Communication

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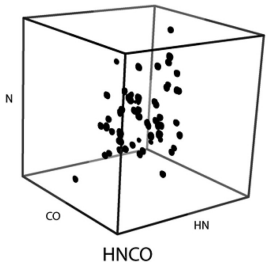
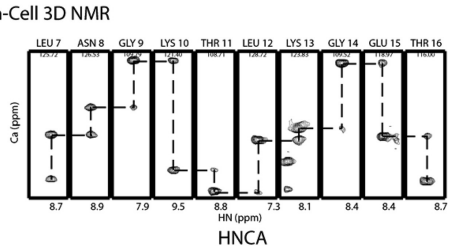


HNCO



HNCA

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Multidimensional NMR Spectroscopy for Protein Characterization and Assignment inside Cells

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The crowded and complex environment in which macromolecules reside within cells can influence their physical properties and, as a consequence, their biological function. This has motivated recent efforts to extend NMR spectroscopy to macromolecular characterization directly in cells. In-cell NMR has the potential to provide specific spectral signatures and structural information, both of which report on molecular properties and intermolecular interactions of cellular proteins which can be associated with protein function. We have completed the backbone resonance assignment of a recombinant protein, GB-1, in-cell without further purification using three fast 3D heteronuclear NMR experiments performed directly on the whole cell slurry.

Recent reports of two-dimensional heteronuclear spectra in whole cells[1−4] indicate that NMR can provide characteristic "fingerprints" for proteins in cells. To date, in-cell NMR spectroscopy of macromolecular components has been confined, however, to 1D and these 2D heteronuclear single quantum coherence (HSQC) experiments, due to inherent sensitivity limitations and time constraints associated with preserving viable cells for study at high field in a typical NMR sample tube. While these experiments show that proteins can be observed in-cell and are useful in some cases in establishing whether the protein is folded, unfolded, or perturbed in this environment, they do not provide sufficient data to assign spectral features de novo. This is a critical step in characterizing macromolecules that have not previously been assigned in vitro or that exhibit conformational changes or intermolecular interactions in the in vivo environment. Assignment of NMR spectra of proteins in-cell requires isotope labeling and heteronuclear 3D NMR experiments even for smaller polypeptides. Conventional methods for these experiments, however, are generally incompatible with in vivo spectroscopy due to the long acquisition times.

Two new advances in NMR have helped to mediate this constraint. The recently developed reduced dimensionality NMR techniques, such as projection reconstruction[5−7] and GFT,[8,9] have substantially increased the acquisition efficiency for multidimensional NMR data and consequently reduced the time required for these experiments. In addition, the development of cryogenic probes has substantially increased the signal-to-noise ratio in the detector, resulting in enhanced sensitivity of NMR experiments. This reduces the number of FIDs required for each data set, allowing shorter acquisition times. Here we report results from three 3D heteronuclear NMR experiments which we have used with cold-probe detection to acquire the data necessary to complete the de novo assignment of the protein backbone of the polypeptide GB-1 in-cell. To our knowledge this is the first report of full backbone assignments for any protein in-cell.

All experiments incorporated data acquisition and processing by the projection reconstruction method.[5−7] With this approach, a 3D spectrum is constructed from data collected radially in the time domain along two sets of orthogonal axes and along additional axes through the origin at selected tilt angles. As with other reduced dimensionality techniques, the t_1 and t_2 evolution times are simultaneously incremented. In practice the data are acquired as 2D data sets, shortening the overall experiment considerably if a limited number of tilt angles are used. The tilt angles sampled, α, are determined by setting the rates at which the evolution times are incremented, which results in planes at $\pm\alpha$ angles for each 2D data set.[5] Signals in the reconstructed 3D spectrum are determined by the intersection of projections from data present in all 2D planes sampled or data contained in all combinations of preselected groupings of the planes.[10]

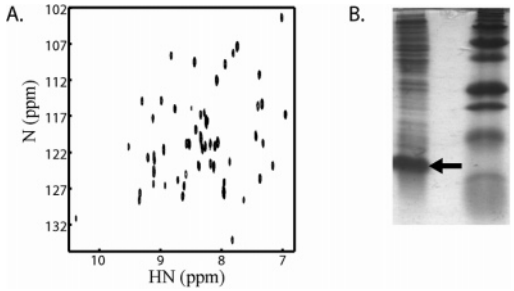

Figure 1. (A) 3D projection reconstruction HNCA spectrum of GB-1 projected along the Ca axis onto the HN,N plane. This spectrum was acquired on a ~20% cell slurry with four scans on the HN, N orthogonal and eight scans at the other angles with a total acquisition time of ~2 h. The final resolution was 407 × 128 × 128. The spectral widths and complex points acquired were 8000 Hz, 1024 points in the direct dimension; 4000 Hz, 64 points in the HN−Ca indirect dimension; and 2100 Hz, 64 points in the HN−N indirect dimension. (B) SDS−PAGE gel showing overexpression of GB-1. The right lane is the molecular weight marker, and the left is a whole cell sample with the GB-1 band identified.

GB-1 is a 56-residue immunoglobulin (IgG) binding domain from the streptococcal protein G. It is commonly used as a model protein for NMR because of its small size and stability. We produced uniformly ^{15}N- and ^{13}C-labeled GB-1 by overexpression in BL21-DE3 cells using the T7 promoter and IPTG induction in 50 mL of fresh minimal media containing [^{15}N]NH_4Cl and [^{13}C]glucose (Cambridge Isotopes, Andover, MA) as sole sources of nitrogen and carbon. After 6 h, the yields of uniformly labeled GB-1 were typical of those observed for GB-1 under the control of the T7 promoter,[11] as illustrated in Figure 1. We estimate the GB-1 produced is labeled at levels >95% with this procedure. For NMR studies, the cells were harvested by centrifugation at 1000*g*, resuspended in ~700 μL of 10% D_2O minimal media, and transferred into a 5 mm NMR tube as a slurry with a density of ~20 vol %. Spectra were collected immediately at 600 MHz on a Varian Inova spectrometer equipped with an H,C,N triple-resonance cold probe. NMR data were processed with NMRpipe,[12] and 3D spectra were reconstructed using software developed by Coggins and Zhou (unpublished software).

The assignment of GB-1 was accomplished using data from the 3D projection reconstruction versions of the HNCA, HNCO, and HA(CA)NH experiments. HNCA is one of the most commonly used

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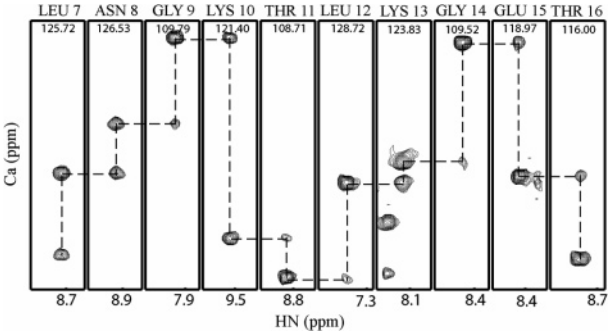

Figure 2. HNCA strip plot showing scalar couplings between the HN and the Ca carbons in the *i* and $i - 1$ positions for residues 7−16. The *y*-axis is ^{13}C chemical shift. The ^{15}N chemical shift for each plane is noted at the top of the strip.

experiments in the sequential assignment process. In this case, we collected an HNCA data set on the bacterial cell sample at 600 MHz in a 5 mm NMR tube in ~2 h, a time frame compatible with cell survival. Figure 1A shows the 3D spectrum projected along the carbon axis. The spectrum is identical to the 10 min 2D ^{15}N-HSQC spectra we have collected in-cell and on purified GB-1 (data not shown). All GB-1 resonances were observed in Figure 1. In the Ca dimension, all *i* and $i - 1$ resonances were also observed, although there are three residues where the Ca chemical shifts are identical. The data were collected with two orthogonals and five tilt angles, at 15°, 30°, 45°, 60°, and 75°, yielding 12 projected planes. We found that five tilt angles were sufficient to observe all of the weaker $i - 1$ resonances for GB-1, with reasonable signal-to-noise and an acceptable number of artifacts. Using more then five tilt angles would have unnecessarily increased the acquisition time, while only three additional angles in this case were not sufficient to observe all $i - 1$ resonances. Usually assignment is done using pairs of experiments such as HNCA and HNCACB because the Ca resonances will often exhibit limited chemical shift dispersion, leading to ambiguous assignments. We chose to use the more sensitive 3D PR−HA(CA)NH experiment to solve the Ca ambiguities on the basis of Ha chemical shifts. This strategy has the additional benefit of providing the data for assignment of Ha resonances. Data collection required ~3 h with the same number of planes as HNCA. Using the Ha chemical shifts to resolve the Ca ambiguities, we were able to sequentially assign the backbone N, HN, Ca, and Ha chemical shifts of GB-1. A sample strip plot associated with the sequential assignment of residues L7−T16 is shown in Figure 2. The remaining backbone CO chemical shifts were obtained using the 3D PR−HNCO experiment. HNCO is more sensitive than HNCA, allowing us to complete the experiment in ~1 h.

These three experiments were sufficient to assign the backbone of GB-1. While the HACANH experiment is suitable for small proteins, such as GB-1, it becomes impractical for larger proteins where deuteration may be necessary or where poor dispersion in Ha generally precludes its use. Large proteins will likely require a different experiment, such as HNCACB or HNCACO, for sequential assignment. In addition, larger proteins may need an experiment that yields only $i - 1$ resonances, such as HNCOCA, to identify peaks where *i* and $i - 1$ resonances are superimposed. In the case of GB-1, the identification of the three substantially unresolved Ca resonances of this type became obvious during assignment. We are currently exploring these additional experiments for in-cell applications.

We have also examined the extent to which protein outside the cells may contribute to the spectra observed. Such peripheral protein might arise from "leaky" cells and accumulate during the longer experiments. Since GB-1 is an IgG binding domain, we were able to use antibodies as extracellular scavengers to sequester any protein outside the cells. In vitro measurement of the ^{15}N-HSQC spectra of GB-1 in the absence and in the presence of generic IgG demonstrated that the characteristic fingerprint resonances were not observable with added antibody, presumably due to the larger molecular mass of the bound complex (data not shown). When IgG was added to in-cell samples prepared for an HNCA experiment, there was no alteration in the spectrum, even at antibody saturation concentrations of 5 mg/mL, indicating that the observed spectrum indeed originates from inside the cell.

In-cell ^{15}N-HSQC NMR spectra have previously been demonstrated to be relatively free from background resonances.[13] However, in our experiments we did observe a small number of cross-peaks that did not correspond to GB-1. These are likely small-molecule metabolites which incorporated the stable isotope labels but exhibit no connectivities to assigned residues. We were pleased to find that the projected spectra were also relatively free of reconstruction artifacts, permitting full backbone assignment with five tilt angles.

We have demonstrated the de novo NMR backbone assignment of the small protein GB-1 from data collected on whole *Escherichia coli* cells. We have accomplished this by combining the advantage of increased sensitivity in cryogenic probes with the fast projection reconstruction data acquisition technique. This marks the opening of the previously inaccessible areas of in-cell protein assignment and 3D heteronuclear NMR study of proteins in their native environments.

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