A Close-up View of the VraSR Two-component System

A MEDIATOR OF STAPHYLOCOCCUS AUREUS RESPONSE TO CELL WALL DAMAGE

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Staphylococcus aureus remains a clinical scourge. Recent studies have revealed that S. aureus is capable of mounting a response to antibiotics that target cell wall peptidoglycan biosynthesis, such as β-lactams and vancomycin. A phosphotransfer-mediated signaling pathway composed of a histidine protein kinase, VraS, and a response regulator protein, VraR, has been linked to the coordination of this response. Herein, we report for the first time on the signal transduction mechanism of the VraSR system. We found that VraS is capable of undergoing autophosphorylation in vitro and its phosphoryl group is rapidly transferred to VraR. In addition, phosphorylated VraR undergoes rapid dephosphorylation by VraS. Evidence is presented that VraR has adopted a novel strategy in regulating the output response of the VraSR-mediated signaling pathway. The VraR effector domain inhibits formation of inactive VraR dimers and, in doing so, it holds the regulatory domain into an intermediate active state. We show that only phosphorylation induces formation of the biological active VraR-dimer species. Furthermore, we propose that damage inflicted to cell wall peptidoglycan could be the main source of the stimuli that VraR responds to due to the tight control that VraS has on the phosphorylation state of VraR. Our findings provide for the first time insights into the molecular basis for the proposed role of VraSR as a “sentinel” system capable of rapidly sensing cell wall peptidoglycan damage and coordinating a response that enhances the resistance phenotype in S. aureus.

A phosphotransfer-mediated signaling pathway in Staphylococcus aureus, namely VraSR2 (for vancomycin-resistance associated sensor/regulator), was shown to mediate resistance to β-lactam antibiotics and vancomycin, antibiotics that target the biosynthesis of cell wall peptidoglycan (cell wall synthesis inhibitors) (1–3). Phosphotransfer-mediated signaling pathways in bacteria link environmental stimuli to cellular responses (4–6). The simplest system, also referred to as two-component system, consists of two conserved modular proteins: a histidine protein kinase (HK) and a response regulator protein (RR) (Fig. 1). The environment stimuli are sensed by the HK, which undergoes autophosphorylation at a conserved histidine residue (7). The cognate RR catalyzes the transfer of the phosphoryl group from HK to a conserved Asp residue of its regulatory domain resulting in its activation (8). Most RR functions as transcription regulators and are divided into three subfamilies based on the homology of their effector domains: the OmpR/PhoB winged helix binding domain, the NarL/FixJ four-helix bundle domain, and the NtrC-ATPase-coupled transcription factors (9). The phosphorylation level of the RR controls the activity of this protein, and it is regulated by the phosphatase activity of the HK or RR itself (10, 11).

Two-component systems have been implicated in antibiotic resistance, such as resistance to vancomycin in Enterococcus faecium (VanSR) (12) and bacitracin in Bacillus subtilis (LiaSR) (13); both, vancomycin and bacitracin, are cell wall synthesis inhibitors. The VraSR system is unique from VanSR and LiaSR systems in that it mediates the S. aureus response to inhibitors of early and late steps in cell wall peptidoglycan biosynthesis (2, 14). This was confirmed by Sorbral et al. (15) and Gardete et al. (16), whereby reduction in the expression of murF or pbp2 (encodes PBP2), two genes, respectively, involved in the synthesis of the peptidoglycan precursor and incorporation of this precursor into the growing peptidoglycan, was shown to cause a rapid increase in the transcription of vraS and vraR. The VraSR system coordinates expression of more than 40 genes in S. aureus exposed to cell wall inhibitors (2). Among the regulated genes are those associated with the biosynthesis of peptidoglycan (17). Clearly, these studies indicate that the VraSR system plays a central role in maintaining the integrity of the cell wall peptidoglycan and coordinating the S. aureus response to cell wall damage, much like an antibiotic resistance mechanism.

Cell wall synthesis inhibitors such as vancomycin and β-lactams are an important group of antibiotics used in treatment of S. aureus infections (18, 19) and the emergence of resistance mechanisms to these antibiotics is a major setback in the fight against these infections (20, 21). The discovery that a two-component system is involved in the S. aureus response to these antibiotics suggests the presence of native defense mechanisms in this organism, which have the potential to evolve into more sophisticated resistance mechanisms if subjected to a constant selective pressure (i.e. constant use of cell wall-synthesis inhibitors) (22). Understanding the signal transduction mechanisms involved in the response to cell wall damage in S. aureus will
provide insights into the system that maintains the integrity of peptidoglycan and the design of new strategies to circumvent antibiotic resistance. Recent efforts on the development of small molecules that inhibit two-component systems have shown that both HK and RR are targetable (23–25).

Herein, we provide for the first time insights into the mechanism of the VraSR-mediated signal transduction pathway. We have investigated the kinetics of VraS autophosphorylation and phosphotransfer processes, together with the phosphotransfer relationship between VraS and VraR. Furthermore, we have probed the mechanisms through which the phosphorylation levels and the output response of VraR are regulated.

EXPERIMENTAL PROCEDURES

Materials and Chemical Reagents

The chemicals were purchased from Sigma or Fisher, unless otherwise stated. Chromatography media and columns were purchased from GE Healthcare. Growth media were acquired from Fisher. The Escherichia coli NovaBlue and BL21(DE3) strains, as well as the cloning and expression plasmids were purchased from Novagen. Restriction enzymes were obtained from New England Biolabs or Stratagene. The genome of S. aureus strain Mu50 was obtained from ATCC.

Cloning, Expression, and Purification of the Target Proteins

Cloning of VraR—The following cloning strategy for vraR did not result in the introduction of tags or extra amino acids. The PCR primers for vraR (direct, 5′-ACGCATATGACGTTTATTATTAATTTGGTGGG-3′ and reverse, 5′-ACGAAAGCTTTTATTATGGATTTGGGAA-3′) were designed to incorporate NdeI and HindIII restriction sites at the 5′ and 3′ ends (italicized sequences), respectively. The vraR gene encoding 209 amino acids was amplified from the methicillin-resistant S. aureus genome, strain Mu50 (ATCC). The amplicon was digested with HindIII and ligated into pET26b vector between the Mscl and HindIII cloning sites. The resulting construct was amplified in E. coli strain NovaBlue. Subsequently, the isolated plasmid DNA was digested with NdeI to remove the DNA stretch between NdeI sites in the pET26b::vraR construct. The digested vector was ligated and used to transform E. coli NovaBlue for amplification of the final construct. The correct sequence of the ligated vraR was confirmed by DNA sequencing and the final pET26b::vraR construct was used to transform E. coli BL21(DE3) cells.

Cloning of vraRN—The following cloning strategy for vraRN did not result in the introduction of tags or extra amino acids. The following direct and reverse primers were designed to amplify the N-terminal domain region of vraR, spanning residues 1 to 133, from the pET26b::vraR construct: direct, 5′-ACGCAATATGACGTTTATTATTAATTTGGTGGG-3′ and reverse, 5′-ACGAAAGCTTTTATTATGGATTTGGGAA-3′ (the restriction sites of NdeI and HindIII are italicized). The amplicon was ligated into pSTBlue blunt-end vector (Novagen) and the construct was used to transform E. coli NovaBlue. The insert was released from the pSTBlue vector by digestion with NdeI and HindIII and cloned into pET26b vector into the corresponding restriction sites. The final construct pET26b::vraRN was used to transform E. coli BL21(DE3).

Cloning of vraRC—The following cloning strategy for vraRC did not result in the introduction of tags or extra amino acids. The C-terminal domain of vraR, spanning residues 141–209 (this sequence was predicted by ExPaSy to form the C-terminal domain, which is in good agreement with other RRs), was amplified from the pET26b::vraR construct using the following PCR primers: direct, 5′-CATATGCGAGATTATGAAATGTTACA-3′ and reverse, 5′-ACGAAAGCTTTTATTATGGATTTGGGAA-3′ (the restriction sites NdeI and HindIII are italicized). The resulting PCR product was digested with the HindIII restriction enzyme and subsequently ligated into pET26b vector between the Mscl and HindIII cloning sites. The resulting construct was used to transform E. coli NovaBlue to amplify the vector. The isolated pET26b::vraRC was treated with NdeI restriction enzyme to remove the DNA stretch between NdeI sites followed by ligation. The correct cloning of the vraRC was determined by DNA sequencing analyses. The final construct pET26b::vraRD55N was used to transform E. coli BL21(DE3) competent cells.

Mutation of Asp-55 to Asn in VraR—The Asp-55 residue of VraR (encoded by GAT) was mutated to Asn (encoded by AAT) by QuikChange Site-directed Mutagenesis (Stratagene) using the following mutagenic primers: 5′-GATTTAATTTTATATGGTTTTATGGAAGAC-3′ and 5′-CTAAATTTAAATTTTAATGAC-3′ (the mutated nucleic acids are italicized in both the primers). As a template we used the pET26b::vraR vector. Successful mutation of the aspartate to asparagine was confirmed by DNA sequencing and the final pET26b::vraRD55N construct was used to transform E. coli BL21(DE3).

Purification of VraR, VraR-D55N Variant, and VraRC—All steps of the purifications were carried out at 4 °C. A seed culture of BL21(DE3) carrying the appropriate expression vector was grown overnight in Luria Bertani medium (Difco). An aliquot of 1 ml from the seed culture was used to inoculate 1 liter of Terrific Broth medium, supplemented with kanamycin (50 μg/ml). Protein expression was initiated at OD600 nm = 0.6 by the addition of isopropyl β-d-thiogalactopyranoside at a final concentration of 1 mM. Cells were allowed to express the target protein for 16 h at 25 °C and then harvested by low speed centrifugation (2,500 × g, 10 min). The resulting pellet was resuspended in 20 mM Tris buffer, pH 7.0, supplemented with 5 mM MgCl2. The protein was liberated by sonication and cell debris was removed by centrifugation at 25,000 × g for 60 min. The resulting supernatant was loaded onto a DEAE-Sepharose column. The VraR protein was eluted with a linear gradient of 500 mM Tris buffer, pH 7.0, supplemented with 5 mM MgCl2. Fractions containing the target protein (VraR, VraR-D55N, or VraR55) were concentrated by Amicon ultracentrifugation membrane (Ultralec 5K or Centriprep Ultracel YM-3, Amicon) and loaded into a heparin-Sepharose affinity column. The target protein (VraR, VraR-D55N, or VraR55) was eluted from the column with a linear gradient of buffer 500 mM Tris buffer, pH 7.0, 5 mM MgCl2.

Purification of VraRN—The purification of VraRN was carried out by a DEAE-Sepharose anion exchange column followed by Sephacryl S-200 size exclusion column chromatogra-
Cloning, Expression, and Purification of GST-VraS—The nucleic acid sequence of vrs encoding for the cytoplasmic domain of VraS, spanning amino acid residues 85 to 347, was amplified from the genome of *S. aureus* Mu50 (ATCC) using the following direct and reverse primers: direct, 5′-ACGG-GATCCATGGAAAGCCTACGTTGGCATTT-3′ and reverse, 5′-AGTTATTTATCGTCTACGAATCCTCTTT-3′. The primers contain restriction enzymes BamHI and SmaI (the italicized sequence). The amplicon (789 bp) was digested with BamHI and SmaI and subsequently cloned into the corresponding restriction sites of the vector pGEX-4T-1 (GE Healthcare), so that vrs is fused at the COOH terminus of the GST. The inserted sequence was confirmed by DNA sequencing. The resulting construct pGEX-4T-1::vrs was transformed into *E. coli* BL21(DE3).

A single colony from *E. coli* BL21(DE3) transformed with pGEX-4T-1::GST-vrs was grown overnight in Luria-Bertani medium, supplemented with 100 µg/ml ampicillin. A 1-ml aliquot from this seed culture was used to inoculate 1 liter of Terrific Broth medium, supplemented with 100 µg/ml ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside and growth media shaken for another 3 h at 7°C. The cells were harvested by centrifugation (2,500 x g, 10 min) and suspended in 1× phosphate-buffered saline buffer, pH 7.4. The soluble content of the cell was liberated via sonication. Cell debris was removed by centrifugation at 25,000 x g for 60 min. Purification of GST-VraS was carried out using a glutathione-Sepharose affinity resin. The target protein was eluted with 10 mM reduced glutathione in 50 mM Tris buffer, pH 8.0.

**Determination of the Oligomerization State of the Target Proteins**

The oligomerization state of the target proteins were analyzed by native polyacrylamide gel electrophoresis (native-PAGE; Tris-glycine system) and size exclusion chromatography (Superdex™ 75 (10/300GL, GE Healthcare) or TSKgel G2000SW_xl (7.8 × 300 mm, 5 µm, TOSOH Biosciences LLC)). Concentrations of VraR or VraRN samples analyzed by native PAGE were 20 or 40 µM, whereas the concentrations of these proteins analyzed by size exclusion chromatography were 100 µM.

**In Vitro Phosphorylation of GST-VraS**

Autophosphorylation of GST-VraS was examined as described previously with minor modifications (12). Briefly, purified GST-VraS (1 µM) was equilibrated in phosphorylation buffer (PB: 50 mM Tris, pH 7.4, 50 mM KCl, 5 mM MgCl₂) supplemented with 10 mM CaCl₂ at a final volume of 100 µl. The reaction was initiated by the addition of [γ-32P]ATP (250 µCi, 10 µM) at 25 °C. Aliquots were removed at different time intervals and the reactions were quenched by addition of 5× SDS sample buffer (SDS-SB: 2.5% SDS, 25% glycerol, 125 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 0.0025% bromphenol blue). Samples were analyzed by 12.5% SDS-PAGE. The gels were dried and subjected to autoradiography. These experiments were repeated three times. The gel were analyzed by NIH Image software (version 1.3). The band intensities were plotted against time and these curves are referred to as progress curves. The rates of phosphorylation were calculated as initial rates from the progress curves.

**Phosphotransfer Reaction**

Phosphorylation of GST-VraS was carried out as described above. The excessive [γ-32P]ATP was removed using a desalting column (Zeba™, Pierce). GST-VraS-phosphate (4 µM) was added to VraR (25 µM) in the PB buffer. The reaction mixture was incubated at 25 °C and samples of 10 µl were removed and quenched with 10 µl of 5× SDS sample buffer. The quenched solutions were analyzed by 15% SDS-PAGE. The gels were dried and subjected to autoradiography. Time-dependent experiments were repeated three times. The quantitative analysis of these experiments was carried out using NIH Image software (version 1.3). The band intensities were plotted against time and the rates of phosphorylation were calculated as initial rates from the progress curves.

**Phosphorylation of VraR by Acetyl Phosphate**

The phosphorylation of VraR by inorganic phosphate was carried out as described previously with minor modifications (26). Briefly, VraR at 40 µM was equilibrated in the PB buffer. Lithium potassium acetyl phosphate (Sigma) was added to a final concentration of 50 mM and the reaction was incubated for 60 min at 37 °C. The phosphorylated and unphosphorylated species were resolved by HPLC (Varian Inc.) using a ProSphere™ HP C4 reverse phase column (5 µm, 300 Å, 4.6 × 250 mm, flow rate 1 ml/min). The extent of VraR phosphorylation was estimated by integrating the surface area under the peak corresponding to VraR-P and comparing it with that of VraR in the absence of phosphorylation. Three independent experiments were performed.

**Phosphatase Activity of GST-VraS**

VraR (25 µM) was phosphorylated by acetyl phosphate as described above and GST-VraS-P was added to a final concentration of 4 µM. The reaction mixture was incubated for 10 min at 25 °C. A control reaction was prepared in parallel, where GST-VraS-P was replaced with buffer. Both reaction mixtures were analyzed by ESI-MS at the Advanced Protein Technology Center, Hospital for Sick Children (Toronto, Canada).

**Limited Trypsin Digestion**

Three reaction mixtures of VraR (40 µM) in the unphosphorylated state, subjected to phosphorylation and subjected to phosphorylation in the presence of 2 ng of DNA (*vraSR* promoter region, 156 bp), were mixed with 0.2 µg/µl trypsin (ProteoExtakt Kit, CalBiochem). The reaction mixtures were incubated at 37 °C. Aliquots of 10 µl were removed at the defined time intervals and digestion was terminated by addition of 10 µl of 6× SDS-PAGE and heating at 60 °C. Samples were analyzed by 15% Tris Tricine SDS-PAGE. Protein bands were cut from the gel, subjected to trypsin digestion, and analyzed by matrix-assisted laser desorption/ionization time-of-flight
Signal Transduction of Cell Wall Damage in S. aureus

Analysis of the Protein Masses by Mass Spectrometry

Electrospray ionization mass spectrometry (ESI) was carried out at the Advanced Protein Technology Center, Hospital for Sick Children (Toronto, Canada). Briefly, molecular masses of protein samples were measured on a QSTAR XL electrospray ionization QTOF mass spectrometer (Applied Biosystems/MD Sciex, Concord, ON, Canada) in positive ion mode. Typically, 5 µl of each sample was injected using an HPLC system (Waters, Milford, MA) and carried over to the nanospray source by a mobile phase of 50% acetonitrile in deionized water containing 0.2% formic acid. The flow rate was kept at 8 µl/min and the capillary voltage was maintained at 3000 eV. The mass spectra obtained were deconvoluted using the Bayesian Protein Reconstruct script that comes with the ABI BioAnalyzer 1.1 software.

The protein identity was confirmed by trypsin digestion followed by MALDI, carried out at the Center for Mass Spectrometry, York University. The protein band excised from SDS-PAGE was subjected to in-gel trypsin digestion (ProteoExtract Kit, Calbiochem) and analyzed by MALDI. Briefly, the digested protein samples were first desalted and concentrated using a Millipore Zip–Tip®. Peptides were eluted off the tip with 1 µl of α-cyano-4-hydroxy-cinnamic acid matrix (10 mg/ml in 60% acetonitrile and 0.3% trifluoroacetic acid). The solution was spotted on a Perseptive Biosystems MALDI target plate. MS and MS/MS experiments were carried out using an AB/SCIEX QStar XL® hybrid quadrupole/time-of-flight mass spectrometer. The identity of the unmodified peptide of interest was confirmed by searching the MS and MS/MS spectra against the NCBI non-redundant data base using MASCOT® software from Matrix Science.

RESULTS

Modular Architecture of VraS and VraR—Analysis of the VraS amino acid sequence indicates that the protein is a typical HK with an N-terminal transmembrane domain and a C-terminal conserved HK core (Fig. 1). The N-terminal transmembrane domain of VraS consists of two membrane spanning regions that are connected through a periplasmic linker. This domain varies widely among HKs (7). The conserved HK core of VraS contains the dimerization domain, which harbors the conserved His residue, and the ATP-binding domain. In this study, we used the conserved HK core of VraS (residues 85–347) fused to GST.

VraR is a two-domain response regulator protein (Fig. 1), composed of a conserved N-terminal regulatory domain (residues 1–117) and a C-terminal DNA-binding domain (residues 141–209), referred to as the effector domain (Fig. 1). Herein, the cloned VraR(N) spans residues 1–133, and cloned VraR(C) spans residues 141–209 (in analogy with other well studied effector domains).

VraR exhibits considerable sequence homology with members of the NarL/FixJ subfamily of RR proteins (Fig. 2). This family of RRs utilizes a helix-turn-helix motif for binding to DNA. Based on sequence alignments with other RRs (Fig. 2), we predicted that Asp-55 is the phosphorylation site in VraR, Asp-9, Asp-10, and Lys-105 are the residues involved in VraR-
dependent phosphotransfer, and Thr-83 and Tyr-102 are the residues involved in phosphorylation-induced activation of VraR (9).

Isolation of the Target Proteins—The isolated target proteins were homogeneous as estimated by SDS-PAGE and Coomassie Blue staining (supplemental materials Fig. S1). The selection of each purification protocol was based either on the isoelectric point of the proteins (selection of ion-exchange columns) and/or the affinity of the protein for a particular matrix.

The identity of the proteins and their molecular masses were confirmed, respectively, by trypsin digestion followed by characterization via MALDI and ESI-MS. The molecular masses for VraR, VraR(F55N), and VraR(D55N), as determined by ESI-MS (standard deviations in these experiments were estimated to be 0.2 Da), were 23,428.5, 14,557.3, and 7,785.3 Da in agreement with their calculated masses (VraR, 23,428.1 Da; VraR(F55N), 7,786.9 Da). The mass of the isolated GST-VraS protein was confirmed by SDS-PAGE to be 55.6 kDa. VraR and VraR(F55N) are monomeric in solution, pH 7.0, as assessed by size exclusion chromatography and native PAGE (data not shown). VraR(F55N) forms dimers in solution, pH 7.0, as estimated by native PAGE and analytical ultracentrifugation (see below).

VraS Autophosphorylation Activity—A time-dependent experiment on autophosphorylation of GST-VraS in the presence of 10 μM ATP (room temperature) indicated a progressive reaction during the first 20 min (Fig. 3A) followed by the saturation of phosphorylation over the next 60 min (Fig. 3B). The phosphorylated GST-VraS was stable for more than 2 h at room temperature (Fig. 3C). The pseudo-first order rate constant of VraS phosphorylation at a saturation concentration of ATP (1 mM) is 0.070 ± 0.005 min⁻¹ (t₁/₂ = 10 min). It is of note that these rates were calculated at room temperature (<25 °C), thus at the physiological temperature (37 °C) the autophosphorylation rate of VraS will be faster (t₁/₂ < 10 min). The autophosphorylation rate of VraS is similar to that of E. faecium vancomycin-resistant HK VanS (0.17 min⁻¹ at room temperature) (12) and E. coli fumarate-stimulated HK DcuS (0.043 min⁻¹ at room temperature) (27). The rate of VraS autophosphorylation indicates that VraS is capable of transducing the stress signal before S. aureus starts to duplicate (duplication time of S. aureus is ~30 min).

Phosphotransfer and VraR Phosphorylation by Small Molecule Phosphodonsors—Incubation of GST-VraS-P with VraR resulted in a rapid transfer of the phosphoryl group to VraR (Fig. 4A), whereby 70% of the phosphoryl group was transferred within 30 s (Fig. 4, A and B). The estimated pseudo-first order rate constant of the phosphotransfer at room temperature is 0.084 ± 0.001 s⁻¹.

VraR underwent phosphorylation by endogenous small molecule phosphodonsors, such as acetyl phosphate (Fig. 4C). However, the phosphorylation rate of VraR by acetyl phosphate is 200-fold slower than the rate of the phosphotransfer process (the estimated rate constant for the phosphorylation of VraR by acetyl phosphate is (3.67 ± 0.03) × 10⁻⁴ s⁻¹ (Fig. 4C and supplemental materials Fig. S2).

The phosphorylation site in VraR was confirmed to be Asp-55 as the VraR-D55N mutant was found to be completely phosphorylation deficient (supplemental materials Fig. S3). Herein, VraR samples that have been subjected to phosphorylation by acetyl phosphate are referred to as VraR-P, to indicate the presence of both unphosphorylated (VraR) and phosphorylated VraR (VraR-P) species in these samples. Typically
the phosphorylation level of VraR by acetyl phosphate under our experimental conditions is 46% as estimated by HPLC chromatography (see “Experimental Procedures”).

VraS Phosphatase Activity—Time-dependent phospho-transfer experiments indicated the disappearance of the VraR-phosphorylated species in less than 4 min (Fig. 4, A and B). The estimated dephosphorylation pseudo-first order rate constant is 0.016 s\(^{-1}\) at room temperature. VraR can be phosphorylated and dephosphorylated by VraS several times with no much effect on the rates of these two processes (supplemental materials Fig. S4).

We attributed the disappearance of the VraR-phosphorylated species to the phosphatase activity of VraS. ESI-MS data acquired on VraR, VraR\(^{\text{P}}\), and VraR\(^{\text{N}}\) in the presence of GST-VraS indicated that the phosphorylated VraR species (Fig. 5B, mass 23,508.5 Da) disappears only in the presence of GST-VraS (Fig. 5C). Similar results were also obtained by HPLC, where the peak representing VraR\(^{\text{P}}\) disappeared when the reaction mixture was incubated with GST-VraS (supplemental materials Fig. S5).

A closer look at ESI-MS data acquired on the VraR\(^{\text{N}}\) sample indicated the presence of a new species with a mass of 23,410.6 Da (Fig. 5B). The molecular mass of this species differs from the unphosphorylated VraR by 18 Da, which corresponds to the mass of a water molecule. This species has also been reported for OmpR (28), where it was proposed to correspond to the dephosphorylated species formed as a result of the release of \(\text{H}_3\text{PO}_4\) into the milieu due to the nucleophilic attack on the phosphorus by either a neighboring lysine (Lys-105, resulting in isopeptide bond formation) or threonine (Thr-83, resulting in ester bond formation). The mass 23,410.6 disappeared in the presence of VraS indicating that formation of the putative isopeptide or the ester bond (intrinsically unstable) will not lead to inactivation of VraR.

The presence of the mass 23,410.6 Da indicates that VraR may catalyze its own dephosphorylation, albeit with much lower efficiency than VraS. In our hands, after the removal of the acetyl phosphate from the phosphorylation reaction, VraR\(^{\text{P}}\) is stable for at least 10 h; it takes about 48 h to completely dephosphorylate VraR\(^{\text{N}}\) (data not shown).

DNA-binding Properties of VraR, VraR\(^{\text{P}}\), and VraR Effector Domain (VraRC)—We investigated the DNA binding properties of VraR using a 156-bp DNA sequence derived from the upstream region of the vraSR promoter (vraSR\(^{\text{prom}}\), −121 to +26) of S. aureus Mu50. These experiments indicated single band shifts. In the case of the electrophoretic mobility shift...
assay experiments involving VraR–P the band shift was slightly retarded at low protein concentrations. Because the experimental conditions for VraRC were the same as for all the proteins, we believe this phenomenon is due to the dissociation of the VraR–P–DNA complex. It is of note that the shape of the binding isotherms for all three proteins is the same. This is an indication that the binding mode of each VraR protein to DNA is the same but only stronger for VraR–P (see below).

We measured a 4-fold increase in the DNA binding affinity when VraR was phosphorylated, $K_d^{VraR} = 5 \pm 2 \mu M$ and $K_d^{VraR}$. Similar effects by phosphorylation were observed in the case of E. coli OmpR (29), B. subtilis PhoP (30), and B. subtilis ComA (31), e.g. binding affinity of the unphosphorylated OmpR increased from 15 to 1.5 $\mu M$ upon phosphorylation. Interestingly, VraRC exhibited similar DNA binding affinity as the full-length VraR ($K_d^{VraRC} = 7 \pm 1 \mu M$) (Fig. 6). This observation suggests that DNA binding is phosphorylation independent, and that the DNA binding is not hindered in VraR.

It is of note that in the presence of VraR or VraRC at concentrations as high as 25 $\mu M$ not all the DNA was recruited (15% of the DNA remains unbound). In contrast, VraR–P was capable of recruiting the entire free DNA from solution at a concentration as low as 6 $\mu M$ (Fig. 6).

Effect of Phosphorylation on VraR Structure—We investigated the effect that phosphorylation has on the structure of VraR by circular dichroism spectroscopy (CD), isoelectric focusing (IEF), and limited trypsin digestion. The CD experiments provided clear evidence of conformational changes in VraR upon phosphorylation (Fig. 7A). In addition, these experiments indicated that phosphorylation-induced conformational changes were associated with an 8% reduction in the VraR $\alpha$-helix content, as estimated by measuring the mean residue ellipticity at 222 nm (Fig. 7A). No such experiments have been reported before on other RRs. However, analyses of the RRs three-dimensional structures in the unphosphorylated and phosphorylated states indicate the presence of local conformational changes in the latter state (9, 11, 32).

IEF revealed the appearance of two additional bands after VraR phosphorylation (Fig. 7B). Because IEF is sensitive to the overall net protein surface charge, and upon phosphorylation the charge on the Asp-55 remains the same (at pH 7.0), this experiment suggests that phosphorylation causes charge redistribution in VraR and the two bands could correspond to two different VraR-P species.

Occurrence of phosphorylation-induced conformational changes was also supported by limited trypsin digestion experiments (Fig. 8). These experiments had very good reproducibility especially in the Tris Tricine buffer system. Two fragments were released upon incubation of VraR with trypsin: fragment I with mass of 14.6 kDa (residues 1 to 133) and fragment II with mass of 11.9 kDa (residues 106–209) (Fig. 8). The VraR-P sample (where VraR-P constitutes about 46% of total VraR under these experimental conditions) was more resistant to trypsin digestion, as indicated by the attenuated release of fragment I. The protective effect of phosphorylation was more pronounced in the presence of DNA; it took more than 15 min to cleave the full-length VraR and fragment I was present in solution even after 90 min. Similar phenomenon has also been reported for OmpR (33, 34). The fragment II (encompassing the effector domain) appeared to be very resistant to trypsin cleavage (even after 2 h) despite the phosphorylation state of VraR or the presence of target DNA. This observation indicates that this domain is not affected by the phosphorylation state of the regulatory domain.

The effect of phosphorylation in the VraR structure was also investigated by native PAGE (Fig. 9A) and size exclusion chromatography (supplemental materials Fig. S6). These experiments indicated that VraR dimerizes upon phosphorylation and this phenomenon is concentration dependent (Fig. 9A and supplemental materials Fig. S6). The apparent dissociation constant for the VraR-P dimer was determined as follows: first we determined the extent of VraR phosphorylation by HPLC, and second, we determined the amount of VraR-P engaged in
dimerization by native PAGE. If this amount constitutes 50% of the total VraR-P species, then the apparent dissociation constant would be equal to the concentration of the VraR-P species in the solution. We carried out native PAGE experiments at different protein concentrations and the apparent $K_d$ was estimated to be $\sim 40 \mu M$ (Fig. 9A).

**Characterization of the VraR-P**—Analyses of the N-terminal domain of VraR (residues 1 to 133) by native PAGE showed that at 20 $\mu M$ the protein resolves mainly as dimeric species (Fig. 9B). Analytical ultracentrifugation experiments also indicated that VraR exists mainly as dimer in solution. The plots of $\ln(\text{Abs})$ versus the radius squared were linear, suggesting that the sample is composed of mainly one species (supplemental materials Fig. S7). The analysis of the global self-association gave an average molecular mass of 25.6 kDa. The ratio of the apparent molecular weight to the molecular weight of the monomer was 1.74:1 suggesting that the solution of VraR mainly contained dimers. The apparent dissociation constant for the dimer was determined to be 8 $\mu M$. These experiments and the native PAGE (Fig. 9B) and IEF (data not shown) data indicate that solutions of VraR in our hands are homogenous; there is no partial unfolding of the protein in our protein solutions.

The pseudo-first order rate constant for the phosphotransfer process catalyzed by VraR was determined to be $0.035 \pm 0.003$ s$^{-1}$, which is 2-fold slower than that of VraR (Fig. 10A). The phosphorylation rate of VraR by acetyl phosphate ($9.64 \times 10^{-5}$ s$^{-1}$) was estimated to be 4-fold slower than that of VraR (Fig. 10B). Unexpectedly, we observed that phosphorylation has a negative effect in the dimerization state of VraR (Fig. 9B). This observation indicates that dimerization interfaces in VraR-P and VraR-P dimers could be different.

with the cytosolic portion of VraS, other studies have shown that these fragments represent well the in vivo enzymatic activities of the full-length histidine kinases (35, 36).

In the second phosphorylation event, the phosphoryl group is transferred from the HK to the cognate RR, in a RR-catalyzed reaction. Our experiments show that VraR-dependent phosphotransfer is rapid, with 70% of it occurring within 30 s (Fig. 4A). This result, taken together with the fast kinetics of VraS autophosphorylation, suggests that the VraSR system is capable of rapidly transducing cell wall stress in vivo. This proposal is in agreement with the in vivo studies (16) and further supports the putative role of the VraSR as a “sentinel” system.

The fast kinetics of the phosphotransfer also suggests that VraS may be the only biologically relevant kinase to VraR, and the VraSR system is the main pathway through which the signal is transduced. Whereas, there could be other HKs that cross-talk with VraSR, the rapid phosphotransfer reaction between VraS and VraR may preclude such contributions. This proposal is supported by a phosphotransfer profiling study carried out in _E. coli_ and _Caulobacter crescentus_, which show that phosphotransfer between cognate HK-RR pairs is 1000-fold faster than for non-cognate pairs (36). In this study, the authors determined the phosphotransfer during 10 s and 60 min. They observed that quantitative phosphotransfer between cognate pairs took place within 10 s, as it is the case for VraS; for the non-cognate pairs the quantitative phosphotransfer took place during 60 min (36). Furthermore, in vivo studies on the VraSR system have shown that inactivation of VraS drastically abolishes the _S. aureus_ response to cell wall damage (2, 3, 16, 37).

Another route of VraR phosphorylation in vivo could result from the acetyl phosphate, a concentration of which is as high as 1.5 mM (38). Our study suggests that even though this is

**FIGURE 6.** DNA binding of VraR (A), VraR-P (B), and VraR (C) to vraSRprom. D, bound DNA in the above experiments (VraR, empty diamonds; VraR-P, filled diamonds; VraR-P, empty triangles) is plotted against the protein concentrations in the assays (A–C). In a typical assay, 2 ng of 5'-γ-32P-end labeled vraSRprom was incubated with different concentrations of VraR for 30 min at 25 °C (the error bars represent the S.D. calculated from three independent experiments).
possible it might not be relevant because in vitro phosphorylation rates by acetyl phosphate are 200-fold slower than phosphorylation by VraS, even at acetyl phosphate concentrations 30-fold higher than in vivo.

Another important aspect of the phosphotransfer-mediated signaling pathways is the switch-off of the output response in the absence of the stimulus. Our discovery that VraR undergoes dephosphorylation only in the presence of VraS suggests that the phosphorylation level of VraR is controlled by VraS and, based on our experimental data, this process is rapid ($t_{1/2} = 2$ min). Indeed, Sobral and colleagues (15) showed that high expression levels of vraS, observed under suboptimal concentrations of the inducer in a S. aureus murF conditional mutant, reverted rapidly under optimal inducer concentrations. However, we cannot exclude the probability that other histidine kinases might also affect the phosphorylation level of VraR.

Phosphorylation-induced Activation of VraR: Indication of a Novel Regulatory Mechanism—Our study shows that VraR undergoes phosphorylation-induced conformational changes that mediate dimerization of the protein. The dimerization interface

![Figure 7](image)

**FIGURE 7.** Phosphorylation-induced conformational changes in VraR. A, circular dichroism spectra of VraR (30 μM, filled circles) and VraR-P (empty circles). The spectra were acquired at 22 °C in 50 mM Tris, pH 7.4, 50 mM KCl and 5 mM MgCl$_2$ buffer. B, IEF gel: lane 1, VraR (20 μM); lane 2, VraR-P (20 μM); lane 3, VraRN (20 μM); and lane 4, ovalbumin (1 mg/ml).

![Figure 8](image)

**FIGURE 8.** Analysis of trypsin digestion products of VraR, VraR-P, and VraR$\text{N}$P in the presence of vraSR$\text{prom}$. Three reaction mixtures of VraR (40 μM) under each condition were digested by 0.2 μg/μl trypsin at 37 °C. Aliquots of 10 μl were removed from the reaction at different time intervals and quenched by the addition of 10 μl of 6× SDS-PAGE loading dye followed by heating at 60 °C. The resulting samples were analyzed by 15% Tris Tricine SDS-PAGE.

![Figure 9](image)

**FIGURE 9.** Native PAGE analysis of VraR-P (A) and VraR$\text{N}$-P (B). VraR or VraRN$\text{N}$ were subjected to phosphorylation by acetyl phosphate (50 μM) for 60 min at 37 °C (d and m denote the dimeric and monomeric species, respectively). In these experiments, VraR- and VraRN$\text{N}$-phosphorylated species constitute 46 and 30%, respectively.

![Figure 10](image)

**FIGURE 10.** Phosphotransfer between VraS and VraRN$\text{P}$. A, VraR or VraRN$\text{P}$ (25 μM) were incubated with GST-VraS-32P (4 μM). Reactions were quenched at different incubation times and analyzed by 15% SDS-PAGE. The gels were analyzed by autoradiography. B, relative phosphorylation VraS and VraRN$\text{P}$ obtained in C (normalized to GST-VraS-P) are plotted against time (empty circles, VraS; filled squares, VraRN$\text{P}$). The error bars in the graph represent the S.D. calculated from three independent experiments.
most likely occurs at the N-terminal domain as the stand alone VraRN forms stable dimeric species in solution ($K_d = 8 \mu M, \Delta G^\circ = -7 \text{ kcal/mol}$), whereas the C-terminal domain remains a monomer. A lack of dimerization in the unphosphorylated VraR suggests that either the N- or C-terminal domain prevents exposure of the dimerization interface in the full-length protein, and that this inhibitory effect is reversed only by phosphorylation of the regulatory domain.

At first glance, our data seem to suggest that VraR employs an activation mechanism that is similar to that of other RRs, where phosphorylation releases the inhibitory activity of the regulatory domain or the effector domain (32, 39, 40). However, when we started to look at the two domains separately a different story began to unveil. VraRN undergoes phosphorylation and dephosphorylation at slower rates than full-length VraR (Fig. 4B). These observations strongly indicate that VraRN adopts an inactive conformation in the unphosphorylated state, contrary to the notion that stand-alone regulatory domains are free of any inhibitory effect and as such they explore active conformations.

The rapid phosphotransfer rates observed for the full-length VraR, in contrast to VraRN, strongly suggests that the regulatory domain is held into an intermediate conformation between active and inactive states in VraR. Population of similar states by unphosphorylated regulatory domains has been reported for the E. coli chemotaxis response regulator CheY in complex with a peptide of its effector protein target, the flagellar switch protein FlIM (41). The intermediate active conformation of the regulatory domain in VraR could be stabilized through interdomain interactions between the regulatory and effector domains, the same interactions that prevent unphosphorylated VraR from dimerizing ($\Delta G^\circ(VraRN-VraRC) < -7 \text{ kcal/mol}$).

It is of note that phosphorylation of VraRN shifts the equilibrium monomer:dimer toward the monomer. This observation could be an indication that a new dimerization interface forms in VraRN-P species. Although it is possible that phosphorylation of VraRN could affect only some of the dimerization elements, leaving the rest intact. This hypothesis is in good agreement with our observations that phosphorylation induces conformational changes in VraR, which are likely to occur in the N-terminal domain.

**Dimerization Is Required for the Biological Activity of VraR**—Based on our experimental data, a working model for the phosphorylation-induced activation of VraR can be proposed, whereby phosphorylation through dimerization of VraR at the N-terminal domain enables two C-terminal domains to come close and bind to the target DNA (Fig. 1). This model is similar to the model proposed for E. coli FixJ (42) and the OmpR family of proteins (11). Indeed, our data show that phosphorylation-induced dimerization of VraR enhances the VraR DNA binding affinity by at least a factor of 4 and that DNA has a stabilizing effect on the dimer. Both factors could play an important role in shifting the equilibrium toward the formation of active VraR species, i.e. VraR dimers in vivo.

The apparent dissociation constant for VraR-P is slightly high (40 $\mu M$) and complete dimerization of VraR-P may not be reachable in vivo. We propose that the initially formed VraR-P dimer species could serve to recruit the monomeric VraR-P to the promoter site. Accumulation of VraR-P and the stabilizing effect that binding to DNA seem to have on the dimer will lead to further dimerization of VraR-P at the promoter site. However, a direct binding of VraR-P on the regulatory sequences followed by dimerization cannot be excluded.

The VraSR two-component system has been shown to modulate the cell wall-biosynthesis pathway in *S. aureus* and is implicated in regulation of more than 40 genes. The question that arises is how the expression of these many genes can be regulated simultaneously in vivo? It has been demonstrated for many RRs that they utilize the presence of multiple regulatory sequences, with a hierarchy in RR binding affinities, in their target promoters to regulate different genes simultaneously (43–45). We propose that VraR could utilize similar regulation mechanisms to control the VraSR regulon.

In summary, our study shows that VraR utilizes intradomain interactions in a unique way to regulate the output response of the VraSR-mediated signaling pathway. The effector domain prevents dimerization of unphosphorylated VraR at the N-terminal domain. In doing so, the effector domain holds the regulatory domain in a conformation that is capable of catalyzing a rapid phosphotransfer, which in turn could result in high levels of phosphorylated VraR in the cell. Our study also suggests that damage inflicted into the cell wall peptidoglycan could be the main source of the extracellular stimuli that VraR responds to, as VraS appears to have tight control in the phosphorylation state of VraR. In addition, the VraR-P dimer represents the biologically active species that could be participating in different gene regulation schemes to facilitate the coordination of the *S. aureus* response to cell wall damage. In all, our study provides for the first time insights into the molecular basis for the proposed role of VraR as a sentinel system capable of sensing cell wall peptidoglycan damage and coordinating a rapid response that enhances the resistance phenotype in *S. aureus* (2, 7).

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