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Methyl Groups as Probes for Proteins and Complexes in In-Cell NMR Experiments

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Abstract: Studying protein components of large intracellular complexes by in-cell NMR has so far been impossible because the backbone resonances are unobservable due to their slow tumbling rates. We describe a methodology that overcomes this difficulty through selective labeling of methyl groups, which possess more favorable relaxation behavior. Comparison of different in-cell labeling schemes with three different proteins, calmodulin, NmerA, and FKBP, shows that selective labeling with [^{13}C]methyl groups on methionine and alanine provides excellent sensitivity with low background levels at very low costs.

Introduction

The recent development of in-cell NMR experiments allows researchers to characterize the conformation and dynamics of proteins and other macromolecules inside their natural environment, the cytoplasm of cells.[1−7] In a previous publication, we investigated the requirements for observing proteins in the bacterial cytoplasm, and we demonstrated that bacteria grown in uniformly ^{15}N-labeled medium do not give rise to significant background signals that obscure the observation of a particular protein.[2] Our in-cell NMR investigations have shown that if an unbound protein reaches a certain threshold level of intracellular expression ($\sim$150 μM),[3] its nuclear magnetic resonances become detectable while other proteins within the cell remain largely unseen because either they are too dilute or they relax too quickly. The sensitivity of in-cell NMR experiments could be further increased approximately 3-fold if methyl groups were used as probes because the three protons coupled to one carbon nucleus offer a greater intrinsic sensitivity than the single amide proton−amide nitrogen pair. Methyl groups offer further additional advantages because of their high mobility and concomitant slow relaxation. Finally, methyl protons, unlike amide protons, do not exchange chemically with water, again yielding higher sensitivity. These features are particularly crucial for studying proteins that exist in larger complexes.[8−11] However, these same advantages, and the abundance of methyl groups in a variety of molecules found within the cell, would likely give rise to high levels of background. In this paper, we describe a strategy for labeling calmodulin, NmerA, and FKBP methyl groups as probes for a variety of in-cell NMR experiments while also minimizing signals of the cellular environment.

Experimental Section

Suspensions of bacteria for in-cell NMR experiments were prepared as described previously by first growing the samples to an optical density of 1.2 in unlabeled LB medium, followed by harvesting and resuspension in minimal M9 medium at an optical density of 1.8. Expression of calmodulin, NmerA, and FKBP was induced by adding 0.4 mM of isopropyl thiogalactoside (IPTG) for 4 h. In the case of full ^{13}C labeling, the M9 medium[12] contained 2 g/L ^{13}C-labeled glucose. Labeling of methyl groups with pyruvate was carried out in M9 medium made with D_2O containing 3 g/L ^{13}C-labeled and protonated pyruvate. For these experiments the bacteria were grown from the beginning in the D_2O/M9/pyruvate medium and were transferred to fresh medium prior to induction. The concentrations of methionine and alanine in the amino acid type-selective experiments were 250 and 100 mg/L,

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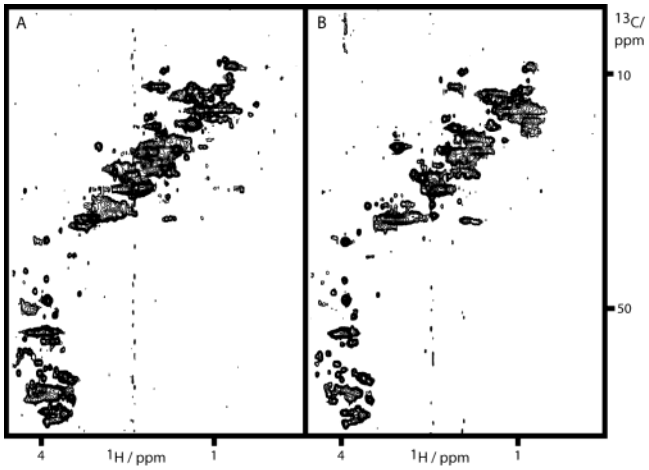

Figure 1. Sections of two $^{13}C,^{1}H$-HSQC spectra taken from a bacterial sample grown on minimal medium containing ^{13}C-labeled glucose with (A) and without (B) overexpression of calmodulin.

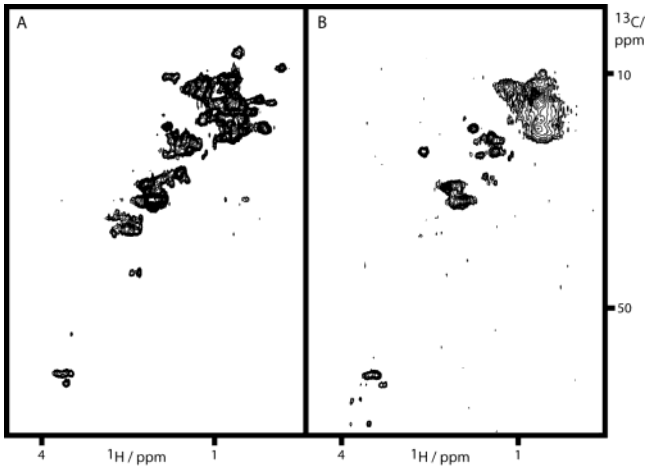

Figure 2. Two $^{13}C,^{1}H$-HSQC spectra of calmodulin measured on minimal medium containing ^{13}C-labeled and protonated pyruvate in D_2O. The bacterial sample in panel A overexpressed calmodulin; the sample in panel B was not induced.

respectively. When alanines were labeled, the M9 medium was supplemented with amino acids and nucleotides according to Cheng et al.;[13] this was later referred to as supplemented M9 medium. For the drug binding studies, 2 mg of phenoxybenzamine hydrochloride (Calbiochem) were added to 50 mL of bacterial culture half an hour before the NMR sample was prepared.

All NMR experiments were measured on a Bruker Avance 500 MHz NMR spectrometer equipped with a cryogenic probe except for the alanine-labeled samples, which were measured with a room-temperature probe. Each spectrum consisted of 1024 complex points in the acquisition dimension and 60 complex points in the indirect dimension. NMR experiments were carried out at 37 °C. Sensitivity comparison with a sample doubly labeled with [^{15}N]lysine and [*methyl*-^{13}C]methionine were carried out by measuring $^{13}C,^{1}H$ and $^{15}N,^{1}H$ heteronuclear single quantum coherence (HSQC) spectra at the same time in interleaved mode.

Results

Full ^{13}C Labeling. To investigate the potential for observing side-chain resonances and in particular methyl groups, we grew a bacterial sample on standard minimal medium containing 2 g/L ^{13}C-labeled glucose and induced calmodulin expression. Figure 1A shows the $^{13}C,^{1}H$-HSQC spectrum of calmodulin in the *Escherichia coli* cytoplasm.

To investigate if the large number of peaks seen in the spectrum are calmodulin peaks or background signals, we repeated this experiment without induction of calmodulin (Figure 1B). The similar patterns of the two spectra suggest that many of the signals are background peaks, most likely from small molecules, rather than calmodulin side-chain resonances. Only in the high-field-shifted region, which does not contain resonances from small molecules, can calmodulin methyl group resonances be identified unambiguously. This very high background level starkly contrasts with the low background level previously observed in $^{15}N,^{1}H$-HSQC-based in-cell NMR applications. The greater abundance of carbon rather than nitrogen in small molecules is the most likely reason for the excessive ^{13}C background level. In addition, many nitrogen-bound protons in these small molecules will exchange very fast with the bulk water, thus effectively broadening their resonances beyond detection. These results demonstrate that full ^{13}C labeling is less useful than ^{15}N labeling for in-cell NMR studies unless resonances with unique chemical shifts (like high-field-shifted methyl groups) are used as probes for the protein.

Selective Labeling with Pyruvate. In previous experiments we were able to obtain background-free NMR spectra when we switched from full ^{15}N labeling to amino acid type-selective labeling, for example with ^{15}N-labeled lysine.[2] We wanted to test whether analogous labeling approaches would decrease the ^{13}C background signal level. Several different labeling schemes have been developed recently that allow for selective protonation of methyl groups in otherwise fully deuterated proteins.[14,15] While originally designed to provide nuclear Overhauser effect (NOE) information for high molecular weight proteins, these schemes could be applied to in-cell NMR applications. Most importantly, a scheme that allows for selective labeling of all methyl groups simultaneously would minimize the costs. Kay and co-workers have published such a method based on the use of protonated and ^{13}C-labeled pyruvate as the sole carbon source in $^{2}H_2O$/M9 medium.[14] They have shown that methyl groups become highly protonated while other side-chain positions remain highly deuterated. Therefore, we tested the effectiveness of pyruvate as the carbon source for in-cell NMR experiments. Panels A and B of Figure 2 compare spectra of *E. coli* grown on M9 medium containing 3 g/L ^{13}C-labeled and protonated pyruvate obtained with and without expression of calmodulin, respectively. While the number of peaks in both spectra is considerably reduced relative to the spectra obtained with glucose-based media, the spectrum measured without induction of calmodulin does show a considerable level of background, consisting mainly of broad, overlapping peaks in the methyl region. In contrast, the peaks obtained with the calmodulin-expressed sample are better defined with a substantially different pattern. Thus, ^{13}C labeling with pyruvate is superior to glucose and the majority of the peaks in Figure 2A are likely to be calmodulin resonances. A quantitative analysis of the spectrum by peak integration is still problematic, however, due to the

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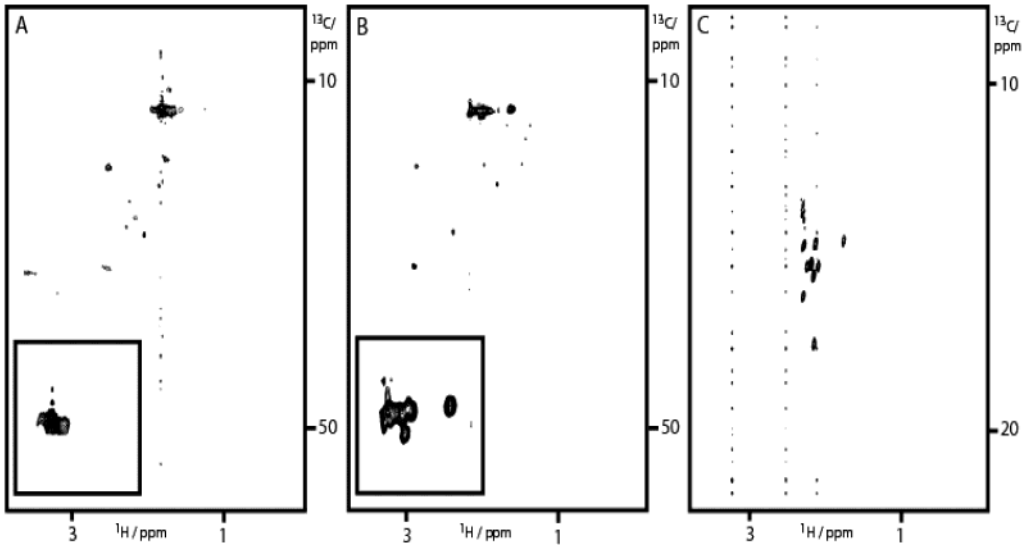

Figure 3. Comparison of background levels in ^{13}C,^{1}H-HSQC spectra of calmodulin samples grown on [*methyl*-^{13}C]methionine without overexpression (A), with overexpression of calmodulin (B), and with a wash step (C). The spectrum in panel C was measured with a smaller spectral width than the spectrum in panel B. Only the free methionine and the nine methionine resonances of calmodulin are visible. The insets in panels A and B show the enlarged peak areas.

overlap of protein resonances with broad background peaks, especially for proteins that reach lower expression levels than calmodulin.

Methionine Labeling. On the basis of this result, we wanted to explore more selective labeling techniques with single amino acid types. Due to their isolated position, methionine methyl groups belong to the slowest relaxing spins in proteins. At the same time, [*methyl*-^{13}C]methionine is considerably less expensive than other methyl group-labeled amino acids. For these two reasons we wanted to explore the possibility of utilizing methionine methyl groups as protein probes for in-cell NMR experiments. Potential background signals could arise from the fact that methionine is a substrate for *E. coli*'s adenosylmethionine synthetase.[16] *S*-Adenosyl-L-methionine, the product of this enzyme, is a methyl donor in a wide variety of reactions, including methylation of DNA, RNA, and proteins. In principle, therefore, *S*-adenosyl-L-methionine holds the potential for a high level of background signal by transferring the ^{13}C-labeled methyl group to many other molecules. Panels A and B of Figure 3 show the comparison of two ^{13}C,^{1}H-HSQC spectra measured with *E. coli* cells that were grown on minimal medium containing [*methyl*-^{13}C]methionine without and with expression of calmodulin, respectively. In the absence of induction, only the very intense peaks of the free methionine and some potential metabolic products with very similar chemical shifts can be observed, demonstrating that the ^{13}C-labeled methyl group of methionine does not create a significant level of background signal. Figure 3B shows that, in addition to the free methionine, only the calmodulin methionine peaks are visible. The spectral width of the spectrum shown in panel C is smaller than the spectra in panels A and B, to better show the expected nine calmodulin peaks.[17]

The peaks of the free methionine and its potential metabolic products in the spectra of Figure 3A,B are the most prominent by far. Very strong peaks in NMR spectra may lead to spectral artifacts and also may overlap with and mask smaller peaks. To test if the amount of free methionine in the in-cell sample could be reduced, the cells were washed in methionine-free minimal medium prior to final sample preparation. The spectrum shown in Figure 3C was obtained with a sample induced in 50 mL of minimal medium containing 12.5 mg of methyl-group-labeled methionine, then harvested and resuspended in 50 mL of methionine-free minimal medium, followed immediately by centrifugation and final sample preparation. The result shows that ~90% of the free methionine (and the other products) can be removed by a single washing step. Longer washing times did not result in significantly better suppression of the free methionine peak, suggesting that most of the free methionine is actually in the extracellular milieu and not contained in the cells.

Detection of methyl groups should significantly increase the sensitivity of in-cell NMR experiments due to the fact that three protons rather than one proton contribute to the signal intensity and due to the slower relaxation. To test if ^{13}C methyl group labeling of proteins indeed increases the sensitivity, we prepared a sample of calmodulin doubly labeled with both [^{15}N]lysine and [^{13}C]methionine and measured both ^{15}N,^{1}H-HSQC and ^{13}C,^{1}H-HSQC spectra simultaneously at 25, 50, and 75 min after induction of protein expression. The three spectra as well as selective traces are shown in Figure 4. In the ^{15}N,^{1}H-HSQC spectrum, only the most flexible lysine that is located at the very C-terminus of calmodulin at 125 ppm is visible in the 25 min spectrum (the strong peak in the spectrum, located at 129 ppm in the ^{15}N dimension, is not a calmodulin resonance[18]). In contrast, the methyl groups of the methionines of calmodulin are all visible even at the earliest time point. Comparison of the intensities at the later time points indicates that detecting methionine methyl groups is indeed roughly 3 times more sensitive than detecting lysine amide protons.

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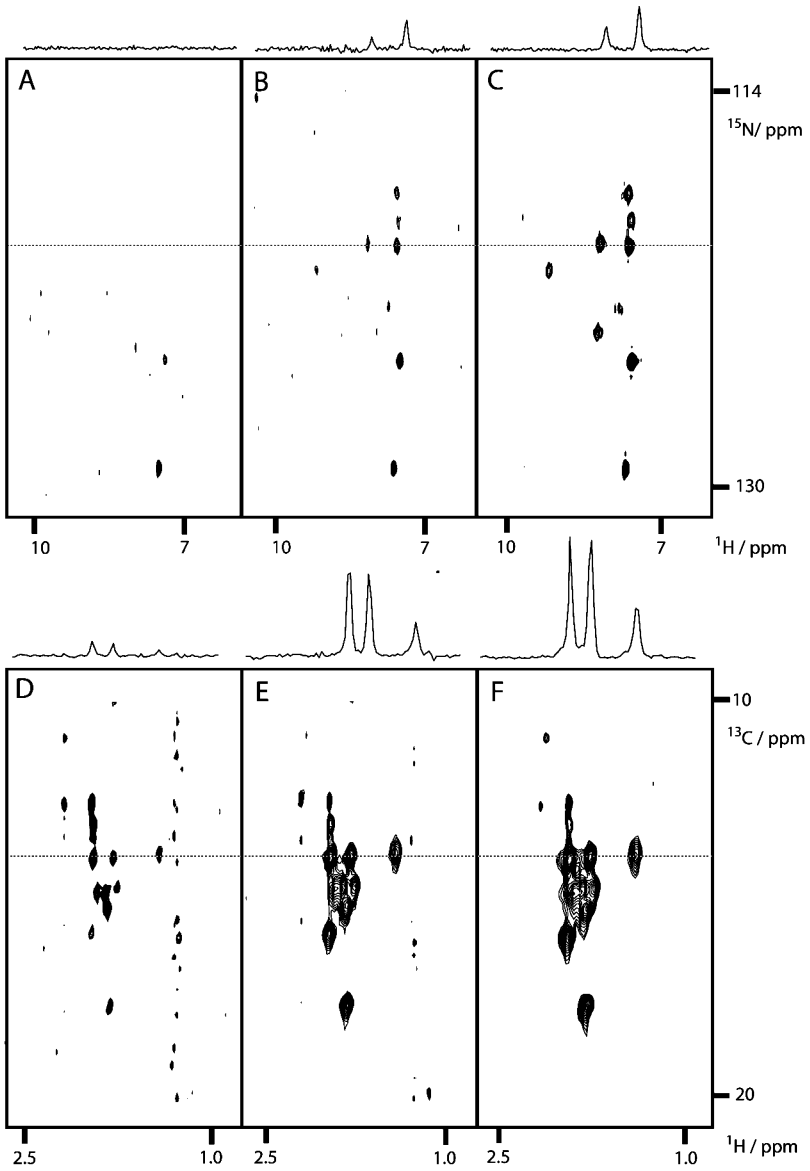

Figure 4. ^{15}N,^{1}H-HSQC spectra (A−C) and ^{13}C,^{1}H-HSQC spectra (D−F) measured with a bacterial sample expressing calmodulin doubly labeled with [^{15}N]lysine and [*methyl*-^{13}C]methionine. Spectra in panels A and D were acquired simultaneously (in an interleaved way) 25 min after induction. Spectra in panels B and E were acquired 50 min after induction. Spectra in panels C and F were acquired 75 min after induction. One-dimensional slices taken at the indicated position are shown above each panel. The strong peak located at 129 ppm in the ^{15}N,^{1}H-HSQC spectrum is not a calmodulin resonance.[18]

Alanine Labeling. The main disadvantage of using methionine as a probe is its low natural abundance in proteins, with just 2.4% of all amino acids. An attractive alternative would be alanine, since it belongs to the most frequent amino acids found in proteins (8.3%), is commercially available in a methyl-group-only ^{13}C-labeled form, and is quite inexpensive. We therefore wanted to test the suitability of alanine for in-cell NMR experiments. Figure 5A shows the ^{13}C,^{1}H-HSQC spectrum of NmerA in the bacterial cytoplasm labeled with [*methyl*-^{13}C]-alanine, and Figure 5B shows the corresponding spectrum of uninduced bacteria, both recorded in standard M9 medium. In addition to strong signals in the methyl region, many additional peaks can be seen throughout the entire spectral width, indicating that significant amounts of background signals exist. This is due to the central role that alanine plays as a precursor in multiple *E. coli* biosynthetic pathways. However, selective labeling with ^{15}N-labeled alanine has been reported with special medium enriched in certain amino acids and nucleotides[13] (supplemented M9 medium). Figure 5C shows a spectrum of NmerA expressed and labeled with [*methyl*-^{13}C]alanine in *E. coli*, and Figure 5D shows a spectrum of the corresponding uninduced control sample grown on the same supplemented M9 medium. The supplemented M9 medium significantly reduces the amount of background signals. Figure 5E shows a section of an in-cell ^{13}C,^{1}H-HSQC spectrum of NmerA measured with a higher resolution, showing the expected 13 alanine peaks of NmerA, the free alanine (highest peak), and a small number of background peaks. These results demonstrate that alanine methyl groups are useful probes for proteins in *E. coli* and that the amount of background peaks can be minimized by use of supplemented M9 medium.

In-Cell NMR of FKBP. On the basis of the results presented above, isotopically labeled methionine and alanine methyl groups are attractive probes for in-cell NMR experiments. As mentioned in the Introduction, methyl groups also offer the advantage of slow relaxation, which combined with their

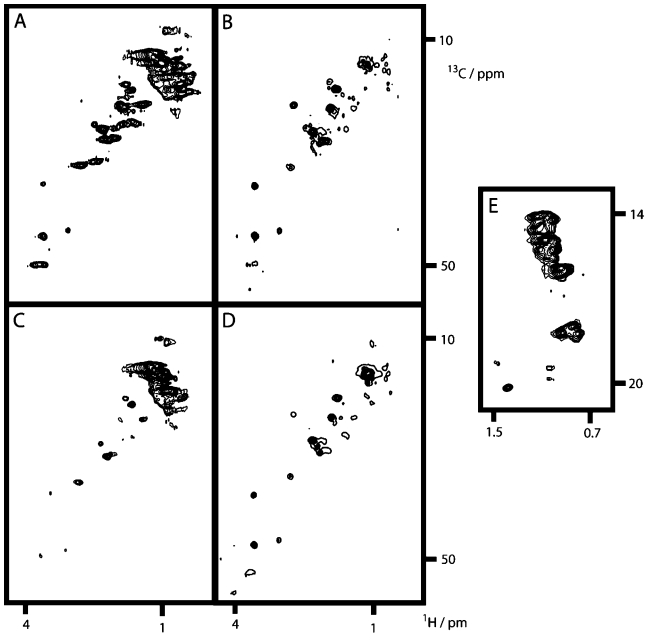

Figure 5. Comparison of in-cell spectra of bacterial samples grown on standard M9 medium (A, B) and supplemented M9 medium (C, D): *E. coli* samples carrying a vector with the NmerA gene with (A) and without (B) induction on standard M9 medium, and *E. coli* samples carrying a vector with the NmerA gene with (C) and without (D) induction on supplemented M9 medium. (E) Section of a ^{13}C,^{1}H-HSQC spectrum of the sample in panel C but measured with a shorter spectral width in the ^{13}C dimension.

intrinsic high sensitivity could make larger complexes become visible. To test this hypothesis, we performed in-cell NMR experiments with the peptidyl−prolyl isomerase FKBP.[19,20] During our initial search for a model system to develop in-cell NMR techniques, we had tested FKBP since it is small, highly soluble, and can be easily overexpressed to high levels in *E. coli* cells. Surprisingly, we were unable to detect any NMR resonances of FKBP in ^{15}N,^{1}H-HSQC experiments with bacterial cells, even with the use of high levels of deuteration and TROSY-based pulse sequences.[21] Disrupting the bacterial membrane by adding lysozyme to the bacterial slurry in the NMR tube, however, allowed us to observe the normal ^{15}N,^{1}H-HSQC of FKBP. This result suggested that FKBP is involved in larger complexes inside the bacterial cell that reduce its tumbling rate sufficiently to broaden its resonance lines beyond the detection limit. Disrupting the cellular membrane releases FKBP from these complexes and makes it observable. Figure 6 shows a ^{13}C,^{1}H-HSQC spectrum of FKBP labeled with methionine in the bacterial cytoplasm. The center of the spectrum contains the methyl group resonances of free methionine as well as some methionine metabolic products. The two very broad peaks indicated by the arrows represent two of the three internal methyl groups of FKBP.[22] A more detailed analysis of these methyl group peaks and the behavior of FKBP inside *E. coli* cells will be given elsewhere. This result demonstrates that the favorable relaxation behavior of methionine methyl groups

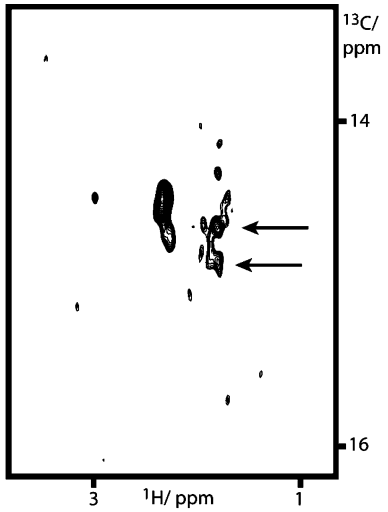

Figure 6. Section of a ^{13}C,^{1}H-HSQC spectrum of FKBP in the bacterial cytoplasm labeled with ^{13}C on the methyl groups of methionines. The very strong peak in the center of the spectrum represents the free amino acid. The arrows mark the resonance positions of two of the three methionine peaks in FKBP.

can indeed be used to observe larger complexes inside cells even if their backbone resonances are unobservable.

Drug Binding. One of the advantages of in-cell NMR experiments is that it can be used to study drug−protein interactions in their natural environment. While amide protons are usually detected in drug binding assays due to their higher chemical shift dispersion, the higher sensitivity of methyl groups would allow researchers to perform these experiments at lower concentrations. For calmodulin, many different drugs have been found that bind to the hydrophobic pocket created by the two halves of the protein.[23] This hydrophobic pocket is lined with methionines, which makes the methionine methyl group an excellent indicator for drug−calmodulin interactions. We expressed calmodulin containing methyl-group-labeled methionine in *E. coli* and added the drug phenoxybenzamine hydrochloride to the minimal medium. In panels A and B of Figure 7, ^{13}C,^{1}H-HSQC spectra of in-cell calmodulin samples without and with phenoxybenzamine are compared. While no differences in chemical shift can be detected, some of the peaks in the spectrum in Figure 7B show increased line broadening, indicating a weak interaction with the drug. This result is in agreement with reports that phenoxybenzamine interacts with calmodulin only in its calcium-bound form[23] and with our own results demonstrating that calmodulin in the bacterial cytoplasm exists mainly in the calcium-free apo form,[3] suggesting that at most a weak interaction should be observable. To investigate if and how much of the drug had been taken up by the bacteria, we harvested the cells by centrifugation and measured a one-dimensional ^{1}H spectrum of the supernatant. As can be seen in Figure 8A, no sign of the drug could be detected. In contrast, the resuspended bacterial pellet showed strong signals of the drug (Figure 8B). Further investigations showed that after cell lysis almost all of the drug was still associated with the cell debris, suggesting that phenoxybenzamine is mainly associated with the bacterial membrane. The high local concentration of phenoxybenzamine near the bacterial membrane is most likely

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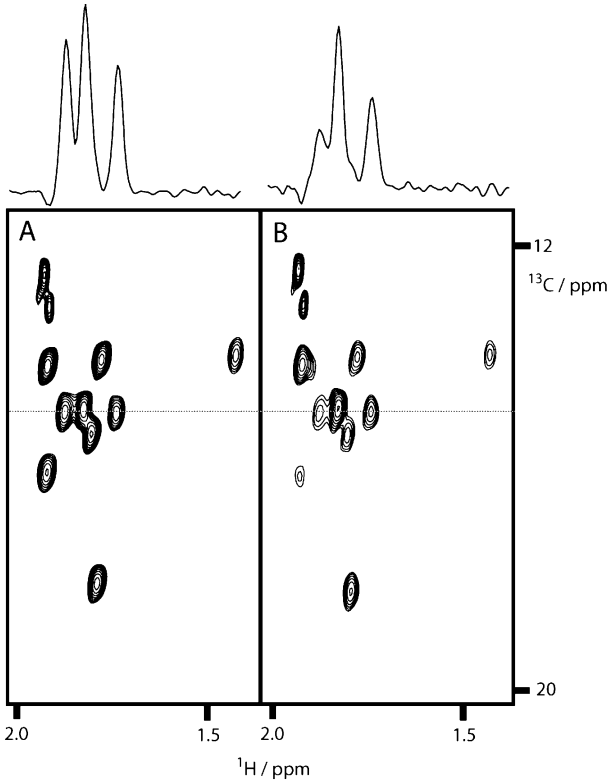

Figure 7. Comparison of two in-cell ^{13}C,^{1}H-HSQC spectra of methionine methyl group-labeled calmodulin. The sample in panel B contained 40 mg/L of the drug phenoxybenzamine hydrochloride while the sample in panel A did not have any additives.

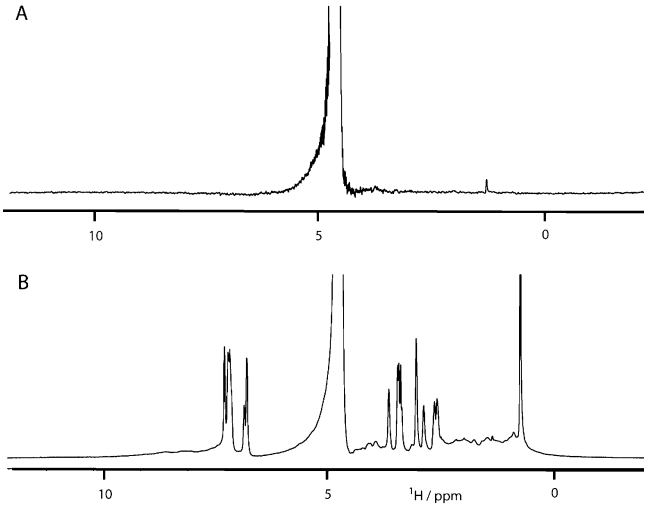

Figure 8. One-dimensional ^{1}H spectra of a bacterial sample expressing calmodulin and containing 40 mg/L of the drug phenoxybenzamine hydrochloride. (A) Supernatant of that sample after harvesting of all cells by centrifugation; (B) resuspended bacteria. The strong resonance lines belong to phenoxybenzamine hydrochloride.

responsible for the observed weak interaction between the calcium-free protein and the drug. While the example reported here—calmodulin expressed in *E. coli*—is not a biologically relevant system, it demonstrates the advantages of in-cell NMR experiments, which are able to detect the protein resonances as well as the drug.

Discussion

The side chains of proteins play the dominant role in defining their functions, conformation, and stability. To study the structure−function relationship of proteins in their intracellular environment by NMR spectroscopy, it is necessary to develop labeling schemes that allow the selective observation of side chains or certain groups of side chains on the protein of interest. In a previous publication we had shown that histidine side chains can be observed by adding labeled histidine to the minimal medium.[24] This allowed us to determine the protonation and tautomerization state of histidines in the intracellular environment. In this paper we have extended these side-chain labeling schemes for in-cell NMR applications to methyl groups. In principle, the detection of methyl groups should be 3 times more sensitive than the detection of amide protons. This ratio, which is based on the number of protons attached to the heteronucleus, is further increased by the slow relaxation of the methyl groups and the fact that they do not chemically exchange with the water. During previous investigations we had estimated that the detection limit for a protein in the intracellular environment is approximately 150 μM.[3] This paper demonstrates that this detection limit extends to much lower levels when methyl groups are used as probes ($\sim$50 μM). Furthermore, for proteins that form large complexes with other cellular components and that cannot be detected by amide-proton-based experiments, methyl-group labeling is an attractive alternative to study their intracellular behavior.

Methyl groups can also be used to investigate the interaction of proteins with drugs in the cellular cytoplasm.[3,7] A recent investigation has found that within a set of 191 crystal structures of protein−ligand complexes 92% of the ligands had a heavy atom within 6 Å of a methyl group, while only 82% had a heavy atom within the same distance of an amide proton.[25] This fact, combined with the considerably higher sensitivity, makes methyl groups very attractive alternatives to amide protons for monitoring protein−drug interactions by in-cell NMR experiments.

The most significant drawback of methyl groups is their—in comparison to amide protons—narrow chemical shift dispersion, which in in-cell NMR experiments is further reduced by the broader line width as compared to in vitro studies. One possible solution to this problem is the reduction of the total number of resonances through selective labeling schemes. In in-cell NMR experiments, the use of selective labeling protocols has the additional advantage of minimizing background signals. In the case of full ^{13}C labeling, the high level of background signal prevents (with the exception of the most high-field-shifted methyl groups) the unambiguous identification of proton methyl group resonances. In contrast, labeling with [*methyl*-^{13}C]methionine produces virtually background-free spectra. [*methyl*-^{13}C]-Alanine also proved to be useful for detecting proteins in the bacterial cytoplasm. Although the alanine spectra show a much higher background signal level than the methionine spectra, the abundance of alanine compared to methionine in proteins makes alanine a very useful probe.

Methionine and alanine advantageously combine low background signal with low labeling costs. In recent years, additional labeling schemes for the methyl groups of valines, leucines, and isoleucines have been proposed based on common precursors, mainly for structural investigations of very large proteins. The use of pyruvate has demonstrated the usefulness of more

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selective labeling schemes by reducing the background level. However, a significant amount of background remained and the labeling costs were considerably higher than the labeling costs with methionine or alanine. These costs would be even higher for the use of α-ketobutyrate or α-ketoisovalerate as precursors for labeling the methyl groups of isoleucines or valines and leucines. An additional problem with labeling schemes that produce more than one labeled amino acid is the higher density of signals that, due to the larger line width of the methyl signals in the intracellular environment, would result in more overlap. Labeling schemes with single amino acids are therefore the most attractive approach for in-cell NMR applications. In addition to methionine and alanine, the δ-methyl group of isoleucine would also be very interesting due to its slow relaxation behavior. Unfortunately, isoleucine selectively ^{13}C-labeled on the δ-methyl group is not commercially available and has to be produced through methyl-group-labeled α-ketobutyrate. However, preliminary experiments with fully ^{13}C-labeled isoleucine have shown that the background signal level would be minimal (data not shown).

Amino acid type-selective labeling techniques with methyl groups have an additional very interesting application: extending in-cell NMR experiments to eukaryotic cells such as yeast, insect cells, or even mammalian cells would be very attractive. However, commercially available media for isotopic labeling of eukaryotic cells are prohibitively expensive. Adding selected labeled amino acids such as [*methyl*-^{13}C]methionine to unlabeled medium for eukaryotic cells creates a very cost-efficient method to observe proteins in these cell types. Initial experiments with insect cells have indeed confirmed that adding [*methyl*-^{13}C]-methionine to the normal medium does not create a significant background signal level (work in progress).

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