High-Resolution Macromolecular NMR Spectroscopy Inside Living Cells

Zach Serber,[†] Adrian T. Keatinge-Clay,[†] Richard Ledwidge,[‡] Alexander E. Kelly,[†] Susan M. Miller,[‡] and Volker Dötsch*[,‡,§]

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

Received November 2, 2000
Revised Manuscript Received January 10, 2001

The role of a protein inside a cell is determined by both its location and its conformational state. While GFP-labeling[1,2] and other powerful fluorescence techniques are widely used to determine the cellular localization of proteins in vivo, these approaches cannot provide detailed information about a protein's three-dimensional state. In principle, NMR spectroscopy is capable of filling this gap and providing information about a protein's conformation inside living cells. While in vivo NMR experiments have been conducted in living organisms ranging from bacteria to humans, the majority of these studies has been limited to examining metabolites (e.g. glucose and ATP) containing NMR-active isotopes, often added extrinsically.[3−6] Theoretically, high-resolution in vivo NMR on macromolecules should be feasible since the rotational diffusion of proteins inside eucaryotic cells is only twice that in water.[7,8] Consequently, many of the proteins currently studied by conventional NMR spectroscopy should be amenable to in vivo NMR.

To test the feasibility of high-resolution NMR spectroscopy of macromolecules in living cells, we have chosen the metal-binding domain of the Tn*501* mercuric ion reductase (MerA) as a model system. MerA is the key protein in the most common bacterial pathway for detoxification of mercurials[9] and is expressed cytoplasmically at levels up to 6% of soluble protein upon induction of the *mer* operon with $HgCl_2$ or organomercurials.[10] MerA proteins from Tn*501* and *E. coli* are highly conserved in sequence and consist of a large, multidomain catalytic core tethered to a small 7 kDa, N-terminal metal-binding domain (NmerA)[11] that we have used in the current study.

To observe a protein in vivo by NMR spectroscopy, its resonances must be separated from the signals produced by every other molecule within the cell. Gronenborn and Clore have shown that ^{15}N-labeled protein signals can be discriminated in *E. coli* cell lysates after removal of the insoluble cell debris and buffer exchange.[12] To distinguish proteins in vivo we have used a combination of selective ^{15}N labeling and overexpression. *E. coli* cells harboring an overexpression plasmid for NmerA were first grown in unlabeled LB medium. Protein production was induced following transfer of the bacteria into ^{15}N-labeled minimal medium. The cells were harvested by gentle centrifugation and placed as a 15−30% slurry into an NMR tube. Figure 1A shows a [^{15}N,^{1}H]-HSQC experiment conducted on this bacterial sample in less than 10 min. This figure demonstrates that high-resolution NMR spectra can be obtained from overexpressed proteins in living bacterial cells. As a comparison, Figure 1B shows an in vitro spectrum of a purified NmerA sample measured with identical parameters. The peak patterns in both spectra are very similar, suggesting that the three-dimensional structures of the protein in vivo and in vitro are nearly identical. Small differences in chemical shifts are, however, observed for residues in the metal-binding loop[13] of NmerA, possibly reflecting differences in the salt composition of the in vitro NMR buffer and the cytoplasm. The in vivo spectrum also contains several strong peaks that are completely absent from the in vitro spectrum. These strong peaks show a much narrower line width than most of the peaks in the in vivo spectrum, suggesting that they might arise from ^{15}N incorporation into small molecules. To test this hypothesis we have prepared an identical uninduced bacterial sample and, as anticipated, the strong peaks observed in the in vivo sample are all present (data not shown). Further differences between both spectra arise from the absence of several weak peaks of the in vitro HSQC from the in vivo spectrum, most likely due to the broader line width of the in vivo sample. In fact, most of these resonances could be observed when the number of scans in the HSQC experiment was increased (data not shown).

To investigate the robustness of in-cell NMR spectroscopy we have carried out a series of control experiments. The main concern is that protein outside the cell tumbles faster and, therefore,

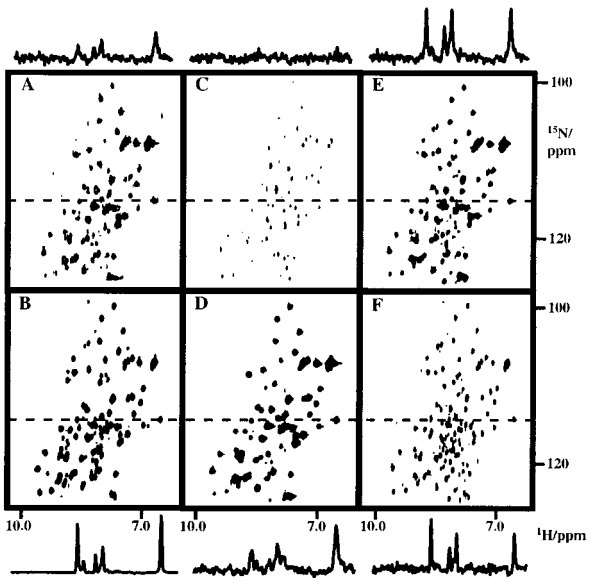

Figure 1. [^{15}N,^{1}H]-HSQC spectra of NmerA in varying conditions: (A) in vivo spectrum of a 15% bacterial slurry, (B) spectrum of a 2 mM purified NmerA sample, (C) supernate of the sample used in (A) after removal of all bacteria by centrifugation and filtration, (D) pellet after resuspension into a 25% slurry, (E) 2 h after adding lysozyme to the sample in (D), (F) supernate of (E) after centrifugation. All experiments were measured on a Bruker Avance 500 MHz NMR spectrometer equipped with a 5 mm triple resonance cryoprobe at 37 °C. Each experiment was measured with 4 scans in less than 10 min.

* Author to whom correspondence should be sent. Phone: (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.

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10.1021/ja0057528 CCC: $20.00
Published on Web 02/15/2001

exhibits sharper lines than protein in the more viscous cytoplasm. Consequently, a small fraction of extracellular protein could contribute disproportionately to, or even dominate, the HSQC spectrum. Particularly, in light of the almost identical chemical shifts between the in vitro and the in vivo sample, great care must be taken to ensure that the protein is indeed inside the bacterial cytoplasm. To examine the contribution of extracellular NmerA, we have removed the bacteria from the sample by centrifugation and filtration of the supernate. The [^{15}N,^{1}H]-HSQC experiment measured with the same parameters as the in vivo spectrum is shown in Figure 1C. Only a few strong peaks that correspond to the strong, nonprotein resonances observed in the in vivo HSQC and an insignificant amount of NmerA can be detected in this spectrum. Furthermore, resuspending the bacterial pellet in fresh minimal medium completely restores the original spectrum (Figure 1D). The spectrum in Figure 1D shows a higher signal-to-noise ratio than the spectrum in Figure 1A because the pellet was resuspended in less buffer than was used for the original sample, further suggesting that the measured signal intensity is directly coupled to the cell density inside the NMR tube.

As additional proof that NmerA is indeed inside the bacteria, we lysed the cells by addition of lysozyme to the NMR tube containing the resuspended pellet. Over time the average line width decreases from 55 to 36 Hz, consistent with the release of the protein from the interior of the cells into the less viscous culture medium (Figure 1E). Comparison with the in vivo spectrum reveals only small changes in chemical shifts of residues in the metal-binding loop. To demonstrate that NmerA is not associated with the cellular debris, we again centrifuged and filtered the sample and repeated the experiment on the supernate as before (Figure 1F), demonstrating that, after cell lysis, the majority of NmerA is in the medium.

The experiments described above clearly demonstrate that NmerA is associated with the bacteria, strongly suggesting that the protein NMR signals we observe originate from protein in the bacterial cytoplasm. However, to rule out the very unlikely possibility that NmerA could be associated with the bacterial outer membrane we took advantage of the metal binding activity of NmerA. The homologous protein MerP binds zinc with a K_d in the low micromolar range[13,14] causing changes in chemical shifts, mainly in the metal-binding loop.[13] NmerA binds divalent cations as well, though exact binding constants have not yet been determined. Nevertheless, changes in chemical shifts of residues in the metal-binding loop are immediately visible upon addition of $ZnCl_2$ to both the purified NmerA sample (Figure 2A and B) and a bacterial sample that had been treated with lysozyme (Figure 2C,D). The intracellular zinc concentration is tightly regulated by specific Zn^{2+} ion transport proteins[15] and presumably does not reach levels sufficient for the majority of NmerA to be in the metal-bound form. Indeed, NmerA expressed in bacteria grown in the presence of zinc did not display chemical shift differences relative to a sample expressed in the absence of zinc. If the NmerA protein were associated with the exterior of intact cells, titrating $ZnCl_2$ into the NMR tube containing an in vivo sample would lead to zinc-binding and concomitant chemical shift changes. When we added $ZnCl_2$ to an in vivo sample, however, no changes in chemical shifts were observed for at least 1 h (Figure 2E), in contrast to the immediate changes in chemical shifts observed in the in vitro and in the lysed samples in the presence of zinc. This strongly suggests that NmerA is inside the cells and cannot complex with the extracellular zinc. After an hour the cells were lysed and changes in chemical shifts characteristic of zinc binding, like those seen in vitro, did occur (Figure 2F).

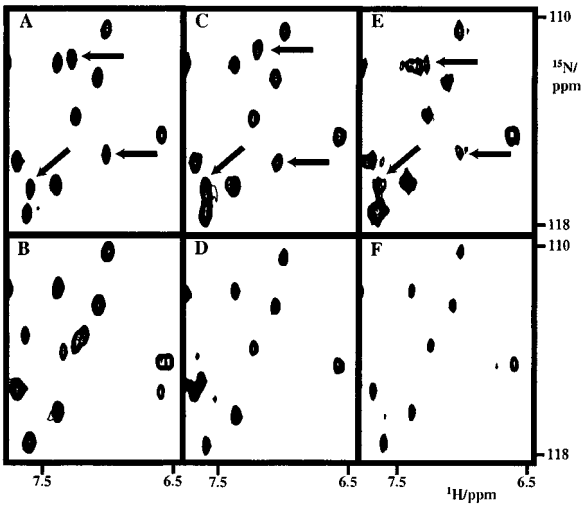

Figure 2. Sections taken from HSQC experiments: (A) Purified 2 mM NmerA in the absence of zinc and (B) after addition of 3 mM zinc, lysed in vivo sample without (C) and with (D) 3 mM zinc, and in vivo sample in the presence of 3 mM zinc (E) and after lysis (F). Peaks that disappear upon addition of zinc are labeled with an arrow.

The NMR experiments described above do not kill the bacteria over the short term as the number of colony-forming units/OD is the same before and after a 40 min HSQC experiment (data not shown). However, during a series of experiments, which lasted several hours, we noticed an increase in the extracellular fraction of NmerA. The increased presence of extracellular NmerA is most likely caused by bacterial cell death and subsequent lysis.

Our data demonstrate that high-resolution spectra of proteins can be observed in the cytoplasm of bacterial cells. The greatest advantage of our novel approach does not lie in solving structures, which can be done more easily in vitro. Rather, in-cell NMR spectroscopy will open new avenues of research into protein conformations in their natural environment, as affected by protein−protein interactions, reversible small molecule binding, and posttranslation modifications. Obviously, it is desirable to extend this technique to eucaryotic systems such as yeast. In addition, one can envision a screen in which the membrane permeability of a potential drug could be measured concurrently with its affinity to a target protein (in-cell SAR by NMR[16]).

Acknowledgment. We thank Kevan Shokat, Carol Gross and members of her laboratory for helpful discussion. 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., A.T.K. and A.E.K. were supported by a NIH Training Grant (GM08284).

JA0057528

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