Dynamic structure of whole cells probed by nuclear Overhauser enhanced nitrogen-15 nuclear magnetic resonance spectroscopy

( Escherichia coli / Bacillus licheniformis / baker's yeast / Friend leukemic cells / Staphylococcus aureus )

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Communicated by R. G. Shulman, February 9, 1977

ABSTRACT The proton-decoupled 15N Fourier transform nuclear magnetic resonance (NMR) spectra of 15N-enriched Escherichia coli, Bacillus licheniformis, baker's yeast, and Friend leukemic cells were obtained. The 15N NMR spectra of whole cells displayed 15N resonances originating from (i) protein backbones with lysine, arginine, and histidine side chains, (ii) ribonucleic acids, (iii) phosphoglycan, and (iv) phospholipids. Several additional amino and amide resonances were observed but not identified. In bacteria and yeast, the cell wall was found to be the site of a relatively mobile group of molecules, whose resonances dominate the proton-decoupled 15N NMR spectra of whole cells. 15N NMR chemical shifts and nuclear Overhauser effects have provided information on the in vivo structure of cell wall phosphoglycan. In Staphylococcus aureus the penta- glycan cross-bridge of cell wall phosphoglycan was found to have a random coil conformation. In B. licheniformis considerable segmental motional freedom was detected in teichuronic acid and phosphoglycan polysaccharide chains in the wall of the intact cell.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for obtaining information on the electronic structure, conformation, solution, and dynamics of molecules and can be applied to systems as complex as biological cell cultures and tissues. The NMR technique has proved to be particularly useful for monitoring the metabolic reactions of small molecules in living cells (1–4). NMR measurements have also provided information on the motional freedom of small molecules in different cellular compartments and cell fractions (5). The NMR spectra of molecules that comprise the structural components of cells are not easily obtained (6).

Although the 1H, 13C, and 31P NMR spectra of complex biological systems are being actively studied, the 15N spectrum of only one cell system, the fungus Ustilago sphaerogena, has been reported (7). The scope of 15N NMR spectroscopy of whole cells remains to be determined with respect to (i) the cellular components that can be studied, (ii) the ability of the 15N-1H nuclear Overhauser effect nulling (8) to simplify complex spectra and provide dynamic information, and (iii) the need of specific isotopic labeling for making specific assignments and reducing the number of observed resonances. 15N NMR spectroscopy appears to be well suited to probing the structural organization of cells for several reasons. The nitrogen atoms are often found in close proximity to the sites of the intermolecular interactions that play important roles in determining the structure and function of biological molecules. 15N chemical shifts are particularly sensitive to molecular conformation and noncovalent bonding interactions and correlations are actively being sought in small molecules (9–11). 15N NMR spectra of structural molecules may be better resolved than 1H and 13C NMR spectra, because the smaller gyromagnetic moment of the 15N nucleus makes its NMR resonances less susceptible to dipolar line broadening. The low sensitivity of 15N can now be overcome by isotopic labeling and the use of high sensitivity Fourier transform and wide gap spectrometers.

In this paper we survey the 15N NMR spectra of a series of 15N-enriched Gram-positive and Gram-negative bacteria, yeast, and murine Friend leukemic cells. These spectra demonstrate the types of cellular components whose 15N resonances can be detected in proton-decoupled 15N NMR of whole cells and indicate which problems in cell organization lend themselves to 15N NMR techniques. Interestingly, the majority of resonances observed in whole cells of bacteria and yeast originate not from small molecule metabolites but from a relatively mobile group of cell wall components. These latter resonances have provided insights into the organization of the Gram-positive bacterial cell wall.

MATERIALS AND METHODS

Isotopically Labeled Compounds. [15N]Ammonium chloride, enriched to 90–95% 15N, was prepared by standard methods (12) from [15N]nitric acid (obtained from the Isotope Separation Plant of the Weizmann Institute). [15N]Glycine (95%) was prepared by the method of Schoenheimer (13). 15N-labeled algal amino acid mixture was prepared from Chlorella pyrenoidosa grown on K15NO3 (14).

Cells, Media, and Growth Conditions. Escherichia coli B/r was grown at 37°C with aeration to the early stationary phase (OD 0.55 at 680 nm) in Spizizen salts medium (15), in which 0.2% (NH4)2SO4 was replaced by 0.1% 15NH4Cl. Bacillus licheniformis ATCC 9945 was grown at 37°C with aeration to the mid-logarithmic (OD 0.21), late logarithmic (OD 0.37), early stationary (OD 0.55), and stationary (4 hr at OD 0.67) phases in 15N-labeled Spizizen salts medium. Staphylococcus aureus H was grown at 37°C with aeration to the late logarithmic phase (OD 0.49) in basal medium (16), in which 0.1% (NH4)2SO4 was replaced with 0.05% NH4Cl and supplemented with the Coutinho and Nutini amino acid/vitamin mixture (17), which contained 15N-glycine (95% 15N, 200 mg/liter) in place of nonlabeled glycine. Baker’s yeast (Saccharomyces cerevisiae) was grown to the stationary phase at 37°C with aeration on basal media, in which 0.1% (NH4)2SO4 was replaced with 0.1% 15NH4Cl. Friend-virus-infected murine leukemic cells (clone 707) (18) were grown in a humidified 5% CO2 atmosphere at 37°C in minimum essential medium with Earl’s salts and antibiotics, in which the amino mixture was replaced with 15N-labeled algal amino acid mixture, supplemented with arginine (100 mg/liter), cysteine (24 mg/liter), methionine (13 mg/liter), threonine (35 mg/liter), phenylalanine (27 mg/liter), and glutamine (240 mg/liter) and harvested after 4 days (3 × 10⁶ cells per ml).

Intact Cell Samples. E. coli and baker’s yeast cells were harvested by centrifugation in the cold (6000 × g, 10 min) and washed three times with 100 ml of distilled water and packed.
amino acids at pH 5.0 and to the chemical shifts reported in the
literature (19, 22). The PE resonance was assigned on the basis of
its disappearance from the $^{15}$N spectrum of the cell envelope
after chloroform/methanol extraction. Only the amide PP4 and
PP7 resonances are exclusively associated with the cell protoplast,
because the other resonances were present in the $^{15}$N NMR spec-
trum of the cell envelope. No free amino acid resonances
were observed and it is likely that the $^{15}$N resonances of
amino acids and other small nitrogenous molecules, with $T_1$
relaxation times longer than 1 sec, had been saturated by the
fast repetition times used in these experiments. The resonances
that were observed were not saturated, because their relative
intensities did not change on decreasing the pulse length from
90° to 30° or increasing the recycle time from 332 to 664 msec.
We conclude that intact cell proton-decoupled $^{15}$N NMR
spectra obtained with fast repetition rates (<1 sec) will be
dominated by the resonances of cellular structural components
associated with the cell wall, rather than by free metabolites.

**B. licheniformis.** In Gram-positive bacteria, the protein- and
phospholipid-rich outer membrane of the cell envelope is re-
placed by a thicker cell wall consisting of peptidoglycan, te-
ichoic acid, and teichuronic acid. The different chemical
composition of the Gram-positive cell wall is amply demonstrated
by the proton-decoupled $^{15}$N NMR spectrum of the
Gram-positive **B. licheniformis** cells (Fig. 2B). The phospha-
tidylylthanolamine resonance is absent. The Lys resonance is
weak and broadened. The Arg1 and Arg2 resonances are also
weak and vary in intensity among different batches of cells.
However, the spectrum displays a relatively intense set of res-
ONAs (AM4, P5, and P9) that originate from cell wall com-
ponents. The P5 resonance is assigned to the acetamido nitrogen
of cell N-acetylglucosamine, N-acetylmuramic acid, and
N-acetylgalactosamine residues of cell wall peptidoglycan and
techuronic acid, because (i) one of the two $^{15}$N resonances
observed in the proton-decoupled natural-abundance $^{15}$N
spectrum of N-acetylglucosamine in aqueous solution corre-
sponds to P5, (ii) cells grown in the presence of nonlabeled
N-acetylglucosamine had a P5 resonance that was significantly
reduced in intensity, and (iii) the P5, P9, and AM4 resonances
are found in the $^{15}$N NMR spectra of the lysozyme digests of
**B. licheniformis** cell wall particles freed of proteins and teichoic
and teichuronic acids.

The assignment of the intact cell $^{15}$N resonances to cell wall
structural components rather than to labile metabolite mol-
ecules is supported by the fact that (i) the intact cell and isolated
cell wall spectra are identical, (ii) two consecutive intact cell
spectra, obtained over the course of 24 hr, did not differ, and
(iii) the spectra of cells harvested in mid-logarithmic phase, late
logarithmic phase, and 4 hr into the stationary phase did not
differ significantly. The observed intact cell resonances were
not partially saturated, because decreasing the pulse angle from
90° to 30° did not change the relative amplitudes of the reson-
ances.

Increasing the temperature of the cell sample to 47° pro-
duced dramatic changes in the **B. licheniformis** cell $^{15}$N spec-
trum, which reflect the thermal denaturation of proteins and
the unfolding of ribonucleic acids. The $^{15}$N NMR spectrum of
**B. licheniformis** cells at 47° (Fig. 3) displays (i) relatively in-
tense Arg1 and Arg2 resonances, (ii) a noninverted histidine-
N$_\alpha$ nitrogen resonance (His) (22), and (iii) a set of ribonucleic
acid resonances (G1, A1, C1, U1, U2, C2, A2, and G3) (14), in
addition to the Lys, AM4, P5, and P9 resonances that were
observed at 27°.

**Baker’s Yeast.** The yeast cell wall consists of proteins and
nitrogen-free polysaccharides (mannans), as compared to the

**RESULTS**

**E. coli.** The proton-nondecoupled $^{15}$N NMR spectrum (Fig.
1A) of $^{15}$N-enriched intact **E. coli** cells consists of a lysine-N,
resonance, a protein backbone amide envelope of resonances
(BB), and an unidentified amino resonance (AM4). The as-
signment of these resonances to globular cellular proteins is
consistent with the similarity of the spectrum to the proton-
nondecoupled spectrum of nonspecifically labeled $^{15}$N-hem-
oglobin (20, 21).

The proton-decoupled $^{15}$N spectrum of **E. coli** cells displays
narrow and inverted resonances originating from protein
side-chain lysine-N$_\alpha$, arginine-N$_\alpha$, and arginine-N$_\gamma$ nitrogen
(Lys, Arg2, and Arg1, respectively), phosphatidylethanolamine
nitrogen (PE), and unidentified amino (AM4) and amide (PP4
and PP7) resonances. The envelope of protein backbone amide
resonances (BB) has been nulled by proton broad band noise
decoupling.

The Lys, Arg1, and Arg2 resonances were assigned by
comparison to natural abundance $^{15}$N NMR spectra of free

![Diagram](https://example.com/diagram.png)
nitrogen-rich cell wall components of bacteria. The proton-decoupled $^{15}$N NMR spectrum of baker’s yeast cells (Fig. 2C) is markedly different from the spectra of bacterial cells. It displays relatively intense protein side-chain group resonances (Lys, Arg1, and Arg2). In place of the narrow peptidoglycan resonance (P5) a broad envelope of peptide resonances (PP1) is observed. Amino resonances (AM3 and AM8), and an unusual noninverted amine resonance (AM9) were observed, but not identified. These resonances apparently originated from the cell wall, because the intact cell spectrum was identical to the $^{15}$N spectrum of cell walls prepared by freeze-thaw osmotic shock (4). Interestingly, the yeast proteins and ribonucleic acids did not undergo thermal denaturation at 47°C, as had occurred in B. licheniformis cells.

**Friend Leukemic Cells.** The proton-decoupled $^{15}$N spectrum of Friend leukemic cells (Fig. 2D) is relatively simple and consists of Lys resonance and an envelope of peptide resonances (PP2). Because these cells were grown on a medium containing nonlabeled arginine, the Arg1 and Arg2 resonances have not been observed. Trypan-blue staining of cells upon completion of the NMR measurements indicated that about 75% of the cells remained viable during 5 hr of spectrum accumulation at 27°C. The simplicity of the spectrum of Friend leukemic cells is consistent with the absence in the mammalian cell of a cell wall, which appears to be the source of many of the resonances observed in the $^{15}$N spectra of microorganisms.

**Selectively Labeled S. aureus Cells.** The $^{15}$N NMR spectrum of S. aureus cells that have been specifically labeled with $^{15}$N-glycine (Fig. 4A) displays a single intense resonance at 267.8 ppm. This resonance is assigned to the cell wall peptidoglycan pentaglycine cross-bridge with a random coil conformation, because (i) an identical resonance is obtained from the isolated cell wall (Fig. 4B), (ii) glycine is the major amino acid component of S. aureus peptidoglycan and forms a pentaglycine cross-bridge, and (iii) the 267.8 ppm chemical shift is identical to that of the middle glycyl residue of N-acetyltetraglycine in aqueous solution and the major resonance of unfolded [glycyl-$^{15}$N]hemoglobin, both of which have random coil, water-solvated conformations (21). Glycyl residues in other conformations would have different chemical shifts, as demonstrated by native [glycyl-$^{15}$N]hemoglobin, whose $^{15}$N chemical shifts range from 256 to 276 ppm (21).

**DISCUSSION**

The Scope of Whole Cell $^{15}$N Spectra. The applicability of $^{15}$N NMR spectroscopy to problems in cell biology ultimately depends on whether the $^{15}$N resonances of a particular nitrogen-containing group can be detected in an intact cell and can be resolved from the resonances of similar nitrogen groups in different types of molecules and from similar molecules in different cellular compartments. The $^{15}$N NMR spectra of intact cells might be expected to display resonances originating from polypeptides and their nitrogenous side chain groups, purines and pyrimidines, amino- and acetamidosugars, phospholipids, and various small nitrogenous metabolite molecules. The intensities of the $^{15}$N resonances of these molecules are determined primarily by their linewidths and $^{15}$N-$^{1}$H nuclear Overhauser effects. These NMR parameters are related to the dynamic structure of the cell, and to a first approximation can be related to a single correlation time ($\tau_c$) which describes isotropic tumbling or segmental motion.

The correlation time ($\tau_c$) dependence of line broadening by $^{15}$N-$^{1}$H dipolar interaction for an $\text{N-H}$ group indicates that the $^{15}$N resonances of molecules with $\tau_c > 100 \text{ nsec}$ will be very broad (>200 Hz), and usually will not be observable. The effect of correlation time on the $^{15}$N-$^{1}$H nuclear Overhauser effect (NOE = proton-decoupled intensity/proton-nondecoupled intensity) indicates that three types of $^{15}$N resonances are expected in proton broad band decoupled spectra of intact cells: (i) resonances that are enhanced and inverted (NOE approximately ~4) corresponding to molecules with $\tau_c < 1 \text{ nsec}$; (ii) resonances of molecules with $\tau_c$ about 5 nsec that are nulled and made unobservable (NOE approximately 0) by proton decoupling; and (iii) resonances that are only slightly attenuated (NOE about 0.9) originating from molecules with $\tau_c > 10 \text{ nsec}$. A fourth possibility is a narrow proton-nondecoupled resonance that is nulled or remains uninverted on proton decoupling, and
that corresponds to relatively mobile molecules coordinated to or in close proximity to paramagnetic ions (23, 24).

Among the components of the cell protoplast, only the proteins have produced observable $^{15}$N NMR resonances. These resonances are nulled on proton decoupling. The nulling of the protein backbone amide $^{15}$N resonances in whole cells could be the result of either all cellular proteins having $\tau_e = \text{approximately 5 nsec and NOE about 0}$ or the averaging out of inverted resonances of fast tumbling proteins with the non-inverted resonances of slower tumbling proteins. The $^{15}$N NMR resonances of free small nitrogenous molecules have either been saturated or their NOEs have been nulled by the presence of trace amounts of paramagnetic ions. The use of large samples of highly $^{15}$N-enriched cells in wide-gap spectrometers will apparently be required to offset the loss of signal-to-noise ratio associated with longer recycle times required to avoid saturation effects. The absence of purine and pyrimidine resonances from intact cell spectra at 27°C indicates that little segmental motion occurs in the tightly hydrogen-bonded, slowly tumbling nucleic acids. Cellular dynamic structure makes the cell protoplast essentially transparent in $^{15}$N-$[^1$H] nuclear Overhauser enhanced $^{15}$N NMR measurements. This restricts $^{15}$N NMR observations of intact cells to cell wall components.

Cell Wall Dynamic Structure In Vivo. The three-dimensional models proposed for cell wall peptidoglycan (25–29) consist of rigid, extensively hydrogen-bonded mosaics of peptidoglycan subunits, while the x-ray diffraction patterns of B. licheniformis and S. aureus cell walls (30) as well as the elastic behavior of bacterial cell walls (31) have indicated that peptidoglycan behaves as a randomly crosslinked polymer with much free rotation. The random-coil conformation of a large percentage of the pentaglycine cross-bridges of S. aureus cell wall peptidoglycan is more consistent with a mobile conformation than with the extensively hydrogen-bonded $\beta$-pleated sheet conformation used in model-building studies (25). The observation of peptidoglycan resonances in the cell walls of intact cells is consistent with the existence of considerable segmental motion, oscillations, conformational jumps, and internal rotations within the native peptidoglycan matrix. Because changes in morphology and tensile strength of the cell wall will most probably affect peptidoglycan $\tau_e$ and the relative intensities of its $^{15}$N-$[^1$H] NOE enhanced $^{15}$N resonances, $^{15}$N NMR promises to be a useful probe of the physiological state of the bacterial cell wall.

Thanks are due to Mrs. Hannah Bayer for able technical assistance and to the Biological Service Unit of the Weizmann Institute for assisting in growth of bacteria. We wish to thank Prof. Charlotte Friend for Friend leukemic cell clone 707. This research was supported in part by National Institutes of Health Grant HL14687-03.

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![FIG. 3. The proton-decoupled 9.12 MHz $^{15}$N NMR spectrum of $^{15}$N-enriched B. licheniformis cells at 47°C. Spectral conditions were as in Materials and Methods. Resonance frequencies (ppm): G1, A1, C1, 177; U1, 218.3; U2, 229.0; C2, 282.0; A2, 297.9; G3, 303.2; His, 207.7; Arg1, 291.2; Arg2, 304.4; Lys, 343.0; AM4, 336.6.](image1)

![FIG. 4. The proton-decoupled 9.12 MHz $^{15}$N NMR spectra at 27°C of S. aureus specifically labeled with $[^1$N]glycine: (A) intact cells, (B) cell walls. Spectral conditions were as in Materials and Methods.](image2)