NMR spectroscopy of the neuronal tau protein: normal function and implication in Alzheimer’s disease

Isabelle Landrieu*, Arnaud Leroy†, Caroline Smet-Nocca‡, Isabelle Huvent‡, Laiiza Amniai*, Malika Hamdane‡, Nathalie Sibille*, Luc Bue†, Jean-Michel Wieruszeski* and Guy Lippens*

1CNRS-UMR 8576 - IFR 147, Lille 1 Science and Technology University, 59655 Villeneuve d'Ascq Cedex, France, ‡Laboratoire de biochimie appliquée, University Paris XI, Faculty of pharmacy, Chatenay-Malabry Cedex, France, and §INSERM U815, Lille 2 University, School of Medicine, 59000 Lille, France

Abstract
NMR spectroscopy was used to explore the different aspects of the normal and pathological functions of tau, but proved challenging because the protein contains 441 amino acids and has poor signal dispersion. We have set out to dissect the phosphorylation patterns of tau in order to understand better its role in the aggregation process and microtubule-binding regulation. Our current knowledge on the functional consequences of specific phosphorylations is still limited, mainly because producing and assessing quantitatively phosphorylated tau samples is far from straightforward, even in vitro. We use NMR spectroscopy as a proteomics tool to characterize the phosphorylation patterns of tau, after in vitro phosphorylation by recombinant kinases. The phosphorylated tau can next be used for functional assays or interaction assays with phospho-dependent protein partners, such as the prolyl cis-trans isomerase Pin1.

Introduction
Deregulation of tau phosphorylation has been linked to the pathogenesis of AD (Alzheimer’s disease). The aggregated tau in PHFs (paired helical filaments) is indeed found to be hyperphosphorylated, although causality has never been ascertained. NFT (neurofibrillary tangle) development is nevertheless correlated with the evolution of the pattern of phosphorylation affecting sequentially various epitopes that can be classified as early- to late-stage [1]. For example, the AT8 monoclonal antibody that recognizes the pSer202/pThr205 epitope can be used in post-mortem biopsies to detect the progression of the tau pathology in the brain and is a late-stage epitope [2]. It would be of particular interest to define how the early phosphorylations could affect the evolution of the subsequent phosphorylation pattern. Additionally, the minimal phosphorylation pattern of tau that would assume its pathological characteristics has not yet been clearly defined. Both of these points would be important steps in the development of new drugs that could target the phosphorylated pathological tau at an early stage, before filament formation. Although the interest in linking specific phosphorylations to disease progression was recognized a long time ago, characterization of these phosphorylations is not a simple task because of the high number of sites in the tau sequence. We have used NMR spectroscopy as an analytical tool to study the various aspects of tau biology [3], with a single residue resolution. All of these experiments are performed in vitro and have necessitated the development of relevant models. In the present paper, we discuss the different tau models that we have used to analyse the functional impacts of tau phosphorylation [4].

NMR spectroscopy of the tau protein
Bio-NMR, previously best known for the structural determination of small globular proteins, has now imposed itself as a method of choice in the emerging field of unstructured protein biochemistry [5]. NMR spectroscopy is still in a phase of fast technological advances, with increased magnetic fields allowing for better resolution and sensitivity, and novel probe heads allowing for smaller volumes to be analysed with good signal to noise ratios. These methodological advances have proved to be important for the study of tau, as they have given access to more complex samples than just the recombinant protein, and this with acceptable amounts of sample, by using concentrations of 10–100 μM in volumes from 150 to 600 μl. The protein observed has to be labelled with stable isotopes (15N, 13C) to be detected and it thus requires the use of recombinant proteins. The basic spectrum acquired by NMR is a [1H,15N]-HSQC (heteronuclear single-quantum coherence) in which every resonance corresponds to an amide function of an amino acid residue of the protein. The [1H,15N]-HSQC of tau is very crowded, because of the size of tau (441 amino acids) and its disordered nature [6]. The spectra acquired on tau samples are, however, of good quality, with sharp lines, owing to the high flexibility of the disordered polypeptide chain. The assignment of the resonances to specific amino
acids in the full-length tau sequence is now established [6–8] and high-field spectrometers have been crucial to overcome the difficulties linked to tau size and its unfolded nature.

**In vitro studies of multi-phosphorylated tau**

Our first attempt to phosphorylate recombinant tau in vitro for NMR analysis was with SYSY cell extract overexpressing the CDK (cyclin-dependent kinase) activator p25 [9]. From this experiment, we indeed detected a phosphorylation event, characterized by a large downfield shift of the [1H,15N]-resonance of the modified residue to an empty region of the spectrum. Even in the cases in which the resonance is not isolated in the two-dimensional spectrum, the typical values of the Ca and Cβ chemical shifts of the phosphoserine and phosphothreonine residues [10] will be sufficient to detect the phosphorylation event in a three-dimensional spectrum. Assignment of these phospho-resonances to specific residues in the sequence can then be performed by classical three-dimensional spectroscopy that links resonances from adjacent residues. pSer^214 was identified as the specific phosphoserine residue of tau detected after incubation with the SYSY cell extract. This site is well known to be preferential for PKA (protein kinase A) [11] and is not a proline-directed site. As we initially hoped using this assay to phosphorylate proline-directed sites through the CDK5–p25 activity described for this cell model [12], this experiment definitively convinced us that it was difficult to target specific serine and threonine residue(s) for phosphorylation using cell extracts. However, this first attempt also taught us a few other lessons. First, proteolytic degradation was hard to control when we added cell extract to tau, probably because its unfolded nature makes tau very sensitive to proteases. Secondly, by checking phosphorylation after Western blotting of our modified tau sample with a combination of antibodies raised against phospho-tau, we detected virtually all of them in our NMR sample. The antibodies being much more sensitive than the NMR spectroscopy, they also detect minor events that remain below the threshold of detection by NMR spectroscopy. NMR is thus not a sensitive method (certainly compared with MS or immunochemistry), but it has the advantage of being quantitative. From the intensity or the integral of a resonance, we can deduce a relative percentage of phosphorylation of a specific residue in the protein sample. This allows us to discriminate the main modifications in the sample from the minor ones, facilitating the interpretation of the impact of these major phosphorylations on tau function.

After this first attempt, we decided that it would be more efficient to use recombinant kinases for in vitro phosphorylation of tau. We first used PKA [13], which is not a proline-directed kinase, but is very active and easy to manipulate. With this kinase, we could demonstrate that the NMR approach allows the monitoring of phosphorylation as a dynamic process, given that the timescale of the enzymatic reaction is adapted to the data acquisition timescale. We have thus been performing ‘in spectrometer’ kinetics of phosphorylation. We were thus able to rank the phosphorylation of tau by PKA, the first resonance appearing being the one of pSer^214, followed by pSer^202/pSer^234 and finally pSer^109/pSer^116. Not only was this more than a good overview of the kinase specificity, but also it allowed us to set up the conditions to prepare tau protein with specific patterns of phosphorylation (for example, a single phosphorylation on Ser^214) for further functional assays such as tubulin polymerization and interaction with taxol-stabilized microtubules. This phosphorylation site is particularly intriguing as it decreases the affinity of tau for the microtubules by two orders of magnitude (from approx. 20 nM to 2.3 μM) [14], but, in our hands, it has little effect on the tubulin polymerization capacity of tau in a turbidity measurement assay. pSer^214-tau is thus an interesting model to characterize the structural modifications induced by this single modification in relation to microtubule binding.

Our main interest was, however, in proline-directed kinases, as they target the regulatory proline-rich region and several sites that were linked to development of the tau pathology. We used a CDK2–CycA3 (cyclin A3) kinase [15,16] to produce a phosphorylation pattern of interest, including the generation of the AT8 and AT180 epitopes. So, although this kinase is not relevant to the pathology, it can be used in a practical way to generate phosphorylations of interest. The alternative is to work with relevant kinases, but this requires more complicated combinations of patterns: reconstitution of the AT180, for example, would require a first step of phosphorylation with CDK5–p25, followed by a second step of incubation with recombinant GSK3β (glycogen synthase kinase 3β) (Figure 1). In the first step, Ser^235 is phosphorylated and will prime GSK3β for the second step, phosphorylation at Thr^231 [17]. Note in Figure 1 that the resonance of pSer^235 has a different co-ordinate in the [1H,15N]-HSQC depending on the phosphorylation status of Thr^231. The split pSer^235 resonance thus indirectly informs on the phosphorylation status of another residue as their relative intensities reflect the fraction in the sample phosphorylated on Thr^231. Although useful, incomplete phosphorylation of Thr^231 will lead to a splitting of pSer^235 resonance and as such complicate the assignment of the sample. We could show that both the AT8 and AT180 are necessary to abolish the tubulin-polimerization capacity of tau [18]. However, tau modified on pThr^212/pSer^235 and pSer^202/pThr^205 still has a high affinity for the microtubules. The situation looks to be the reverse of pSer^214-tau, which has lost affinity for the pre-assembled microtubules, but maintains its potential to assemble tubulin into microtubules. The CDK3-phospho-tau is thus another good model to investigate the conformational modifications that could be responsible for a non-functional binding to the microtubules.

As an example of the interaction studies that can be performed with NMR spectroscopy, we detail the characterization of tau interaction with the phospho-binding protein Pin1. In relation to the tau pathology, Pin1 was shown to be able to restore the capacity of phospho-tau to assemble tubulin into microtubules [19] and also to stimulate
In vitro phosphorylation of tau to reconstitute the AT180 epitope

Superimposition of regions of the \([^{1}H,^{15}N]\)-HSQC of CDK5–p25 phospho-tau-(165–441) (black), CDK5–p25 followed by GSK3β phospho-tau-(165–441) (violet) and CDK2–CycA3 phospho-tau-(208–324) (grey) centred on the resonances of pThr231 and pSer235. Spectra were acquired on samples in 25 mM sodium phosphate buffer, 25 mM NaCl, 2.5 mM EDTA and 2 mM dithiothreitol (pH 6.8), on a 600 MHz equipped with a cryoprobe (sample volume 600 \(\mu l\)).

deep phosphorylation of tau by PP2A (protein phosphatase 2A) [20]. These roles could confer neuroprotection, and this was confirmed in vivo by a Pin1-knockout mouse developing an age-related tauopathy [21]. Our long-term goal has been to understand the molecular mechanisms that could explain Pin1 function [22,23]. Here, we used our experimental set-up based on in vitro phosphorylation and NMR spectroscopy to probe the interaction between Pin1 and CDK2–CycA3 phospho-tau. In addition to the CDK2–CycA3 phospho-tau, other models to mimic a multiply phosphorylated substrate, namely phosphorylated peptides of tau and tau-Glu10, were also used.

A mixture of unlabelled WW (Trp-Trp) and catalytic Pin1 domains was added to \(^{15}N\)-labelled CDK2–CycA3 phospho-tau. The resonances of several phospho-residues in the \([^{1}H,^{15}N]\)-HSQC of phospho-tau disappeared upon addition of the Pin1 protein (Figure 2). Two mechanisms can explain how a resonance will broaden upon interaction. First, interaction with a large object will induce a loss of local mobility and of global tumbling rate, as we clearly observed in the case of tau interaction with microtubules [24,25]. The second possibility comes from an ‘intermediate dynamics’ of interaction on the NMR timescale. It means that the resonance is ‘blurred’ between the resonance of the bound and free states of the molecule and thus is broadened. We frequently observe this kind of dynamics for the \(K_d\) value of the interaction in the 10–100 \(\mu M\) range. Several phospho-residues are bound to the catalytic and WW domain phospho-binding sites of Pin1 during the acquisition time of the spectrum, which means that Pin1 is dynamically sampling several phospho-sites with a low affinity. Another example of a multi-phosphorylated substrate bound by a single-site receptor in a dynamic equilibrium is provided by the binding of the kinase inhibitor Sic1 by its Cdc4 receptor upon phosphorylation of its multiple CDK sites [26]. We can thus define a low specificity of Pin1 for the various phospho-sites, with a preference for phosphothreonine residues over phosphoserine. Addition of an excess of Pin1 led to the broadening of all the phospho-resonances, except those of pSer235 and pSer404 whose resonances are less affected. These results with a complete unfolded multi-phosphorylated substrate can be compared with our previous work with a series of phospho-peptides from the tau sequence [27,28], ranging from 12 to 40 amino acid residues. The use of peptides has the advantages of ensuring homogeneity of the phosphorylation and, of course, of a much simplified spectrum for the analysis.

At least for unphosphorylated tau peptides, their resonances in the \([^{1}H,^{15}N]\)-HSQC spectra from \(^{15}N\) natural abundance superimposed very well on the corresponding resonances of the residues embedded in the full-length protein [6], leading to the proposition that tau behaves as a collection of peptides. The perfect superposition of the \([^{1}H,^{15}N]\)-HSQC spectra of the peptides and of the same peptide sequence embedded in the full-length protein indicates that the residues sample the same conformational space in both peptide and full-length protein. For the phospho-peptides, that conclusion is less obvious. Superimposition of \([^{1}H,^{15}N]\)-HSQC of the phospho-peptide and the full-length tau protein does not always match perfectly. This could be due to the fact, as explained in the case of pSer235 (Figure 1), that adjacent phosphorylation(s) will influence the resonance chemical shifts of each other. The effect can affect residues located four
amino acids apart, a rather long-distance effect if we compare with our experience with point mutations that usually do not affect residues located further than two residues away (in the tau protein). So, on the point of the comparison of the conformation of the phospho-peptides or the phospho-protein, we remain cautious and cannot conclude, as was the case for the unphosphorylated peptides, that they are identical.

However, if we compare the interaction studies that we have performed with the tau phospho-peptides and the full-length phospho-tau as Pin1-interacting partners, we can draw similar conclusions. In both cases, Pin1 did indeed interact with several phospho-sites, with a range of affinities corresponding to \( K_d \) values in the order of 100–1000 \( \mu M \). As for pSer\(^{235} \), we indeed found a low affinity with a \( K_d \) of 1 m\( M \) in our phospho-peptide screen [27]. The affinity for a simple phospho-dipeptide pThr-Pro (\( K_d \) of 180 \( \mu M \)) was approx. 3-fold better than for a pSer-Pro dipeptide (\( K_d \) of 580 \( \mu M \)).

In conclusion, the phospho-peptides are also a good model to observe the specificity of a phospho-binding protein with regard to tau phospho-residues. However, the interaction of the WW domain of Pin1 with the full-length tau phospho-protein allows us to study a dynamic complex formed by the interaction of a single phospho-binding module with several low-affinity sites in an ensemble of disordered states.

Finally, we also used a tau-Glu\(^{10} \) protein [29,30] as an interacting partner for Pin1. We observed several chemical-shift perturbations in the [\( ^{1}H,^{15}N \)]-HSQC of [\( ^{15}N \)]-Pin1 upon addition of unlabelled tau-Glu\(^{10} \), indicative of an interaction. However, the perturbations were weak and localized to the Pin1 catalytic domain (Figure 3), whereas there were none for the resonances corresponding to residues of the WW domain. By comparison, a phospho-peptide added similarly at a 1:1 stoichiometry would induce most perturbations in resonances of the WW domain residues and an excess of 20 equivalents of phospho-peptide is necessary to reproduce the chemical shift modifications in the catalytic domain. There is no interaction of Pin1 with a short T231E-tau peptide. Furthermore, addition of an excess of phosphate to [\( ^{15}N \)]Pin1-tau-Glu\(^{15} \) did not completely disrupt the interaction. These data agree with previous studies that had shown a residual peptidylprolyl isomerase activity of Pin1 with a Glu-Pro substrate [31]. However, we concluded that the interaction of Pin1 with tau-Glu\(^{15} \) does not resemble the one with a phospho-substrate and we thus do not consider tau-Glu\(^{15} \) to be a valid model to study interaction with phospho-dependent protein partners.

**Interaction of tau with heparin**

To finish this short survey of our experience with several models of tau multiple phosphorylations, we mention the interaction of tau with heparin [32]. This heparin binding could represent another model of multiple phosphorylations of tau, as it similarly introduces negative charges. In addition to the analysis of the heparin-binding sites and phosphorylation patterns, we have been characterizing the local and global conformational changes induced by phosphorylation and heparin binding to compare their effects. From the basic information of the carbon chemical shifts, the propensity of specific regions of the protein to adopt residual secondary structures can be derived.

---

**Figure 3 | Mapping by NMR of the interaction site of Pin1 with tau-Glu\(^{10} \) or a phospho-peptide**

Histogram of the chemical shift perturbations (\( \Delta C.S. \)) obtained after addition to [\( ^{15}N \)]Pin1 of an equimolar amount of tau-Glu\(^{10} \) (blue) or a 12-amino-acid peptide phosphorylated at Thr\(^{212} \) and Ser\(^{214} \) (full-length tau numbering) (pink), reported along the Pin1 sequence. The residues with broadened resonances observed after the addition of tau-Glu\(^{10} \) are represented with a horizontal red bar. The structure of the Pin1 protein (PDB code 1PIN [33]) is represented as a ribbon, with residues experiencing a shift upon addition of tau-Glu\(^{10} \) of between 0.04 and 0.1 coloured orange and those with a shift above 0.1 in red. Residues with broadened resonances are in blue. In the ligand-binding site, a sulfate molecule and a proline residue are represented as spheres, with a sulfur atom in yellow, oxygen atoms in red and carbon atoms in green. The WW domain of Pin1 and its catalytic domain are labelled WW and Cat respectively.
To this end, the chemical shifts of the experimental $\alpha$C atoms are compared with the values of the corresponding amino acids in random coil polymers. To characterize the global conformational changes, PRE (paramagnetic relaxation enhancement) can be used, with introduction of nitroxide labels attached on cysteine residues that will switch off the resonance signals of the residues in spatial proximity. Even a transient interaction in the protein can then be detected. Although these structural characterizations are of little interest themselves, they could reflect the conformation of the bound protein and be important parameters for the nucleation of the aggregates. We proposed previously that the negative charges introduce by heparin could neutralize the lysine residues located next to the hydrophobic peptides in the repeat regions, R2 and R3 (Lys$^{280}$ and Lys$^{311}$), allowing the stacking of segments that will form the core of the aggregates [32]. Phosphorylation in the regions flanking the microtubule-binding domain could similarly neutralize key lysine residues, facilitating the tau–tau interaction.

Concluding remarks
We believe that the use of well-characterized multi-phosphorylated tau samples will be of importance to define a ‘phospho-code’ that would link specific phosphorylation to functional output. In addition to its capacity to provide a complete map of all the phosphorylations in a single experiment, we hope that we have demonstrated the capacity of NMR spectroscopy to characterize interactions with a per residue resolution. This proves particularly important in facing the challenge offered by the dynamic complexes formed by unfolded and multi-phosphorylated proteins. Finally, NMR can also provide information on structures, even in unfolded proteins, that could prove to be important to define the characteristic of interacting regions. However, to be able to understand how specific phosphorylations influence aggregation or microtubule binding, results are lacking on the structures of these gigantic complexes.

Acknowledgements
We thank Professor K. Kosik (University of California Santa Barbara, Santa Barbara, CA, U.S.A.) and Professor R. Brandt (University of Osnabruck, Osnabruck, Germany) for providing CDKS-p25 and tau-Glu$^{10}$ respectively.

Funding
This work was supported by the Agence Nationale de la Recherche [grant number ANR-05-blanc-6326-01]. The NMR facilities were funded by the Région Nord, CNRS (Centre National de la Recherche Scientifique) Pasteur Institute of Lille, European Community (Fondo Europeo de Desarrollo Regional), French Research Ministry and the University of Sciences and Technologies of Lille I. A. W. was supported by a predoctoral fellowship of the CNRS/Région Nord-Pas de Calais (France).

References
5 Dyson, H.J. and Wright, P.E. (2006) According to current textbooks, a well-defined three-dimensional structure is a prerequisite for the function of a protein. Is this correct? JUBMB Life 58, 107-109


Received 5 January 2010
doi:10.1042/BST0381006