Conformation of the Phosphate d-Alanine Zwitterion in Bacterial Teichoic Acid from Nuclear Magnetic Resonance Spectroscopy†

Ravindranath Garimella, Jeffrey L. Halye, William Harrison, Phillip E. Klebba, and Charles V. Rice*

Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Room 208, Norman, Oklahoma 73019

Received March 24, 2009; Revised Manuscript Received July 7, 2009

ABSTRACT: The conformation of d-alanine (d-Ala) groups of bacterial teichoic acid is a central, yet untested, paradigm of microbiology. The d-Ala binds via the C-terminus, thereby allowing the amine to exist as a free cationic NH₃⁺ group with the ability to form a contact ion pair with the nearby anionic phosphate group. This conformation hinders metal chelation by the phosphate because the zwitterion pair is charge neutral. To the contrary, the expulsion of cationic antimicrobial peptides (CAMPs) is attributed to the presence of the d-Ala cation; thus the ion pair does not form in this model. Solid-state nuclear magnetic resonance (NMR) spectroscopy has been used to measure the distance between amine and phosphate groups within cell wall fragments of Bacillus subtilis. The bacteria were grown on media containing [¹⁵N] d-Ala and β-chloroalanine racemase inhibitor. The rotational-echo double-resonance (REDOR) pulse sequence was used to measure the internuclear dipolar coupling, and the results demonstrate (1) the metal-free amine-to-phosphate distance is 4.4 Å and (2) the amine-to-phosphate distance increases to 5.4 Å in the presence of Mg²⁺ ions. As a result, the zwitterion exists in a nitrogen−oxygen ion pair configuration providing teichoic acid with a positive charge to repel CAMPs. Additionally, the amine of d-Ala does not prevent magnesium chelation in contradiction to the prevailing view of teichoic acids in metal binding. Thus, the NMR-based description of teichoic acid structure resolves the contradictory models, advances the basic understanding of cell wall biochemistry, and provides possible insight into the creation of new antibiotic therapies.

The cell wall of Gram-positive bacteria is composed of teichoic acids (lipoteichoic and wall teichoic acid, TAs) embedded in a peptidoglycan matrix (PG, Figure 1) (1−3). These biomolecules protect the bacterium from attack, transport nutrients, participate in cell-to-cell signaling, assist cell division, and aid in the attachment of bacteria to a substrate (2−11). Due to its role in bacterial infection and adhesion (7, 10, 12−17), teichoic acid has arisen as a potential target for antibiotic therapies (15−24). Assembly and transport events have been linked to several genes and their associated proteins. For Bacillus subtilis (B. subtilis), the teichoic acid backbone is composed of poly(glycerol phosphate), and the genes are denoted tagABCCEFEGH (25−27). Poly(ribitol phosphate) forms the backbone of teichoic acid in B. subtilis W23 (25, 26). Here, the necessary proteins are produced by genes tarABJIKLDF. Efforts are under way to identify the structure of the protein products and thereby aid the development of inhibitors to the teichoic acid synthetic pathway (13, 14).

Teichoic acids have the same general structure (Figure 2); however, differences are seen in the anchoring groups and backbone composition (1, 3, 32). One form of TA is found anchored to the cell wall peptidoglycan (33, 34). For B. subtilis wall teichoic acid (wall TA or WTA), d-alanine (d-Ala) and glucose groups are attached to the poly(glycerol phosphate) backbone (Figure 2). WTA from Staphylococcus aureus (S. aureus) has a poly(ribitol phosphate) backbone with N-acetylglucosamine (NAG) in place of glucose. In addition to WTA, TA can have lipid tails that anchor the polymer to the phospholipid bilayer. This lipoteichoic acid (LipoTA or LTA) has a poly(glycerol phosphate) with d-Ala, NAG, and hydroxyl groups decorating the backbone (25, 35). The polyphosphate in both WTA and LTA is a long chain of 20−50 highly charged repeat units (36−39). While the NAG and hydroxyl side chains are charge neutral, an abundance of anionic character is created by phosphodiester, only partially neutralized by the cationic d-Ala side chains. Charge neutralization is incomplete (10), giving teichoic acids a net negative charge and allowing them to form ionic bonds with surrounding fluids, dissolved ions, and the PG matrix. Gram-positive bacteria rely on the PG and TAs to chelate many types of metals from the extracellular milieu (28, 30−32, 40, 41). The PG and TAs assist metal ion homeostasis across bacterial membranes by metal binding/uptake in the cell wall. TA is nearly 100% responsible for Ca²⁺ adsorption whereas it contributes 50% for K⁺, Na⁺, and Mg²⁺ binding (42). In addition, cadmium competitively displaces Ca²⁺ from the cell wall, likely due to the metal selectivity of teichoic acid (43). These binding observations can all be attributed to interactions with the diverse chemical functional groups of PG and TA.

These experiments offer a limited, macroscopic, view of metal binding. While the chemical functional groups have been identified, a complete explanation of the competitive binding trends requires structural data. A structural model has remained out of reach with the exception of one report by the Baddiley group. In the early 1970s, TAs were mixed with Mg²⁺ and analyzed with X-ray photoelectron spectroscopy, a technique which measures...
the energy of electrons ejected from the Mg$^{2+}$ atom. Because the ejected electrons are also involved in chemical bonds, TA interactions alter the electronic energy, a perturbation detectable via XPS. Their results, reported in 1973 (44), were interpreted as monodentate Mg$^{2+}$ binding by a single oxygen of the phosphate group. Additionally, the cationic α-NH$_3$ of D-Ala side chains of TA were suspected to inhibit metal binding by charge neutralization of the nearby phosphate. However, nuclear magnetic resonance (NMR) data from our group support a revised chelation model (45).

A different paradigm for teichoic acid structure is used to explain the repulsion of cationic antimicrobial peptides (CAMPs) by the bacterial cell wall. CAMPs bind to the anionic phospholipid bilayer and disrupt membrane integrity, resulting in bacterial death. The D-Ala groups of TA use the α-carboxyl for attachment to the phosphodiester backbone (Figure 2), an arrangement which leaves a positively charged α-amine group, which allows for a potential repulsive interaction with CAMPs. In wild-type strains, new CAMP-based antimicrobial therapies must overcome this D-Ala repulsion. Presently, we report rotational-echo double-resonance (REDOR) NMR data, whose interpretation creates a structural model in which the supposed salt bridge does not exist, a model which indicates separation by water molecules. More importantly, the presence of D-Ala does not prevent this site from chelating Mg$^{2+}$.

EXPERIMENTAL PROCEDURES

Sample Preparation. To produce cell walls with $^{15}$N D-alanine labels, a 100 mL culture of B. subtilis was grown with defined media containing 10 mg of unenriched l-alanine and 10 mg of $^{15}$N D-Ala (99%, Isotec) and harvested during the stationary phase. The alanine racemase inhibitor β-chloro-D-alanine (500 μg/mL) was added to prevent racemic conversion of the $^{15}$N D-Ala. (46) Additional details of the growth media and the bacterial growth curve are included in the Supporting Information. Pepsin wall (WTA + peptidoglycan) samples were obtained from cells isolated from media using the following protocol (47–49). Cells were disrupted using a French press (14000 psi) and washed with doubly distilled water. The cell wall was then collected by centrifugation at 10000g for 20 min and then 2% SDS (37°C, 30 min) to remove the cytoplasmic membrane. The pellet was washed with ddH$_2$O and resuspended in 20 mM sodium acetate, pH 4.0. The sample was subsequently treated with pepsin (100 μg/mL) for 2 h. Nucleic acid and protein removal was verified by spectroscopic analysis (260 and 280 nm). Prior to NMR analysis, the pellet was washed with ddH$_2$O and dried by lyophilization.

NMR Experiments. Solid-state NMR experiments were performed using a three-channel NMR spectrometer (1H= 300 MHz, UnityInova; Varian, Inc.) and a 2 mm, three-channel, magic-angle-spinning (MAS) NMR probe (Doty probe with a revolution NMR spinning module). Drive and bearing gas were provided by dry, compressed air. The $^{15}$N chemical shifts were referenced to external glycine. Data acquisition and processing
were accomplished with VNMRJ (version 1.1D) provided by Varian, Inc. REDOR data were collected using a sample spinning rate of 9000 Hz, cross-polarization (CP) contact time of 2000 μs, and 1H decoupling at a power level of 50 kHz. The rf powers for the 15N and 31P channels were 50 kHz each. The XY-8 phase cycling scheme was used on both the 15N and 31P channels.

Distance determinations arise through the measurement of 15N–31P dipolar coupling. Although traditional CPMAS (cross-polarization under magic-angle spinning) averages away all dipolar interactions, one can turn to the solid-state NMR experiments, REDOR, to restore the dipolar interaction effects. Detailed mathematical and pictorial descriptions of REDOR have been reported (54–57). The dipole–dipole interaction causes transverse dephasing of the magnetization, reducing the signal intensity. Subtracting the “dephased echo” spectrum (S_d) from the full echo (S_0) spectrum provides a difference spectrum (ΔS), which is used to measure the dipolar coupling. The distance between 15N and 31P can be found by relating the intensity of the total signal reduced exclusively by dipolar interaction. A factor is applied by dividing ΔS by S_0 thereby giving the fraction of the total signal reduced exclusively by dipolar interaction. A plot of ΔS/S_0 vs dephasing time yields the REDOR dephasing curve. The distance “r” is found by fitting the REDOR curve data to Bessel equations, which computationally provide an analytical solution to the nuclear spin Hamiltonians (50, 51). This fitting is accomplished with the SIMPSON simulation software package (52), a method we have used previously to study metal binding of Li⁺ ions in polymer electrolytes (52, 53). The SIMPSON program (58) calculates a theoretical dephasing curve based on the nuclear spin system and the dipolar coupling constants. The distance between the interacting spins can be extracted from the strength of the dipole–dipole coupling using D = (μμ_h/4π)(γ_1γ_2/μ_0ε_0) r³, where D is the dipolar coupling constant, μ_0 is the permittivity of free space, γ_1 is the magnetogyric ratio of the dephasing nucleus (31P), γ_2 is the magnetogyric ratio of the observe nucleus (15N), and r is the internuclear distance. Dipolar coupling constants were determined from multiple SIMPSON(58) simulations, each generating a theoretical REDOR dephasing curve. Using SIMPSON simulations to determine distance measurements and the accuracy of said measurements is well established. Although the error associated with these measurements cannot be measured quantitatively, the change in the dipolar coupling constant can be used to show a definitive change in the binding environment when metals are added to the cell wall samples. Additionally, a visual inspection of the theoretical REDOR curves shows that discrimination between measurements of ±0.1 Å is well within reason.

RESULTS

In Figure 3, the REDOR S_0 and S_r spectra are presented, showing two resolved signals for the labeled 15N-D-Ala amide and amine environments. In this sample, the amide signal is from the PG and the amine signal is from D-Ala of TA. The metal-free amine data points can be described by a theoretical REDOR curve with an ~4.4 Å N–P distance (Figure 4). The metal-free amide data (Figure 5) show that there is negligible heteronuclear dipolar coupling between the peptidoglycan amide sites and the neighboring teichoic acid phosphate groups. As our interest lies with the nearest neighboring heteronuclear dipolar coupling interactions, Figures 4 and 5 only show data within the range of the first 15 ms of dephasing time. SIMPSON simulations demonstrate that the first dephasing nucleus dominates this region.

The implication of these distance measurements is shown in Figure 6. First, the amine signal, arising solely from teichoic acid, can be used to determine if the amine actually forms a contact ion pair, the current paradigm for metal chelation by TAs. From energy-minimized structures (45), we know the amine–phosphate distance in the contact ion pair configuration is approximately 3.2 Å (model 1, Figure 6). Thus, the 4.4 Å amine–phosphate distance is inconsistent with the contact ion pair model. Therefore, a second model has been constructed to illustrate the 4.4 Å nitrogen–phosphorus distance. This new configuration provides space to allow for water molecules between the amine and phosphate groups (model 2, Figure 6).

These data refute the existence of the supposed contact ion pair. Nevertheless, the charges are close enough to each other to allow for partial charge neutralization which may affect metal binding. Therefore, 15N(31P) REDOR NMR experiments were performed to measure the N–P distance after the 15N-D-Ala labeled pepsin wall was mixed with MgCl₂. The metal-bound data show a longer internuclear distance of ~5.0 Å. The metal-bound amide data,
much like the unbound data, necessitate further experimentation in order to fully understand and confirm the decrease in N–P distance to \( \sim 6.0 \) Å. The metal-bound amine and amide data are also presented in Figures 4 and 5 and, when compared to the metal-free REDOR data, show that the metal cation pushes the amine farther away from the phosphate group, an orientation that should minimize (+) charge repulsions. We know from the 1-D \(^{31}\)P CPMAS data that the phosphate CSA tensors are perturbed, a clear indication of metal binding (45). Combined with the present study, the solid-state NMR data contradict the current view of TA metal binding, in which the positively charged \( \text{D-Ala} \) neutralizes the phosphate anion and frustrates metal binding at this site. An energy-minimized model was created with an octahedral-coordinated hydrated bidentate \( \text{Mg}^{2+} \) cation with water molecules added to replicate charge shielding (Figure 7). The model shows a change in phosphate structure, with the two chelating oxygens now facing away from the amine, increasing the \( \text{NH}_3^+ - \text{Mg}^{2+} \) distance.

**DISCUSSION**

Ion pairs further can exist as three groups: salt bridge, nitrogen–oxygen (N–O) bridge, or a long-range ion pair (Figure 8) (90, 91). The strongest interaction of these three would be the salt bridge, where the distance between at least one nitrogen–oxygen pair is within 4 Å. Likewise, the distance between the charged group central atoms (N–C in Figure 8 and N–P in teichoic acid) is also less than 4 Å. The nitrogen–oxygen bridge geometry is similar to the salt bridge, except that the central atoms of the charged groups no longer lie within 4 Å. In line with this trend, the long-range ion pair shows a geometry...
in which neither the charged group central atoms nor the nitrogen–oxygen pairs lie within the 4 Å distance. Additionally, a distance of less than 3.5 Å allows for the inference of a hydrogen-bonded salt bridge. These criteria outline a fairly specific molecular geometry, allowing for a particularly strong bonding interaction. This configuration results in protein–peptide stabilization or destabilization depending on the location and type of ion pairing interaction. Because these criteria deal with the interaction between two oppositely charged molecular species, they can be applied to the d-Ala–phosphate interactions of wall teichoic acid. With these criteria in mind, one can infer ion pairs of the nitrogen–oxygen bridging group based on our experimental NMR data. The 31P{31P} REDOR NMR data show that the cationic d-Ala and the anionic phosphate oxygen distance is ~4.4 Å.

Electrostatic forces play a fundamental and fairly predictable role in chemical interactions. In the case of d-Ala bound to teichoic acid, the labile nature of the charged cationic amine in close proximity to the anionic phosphate group(s) should result in the high probability of ion pairing. This configuration would neutralize the d-Ala cation. However, this model disagrees entirely with current experimental data which show that the presence of d-Ala in the cell wall of Gram-positive species provides resistance to CAMPs through a charge repulsion mechanism (64). This apparent contradiction can be explained by repeating REDOR-based distance measurements in the presence of Mg²⁺ because metals cations are required for bacterial survival and are found in relative abundance in nature. Thus, the analysis of REDOR NMR data collected on the bacterial cell wall in the presence of Mg²⁺ provides a more realistic insight into the structure and interactions of both peptidoglycan and teichoic acid.

Due to the phosphate pKₐ of 2.1, TA metal coordination involves anionic PO₄⁻ ions at the majority of naturally occurring pH values. The fraction of PO₄⁻ anions is lowered by 80% at pH 1.5, which decreases Mg²⁺ binding by 75% (28). However, it is important to consider the pKₐ of the amine of d-Ala, 9.69. At pH < 9, the alanyl amino group exists as NH₃⁺, perhaps forming an ion pair with the phosphate anion (Figure 2), possibly hindering the ability of the phosphate to participate in metal binding. In a seminal 1970 paper, Baddiley showed that chemical removal of d-alanine increased Mg²⁺ binding by 60% (31). Conversely, removal of the entire TA polymer from cell walls of S. aureus did not completely eliminate Mg²⁺ binding. This observation led to the conclusion that, in addition to TA, PG binds metal ions. Research over the next decade served to reveal the competitive binding nature of the cell wall. Addition of NaCl and KCl had little effect on Mg²⁺ concentration while CaCl₂ displaced Mg²⁺ in the cell wall (28).

Hoover and Gray studied a heat-sensitive strain of S. aureus showing that, without WTA, bacteria were susceptible to heat-induced death (59). Enhanced survivability from the presence of TA was linked to Mg²⁺ binding in the cell wall. As Ca²⁺ preferentially displaces magnesium, use of CaCl₂ also led to cell death. Later, Beveridge used electron microscopy and heavy metal staining to show metal binding to PG, presumably to the carboxyl groups (60). In 1980, Beveridge and Murray removed TA from B. subtilis cell walls to analyze the binding affinities for various alkali and alkali earth metal ions. Comparison with intact cell walls showed that Ca²⁺ binding requires TA, whereas Mg²⁺, K⁺, and Na⁺ are bound by both TA and PG (42). Thus, the cell wall binding site was assigned to the carboxyl groups of the glutamic acid (Glu), diaminopimelate (Dap), and terminal d-Ala regions.

We show that a portion of the metal binding pocket involves phosphate anions near the d-Ala groups. Using electrostatics, it is possible to evaluate the forces between the zwitterion using $F = Q_1Q_2/4\pi\varepsilon_0r^2$, where $Q_1$ and $Q_2$ are the charges, $\varepsilon_0$ is the vacuum permittivity, $\varepsilon$ is the dielectric constant, and $r$ is the distance between charges. The salt bridge ion pair, without water separation, has a dielectric constant of 1 and, thus, should be favored over the nitrogen–oxygen ion pair, where the water has a dielectric constant of 78. Our data show a much different situation, explained by the entropy-driven formation of a solvent cage around the charges, which provides the negative Gibbs energy necessary for solvation. The divalent Mg²⁺ ion incurs preferential chelation by the phosphate, producing repulsion between the cation and the d-Ala, which results in the longer N–P distance. In the solvent-separated model, the ability to separate the charges with two water molecules results in greater shielding and a lower repulsive force. These structures provide a potential drug target; compounds that sequester metals are antimicrobial (61, 62) given that the lack of metals causes cell wall destabilization, ultimately leading to membrane disruption and cell death.

A recent article by Karl Mueller’s research group is an excellent example of how solid-state NMR spectroscopy can impact structural biology paradigms (63). Here, 31P CPMAS spectra of mononucleotide deoxyadenosine monophosphate were collected to determine if it binds to the surface of aluminum oxide in a monodentate or a bidentate fashion. CSA tensors for
the phosphate demonstrated a bidentate binding, which was confirmed with computational chemistry calculations using Gaussian03. Subsequent $^{27}$P-$^{27}$Al REDOR studies confirmed that binding occurs at the octahedral aluminum oxide site rather than the tetrahedral site.

CAMP repulsion by teichoic acid due to cationic alanine groups is a structural model that was developed to explain genetic mutation studies (64). Mammalian production of CAMPs is an important means of protecting the host from microbial attack. The peptides contain 12–80 residues whose conformation is stabilized by cysteine–cysteine (Cys) disulfide bridges. The numerous arginine (Arg) and lysine (Lys) amino acids impart cationic properties that are only partially offset by anionic aspartate (Asp) and glutamate (Glu) residues. The basis for $\delta$-Ala addition to teichoic acid is attributed to the anionic aspartate (Asp) and glutamate (Glu) groups. This model was developed to explain repulsion of cationic antimicrobial peptides by the $\delta$-Ala group.

CONCLUSIONS

$B.\ subtilis$ cell walls (teichoic acid and peptidoglycan) were labeled with $^{15}$N $\delta$-Ala and analyzed with solid-state NMR spectroscopy. Phosphorus–nitrogen distance measurements show that the zwitterion exists as a probable nitrogen–oxygen ion pair, contradicting the models used to explain metal binding trends. Subsequent measurements in the presence of Mg$^{2+}$ ions reveal that metals bind in this area and the resultant charge repulsion pushes the $\delta$-Ala further away from the phosphate. These data support the model of teichoic acid structure used to explain repulsion of cationic antimicrobial peptides by the $\delta$-Ala group.

ACKNOWLEDGMENT

We thank Prof. Eric Brown (McMaster University) for helpful insight into teichoic acid biochemistry. Likewise, we thank Prof. Andreas Peschel (University of Tubingen) for helpful comments. We also thank Roger Frech (University of Oklahoma) for helpful discussions regarding solvent-separated ion pairs.

SUPPORTING INFORMATION AVAILABLE

The $B.\ subtilis$ synthetic medium (BSSM) is described and the growth curve provided as a collection (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES