Purification and Characterization of VanR and the Cytosolic Domain of VanS: A Two-Component Regulatory System Required for Vancomycin Resistance in *Enterococcus faecium* BM4147†

Gerard D. Wright,‡ Theodore R. Holman,‡ and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Resistance to the glycopeptide antibiotic vancomycin requires five genes. Two of these, *vanR* and *vanS*, have sequence homology to cytoplasmic response regulatory (VanR) and transmembrane sensory (VanS) proteins of two-component regulatory systems used to sense and transduce environmental signals. We report the overproduction and purification to homogeneity of VanR (27 kDa) and of a fusion protein of VanS (residues 95–374, the cytosolic domain) to the maltose binding protein (MBP), yielding a MBP–VanS protein of 76 kDa. The MBP–VanS fusion protein displayed an ATP-dependent autophosphorylation on a histidine residue with a rate of 0.17 min^{-1} and a phosphorylation stoichiometry of 10–15%. ^{32}P-PhosphoMBP–VanS transferred the phosphoryl group to VanR. ^{32}P-PhosphoVanR showed chemical stability anticipated for an aspartyl phosphate and was relatively stable to hydrolysis ($t_{1/2}$ = 10–12 h). Thus, the vancomycin resistance operon appears to have collected and specifically tailored the His kinase and Asp phosphoryl receptor of two-component signal transduction logic for sensing extracellular vancomycin and turning on structural genes, *vanA* and *vanH*, to make altered peptidoglycan structures such that vancomycin does not bind.

Resistance to antibiotics continues to present a major problem in health care (Cohen, 1992; Neu, 1992). Pathogenic bacteria have acquired resistance to virtually all clinically useful antibiotics including most recently the glycopeptide vancomycin in clinical isolates of *Enterococci* (Courvalin, 1990). Vancomycin is an antibiotic in wide clinical use for the treatment of Gram-positive infections (Wilhelm, 1991). The antibiotic functions by forming a tight noncovalent interaction with C-terminal D-Ala-D-Ala moieties of peptidoglycan units that comprise the barrier to osmotic lysis in the bacterial cell wall [for reviews, see Barna and Williams (1984) and Reynolds (1989)]. Formation of this antibiotic–cell wall complex is mediated through a series of five hydrogen bonds and results in the inhibition of the essential cell wall polymerization activities, transglycosylation and transpeptidation. The result of this inhibition is the failure to achieve effective elongation and cross-linking of the peptidoglycan which contributes to cell death.

Vancomycin resistance in *Enterococcus faecium* has been shown to require five genes: *vanR*, *vanS*, *vanH*, *vanA*, and *vanX*, located contiguously on transposon Tn*1546* (Arthur et al., 1992c). The *vanH* gene encodes a D-specific α-ketoacid dehydrogenase (Arthur et al., 1991; Bugg et al., 1991b), and *vanA* encodes a protein with low levels of D-Ala-D-Ala ligase activity but which is capable of efficient depsipeptide synthesis, unlike known D-Ala-D-Ala ligases (Bugg et al., 1991a; Dutka-Malen et al., 1990): the function of *vanX* remains unknown. This suggests that vancomycin-resistant bacteria synthesize cell walls terminating in a depsipeptide of the form *N*-acyl-D-Ala-*O*-D-X rather than the amide *N*-acyl-D-Ala-D-Ala generally found (Wright & Walsh, 1992). Indirect microbiological evidence (Arthur et al., 1992a) and more recently direct identification of the depsipeptide component of the peptidoglycan in vancomycin-resistant *E. faecium* (Messer & Reynolds, 1992) and *E. faecalis* (Handwerger et al., 1992) have demonstrated that D-lactate is indeed incorporated into the C-terminal position of the muramyl pentapeptide. This provides the molecular rationale for observed vancomycin resistance as biosynthesis of an ester in place of an amide results in loss of a critical N–H hydrogen–antibiotic carbonyl oxygen interaction involved in the H-bond network required for vancomycin binding. This results in at least a 1000-fold increase in the K_d for vancomycin binding, thereby conferring resistance to the antibiotic (Bugg et al., 1991b).

In addition to the VanA and VanH proteins, enteroccocal resistance to vancomycin also requires the *vanR* and *vanS* gene products. On the basis of sequence homologies to other proteins (Arthur et al., 1992b), the *vanR* and *vanS* genes appear to encode a two-component regulatory system common in bacteria for sensing and responding to external stimuli [for reviews, see Bourret et al. (1991), Gross et al. (1989), Stock et al. (1989, 1992), and Wanner (1992)]. Other two-component systems include the chemotaxis proteins CheY and CheA, the osmoregulatory proteins OmpR and EnvZ, the nitrogen regulation proteins NtrC (NR_I) and NtrB (NR_{II}), and the phosphate regulatory proteins PhoB and PhoR. These systems comprise a histidine kinase (e.g., CheA, EnvZ, NtrB, PhoR), generally membrane-bound, which undergoes ATP-dependent autophosphorylation on a cytoplasmic His residue in response to a stimulus. The phosphorylated sensor protein is then able to transfer the phosphoryl group to an aspartyl residue of a soluble response regulator protein which typically either acts as a transcriptional regulator (e.g., OmpR, NtrC, PhoB) or, in the case of CheY, interacts with another protein component (flagellar motor). The nature of these two-component systems allows directional transfer of information from the external environmental to the cell interior. This permits the cell to react to outside stimuli by using phos-

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* Correspondence should be addressed to this author. Phone: 617-432-0930. Fax: 617-738-0516.

‡ These authors contributed equally to this work.

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photransfer chemistry to propagate the signal, in formal analogy to eukaryotic phosphate cascades and regulation of transcription (Hunter & Kain, 1992).

If the sequence homology between VanR and VanS and the corresponding partner proteins of other two-component regulatory systems can be extended to biological function, then VanS is a membrane-bound sensor protein which is autophosphorylated on a cytosolic His residue in response to an external stimulus (presence of vancomycin?). The phosphoryl group would then be transferred to an Asp residue of VanR (the response regulator) to activate transcription of the *vanH*, *vanA*, and *vanX* genes. In this report, we describe the overproduction of VanR and of a fusion protein with the cytosolic domain of VanS, the purification of these proteins, and the identification of VanS as a His protein kinase and VanR as an acceptor of the phosphoryl group from VanS.

MATERIALS AND METHODS

Preparation of Overproducing Constructs. A plasmid suitable for overproduction of VanR was prepared by the expression cassette polymerase chain reaction (PCR) method (MacFerrin et al., 1990). PCR primers were designed to incorporate the ribosome binding site of bacteriophage T7 gene 10 and a unique *Xba*I site 5′ to the initiation codon of *vanR* and a unique *Hin*dIII site 3′ to the gene. These primers were used to amplify the *vanR* gene from plasmid pAT89 (Arthur et al., 1992b). The isolated fragment was treated with *Xba*I and *Hin*dIII and ligated into M13mp19 cut with the same restriction enzymes and sequenced to ensure no mutations had occurred during the polymerase reaction. The *vanR* gene was then excised and ligated into vector pKK223-3* (Zawadzke et al., 1991) to place the *vanR* gene under control of the IPTG-inducible *tac* promoter, and this new plasmid, pTH1, was transformed into *Escherichia coli* W3110.

Since we anticipated difficulties in purification of full-length VanS due to two predicted membrane-spanning regions in the protein (Arthur et al., 1992b), we made a plasmid in which the cytosolic C-terminal domain, beginning with Met95, was fused to the gene for the maltose binding protein (MBP). PCR primers were designed to introduce a *Bam*HI site 5′ to Met 95 and a *Xba*I site 3′ to the stop codon. The gene was amplified by PCR, inserted into M13mp19, and sequenced to ensure no mutations had occurred during the polymerase reaction. Plasmid pMAL-c2 (New England Biolabs) was treated with *Bam*HI and *Hin*dIII and ligated with the *vanS* fragment treated with the same restriction enzymes. This construct places MBP–VanS fusion downstream from an IPTG-inducible *tac* promoter. The plasmid pMAL–VanS was used to transform *E. coli* W3110.

Purification of Proteins. All steps were carried out at 4 °C. Purification of VanR and MBP–VanS was monitored by SDS–PAGE.

(*a*) *VanR. E. coli* W3110/pTH1 was grown at 30 °C in Luria broth (1% tryptone, 1% NaCl, and 0.5% yeast extract) containing ampicillin (100 μg/mL) to an absorbance of 0.5–0.6 at 595 nm. IPTG was then added to a final concentration of 1 mM, and the cells were grown an additional 2 h. Cells were harvested by low-speed centrifugation (2500*g*, 10 min), washed with 50 mM, Tris pH 8, and resuspended in 25 mL of 50 mM HEPES, pH 7.2, 500 mM NaCl, and 5 mM $MgCl_2$. Cells were disrupted by two passages through a French press at 15 000 psi, and cell debris was removed by centrifugation at 25000*g* for 30 min.

The supernatant was brought to 40% in ammonium sulfate and centrifuged at 12000*g* for 30 min. The resulting pellet was resuspended in 5 mL of 50 mM HEPES, pH 7.2, and 5 mM $MgCl_2$ (loading buffer) and applied to an Ultragel AcA 54 (LKB) gel filtration column (1000 × 2.5 cm) which was preequilibrated with loading buffer. Fractions were collected and analyzed by SDS–PAGE. Fractions containing VanR were pooled and loaded onto a Hi-Load Q (Pharmacia) FPLC column equilibrated with 50 mM HEPES, pH 7.2, and 5 mM $MgCl_2$; VanR was not bound by the column. Fractions containing VanR were pooled and concentrated over an Amicon PM 10 ultrafiltration membrane. VanR (20 mg/L) was judged to be greater than 90% pure and showed a molecular mass of 26 000 Da by SDS–PAGE and gel filtration. The molecular mass was confirmed by matrix-assisted laser desorption mass spectrometry (MALDMS) (26 534 Da). These results are consistent with the predicted molecular mass of the full-length protein.

(*b*) *MBP–VanS. E. coli* W3110/pMAL–VanS was grown at 37 °C in Luria broth, induced with IPTG, and harvested 2 h after induction. Cells were resuspended in 25 mL of 50 mM Tris, pH 7.5, containing 300 mM NaCl, 2 mM EDTA, 2 mM DTT, and 0.1 mM PMSF and disrupted in a French press as described above. The solution was cleared by centrifugation at 25000*g* for 30 min and the supernatant fluid adjusted to 20% saturation with ammonium sulfate. The solution was centrifuged at 12000*g* for 30 min and the supernatant fluid dialyzed against 25 mM NaCl/50 mM Tris, pH 7.5. The dialysate was loaded onto a column (9 × 2 cm) of amylose resin (New England Biolabs) equilibrated with 25 mM NaCl/50 mM Tris, pH 7.5, at 1 mL/min. The column was washed with 60 mL of equilibration buffer and the MBP–VanS eluted by a wash with 10 mM maltose, 25 mM NaCl, and 50 mM Tris, pH 7.5. Fractions containing MBP–VanS were identified by SDS–PAGE, pooled, and dialyzed against 25 mM NaCl/50 mM Tris, pH 7.5. The MBP–VanS (85–90 mg) was judged to be 90–95% pure and showed a molecular mass of 75 000 by SDS–PAGE which was confirmed by MALDMS (75 696 Da). These results are consistent with the predicted molecular mass of the fusion protein.

Autophosphorylation of MBP–VanS. Autophosphorylation of VanS was performed in a final volume of 5 μL containing 2 μL of MBP–VanS (100–200 pmol) and 2 μL of 2.5× autophosphorylation buffer (final concentrations: 50 mM Tris, 50 mM KCl, and 1 mM $MgCl_2$, pH 7.4). The reaction was initiated by the addition of 1 μL of [γ-^{32}P]ATP (5 mM, 400–800 cpm/pmol) and incubated at room temperature, usually for 60 min. The reaction was quenched by the addition of 5 μL of SDS-stop solution (125 mM Tris, pH 6.8, 2.5% SDS, 2 mM EDTA, 0.0025% bromophenol blue, and 25% glycerol) and applied to a 10% SDS–polyacrylamide gel. Either gels were dried and subjected directly to autoradiography (and/or phosphorimage analysis using a Molecular Dynamics PhosphoImager 400), or gels were stained for 10 min in 0.2% Coomassie brilliant blue R, 50% ethanol, and 10% acetic acid at room temperature, followed by two destaining steps in 25% ethanol/10% acetic acid for 10 and 15 min, and then bands corresponding to MBP–VanS were excised with a razor blade and counted in 5 mL of liquid scintillant.

Phosphoamino Acid Analysis. Standard phosphohistidine was prepared by reaction of potassium hydrogen phosphoramidate (Sheridan et al., 1971) with histidine followed by purification over Dowex-1 (Cl^- form) (Hultquist et al., 1966). No effort was made to separate 1-phosphohistidine from 3-phosphohistidine. MBP–VanS (0.1 pmol) and CheA (0.02 pmol, generous gift of L. Alex and M. Simon) were autophosphorylated as described above; a control reaction without protein was also treated similarly. After 60-min incubation, 100 μL of 3 M KOH was added. The samples were heated

for 5 h at 60 °C and cooled, and potassium ions were precipitated by the addition of 10 μL of concentrated $HClO_4$. The supernatant fluid was clarified by centrifugation, and 10 μL was spotted onto the corner of a 20 × 20 cm aluminum-backed silica gel plate. The dried samples were subjected to 2D-TLC using *tert*-butyl alcohol/methyl ethyl ketone/acetone/methanol/water/ammonium hydroxide (10:20:20:5:40:5) in the first dimension and isopropyl alcohol/acetic acid/water (20:1:5) in the second dimension (Smith et al., 1978) followed by analysis by autoradiography.

Phosphorylation of VanR by PhosphoMBP–VanS. MBP–VanS (100–200 pmol) was autophosphorylated for 60 min with 1 mM [γ-^{32}P]ATP in a volume of 5–10 μL. Excess [γ-^{32}P]ATP was removed by two successive spin columns (Penefsky, 1977) consisting of 500 μL of Sephadex G-50 equilibrated in 50 mM Tris, 50 mM KCl, and 1 mM $MgCl_2$, pH 7.4. Phospho transfer to purified VanR (10–700 pmol) was performed at room temperature. The transfer reaction was quenched by the addition to stop solution consisting of 1 volume of 125 mM Tris, pH 6.8, 2.5% SDS, 5 mM EDTA, 0.0025% bromophenol blue, and 25% glycerol and applied to a 10% SDS–polyacrylamide gel. Gels were dried and subjected to phosphorimage analysis as described above. The amount of [^{32}P]phosphoVanR was determined by excision of the VanR band with a razor blade and by counting in 5 mL of liquid scintillant.

HPLC Purification of PhosphoVanR and PhosphoMBP–VanS. Proteins were phosphorylated as described above and injected directly onto a Bio-Rad Bio Sil Sec-125 (300 × 7.8 mm) gel filtration column with 50 mM Tris, 50 mM NaCl, and 5 mM EDTA, pH 8.0, buffer and separated at a flow rate of 1 mL/min. Phosphoproteins were monitored by UV absorption at 214 nm and with a Beckman Model 171 in-line radioisotope detector. Under these conditions, MBP–VanS eluted at 6 min and VanR at 8 min. Phosphoproteins were collected and stored at −20 °C.

Determination of the Half-Life of PhosphoVanR. HPLC-purified [^{32}P]phosphoVanR was purified in the presence of EDTA; half-life studies were performed following the addition of $MgCl_2$ and Tris, pH 8, to final concentrations of 10 and 100 mM, respectively. The decomposition reaction mixture (75 pmol of VanR, 15-μL total volume) was allowed to proceed at room temperature and quenched with stop solution (10 μL) at incremental time points. The reactions were then subjected to standard SDS–PAGE separation (15%) for detection of the remaining [^{32}P]phosphoVanR. The gels were dried, analyzed, and quantitated by phosphorimagery as described above.

Similarly, the decomposition of [^{32}P]phosphoVanR was also determined by measurement of free phosphate released, as detected by thin-layer chromatography (TLC). [^{32}P]PhosphoVanR was incubated as described above, but reactions were quenched with EDTA alone (100 mM final concentration) and spotted on poly(ethylenimine) (PEI)–cellulose TLC plates (Sigma). The plates were developed in 0.5 M LiOH/2 M HCOOH, air-dried, and analyzed by phosphorimaging.

Formation of ATP from [^{32}P]PhosphoMBP–VanS (Back-Reaction). HPLC-purified [^{32}P]phosphoMBP–VanS (2.5 μL, 2–3 pmol) was incubated with ADP (2 μL) in 50 mM Tris, pH 7.4, 50 mM KCl, and 5 mM $MgCl_2$ for 5–10 min, spotted on PEI–cellulose TLC plates, and developed as indicated above. ATP formation was monitored by phosphorimaging or by scraping the appropriate regions (identified by autoradiography) into scintillation vials and counting radioactivity in the presence of 5 mL of scintillant.

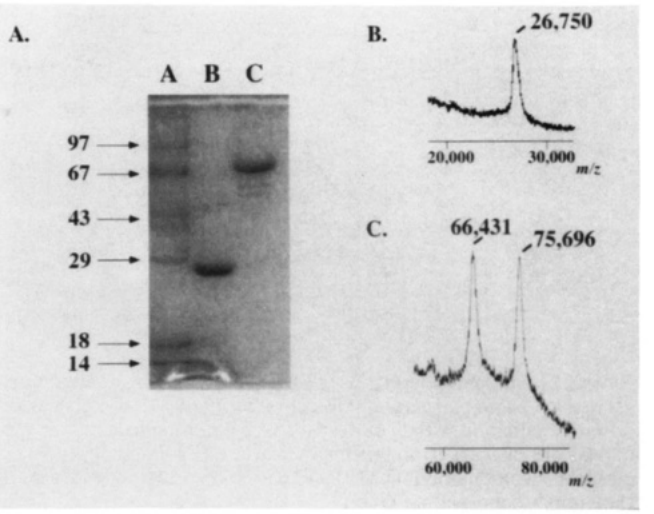

FIGURE 1: Overproduction and purification of VanR and MBP–VanS. (A) 11% SDS–polyacrylamide gel of purified proteins: lane A, molecular mass standards (in kilodaltons); lane B, VanR; lane C, MBP–VanS. (B) MALDMS spectrum of purifed VanR. (C) MALDMS spectrum of MBP–VanS; bovine serum albumin was added as an internal reference.

Miscellaneous Methods. Protein concentration was determined by the method of Bradford (1976). N-Terminal sequence analysis of VanR was determined by blotting VanR from a 10% SDS–poylacrylamide gel onto a poly(vinylidene fluoride) sheet (Immobilon, Millipore) as previously described (Matsudaira, 1990). Sequencing was performed by the microchemistry facility at the Dana Farber Cancer Institute (Boston, MA). Kinetic analysis was performed using the KinetAsyst software package (IntelliKinetics, State College, PA). MALSMS was performed on a time-of-flight mass spectrometer, Finnigan Lasermat (San Jose, CA), using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) acid as the matrix by E. Petit (Finnigan MAT, New Bedford, CT). Samples were mixed with matrix and spotted onto a gold target, and the mass spectrum was obtained by summing the data from 11–15 pulses of a 337-nm nitrogen laser.

RESULTS

Overproduction of VanR and MBP–VanS. Using the expression cassette PCR method (MacFerrin et al., 1990), we prepared the plasmid pTH1, which gave strong overproduction of VanR (≈ 4% of soluble protein). Three-step purification of VanR gave 20 mg of pure protein per liter of cell culture. VanR showed an estimated molecular mass of approximately 27 kDa by SDS–PAGE (Figure 1A) and a more accurate molecular mass of 26 750 Da by matrix-assisted laser desorption mass spectrometry (MALDMS) (Figure 1B), consistent with the predicted molecular mass of 26 625 Da. The native molecular mass of purified VanR was determined to be 27 000 Da by HPLC on a Bio Sil Sec-125 size-exclusion column, using 50 mM Tris, pH 8.0, and 200 mM NaCl as eluant, indicating that VanR, as isolated, is a monomer. N-Terminal sequence analysis of VanR gave the sequence MSDKIL, identical to the predicted N-terminal six amino acids (Arthur et al., 1992b).

We anticipated difficulty in overproducing full-length VanS due to two predicted membrane-spanning segments in the N-terminal third of the protein (Arthur et al., 1992b). Overproduction of other bacterial sensory component transmembrane His kinases has resulted in proteins of limited solubility (Igo et al., 1989a; Jin et al., 1990). In order to avoid potential difficulty in resolubilization of full-length VanS, we elected to overproduce the predicted soluble cytoplasmic

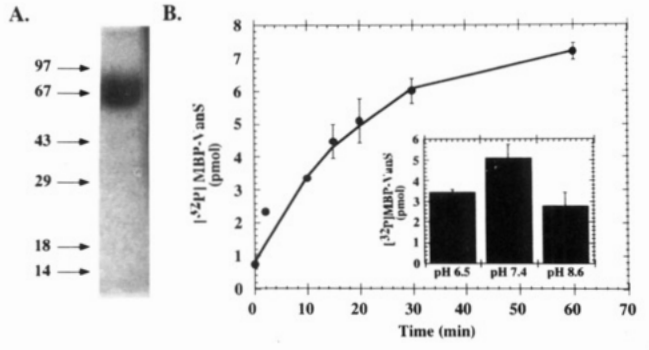

FIGURE 2: Autophosphorylation of MBP–VanS. (A) Autoradiogram of an 11% SDS–polyacrylamide gel of purified MBP–VanS after 60-min incubation with 1 mM [γ-^{32}P]ATP; the molecular mass in kilodaltons of marker proteins is indicated. (B) Time dependence of autophosphorylation of MBP–VanS. Inset: pH dependence of the autophosphorylation reaction.

Scheme I

$$\text{MBP–VanS} + \text{ATP} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{MBP–VanS•ATP} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{Phos–MBP–VanS•ADP}$$

$$\text{Phos–MBP–VanS•ADP} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{Phos–MBP–VanS} + \text{ATP}$$

C-terminal domain of VanS beginning at Met95. The resultant 290 amino acid, 32.8-kDa protein would include His164 which is predicted to be the site of autophosphorylation by homology overlap of the VanS sequence with other sensor proteins from 2-component regulatory systems (Arthur et al., 1992b). We also elected to produce the VanS fragment as a fusion protein with the maltose binding protein (MBP) both to assist in folding and to enable affinity purification. PCR primers were prepared which included an in-frame *Bam*HI site for cloning into the vector pMAL-c2, placing VanS at the C-terminus of fusion with MBP. The construct pMAL–VanS overproduced the fusion protein (MBP–VanS) and gave 85–90 mg of pure protein per liter of cell culture after affinity chromatography over an amylose column. The protein showed a molecular mass of approximately 76 kDa by SDS–PAGE (Figure 1A) and a more accurate molecular mass of 75 696 Da by MALDMS, consistent with the predicted molecular mass of 76 141 Da (Figure 1C). Attempts to cleave the MBP–VanS fusion protein using factor Xa did not liberate an intact VanS fragment, and therefore all experiments were performed the with intact fusion protein.

MBP–VanS Autophosphorylates on a His with ATP. Incubation of MBP–VanS with [γ-^{32}P]ATP and Mg^{2+} resulted in transfer of the ^{32}P-labeled phosphoryl group to the protein (Figure 2A). Phosphorylation was essentially complete after 60 min (Figure 2B) and was maximal at pH 7.4 (insert, Figure 2B). Phosphorylation was inhibited by EDTA. KCl stimulated phosphorylation at a concentration of 50 mM but was inhibitory at higher concentrations (not shown). The kinetics of autophosphorylation were analyzed according to the reaction in Scheme I. Autophosphorylation was allowed to proceed over the first 10 min where the reaction is first-order in ATP, and quenched to terminate the reaction. The dependence of k_{obs} on ATP concentration was half-maximal at 620 ± 90 μM (K_{ATP}) and showed a maximal rate of 0.23 ± 0.02 pmol min^{-1} (μg of protein)$^{-1}$ (Figure 3). Therefore, the overall apparent pseudo-first-order rate of autophosphorylation, k_{phos}, which incorporates the autophosphorylation step, k_3, and ATP binding, k_1 and k_2, can be determined by multiplying the

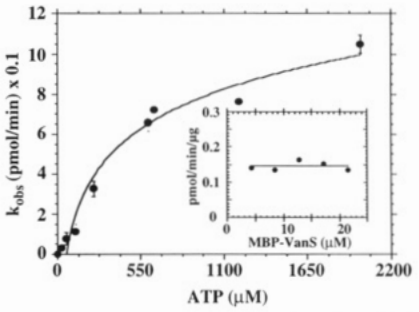

FIGURE 3: Dependence of the rate of autophosphorylation of MBP–VanS on ATP concentration. The observed pseudo-first-order rates of autophosphorylation (k_{obs}) were determined as indicated under Materials and Methods. Inset: Dependence of the specific activity of autophosphorylation on MBP–VanS concentration.

maximal rate by the reciprocal concentration of MBP–VanS to give 0.017 min^{-1}. In the case where ATP is saturating (where we estimate k_{phos}), we expect that $k_{phos} \approx k_3$. Since only 10% of MBP–VanS is phosphorylated (vide infra), the actual fraction of demonstrably active protein is 0.1 MBP–$VanS_{total}$, and the actual rate of phosphorylation is 0.17 min^{-1}. Since phosphorylation of MBP–VanS was independent of protein concentration (Figure 3, inset), MBP–VanS autophosphorylation appears to be an intramolecular reaction; however, this requires further study, considering EnvZ and CheA have recently been shown to proceed through an intermolecular reaction (Yang & Inouye, 1992; Wolfe & Stewart, 1993).

Given the primary sequence homology between VanS and other sensor kinases, we expected a His residue to be autophosphorylated. Consistent with this expectation was the observation that phosphoMBP–VanS was stable at neutral pH but was rapidly dephosphorylated under acidic conditions (not shown). Hydrolysis of [^{32}P]phosphoMBP–VanS in 3 M KOH (to prevent hydrolysis of a phosphoramidate bond) followed by two-dimensional TLC showed a new spot by autoradiography which was absent in the control with [γ-^{32}P]-ATP alone (not shown). A spot at the same location was observed after base hydrolysis of [^{32}P]phosphoCheA, which is known to be phosphorylated on N-3 of a His residue (His48) (Hess et al., 1988). Furthermore, the new spot corresponded to the migration of authentic *N*-phosphohistidine. Therefore, we conclude that MBP–VanS autophosphorylates on a His residue.

Analysis of the extent of ^{32}P incorporation into MBP–VanS revealed a stoichiometry of 10–15% over a 1-h incubation using saturating levels of [γ-^{32}P]ATP. This value was obtained by counting of SDS–polyacrylamide gel slices followed by localization of the protein bands by Coomassie staining. This treatment uses incubation with acetic acid solutions, and we estimate by measurement of HPLC-purified phosphoVanS that approximately 25% of the acid-labile phosphate is lost. Therefore, the stoichiometry reported is lower estimate of the actual loading. We attempted to obtain stoichiometries by filter binding assays under both acidic (Hess et al., 1991) and basic (Wei & Matthews, 1990) conditions; however, these assays, while qualitatively validating autophosphorylation, did not give results as reproducible as the gel slice method. Preincubation of MBP–VanS with stoichiometric amounts of VanR followed by HPLC purification of MBP–VanS and reevaluation of ^{32}P incorporation from [γ-^{32}P]ATP did not show an increase in ^{32}P loading over a control reaction without VanR preincubation, indicating that the low stoichiometry observed was not due to purification of already phosphorylated MBP–VanS from the cell supernatent. Additionally, treatment of as-isolated MBP–VanS (5 nmol) with 6 M HCl

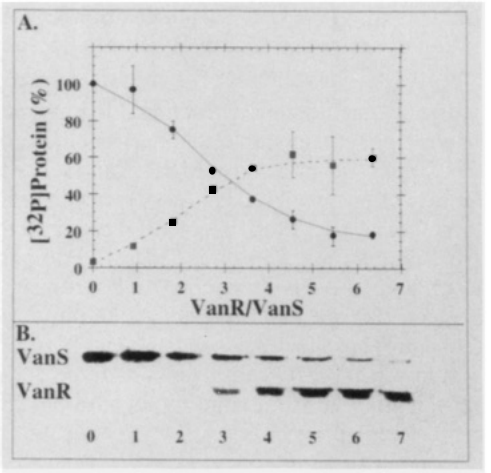

FIGURE 4: Titration of phosphoryl transfer of [^{32}P]phosphate from phosphoMBP–VanS to VanR. (A) Transfer as a function of protein ratio. The amount of phosphoprotein was determined by phosphorimage analysis of 11% SDS–polyacrylamide gels. PhosphoMBP–VanS (●); phosphoVanR (■). (B) Example of a phosphorimage of an 11% SDS–polyacrylamide gel used for determination of the data in (A). Numbers correspond to the molar ratio of VanR to MBP–VanS as indicated in (A).

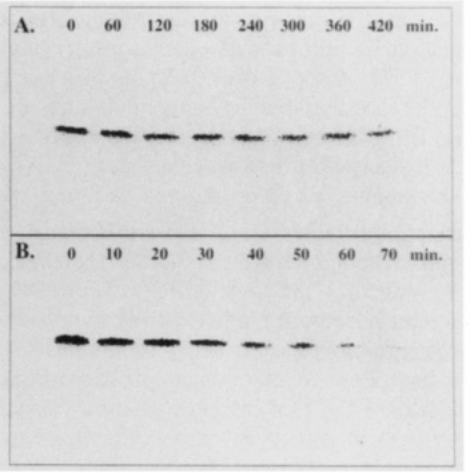

FIGURE 5: Determination of the half-life of [^{32}P]phosphoVanR. Phosphorimages of 11% SDS–polyacrylamide gels of HPLC-purified [^{32}P]phosphoVanR. (A) [^{32}P]PhosphoVanR (100 pmol) + 10 mM $MgCl_2$, 50 mM NaCl, and 100 mM Tris, pH 8. (B) [^{32}P]-PhosphoVanR (100 pmol) + MBP–VanS (25 pmol) + 5 mM $MgCl_2$, 50 mM NaCl, and 100 mM Tris, pH 8.

followed by determination of phosphate by a colorimetric reaction (Lanzetta et al., 1979) did not demonstrate any release of P_i.

[^{32}P]PhosphoMBP–VanS Transfers the Phosphoryl Group to VanR. Incubation of purified VanR with [^{32}P]phospho-MBP–VanS resulted in rapid transfer of ^{32}P radioactivity to VanR (Figure 4). No phosphoryl transfer occurred when VanR was incubated with [^{32}P]phosphoCheA (not shown). Transfer was too rapid to permit accurate determination of the rate. Phosphoryl transfer was dependent on VanR concentration and was essentially complete with VanR in 5-fold excess of phospho-MBP–VanS with approximately 50–75% of ^{32}P transferred from MBP–VanS to VanR (Figure 4A). This indicates a stoichiometry of roughly 0.15 [^{32}P]phosphate covalently bound per VanR molecule assuming a 1 to 1 ratio of phosphate to VanR. This 15% stoichiometry was verified by excision of the protein band and counting the radioactivity associated with VanR. Phosphoryl transfer was also dependent on the presence of divalent metal and was inhibited by EDTA.

Half-Life of [^{32}P]PhosphoVanR Is Affected by the Presence of MBP–VanS. Isolation of [^{32}P]phosphoVanR and separation from MBP–VanS (phospho and dephospho forms) by HPLC permitted the investigation of kinetic parameters in the absence of interfering MBP–VanS or ATP. The protein–[^{32}P]phosphate linkage was highly unstable in NaOH (0.25 M) and NH_2OH (0.88 M), yet stable in HCl (0.1 M), consistent with the presence of an acyl phosphate as anticipated by homology of the primary sequence of VanR with other response regulators (Arthur et al., 1992b) such as NtrC and CheY, which have been demonstrated to be phosphorylated on an Asp residue [see Weiss and Magasanik (1988) and Sanders et al. (1989), respectively].

The first-order rate of hydrolysis of the acyl phosphate of phosphoVanR was monitored using HPLC-purified protein (Figure 5). [^{32}P]PhosphoVanR, in the presence of Mg^{2+} (10 mM), demonstrates a $t_{1/2}$ of 820 ± 240 min as observed directly by SDS–PAGE and a $t_{1/2}$ of 550 ± 50 min by release of ^{32}P-labeled inorganic phosphate as determined by TLC. In the absence of Mg^{2+}, the $t_{1/2}$ increased to approximately 50 h. Addition of MBP–VanS in the absence of ATP accelerates decomposition of [^{32}P]phosphoVanR approximately 6-fold ($t_{1/2}$ of 120 ± 40 min, by SDS–PAGE; 140 ± 10 min by phosphate release). The dephosphorylation was unaffected by the addition of ATP or ADP at a concentration of 10 mM, with or without MBP–VanS present.

[^{32}P]PhosphoMBP–VanS Forms [γ-^{32}P]ATP in the Reverse Reaction with ADP. Incubation of [^{32}P]phosphoMBP–VanS with ADP resulted in the time-dependent formation of ATP, indicating that the autophosphorylation reaction was readily reversible. The concentration of ADP required for half-maximal transfer of ^{32}P from MBP–VanS, K_{ADP}, was 0.20 ± 0.04 mM, and the pseudo-first-order rate of transfer, k_{dephos}, was 0.038 ± 0.002 min^{-1}. As indicated above, under the conditions used to determine k_{dephos}, ADP is saturating; therefore, $k_{dephos} \approx k_4$. This permits an estimate of the internal equilibrium, k_3/k_4, to be 4.5.

DISCUSSION

Sequential exchange of phosphoryl groups between proteins constitutes a major paradigm of biological information transfer. In eukaryotes, phosphoryl-transfer cascades are central in cellular responses to stimuli including mitogens and therefore play a crucial role in many forms of cancer. Prokaryotic phospho-transfer cascades are so far limited to two cases: (1) the vectorial transport of carbohydrates into the cell by coupling phosphoenolpyruvate hydrolysis to sugar transport (Meadow et al., 1990); (2) two-component regulatory systems. The phosphoenolpyruvate:sugar phosphotransferase systems generally consist of three protein components: (1) enzyme I which is phosphorylated on a His residue in a phosphoenolpyruvate-dependent fashion; (2) a phosphocarrier protein (HPr) which accepts the phosphoryl group from enzyme I, also becoming phosphorylated on a His residue, and transfers the phosphoryl group to a His residue and then a Cys residue of a membrane-bound sugar-specific permease, enzyme II (Meadow et al., 1990; Pas et al., 1991). Transfer of the PO_3^{2-} moiety to the sugar ensures capture of the carbohydrate in the cell.

The second family of examples of sequential phosphoryl transfer in prokaryotes is the set of two-component regulatory systems which consist of a ligand-activated His autokinase and a downstream response regulator which accepts the migrating phosphoryl group on an Asp residue (Stock et al., 1989). In most two-component systems (the chemotactic response proteins CheY and CheB are exceptions), the phosphorylated regulators act as transcriptional regulators to activate, or repress, the production of proteins in a response

relevant to the stimulus, e.g., transcription of the phosphate (Pho) regulon by phosphorylated response regulator PhoB (Wanner, 1987). Control of the response time is presumed to be regulated either by the inherent lability of the acyl phosphate form of the regulator or by phosphatases which accelerate hydrolysis of the acyl phosphate (e.g., CheZ in the case of chemotaxis, EnvZ in osmoregulation).

Transcriptional regulation of the *vanH*, *vanA*, and *vanX* genes required for high-level resistance to vancomycin requires two gene products, VanS and VanR (Arthur et al., 1992b). These proteins have been predicted to be members of a two-component regulatory system solely on the basis of primary sequence homology to other such prokaryotic regulatory proteins. This is the first instance where a two-component regulatory system has been implicated in regulation of antibiotic resistance. We now have overproduced and purified the cytosolic domain of VanS and full-length VanR in an effort to characterize the vancomycin-dependent mechanism of signal transduction. The VanS cytosolic domain (purified as a fusion to maltose binding protein) is phosphorylated in the presence of ATP at a rate of 0.17 min^{-1}, and phosphoamino acid analysis identified the modified residue as a His. On the basis of primary sequence homology with other bacterial His kinases of two-component regulatory systems, we predict that His194 is phosphorylated in VanS.

Phosphorylation was saturable with ATP; however, only 10–15% of MBP–VanS was phosphorylated. The low stoichiometry observed with MBP–VanS was not due to isolation of the fusion protein from cell extracts as 85–90% phospho-MBP–VanS because the stoichiometry remained unchanged after incubation with purified VanR and no detectable phosphate was released by treatment with acid to hydrolyze a histidyl–phosphoramidate bond. In contrast, the stoichiometry of fully cytosolic His kinases has been reported as greater than 30% for NtrC (Weiss & Magasanik, 1988) and as much as 100% for CheA (Hess et al., 1987). Other autophosphorylation studies of bacterial sensor His autokinases have been qualitative, and their stoichiometry is not known. The low stoichiometry of MBP–VanS autophosphorylation may be because it is only a fragment of the full-length transmembrane protein. Only a small portion of the MBP–VanS population may be active either due to improper folding of the VanS domain in the fusion protein during expression or due to an intrinsic low basal level of functional conformers. Full-length VanS is expected to be comprised of two membrane-spanning regions, an extracellular sensory domain and a cytosolic kinase domain. Full stoichiometric loading of VanS with one phosphoHis group in an ATP-dependent manner may require a conformational change in the kinase domain brought about by interaction of the signaling domain with its extracellular (as yet unidentified) ligand. That could be a ligand-induced oligomerization (e.g., dimerization) of membrane-bound VanS reminiscent of ligand-induced transphosphorylation of dimerizing eukaryotic receptors such as the EGF receptor or insulin receptor where the cytoplasmic kinase domain autophosphorylates multiple tyrosines [e.g., see Treadway et al. (1990)]. Other sensory proteins of two-component regulatory systems, EnvZ and CheA, have been shown to be active as dimers and involved in intersubunit phosphorylation (Yang & Inouye, 1992; Wolfe & Stewart, 1993). Furthermore, addition of some as yet unidentified protein component may be required to achieve maximal stoichiometry and rate of autophosphorylation. As precedent, we note the observation that CheA phosphorylation is stimulated 10-fold by reconstitution with the partner protein CheW and the aspartate receptor Tar (Borkovich & Simon, 1990; Ninfa et al., 1991). The purification and perhaps reconstitution of active full-length VanS into liposomes will be required to test this hypothesis.

Even with substoichiometric, fractional loading of MBP–VanS, we were nonetheless able to determine initial kinetic parameters for phosphorylation of MBP–VanS. The pseudo-first-order rate of phosphorylation (k_{phos}) was determined to be 0.17 min^{-1}. The concentration of ATP required for half-maximal phosphorylation, K_{ATP}, was 620 μM. PhosphoMBP–VanS was capable of ADP-dependent ATP synthase activity with a K_{ADP} of 200 μM and a rate of 0.038 min^{-1} (k_{dephos}). The rate of autophosphorylation by the cytoplasmic VanS domain is a low basal rate, and it remains to be determined how much this may be stimulated by an external signaling ligand in full-length VanS.

Phosphorylated MBP–VanS rapidly transferred the phosphoryl group from His to VanR. The sensitivity of [^{32}P]-phosphoVanR to alkali and hydroxylamine, and the knowledge that Asp is the phosphate acceptor residue in the regulators CheY (Sanders et al., 1989) and NtrC (Weiss & Magasanik, 1988), suggests that Asp53 of VanR is phosphorylated by MBP–VanS. It is not yet known which partner is the substrate and which is the enzyme in engineering this His to Asp intermolecular phosphoryl transfer. Unlike many other response regulators, phosphoVanR is relatively stable to hydrolysis in the presence of divalent cations with $t_{1/2} \approx 8$–13 h compared with phosphoCheY at 6–15 s (Hess et al., 1988), phosphoNtrC at 4 min (Weiss & Magasanik, 1988), and phosphoOmpR at 90 min (Igo et al., 1989b). In the absence of Mg^{2+}, the $t_{1/2}$ increases to $\approx$50 h. Similar increases have also been observed for NtrC in which the $t_{1/2}$ is 1.8 h without Mg^{2+} and 5.5 h in 0.1% SDS (Weiss & Magasanik, 1988); however, the overall relative stability is less than that for VanR. It is evident that the $t_{1/2}$ of these phospho regulatory proteins varies widely due to factors not yet recognized and further studies will be required. Addition of MBP–VanS dramatically reduces the stability of phospoVanR ($t_{1/2} \approx 2$ h); however, ATP did not accelerate VanS-dependent dephosphorylation as has been noted in other two-component systems. The increased lability of phosphoVanR in the presence of MBP–VanS was not accompanied by an accumulation of phospho-MBP–VanS (back-reaction), and half-lives separately calculated by release of phosphate correlated with those measured by the decrease in phosphoVanR, demonstrating that MBP–VanS was acting to induce [^{32}P]phosphoVanR hydrolysis. The phosphatase activity in other two-component regulatory systems is highly dependent on the specific biological system; phosphoCheY requires only CheZ, phosphoOmpR requires both EnvZ and ATP (or an ATP analogue), and phosphoNtrC dephosphorylation requires NtrB, P_{II}, and ATP. The exact reasons for these differences remain elusive; however, they may well play a role in the overall regulation of each individual system, in vivo.

Phosphorylation of response regulators by sensors other than their partner His kinase (cross-talk) has been established in the case of OmpR (Igo et al., 1989a) and in the case of the Pho regulon in *E. coli* [reviewed in Wanner (1992)]. There is also some indirect evidence for cross-talk in phosphorylation of VanR in *Enterococcus faecium*. A *vanS* deletion mutant was vancomycin resistant and directed transcription of a reporter gene downstream from the *van* gene cluster, suggesting possible cross-talk between VanR and another protein (Arthur et al., 1992b). We tested for phosphorylation of VanR by [^{32}P]phosphoCheA; however, VanR did not accept a ^{32}P label from [^{32}P]phosphoCheA, demonstrating the specificity of protein–protein recognition in phosphoryl transfer. The

importance of cross-talk in transcription of the *van* genes in *Enterococcus* remains to be elucidated and is relevant when considering possible targets of inhibition to circumvent vancomycin resistance.

CONCLUSIONS AND FUTURE PROSPECTS

We have demonstrated by overexpression, purification, and kinetic analysis of VanR and the cytosolic domain of VanS that these proteins are members of a two-component regulatory system that uses a phosphoryl-transfer mechanism of signal transduction. VanS has autophosphorylation His kinase activity, and this phosphohistidyl residue is rapidly attacked by VanR to give, presumably, a β-aspartyl acylphosphate characteristic of the response regulator components. PhosphoVanR is a long-lived phosphoprotein which may be relevant for extended transcription of the vancomycin resistance operon in *Enterococcus faecium* and the maintenance of vancomycin-resistant peptidoglycan structures. Future work directed toward the characterization of full-length VanS to probe the effects of potential ligands such as vancomycin or cell wall autolysis fragments on the rate and stoichiometry of autophosphorylation will provide information regarding the importance of the external signaling domain in the cytoplasmic domain receptor response. In addition, the precise mechanism and specificity of interprotein phosphoryl transfer and of phosphoVanR dephosphorylation and the equilibrium position to probe the energetics of the signaling system will be analyzed. The ability to isolate stable phosphoVanR should permit analysis of the possible role of VanR with its predicted helix–turn–helix DNA binding domain in vancomycin resistance gene transcriptional activation.

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