NEWS & VIEWS

STRUCTURAL BIOLOGY

Inside the living cell

David S. Burz and Alexander Shekhtman

Proteins work properly only if they have the correct three-dimensional atomic structure. It is now possible to look at the structures and dynamics of these biological macromolecules as they function inside cells.

The development of structural biology was historically based on the principle of divide and conquer — individual proteins were purified to homogeneity and their atomic structures were solved *in vitro* by using either X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. This approach was tremendously successful, and led to the creation of a protein-structure databank that currently contains more than 50,000 structures.

But relating *in vitro* protein structures to biological processes that occur inside the cell is not a trivial task. A traditional approach to solving this problem entails mutating a protein's structure at certain sites based on its *in vitro* structure and observing the effects of these changes on the cell. This low-resolution validation of high-resolution structures may still lead to situations where the *in vitro* structure does not fully represent the physiologically active protein structure under the conditions present in a cell. The work presented by Sakakibara *et al.*[1] and Inomata *et al.*[2] (pages 102 and 106 of this issue) reveals new ways to solve the structures of proteins as they exist inside living cells, ushering in a fresh era of structural biology.

To determine how protein structures are influenced by their intracellular environment, in-cell NMR spectroscopy was developed[3]. NMR spectroscopy allows one to directly observe NMR-active isotopes of atomic nuclei within any NMR-inactive environment, and can thus be used to analyse isotopically labelled biomolecules inside unlabelled cells. To date, two approaches have been used to deliver labelled proteins into unlabelled bacterial and animal cells. In the first case, target proteins are produced inside the bacteria by growing them on isotopically labelled media; in the second, labelled proteins are microinjected into large cells such as *Xenopus* oocytes (frog eggs)[4]. In these instances, in-cell NMR spectra suggested that protein structures inside cells are very similar to those solved *in vitro*. The devil, though, is in the detail.

Changes in protein structure that are caused by specific interactions with well-defined binding partners can be identified by solving *in vitro* structures of the protein complexes. A more difficult problem is to address how the numerous, nonspecific, low-affinity interactions, which are omnipresent in cells, affect a protein's structure. Living cells are extremely crowded environments owing to the high (20–30% by volume) concentration of macromolecules they contain, and this results in small but potentially important changes in protein structure. The papers published in this issue[1,2] make significant steps towards understanding these changes.

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. Sakakibara *et al.*[1] shortened this time to 2–3 hours by applying a well-known but seldom-used modification of NMR experiments, and thereby determined the three-dimensional structure of a putative heavy-metal-binding protein, TTHA1718, expressed inside bacterial cells (Fig. 1a). Their procedure, as described in detail in the paper, may well become a new standard for in-cell NMR.

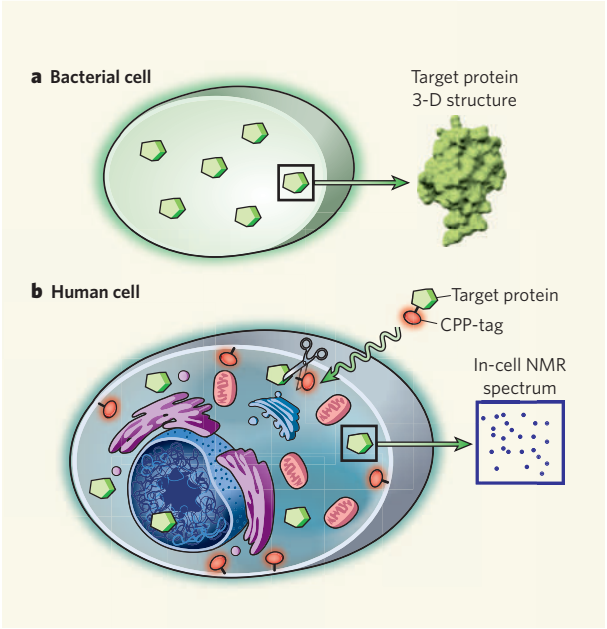

Figure 1 | Analysis of proteins using in-cell NMR spectroscopy. **a**, Sakakibara *et al.*[1] show that atomic structures of proteins expressed inside bacterial cells can be solved by in-cell NMR spectroscopy. **b**, Inomata *et al.*[2] fused a target protein with a cell-penetrating peptide (CPP-tag) to transfer stable-isotope-labelled proteins into various human cells and collect in-cell NMR spectra at atomic resolution. This approach has not yet been used to determine protein structures but has been applied by the authors to study protein dynamics.

Comparing the *in vitro* and in-cell structures of TTHA1718 revealed that, despite marked similarities, there are structural differences, mostly concentrated in the heavy-metal-binding site and in loop regions that undergo dynamic changes as the protein functions. In contrast to the changes in the binding site, which can be explained by metal ions present in the bacterial cytosol, the structural changes seen in the dynamic loop are probably due to molecular crowding and the viscosity of the cytosol that are characteristic of the cell interior. It will be interesting to discover whether this phenomenon is seen when further in-cell NMR structures become available.

Extending in-cell NMR to study proteins inside human cells presents a further challenge. In general, protein production inside these cells does not reach high enough levels for atomic-resolution in-cell NMR spectra to be collected. The microinjection technique is laborious, and up to now has been limited to large cells such as *Xenopus* oocytes. Inomata *et al.*[2] describe an innovative method that avoids using microinjection and makes in-cell NMR in human cells possible. By fusing the labelled target protein with a cell-penetrating peptide (Fig. 1b), the target protein can be delivered into the cells, where the peptide tag is then snipped off, allowing the free, labelled target protein to disperse uniformly. Importantly,

NATURE|Vol 458|5 March 2009

the authors found that the tag binds to large intracellular structure(s), making it invisible to NMR and thus simplifying the in-cell NMR spectrum of the target protein.

This method opens the door to determining protein structures inside human cells in the near future, but has already been used by Inomata *et al.* to study in-cell protein dynamics. For example, macromolecular crowding inside the cell should stabilize protein structures. Inomata *et al.* show that, for at least one protein, ubiquitin, introduced into a human cell at physiological concentrations, the opposite is true. Ubiquitin becomes more dynamic in its reactivity and less structured, presumably due to nonspecific, low-affinity interactions with its binding partners. This unexpected result highlights the importance of studying proteins inside living cells.

In-cell NMR is limited by the concentration and structural stability of the protein that can be attained inside cells. In addition, some proteins could be difficult to deliver into the cytosol by fusing them with cell-penetrating peptides. Very large proteins, or proteins that bind to large cellular structures, become invisible and cannot be effectively studied by in-cell NMR. The lifetime of the cells is also a limiting factor, because cell breakdown results in protein leaking away into solution, the condition of *in vitro* NMR. Despite these pitfalls, we are left with an enormous number of proteins that can be studied.

Exploring protein structures and dynamics at the atomic level inside living cells will provide results that cannot be obtained using standard *in vitro* techniques. The comparative simplicity of the in-cell method allows for a myriad of applications. The regulation of metabolic or signal-transduction pathways, mediated by biomolecular interactions, can now be studied in detail. Drug screening using in-cell NMR can function as an *in vivo* assay at atomic resolution, providing information about drug delivery inside the cell, where the drug binds, and whether there is a notable difference between how it binds *in vivo* and *in vitro*. More exotic applications include the study of intrinsically unstructured and amyloid-forming proteins in neurons — such proteins having been implicated in neurodegeneration — or of labelled protein probes in diseased tissue. The ability to observe the structures of proteins in their native environment releases the constraints that have previously limited study of protein structure and dynamics to the test tube. Now that structural biology has moved into the cell, it is likely to stay there. ■

David S. Burz and Alexander Shekhtman are in the Department of Chemistry, State University of New York at Albany, 1400 Washington Avenue, Albany, New York 12222, USA.
e-mail: ashekhta@albany.edu

1. Sakakibara, D. *Nature* **458,** 102–105 (2009).
2. Inomata, K. *et al. Nature* **458,** 106–109 (2009).
3. Serber, Z. *et al. J. Am. Chem. Soc.* **123,** 2446–2447 (2001).
4. Selenko, P. *et al. Proc. Natl Acad. Sci. USA* **103,** 11904–11909 (2006).

CONDENSED-MATTER PHYSICS

Carbon conductor corrupted

Michael S. Fuhrer and Shaffique Adam

Atomically thin sheets of graphite are metal-like conductors — until they react with hydrogen, when they become insulators. This curious effect could be an excellent model for studying metal–insulator transitions.

In most solids, electrons behave much like particles of matter: they have a mass, and they speed up and slow down in response to forces. But in graphene — the single-atom-thick sheet of carbon that constitutes the basic building block of graphite — electrons move as if they have no mass[1,2], and so behave more like photons. In other words, although electrons in graphene can change their momentum and energy, they cannot speed up or slow down. One would therefore intuitively think that electron flow (electrical current) in graphene could never be completely blocked. But reporting in *Science*, Elias *et al.*[3] show that, when graphene reacts with a small amount of hydrogen, its electrons become stuck and the carbon sheet becomes an insulator.

The band structure of a material describes which energy states can be occupied by the material's constituent electrons. Just like other fermions, electrons in a solid fill up the lowest-energy bands first, before filling up bands at higher energies, like water filling a bath. If electrons at the top surface — the Fermi level — of the resulting electron sea can slosh about into the unoccupied part of the band structure, then the solid is said to be a metal, in which electrical currents are created by electrons moving from one momentum state to another (Fig. 1a). But if the electrons are not free to move because of a gap in the band structure, then the solid is a 'band insulator' (Fig. 1b).

There is, however, another way to make an insulator. Electrons are quantum-mechanical objects that behave as waves. Constructive interference of electron waves near any imperfections — disorder — in a solid creates standing waves (Fig. 1c) that are localized in real space, effectively 'freezing' the electron sea[4]. A long-standing conjecture in physics states that electrons in any two-dimensional system will become localized by any disorder, no matter how small. Because all two-dimensional electronic systems contain some disorder, all such systems should therefore be insulators[5]. In practice, however, very low temperatures and/or large samples are needed to reveal this insulating behaviour, and even then the effect is not always seen.

So how does graphene fit into all this? The band structure of graphene can be thought of as a cone balancing on its tip, atop the point of another cone (Fig. 1d). Because there is no bandgap, graphene is a metallic conductor. But graphene differs from other metals when its Fermi level lies at the Dirac point — the point where the cones touch. There, the top surface of the electron sea becomes vanishingly small. One consequence is that, in contrast to disorder-free metals (which would have infinite conductivity), perfect sheets of graphene are expected to have a conductivity of $4e^2/\pi h$, where e is the electronic charge, and h is Planck's constant. Furthermore, graphene is thought to be the only exception to the localization conjecture: if it contains only 'smooth' disorder in which there are no sharp changes between neighbouring carbon atoms, then graphene remains metallic, and even quantum interference cannot localize its electrons[6].

In reality, graphene is found to have a sample-dependent, finite minimum conductivity that is always greater than $4e^2/\pi h$ (ref. 1), thus creating a mystery — how could the disorder present in real graphene increase the conductivity above the theoretical value for perfect graphene? Disorder normally increases the scattering of electrons, which decreases conductivity.

The answer lies in the nature of the disorder. Most graphene samples are dirtied by charged impurities that lie near the graphene sheet, either on the surface or in the nearby substrate[7]. These charges have two effects: they create a smooth disorder that scatters electrons (which reduces conductivity), but they also either attract or repel electrons, creating local electron-rich or electron-poor 'puddles' whose Fermi levels lie above or below the Dirac point. The electrons in these puddles increase the conductivity of the sample, counteracting the decrease due to scattering[8].

By adding hydrogen to graphene, Elias *et al.*[3] were able to study a fundamentally different sort of disorder. By reacting all the carbon atoms in a graphene sheet with hydrogen, so that each carbon becomes bonded to a single hydrogen atom, the authors made a new kind of two-dimensional crystal. This material, known as graphane, is expected to be a conventional band insulator[9].

But at lower doses, hydrogen probably bonds randomly to only a few carbon atoms. Unlike charged impurities, which cause a smooth disorder, hydrogen creates sharply varying disorder, because a carbon atom bonded to hydrogen is very different from its neighbours. The authors[3] observed that their partially hydrogenated graphene had greatly reduced minimum conductivity, which varied with temperature and tended towards zero at low temperatures — the signature of an insulator. This is in contrast to graphene containing