Seeing Proteins inside Living Cells

Structures and Dynamics of Proteins by In-cell NMR

Investigating proteins "at work" in a living environment at atomic resolution is a major goal of molecular biology. In-cell NMR spectroscopy yields multi-dimensional NMR spectra of macromolecules in living cells [1, 2]. Now, for the first time the three-dimensional structure of a protein has been determined exclusively on the basis of information obtained in living cells [3], and stable isotope labeled proteins have been delivered into human cells using cell-penetrating peptide tags [4].

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Introduction

Proteins in living cells work in an extremely crowded environment where they interact specifically with other proteins, nucleic acids, co-factors and ligands. Methods for the three-dimensional (3D) structure determination of purified proteins in single crystals or in solution are widely used and have made very valuable contributions to understanding many biological processes. However, replicating the cellular environment *in vitro* is difficult. *In vivo* observations of three-dimensional structures, dynamics and interactions of proteins are required for fully understanding the structural basis of their functions inside cells.

The non-invasive character of NMR spectroscopy and its ability to provide data at atomic resolution make NMR ideally suited for the task. For instance, *in vivo* NMR spectroscopy focuses on the observation of metabolites and metal ions in systems ranging from suspensions of bacteria and other cells to entire perfused organs. Magnetic resonance imaging can provide information about entire organisms. The chemical shift of an NMR-active nucleus is highly sensitive to changes in its chemical environment. This makes NMR spectroscopy an excellent tool for studying the interaction of biological macromolecules with binding partners, ranging from other macromolecules to small ligands and medically important drugs. Recently, these advantages of NMR spectroscopy have been combined to obtain information about

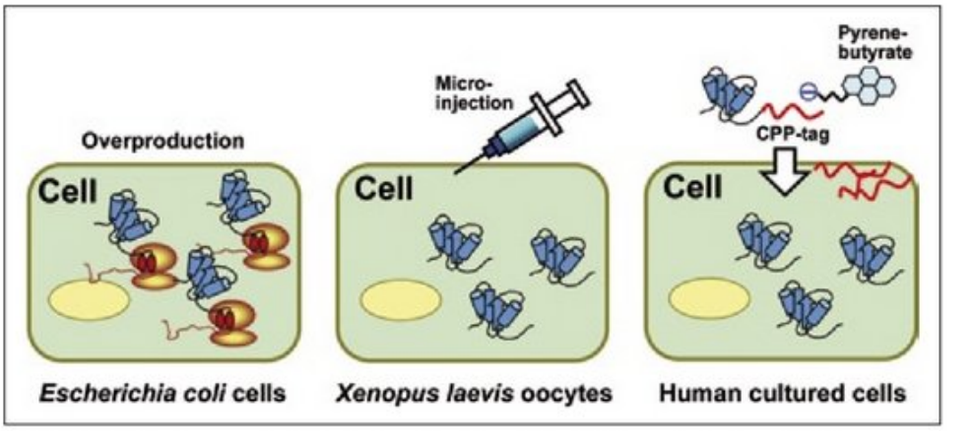

Fig. 1: Methods to incorporate stable isotope labeled proteins in living cells for in-cell NMR.

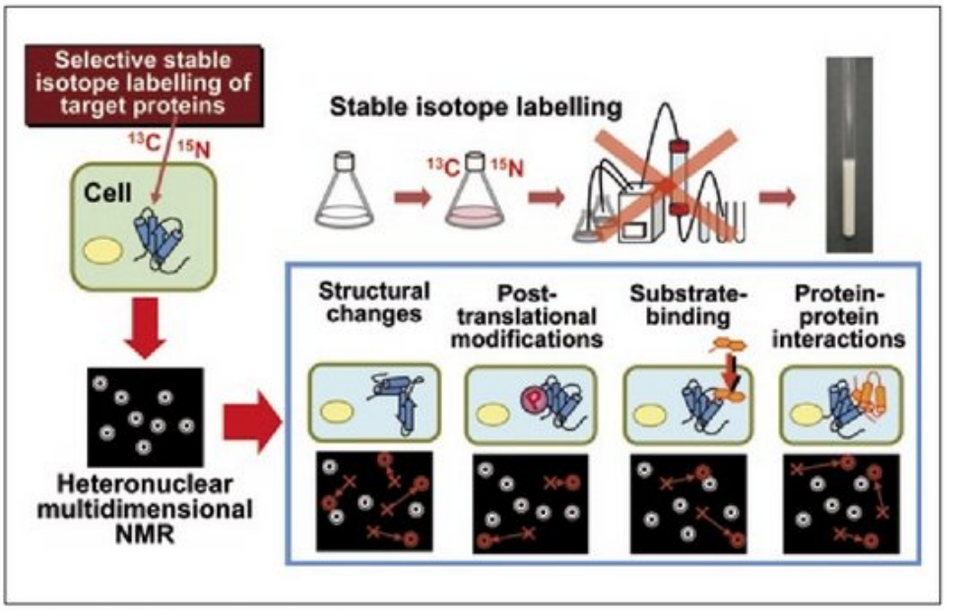

Fig. 2: In-cell NMR principles and applications

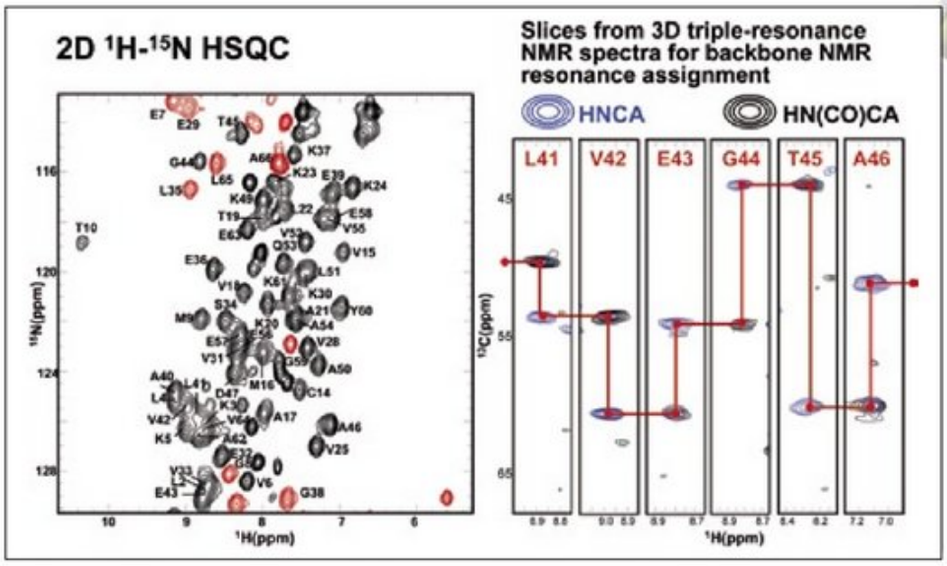

Fig. 3: In-cell NMR spectra of the putative heavy-metal binding protein TTHA1718 [3]

the conformation and dynamics of biological macromolecules inside living cells.

In-cell NMR Spectroscopy

NMR methods have been developed that enable the observation of proteins inside living bacterial cells [1, 2]. These in-cell NMR experiments can be used to study proteins in their natural surroundings. Detection of proteins in the bacterial cytoplasm relies on labeling the protein of interest with NMR active stable isotopes such as ^{13}C and ^{15}N. Two methods have been established to deliver labeled proteins into unlabeled bacterial and animal cells. Target proteins can either be produced within bacterial cells by growing them on isotopically labeled media or microinjected into individual large cells such as *Xenopus* oocytes (frog eggs).

Changes in protein structure caused by specific interactions with well-defined binding partners can be identified by solving structures of the protein complexes *in vitro*. A more challenging problem is to address how the omnipresent, non-specific, low-affinity interactions in cells affect the structure of a protein. Living cells are crowded environments in which 20–30% of the volume is filled by macromolecules. This results in small but potentially important changes in protein structure. Significant steps towards understanding these changes have been made [3, 4].

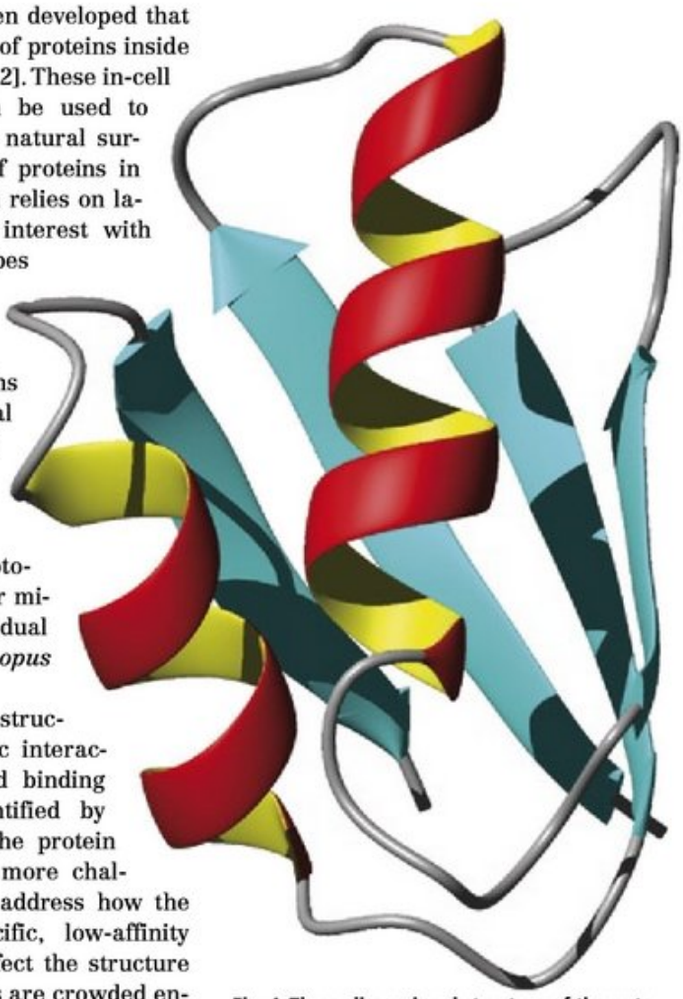

Fig. 4: Three-dimensional structure of the putative heavy-metal binding protein TTHA1718 determined in living *E. coli* cells.

Protein Structure Determination inside Cells

Until recently, the low sensitivity and short life time of the samples have prevented the acquisition of sufficient structural information to determine protein structures by in-cell NMR. *De novo* NMR protein structure determination in living cells requires methods for resonance assignment that do not rely on information obtained *in vitro*, and distance information derived from nuclear Overhauser effects (NOEs), where broadened lines result in severely overlapped cross peaks.

Now, Sakakibara *et al.* [3] have presented the first 3D protein structure calculated exclusively on the basis of information obtained in living cells. The structure of the putative heavy metal-binding protein TTHA1718 from *Thermus thermophilus* HB8 overexpressed in E. coli cells was solved by in-cell NMR. A major hurdle to determining in-cell NMR structure is the limited lifetime of the cells inside the NMR sample tube. Standard NMR experiments usually require 1–2 days of data collection, which is an unacceptably long time for live cells. This time could be shortened to 2–3 hours by preparing a fresh sample for each experiment and by applying a nonlinear sampling scheme in combination with maximum entropy processing for the indirectly acquired dimensions.

The expected NMR resonances for the backbone and the majority of side-chain NMR resonances were observed and assigned. Assignments of NOEs with side chain methyl groups have a large impact on the structure calculation. NMR mea-

surements in *E. coli* cells of TTHA1718 with selectively $^{1}H/^{13}C$-labelled methyl groups of alanine, leucine and valine yielded 77% of all long-range distance restraints. 3D ^{15}N-separated NOESY and 3D ^{13}C-separated NOESY spectra measured on uniformly labelled *E. coli* samples provided further NOE-derived distance restraints. On the basis of this data, a high-quality structure of the protein with a precision of the backbone atom positions better than 1 Å could be calculated with the program CYANA.

Comparing the *in vitro* and in-cell structures of TTHA1718 revealed that, despite marked similarities, there are structural differences, mostly concentrated in the putative heavy metal-binding site and in dynamic loop regions. The structural changes in the binding site are probably induced by metal ions present in the bacterial cytosol, whereas those observed in the dynamic loop could be due to molecular crowding and the viscosity of the cell interior. It will be interesting to confirm this phenomenon in future in-cell protein structures.

In-cell NMR of Proteins inside Human Cells

Extending in-cell NMR to study proteins inside human cells presents a further challenge. In general, protein production inside these cells is insufficient for atomic-resolution in-cell NMR spectra to be collected, and the artificial delivery of labeled proteins by microinjection is cumbersome and limited to extraordinarily large cells such as oocytes. Inomata *et al.* [4] introduce an innovative method that enables in-cell NMR in human cells by which the target protein is delivered into the cells by tagging it with a cell-penetrating peptide. The target protein is released in the cell by cleaving off the tag that becomes invisible for NMR by binding to large intracellular structures. Inomata *et al.* [4] use their approach to study in-cell protein dynamics.

Conclusions

The in-cell NMR approach can thus provide accurate high-resolution structures of proteins in living environments. Rapid data collection using nonlinear sampling and selective protonation at methyl groups to enable the identification of unambiguous long-range NOE interactions were key for the success of this approach [3].

New avenues have been laid for the investigation of protein conformations at atomic resolution and how they change in response to biological events in living environments. In particular, this approach provides the tools to study the effects of molecular crowding in the cytosol, the conformations of proteins that are intrinsically disordered *in vitro*, and the 3D structures of proteins that are otherwise unstable and difficult to purify to be investigated in living cells.

In combination with the protein delivery method [4] this method opens the door to determining protein structures inside human cells in the near future.

References

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