Tetrameric architecture of an active phenol-bound form of the AAA$^+$ transcriptional regulator DmpR

Kwang-Hyun Park$^{1,6}$, Sungchul Kim$^{2,6}$, Su-Jin Lee$^{1,3}$, Jee-Eun Cho$^1$, Vinod Vikas Patil$^{1,3}$, Arti Baban Dumbrepatil$^1$, Hyung-Nam Song$^1$, Woo-Chan Ahn$^1$, Chirlmin Joo$^{2,6}$, Seung-Goo Lee$^4$, Victoria Shingler$^5$ & Eui-Jeon Woo$^{1,3}$

The Pseudomonas putida phenol-responsive regulator DmpR is a bacterial enhancer binding protein (bEBP) from the AAA$^+$ ATPase family. Even though it was discovered more than two decades ago and has been widely used for aromatic hydrocarbon sensing, the activation mechanism of DmpR has remained elusive. Here, we show that phenol-bound DmpR forms a tetramer composed of two head-to-head dimers in a head-to-tail arrangement. The DmpR-phenol complex exhibits altered conformations within the C-termini of the sensory domains and shows an asymmetric orientation and angle in its coiled-coil linkers. The structural changes within the phenol binding sites and the downstream ATPase domains suggest that the effector binding signal is propagated through the coiled-coil helixes. The tetrameric DmpR-phenol complex interacts with the $\sigma^{54}$ subunit of RNA polymerase in presence of an ATP analogue, indicating that DmpR-like bEBPs tetrarsers utilize a mechanistic mode distinct from that of hexameric AAA$^+$ ATPases to activate $\sigma^{54}$-dependent transcription.
The AAA⁺ family of ATPases is involved in various essential cellular processes. The bacterial enhancer binding (bEBP) subgroup of AAA⁺ proteins couple ATPase hydrolysis to initiation of transcription by σ54-RNA polymerase (σ54-RNAP). Many bEBPs belong to two-component systems, in which a membrane-bound histidine kinase senses and transfers a signal from the environment to a corresponding response regulator to allow σ54-dependent promoter activity. In contrast, some bEBPs are single-component sensory regulators that directly bind effector molecules to achieve the same outcome. DmpR (dimethyl phenol regulator) from Pseudomonas putida KCTC 1452 (also known as CapR) is a single-component bEBP that serves as a sensor of phenolic compounds. In habitats contaminated by phenol and other aromatic pollutants, catabolism of these compounds is mediated by tightly regulated operons that encode specialized suites of enzymes necessary for the sequential breakdown of recalcitrant compounds (e.g., toluene, xylene, cresols and other aromatic ring-containing hydrocarbons). DmpR has also been widely used in engineering of bacteria and the development of whole-cell biosensors.

As is typical of bEBPs, DmpR consists of three domains: (1) a sensory domain consisting of a vinyl-4-reductase (V4R) scaffold that functions in binding of an aromatic effector molecule; (2) a conserved central AAA⁺ ATPase domain bearing the bEBP-specific GAFTGTA motif that is involved in coupling ATP hydrolysis to the restructuring of σ54-RNAP; and (3) a DNA binding domain that interacts with the palindromic upstream activating sites (UASs) situated ~100–200 bp upstream from the σ54 promoter. The B-linker that connects the sensory domain and the ATPase domain plays an important role in relaying the effector binding signal to allow ATP hydrolysis. In hexameric bEBPs with ring structures, higher-ordered oligomers induce formation of the catalytic active site at the interface between adjacent ATPase subunits. DmpR share high sequence homology with other aromatic-responsive bEBPs, such as XylR, TnrR, PoxR and MopR. In the absence of phenol, a majority of the molecules (~75%) exhibited four-step photobleaching upon addition of phenol, while ~17% of the molecules exhibited two-step photobleaching, indicating that phenol promotes an increase of the tetrameric subpopulation before addition of phenol due to the binding of E. coli derived aromatic metabolites as has been observed for other aromatic hydrocarbon binding proteins.

To confirm tetramer formation upon phenol binding, we used single-molecule photobleaching (SMPB). We generated a fusion containing fluorescent eGFP and N-terminally 6 × His-tagged DmpR (Fig. 1a). The fusion protein was surface-immobilized using a biotinylated anti-GFP antibody. Stepwise bleaching signals from eGFP were recorded using total internal reflection fluorescence (TIRF) microscopy. A TIRF image of eGFP-DmpR fluorescence showed clearly separate fluorescent spots (Fig. 1c). Discrete steps were observed from individual eGFP-DmpR fluorescence time traces (Fig. 1d). Although there were ~18% of protein aggregates (Fig. 1e), eGFP-DmpR exhibited a photobleaching distribution that corresponds to a mixture of multiple oligomeric states. In the absence of phenol, a major fraction of molecules (~32%) showed two-step photobleaching, which is indicative of dimers. One-step (monomers), three-step (trimers) and four-step (tetramers) photobleaching were observed in around 11, 14 and 19% of the population, respectively (Fig. 1f). One-step bleaching from dimeric eGFP-DmpR and less-than-four-step bleaching from tetrameric eGFP-DmpR could originate from incomplete eGFP maturation. The eGFP maturation was estimated to be 85% from the ratio between a protein concentration measured from the 280-nm absorbance and an eGFP fluorophore concentration measured from 488-nm absorbance. There were hardly any oligomers that underwent more than five photobleaching steps within the populations. Upon the addition of phenol, a majority of the molecules (~34%) exhibited four-step bleaching, while ~17% of the molecules exhibited two-step bleaching, indicating that phenol promotes an increase of the tetrameric population at the expense of the dimer population. No change was observed upon the addition of ATP (Fig. 1h–j). Together, these results show that phenol promotes tetramer formation by DmpR and this oligomerization is independent of ATP.

**Results**

**Phenol promotes tetrameric association.** Upon the addition of a phenolic ligand, DmpR forms oligomers which are required to promote transcription. We first examined the formation of oligomers in response to the addition of phenol using purified full-length DmpR bearing an N-terminal 6 × His tag (DmpRWT, purity >95%; 66 kDa). As assessed by blue native (BN)-PAGE analysis, in the absence of phenol, DmpRWT appeared as a mixture of dimers (~132 kDa) and tetramers (~264 kDa). When incubated with 1 mM phenol, the band corresponding to the dimer shifted to reflect the higher molecular weight of the DmpRWT tetramer (Supplementary Fig. 1a). A change in the oligomeric state of DmpRWT by phenol was also observed in size exclusion chromatography (SEC) and in dynamic light scattering (DLS), respectively (Supplementary Fig. 1b, c). Addition of ATP analogues (ANP-PNP or ATPyS), or DNA containing its specific binding sites (upstream activating sequences, UASs) did not change the tetrameric association of DmpRWT (Supplementary Fig. 1d). DmpRWT exhibited a marginal increase in DNA binding activity in the presence of phenol (K₅₀ value ~ 387 nM) as compared to the absence of phenol (~476 nM) (Supplementary Fig. 1e). Consistent with these findings, multi-angle light scattering (MALS) analysis also showed a protein peak with a molecular weight of ~280 kDa upon the addition of phenol in both the presence and the absence of ATPyS, indicating that DmpRWT predominantly forms a tetramer in response to phenol (Supplementary Fig. 1f). The presence of a tetrameric subpopulation before addition of phenol presumably resulted from binding of E. coli derived aromatic metabolites as has been observed for some other aromatic hydrocarbon binding proteins.

**Structure of the tetrameric DmpRAD-phenol complex.** Purification and crystallization of DmpRWT was hampered by a limited amount of full-length protein due to its low solubility and aggregation as inclusion bodies in E. coli. Based on a solubility profile analysis and preliminary tests, we designed a truncated DmpRAD version of DmpRWT, which has an N-terminal 6 × His tag and a C-terminal maltose binding protein (MBP) tag (DmpRADWT). However, the MBP tag hindered the crystallization of DmpRWT. The MBP tag was therefore removed, resulting in DmpRADWT. Native mass spectrometry analysis indicated that DmpRADWT forms tetramers in solution. Crystallization of DmpRADWT yielded a tetrameric structure of DmpRADWT-phenol complex.

The structure of DmpRADWT-phenol complex shows that the phenol molecule is located in the center of the tetrameric complex. The phenol molecule is bound to the central AAA⁺ ATPase domain, which is responsible for the ATPase activity of DmpR. The phenol molecule is also bound to the sensory domain, which is responsible for the activation of the ATPase domain. The sensory domain contains a conserved motif, the GAFTGTA motif, which is characteristic of bEBPs. The sensory domain also contains a conserved cysteine residue, which is involved in the binding of the phenol molecule.

**Discussion**

The structure of DmpRADWT-phenol complex provides insights into the mechanism of DmpR activation by phenol. The phenol molecule is located in the center of the tetrameric complex, which suggests that the phenol molecule is the effector molecule that activates DmpR. The phenol molecule is bound to the central AAA⁺ ATPase domain, which is responsible for the ATPase activity of DmpR. The phenol molecule is also bound to the sensory domain, which is responsible for the activation of the ATPase domain. The sensory domain contains a conserved cysteine residue, which is involved in the binding of the phenol molecule. This suggests that the phenol molecule induces a conformational change in the sensory domain, which in turn activates the ATPase domain.

**Conclusion**

The results presented here provide insights into the mechanism of DmpR activation by phenol. The phenol molecule binds to the central AAA⁺ ATPase domain and the sensory domain, which suggests that the phenol molecule is the effector molecule that activates DmpR. The structure of DmpRADWT-phenol complex provides insights into the mechanism of DmpR activation by phenol. The phenol molecule induces a conformational change in the sensory domain, which in turn activates the ATPase domain.
DmpR derivative (aa 18–481) that is soluble and produced at sufficient levels in *E. coli*. This truncated protein, DmpRΔD has an N-terminal 6×His tag that replaces the first 15 residues, lacks the DNA binding domain, and carries serine substitutions of two cysteine residues (C119S/C137S) that were anticipated to be located at the protein surface. DmpRΔD has a phenol binding affinity ($K_D \approx 12 \mu M$) similar to full-length DmpR ($K_D \approx 16 \mu M$)\(^1^9\) and likewise exhibits tetrameric oligomerization in the presence of phenol (Supplementary Fig. 2a, b).

The determined crystal structure of DmpRΔD shows a phenol molecule bound to the sensory domain of each monomer. The sensory and ATPase domains are connected by an ~35 Å long

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**Fig. 1 Single-molecule stoichiometry measurements of DmpR oligomers.**

*Fig. 1a* Domain organisation of the eGFP-DmpR protein used for single-molecule photobleaching (SMPB). *Fig. 1b* Schematic overview of the experimental design of SMPB assays. *Fig. 1c* A representative EMCCD image including four major species of eGFP-DmpR proteins (monomer, dimer, trimer and tetramer). Asterisks represent the signal from presumable protein aggregates. All data are representative of five replicates with similar results. Scale bars, 5 μm. *Fig. 1d* Representative time trajectories of the eGFP emission signals. The stoichiometry of the eGFP-DmpR proteins was determined by counting the number of eGFP photobleaching steps. Light blue lines are eGFP emission traces. Pink lines represent stepwise fits of the traces. *Fig. 1e* A representative time trajectory of the signal from presumable protein aggregates. *Fig. 1f–j* Distribution of photobleaching steps of eGFP-DmpR. The pie graphs above the histograms depict the ratio of dimers and tetramers for each condition. Events with more than eight photobleaching steps were categorised as aggregates (A). Data are presented as mean ± SD from three independent experimental replicates with $n \geq 180$ individual molecule (Counts).
helical B-linker. The protomer topology exhibits a ‘dumbbell-like’ structure with approximate dimensions of 110 × 55 × 70 Å (Fig. 2a). The DmpRΔD-phenol complex is a dimer-of-dimers with overall dimensions of 150 × 75 × 70 Å (Fig. 2b). The two protomers (P1 and P2) form an elongated intertwined P1/P2 dimer through extensive interactions between the related sensory domains and parallel coiled-coil B-linkers in a head-to-head orientation with a buried surface area of 2895 Å² (Fig. 2c). The two dimers—P1/P2 and P3/P4—form the tetramer, which has an antiparallel head-to-tail assembly that places the four ATPase domains at the central core of the complex. The complex, with dimeric sensory domains at either end, adopts an overall elliptical rod-like shape. Since the two DmpRΔD C-termini are located next to each other due to the twofold symmetry, the DNA binding domains that are missing in truncated DmpRΔD would be present as pairs, and those from the P1 and P3 protomers would be on one side and those from the P2 and P4 protomers would be on the opposite side of the centre of the complex, as depicted in Fig. 2d.

The formation of the DmpRΔD-phenol complex buries a surface area of ~26,800 Å² (33% of the combined surfaces) between the protomers. The P1/P2 sensory domain dimer packs against the ATPase domains in the P3/P4 dimer in such a way that the Val53 and Ile58 residues in the P1 sensory domain interact with Phe312 in the GAFTGA motif (aa 310–315) in the P3 ATPase domain (Supplementary Fig. 3a). Residues Glu210 and Glu214 in the P1 protomer B-linker interact with Thr316 and Arg319 of the GAFTGA loop within the ATPase domain of the P4 protomer (Supplementary Fig. 3b). The same pattern is observed for the P2 and P3 protomers. The pairs of ATPase domains within the P1/P2 and P3/P4 dimers do not interact with each other (Supplementary Fig. 3c), whereas the ATPase domains in the P1 and P2 protomers interact with those in the P3 and P4 protomers, respectively, through the twofold symmetry observed...
between the α-helical P1/P3 and P2/P4 subdomains (Supplementary Fig. 3d).

Phenol-bound sensory domain and B-linker. The sensory domain of DmpR shows a core (β/α)₄ barrel scaffold with a bound phenol and zinc ion (Fig. 3a). Each N-terminal region, comprised of residues 18–45 in each sensory domain, intertwines with the other sensory domains to yield a tightly interlocked homodimer. The phenol is located in an enclosed cavity (24–36 Å³ in volume) formed by an antiparallel hairpin motif. The cavity is primarily lined by hydrophobic residues, including Phe93, Trp128, Tyr170, and Tyr159. A strictly conserved Trp128 residue is located between the phenol-binding site and the zinc-binding site, while the zinc is coordinated by residues Cys151, Glu172, Cys177 and Cys185 (Fig. 3b). The hydroxyl group of the phenol is located between His100 and Trp128, indicating a ligand-positioning function of these residues. His100 is conserved in other phenol-responsive regulatory proteins, such as PoxR and MopR, while it is substituted by tyrosine in the toluene/xylene-responsive XylR (Fig. 3c). Interestingly, the electron density of the phenol is strong in the P1 protomer, whereas it is weak in the P2 protomer (Supplementary Fig. 4a, b). The same pattern is also observed in the P3/P4 dimer. Given its location inside a closed pocket, the weak electron density suggests low occupation by phenol in the binding cavities of the P2 and P4 protomers, which is associated with the altered conformations of the two protomers and the asymmetric shape of the B-linkers (see below).

The B-linker connects each lobe of the sensory and ATPase domains to form a linear helix with leucine residues at positions 212, 215, 219 and 222 creating a hydrophobic stripe on one side of the helix in the amphipathic structure. These strips of leucine residues in the two B-linkers adopt a coiled-coil architecture in the dimer and exhibit knobs-into-holes packing typical of leucine zippers (Fig. 3d). At the end of the B-linker, the helix connects to a flexible loop region consisting of residues 227–240 that has a high B-factor (~27 Å) and a sharp angle that extends into the ATPase domain (Fig. 3e).

The ATPase domain and its tetramer-dependent activity. The ATPase domain consists of a typical α/β subdomain (aa 236–401)
and an α-helical subdomain (aa 402–481). The GAFTGA motif (aa 310–315) of the P1 ATPase domain is located close to the P3 sensory domain (aa 53–59) and the P4 B-linker helix (aa 209–213) (Supplementary Fig. 5a). The GAFTGA regions exhibit conformational variation among the DmpR protomers, indicating their flexibility (Supplementary Fig. 5b). All of the ATPase domains have an overall structure similar to that of the ADP-bound form of PspF27. Although the crystallization of DmpRΔS occurred in the presence of 3 mM AMP-PNP, no electron density corresponding to a nucleotide was observed, suggesting that the GAFTGA conformations in this structure may reflect an inactive state that is poised to bind ATP. The putative ATP binding site (cavity volume of ~26 Å³) lies at the interface between the α/β subdomain and the α-helical subdomain and is spatially placed so that residues Glu232 and Tyr233 from the flexible loop that connects the B-linker and the ATPase domain could potentially interact with an ATP molecule (Supplementary Fig. 5c)28, Arg223, which is conserved in the B-linkers of aromatic-sensing DmpR-like bEBPs, is located in the proximity of the putative ATP binding site of the adjacent protomer (Supplementary Fig. 5d).

To investigate the connection between oligomerization and ATPase activity, we purified additional truncated derivatives of DmpR (Fig. 4a). BN-PAGE analysis of these derivatives after incubation with ATP or ATPγS revealed that both the ATPase domain alone (DmpRΔC) and the ATPase domain attached to the B-linker (DmpRBC) exhibited a monomeric conformation. However, the truncated protein lacking only the sensory domain (DmpRΔS) displayed a tetrameric conformation even in the absence of phenol (Fig. 4b). Similarly, SEC-MALS analysis showed a peak corresponding to a protein with a molecular weight of ~164 kDa, indicating that DmpRΔS predominantly forms tetramers in solution (Fig. 4c). The trace band of higher molecular weight observed in BN-PAGE in DmpRΔS, but not in DmpRC, DmpRBC or DmpRWT, is likely an artefact caused by non-native self-interaction of the sensory domain deleted DmpR protein. These results show that the ATPase domain of DmpRΔC alone, or when attached to the B-linker (DmpRBC), does not multimerize despite the major contribution of the ATPase domain to tetramer formation by DmpRΔS. These findings additionally suggest the involvement of the DNA-binding domain in tetramer formation, possibly through the pairing of the DNA-binding domains29. Next, we investigated the ATPase activity of all the DmpR derivatives to assess the correlation between oligomerization and ATPase activity. The DmpRWT and DmpRΔS proteins exhibited ATP hydrolysis in the presence of phenol, but they exhibited only marginal ATP hydrolysis in the absence of phenol (Fig. 4d). In contrast, the monomeric DmpRΔC and DmpRΔC derivatives did not show any ATP hydrolysis activity, while the tetrameric DmpRΔS protein exhibited efficient ATPase activity irrespective of the addition of phenol (Fig. 4e). These results suggest that a tetrameric configuration is essential and sufficient for the ATPase domains of DmpR to hydrolyse ATP.

**Alteration of the conformations within an asymmetric shape.** Conformational changes of DmpR were revealed when the
protomer structures were overlapped. Superimposition of the P1 protomer, which has a high phenol occupancy, onto that of the P2 protomer, which has a low phenol occupancy, uncovered interesting structural features. The volume of the phenol-binding pocket in the P1 protomer was 23.59 Å³, whereas it was 36.91 Å³ in the P2 protomer due to marginal shifts in the residues lining the pocket, including Tyr90, Phe93, His100, Val113, Phe122, Tyr159 and Phe170 (Supplementary Fig. 6a). The N-terminal region, which is involved in the interlocking of dimers (aa 18–39), is located ~2.9 Å further away from the phenol-binding site in the P1 protomer than in the P2 protomer. The helices in the B-linker also differ, with those from the P2 protomer adopting an orientation offset by ~24° compared to that of the corresponding helix from the P1 protomer, and as a result, the dimer exhibits a notably asymmetric configuration (Fig. 5a). The same pattern of conformational variation was observed in the P3/P4 dimer across the diagonal of the complex (P1/P2, r.s.m.d. = 3.7 Å, 437C°; P1/ P3, r.s.m.d. = 0.9 Å, 443C°; and P1/P4, r.s.m.d. = 4.0 Å, 443C°).

The significant shift in the B-linker is associated with helix α6 in the sensory domain; Lys200 is involved in a charged interaction with Glu167 in the P1 protomer at a distance of 2.4 Å, while Phe203 is shifted 1.8 Å further away from the sensory domain in the P1 protomer than in the P2 protomer. Asp206 from the P1 protomer is involved in a charged interaction with Arg60 in the sensory domain, whereas the same residue in the P2 protomer points outside of the helix and is closer to Arg67 (Fig. 5a). Interestingly, the position of the α6 helix exhibits significant variation among the DmpR, PoxR and MopR structures despite the high structural similarity in other regions of the sensory domain (PoxR, r.s.m.d. = 1.1 Å, 196C°; MopR, r.s.m.d. = 0.9 Å, 158C°) (Supplementary Fig. 6b)11,12. Helix α6 in MopR shows a completely opposite trajectory to that observed in DmpR, demonstrating the flexibility of this helical region among the subfamily members (Fig. 5b).

The closest structural analogue of the DmpR monomer is NtrC1, which is a βEBP member of a two-component system (Z score = 28.9; r.s.m.d. = 1.7 Å for 247 C°). Superimposition of the ATPase domains of DmpRΔD with those of inactive NtrC1 (PDB ID, 1ny5) highlights the significantly altered orientation of their B-linkers. With respect to the ATPase domain, the cognate B-linkers are displaced by ~135° despite the high structural similarity of each module (B-linker, r.s.m.d. = 1.3 Å, 21 C°; ATPase domain, r.s.m.d. = 2.4 Å, 243 C°) (Fig. 5c). A recent report showed that the central AAA+ domain and part of the B-linker of apo DmpR forms a homodimer with a face-to-face orientation in the ATPase domain28. Given the head-to-head geometry of the tightly intertwined sensory domains of DmpR and the dimeric features of the coiled coil B-linker helices, the apo dimer of DmpR may adopt a configuration similar to that of the

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**Fig. 5 Conformational changes within DmpRΔD.**

a) Superimposition of the sensory domains of the P1 protomer (light blue) and the P2 protomer (wheat) to highlight the asymmetry. The region showing significant structural changes is indicated by a dotted box. The movement of several residues in the region connecting helix α6 and the B-linker (blue sticks in the P1 protomer and white sticks in the P2 protomer). The changes in each residue are indicated by the dotted arrows. b) Structural flexibility of helix α6 in the sensory domains of MopR (PDB ID, 5kbi, light pink), PoxR (PDB ID, 5fru, yellow) and DmpR (light blue) is represented within a dotted circle. c) Superimposition of the DmpR protomer (light blue) onto that of NtrC1 (PDB ID, 1ny5, green) with respect to the ATPase domains to highlight the flexible region and the different trajectories of the B-linker and ATPase domain. d) Model of the conformational change of the transition from the inactive DmpR dimer to the tetrameric complex upon the binding of phenol.
inactive dimer of NtrCl or NtrX (Supplementary Fig. 6c)\textsuperscript{30,31}. Overall, the spatial variation in the phenol binding pocket, the phase shifts of the residue interactions in helix a6 and the asymmetric angle and trajectory of the B-linker of DmpR indicate propagation of structural changes and modulation of downstream domain interactions through the B-linker upon phenol binding (Fig. 5d) (see below).

**Interaction between tetrameric DmpR and $\sigma^{54}$.** Activation of transcription involves a physical interaction between the bEBP and $\sigma^{54}$-RNAP, specifically through the N-terminus region (aa 1–56) of the $\sigma$-factor\textsuperscript{32}. We examined the interaction of the ligand-bound DmpR complex with $\sigma^{54}$ using far-Western blotting\textsuperscript{33} (Supplementary Fig. 7a). A band corresponding to the size of the $\sigma^{54}$ protein was detected only when DmpRWT was incubated in the presence of phenol and ATPγS (Supplementary Fig. 7b), while the ATPase activity of DmpRWT did not change upon addition of the $\sigma^{54}$ protein (Supplementary Fig. 7c). We next measured the interaction of DmpRWT with the $\sigma$-factor using isothermal titration calorimetry (ITC) with $\sigma^{54}$ (1–119)-CPD. The $\sigma^{54}$ (1–119)-CPD protein comprises the N-terminal residues of $\sigma^{54}$ (aa 1–119) fused to a C-terminal cysteine protease domain (CPD) that allowed better expression and purification (Supplementary Fig. 7d). Consistent with the far-Western data, DmpRWT interacted with the N-terminal peptide of $\sigma^{54}$ only in the presence of phenol and ATPγS ($K_D = 4 \mu$M; Supplementary Fig. 7e). The stoichiometry of the ITC binding curve ($n = 0.86 \pm 0.022$) indicates a 1:1 molar ratio for the interaction between $\sigma^{54}$ (1–119)-CPD and tetrameric DmpRWT.

To visualise the interaction of DmpR with $\sigma^{54}$ (1–119)-CPD and confirm the stoichiometry of the complex, we used single-molecule fluorescence imaging\textsuperscript{34}. In the first series of experiments, biotinylated $\sigma^{54}$ (1–119)-CPD was surface-immobilized through a biotin-streptavidin interaction, and then stepwise photobleaching signals from $\sigma^{54}$ (1–119)-CPD bound eGFP-DmpRWT were recorded using TIRF microscopy (Fig. 6a and Supplementary Fig. 8a). The number of binding events between eGFP-DmpRWT and $\sigma^{54}$ (1–119) significantly increased upon the addition of phenol and ATPγS (Fig. 6b and Supplementary Fig. 8b). As assessed by real-time imaging, the majority of eGFP-DmpRWT bound to $\sigma^{54}$ (1–119) exhibited four-step bleaching under these conditions (Fig. 6c, d and Supplementary Fig. 8c). The fractions of the monomeric, dimeric and trimeric states could be attributed to the incomplete maturation of the eGFP fluorophore\textsuperscript{22,24,25,35}. These results show that when associated with phenol and ATPγS, tetrameric DmpR efficiently interacts with the $\sigma^{54}$ peptide.

As a complementary approach, we reversed the order of the interaction by immobilizing eGFP-DmpRWT in the presence of phenol and ATPγS using biotinylated anti-GFP antibody. Cy5-labelled $\sigma^{54}$ (1–119)-CPD was then added to assess the interaction between DmpR and the $\sigma^{54}$ (1–119) Peptide and determine which oligomeric state(s) of DmpR can interact with $\sigma^{54}$ (Fig. 6e and Supplementary Fig. 8d, e). Binding of Cy5-labelled $\sigma^{54}$ (1–119)-CPD co-localized with surface-immobilized eGFP-DmpR, indicating a highly specific interaction. eGFP-DmpRWT binding, which was observed at a location where $\sigma^{54}$ (1–119) was pre-bound (Fig. 6d), further revealed that tetrameric DmpR specifically interacts with the $\sigma^{54}$ (1–119) peptide (Fig. 6g). Taken together, the single-molecule data suggests that in the presence of phenol and ATP, tetrameric DmpR binds $\sigma^{54}$ to activate transcription by $\sigma^{54}$-RNAP.

**Discussion**

Research on the activation mechanism of DmpR has been hindered due to the ambiguity of the oligomeric state of its transcription-promoting active form. DmpR has been widely believed to form hexamers\textsuperscript{13}, primarily based on its similarity to ring-structured hexameric bEBPs such as NtrC and PspR\textsuperscript{36,37}. Although many AAA$^+$ ATPases function as hexamers, the active oligomeric state of DmpR-like bEBPs remained unclear. Thus, the discovery of the tetrameric configuration of DmpR and its demonstrated ability to interact with the $\sigma^{54}$ factor provided by this study represents an important step for an increased understanding of the activation mechanism of DmpR-like single component bEBPs. Interestingly, the GAF-TGα motif loops in the ATPase domains are located some distance from one another in the tetrameric architecture of DmpR with a perpendicularly two-fold symmetry, whereas the GAF-TGα loops are close together in the centre of the ring-like hexamer, indicating an altered mode of binding to $\sigma^{54}$. The interaction of the DmpR tetramer with $\sigma^{54}$ in a 1:1 ratio implies that the initial binding to $\sigma^{54}$ likely occurs through a GAF-TGα motif in a single ATPase domain. Such an interaction could plausibly cause a steric hindrance in the complex to prevent further interactions or trigger an allosteric change in the tetramer that would allow it to assume a configuration optimally poised to activate $\sigma^{54}$-RNAP; these two mechanistic alternatives require further investigation. Given the asymmetric configuration between two monomers in a dimer and the absence of ATP molecule in the crystal structure, the dynamic DmpR tetramer probably undergoes conformational change in the process of binding and/or hydrolyzing ATP that accompanies its interaction with $\sigma^{54}$.

The structural features of ligand-bound DmpR exhibit remarkable similarity to those of histidine kinases (HKs), which are sensory components of the bacterial two-component system. The sensing of environmental changes through a dimeric N-terminal domain, the shifts of the coiled-coil linker helices in the middle of the molecule, and the modulation of ATPase activity by alterations in the positioning and orientation of a downstream domain are all reminiscent of the internal signal relay mechanism observed in HKs\textsuperscript{2}. The coiled-coil architectures of the GAF, HAMP and PAS linker domains in HKs are known to be crucial for oligomerization, signalling and the regulation of their activity\textsuperscript{39}. Although the exact mechanism of signal propagation through coiled-coil helices in HKs is still under debate (e.g., an axial rotation, axial tilt (scissor) or axial shift (piston) mechanism), typically, HKs exhibit two distinct structural conformations: an “off” state that imposes conformational restraints on the downstream domains and a dynamic “on” state that releases those conformational restraints, allowing the downstream domains to carry out ATPase functions\textsuperscript{39}. Intriguingly, the helical motifs that connect to the DHP domains in HKs reportedly exhibit asymmetric conformations\textsuperscript{40}, as observed in the DmpRΔD-pheno phenol structure. Given that the symmetric to asymmetric “flip-flop” transition within a homodimer is a well-known signal transduction mechanism in many HKs\textsuperscript{41–43}, DmpR-like bEBPs may utilize a similar mechanism for signal transduction upon sensing aromatics. In particular, the formation of tetramers and the constitutive ATPase activity of the DmpRAS protein support the notion that the tightly-bound dimeric sensory domains of the full-length protein restrain the downstream domains to prevent tetramer formation in the absence of phenol, which explains the negative regulation of activity mediated by the sensory domain of DmpR. The tightly interlocked sensory domains, which are also observed in the PoxR and MopR structures\textsuperscript{13,15}, may also be the key structural element that would prevent hexamer formation and thus set DmpR and its homologues apart from the other typical hexameric AAA$^+$ ATPases.

In the absence of phenol, DmpR may form a dimer in such a way that the tightly intertwined sensory domains with a head-to-head geometry impose a conformational constraint on the coiled-
Photobleaching steps were categorized as aggregates (A).

The observation of features common between the first (HK) and second (response regulator, e.g., NtrC) protein that make up two-component systems suggests that DmpR may have combined the sensing and the regulation modules of each protein into one protein to ensure simple and efficient detection of small lipophilic ligands that can freely diffuse through the membrane layer. The formation of a DmpR tetramer in the presence of phenol and the absence of ATP indicates that ATP binding and hydrolysis, known to be prerequisites for transcriptional activation, are not required for subunit association. It thus appears plausible that ATP is bound to DmpR after oligomerization, and the energy from ATP hydrolysis is subsequently utilized for coordinating the binding and restructuring of σ^{54}-RNA polymerase through the structural rearrangement of the GAFGTGA loop. Because it is structurally distinct from ring-forming hexameric AAA^{+} bEBPs, the interaction of a tetrameric complex with σ^{54} represents a unique mechanistic mode of DmpR-like bEBPs in terms of σ^{54} dependent transcriptional activation.

**Methods**

**Cloning and protein purification.** DNA encoding DmpR (Accession No. AAP46187.1) was amplified by PCR from Pseudomonas putida KT2440 (Accession No. AF515710). Fragments spanning codons 1–563 (wild type), 18–481 (DmpRΔL), 205–563 (DmpRΔS), 205–481 (DmpRΔL) and 232–481 (DmpRΔS) were cloned into the pProEX HTa cloning vector, which has an N-terminal His tag (Invitrogen), via the BamHI and HindIII restriction sites. The DmpR cysteine mutant (C119S) was generated using a site-directed mutagenesis kit (Enzynomics) and verified by DNA sequencing (Sodgent). The σ^{54} gene sequence, (accession no. WP_003255133) including the σ^{54} and σ^{54}(1–119) gene cassettes, was cloned into the pET22b expression vector via the Ndel/HindIII sites, and the CPD coding region was inserted in-frame using the HindIII/XhoI sites to generate the σ^{54}(1–119)-CPD expression construct. The eGFP (FPbase ID. R9NL8) gene was fused with pProEX HTa-cloned DmpR by a ligation-independent cloning method. Detailed cloning primer information is listed in Supplementary Table 1.

For DmpR purification, the His-tagged wild type, substituted, and eGFP-tagged DmpR variants were produced using E. coli strain BL21-CodonPlus (DE3)-RIL (Agilent Technologies, #230245), which was cultured at 30 °C, with expression induced with 1 mM IPTG. The cells were harvested, lysed and centrifuged. The supernatant was then applied to a His-Trap HP column (GE Healthcare) in elution buffer composed of 30 mM Tris-HCl (pH 7.5), 250 mM NaCl, 3 mM β-mercaptoethanol, 1 mM PMSF, 250 mM imidazole and 5% glycerol. The peak fractions were applied to a Superdex 200 Increase 10/300 column (GE Healthcare) in a final elution buffer composed of 30 mM Tris-HCl (pH 7.5), 50 mM NaCl, 3 mM β-mercaptoethanol and 5% glycerol. For eGFP purification, His-tagged wild type σ^{54} and its variants were expressed as described above. The supernatants were
applied to His-Trap HP columns (GE Healthcare) with elution buffer composed of 30 mM Tris-HCl (pH 7.5), 500 mM NaCl, 3 mM β-mercaptoethanol, 1 mM PMSE, 1 mM DTT, 250 mM sucrose and 5% glycerol. To remove the CDP Tim4-CYP-CDP protein was incubated in 1 μM phytic acid overnight at 25 °C. Peak fractions were applied to a Superdex 200 Increase 10/300 column (GE Healthcare) in a final elution buffer composed of 30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 3 mM β-mercaptoethanol, 1 mM DTT and 5% glycerol.

To add the biotin and Cy5 fluorescent dye to eD41-119-CDP, purified eD41-119-CDP was reduced in phosphate-buffered saline (PBS) with 10 mM DTT for 2 hours at 25 °C. The reduced protein was buffer-exchanged into PBS without DTT using PD MiniTrap G-10 (GE Healthcare) and labelled with either poly(ethylene glycol) [N-(2-aminoethyl)-N-methyl]ethylene 2-(bismaleimido)ethane (Sigma-Aldrich, cat# 757748) or Cy5 Maleimide Mono-Reactive Dye (Sigma, cat# GEPA15131) for 2 hours at room temperature followed by incubation at 4 °C overnight. The labelled eD41-119-CDP preparations were then purified by SEC with a Superdex 200 100/300 GL column. The fractions containing labelled proteins were concentrated using Amicon® Ultra Centrifugal Filters, pooled in PBS with 50% glycerol, snap-frozen in liquid nitrogen and stored at −80 °C.

Single-molecule TIRF imaging and data acquisition. A prism-type total internal reflection microscope was used for the SMPB experiments. The eGFP derivative was excited with a 473-nm laser (Coherent, OBIS LX 75 mW). Cy5 was excited using a 637-nm laser (Coherent, OBIS 637 nm LX 140 mW). To obtain time traces, eGFP was excited as weakly as possible to minimize their rapid photobleaching during the time range of the measurement. The fluorescence signals of the molecules were collected using an inverted microscope (Olympus, IX-73) with a ×60 water immersion objective (Olympus, ULSAPo60xW). To block the 473-nm scattering was blocked with a notch filter (Semrock, 488/532/635 nm, NF01-488/532/635). Subsequently, the Cy5 signals were spectrally split with a dichroic mirror (Chroma, 635dctx) and imaged with the help of an electron multiplying EMCCD camera (Andor Technology, iXon 897). The data were obtained in either single-colour or dual-colour mode.

To eliminate the non-specific adsorption of proteins onto the quartz surface, pinenna-etched slides (Flukenbeiner) were passivated with a mixture of mPEG-SV (5 kDa, Lysan Bio Inc.) and Biotin-PEG-SVA (5 kDa, Lysan Bio Inc.) in the first PEylation treatment, and then M(PEG)4 Methyl-PEG-NHS-Ester reagent (ThermoFisher Scientific) was used for the second treatment as described previously. To improve the surface morphology, the assembled microfluidic flow chambers were subsequently incubated with 5% Tween-20 (v/v in T50 buffer containing 10 mM Tris, pH 8.0, and 50 mM NaCl) for 10 min, followed by a wash step with 100 µL of T50 buffer. Afterwards, the slides were incubated with 50 µL of streptavidin (0.1 mg/mL in T50 buffer, S888, Invitrogen) for 5 min, followed by a wash step with 100 µL of phosphate-buffered saline (PBS).

For the single-molecule photobleaching (SMPB) assay, 50 µL of 1 mg/mL anti-GFP (biotin) goat polyclonal antibody (pAb) (Abcam, ab6658) was injected into the chambers and incubated for 5 min prior to a wash step with 100 µL of phosphate-buffered saline (PBS). One microlitre of 10 mM eGFP-DmpR-WT was incubated in each well of a 384-well plate containing 50 mM MgCl2, 1% dextrose monohydrate (w/v, Sigma, D9559) and 1 mM Trolox ((±)-6-Maleimide Mono-Reactive Dye (Sigma-Aldrich, cat# 757748) or Cy5 Maleimide Mono-Reactive Dye (Sigma, cat# GEPA15131)) for 2 hours at room temperature followed by incubation at 4 °C overnight. The labelled eD41-119-CDP preparations were then purified by SEC with a Superdex 200 100/300 GL column. The fractions containing labelled proteins were concentrated using Amicon® Ultra Centrifugal Filters, pooled in PBS with 50% glycerol, snap-frozen in liquid nitrogen and stored at −80 °C.

Size exclusion chromatography and dynamic light scattering. DmpR (20 µM) and phenol (0.5 mM) were incubated at 25 °C for 20 min in PBS buffer. Before centrifugation, the supernatant was applied to a SEC or DLS system. SEC analysis was performed using a Superdex 200 increase 10/300 column with an AKTA FPLC system (GE Healthcare). DLS analysis was performed using a Zetasizer Ultra (Malvern), fitted with a 10-mW 632.8 nm laser with scattering angle of 173° in air and set at a 90° scattering angle.

Molecular docking modelling. The inactive DmpR dimer was modelled using the A. aeolicus NtrC1 in complex with ADP (PDB ID, 1n5y) as the template. The dimeric NtrC1 structure was truncated so that only the ATPas domain was retained. The docking of ADP to the DmpR structure with loop modelling was performed using the SwissDock server. The initial models were subjected to energy minimization followed by 1 ps of molecular dynamics at 300 K after equilibration. They were finally minimized to a maximum derivative with 1.0 kcal per step using the Discover module in the Insight II program (Accelrys) with the AMBER force field.

ATPase assay. The ATPase reactions were initiated by adding 5 mM MgCl2 into a mix containing 200 mM DmpR protein, 30 µM ATP, [γ32P] ATP (5 Ci/mmol) or/and 1 µM phenol or/and 1 mM MgCl2. The reactions (20 µL) were incubated at 25 °C for 20 min and then terminated by the addition of 10 mM EDTA. The radiolabelled reaction products (1.5 µL) were separated with polyethyleneimine-cellulose thin-layer plates (Merck) in 0.325 M phosphate buffer (pH 3.5) and visualised using a FLA-5100 phosphorimager (Fujifilm).

Isothermal titration calorimetry. The ITC experiments were conducted using a Microcal Auto-ITC200 at 25 °C at the Korea Basic Science Institute (KBSI). The DmpR solution (20 µM) was titrated with 100 µM phenol in the calorimetric cell was titrated with the phenol ligand (100 µM), cognate DNA with specific US sequences (100 µM), or eD41-119-CDP protein (400 µM) as the reactant. The data were analysed with the Microcal Origin software package (GE Healthcare).

Far-Western blot assay. Purified eD41 protein (0.25 µg) was resolved by 10% SDS-PAGE and electro-transferred onto PVDF membranes (GE Healthcare). The eD44 bound to the membrane was refolded in incubation in 6 M–0.1 M guanidine-HCl in AC buffer (10% glycerol, 100 mM NaCl, 20 mM Tris, 0.5 mM EDTA, 1 mM DTT and 0.1% Tween-20) supplemented with 100 µg/mL DmpR protein in a 0.16 mg/mL final protein concentration in AC buffer at room temperature. The membrane was washed with AC buffer supplemented with 5% milk powder for 2 hours at 4 °C prior to incubation with 500 µg/mL His-tagged DmpR bait protein at 4 °C overnight. The membrane was subsequently washed and incubated for 1 hour with His-tag antibody (Invitrogen, #MA1-21313, 3000-fold dilutions) in phosphate-buffered saline with Tween-20 (PBST) with 3% milk powder. After washing with PBST, the membrane was incubated for 1 hour with the anti-mouse secondary antibody (Sigma, #A3362, 30,000-fold dilutions).
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Author contributions

K.-H.P., S.K. and E.-J.W. conceived the study. S.-G.L. and V.S. provided scientific and experimental suggestions. K.-H.P., S.K., S.-J.L., J.-E.C., H.-N.S., A.B.D. and W.-C.A. performed the protein purification and/or crystallization. The structural data analysis and refinement were performed by K.-H.P. and E.-J.W. The biochemical experiments were performed by K.-H.P., S.-J.L., V.V.P. and W.-C.A., and the single-molecule
fluorescence analysis was performed by S.K. and C.J. The manuscript was written by K.-H.P., S.K., V.S. and E.-J.W. with input from all authors.

**Competing interests**
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to C.J. or E.-J.W.

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