Evaluation of Parameters Critical to Observing Proteins Inside Living *Escherichia coli* by In-Cell NMR Spectroscopy

Zach Serber,[†] Richard Ledwidge,[‡] Susan M. Miller,[‡] and Volker Dötsch*[,‡,§]

Contribution from the Graduate Group in Biophysics, Department of Pharmaceutical Chemistry, Department of Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, California 94143

Received May 25, 2001. Revised Manuscript Received July 6, 2001

Abstract: Our recently developed in-cell NMR procedure now enables one to observe protein conformations inside living cells. Optimization of the technique demonstrates that distinguishing the signals produced by a single protein species depends critically on protein overexpression levels and the correlation time in the cytoplasm. Less relevant is the selective incorporation of ^{15}N. Poorly expressed proteins, insoluble proteins, and proteins that cannot tumble freely due to associations within the cell cannot yet be observed. We show in-cell NMR spectra of bacterial NmerA and human calmodulin and discuss limitations of the technique as well as prospects for future applications.

Introduction

Of all methods currently available for obtaining high-resolution structures of biological macromolecules, NMR is the only one that can provide this information in solution under near-physiological conditions.[1,2] However, even NMR structures are still determined in vitro, and often buffer conditions are not selected for their closest match to the natural environment of the protein but to optimize experimental parameters such as solubility and sensitivity or to minimize NMR buffer signals that could interfere. A recent survey of buffer conditions used for NMR structure determinations showed that 27% of all structures were determined in unbuffered (or autobuffered) solutions, 50% in phosphate, 10% in acetate buffer and 9% in tris buffer. [3] Depending on the natural host cell and the exact cellular compartment, these NMR buffer conditions can be substantially different from a protein's natural environment and may influence its structure and dynamics. Furthermore, interactions with other cellular (macro)molecules and posttranslational modifications can alter the conformation. In principle, NMR spectroscopy, as a noninvasive spectroscopic technique, should be able to provide information about the structure and dynamics of biological macromolecules inside living cells. Indeed, in vivo NMR and magnetic resonance imaging are well-established fields that use NMR spectroscopy to obtain information from living organisms ranging from cell suspensions to human beings.[4−8] These studies, however, have mainly focused on small molecules, which can be distinguished from all other molecules in the cell either because they are the most abundant or because they have been isotopically labeled. Clearly, having an equivalent of these in vivo NMR experiments for macromolecules would be of great interest. Toward this goal, we have begun to develop techniques to enable us to collect high-resolution NMR data on proteins expressed inside living *Escherichia coli* bacteria, and the first successful experiment with the small bacterial protein NmerA was published in a recent paper.[9] In addition, in-cell NMR spectra of osmoregulated glucans in the periplasm of *Ralstonia solanacearum* were recently reported.[10,11] These in-cell NMR experiments now open new avenues to characterize the conformation and dynamics of proteins and other biological macromolecules in their natural environment. Here we describe our strategy to obtain these in-cell NMR spectra of NmerA and discuss further possible improvements of the technique.

Experimental Section

Protein Overexpression. The N-terminal metal-binding domain of MerA containing amino acids 1−69 was cloned into a pET-11a vector (Stratagene) by standard PCR techniques. BL21 DE3 *E. coli* bacteria were transformed with the plasmid and selected for transformation on an ampicillin plate. The cells were grown in different media at 37 °C in a rotary shaker. Unless stated otherwise, cells were first grown in 170 mL of LB medium to an optical density of 1.2 and harvested by centrifugation at 850*g* for 20 min. The pellet was then resuspended in 125 mL of a different medium and induced with 0.4 mM IPTG. Four hours post-induction, the bacteria were harvested by gentle centrifugation (170*g* for 25 min), which formed an easily dislodged, poorly packed pellet at the bottom of a conical tube. We used a wide-bore glass pipet

* To whom correspondence should be addressed. Telephone: (415) 502-7050. Fax: (415) 476-0688. E-Mail: volker@picasso.ucsf.edu.
[†] Graduate Group in Biophysics.
[‡] Department of Pharmaceutical Chemistry.
[§] Department of Cellular and Molecular Pharmacology.

(1) Kremer, W.; Kalbitzer, H. R. In *Methods in Enzymology*; James, T. L., Schmitz, U., Dötsch, V., Eds.; Academic Press: San Diego, 2001; Vol. 339, pp 3−19.
(2) Stoll, V. S.; Blanchard, J. S. *Methods Enzymol.* **1990**, *182*, 24−38.
(3) Hubbard, J.; Johnson, P.; Ong, B.; Laddes, J.; King, G.; MacLachlan, L. K. *42nd ENC*; Orlando, Florida, 2001.
(4) Li, C. W.; Negendank, W. G.; Murphy-Boesch, J.; Padavic-Shaller, K.; Brown, T. R. *NMR Biomed.* **1996**, *9*, 141−155.
(5) Kanamori, K.; Ross, B. D. *J. Neurochem.* **1997**, *68*, 1209−1220.
(6) Bachert, P. *Prog. Nucl. Magn. Reson. Spectrosc.* **1998**, *33*, 1−56.
(7) Spindler, M.; Saupe, K. W.; Tian, R.; Ahmed, S.; Matlib, M. A.; Ingwall, J. S. *J. Mol. Cell. Cardiol.* **1999**, *31*, 2175−2189.
(8) Gillies, R. J. *NMR in Physiology and Biomedicine*; Academic Press: San Diego, 1994.
(9) Serber, Z.; Keatinge-Clay, A. T.; ledwidge, R.; Kelly, A. E.; Miller, S. M.; Dötsch, V. *J. Am. Chem. Soc.* **2001**, *123*, 2446−2447.
(10) Wieruszeski, J.-M.; Bohin, A.; Bohin, J.-P.; Lippens, G. *J. Magn. Reson.* **2001**, *151*, 118−123.
(11) Lippens, G.; Bohin, J.-P. In *NMR in Supramolecular Chemistry*; Pons, M., Ed.; Kluwer Academic Publishers: 1999; pp 191−226.

10.1021/ja0112846 CCC: $20.00
Published on Web 08/16/2001

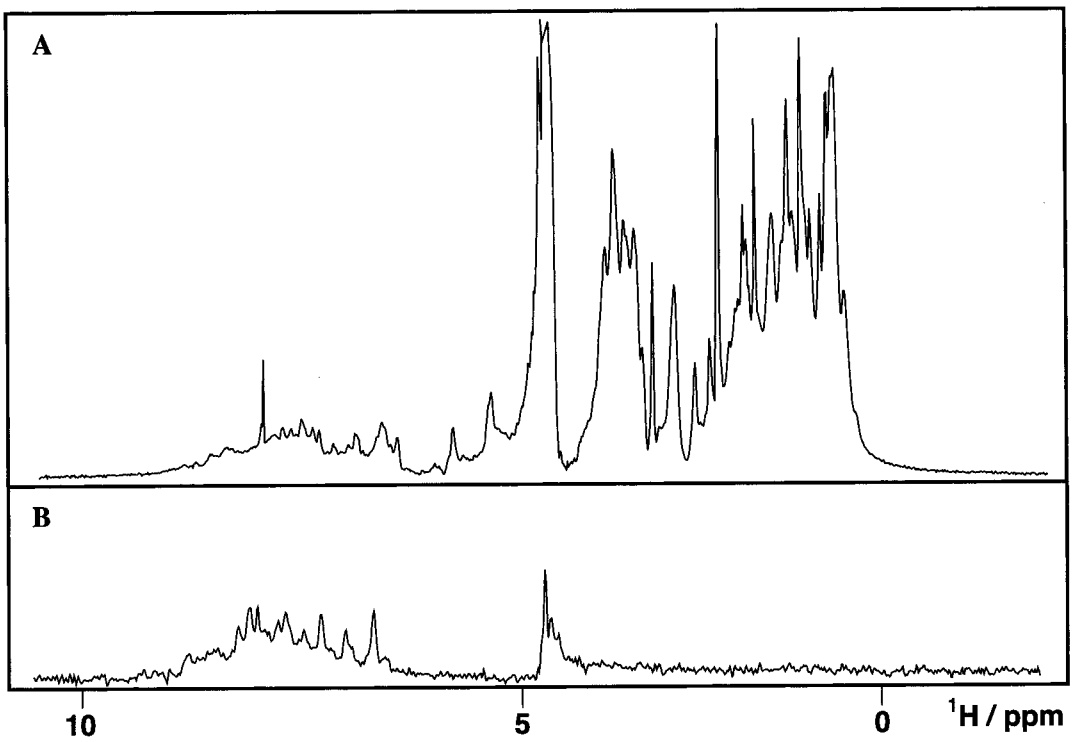

Figure 1. Comparison of an (A) unfiltered one-dimensional ^{1}H spectrum with an (B) ^{15}N-filtered one-dimensional spectrum of the same living *E. coli* sample following 4 h of overexpression of NmerA in ^{15}N-labeled minimal medium.

to suction out the bacterial pellet from the bottom and to place 460 μL into a 5 mm NMR tube already containing 40 μL of deuterium oxide. We deliberately put a small air bubble into the bacterial slurry to mix and homogenize the sample by carefully inverting the tube back-and-forth. For the investigation of the influence of the overexpression level, different cultures were centrifuged exactly 10 min, 30 min, 1 h, and 2 h post induction. Sample preparation took 30 min and was performed at 4 °C. Prior to insertion of the NMR tube into the magnet, 5 μL were removed and flash frozen for subsequent analysis by SDS PAGE. Samples that were selectively labeled with ^{15}N on lysines were produced by expressing the protein in minimal medium containing 100 mg/L of the labeled amino acid.

NMR Spectroscopy. All NMR experiments were measured on a Bruker 500 MHz Avance NMR instrument equipped with a triple resonance cryoprobe. Due to the insensitivity of the bacterial sample to shimming, we used a separate sample of the same height containing the supernatant of the harvested cells to shim. All HSQC experiments were measured at 37 °C with a standard FHSQC pulse sequence employing WATERGATE for water suppression.[12] In the ^{1}H acquisition dimension, 1024 complex data points with a t_{2max} of 80 ms were recorded. In the indirect ^{15}N-dimension, 60 complex points with a t_{1max} of 41 ms were measured. Unless stated otherwise, all spectra were collected with four scans per increment. The total measurement time per experiment was less than 10 min. All spectra were transformed using the XWINNMR software package (Bruker).

Results

The Effect of the Polymerase Inhibitor Rifampicin on Background Signals. Detection of NMR spectra of proteins inside living cells differs from in vitro protein NMR experiments in several ways. Instead of dissolving the protein in an homogeneous aqueous buffer solution, proteins inside living cells are in an inhomogeneous environment that contains hundreds of different protein species, nucleic acids, lipids and a huge arsenal of small molecules. Figure 1A shows a one-dimensional spectrum of living *E. coli* demonstrating the high density of proton resonances. The greatest obstacle for in-cell NMR experiments is to selectively distinguish a particular protein's resonances from the resonances of all other molecules inside the cell. One way to achieve this selectivity is to incorporate an NMR-active isotope, such as ^{15}N, through overexpression. Figure 1B shows a ^{15}N-filtered one-dimensional spectrum of the same sample as in Figure 1A. Clearly, this isotope-filtering drastically reduces the signal density. To minimize the ^{15}N incorporation into other proteins and other cellular molecules, we originally used a two-step protocol. Cells harboring the expression plasmid were first grown in unlabeled LB medium. After being harvested by centrifugation, they were resuspended in ^{15}N-labled minimal medium. Ten minutes after resuspension, the cells were induced with 0.4 mM IPTG. Forty minutes after induction, the RNA polymerase inhibitor rifampicin was added to the bacterial culture to a concentration of 35 μM. Rifampicin suppresses the production of all bacterial proteins, while our protein of interest, NmerA, is under the control of a T7 promoter. The polymerase of the bacteriophage T7 is not affected by the drug, which enables the selective expression of a single protein in bacteria.[13−15]

To evaluate the effect of suppressing the bacterial protein production by rifampicin, we have expressed NmerA in the presence and in the absence of the drug while leaving all other parameters unchanged. The two HSQC experiments obtained with the in-cell NmerA samples expressed in the absence and presence of rifampicin are shown in Figure 2, A and B, respectively. In addition, an in vitro HSQC spectrum of purified NmerA is shown in Figure 2C. Comparison of all three spectra shows that they are very similar. Both in-cell HSQC spectra

(12) Mori, S.; Abeyguawardana, C.; Johnson, M. O. N.; Zijl, P. C. M. v. *J. Magn. Reson. B* **1995**, *108*, 94−98.

(13) Sippel, A.; Hartmann, G. *Biochim. Biophys. Acta* **1968**, *157*, 218−219.

(14) Richardson, J. P.; Greenblatt, J. In *Escherichia coli and Salmonella*; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; Vol. 1, pp 822−848.

(15) Campbell, E. A.; Korzheva, N.; Mustaev, A.; Murakami, K.; Nair, S.; Goldfarb, A.; Darst, S. A. *Cell* **2001**, *104*, 901−912.

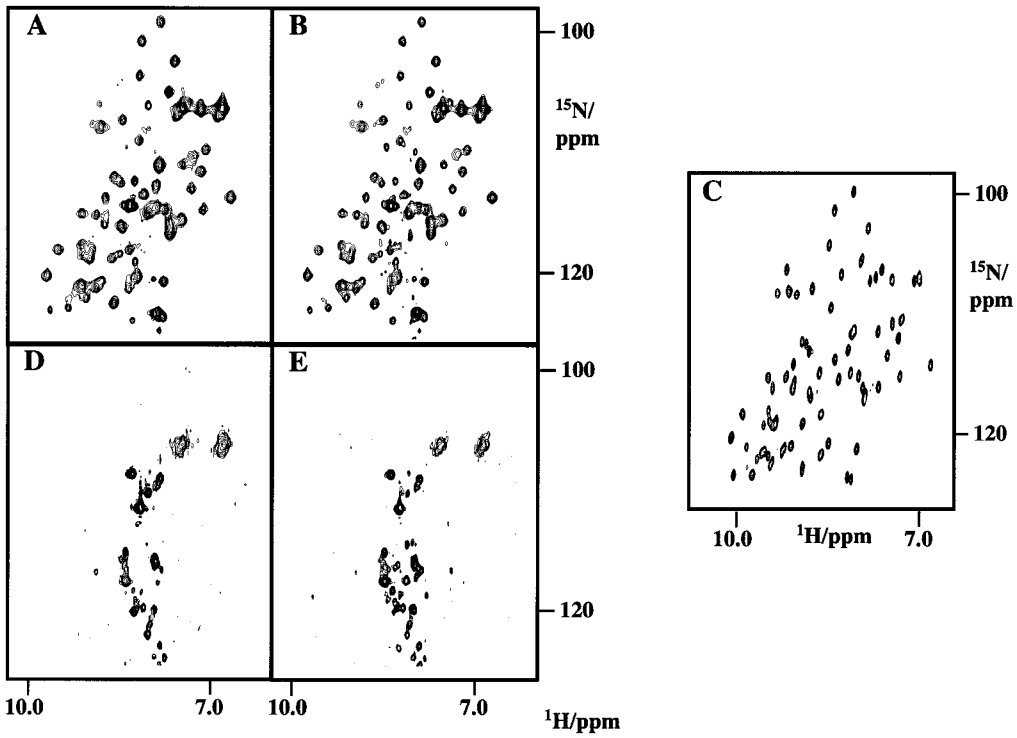

Figure 2. Comparison of in-cell HSQC spectra in the absence or presence of 35 μM rifampicin and 400 μM IPTG. All spectra were recorded with four scans per increment. (A) Induced bacteria without rifampicin. (B) Induced bacteria with rifampicin. (C) An in vitro HSQC of a purified NmerA sample. (D) Uninduced bacteria without rifampicin. (E) Uninduced bacteria with rifampicin.

contain, in addition to the protein resonances of NmerA, several sharp NMR signals in the range of 8−8.5 ppm. The sharpness of these lines suggests that they do not originate from protein signals but from the incorporation of ^{15}N into small molecules such as amino acids. Interestingly, both spectra contain almost identical artifacts but do not show any signs of additional protein resonances. This result suggests that rifampicin is not necessary to suppress potential NMR signals of bacterial proteins. To further investigate the influence of rifampicin on the ^{15}N incorporation into small organic molecules, we produced two samples as described above. However, this time the bacterial samples were not induced. The resulting HSQC spectra of these noninduced samples are shown in Figure 2D for a sample without rifampicin and in 2E for a sample containing rifampicin. Like the spectra of the induced samples, both spectra are very similar with even a slight increase in the number of NMR signals in the rifampicin sample, suggesting that addition of rifampicin to bacterial samples does not have any effect on the suppression of background NMR signals in in-cell NMR experiments. Differences in the number and intensity of background signals observed in all four spectra are most likely due to changes in the bacterial metabolic state caused by IPTG and rifampicin.

The Effect of Switching the Medium between the Bacterial Growth and the Protein Expression Phase. We investigated the influence of switching the medium from unlabeled LB medium to ^{15}N-labled minimal medium prior to induction. Three different protocols were used to produce in-cell NMR samples of NmerA. First, we grew the bacteria in ^{15}N-labeled minimal medium to an optical density of 0.8 and induced the expression of NmerA by addition of IPTG in the same medium. Second, we grew the bacteria in ^{15}N-labeled minimal medium to an optical density of 0.8, harvested them by centrifugation at 850*g* and resuspended them in fresh ^{15}N-labeled minimal medium before induction with IPTG. Finally, we grew the bacteria in LB medium, harvested them by centrifugation, and resuspended them in ^{15}N-labeled minimal medium to the same optical density as the previous sample. The resulting HSQC spectra of all three different samples are shown in Figure 3. All three spectra show a very similar level of background signals, suggesting that switching the type of medium prior to induction has a negligible effect on the suppression of these signals. However, the spectra show large differences in the intensity of the protein peaks. The sample obtained by growing and expressing the protein in the same minimal medium clearly exhibits the lowest sensitivity. Switching the medium to fresh ^{15}N-labeled minimal medium prior to induction increases the spectral quality severalfold. The type of medium used to grow the bacteria in the first phase before induction seems to have only a very small influence on the resulting spectrum, with the sample that was initially grown in LB medium showing a slightly higher sensitivity than the spectrum that was grown in minimal medium.

Investigation of the Influence of the Overexpression Level. The combined results of the rifampicin experiments and the studies of changing the media suggest that the amount of background signals that arises from ^{15}N incorporation into other cellular components is small and is insensitive to the specific growth and induction protocol used. This implies that the most important factor for observing proteins inside living bacterial cells is the behavior of the individual protein. While interactions between the protein and cellular components, as well as its intracellular stability, are protein-specific parameters that must be investigated in each individual case, the overexpression level is a general parameter that will influence the quality of in-cell NMR experiments of all proteins. We tested the lower limit for the observation of overexpressed proteins inside living bacteria by inducing NmerA for varying amounts of time. The resulting spectra are shown in Figure 4, and Figure 5 shows a gel that demonstrates the level of NmerA overexpression that corresponds to the spectra in Figure 4. Ten minutes after induction the in-cell HSQC shows only some background signals (Figure

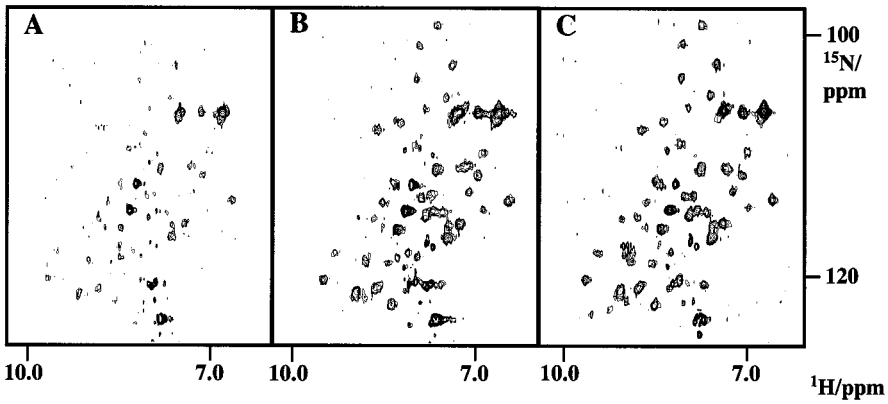

Figure 3. Influence of the bacterial growth protocol on the quality of the resulting NMR spectra. (A) In-cell HSQC of an NmerA sample. The same ^{15}N-labeled minimal medium was used to grow the bacteria to an optical density of 0.8 and for expressing the protein following induction with 0.4 mM IPTG. (B) The bacteria were harvested after reaching an optical density of 0.8 in ^{15}N-labeled minimal medium by centrifugation and were resuspended in fresh ^{15}N-labeled minimal medium followed by induction with IPTG. (C) The cells were grown in unlabeled LB medium, harvested by centrifugation, and resuspended in ^{15}N-labeled minimal medium for protein expression. In all three cases the bacteria were harvested 4 h after induction.

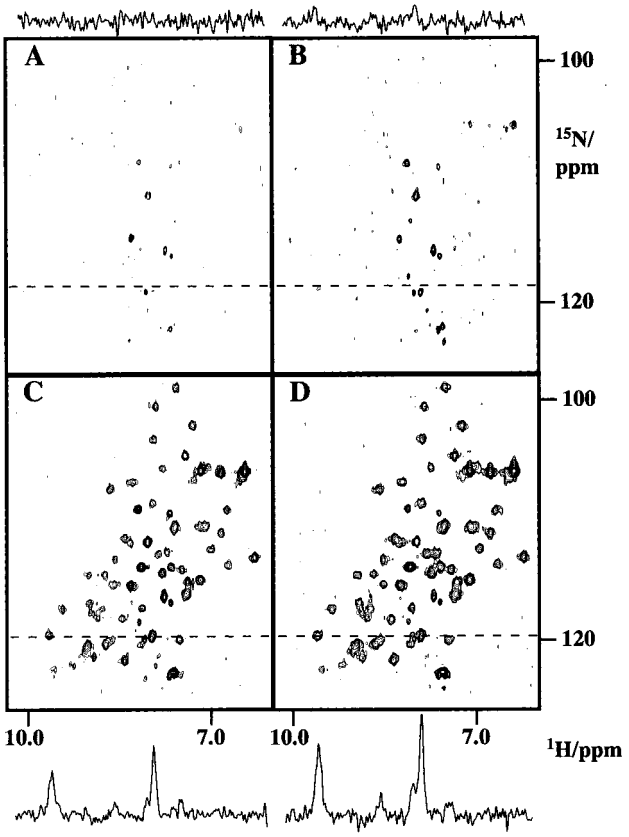

Figure 4. In-cell HSQC spectra of NmerA collected after varying times following induction of protein expression on ^{15}N-labeled minimal medium. (A) HSQC spectrum recorded after 10 min, (B) after 30 min, (C) after 1 h, and (D) after 2 h of induction. One-dimensional cross sections taken at the position indicated by the dotted line are shown as well.

4A), and NmerA cannot be detected on the gel. After 30 min some weak protein resonances become visible in the HSQC spectrum (Figure 4B), and a faint band of NmerA appears. One hour post-induction all resonances seen in in-cell NMR experiments of NmerA are visible, and after 2 h the signals become stronger. The corresponding gel lanes show a strong NmerA band. For a better comparison of the signal-to-noise ratios, one-dimensional cross-sections along the acquisition dimension taken at the position of the dotted line are shown for each spectrum. Protein levels inside the bacteria remain fairly constant over the course of these NMR experiments, given that further tests revealed that detectable amounts of protein could not be induced by addition of IPTG to a bacterial sample inside an NMR tube even after 4 h.

The spectrum shown in Figure 4B was measured with four scans per increment, as always, and establishes the lower detection limit for in-cell NMR experiments. Although, the intensity of the bands is only approximately related to the intracellular concentration of the protein, we estimate from the NmerA band in lane B in Figure 5 that the detection limit for a protein in in-cell NMR experiments is only a few percent of the total amount of soluble protein. Furthermore, we estimate from these data that a 5% overexpression level is sufficient to provide high-quality in-cell NMR spectra.

Improvement of Spectral Quality by Expression in Labeled, Rich Media. On the basis of the experiments described above it is evident that the overexpression level is one of the most important factors influencing the spectral quality of in-cell NMR experiments. To investigate if the quality of the spectra can be enhanced by expressing the protein in rich, labeled media we grew the bacteria in LB medium to an optical density of 1.2. After we harvested the bacteria by centrifugation, half of the pellet was resuspended in standard ^{15}N-labeled minimal medium and the other half in ^{15}N-labeled rich medium. This rich medium was produced from 13.3 g/L of 98% ^{15}N-labeled and 97% deuterated algae extract (Celtone-dN, Martek) dissolved in H_2O. Overexpressing proteins in bacteria grown in deuterated media dissolved in H_2O has been shown to give approximately 80% deuteration on methyl groups and 50% deuteration on the α-protons leading to a 2-fold reduction of the proton T_2 relaxation rate.[16] The HSQC spectra of both in-cell samples are shown in Figure 6. The spectrum of NmerA expressed in the rich medium clearly shows a 2−3-fold higher sensitivity. This higher sensitivity can be attributed both to the higher protein expression level in the rich medium as well as to the effect of the deuteration. The comparison of one-dimensional cross section through peaks of the HSQC spectra shows a reduction in the amide proton line width from an average of 55 Hz in the nondeuterated sample to 40 Hz in the partially deuterated sample. A more detailed analysis of the

(16) Markus, M. A.; Dayie, K. T.; Matsudaira, P.; Wagner, G. *J. Magn. Reson. B* **1994**, *105*, 192−195.

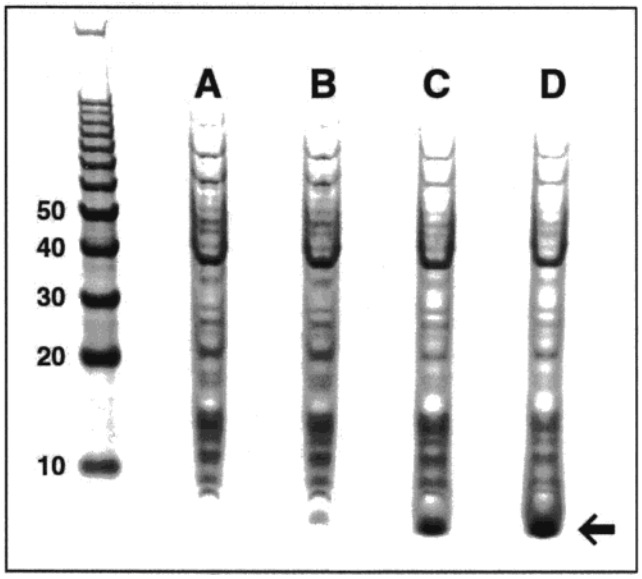

Figure 5. SDS-polyacrylamide gel (12%) of 2 μL samples taken from the NMR samples of Figure 4. The letters correspond to the letters of the HSQC spectra. A molecular weight marker is shown at the left-hand side. The arrow marks the location of the NmerA band.

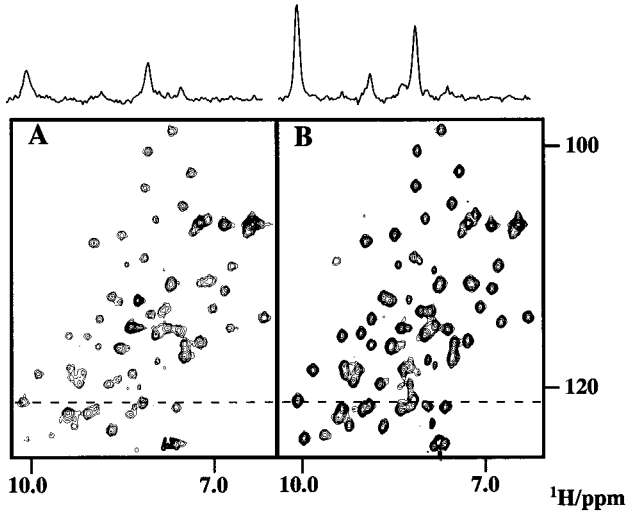

Figure 6. Comparison of the quality of in-cell NMR spectra of NmerA which were obtained by protein expression in (A) ^{15}N-labeled minimal medium and (B) 98% ^{15}N-labeled, 97% deuterated rich medium (Celtone-dN, Martek). In both cases the samples were grown in unlabeled LB medium before they were transferred to the labeled media for protein expression. One-dimensional cross sections taken along the acquisition dimensions at the position indicated by the dotted line are shown on top of both spectra.

deuteration effect on sensitivity and the use of TROSY-type experiments will be presented elsewhere.

Selective Amino Acid Labeling. The larger line width of the in-cell NMR spectra causes greater peak overlap relative to in vitro spectra. One potential method to overcome this problem is selective ^{15}N-labeling of only certain types of amino acids.[17] This method is particularly powerful if only a certain type of amino acid is of interest, for example a residue in the active site of an enzyme. Figure 7 A shows an in-cell HSQC experiment of NmerA expressed in standard, unlabeled minimal medium that was supplemented with 0.1 g/L of ^{15}N-labeled lysine (CIL). The spectrum contains six peaks, five of which correspond to the five lysines of NmerA. The sixth and by far

(17) Waugh, D. S. *J. Biomol. NMR* **1996**, *8*, 184−192.

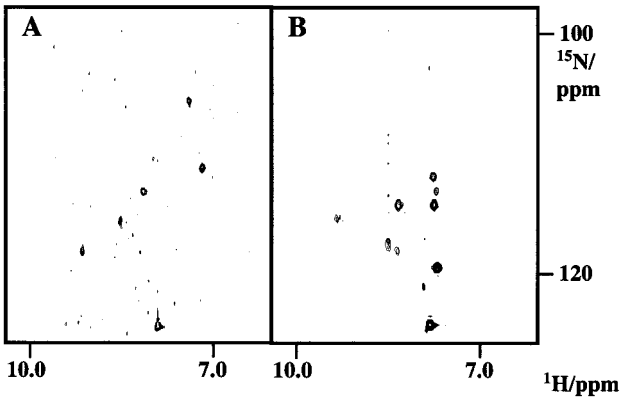

Figure 7. In-cell HSQC-spectra of selectively ^{15}N-lysine labeled NmerA (A) and human calmodulin (B). The calmodulin spectrum was measured with 16 scans per increment.

strongest peak represents a metabolic product of ^{15}N-labled lysine. As a second example, Figure 7B shows an in-cell HSQC spectrum of human calmodulin selectively labeled on lysines. In addition to the expected seven resonances, some minor peaks are visible, which might represent protein species with different metal ions in the four binding sites. A more detailed analysis of the in-cell spectra of calmodulin will be given elsewhere.

These experiments demonstrate that selective amino acid labeling and selective observation of certain types of amino acids in living cells is possible without any background signal with the exception of one metabolic product of lysine. However, not all types of amino acids are good candidates for selective ^{15}N-labeling in *E. coli* BL21 cells. Some amino acids are precursors for other amino acids, and aminotransferases can transfer (^{15}N-labeled) amino groups between amino acid types.[17] Lysine as well as other end products of biosynthetic pathways in *E. coli*, however, can be used. Selective labeling of other amino acid types requires special *E. coli* strains that are auxotrophic for particular amino acids.

Discussion

In-cell NMR spectroscopy provides a new tool for the characterization of protein conformations in their natural environment. In the experiments described in this paper we have tested several different expression and labeling schemes to optimize the sensitivity of the NMR measurements. Initially, our main concern was that growing the bacteria and expressing the protein in ^{15}N-labeled medium will result in the labeling of hundreds of proteins, nucleic acids, and small molecules with ^{15}N, which could cause so many background NMR signals that the identification of the peaks belonging to the protein of interest would be impossible. Surprisingly, however, only a very small number of background signals, mainly arising from ^{15}N-incorporation into small molecules such as amino acids, can be detected in the in-cell HSQC spectra. Furthermore, the addition of rifampicin, a drug that inhibits the bacterial RNA polymerase but not the T7 RNA polymerase did not have any effect on the spectrum. Even growing the bacteria in ^{15}N-labeled media prior to induction did not affect the amount of background signal significantly. These results demonstrate that, at least in *E. coli* BL21, potential background signals from cellular components are not a limiting factor and the quality of the NMR spectra does not critically depend on the explicit growing and expression scheme used. Earlier work by Clore and Gronenborn had shown that overexpression of proteins in ^{15}N-labeled medium followed by cell lysis, buffer exchange to a suitable NMR buffer, and concentration of the protein resulted in virtually background-

free HSQC spectra.[18] Our results demonstrate that, even in living cells, no background signals from bacterial macromolecules are detected and the only background signals are caused by some small molecules that become ^{15}N-labeled.

The results above further indicate that the overexpression level seems to be a crucial parameter. The degree of overexpression level is very sensitive to the media and the growth and expression protocol used. When we expressed the protein in the same batch of ^{15}N-labeled minimal medium that we had used to grow the bacteria, the expression level and the sensitivity of the NMR experiments were far reduced compared to those with a protocol that uses different media for bacterial growth and for protein expression. Furthermore, the sensitivity of in-cell NMR experiments can be enhanced 2−3-fold if rich labeled media are used during the protein expression phase.

Although our experiments have revealed that the same type of medium can be used for both growth phase and expression phase, we prefer LB medium for the growth phase and switching media to the desired expression medium prior to induction. This protocol allows us to minimize the costs for producing in-cell NMR samples because expensive labeled media are used only during the protein expression phase. At the same time, the use of fresh medium just prior to induction optimizes the expression level and increases the quality of the spectra.

In addition to the overexpression level, the rotational correlation time of a protein in the cytoplasm is a crucial parameter that determines the sensitivity of in-cell NMR experiments. An increase in viscosity slows down the rotational tumbling of the molecules, which leads to faster relaxation and broader resonance lines. However, fluorescence anisotropy studies [19] and NMR relaxation experiments [20] have revealed that the viscosity of the cytoplasm of bacteria, yeast, and mammalian cells differs only by a factor of 1.2−2 from that of water.[21,22] Due to the linear relationships between viscosity, rotational correlation time, and molecular mass of a protein, this increase in viscosity means that the "apparent molecular mass" of a protein in the cytoplasm will increase by a factor of 1.2−2 relative to its real mass. The recent introduction of TROSY and related techniques into the field of high-resolution NMR spectroscopy has dramatically extended the molecular-weight range of macromolecules to which NMR spectroscopy can be applied.[23,24] TROSY-type experiments require high levels of deuteration on the aliphatic carbons. For in-cell NMR experiments based on the detection of amide protons, 100% deuteration cannot be achieved because this requires expressing the protein in D_2O, which will also exchange the amide protons. In principle, it is possible to back-exchange the amide protons by harvesting the bacteria and resuspending them in H_2O-based media some time after the initiation of expression; however, some amide protons located in regular secondary structure elements might exchange too slowly relative to the lifetime of the protein inside the bacteria or the bacteria themselves. However, by dissolving deuterated algae extract in H_2O, a high level of approximately 80% deuteration can be achieved while retaining the amide protons. This deuteration level is sufficient for many TROSY-type applications, which extends the applicability of in-cell NMR experiments. However, not all proteins will tumble freely in the cytoplasm. Instead, they might interact with other cellular components such as DNA, membranes, or other proteins. In this case, a protein's rotational correlation time will be further increased, which can result in the broadening of its resonances beyond detection. In-cell NMR experiments are, therefore, most likely to be successful with proteins whose role is not to bind to other large cellular components.

In addition to the tumbling rate of the protein, other factors influence the line shape of the protein resonances in in-cell NMR spectra. In contrast to the homogeneous environment of a purified protein sample, the protein in the cytoplasm experiences an inhomogeneous surrounding. Even in the absence of any direct interaction with other cellular components, resonance lines in in-cell NMR experiments are broader due to the introduction of magnetic susceptibility gradients caused by differences in the magnetic susceptibility of the surrounding cellular components.[10,25] In addition, improving the overall homogeneity of the sample by shimming is difficult because the lock signal becomes very insensitive to changes in the shim values. This effect becomes worse with higher cell densities in the NMR tube. The rotational correlation time of the protein only depends on the cytoplasmic viscosity and not on the macroscopic viscosity of the entire sample, and a denser packing of the bacteria in the NMR tube should, therefore, result in more signal. However, in our experience, the spectral quality deteriorates at higher cell density due to broader lines, which is most likely caused by an uneven distribution of the cell density in the NMR tube. In our experience, a 20−30% bacterial slurry (measured as the volume of the pellet following hard centrifugation) provides the optimum between maximizing the signal intensity and obtaining a reasonable line width. At that concentration, a uniform cell distribution can be maintained for at least 4 h with only little sedimentation.

The final goal of in-cell NMR experiments is to investigate the conformation and dynamics of proteins in their natural environment. The relative insensitivity of NMR spectroscopy, however, currently requires the overexpression of the investigated protein to levels of at least 5% of total soluble protein. This overexpression changes the protein concentration in the bacterial cytoplasm relative to its natural state and can potentially also influence the behavior of a protein. NmerA is the N-terminal domain of the bacterial detoxification protein MerA that accumulates in the bacterial cytoplasm to levels of up to 6% of total soluble protein in response to mercurials.[26−28] This naturally occurring high expression level allows us to observe it under "near natural" conditions. However, the effect of overexpression and its consequences for the behavior of the investigated protein have to be considered for each individual protein. Another factor that can change the cytoplasmic environment is the high cellular density in the NMR tube. This high density leads to oxygen starvation for the bacteria, switching them to an anaerobic state, which changes the metabolism of the bacteria and influences the intracellular pH. These problems, however, can be solved by employing modified NMR tubes or bioreactors for the NMR experiments that can be used to exchange media and provide the bacteria with oxygen. Several

(18) Gronenborn, A. M.; Clore, G. M. *Protein Sci.* **1996**, *5*, 174−177.
(19) Dayel, M. J.; Hom, E. F. Y.; Verkman, A. S. *Biophys. J.* **1999**, *76*, 2843−2851.
(20) Williams, S. P.; Haggle, P. M.; Brindle, K. M. *Biophys. J.* **1997**, *72*, 490−498.
(21) Fushimi, K.; Verkman, A. S. *J. Cell Biol.* **1991**, *112*, 719−725.
(22) Moonen, C. T. W.; Zijl, P. C. M. v.; Bihan, D. L.; DesPres, D. *Magn. Reson. Med.* **1990**, *13*, 467−477.
(23) Pervushin, K.; Riek, R.; Wider, G.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12366−12371.
(24) Riek, R.; Wider, G.; Pervushin, K.; Wüthrich, K. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4918−4923.
(25) Springer, C. S. In *NMR in Physiology and Bionedicine*; Gillies, R. J., Ed.; Academic Press: San Diego, 1994; pp 75−99.
(26) Misra, T. K.; Brown, N. L.; Haberstroh, L.; Schmidt, A.; Goddette, D.; Silver, S. *Gene* **1985**, *34*, 253−262.
(27) Fox, B.; Walsh, C. T. *J. Biol. Chem.* **1982**, *257*, 2498−2503.
(28) Miller, S. M. *Essays Biochem.* **1995**, *34*, 17−30.

different designs for these bioreactors have already been used for in vivo spectroscopy with small molecules.[29−31]

The biggest challenge for in-cell NMR spectroscopy is to increase the sensitivity and spectral quality of the experiments. Currently, the resolution is compromised by the large line width of the peaks. As discussed above, several factors contribute to the observed line width, and potential solutions exist. In the case of NmerA, relaxation measurements have demonstrated that the rotational correlation time is similar to the one observed in an in vitro sample (Serber et al., manuscript in preparation). Consequently, the increase in line width is not caused by the higher viscosity of the bacterial cytoplasm but by other factors, linked to the inhomogeneity of the sample. As discussed above, magnetic susceptibility gradients contribute to the observed line width. It has been demonstrated for other inhomogeneous systems, such as organic compounds bound to beads or tissue samples in traditional in vivo NMR spectroscopy, that the contribution of these susceptibility gradients can be eliminated by magic angle spinning.[10,32,33] Other factors, such as the overall homogeneity of the sample, could be improved if suitable shimming protocols could be developed.

In conclusion, we have demonstrated that the observation of the conformation and the dynamics of proteins in the bacterial cytoplasm is possible, making NMR a new high-resolution tool for studying proteins in vivo. Comparison of chemical shifts for amino acids in the metal-binding loop of the protein has already revealed slight differences from the in vitro state. Furthermore, preliminary relaxation experiments and line width analysis suggest that resonances in this metal-binding loop relax faster than the average backbone nitrogen resonances. While the same trend is observed in vitro, it is less pronounced. These differences in chemical shifts and relaxation rates might reflect biologically relevant variations in local conformation and dynamics.

Acknowledgment. We thank Alexander Kelly, Horng Der Ou, Helen Lai, and Anson Nomura for discussion and critical comments. Financial support was provided by NIH (GM56531-02), the UCSF Innovations in Basic Sciences Award, the Sandler Family Supporting Foundation, and NSF (MCB-9982596). Z.S. was supported by an NIH Training Grant (GM08284).

JA0112846

(29) Egan, W. M. *The Use of Perfusion Systems for Nuclear Magnetic Resonance Studies in Cells.*; CRC Press: Boca Raton, FL, 1987; Vol. 1.

(30) Szwergold, B. S. *Ann. Rev. Physiol.* **1992**, *54*, 775−798.

(31) Cohen, J. S.; Lyon, R. C.; Daly, P. F. *Monitoring Intracellular Metabolism by Nuclear Magnetic Resonance*; Academic Press: San Diego, 1989; Vol. 177.

(32) Dhalluin, C.; Boutillon, C.; Tartar, A.; Lippens, G. *J. Am. Chem. Soc.* **1997**, *119*, 10494−10500.

(33) Jelinek, R.; Valente, A. P.; Valentine, K. G.; Opella, S. J. *J. Magn. Reson.* **1997**, *125*, 185−187.