Solid-state NMR Studies of *Klebsiella pneumoniae* Grown under Nitrogen-fixing Conditions*

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The carbon and nitrogen metabolism of *Klebsiella pneumoniae* M5a1 has been characterized using 13C and 15N labeling with detection by cross-polarization magic-angle spinning solid-state NMR. Cells grown on ammonium typically require some 20 h to derepress fully for nitrogenase when transferred to medium devoid of any source of fixed nitrogen. We have established that during this period some cellular proteins are catabolized with the liberated nitrogen being used for the synthesis of purines needed for formation of ribosomal RNA. The 20-h derepression period can be shortened to 6 h by the introduction of fixed nitrogen in certain specific forms. Serine is the most successful agent we have examined for shortening the derepression period and glycine among the least successful. We attribute this difference to the advantage of serine over glycine in providing both specific and nonspecific carbon and nitrogen sources for complete purine synthesis. These determinations were made by tracing the metabolism of 13C- and 15N-labeled chemical bonds from the 2 amino acids during derepression.

Lindsay (1) observed that the 15-20 h diauxic lag period for growth of *Klebsiella pneumoniae* on N2 following exhaustion of supplied fixed nitrogen such as ammonium, was considerably shortened by the addition of certain amino acids. Brill and co-workers (2, 3) subsequently made use of this fact in their studies of the regulation and genetics of nitrogenase and developed a protocol which involved the addition of 0.5 mM L-serine to the growth medium. This reduced the derepression period to 6–8 h.

The role serine plays in hastening derepression has never been totally understood. It might be simply to alleviate insufficient intracellular serine needed for synthesis of nitrogenase during derepression. However, serine is present in only an average amount within nitrogenase (4). In addition, some amino acids more difficult for the organism to synthesize than serine have no comparable influence on shortening the time of derepression (1).

In the early stages of derepression, cells of *K. pneumoniae* are nitrogen starved and under metabolic stress (5). The effect of serine, and other amino acids able to shorten the derepression period, might be interpreted in terms of relative uptake and utilization of serine as a general source of nitrogen during the stress period. This does not explain, however, why serine is more effective than, say, aspartate and glutamate (see Miniprint Supplement), both of which are taken up by the cell and are excellent sources of nitrogen via transamination.

To establish the role that serine plays in the derepression of nitrogenase, we have performed solid-state 13C and 15N NMR experiments on intact lyophilized solid samples of *K. pneumoniae*, grown under N2 fixing and nonfixing conditions, in the presence of both specific and nonspecific 13C and 15N labels. The resulting spectra are sufficiently detailed that the total flow of carbon and nitrogen from serine into purines and proteins can be established. These results lead to the conclusion that serine's advantage over other amino acids in shortening derepression lies in its suitability for supplying both carbon and nitrogen for the purines needed for ribosomal RNA production preceding nitrogenase synthesis.

**MATERIALS AND METHODS**

Chemicals—L-[2-13C,15N]Serine (90 atom % 13C; 95 atom % 15N), L-[3-13C]serine (99.7 atom % 13C), [2-13C,15N]glycine (92 atom % 13C; 99 atom % 15N), and [15N]ammonium acetate (96 atom % 15N) were obtained from Merck Stable Isotopes, Montreal, Canada; and [15N]nitrogen gas (95 atom % 15N) from Monsanto Research Co., Mound Facility, Miamisburg, OH.

Culture Methods—*K. pneumoniae* strain M5a1 was a gift from Dr. Winston Brill (formerly at the University of Wisconsin, now at Agracetus Co.). The medium described by Yoch and Pengra (6) was used as the basal medium and was supplemented by 1 mg/ml ammonium acetate for growth of cells under nonnitrogen fixing conditions.

Derepression experiments were performed by the procedure of Brill et al. (2). These experiments involved growth of cells under nonnitrogen fixing conditions, followed by suspension of the cells in a nitrogen-free medium in an open-atmosphere fermenter continuously sparged with natural-abundance nitrogen gas. After a 1.5-h incubation to ensure that trace ammonium was depleted from the medium, L-serine (0.5 mM) or other amino acids were added and the lag times measured for derepression of nitrogenase and onset of cellular growth.

Cells to be used for solid-state NMR analysis were harvested as a function of time after addition of amino acid, washed with 0.025 M potassium phosphate, pH 7.0, centrifuged, frozen in liquid nitrogen, and lyophilized.

**Preparation of Crude Extracts and Assay of Nitrogenase Activity**—Crude extracts were made following the procedure of Brill et al. (2). Nitrogenase activity of crude extracts was monitored using an acetylethylene reduction assay (7). Specific activity of nitrogenase was defined as micromoles of ethylene formed per min per mg of protein. Protein concentration was determined by a microbiuret method with bovine serum albumin as the standard (8).

**Magic-Angle Spinning NMR**—15N NMR spectra were obtained at 20.3 MHz using matched spin-lock cross-polarization transfers with 2- or 5-ms contacts and 35 kHz H1's (9). The chemical shift scale is in parts per million downfield from external solid ammonium sulfate. The dried samples were contained in a cylindrical double-bearing rotor spinning at 3.2 kHz. A few spectra were obtained at 9.07 MHz with a single-bearing rotor spinning at 1.6 kHz. Technical details of the spinning and cross-polarization procedures are reported elsewhere (10, 11). Fast cross-polarization rates for protonated nitrogens, long
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RESULTS AND DISCUSSION

Nitrogen from Proteins—The CPMAS\(_{9.07\text{ MHz}}\) \(^{15}\text{N}\) NMR spectrum of uniformly \(^{15}\text{N}\)-labeled intact K. pneumoniae is shown in Fig. 1 (left). The bacterium was grown in the presence of \(^{15}\text{NH}_3\) and is essentially 100\% enriched in \(^{15}\text{N}\). The resulting spectrum (obtained under low-speed spinning conditions which produce the mechanical spinning sidebands) exhibited peaks at the extremes of the spectrum showing label in purine and peptide nitrogen, as well as protein side chain nitrogen in histidyl, arginyl, and lysyl residues (17, 18). Cells produced in this manner were then transferred into fresh nitrogen-free medium contained within a closed-atmosphere fermenter built for long term \(^{15}\text{N}_2\) labeling experiments (see Miniprint Supplement for details). The cells were deprived for nitrogenase under an anaerobic atmosphere composed of 30\% \(^{14}\text{N}_2\) and 70\% helium, with the addition of 0.5 mM L-serine 1.5 h after transfer. Under these conditions, cells harvested at the end of one doubling period contain only half as much \(^{15}\text{N}\) on a per mg cellular dry weight basis as the starting cells. Thus the \(^{15}\text{N}\) NMR spectrum of a sample of final cells with twice the weight of a sample of starting cells permits direct comparisons to be made (Fig. 1, left and middle). We observe a diminution in the peptide-peak intensity, along with a corresponding increase in purine-peak intensity for the final cells relative to those of the starting cells. The complementary experiment, using an \(^{14}\text{N}\) inoculum and performing \(^{15}\text{N}_2\) fixation within the closed-atmosphere fermenter, leads to an \(^{15}\text{N}\) NMR spectrum which shows a smaller purine signal than that observed under uniform labeling conditions (Fig. 1, left and right).

We interpret the above results as evidence of the need for enhanced ribosomal RNA synthesis by K. pneumoniae during derepression of nitrogenase. With limited fixed nitrogen available from the medium, this synthesis depends on relatively slow catabolism of existing proteins. Not only must the cells synthesize ribosomal RNA for the nitrogenase needed to fix nitrogen and restore depleted nitrogen pools; they must also increase ribosome production to begin rapid growth (19, 20). By the time nitrogen fixation begins to provide an appreciable source of nitrogen, a smaller percentage of newly fixed nitrogen is needed for additional purine synthesis than at earlier times in the adaptive period because existing proteins have been utilized for this purpose.

Nitrogen from Serine and Glycine—The \(^{15}\text{N}\) NMR spectrum of K. pneumoniae derepressed for 3 h in the presence of \(\text{l-[\(^{13}\text{C},^{15}\text{N}\)]serine (Fig. 2, bottom left)} appears much like that from the non-specific label (Fig. 1, left). This shows that most serine nitrogen is scrambled and used as a general source of nitrogen. However, the appearance of two DCPMAS \(^{15}\text{N}\) difference peaks (Fig. 2, top left) indicates that some serine is used for products which retain the original labeled \(^{13}\text{C},^{15}\text{N}\) chemical bond of serine. (Details of the DCP experiment and its interpretation may be found in the Miniprint Supplement.) Based on a previous \(^{15}\text{N}\) NMR study of glycine metabolism (21), we assign the 220-ppm DCP signal to the N(7)-C(5) bond in...
and N-9 are derived from the general nitrogen pool. Serine protein directly, or it can be used to produce purines after purine ring position. The observed DCP signals show that tase, releasing labeled ammonium. The weak DCP signals in which is known generally to be a necessary requirement for purines, and the 90-ppm signal to a(C)-amide-N bonds of the glycine transported into the cell is directly incorporated into either proteins or purines, giving rise to strong DCP spectrum of the cells, as well as 13C due to uptake and metabolism of the labeled serine. The difference spectrum, generated by subtracting the natural-abundance background from the labeled-serine spectrum, is considerably simpler (Fig. 3, top left). This indicates specific incorporation of label. Line assignments can be made using known chemical shifts (21). Label from the C-2 carbon of serine ends up primarily in three sites. Labeled serine is incorporated directly into proteins (62 ppm), or is converted first to glycine, with label then appearing in glycyl residues of proteins (45 ppm) and in purines (119 ppm) synthesized from glycine (see Miniprint Supplement, Fig. S6). A minor negative-going peak appears in the difference spectrum near 80 ppm; we attribute this peak to a slightly different level of carbohydrates in the natural-abundance control sample compared to the labeled analytical sample.

The corresponding difference spectrum of K. pneumoniae grown on L-[3-13C]serine shows a totally different specific labeling pattern (Fig. 3, top right). The labeling patterns for the above two labeled-serine experiments are consistent with seryl and glycylic fragment insertions into proteins and purines. Conversion of endogenous serine to glycine involves transfer of the C-3 carbon of serine into the tetrahydrofolate cofactor system, where it is routed into a number of metabolites (Fig. 3, top right; see Miniprint Supplement, Fig. S6, for descriptive pathway).

We claim that the combination of metabolic pathways available to serine is the explanation for serine's effectiveness in shortening derepression time (Miniprint Supplement, Table I) because complete purine synthesis still depends on a ready source of glycine. Glycine itself does not shorten the derepression time because glycine is not an adequate source of general nitrogen.

**Fig. 2.** CPMAS 20.3-MHz 15N NMR spectra of intact lyophilized cells of K. pneumoniae grown under nonnitrogen fixing conditions and then derepressed for 3 h in media sparged with 14N2 and containing either L-[2-15N]serine (bottom left) or natural-abundance serine plus [2-15N, 14N]glycine (bottom right). The vertical display on the right has been increased by a factor of 3 relative to that on the left. The spectra at the bottom of the figure were obtained using the pulse sequence of Fig. S4 (Miniprint Supplement) with a proton-nitrogen Hartmann-Hahn match of 2 ms, a nitrogen spin lock of 3 ms, and the carbon radiofrequency field off resonance. The spectra at the top of the figure are the double cross-polarization difference spectra generated using the pulse sequence of Fig. S4 (Miniprint Supplement) with the carbon radiofrequency field alternatively off and on resonance.

The difference spectrum, generated by subtracting the natural-abundance spectrum of the labeled-serine spectrum, is considerably simpler (Fig. 3, top right). This indicates specific incorporation of label. Line assignments can be made using known chemical shifts (21). Label from the C-2 carbon of serine ends up primarily in three sites. Labeled serine is incorporated directly into proteins (62 ppm), or is converted first to glycine, with label then appearing in glycyl residues of proteins (45 ppm) and in purines (119 ppm) synthesized from glycine (see Miniprint Supplement, Fig. S6). A minor negative-going peak appears in the difference spectrum near 80 ppm; we attribute this peak to a slightly different level of carbohydrates in the natural-abundance control sample compared to the labeled analytical sample.

**Fig. 3.** CPMAS 50.3-MHz 13C NMR spectra of intact lyophilized cells of K. pneumoniae derepressed for 3 h in media sparged with 14N2 and containing either L-[2-15C, 14N]serine (bottom left) or L-[3-13C]serine (bottom right). The difference spectra shown at the top of the figure were generated by subtracting an appropriately scaled natural-abundance 13C NMR spectrum.
FIG. 4. CPMAS 50.3-MHz 13C NMR spectra of intact lyophilized cells of K. pneumoniae derepressed for 3 h in media sparged with 14N2 and containing L-[2-13C,15N]serine (left), L-[2-13C,15N]serine plus unlabeled ammonium (middle), or L-[2-13C,15N]serine plus unlabeled glycine (right). The DCP difference spectra shown at the top of the figure arise from those carbons which are directly bonded to 15N's.

The use of the carbons of serine for both specific and general synthesis is fully consistent with the analysis of the nitrogen metabolism of serine discussed in the previous section.

Carbon from Serine: Double Cross-Polarization Spectra—The DCPMAS 13C NMR spectra of K. pneumoniae grown on L-[2-13C,15N]serine (Fig. 4) support our analysis of the difference spectra. In the DCP experiment only those carbons directly bonded to 15N's generate a signal. The three 13C DCP signals are assigned to labeled seryl and glycyl residues of proteins and to purines synthesized from labeled glycine. The observed DCP signals are therefore consistent with intact incorporation of labeled bonds into purines and proteins.

In addition, a minor DCP peak is observed in the carbonyl region (Fig. 4, top left). However, this particular signal is not due to incorporation of an intact labeled chemical bond, but rather to scrambled carbon and nitrogen levels forming a labeled bond within newly synthesized proteins of the cell. Since the general nitrogen pool is small during derepression, it becomes virtually 100% 15N enriched as a result of the active nitrogen scrambling of serine, established earlier. Thus, single-carbon 13C incorporations can produce 13C-15N bonds. We confirm this interpretation of the carbonyl-carbon DCP peak by observation of a marked diminution in the peak's intensity in the spectrum of K. pneumoniae grown in the presence of L-[2-13C,15N]serine plus natural-abundance ammonium (Fig. 4, top center). The remaining three DCP peaks retain most of their intensity under these conditions, consistent with labeled bond incorporations.

The DCPMAS 13C NMR spectra of K. pneumoniae derepressed for 3 h in the presence of L-[2-13C,15N]serine plus natural-abundance glycine (Fig. 4, top right) characterizes the changes that can occur in carbon routing by an alteration in the levels of intermediates. For instance, in the presence of exogenous glycine, cells transport enough glycine to activate a glycine-cleavage reaction pathway (22, 23). Double-labeled serine is used sparingly as a precursor for glycyl residue insertions into proteins (Fig. 4, top right, 45 ppm). Instead, the glycine derived from serine is primarily directed into the tetrahydrofolate cofactor system pathway, resulting in label from the C-2 carbon of serine appearing in the C-2 and C-8 carbons of purines. Again, these 13C single-carbon insertions result in the formation of 13C- and 15N-labeled bonds because the scrambled 15N of serine has isotopically saturated the nitrogen pool. In contrast, the DCP difference spectrum (not shown) of K. pneumoniae grown for 8 h in the presence of L-[2-13C,15N]serine plus natural-abundance glycine has significant intensity only at 120, 60, and 45 ppm. Only three peaks are observed because at this stage of growth 14N2 fixation has sufficiently diluted the general 15N pool to a degree that single-carbon incorporations no longer generate 13C- and 15N-labeled bonds. (A discussion of the fate of double-labeled glycine under these conditions is presented in the Miniprint Supplement.)

Even though K. pneumoniae was grown in media containing 2% sucrose, the carbons of labeled serine are heavily used, producing specific enrichments of 25-50% for some purine ring carbons. We attribute the heavy use of labeled carbon to pressure for the nitrogen of serine for purine synthesis during derepression. The fact that the C-3 carbon of serine can be readily used in purine synthesis probably contributes to the effectiveness of serine in shortening the time for derepression.

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A closed-atmosphere fermenter system (Figure 3) was used for long-term 13C labeling experiments. This system is capable of maintaining a high degree of homogeneity, and features real-time monitoring of the gas phase within the 20-L fermenter, as well as provisions for the controlled addition of carbon label, and partial removal of 13CO₂ headspace. Harvesting of cells at any time during an experiment is also possible without interruption of labeling.

Typical 1H NMR spectra of intact lyophilized cells of K. pneumoniae grown under 13CO₂ fixing conditions in this fermenter show that the incorporation of 13C label within cells could be detected as early as 2 hours after the addition of series (Figure 3). Maximum cell growth, as well as incorporation of 13C label occurs about 4-5 hours after addition of series, and production of nitrogenase reaches a maximum between 6 and 8 hours after addition (Figure 3). The observed reduction in growth rates after 8 hours was most likely due to an accumulation of inhibitory gases (e.g.) within the fermenter.

Effect of various agents on the production of K. pneumoniae

Several amino acids were able to induce the increase needed for K. pneumoniae to make 13C-labeled amino acids under anaerobic conditions (Table 1). We found that L-serine was the most successful derepressing amino acid, while amino acids like L-serine and L-lysine, which can readily be removed by the cell at later times, are less effective. L-serine and L-lysine had a negative effect. The remaining amino acids tested proved negative.

TABLE 1

Effect of amino acids on derepression of K. pneumoniae

| Amino acid | Control | 0.01 M L-serine | 0.01 M L-lysine | 0.01 M L-lysine
|------------|---------|----------------|----------------|----------------|
| Absorbance | 0.000   | 0.000          | 0.000          | 0.000          |
| 6 hours after | 0.150   | 0.150          | 0.150          | 0.150          |

Double-label 13C NMR experiments were performed using a double-label 13C NMR apparatus to study the incorporation of 13CO₂ into cellular material. The apparatus was a modified 1H-13C NMR spectrometer equipped with a 5-mm double-resonance probe.

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Figure 33. CP/MAS 2D-NOESY 13C NMR spectra of intact lyophilized cells of K. pneumoniae harvested at indicated times from the fermentor of Figure 31 during 13CO2 fixation. Mechanical sample spinning prevented sidebands with opposite sense of the spectra. The spectra of the 20-hour sample match that of Figure 31 (right, top inset). Each spectrum was obtained with a 5-msec proton-pulse excited Hartmann-Hahn spin lock.

Figure 34. Variation with time of 13CO2 and 13CO in the closed-atmosphere fermenter of Figure 31 during growth of K. pneumoniae under 13CO2-fixing conditions (top). The reduction in CO2 level after 9 hours resulted from venting of the fermentor atmosphere through the exhaust valve (see Figure 31). High CO2 levels did not inhibit growth. Growth of cells harvested at 600 ml and subsequent activity (ethanol production) of crude extracts of harvested cells are shown at the bottom of the figure.

Figure 35. CP/MAS 2D-NOESY 13C NMR spectra (left) and CP/MAS 30.6-MHz 13C NMR spectra (right) of intact lyophilized cells of K. pneumoniae grown for 8 hours on medium containing 13CO2 plus natural-abundance L-arginine plus 15NH4Cl. A natural-abundance 13C NMR spectrum is also shown. The natural-abundance 13C NMR spectrum (not shown) consists of a peak at resonance at 100 ppm about one-tenth the intensity of that from the glycerol label.

Figure 36. Labeling pathways of glycerol and serine metabolism.