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THE DYNAMIC STRUCTURE OF THE *ESCHERICHIA COLI* CELL ENVELOPE AS PROBED BY ^{15}N NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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Summary

Proton decoupled ^{15}N NMR spectroscopy is shown to be a useful tool for probing the dynamic structure of the bacterial cell envelope. The proton decoupled ^{15}N NMR spectra of *Escherichia coli* whole cells, cell envelopes and outer membranes were obtained and displayed resonances originating from protein side-chain groups, phosphatidylethanolamine, and peptidoglycan. Removal of phospholipids from the cell envelope resulted in a decrease in the motional freedom of peptidoglycan and cell envelope proteins. The mobility of the protein Arg side-chain groups is increased in the absence of peptidoglycan. These data provide insights into the effect of supramolecular organization on the dynamic structure of the *E. coli* cell envelope.

Introduction

The origin of the cylindrical shape of the cell wall of rod-shaped bacteria is obscure [1]. It is not directly related to the chemical composition of the peptidoglycan layer in either gram-positive or gram-negative bacteria [2,3]. Non-covalent bonding, electrostatic and hydrophobic interactions between peptidoglycan subunits and accessory molecules and other cell wall components might contribute to the shape and tensile strength of the cell wall. However, this hypothesis has not been explored due to the lack of physico-chemical information on the bacterial cell envelope. Density measurements, [4] electron microscopy, [5] X-ray diffraction, [6] infra-red spectroscopy [4] and model-building studies [4,7—10] of peptidoglycan films have not provided many insights into the in vivo nature of the polymer. Infrared spectroscopic measurements have provided information on the overall structure of proteins in dried films of *Escherichia coli* cell envelope components [11,12].

Nuclear Overhauser-enhanced nitrogen-15 nuclear magnetic resonance

spectroscopy is a useful tool for probing the physical properties of molecules in complex biological systems [13]. The linewidths and ^{15}N-^{1}H Nuclear Overhauser effect * of ^{15}N NMR resonances can provide information on the dynamic structure of cell components. In this communication we report that ^{15}N NMR spectroscopy has been used to follow changes that occur in the mobility of *E. coli* cell envelope proteins, phospholipids and peptidoglycan upon the removal of various cell envelope components.

Methods

[^{15}N]Ammonium chloride enriched to 90—95% ^{15}N, was prepared by standard methods [14] from [^{15}N]nitric acid (obtained from the Isotope Separation Plant of the Weizmann Institute).

E. coli B/r was grown at 37° C with aeration to the early stationary phase (*A* 0.55, 680 nm) in Spizizen salts medium [15], in which 0.2% $(NH_4)_2SO_4$ was replaced by 0.1% [^{15}N]NH_4Cl. The *E. coli* were harvested by centrifugation in the cold (6000 × *g*, 10 min) and washed three times with 100 ml distilled water. To prepare cell envelopes *E. coli* (50—100 mg/ml in 20 ml of distilled water) were disrupted in a Braun Tissue homogenizer by shaking with 20 ml of glass beads at maximum speed for 3 min at 4° C. The cell envelopes were separated from the glass beads by decanting, centrifuged (30 000 × *g*, 5 min) and washed three times with distilled water. An *E. coli* membrane fraction was prepared from lysozyme-EDTA spheroplasts by the method of Mizushima and Yamada [16] and corresponded to their Prepration II. Phospholipids were removed from *E. coli* envelope by the method of Henning, Hohn and Sonntag [17]. Lipopolysaccharide (0.8 g) was obtained from *E. coli* cells (30 g) by the phenol/water extraction procedure of Nakamura and Mizushima [18]. Samples for NMR measurements were prepared by packing the cell or cell envelope preparation in a 10 mm sample tube by centrifugation (10 000 × *g*, 1 h).

^{15}N NMR spectra were obtained on a Bruker HFX-90 spectrometer operating at 9.12 MHz. Field stabilization was achieved by locking on the deuterium signal of $^{2}H_2O$ contained in the 5 mm concentric sample tube. ^{15}N chemical shifts were determined from an external reference of 2 M $^{15}NH_4Cl$ in 2 M HCl. However, the spectra and chemical shifts are presented in terms of the $H^{15}NO_3$ chemical shift scale of Roberts [19], using a conversion factor δ_{HNO_3} of NH_4Cl = −352.7 ppm.

Results

The proton decoupled ^{15}N NMR spectrum of intact *E. coli* cells (Fig. 1A) displays resonances at 258.5, 264.2, 291.2, 304.4, 335.6, 343.0 and 350.0 ppm. The resonances are relatively intense and narrow (<20 Hz) and are inverted, as a result of a large negative Nuclear Overhauser effect, which indicates that they originate from relatively mobile groups [13]. Assignments of these resonances can be made by comparison of chemical shifts to those of standard compounds and model systems, as well as by measurements on

* Nuclear Overhauser effect = (proton decoupled intensity)/(proton non-decoupled intensity).

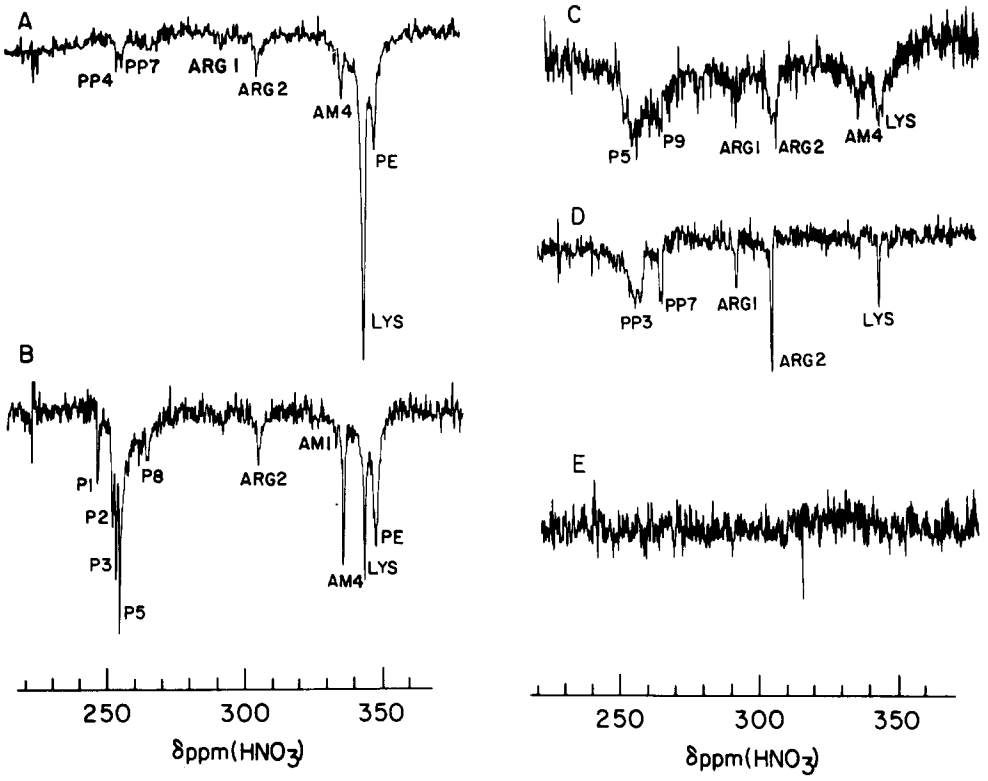

Fig. 1. The ^{15}N NMR spectra of ^{15}N-labelled *E. coli*. A, whole cells; B, cell envelopes; C, cell envelope minus phospholipids; D, the fraction of cell envelope minus phospholipids soluble in 4% SDS at 100°C; E, lipopolysaccharides. Spectral conditions: 90° pulse angle; 0.672 s repetition rate; 50 000 accumulations; 3000 Hz spectrum range; ±0.08 ppm resolution; 0.9 Hz exponential decay filter; 27°C. Resonance frequencies (ppm): Proteins, PP3 (255.0), PP4 (258.5), PP7 (264.2), Arg1 (291.2), Arg2 (304.4), Lys (343.0); Peptidoglycan, P1 (245.5), P2 (250.5), P3 (251.3), P5 (253.4), P8 (263.4), AM4 (335.6); Phospholipids, phosphotidylethanol (350.0).

isolated cell fractions. Comparison of chemical shifts to natural abundance ^{15}N NMR resonances of nitrogenous amino acid side-chain groups, as well as the published chemical shifts [19], indicated that the 291.2, 304.4 and 343.0 ppm resonances originate from Arg-N_ϵ, Arg-$N_{\omega,\omega'}$ and Lys-N_ω nitrogens of cellular proteins. The 255.0 and 258.5 ppm resonances occur in the region of polypeptide amide groups [13,19]. The 335.6 and 350.0 ppm resonances apparently originate from the cell envelope, since in the proton decoupled ^{15}N NMR spectra of *E. coli* cell envelopes (Fig. 1B) their intensities increase more than 2-fold compared to protein side-chain group resonances. The 350.0 ppm resonance disappears from the cell envelope spectrum when phospholipids are removed from the cell envelope by chloroform/methanol extraction. The 350.0 ppm is assigned to the ammonium group of phosphatidylethanol, since phosphatidylethanol is the major nitrogenous phospholipid present in *E. coli* cell envelopes [20]. The 335.6 ppm resonance is absent from the spectrum of the cell envelope solubilized in 4% sodium dodecyl sulphate (SDS) (Fig. 1D) which contains all the cell wall components with the exception of the peptidoglycan

rigid layer. The 335.6 ppm resonance is also absent from the spectrum of the peptidoglycan fractions of gram-positive *Bacillus licheniformis* cell walls [22]. The 335.6 ppm resonance is tentatively assigned to the meso-diamino pimelic acid amino groups of non-crosslinked peptidoglycan groups in the cell wall rigid layer.

Cell envelope fractionation experiments also provide information on the relationship between cell wall supramolecular structure and the ^{15}N NMR parameters of cell wall components. The proton decoupled ^{15}N NMR spectrum of *E. coli* cell envelopes display a set of relatively intense resonances at 245.5, 250.5, 251.3, 253.4 and 263.4 ppm, which are absent from the spectrum of whole cells (Fig. 1A) and peptidoglycan-free SDS soluble fraction of the cell envelope (Fig. 1D) and the cell envelope outer membrane. These resonances occur at exactly the same positions as those of the lysozyme digests of gram-positive cell walls [22]. These resonances are assigned to peptidoglycan amide groups. It is interesting to note that removal of phospholipids from the *E. coli* cell envelopes (Fig. 1C) led to a broadening and loss of intensity of the peptidoglycan amide and amino resonances, as well as of protein side-chain group resonances. Another interesting aspect of these spectra is that in the absence of the peptidoglycan rigid layer (Figs. 1D and 2), the cell envelope proteins display narrow and intense Arg-N_{ϵ} and Arg-$N_{\omega,\omega'}$ resonances, as well as an envelope of protein amide resonances at about 255.0 ppm, which resembles those of mobile proteins with respect to band shape and maxima [13].

Although *E. coli* cell envelope lipopolysaccharide layer contains ethanolamine, glucosamine and *N*-acetylglucosamine groups [23], no resonances characteristic of these groups were observed in the spectrum of the cell outer membrane. No resonances were observed in the proton-decoupled ^{15}N NMR spectrum of a sample of *E. coli* lipopolysaccharide (Fig. 1E).

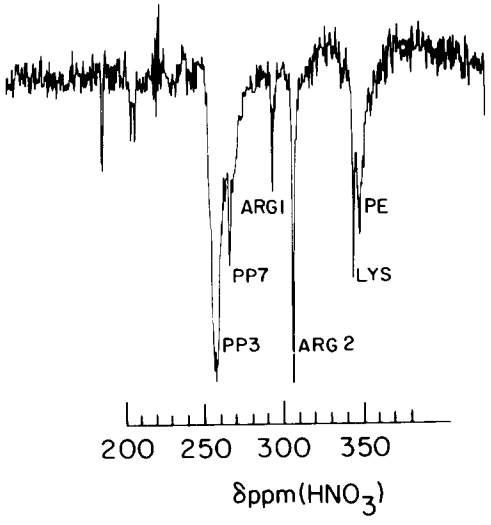

Fig. 2. The ^{15}N NMR spectra of the outer membrane of ^{15}N-labelled *E. coli*. Spectral conditions: 90° pulse angle; 0.336 s repetition rate; 80 000 accumulations; 6000 Hz spectrum range; +0.16 ppm resolution; 1.8 Hz exponential decay filter; 27°C. Resonance frequencies as in Fig. 1.

Discussion

The ^{15}N NMR spectra of *E. coli* whole cells and cell envelopes are much simpler and better resolved than the ^{1}H and ^{13}C NMR spectra of whole cells, cell walls and cell membranes [24,25,26,27]. The technique is therefore very suitable to the study of *E. coli* cell envelope components.

The ^{15}N-^{1}H Nuclear Overhauser effect of a ^{15}N resonance increases from −3.9 to 0.9 as the correlation time of a molecule increases from 0.1 ns to 100 ns (Fig. 3) at 9.12 MHz. The increase in the intensity of an inverted ^{15}N resonances corresponds to an increase in the motional freedom of that molecular group. The linewidths ($\Delta\nu_{1/2}$) of the ^{15}N resonances of N-H groups with a bond length of 0.8 Å rapidly increase as the correlation time of the molecule exceeds 5 ns (Fig. 3). The relative intensities of proton decoupled ^{15}N resonances can be used as mobility probes. The dependence of the motional freedom of the *E. coli* cell envelope components on the structural organization of the cell envelope can be observed as changes in the intensities and linewidths during cell envelope fractionation.

The appearance of a set of relatively narrow and inverted peptidoglycan ^{15}N resonances in the cell envelope, but not in the whole cell, indicates that motional freedom of peptidoglycan has increased during the preparation of the cell envelope. The increase in the mobility of peptidoglycan might arise from (1) the action of autolysins released on cell disruption, (2) reduction of mechanical stress, resulting from cell tugor on the envelope and (3) the separation of the rigid layer from the cytoplasmic membrane [28].

The reduction in mobility of the cell envelope peptidoglycan and protein components upon removal of the phospholipids provides a clear indication that

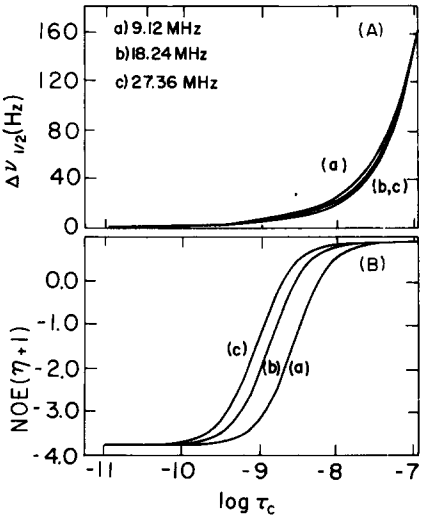

Fig. 3. A , the effect of correlation time (τ_c) on half-height linewidth ($\Delta\nu_{1/2}$) of dipolar broadened ^{15}N resonance of an N-H group with r = 0.80; B, the effect of correlation time (τ_c) on the ^{15}N-^{1}H Nuclear Overhauser effect as calculated from Eqns. 19, 37 of ref. 29 and Eqn. 2 of ref. 30.

one of the functions of phospholipids is to promote dynamic motions in the cell envelope. The changes in the protein side chain group resonances reflects either denaturation or aggregation of cell wall proteins in the absence of phospholipids. Changes in peptidoglycan resonances could arise from either the direct electrostatic interaction of peptidoglycan with phospholipids or the interaction with the cell wall proteins that have aggregated or undergone denaturation.

The narrowing of the Arg side-chain group resonances and the appearance of an inverted envelope of protein backbone resonances in the outer membrane and SDS soluble envelope fractions are indicative of an increase in the population of proteins with unfolded, mobile conformations. One possible explanation for the increase in the fraction of unfolded proteins in the outer membrane would be an immobilizing interaction between the outer membrane proteins and the rigid layer, which is disrupted during the preparation of the outer membrane.

These ^{15}N NMR spectra of whole cells and cell envelope fractions have demonstrated that the degree of motional freedom found in cell envelope fractions are not necessarily those that exist in the intact cell.

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