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High-Resolution Heteronuclear Multidimensional NMR of Proteins in Living Insect Cells Using a Baculovirus Protein Expression System

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Supporting Information

ABSTRACT: Recent developments in in-cell NMR techniques have allowed us to study proteins in detail inside living eukaryotic cells. In order to complement the existing protocols, and to extend the range of possible applications, we introduce a novel approach for observing in-cell NMR spectra using the sf9 cell/baculovirus system. High-resolution 2D $^{1}H-^{15}N$ correlation spectra were observed for four model proteins expressed in sf9 cells. Furthermore, 3D triple-resonance NMR spectra of the *Streptococcus* protein G B1 domain were observed in sf9 cells by using nonlinear sampling to overcome the short lifetime of the samples and the low abundance of the labeled protein. The data were processed with a quantitative maximum entropy algorithm. These were assigned *ab initio*, yielding approximately 80% of the expected backbone NMR resonances. Well-resolved NOE cross peaks could be identified in the 3D ^{15}N-separated NOESY spectrum, suggesting that structural analysis of this size of protein will be feasible in sf9 cells.

In living cells, a variety of soluble macromolecules exist in a very crowded environment, thus influencing the kinetics and thermodynamics of protein folding and various binding events.[1] *In situ* observation of proteins is therefore indispensable for explicit understanding of the functions of proteins inside cells.

In-cell NMR is currently the only approach that can provide structural information of proteins inside cells at atomic resolution. Since in-cell NMR was first applied to proteins inside *E. coli* cells,[2] this has been used to detect conformational changes, dynamics, interactions, and high-resolution 3D structures in living bacterial cells.[3−10] Recently, bacterial in-cell NMR has been used for investigating molecular crowding effects and to gain new insights about protein dynamics, structural stability, and diffusion in the crowded intracellular environment.[11,12]

In eukaryotic cells, in-cell NMR studies were first performed by injecting proteins into *Xenopus laevis* oocytes or eggs.[13,14] For cultured mammalian cells, cell-penetrating peptides[15] and pore-forming toxin[16] have instead been used to deliver proteins. These approaches enabled *in situ* observations of post-translational modification,[17] protein−ligand interactions, and protein folding stability[15] in eukaryotic cells. Applications in drug development are also anticipated.[18] In comparison to the bacterial in-cell NMR protocols, in which proteins of interest are overexpressed in the host cells, these protocols for eukaryotic cells have the advantage that the resulting spectra are background-free. However, they require relatively large quantities of purified and concentrated proteins, thus preventing their application to proteins that are difficult to purify and/or unstable. Alternative approaches utilizing the intrinsic protein expression systems of the host cells have therefore been awaited. Very recently, Bertrand et al. reported the observation of in-cell NMR spectra of ubiquitin heterologously expressed in *Pichia pastoris*,[19] which provided new insights into the properties of yeast intracellular vesicles. In this Communication, we demonstrate eukaryotic in-cell NMR in the sf9 cell/baculovirus system. Expression in insect cells has the ability to produce and fold proteins of higher eukaryotic species correctly, and is widely used for efficiently obtaining $^{13}C/^{15}N$-labeled proteins.[20]

As model systems, we used four proteins, *Streptococcus* protein G B1 domain (57 a.a., henceforth referred to as GB1), *T. thermophilus* HB8 TTHA1718 (66 a.a.), rat calmodulin (148 a.a., henceforth referred to as CaM), and human HAH1 (68 a.a.). Baculoviruses encoding each gene were constructed using the Bac-to-bac system (Invitrogen). For culturing sf9 cells and ^{15}N- or $^{13}C/^{15}N$-labeling, we used Sf900II (GIBCO) containing 10% FBS, penicillin, and streptomycin (henceforth referred to as Sf900II+) and Bioexpress 2000 (C.I.L.) media with 10% FBS (henceforth referred to as B.E.2000+), respectively.

The experimental scheme is presented in Supporting Information Figure S1. Since there is a time lag of hours from the inoculation of a baculovirus to the protein expression,[21] the timing of the replacement Sf900II+ with labeled B.E.2000+ was

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optimized so as to achieve the best contrast between the signals of the target proteins and the background. This optimization substantially improved the quality of the spectra without decreasing the labeling efficiency (Figures S2 and S3). Next we examined the stability of the sf9 cells. The virtual identity of the spectra recorded immediately after sample preparation and after 3.5 h in an NMR tube at 28 °C shows that sf9 in-cell NMR samples are stable for at least 3.5 h (Figure S4). The viability of the sf9 cells after 3.5 h of NMR measurements was confirmed to be 90 ± 3% (GB1), 87 ± 3% (TTHA1718), 83 ± 5% (CaM), and 86 ± 1% (HAH1) by trypan-blue staining. The expression of these proteins was found to be cytoplasmic (Figure S5).

Figure 1a shows the 2D ^{1}H−^{15}N HSQC spectrum of sf9 cells expressing GB1. The concentration of GB1 in the sf9 samples

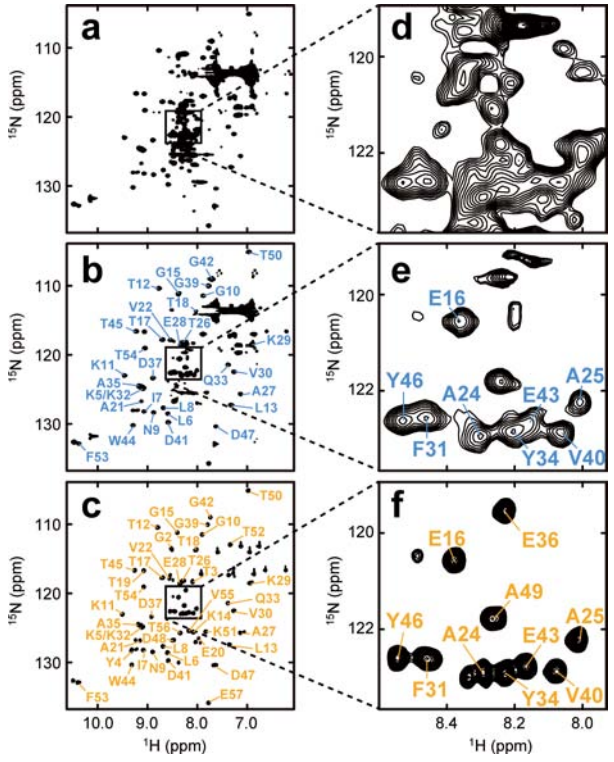

Figure 1. (a) 2D ^{1}H−^{15}N HSQC spectrum of ^{15}N-labeled sf9 cells expressing GB1. (b) 2D difference spectrum produced by subtracting the background signals from the spectrum shown in (a). (c) 2D ^{1}H−^{15}N HSQC spectrum of the purified ^{15}N-labeled GB1. A region of the spectra shown in (a−c) is magnified in (d−f), respectively. In panels b, c, e, and f, cross peaks are labeled with their corresponding residue assignments.

was estimated to be ∼200 μM by SDS-PAGE. Most of GB1 cross peaks were well-resolved and showed virtually identical chemical shifts in comparison with the *in vitro* spectrum (Figure 1c), suggesting the proper folding of GB1 in the sf9 cells. In order to remove the background signals, we prepared a baculovirus in which no genes were expressed from the polyhedrin promoter, and then measured spectra of sf9 cells inoculated with this "reference" virus (Figure S6b). The background signals due to endogenous or baculovirus-derived proteins in the "reference" spectra were observed with high reproducibility (Figure S7). By subtracting one of the "reference" spectra from the "protein-expressing" spectrum, the background was successfully removed (Figure 1b), and even GB1 cross peaks that were buried within the high background regions could be identified (Figure 1d−f).

It is crucial in in-cell NMR studies to ensure that the proteins observed in the spectra are indeed inside the living cells.[22,23] Most ^{1}H−^{15}N HSQC cross peaks disappeared upon removal of the sf9 cells by gentle centrifugation after the measurement shown in Figure 1a (Figure S4f), whereas the lysate of the harvested cells yielded a spectrum similar to the in-cell NMR spectrum (Figure S4d). These results were corroborated by SDS-PAGE (Figure S4g), demonstrating that the contribution of extracellular proteins to the observed signals is negligible.

By employing a virtually identical experimental scheme, 2D ^{1}H−^{15}N HSQC difference spectra of TTHA1718 (Figure 2a), HAH1 (Figure 2b), and CaM (Figure 2c) were obtained. The 2D ^{1}H−^{15}N HSQC in-cell NMR spectra before the background subtraction are shown in Figure S2d, f, and h, respectively.

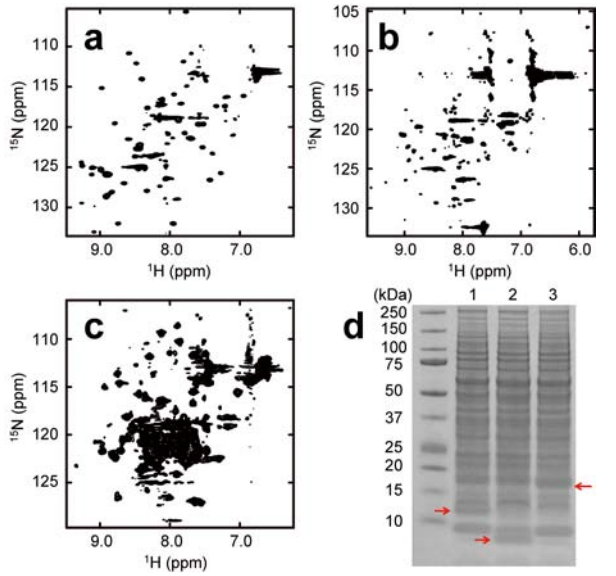

Figure 2. 2D ^{1}H−^{15}N HSQC spectra of ^{15}N-labeled sf9 cells expressing TTHA1718 (a), HAH1 (b), and CaM (c). In each spectrum, background signals have been subtracted. (d) SDS-PAGE with Coomassie staining performed on the TTHA1718, HAH1, and CaM in-cell NMR samples (corresponding to lanes 1, 2, and 3, respectively). Red arrows indicate the positions of these three proteins.

All of the eukaryotic in-cell NMR studies reported so far have utilized resonance assignments, which were transferred from those obtained *in vitro*. If the proteins of interest experience large structural changes in cells, large chemical shift changes must be expected, which would hinder the transfer of *in vitro* assignments to in-cell spectra. The possibility to perform detailed analysis in sf9 cells relies on whether resonance assignments can be obtained from the in-cell spectra. Therefore, we recorded three 3D triple-resonance NMR experiments (HNCA, HN(CO)CA, and HNCO) for backbone resonance assignment of ^{13}C/^{15}N-labeled sf9 cells expressing GB1. Since a drastic reduction of the experimental time is necessary because of the short lifetime of the live cell samples, all the 3D NMR experiments were measured with a nonlinear sampling scheme in the indirect dimensions.[10,24−26]

For the data processing, we used a novel Quantitative Maximum Entropy (QME) reconstruction[27−30] (to be published elsewhere). This provides improved results in comparison to the maximum entropy (MaxEnt)[31] reconstruction in the Azara v2.8 software (W. Boucher, www.bio.cam.ac.uk/azara). In particular, while one has to arbitrarily determine the Lagrange multipler, λ,

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in the original MaxEnt reconstruction, in the QME calculation one determines it by seeking the extremum of an approximated conditional probability distribution of the experimental data given λ. Hence, this achieves the optimal λ for an entire spectrum, ameliorating some of the problems faced when reconstructing spectra such as those in which there is wide dynamic range, as is often the case in in-cell spectra (Figures S8 and S9).

With these techniques, the duration of each 3D experiment was reduced to ~3.5 h. A fresh in-cell NMR sample was prepared for each experiment. Selected $^{1}H-^{13}C$ strips from the HNCA, HN(CO)CA, and HNCO spectra are shown in Figure 3.

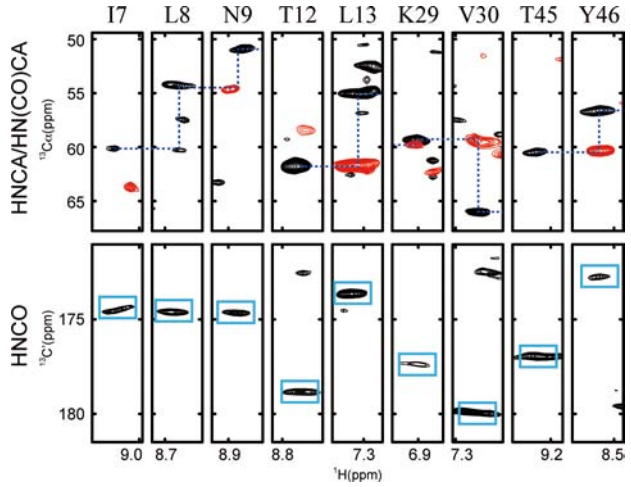

Figure 3. Selected $^{1}H^{N}-^{13}C$ strips extracted from the 3D HNCA (black) and HN(CO)CA (red) spectra (overlaid, upper panel) and the 3D HNCO spectrum (lower panel) of sf9 cells expressing GB1. Each strip corresponds to the ^{15}N frequency of the residue indicated. In the HNCA/HN(CO)CA spectra, sequential connectivities are represented by dashed blue lines. In the HNCO spectrum, cross-peaks due to inter-residual correlations are indicated by cyan boxes.

In the HNCA spectrum, 40 out of 56 (71%) intra-residue and 13 out of 55 (23%) inter-residue correlations could be identified. Additionally, 20 (total 33 out of 55 (60%)) inter-residue correlations were identified in the HN(CO)CA spectrum. Consequently, we unambiguously assigned 44 out of 56 (78%) backbone ^{1}H, $^{13}C^{\alpha}$, and ^{15}N resonances of GB1 in living sf9 cells (Figure 2a). In the HNCO spectrum, 44 out of 55 possible inter-residue correlations were identified. The backbone resonances assigned are summarized in Figure S10 and Tables S1 and S2. Small chemical shift differences between *in vitro* and in-cell are found in the loop regions (Figure S11), and may be due to the effects of viscosity and molecular crowding in the cytosol.

The nonlinear sampling scheme and QME processing were also used for 3D triple-resonance experiments for side-chain assignments as well as 3D nuclear Overhauser enhancement spectroscopy (NOESY) experiments. Experiments for side-chain assignments showed very poor results (data not shown) mainly due to the rapid relaxation of transverse ^{1}H and/or ^{13}C magnetization, due to the increased rotational correlation time of proteins caused by the high viscosity of the cytosol.[22,32] In contrast, NOESY experiments showed more favorable spectra. Figure 4 shows some of the $^{1}H^{N}-^{1}H$ cross sections from the 3D ^{15}N-separated NOESY spectrum of GB1-expressing sf9 cells. Even with the short duration (~3.5 h) experiment, many well-resolved cross peaks were observed. In addition to sequential $^{1}H^{N}-^{1}H^{N}$ NOEs, well-resolved cross peaks could be observed at

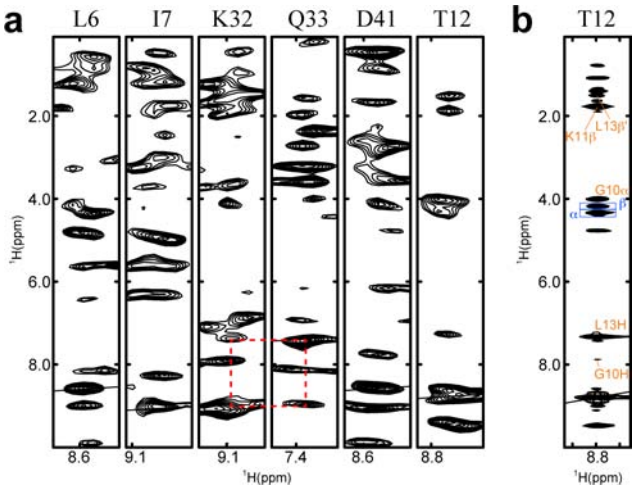

Figure 4. (a) Selected $^{1}H^{N}-^{1}H$ cross sections extracted from the 3D ^{15}N-separated NOESY spectrum of sf9 cells expressing GB1. Each strip corresponds to the ^{15}N frequency of the residue indicated. Sequential $^{1}H^{N}-^{1}H^{N}$ NOEs are represented with dashed red lines. (b) $^{1}H^{N}-^{1}H$ cross-section (corresponding to the ^{15}N frequency of the Thr12 residue) of the same experiment measured *in vitro*. The cross peaks due to inter-residue NOEs are assigned in orange. Intra-residue NOEs are indicated by blue boxes and annotated.

positions similar to those in the *in vitro* spectrum, suggesting the feasibility of collecting NOE-derived distance restraints for proteins in sf9 cells.

The cost of growth media is an issue when labeling proteins in eukaryotic cells, even though only 5 mL of culture is needed for one in-cell NMR sample. Commercially available media for labeling are expensive, and usually their compositions are undocumented. Further cost reduction would be possible with more cost-effective cell culture media.[33,34]

In conclusion, we have developed a novel approach for eukaryotic in-cell NMR experiments using the sf9 cell/baculovirus system. This provides the tools needed to study proteins that are unstable and therefore difficult to purify or proteins that are difficult to express in prokaryotic protein expression systems to be investigated in eukaryotic cells. Eukaryotic proteins that require post-translational modifications will also be good candidates for this approach. Recently, solid-state NMR has been used to investigate proteins in bacterial cells,[35,36] and in one publication integral membrane proteins as well as endogenous membrane-associated molecular components, such as carbohydrate species, were investigated.[36] Although there are some differences—with some modifications being distinct (e.g., glycosylation), and some aberrant modifications due to overexpression of foreign proteins (e.g., phosphorylation)—the use of the sf9 cell/baculovirus system may permit the *in situ* observation of mammalian membrane proteins with authentic glycosylation by employing transgenic insect cell approaches.[37]

A large majority of backbone resonances of GB1 in sf9 cells were assigned exclusively from the in-cell NMR spectra, which is, to the best of our knowledge, a world-first achievement in eukaryotic cells. Here the QME data-processing played a crucial role in producing improved quality spectra from 3D NMR data with reduced sampling points.

Furthermore, a 3D ^{15}N-separated NOESY spectrum of relatively good quality could be measured. Conventionally, the interpretation of NOEs needs side-chain resonance assignments,

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which were difficult to obtain. However, an alternative computational approach involving simultaneous structure calculations exclusively from 3D NOESY spectra[38] has been proposed for backbone and side-chain resonance assignment. Indeed, this has proved to be useful for filling gaps in the side-chain resonance assignments of proteins in *E. coli* cells (data not shown). This automated approach might make in-cell protein structure determination from NOE-derived distance restraints in living sf9 cells possible. As has proven to be the case in *E. coli* cells,[9] methyl-selective protonation,[39] which will be available by using media containing regioselectively $^{2}H/^{13}C/^{15}N$-labeled amino acids, is expected to have a large impact on the feasibility of such structural analysis in the future.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and methods and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

[§]On leave from Ziosoft Inc.

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