Studying Posttranslational Modifications by In-Cell NMR

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Functional in vivo investigation of posttranslational modifications is a problem that a number of analytical techniques are trying to tackle. Below, we briefly discuss the breakthroughs and challenges in placing NMR spectroscopy on the map, as illustrated by a recent report by Selenko et al. (2008).

The cell- and time-wise invariable nature of the genome of an organism renders genomics a better-defined discipline than proteomics. Indeed, even a definition of proteomics is subject of debate (Hunt, 2002), as protein content can vary tremendously from one cell type to another, and at different stages of organism development even within the same cell type. Moreover, proteins are subject to posttranslational modifications (Walsh et al., 2005), of which phosphorylation is one of the best studied (Fischer, 1993; Krebs, 1993). The addition or removal of a phosphate group from the side chain of a Ser, Thr, or Tyr residue by a kinase or phosphatase residue, respectively, can dramatically modify the stability or localization of the protein, and directly affect its interactions with binding partners and/or enzymatic efficiency. A number of human diseases is linked to phosphorylation abnormalities. Therefore, deciphering the phospho-code in terms of molecular function has been, and likely will remain, a major research effort.

Mass spectrometry (MS), due to its exquisite sensitivity and powerful resolution, has been one of the methods of choice to detect protein posttranslational modifications. In principle, the method relies on the preliminary digestion of the protein sample, and subsequent analysis of the resulting peptides. However, if different phosphorylation sites are in close proximity and on the same peptide after trypsin digestion, MS cannot identify the exact location of these sites. MS/MS might give the correct solution, but is hampered by the labile nature of the phosphate groups. The quantitative analysis is also unreliable due to phosphate instability, and differential ionization efficiencies of the peptides. Therefore, MS usually has to be complemented with other biochemical techniques to give a precise picture of a phosphorylated protein.

Recognizing the limitations of available techniques, we introduced the use of NMR spectroscopy as a method to decipher the complex phosphorylation pattern of the neuronal microtubulin associated Tau protein, that with its thirty phosphorylation sites is a true analytical challenge (Landrieu et al., 2006). Abnormal phosphorylation of Tau is associated with its aggregation and Alzheimer's disease (AD). The method relies on using a uniform ^{15}N-labeled protein sample to record a 2D HSQC spectrum, where each signal represents the amide moiety of a single amino acid of a given protein. As the position of each peak in this HSQC spectrum (i.e., its chemical shift) is extremely sensitive to H^N proton environment, a Ser or Thr side chain phosphorylation leads to chemical shift change. Therefore, NMR can be used to monitor the incorporation of a phosphate, both qualitatively, by the assigning peaks shifted, and quantitatively, by integrating peak intensity.

Selenko et al. now apply a similar approach to study phosphorylation of a small peptide derived from the viral SV40 T antigen by the Casein kinase 2 (CK2) (Selenko et al., 2008). CK2 phosphorylates two adjacent sites of the SV40 T antigen, Ser111 and Ser112, and the exact phosphorylation state of the peptide modulates nuclear import properties of the full-length protein. Upon in vitro phosphorylation of the peptide (used here as a B1G-fusion to allow efficient bacterial expression and isotope labeling), they observed that CK2 phosphorylates the peptide in a defined order, with Ser111 requiring prior phosphorylation of Ser112. The Ser112 phosphorylated peptide could be observed by NMR while the reaction was proceeding, which would not be possible if the peptide existed in a complex with large CK2 (due to the size-limit effect of NMR spectroscopy). Based on this observation, they conclude that the same kinase molecule does not hold onto the monophosphorylated peptide en route to double phosphorylation. Rather, CK2 releases pSer112-peptide that subsequently becomes a substrate for another kinase molecule. Since the second site is less optimal, Ser111 phosphorylation occurs only when most of the unphosphorylated peptide is consumed. Would it have been possible to arrive at this conclusion without NMR spectroscopy? Not as directly and as elegantly. Monitoring reaction kinetics by HPLC separation (and quantification) of modified and unmodified peptides is relatively simple, but would not directly demonstrate the dissociation of the pSer111 peptide from CK2. The same mutant peptide used by the authors together with MS might allow one to sort out the sequential mechanism, but not in such a direct manner as by NMR spectroscopy.

Beyond the in vitro assay, the authors go further by exploring the function of CK2 in a more in-cell-like environment, by injecting ^{15}N labeled proteins into *Xenopus* oocytes. This procedure is built upon previous in-cell NMR efforts (Lippens and Bohin, 1999; Wieruszeski et al., 2001; Serber et al., 2001; Selenko et al., 2006; Sakai et al., 2006). Here, Selenko et al. proceed in a two-step manner, by first adding the SV40 T antigen peptide to *Xenopus* egg extracts, and then studying the same peptide after injection into intact *Xenopus* oocytes. They demonstrate that CK2 activity in *Xenopus* egg extracts is identical to one observed for purified kinase, suggesting that molecular crowding does not play a significant role in terms of either specificity or kinetics. Since egg extract contains activated

Cdk1 kinase, they additionally observed phosphorylation of Ser124 in the same peptide. In the *Xenopus* oocytes, however, only CK2 is active before maturation, and the same sequential phosphorylation pattern was observed as in the cell extract. The activation of Cdk1, upon incubation with progesterone, does lead to Ser124 phosphorylation, as observed directly by NMR. Interestingly, the injection of 50 μM SV40 T antigen peptide does not interfere with maturation, suggesting that Cdk1 is not saturated by this massive presence of a novel substrate. This study highlights the ability of NMR spectroscopy to probe both kinase activity and phosphorylation as a posttranslational modification.

Both NMR spectroscopy and MS have their given sets of advantages and disadvantages when used to monitor the process of phosphorylation. However, neither technique is limited to studying only this single type of posttranslational modification. Sensitivity of MS allows detection of a covalent modification that changes the mass by as little as 1 Da. NMR is also not limited to detection of phosphorylation, although the chemical shift changes introduced by other posttranslational modifications are expected to be smaller. For example, acetylation and methylation of side chains or Arg and Lys have only a minor effect on the backbone resonances (C. Smet and G.L., unpublished data), and we recently showed that the addition of an N-acetyl glucosamine on the Ser side-chain oxygen actually led to a larger chemical shift change for the neighboring residue H^N (Dehennaut et al., 2008), forcing us to use side-chain resonances to unambiguously assign the modified residue.

It is worth pointing out that NMR spectroscopy should bring a decisive advantage, not easily challenged by any other single technique, in combining the identification of posttranslational modification sites with the analysis of its structural and functional consequences. Indeed, NMR is extremely powerful in mapping of molecular interactions, many of which are mediated by posttranslational modifications. And, whereas X-ray crystallography has given exquisite insights into the structural changes that accompany phosphorylation, (see work on activated Cdk2 kinase as an example; Russo et al., 1996), NMR could be ideally suited to estimate the (subtle) structural changes and/or novel interaction sites brought about by posttranslational modifications in the natively unfolded regulatory stretches of many proteins.

As for in-cell spectroscopy, we lack hindsight at the moment to predict whether it will lead to novel understanding of the cell functioning. For example, crowding emerges as an important factor determining association of molecular complexes. Although these effects can be studied by in-cell NMR, they can also be introduced by addition of voluminous macromolecules to the buffer system. Thus, earlier studies demonstrated that although partial folding of Flim protein was indeed observed upon its overexpression within the bacterial cytoplasm (Dedmon et al., 2002), protein aggregation was also shown to be accelerated by addition of crowding agents, such as ficoll (van den Berg et al., 1999). The *Xenopus* oocytes clearly offer an advantage over those earlier studies, since they represent a eukaryotic cellular system, containing many of the enzymes responsible for posttranslational modifications, and allowing injection of a well-controlled amount of labeled protein. However, the low sensitivity of NMR spectroscopy remains an obstacle to reaching submicromolar intracellular concentrations required to mimic a more realistic cellular biology picture. Embedding the oocytes in a 20% ficoll solution extends their lifetime, and hence the ability to work at lower protein concentrations (Bodart et al., 2008). Clearly deconvoluting the effects of specific cellular factors on the NMR spectrum remains an outstanding challenge.

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