Effects of Binding and Bactericidal Action of Vancomycin on *Bacillus licheniformis* Cell Wall Organization as Probed by ¹⁵N Nuclear Magnetic Resonance Spectroscopy

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The effects of binding and the bactericidal action of vancomycin on the arrangement and mobilities of cell wall polymers in *Bacillus licheniformis* were investigated by ¹⁵N nuclear magnetic resonance spectroscopy. The bactericidal action of vancomycin led to reduced mobilities of cell wall teichoic acid and teichuronic acid in surviving cells. The decrease in teichoic acid mobility was also observed upon binding of vancomycin to *B. licheniformis* cells and resulted from a specific interaction between the antibiotic and teichoic acid, rather than from electrostatic contraction of the cell wall. The reduction in teichuronic acid mobility appeared to be related either to the elastic contraction of the cell wall resulting from loss of cell turgor or to separation of the cell wall from the protoplast membrane. No spectral changes associated with cell wall autolysis or alterations in cell wall composition, amidation, and cross-linking were found in vancomycin-treated *B. licheniformis* cells. Binding of vancomycin to *Micrococcus lysodeikticus* cell walls led to a decrease in mobility of C-terminal D-alanine residues but was accompanied by an increase in the mobilities of other peptidoglycan residues. The possible contributions of changes in the arrangements of cell wall polymers to the lethal action of vancomycin is discussed.

The bactericidal actions of many antibiotics are associated with the inhibitions of cell wall biosynthesis that result from the binding of the antibiotics to enzymes and intermediates along the cell wall biosynthetic pathway (32). Rogers and a co-worker have suggested (28, 29) that when cell wall biosynthesis is inhibited, the natural ongoing autolysis of the cell wall is no longer in balance with the insertion of new cell material. Hydrolytic cleavage of peptidoglycan glycosidic and peptide bonds proceeds until the mechanical properties of the cell wall that are responsible for osmotic protection of the protoplast membrane are lost and the membrane is damaged. The role of autolysins in the actions of penicillin and vancomycin has been amply demonstrated by the loss of the lethal actions of the antibiotics when cell wall autolysins are inhibited (1, 28, 29, 32, 34). Vancomycin forms strong complexes with gram-positive bacterial cell walls and lipid-soluble cell wall intermediates and peptidoglycan precursors in the protoplast membrane (13, 18, 23, 24, 26, 37). The inhibition of peptidoglycan biosynthesis by vancomycin in cell-free systems is restricted to its complexing with lipid-soluble intermediates in the membrane fraction (12, 13, 18). However, in the whole cell, part of the bactericidal effect of vancomycin may also

be associated with the strong binding of vancomycin to the cell wall (2, 3, 13). The binding of highly charged vancomycin molecules (2, 3) to the amphoteric polyelectrolyte gel-like cell wall (22) is likely to result in changes in the three-dimensional arrangements of the cell wall polymers and in the contacts between the cell wall and protoplast membrane, which could affect the relative rates of cell wall autolysis and the insertion of new cell wall material. The activity of the major cell wall autolysin, *N*-acetylmuramoyl-L-alanine amidase (in *Bacillus licheniformis* and *B. subtilis*), has been shown to be related to the recognition of cell wall teichoic and teichuronic acids and to more subtle structural features of the cell wall (11, 15, 16, 20, 29). It is therefore of interest to determine whether the binding and bactericidal action of vancomycin do indeed lead to changes in the arrangements and mobilities of cell wall polymers.

Although spin-labeling mobility measurements of peptidoglycan precursors have been used to demonstrate the binding of vancomycin to lipid-soluble intermediates of cell wall biosynthesis (18), arrangements and mobilities of individual polymers in vancomycin-treated cell walls have not as yet been probed. In fact, very little is known about the organization of the

native cell wall due to a lack of suitable physical probes. Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for probing the conformations and motions of macromolecules, and it might complement biochemical and electron microscopic studies of cell wall organization. Unfortunately, the ^1H and ^{13}C NMR spectra of intact cells and cellular organelles have proved too complex to be used in studies of arrangements of mobile polymeric cellular components (4, 8, 10). Recently, however, relatively simple and well-resolved ^{15}N NMR spectra have been obtained from a variety of ^{15}N -labeled intact cells (19), and this technique has been used to probe the dynamic structure of the gram-negative bacterial cell envelope (17). In the case of gram-positive bacteria, changes in the conformations and mobilities of cell wall polymers can be determined in a straightforward manner by simple comparison of the relative intensities of cell wall resonances in ^{15}N NMR spectra of ^{15}N -enriched intact cells (17, 19; A. Lapidot and C. S. Irving, in B. Pullman (ed.), *11th Jerusalem Symposium on Nuclear Magnetic Resonance Spectroscopy in Molecular Biology*, in press).

Insights into the local organization of the cell wall polymers can be deduced from the degrees of motional freedom of peptidoglycan peptide chains and acidic polysaccharide chains. In the present study, the effects of binding and the bactericidal action of vancomycin on the mobilities and arrangements of the cell wall polymers of ^{15}N -enriched *B. licheniformis* and *Micrococcus lysodeikticus* have been studied by this technique.

MATERIALS AND METHODS

Strains. The strains used in this study were *B. licheniformis* ATCC 9945 and *M. lysodeikticus* NCTC 2665.

^{15}N -labeled growth media and reagents. [^{15}N] ammonium chloride, enriched 90 to 95% ^{15}N , was prepared by standard methods (30) from [^{15}N]nitric acid (obtained from the Isotope Separation Plant of the Weizmann Institute of Science, Rehovot, Israel). ^{15}N -labeled minimum salts medium was Spizizen salts medium (31) in which 0.2% $(\text{NH}_4)_2\text{SO}_4$ was replaced by 0.1% $^{15}\text{NH}_4\text{Cl}$. Reduced ^{15}N -labeled minimum salts medium contained only 0.05% $^{15}\text{NH}_4\text{Cl}$. ^{15}N -amino acid mixture was obtained from the protein hydrolysate of bakers' yeast grown at 37°C with aeration on ^{15}N -labeled minimum salts medium supplemented with glucose (5 g/liter) and Difco yeast carbon base (10 g/liter). The amino acid mixture was purified by ion-exchange chromatography on Dowex H-50. The composition of the ^{15}N -amino acid mixture, as determined on a Beckman amino acid analyzer, was (in milligrams per gram): lysine, 14.4; histidine, 3.5; arginine, 5.0; aspartate, 0.2; threonine, 0.6; serine, 0.3; glutamate, 2.2; proline, 3.8; glycine, 100; alanine (Ala), 130; cys-

teine, 41; valine, 10.7; methionine, 2.2; leucine, 14.0; tyrosine, 2.6; phenylalanine, 8.1. ^{15}N -amino acid medium consisted of reduced ^{15}N -labeled minimum salts medium supplemented with ^{15}N -amino acid mixture (1 g/liter) and the Coutinho and Nutini vitamin mixture (7, 21). Vancomycin (Eli Lilly & Co., Indianapolis, Ind.) was dissolved in distilled water and used without further purification.

Growth conditions and sample preparation.

For liquid media, the inoculum was taken from a freshly streaked plate, and a liquid starter culture (100 ml) was left shaking for 18 h and then added to 1 liter of growth medium to give an optical density (OD_{680}) at 680 nm of 0.01 to 0.02.

B. licheniformis was grown at 37°C with shaking and aeration in 5-liter conical flasks containing 1 liter of ^{15}N -labeled minimum salt medium. *M. lysodeikticus* was grown at 30°C with shaking in ^{15}N -amino acid medium. Growth was followed by measurement of extinction at 680 nm by means of a side arm fixed to the growth flask. Upon completion of growth, 4% sodium dodecyl sulfate was added to the culture media to inactivate autolysins (21, 28), shaking was continued for 10 min at 37°C, and the bacterial cells were rapidly harvested by centrifugation (Sorvall RC2-B rotor, 10,000 rpm, 10 min) at 37°C. The cell pellet was washed three times with distilled water at 37°C to remove traces of sodium dodecyl sulfate. Cell walls were prepared by mechanical disruption of cells in a Braun tissue homogenizer by shaking at maximum speed with 0.10- to 0.11-mm-diameter Ballatoni-glass beads (B. Braun Melsungen, Ballatoni no. 541540) for 3 to 5 min at 4°C. The supernatant was decanted from the glass beads and centrifuged ($1,000 \times g$, 10 min, at 4°C) to remove unbroken cells. Cell walls were obtained from the supernatant by centrifugation at $20,000 \times g$ for 15 min at 4°C and washed five times in water, until a cell wall suspension devoid of whole cells was obtained. The absence of whole cells was assured by phase-contrast microscopy (2, 29), and the pH was adjusted to pH 7 by addition of concentrated HCl, when needed. Intact cells and cell walls were packed into 10-mm NMR tubes (Wilmar, no. 513-7PP) to heights of 2.5 cm by centrifugation at 3,000 rpm (IEC HN-S centrifuge) for 0.5 and 2 h, respectively, after which the supernatants were clear. Cell wall lysozyme digests were prepared by suspending 1 to 2 cm^3 of packed cell walls in 10 ml of distilled water containing 2 to 3 mg of salt-free lysozyme (Worthington Biochemical Co.) and incubating at pH 7.0 for 5 h at 37°C with gentle shaking. The reaction mixture was boiled for 5 min to precipitate proteins and centrifuged ($15,000 \times g$, 15 min). The supernatant was concentrated to 1 cm^3 by rotatory evaporation, after which the pH was adjusted to 7 by addition to concentrated HCl or NaOH.

Bactericidal action of vancomycin on *B. licheniformis*. Vancomycin (5 $\mu\text{g}/\text{ml}$) was added to a 1-liter culture of *B. licheniformis* in midlog growth phase (OD_{680} , 0.18), and cell growth was continued for 2 h, after which lysis began (Fig. 1); lysis was determined by microscopic observations. Vancomycin-treated cells were harvested as described above. Normal control cells in a parallel growth (Fig. 1) were harvested (OD_{680} , 0.42) at the same time as the vancomycin-treated cells. ^{15}N NMR measurements were

FIG. 1. The effect of vancomycin when added to growing cultures of *B. licheniformis* (ATCC 9945). Concentration of vancomycin: ●, none; ○, 5 µg/ml.

made on the intact cells, isolated cell walls, and cell wall lysozyme digests of both vancomycin-treated and control cells.

Partial autolysis of *B. licheniformis* cells. *B. licheniformis* cells were grown to midlog phase as described above, but they were harvested without addition of sodium dodecyl sulfate and were then packed into an NMR tube.

Binding of vancomycin to *B. licheniformis* cells. A 1-liter culture of *B. licheniformis* was harvested in midlog phase (OD₆₈₀, 0.31). After a ¹⁵N NMR spectrum of the cell pellet was obtained, the pellet was suspended in 5 ml of distilled water containing 1 mg of vancomycin and repacked. Practically quantitative binding of vancomycin had occurred, as judged by the OD₂₈₀ of the supernatant (24). ¹⁵N NMR measurements were repeated on the vancomycin-treated cell pellet.

Binding of vancomycin to *M. lysodeikticus* cell walls. *M. lysodeikticus* cells were prepared from a 1-liter culture (OD₆₈₀, 0.23), and cell walls were prepared by mechanical disruption for 5 to 8 min as described above for *B. licheniformis*. After a ¹⁵N NMR spectrum of the cell wall sample was obtained, the cell wall pellet was suspended in 5 ml of distilled water containing 10 mg of vancomycin and then repacked. Quantitative adsorption of vancomycin was established by the OD₂₈₀ of the supernatant. ¹⁵N NMR measurements were repeated on the vancomycin-treated cell wall pellet.

¹⁵N NMR-measurements. ¹⁵N NMR experiments were performed on a Bruker WH-90 Fourier transform spectrometer operating at 9.12 MHz. Proton-broad-band-noise-decoupled Fourier-transformed spectra consisting of 2,000 data points were obtained with the following spectrometer conditions: 90° pulse angle of 28-µs duration, 2-KHz spectral width, 10,000 accumulations, 2-Hz exponential filter, and digital resolution of 0.976 Hz.

Field stabilization was accomplished by locking on the deuterium resonance of D₂O in a 5-mm-diameter concentric tube inserted into the 10-mm-diameter

sample tube. A sample of 4 M ¹⁵NH₄Cl in 2 M HCl in a 2-mm concentric tube inserted into the 5-mm tube of D₂O provided an external reference signal at 350.89 ppm upfield from H¹⁵NO₃. Chemical shifts were estimated to be accurate to ±0.05 ppm.

Sample temperatures were maintained by a Bruker temperature control unit and measured directly by immersion of the thermocouple into the 5-mm concentric tube containing D₂O.

To obtain the chemical shifts, half-height line widths, and relative intensities of overlapping ¹⁵N resonances, the resonances and shapes were simulated by the superposition of Lorentz lines with variable heights, half-height line widths, and chemical shifts, using interactive graphics computer programs written in SPEAKEZ. Best fits were judged by visual comparison of experimental and simulated band shapes on a Tektronic 4014 visual display unit. Chemical shifts, line widths, and relative intensities were accurate ±0.05 ppm, 20%, and 5%, respectively.

RESULTS

Action of vancomycin on *B. licheniformis* cells. The proton-decoupled, 9.12-MHz ¹⁵N NMR spectrum of ¹⁵N-enriched, normal *B. licheniformis* cells harvested in midlog growth phase displayed five well-resolved, inverted resonances with linewidths of 10 to 20 Hz, in addition to the narrow resonance of the external ¹⁵NH₄Cl reference at 350.89 ppm (Fig. 2A).

The resonances of *B. licheniformis* cells have been assigned to the acetamido groups of teichuronic acid *N*-acetylglactosamine residues (252.8 ppm), the amidated carboxylate groups of peptidoglycan D-glutamate and *meso*-diaminopimelic acid residues (266.8 ppm), the N-terminal free amino group of the D-Ala residues of teichoic acid (335.6 ppm), the free amino group of non-cross-linked *meso*-diaminopimelic acid residues of peptidoglycan (337.9 ppm), and the free amino groups of protein lysine side chains or amino sugars (343.6 ppm) (Lapidot and Irving, in B. Pullman (ed.), *11th Jerusalem Symposium on Nuclear Magnetic Resonance Spectroscopy in Molecular Biology*, in press). *B. licheniformis* cell walls (Fig. 2B) displayed the same resonances as were observed in the intact cell; however, their absolute intensities were greatly reduced, as a result of either difficulties in packing of the cell walls or changes in organization of cell wall polymers. The *B. licheniformis* cell wall lysozyme digest spectrum at pH 7.0 displayed a spectrum (Fig. 2C) that contained 13 resonances in the amide region (240 to 270 ppm). Resonances of Ala residues occur in the 245- to 251-ppm region, D-glutamate and *meso*-diaminopimelic acid peptide groups adjacent to free carboxylic acid groups occur in the 251- to 252-ppm region, the acetamido groups of glycan *N*-acetylmuramic acid residues and teichuronic acid

FIG. 2. Effect of bactericidal action of vancomycin on the dynamic properties of cell wall polymers. *B. licheniformis* cells, harvested when vancomycin-induced cell lysis reached 18% (see Fig. 1) displayed significant differences in their ^{15}N NMR spectra (D), when compared with nontreated cells (A). Differences were not observed in ^{15}N NMR spectra of isolated cell walls (E) and cell wall lysozyme digests (F) of vancomycin-intoxicated cells when compared with the cell wall (B) and lysozyme digest (C) spectra of nontreated cells. Spectra were obtained at 27°C from 1- to 2-cm^3 pellets of packed cells, 0.5- to 1-cm^3 pellets of packed cell walls, and the soluble lysozyme digest (from 1 cm^3 of packed cell walls) at pH 7.0. Exponential filters of 2, 5, and 1 Hz were used for cell, cell wall, and lysozyme digest spectra, respectively.

N-acetylgalactosamine residues occur between 252 and 254 ppm, *D*-glutamate and *meso*-diaminopimelic acid groups are found in the 255- to 256-ppm region, and the amidated carboxylate groups of *D*-glutamate and *meso*-diaminopimelic acid occur between 266 and 269 ppm (Lapidot and Irving, in press). The relative intensities of these resonances provide a sensitive fingerprint of the relative amounts of acidic polysaccharides in the cell wall, the degree of cross-linking of peptidoglycan peptide chains, the degrees and sites of amidation, and the mobilities of the cell wall fragments (Lapidot and Irving, in press). The relative intensities of the resonances in the lysozyme digest spectra were obtained by computer simulation and are given in Table 1.

The ^{15}N NMR spectrum of *B. licheniformis* cells treated with a lethal dose of vancomycin ($5\text{ }\mu\text{g/ml}$) in midlog growth phase and harvested after 20% of the cells had lysed showed (see below) significant differences in the relative intensities of the five intact cell resonances (Fig. 2D). The relative intensities of the 252.8; 266.8; 335.6; and 337.9-ppm resonances changed from 1.4, 1.0, 5.4, and 0.6, respectively, in nontreated cells (Fig. 2A) to 0.8, 1.0, 2.6, and 0.6 in the

TABLE 1. Relative signal intensities (normalized with respect to 253.5 ppm) of the ^{15}N NMR of the cell wall lysozyme digests of normal and vancomycin-treated *B. licheniformis* cells, at pH 7.0, 27°C

Resonance (ppm)	Relative intensity in:	
	Normal cells	Vancomycin-treated cells
245.6	0.23	0.26
246.0	0.20	0.20
248.7	0.11	0.12
249.3	0.24	0.24
250.4	0.25	0.25
251.1	0.24	0.22
252.0	0.22	0.22
252.8	0.39	0.32
253.5	1.00	1.00
254.2	0.37	0.36
256.6	0.21	0.19
267.3	0.26	0.24
268.9	0.23	0.22

vancomycin-treated cells (Fig. 2D) (cell signal intensities are normalized with respect to the 266.8-ppm amide resonances, which have constant relative signal intensities with respect to the external reference of 350.89 ppm). This in-

dicates that as a result of the bactericidal action of vancomycin, a considerable reduction had occurred in either the concentrations or the mobilities of the acidic polysaccharides (252.8 and 335.6 ppm) with respect to peptidoglycan (266.8 and 337.9 ppm).

On the other hand, the relative signal intensities in the lysozyme digest spectra (Fig. 2C) of treated and nontreated cells were remarkably similar, as shown by computer simulation (Table 1). The similarity of the two spectra indicated that changes had not occurred in the primary structure, amidation, or degree of cross-linking of cell wall peptidoglycan nor in the relative amounts of peptidoglycan and teichuronic acid in the lysozyme-susceptible fraction of the cell wall.

Teichuronic acid mobility. The similarity of the lysozyme digest spectra of vancomycin-treated and control cells indicates that the reduction in the intensity of the teichuronic acid resonance (252.8 ppm) in the treated cells must have resulted from a change in mobility, rather than a change in chemical composition. A change in mobility can result from either direct complex formation with vancomycin or a rearrangement of cell wall polymers. The fact that the cell wall spectra of treated and nontreated cells were very similar (Fig. 2B and D) indicated that direct complex formation with vancomycin, which would have persisted upon going from intact cells to isolated cell walls, was not the cause of the change in mobility of teichuronic acid in the native cell wall. The dynamic properties of the teichuronic acid chains that were affected by the bactericidal action of vancomycin must have been related to an arrangement of the polymers that was dependent on either contact of the cell wall with the protoplast membrane or cell turgor, both of which were lost during the isolation of the cell wall.

If the change in mobility of teichuronic acid chains in vancomycin-treated cells was indeed related to elastic contraction of the cell wall resulting from a loss of cell turgor or to separation between the cell wall and the protoplast membrane, then plasmolysis of *B. licheniformis* cells (21, 36) would be expected to produce similar spectral changes. This was the case. Plasmolysis of *B. licheniformis* cells in 2 M sucrose led to a marked reduction in the intensity of the 252.8-ppm teichuronic acid resonance (Fig. 3A), which resembled the effect of the bactericidal action of vancomycin on the *B. licheniformis* cell spectrum.

Teichoic acid mobility. Since the teichoic acid resonance (335.6 ppm) was difficult to detect in isolated cell walls and lysozyme digest, it was not possible to use the cell wall and lyso-

FIG. 3. Effect of plasmolysis and electrostatic contraction on dynamic properties of cell wall polymers of *B. licheniformis* cells. The ^{15}N NMR spectrum of *B. licheniformis* cells in 2 M sucrose (A) shows a significant reduction in the intensity of the 252.8-ppm teichuronic acid resonance, whereas no changes were observed in the ^{15}N NMR spectrum of *B. licheniformis* cells in 0.13 osmol of NaCl per kg (B). ^{15}N NMR spectra were obtained on a Bruker HFX-10 Fourier transform NMR spectrometer.

zyme digest spectra to determine the origin of the reduction in the teichoic acid resonance in the intact cell spectra of vancomycin-intoxicated *B. licheniformis*. However, insights into the effect of vancomycin on teichoic acid mobility could be obtained by investigating the effects of binding of vancomycin to walls of intact cells.

Upon binding of the equivalent of a lethal dose of vancomycin to a suspended pellet of *B. licheniformis* cells whose autolysis had been inactivated before harvesting, the relative intensities of the 252.8; 266.8; 335.6, and 337.9-ppm resonances changed from 1.56, 1.0, 4.25, and 1.0, respectively, in the normal cells (Fig. 4A) to 1.50, 1.0, 3.50, and 1.1 after vancomycin binding (Fig. 4B). The binding of vancomycin led to a selective reduction in the relative intensity of the teichoic acid resonance (335.6 ppm), which was

FIG. 4. Binding of vancomycin (1 mg, equivalent to a lethal dose of $1 \mu\text{g/ml}$) to *B. licheniformis* cells (1 cm^3 of packed cells; $\sim 300 \text{ mg}$, dry weight) whose autolysins had been inactivated before harvesting led to a reduction in the relative intensity of the 335.6-ppm teichoic acid resonance in the intact cell ^{15}N NMR spectrum.

sufficient to explain the reduction in the relative intensity of the teichoic acid resonance in the spectrum of cells grown in the presence of vancomycin. The reduction of mobility of teichoic acid may have resulted from either the direct binding of vancomycin to teichoic acid or from rearrangement of cell wall polymers resulting from electrostatic contraction of the cell wall associated with binding of a highly charged molecule to a polyelectrolyte gel. Electrostatic contraction of cell walls of *B. licheniformis* cells resulting from suspension of intact cells in 0.13 osmol of NaCl per kg (4 g of NaCl per liter) at pH 5.0 (22) did not lead to any observable changes in the intact cell spectrum (Fig. 3B). It is unlikely that the spectral changes produced by either the binding or the bactericidal action of vancomycin was caused by electrostatic contraction of the cell wall.

Plasmolysis of *B. licheniformis* cells also did not lead to a significant reduction in the relative intensity of teichoic acid resonance. The change in teichoic acid mobility that occurred as a result of the bactericidal action of vancomycin probably resulted from direct formation of a teichoic acid-vancomycin complex.

Cell wall autolysis. To determine whether the changes observed in the intact cell spectra of vancomycin-treated cells were related to cell wall autolysis, the ^{15}N NMR spectrum of partially autolyzed cells was examined. *B. licheniformis* cells whose cell walls had been partially

autolyzed due to failure to inactivate autolysis on harvesting showed a different type of cell wall spectrum (Fig. 5). Many of the peptidoglycan peptide resonances found in the lysozyme digest spectrum (Fig. 2C) but not in the spectrum of normal intact cells (Fig. 2A) were seen in the spectrum of partially autolyzed cells (Fig. 5). The reduction in intensity and splitting of the 266.8-ppm resonance observed on going from intact cell (Fig. 2A and D) to lysozyme digest (Fig. 2C and F) spectra was also readily seen in the partially autolyzed cell spectrum (Fig. 5).

These spectral changes were consistent with a loss of cell wall rigidity and an increase in the mobility of the peptidoglycan peptide chains associated with a reduction in covalent cross-linking of glycan strands resulting from autolytic cleavage of *N*-acetylmuramic acid L-Ala bonds. It is remarkable that none of the spectral changes resulting from cell wall autolysis could be observed in the spectrum of vancomycin-treated cells, which were on the verge of cell lysis.

Binding of vancomycin to *M. lysodeikticus* cell walls. The mode of action of vancomycin on cell walls containing tight peptidoglycan groups and large amounts of acidic polysaccharides, as in the case of *B. licheniformis*, might differ from the action of vancomycin on a cell wall composed almost entirely of "loose" peptidoglycan, as occurs in *M. lysodeikticus*. In *M. lysodeikticus*, not all *N*-acetylmuramic acid residues are substituted with peptide chains, and long oligopeptide chains serve as bridges (12). The ^{15}N NMR spectrum of *M. lysodeikticus* cell walls (Fig. 6A) displayed a complete set of peptidoglycan peptide resonances and was very similar to the lysozyme digest spectrum (22). The

FIG. 5. Effect of partial autolysis on dynamic properties of cell wall polymer of *B. licheniformis* cells. Failure to inactivate autolysins before harvesting *B. licheniformis* cells in midlog growth phase led to the appearance of additional peptidoglycan resonances in the intact cell spectrum.

FIG. 6. Binding of vancomycin (10 mg, equivalent to a lethal dose of 10 $\mu\text{g}/\text{ml}$) to cell walls obtained from *M. lysodeikticus* cells (2 cm^3 of packed cells) led to a reduction in the $\sim 246\text{-ppm}$ C-terminal D-Ala resonance and an increase in intensity and narrowing of other cell wall resonances.

large amount of intense resonance observed in the cell wall spectrum was a direct result of the high degree of mobility found in the loose peptidoglycan matrix. The binding of the equivalent of a lethal dose of vancomycin to *M. lysodeikticus* cell walls (Fig. 6B) resulted in both a narrowing and an increase in intensity of almost all of the peptidoglycan peptide resonances. Only the resonance of the C-terminal D-Ala (245.6 ppm) group was lost upon vancomycin binding.

DISCUSSION

Best and Durham (2, 3) have shown that vancomycin absorbs to cell walls of *B. subtilis* via ionic interactions, involving cell wall acid groups and one or more basic groups in vancomycin. It has been assumed that the high-affinity binding sites of vancomycin in cell walls are D-Ala-D-Ala residues in non-cross-linked, peptide glycan segments in unfinished walls (25-26). Perkins and Nieto (26) noted that vancomycin bound to cell walls could be easily extracted by 10 mM Mg^{2+} from *B. licheniformis* cell walls but not from *M. lysodeikticus* cell walls and attributed the difference to nonspecific binding of vancomycin to *B. licheniformis* walls, which have fewer D-Ala-D-Ala sequences. ^{15}N NMR measurements reported here indicate that the binding of vancomycin to *B. licheniformis* cell walls is selective and involves mainly teichoic acid. The binding of vancomycin to teichoic acid readily accounts for the ability of Mg^{2+} to extract vancomycin bound to *B. licheniformis* cell walls, but not that bound to *M. lysodeikticus* walls. Magnesium ions have a high affinity for teichoic acid and would be expected to displace bound vancomycin. Since *M. lysodeikticus* cell walls

lack teichoic acids, extraction with Mg^{2+} would have little effect on vancomycin binding.

The binding of vancomycin to D-Ala-D-Ala sequences has been demonstrated for model peptide systems (5, 6, 37) and for cell wall precursors (18), but not as yet for intact cell walls. The specific reduction in the amplitude of C-terminal D-Ala ^{15}N resonance in the *M. lysodeikticus* cell wall provides evidence for direct interaction of vancomycin with at least the C-terminal D-Ala residues, if not the entire D-Ala-D-Ala sequence. The binding of vancomycin to *M. lysodeikticus* cell walls also results in an increase in the mobility of peptidoglycan. The only likely explanation for this effect is that in a loosely covalently cross-linked peptidoglycan matrix, electrostatic interactions involving the glycyl carboxylate groups and L-lysine amino groups contribute to mobility of peptide chains and cell wall rigidity. The binding of highly charged vancomycin to cell walls somehow disrupts these electrostatic interactions and leads to a further loosening of the peptidoglycan matrix.

Shrinkage of whole gram-positive bacteria cells and isolated cell walls can result from either electrostatic contraction of the amphoteric, polyelectrolyte gel-like cell wall or from elastic contraction of the cell wall associated with the loss of cell turgor. It was proved difficult to distinguish between these two effects (22). The present ^{15}N NMR measurements have shown that salt-induced contraction of cell walls can be readily distinguished from plasmolysis. Durham et al. (9) observed shrinkage of whole *B. cereus* cells shortly after exposure to vancomycin and attributed it to elastic contraction of the wall after loss of cell turgor. Marquis (22) suggested that the antibiotic-induced process is more closely related to the type of contraction which occurs during plasmolysis in concentrated solution, rather than to salt-induced contraction of the cell wall. The spectral changes observed in the ^{15}N NMR spectra of vancomycin-intoxicated *B. licheniformis* resemble those associated with plasmolysis and support the suggestions of Durham et al. (9) and Marquis (22). However, the present ^{15}N NMR experiments cannot distinguish between spectral changes resulting from the loss of cell turgor accompanying the damage to the permeability properties of the protoplast membrane and spectral changes resulting from dissociation of the cell wall from the protoplast membrane. Vancomycin has a high affinity for both lipids and acidic molecules (3) and may be uniquely suited to disrupting interactions between the protoplast membrane and the acidic cell wall polymers.

The possibility that vancomycin inhibits the insertion of new cell wall material or accelerates

cell wall autolysis by binding to the cell wall in a way that alters the organization of cell wall polymers has not been previously considered, since it has not been possible to probe the arrangements of cell wall polymers. The present ^{15}N NMR results showing that binding of vancomycin to *B. licheniformis* cell walls affects the mobility of teichoic acid indicate that investigation of additional modes of action of antibiotics should be considered, since the roles of teichoic and teichuronic acids in regulating the activities of cell wall autolysins have often been demonstrated (13, 18, 24, 26, 35).

The direct role of autolysins in the death of vancomycin-intoxicated *B. licheniformis* cells has been supported by the fact that the action of vancomycin on *B. licheniformis* with low levels of autolysins is bacteriostatic (1, 28, 29, 33, 34). The lack of any detectable signs of autolytic damage in walls of vancomycin-treated *B. licheniformis* cells examined by ^{15}N NMR suggests that (i) the degree of autolysis required for cell lysis is so small that it could not be detected, (ii) extensive autolysis is limited to a very small region of the cell wall, (iii) autolysis does not occur gradually but has an induction period after which it is rapid, or (iv) damage to the permeability barrier and the ensuing cell lysis are due to other processes in addition to autolysis. The first possibility is unlikely, since considerable cell wall damage could be detected in partially autolyzed cells, which had not yet undergone cell lysis. The present ^{15}N NMR measurements rule out the possibility of a buildup of widespread cell wall autolysis that precedes cell lysis.

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