein-protein interaction because the 1H-15N HSQC spectrum of PmrAN in complex with MTSL-labeled PmrDC54 with a reduced proxyl group was similar to that of PmrD, so Cys-54 was able to bind with MTSL. However, Cys-27 of PmrAN did not show any shift perturbation on adding MTSL, so Cys-27 is unlikely to bind to MTSL. We therefore produced two mutants, C17S/C35S on PmrD (PmrDC54) and D56C on PmrAN (PmrAN D56C), for PRE experiments. Spin labeling did not change the protein-protein interaction because the 1H-15N HSQC spectrum of PmrAN in complex with MTSL-labeled PmrDC54 with a reduced proxyl group was similar to that of PmrAN in complex with unlabeled PmrD (Fig. 6A) and vice versa. The unpaired electron of MTSL caused line-broadening effects on nearby residues in a distance-dependent manner. Line-broadening effects on the 15N-labeled PmrAN were observed in the complexes with MTSL-labeled PmrDC54 (Fig. 6A). The superimposed spectra of the reduced (black) and oxidized (red) states showed significant R2 relaxation enhancement effects. With the spin label on Cys-54 of PmrD, the signals on several residues of PmrAN, such as Asp-82, Leu-84, Val-100, Phe-103, and Leu-105, were significantly reduced in the oxidized state (Fig. 6, A and B). Residues Asp-82, Leu-84, Val-100, and Phe-103 were all close to the active site of PmrAN, as seen in the crystal structure. With MTSL attached to D56C of PmrAN, three peaks corresponding to PmrD residues Gly-24–Ala-25 and the side chain amide peak of Trp-3 were severely attenuated (data not shown). These data suggest that the interaction brings the binding surface of PmrD (the open β-barrel area) and the active site of PmrAN close to each other. In conclusion, the observed intermolecular PRE data are generally consistent with the HADDOCK-derived complex structure (Fig. 6C) and hence can provide an independent biophysical method to confirm the long range distance information between corresponding regions of the complex model.

**DISCUSSION**

Several crystal structures of receiver domains of response regulators have been solved (43–46), but little structural information is available regarding how they interact with each other in multiprotein complexes. As well, knowledge of the structure of the receiver domain in complex with the connector protein is limited. Several groups have reported that the active site from the receiver domain of the response regulator is widely used for intermolecular protein-protein interactions with histidine kinase. Here, we present the crystal structure of PmrAN, in which a hydrogen bond couples with the carbonyl oxygen atom of Arg-81 situated on the β4-α4 loop and side chain of the aromatic residue Tyr-98, which is similar to the “T-loop-Y”
model observed for the activation mechanism of a response regulator (47). In the model, the conformation of the β4-α4 loop was gated by phosphorylation and the position of the active site residue Thr-79, thus resulting in an inward orientation of the aromatic switch residue (47). Next, we demonstrated that PmrAN uses a similar surface to interact with the TCS connector protein PmrD (Fig. 7). Therefore, PmrD is likely a competitive inhibitor of histidine kinase because it binds with the response regulator at a similar interface surrounding the phosphorylation site (48). In the PmrD-PmrAN complex model, the phosphoacceptor Asp-51 faces the open mouth area of the β-barrel of the PmrD structure, which reflects the protection mode of the connector protein on the phosphorylated response regulator. The orientation of PmrAN relative to PmrD can be attributed to three specific interactions. The first interaction is between the β1-α1 loop region of PmrAN and loop 4 and N-terminal β1 in PmrD. The second interaction is between the β1-α1 loop of PmrAN and loop 2 of PmrD. The third interaction is between the β4-α4 loop and β3-α3 loop containing the Asp-51 phosphorylation site of PmrAN and C-terminal β6, loop 6, N-terminal β1, and β3 of PmrD. Interactions 2 and 3 create an environment that hides the phosphoion analog of BeF₃ between PmrD and PmrAN. The binding interface of PmrAN is also similar to that observed in the crystal structure of the DHp domain in the ThkA-TrkA complex and Spo0F in the Spo0B-Spo0F complex from Bacillus subtilis, in which TrkA and Spo0F are the response regulators. In the PmrD-PmrAN complex, the phosphoacceptor Asp-51 in PmrA faces the His-70 of PmrD, similar to the phosphodonor His in the HK DHp domain (His-547 in ThkA) (48, 49). Hydrogen bonds and electrostatic interactions are important for stability and specificity of protein-protein interactions (50, 51). Consistently, we identified seven intermolecular hydrogen bonds and five salt bridges in our HADDOCK complex model (Fig. 5C), which emphasizes the role these interactions play in the recognition and specificity of PmrD connector proteins.

With response regulators, the phosphatase reaction is generally considered to proceed essentially as a reversal of the phospho-His phosphotransfer reaction, with water or hydroxide taking the place of the histidine imidazole side chain (52). Some of the histidine kinases have phosphatase activities toward their respective phosphorylated response regulators. The dephosphorylated kinases could promote a transition state with water in place of the phosphorylated histidine side chain, leading to hydrolysis instead of phosphorylation (53). For example, the structural basis of the response regulator Spo0F dephosphorylation by RapH phosphatases was revealed by the RapH-Spo0F crystal structure (Fig. 7B). In the structure, the side chain of RapH Gln-47 inserts into the Spo0F active site. RapH Gln-47 may orient water for direct in-line hydrophilic attack on the Spo0F phosphoaspartate 54 phosphorous atom and thus cause hydrolysis of the phosphoryl group (54). In these cases, the interactions are primarily responsible for the specific histidine
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The receiver domain of PmrA could form a cooperative, intermolecular dimer of a response regulator (57). Therefore, the steric hindrance effect provided by PmrD could prevent the dephosphorylation of PmrA mediated by the cation. As well, nucleophilic attack by a water molecule is the likely mechanism of autodephosphorylation in response regulators (58, 59). The side chain of His-70 in PmrD likely maintains the phosphoryl group stability by sterically inhibiting access of the water molecule, which executes a nucleophilic attack on the phosphorus and causes hydrolysis of the phosphoryl group. These interactions may explain how PmrD stabilizes the activated PmrAN protein and thus prevents dephosphorylation of the receiver domain of a response regulator, thus precluding close contact of phosphatase with the phosphorylation site on PmrAN. Hence, PmrD might inhibit dephosphorylation of the response regulator by acting as a simple physical blockade to sterically block access to the phosphoacceptor Asp residue on the receiver domain of the response regulator, thus precluding close contact of phosphatase with the phosphorylation site on PmrAN.

kinase-response regulator or connector-response regulator binding.

The receiver domain of PmrA could form a cooperative, intermolecular dimer of a response regulatory component that interacts to convert an inherently weak set of protein-protein interactions into a stronger binding force that permits the formation of a functional viable complex. Activation of a response regulator by a “pre-existing population shift” may be a general mechanism for two-component signaling (55). Selective binding to a low populated conformation hidden with traditional structural methods may be a general model for ligand binding (56). Therefore, PmrD may selectively bind to the phosphorylated higher energy active conformation of PmrAN and prevents its dephosphorylation by phosphatase such as PmrB.

In our previous study, we proposed a model of the PmrD-PmrAN protein complex and examined how PmrD regulates phospho-PmrA. Here, we propose that PmrD is a phosphatase inhibitor that specifically binds the phosphorylated active site and acts as a molecular barricade that inhibits interaction between the phosphatase and phosphorylatable Asp residue of PmrA and thus prevents intermolecular dephosphorylation. PmrD might inhibit dephosphorylation by sterically hindering the transfer of phosphate from Asp-51 of PmrA to phosphatase PmrB. The large size of PmrD and its predicted binding interface with PmrA are consistent with the model that inhibition occurs by a steric hindrance. The model shows three H-bonds between PmrD and residues near phosphorylation sites of PmrAN. For example, the HD1 of His-70 in PmrD formed H-bonds with the carbonyl group of Gly-53 in PmrAN. In addition, the HE2 of His-70 in PmrD formed an H-bond with OD1 of Asp-9 in PmrAN (Fig. 7C). A divalent cation close to the active site is required to add or remove phosphoryl groups in the receiver domain of a response regulator (57). Therefore, the steric hindrance effect provided by PmrD could prevent the dephosphorylation of PmrA mediated by the cation. As well, nucleophilic attack by a water molecule is the likely mechanism of autodephosphorylation in response regulators (58, 59). The side chain of His-70 in PmrD likely maintains the phosphoryl group stability by sterically inhibiting access of the water molecule, which executes a nucleophilic attack on the phosphorus and causes hydrolysis of the phosphoryl group. These interactions may explain how PmrD stabilizes the activated PmrAN protein and thus prevents dephosphorylation of phosphate on Asp-51 from a phosphatase such as PmrB. PmrD may inhibit dephosphorylation of the response regulator by acting as a simple physical blockade to sterically block access to the phosphoacceptor Asp residue on the receiver domain of the response regulator, thus precluding close contact of phosphatase with the phosphorylation site on PmrAN.

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Acknowledgments—The NMR spectra were obtained at the High Field Nuclear Magnetic Resonance Center supported by the National Research Program for Genomic Medicine. We thank Laura Smales for copyediting the manuscript. We are grateful for access to synchrotron radiation beamlines BL13B1 at the National Synchrotron Radiation Research Center in Taiwan.
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