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NMR Analysis of a Tau Phosphorylation Pattern

Isabelle Landrieu,[†] Ludovic Lacosse,[†] Arnaud Leroy,[†,‡] Jean-Michel Wieruszeski,[†] Xavier Trivelli,[§] Alain Sillen,[†] Nathalie Sibille,[†] Harald Schwalbe,[||] Krishna Saxena,[||] Thomas Langer,[||] and Guy Lippens*[,†]

Contribution from the CNRS UMR 8525, Institut Pasteur de Lille, 59019 Lille Cedex, France, Laboratoire de Biochimie Appliquée, Faculté de Châtenay-Malabry (Paris XI), Tour D4, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry Cedex, France, IFR118, UMR8576, Université des Sciences et Technologies de Lille I (USTL), Lille, France, and Johann Wolfgang Goethe-University, Center for Biomolecular Magnetic Resonance, Institute for Organic Chemistry and Chemical Biology, Marie Curie-Str. 11, 60439 Frankfurt am Main, Germany

Received July 13, 2005; E-mail: Guy.lippens@pasteur-lille.fr

Abstract: The phosphorylation of the neuronal Tau protein modulates both its physiological role of microtubule binding and its aggregation into paired helical fragments observed in Alzheimer's diseased neurons. However, detailed knowledge of the role of phosphorylation at specific sites has been hampered by the analytical difficulties to evaluate the level of site-specific phosphate incorporation. Even with recombinant kinases, mass spectrometry and immunodetection are not evident for determining the full phosphorylation pattern in a qualitative and quantitative manner. We show here that heteronuclear NMR spectroscopy on a ^{15}N labeled Tau sample modified by the cAMP dependent kinase allows identification of all phosphorylation sites, measures their level of phosphate integration, and yields kinetic data for the enzymatic modification of the individual sites. Filtering through the ^{15}N label discards the necessity of any further sample purification and allows the in situ monitoring of kinase activity at selected sites. We finally demonstrate that the NMR approach can equally be used to evaluate potential kinase inhibitors in a straightforward manner.

Introduction

Tau is a neuronal microtubule associated protein (MAP) that regulates the polymerization of tubulin and the stability of the resulting microtubules. When isolated, it is a natively unstructured protein, very soluble, and devoid of enzymatic activity. Six isoforms of Tau are present in the human brain, ranging from 352 to 441 amino acid residues, and obtained by alternative splicing from a single gene.[1] The shortest isoform of Tau is mainly expressed in foetal tissues, while all six isoforms can be found in an adult brain. Tau interacts with the microtubule via its microtubule-binding domain, located in the C-terminal half of the protein and constituted by three or four pseudo-repeats of approximately 30 amino acids. The microtubule-binding region is flanked upstream by a basic proline-rich regulatory region, which can enhance the binding.[2,3] In the cellular context, the biological activity of Tau is regulated by its degree of phosphorylation, where modification of most sites tends to weaken microtubules binding. Both in vivo and in vitro, Tau is a substrate for many kinases, including PKA.

Beyond its physiological role, Tau is the major component of the paired helical filaments (PHF) that form the intracellular neurofibrillary tangles (NFT) in neurons in the pathological situation of Alzheimer's disease (AD). Tau in PHF is hyperphosphorylated and potentially phosphorylated on sites unique to the disease.[4−6] Mass spectrometry and immunodetection with specific antibodies have identified 25 major phosphorylation sites on PHF−Tau isolated from patients with AD.[7−9] Unlike a normal Tau that contains 2−3 phosphate groups, the hyperphosphorylated Tau in PHF contains 5 to 9 mol of phosphate/mol of the protein.[10] However, overlap exists between the AD and normal adult patterns of phosphorylation, despite quantitative differences in the level.[11] In the same sense, in mitotic cells where the microtubules show a high dynamic instability,[12] Tau exhibits a higher degree of phosphorylation than that in differentiated neurons, suggesting that an erroneous re-entry in the cell cycle of the neurons might be related to the disease.[13]

[†] Institut Pasteur de Lille.
[‡] Faculté de Châtenay-Malabry (Paris XI).
[§] Université des Sciences et Technologies de Lille I (USTL).
[||] Johann Wolfgang Goethe-University.

(1) Buée, L.; Bussiere; T.; Buée-Scherrer, V.; Delacourte, A.; Hof, P. R. *Brain Res. Rev.* **2000**, *33*, 9.
(2) Goode, B. L.; Denis, P. E.; Panda, D.; Radeke, M. J.; Miller, H. P.; Wilson, L.; Feinstein, S. C. *Mol. Biol. Cell* **1997**, *8*, 353.
(3) Chau, M. F.; Radeke, M. J.; de Ines, C.; Barasoain, I.; Kohlstaedt, L. A.; Feinstein, S. C. *Biochemistry* **1998**, *37*, 17692.
(4) Sergeant, N.; Delacourte, A.; Buée, L. *Biochim. Biophys. Acta* **2005**, *1739*, 179.
(5) Stoothoff, W. H.; Johnson, G. V. *Biochim. Biophys. Acta* **2005**, *1739*, 280.
(6) Kosik, K. S.; Shimura, H. *Biochim. Biophys. Acta* **2005**, *1739*, 298.
(7) Hasegawa, M.; Morishima-Kawashima, M.; Takio, K.; Suzuki, M.; Titani, K.; Ihara, Y. *J. Biol. Chem.* **1992**, *267*, 17047.
(8) Morishima-Kawashima, M.; Hasegawa, M.; Takio, K.; Suzuki, M.; Yoshida, H.; Watanabe, A.; Titani, K.; Ihara, Y. *Neurobiol. Aging* **1995**, *16*, 365−380.
(9) Hanger, D. P.; Betts, J. C.; Loviny, T. L.; Blackstock, W. P.; Anderton, B. H. *J. Neurochem.* **1998**, *71*, 2465.
(10) Köpke, E.; Tung, Y. C.; Shaikh, S.; Alonso, A. C.; Iqbal, K.; Grundke-Iqbal, I. *J. Biol. Chem.* **1993,** *268*, 24374.

Because hyperphosphorylation compromises the Tau−microtubule interaction and can induce in vitro assembly of Tau into PHF,[14] the involved kinases are considered potential therapeutic targets.[15] However, any screen for inhibitors that selectively would inhibit the phosphorylation sites responsible for the pathology of AD,[16] requires the preliminary establishment of the link between individual kinases or combinations of them and the generated phosphorylation pattern, both in qualitative (what sites?) and quantitative (to what extent?) terms. Even when the enzymatic reaction is executed in a test tube with a well-known protein substrate and recombinant kinase, current analytical methods still face serious shortcomings. Despite impressive methodological developments, the necessity to preprocess the sample, the tendency of phosphorylation sites on Tau to cluster, and the technical difficulties inherently associated with the detection and quantification of the highly charged phospho-peptide prevent mass spectrometry from being a routine analytical tool to establish a precise phosphorylation pattern.[17−20] Immunodetection by antibodies is equally limited by the requirement of a comprehensive antibody library against all possible epitopes and by the absence of a fully phosphorylated standard for every combination of sites in order to quantify the level of phosphorylation. New analytical methods able to rapidly identify the phosphorylation sites for a given kinase or combination of kinases and quantify their extent of phosphate incorporation are still needed. We argue that NMR spectroscopy can fill this gap, by providing unbiased and unambiguous identification of the phosphorylated residues, as well as data on the reaction kinetics at the different sites. Moreover, the technique uniquely allows the analysis of the structural consequences that accompany certain phosphorylation events. As a demonstration, we present here our results on the Tau protein phosphorylation by the cAMP dependent protein kinase (PKA) and present kinetic data for each of the PKA generated phosphorylation sites.

Experimental Section

Preparation of Recombinant Proteins. Proteins were produced in *Escherichia coli* using the pET15B vector and were fused at their N-terminus to a histidine tag (Novagen). Tau 441 mainly used in this study is the longest Tau isoform comprising 441 amino acid residues. Tau 352 is the shortest isoform that has no N-terminal insert and three tandem repeats at the C-terminus. Tau[163−441]C2S is a fragment of Tau with both Cys residues (Cys291 and Cys322) mutated to Ser, generated by PCR amplification of the corresponding cDNA coding sequence, and cloned in the pET15b vector in the same manner as the cDNA for Tau 441 or Tau 352. Isotope labeling was performed by growing cells in a rich LB medium until $OD_{600} = 0.6-0.8$ before switching to half the volume of modified minimal M9 medium,[21] containing ^{15}N NH_4Cl and/or ^{13}C glucose. Purification of the recombinant Tau protein Tau 441, Tau 352, and Tau[163−441]C2S includes 15 min of heating at 75 °C, followed by affinity purification on Ni-NTA resin (HiTrap column, GE Healthcare). Purity of the fractions was checked by 12% SDS-PAGE. The pooled fractions containing the histidine-tagged recombinant protein were dialyzed (cutoff 3000 Da) in two changes against 50 mM ammonium carbonate, 1 mM DTT before lyophilization. The lyophilized powder was directly solubilized in the kinase buffer. The concentration of recombinant Tau was determined at 280 nm. PKA was prepared as described.[22]

In Vitro Tau Phosphorylation. Recombinant Tau protein (10 μM) was incubated with 0.1 μM or 1 μM recombinant PKA catalytic subunit in a buffer containing 5 mM ATP, 12.5 mM $MgCl_2$, 50 mM Hepes pH 8.0, 55 mM NaCl, 5 mM DTT, at 30 °C. A Tau sample in the same buffer without adding the enzyme was used to obtain a control HSQC spectrum. The reaction was heat-inactivated at 75 °C for 10 min and dialyzed against 50 mM ammonium carbonate, 1 mM DTT before lyophilization. The powder was suspended to obtain a sample of 100 μM Tau protein in 25 mM Na_2HPO_4/NaH_2PO_4 pH 6.9, 25 mM NaCl. NMR samples were filtered at 0.2 μM. When the phosphorylation reaction was directly followed in an NMR tube, we used the same buffer conditions but followed the reaction at 20 °C while acquiring the spectrum directly on the 10 μM Tau sample. Following heat inactivation of the PKA by directly heating the NMR tube, a control spectrum was recorded.

Isoelectrofocalization in Native Conditions. Isoelectric focusing homemade gels containing 8% (w/v) acrylamide and 3.3% (w/v) bisacrylamide as cross-linker and 2% (w/v) pH 3−10 ampholines (Fluka) were used with a 20 mM NaOH cathodic buffer and a 10 mM H_3PO_4 anodic buffer. Before migration of the samples, the ampholines are electrofocalized at 200 V for 0.5 h. Samples were prepared by suspending 12 μg of lyophilisated Tau in 10 μL of sample buffer (20% glycerol, 2% ampholines pH 3−10). Electrofocusing of the samples required the combination of one night at 400 V followed by 1 h at 600 V, at 4 °C.

NMR Spectroscopy. NMR measurements were performed at 293 K on a Bruker Avance 600 MHz spectrometer with a triple resonance cryogenic probehead or a Bruker Avance 800 MHz spectrometer with a regular triple resonance probe (Bruker, Karslruhe, Germany). 2D $^1H-^{15}N$ HSQC spectra were used to monitor Tau phosphorylation, and 1D versions of the same HSQC spectrum, to screen the kinase inhibitors. Two-dimensional spectra were acquired with 1024 complex points for 13 ppm in the proton dimension and 128 complex points for 30 ppm in the ^{15}N indirect dimension. The water signal was suppressed using the Watergate scheme.[23] The SOFAST-HMQC sequence[24] gave optimal *S*/*N* for a fixed total acquisition time with a 50 ms recycling delay. Peak integrals were determined for 10 isolated peaks that do not change upon phosphorylation, thereby defining an absolute integral scale for an amide function. Correlation peaks for phosphorylated residues were integrated and compared to this first value, to measure phosphorylation levels of the given serine residue. Resonance assignments of phosphorylated residues of Tau were achieved using the CBCANH, CBCA(CO)NH,[25] and HNN[26] triple resonance experiments with standard Bruker pulse programs. Acquisition parameters were 1024 (^1H), 47 (^{15}N), and 72 (^{13}C) complex points and 16 or 8 scans per increment. The in situ phosphorylation assay was performed in a regular 5 mm NMR tube, at 20 °C, with a sample containing 10 μM ^{15}N-labeled Tau and 0.1 μM PKA in the standard phosphorylation buffer. A regular HSQC pulse sequence was acquired on a Bruker Avance 800 MHz

(11) Wischik, C. M.; Edwards, P. C.; Lai, R. Y.; Gertz, H. N.; Xuereb, J. H.; Paykel, E. S.; Brayne, C.; Huppert, F. A.; Mukaetova-Ladinska, E. B.; Mena, R.; et al. *Neurobiol. Aging* **1995**, *16*, 409.
(12) Drewes, G.; Ebneth, A.; Mandelkow, E. M. *Trends Biochem. Sci.* **1998**, *23*, 307.
(13) Vincent, I.; Rosado, M.; Davies, P. *J. Cell Biol.* **1996**, *132*, 413.
(14) Alonso, A.; Zaidi, T.; Novak, M.; Grundke-Iqbal, I.; Iqbal, K. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 6923.
(15) Lau, L. F.; Seymour, P. A.; Sanner, M. A.; Schachter, J. B. *J. Mol. Neurosci.* **2002**, *19*, 267.
(16) Ahn, J. S.; Musacchio, A.; Mapelli, M.; Ni, J.; Scinto, L.; Stein, R.; Kosik, K. S.; Yeh, L. A. *J. Biomol. Screen* **2004**, *9*, 122.
(17) McLachlin, D. T.; Chait, B. T. *Curr. Opin. Chem. Biol.* **2001**, *5*, 591.
(18) Mann, M.; Ong, S. E.; Gronborg, M.; Steen, H.; Jensen, O. N.; Pandey, A. *Trends Biotechnol.* **2002**, *20*, 261.
(19) Zeller, M.; Konig, S. *Anal. Bioanal. Chem.* **2004**, *378*, 898.
(20) Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; Gygi, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6940.
(21) Marley, J.; Lu, M.; Bracken, C. *J. Biomol. NMR* **2001**, *20*, 71.
(22) Langer, T.; Vogtherr, M.; Elshorst, B.; Betz, M.; Schieborr, U.; Saxena, K.; Schwalbe, H. *ChemBioChem* **2004,** *5*, 1508.
(23) Potto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661.
(24) Schanda, P.; Brutscher, B. *J. Am. Chem. Soc.* **2005**, *127*, 8014.
(25) Grzesiek, S.; Bax, A.; Hu, J. S.; Kaufman, J.; Palmer, I.; Stahl, S. J.; Tjandra, N.; Wingfield, P. T. *Protein Sci.* **1997**, *6*, 1248.
(26) Weisemann, R.; Ruterjans, H. Bermel, W. *J. Biomol. NMR* **1993**, *3*, 113.

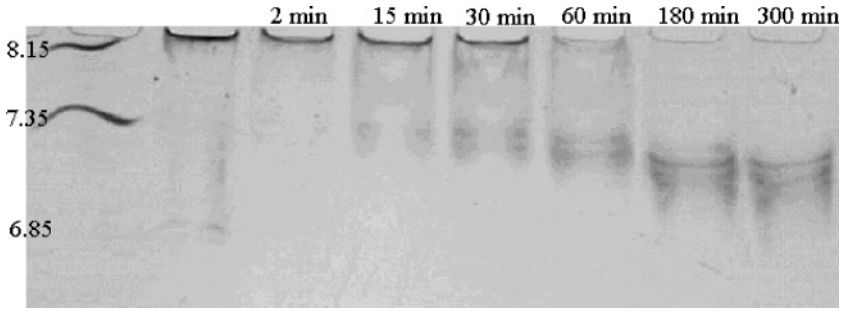

Figure 1. Isoelectrofocusing-PAGE with pH 3−10 ampholines of differentially phosphorylated Tau. In vitro PKA phosphorylated Tau (enzyme/substrate molar ratio 1:10) was fractionated according to its global charge. Samples correspond to various incubation periods, as indicated above the gel. Lane 1 is an unphosphorylated control Tau sample. A pH marker is used as a standard to estimate the pI of the fractionated molecules: lentil pI 8.15, myoglobin basic band pI 7.35, myoglobin acidic band pI 6.85.

spectrometer, with 256 scans per increment. At the end of the experiment, the sample was heated for 15 min at 70 °C, and the same spectrum was recorded a second time. ^{15}N traces were extracted at the final position of the phospho-Ser ^{1}H frequencies for both spectra. The interferogram of the control spectrum was multiplied by an exponentially increasing function $(1 - \exp(-kt))$, where t represents the time scale set by an individual increment, and the kinetic parameter k was optimized to obtain a best fit between both traces. Proton chemical shifts were measured in parts per million (ppm) and referenced relative to the methyl proton resonances of the internal standard trimethylsilyl propionate. 3D-spectra processing and peak picking were performed using the SNARF program (Frank Van Hoesel, Groningen, The Netherlands).

Assay of the Kinase Inhibitors. Recombinant Tau protein (10 μM) was incubated overnight with 0.01 μM recombinant PKA catalytic subunit in a buffer containing 30 μM ATP, 12.5 mM $MgCl_2$, 50 mM Hepes pH 8.0, 55 mM NaCl, 5 mM DTT, at 30 °C. The reaction was heat-inactivated at 75 °C for 10 min and spun at 14000 *g* for 10 min. A 1D-NMR spectrum was acquired on the crude enzymatic reaction mixture without further purification or concentration of the sample. Inhibitors were H-89[27] or *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCl (K_i = 48 nM for PKA), HA-1004[28] or *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide·2HCl (PKA K_i = 2.3 μM), and Staurosporine[29] (PKA K_i = 7 nM), purchased from Biomol (Exeter, UK). LiCl, a selective inhibitor of GSK3β, was used as a control. Inhibitors were added at 2 μM for 15 min before the reaction was started by addition of the PKA enzyme. Staurosporine required DMSO for solubility, so the final concentration of DMSO in the enzymatic reaction was 1%. A control experiment with 1% DMSO without staurosporin proved the absence of effect of the DMSO alone.

Results

In Vitro Phosphorylation of Tau by PKA. Unphosphorylated recombinant Tau was incubated in kinase buffer in the presence of a catalytic amount of recombinant PKA. Samples were removed from the reaction vessel at different moments and were subjected to isoelectric focusing electrophoresis (IEF) to determine the time frame of the reaction for further NMR experiments. When the same samples were fractionated on SDS-PAGE to check for potential degradation of the Tau protein during incubation, no proteolysis was observed. The IEF electrophoresis showed an acidification of the Tau protein, characterized by a faster electrophoretic migration of the phosphorylated Tau protein. At a molar ratio of 1:10 enzyme/substrate, we could already detect the enzymatic phosphorylation after a few minutes, and further acidification of the sample was clearly visible after 15 min (Figure 1). The acidification of the Tau sample after 5 h of incubation with PKA corresponds to an estimated decrease of its pI of about one pH unit. The electrophoretic profile was almost similar between 3 and 5 h of incubation with PKA, showing that the reaction reaches a plateau. IEF hence sets a time scale for the enzymatic reaction but cannot provide accurate information on the nature of the sites.

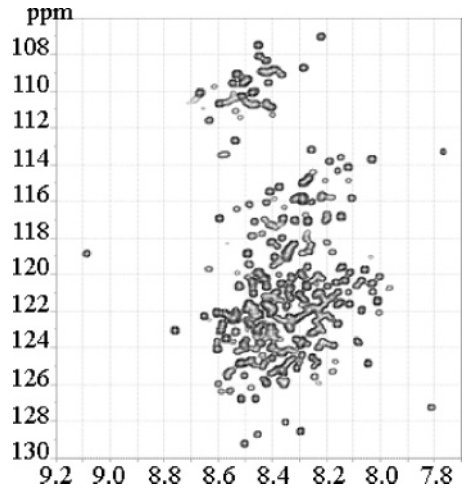

Figure 2. PKA-phosphorylated Tau ^{1}H−^{15}N HSQC. ^{15}N-Tau was incubated during 15 min in the presence of PKA, at a molecular enzyme/substrate ratio of 0.1 μM/10 μM. The spectrum was acquired following buffer exchange and sample concentration as described in the Experimental Section. The phospho-Ser214 resonance (9.09;118.6) is the only signal detected above 8.8 ppm.

NMR Kinetics of the Phosphorylation Reaction. ^{1}H−^{15}N HSQC spectra of ^{15}N-Tau incubated for various time points as previously defined by IEF were recorded and compared to a control HSQC of the Tau protein incubated in the same reaction buffer in the absence of PKA enzyme. The phosphorylated serine or threonine residues are readily detected as phosphorylation shifts their amide proton resonance downfield 8.8 ppm, to an empty region of the ^{15}N-Tau HSQC spectrum (Figure 2).[30] In the first spectrum, we already noted many such signals, and overnight incubation of ^{15}N-Tau with PKA generated an estimated 15 sites of which 6 were intense. Because the high enzyme-to-substrate ratio might lead to nonspecific phosphorylation, we decreased the enzyme/substrate molar ratio to 1:100

(27) Chijiwa, T.; Mishima, A.; Hagiwara, M.; Sano, M.; Hayashi, K.; Inoue, T.; Naito, K.; Toshioka, T.; Hidaka, H. *J. Biol. Chem.* **1990**, *265*, 5267.
(28) Hidaka, H.; Inagaki, M.; Kawamoto, S.; Sasaki, Y. *Biochemistry* **1984**, *23*, 5036.
(29) Omura, S.; Sasaki, Y.; Iwai, Y.; Takeshima, H. *J. Antibiot.* **1995**, *48*, 535.
(30) Bienkiewicz, E. A.; Lumb, K. J. *J. Biomol. NMR* **1999**, *15*, 203.

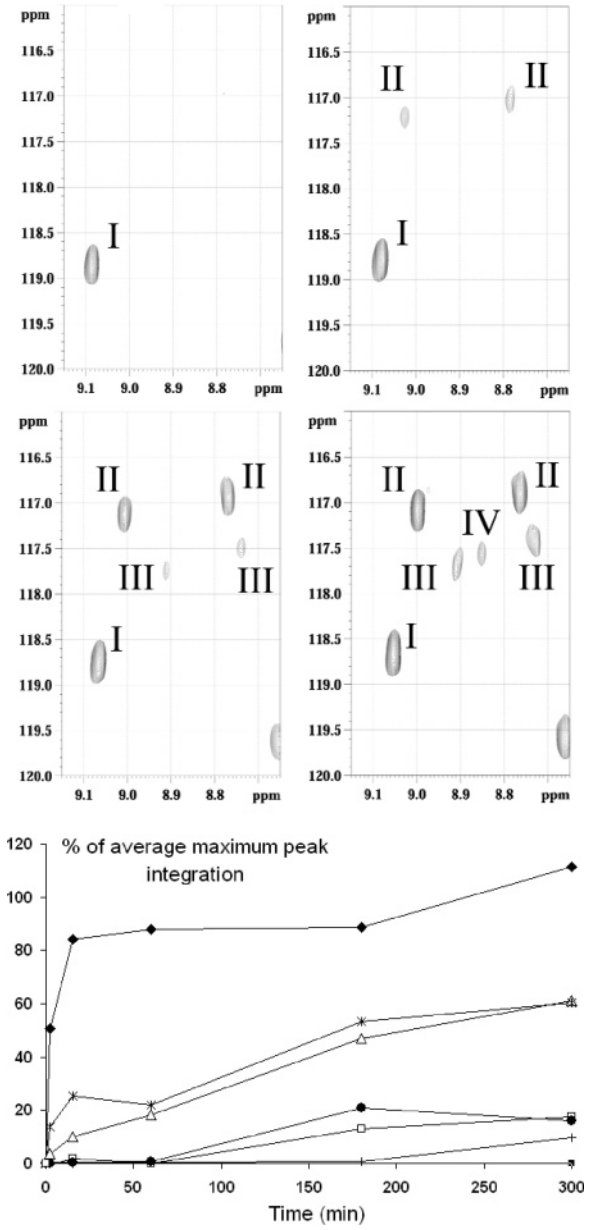

Figure 3. (Upper panels) Appearance of the phospho-Ser resonances during the time course of the Tau phosphorylation reaction by PKA, after 15 min (upper left), 60 min (upper right), 180 min (lower left), and 300 min (lower right) of incubation with an enzyme/substrate molar ratio of 0.1 μM:10 μM. The spectra were acquired following buffer exchange and sample concentration as described in the Experimental Section. (Lower panel) Integrated peak volumes as a function of the reaction time. (Diamonds) phospho-Ser214 (I), (stars) phospho-Ser208 (II), (triangles) phospho-Ser324 (II), (circles) phospho-Ser416 (III), (squares) phospho-Ser409 (III), and (crosses) phospho-Ser356 (IV)

and noticed that PKA phosphorylation of Tau slows down and becomes almost sequential, thereby facilitating the identification of individual resonances. When phosphorylated for 15 min in these conditions, a single peak (class I) at 9.08/118.7 ppm was detected, with an intensity close to that of a fully occupied site (Figures 2 and 3).

This first phosphorylation site was readily identified as phospho-Ser214, as we noted the concomitant disappearance of the Ser214 resonance, previously assigned as a rather isolated resonance in the overcrowded Tau spectrum.[31,32] Upon prolonging the phosphorylation reaction for 1 h, two additional resonances (class II) appeared close to the phospho-Ser214. After 5 h, a total of 6 resonances are detected in the region of the spectrum located between 8.8 and 9.2 ppm. The class II peaks obtained an intensity close to 60%, whereas the last three (class III) that only appeared after 3 to 5 h of incubation did not reach more than 20% of full occupation (Figure 3). The appearance of new resonances corresponding to phosphorylated serine/threonine residues was correlated with chemical shift perturbations in the crowded region of Tau resonances, but previous assignments were not sufficient to unambiguously assign them.

Identification of the Phosphorylation Sites. A sample of ^{15}N,^{13}C Tau phosphorylated on the six expected phosphorylation sites was prepared for further assignment through triple resonance spectroscopy. CBCANH and CBCA(CO)NH spectra were used to identify the nature of every phosphorylated amino acid and of their preceding residue. Because of its unstructured nature, random coil carbon NMR shifts had been found to be generally valid for Tau,[31,32] and the same is true for the phosphorylated protein. On the basis of the observed carbon chemical shift values (57.9 $\pm$ 0.5 ppm for Cα, 66.2 $\pm$ 0.5 ppm for Cβ), we conclude that all the phosphorylated residues correspond to serines.[30] Four of the six phosphorylated Ser have a glycine at the $i - 1$ position, whereas the two remaining phospho-Ser are preceded by a Pro and Leu, respectively (Table 1, Figure 4). Extracting the Cβ plane centered around 66.2 ppm from the same CBCA(CO)NH experiment allowed identification of the amide resonances of the residues that have the phospho-Ser in the ($i - 1$) position. As expected, six resonances were found (Figure 4, green correlation peaks), but the poor resolution of the carbon dimension combined with extreme spectral degeneracy, with all phospho-Ser Cα and Cβ carbon frequencies resonating within 1 ppm, hindered a direct connection of those residues to their exact neighbor. A 3D HNN experiment[26] did allow connection of the amide cross-peak of the phospho-Ser residues with the amide nitrogen of the following residue (Figure 4). A triplet of resonances around a phospho-Ser were thus identified following this scheme and mapped onto the Tau primary sequence (Table 1).

A fragment of Tau, Tau[163−441]C2S, corresponding to the sequence between amino acid residues 163 and 441 of full length Tau 441 and the shortest Tau isoform Tau 352 were phosphorylated under the same conditions as full length Tau 441 (data not shown). The characteristic resonance pattern observed in both cases, identical to the one observed for full length Tau 441, confirms that all serine residues phosphorylated by PKA are located in the C-terminal part of Tau but not in the repeat R2 (Lys274 to Val306). Peak intensity perturbation of the phospho-Ser neighboring residues was additionally used to confirm the assignments. For example, the gradual increase of phospho-Ser416 peak intensity was coupled to a concomitant decrease of the previously assigned Gly415 resonance intensity. Phosphorylation of a serine generally shifted the resonances of its nearest neighbors, but not beyond one or two residues up- or downstream. A noticeable exception is Ser324 in the third repeat, whose phosphorylation leads to a small but significant shift of the Lys317 and Val318 cross-peaks (Figure 4).

(31) Lippens, G.; Wieruszeski, J. M.; Leroy, A.; Smet, C.; Sillen, A.; Buée, L.; Landrieu, I. *ChemBioChem* **2004**, *5*, 73.
(32) Smet, C.; Leroy, A.; Sillen, A.; Wieruszeski, J. M.; Landrieu, I.; Lippens, G. *ChemBioChem* **2004**, *5*, 1639.

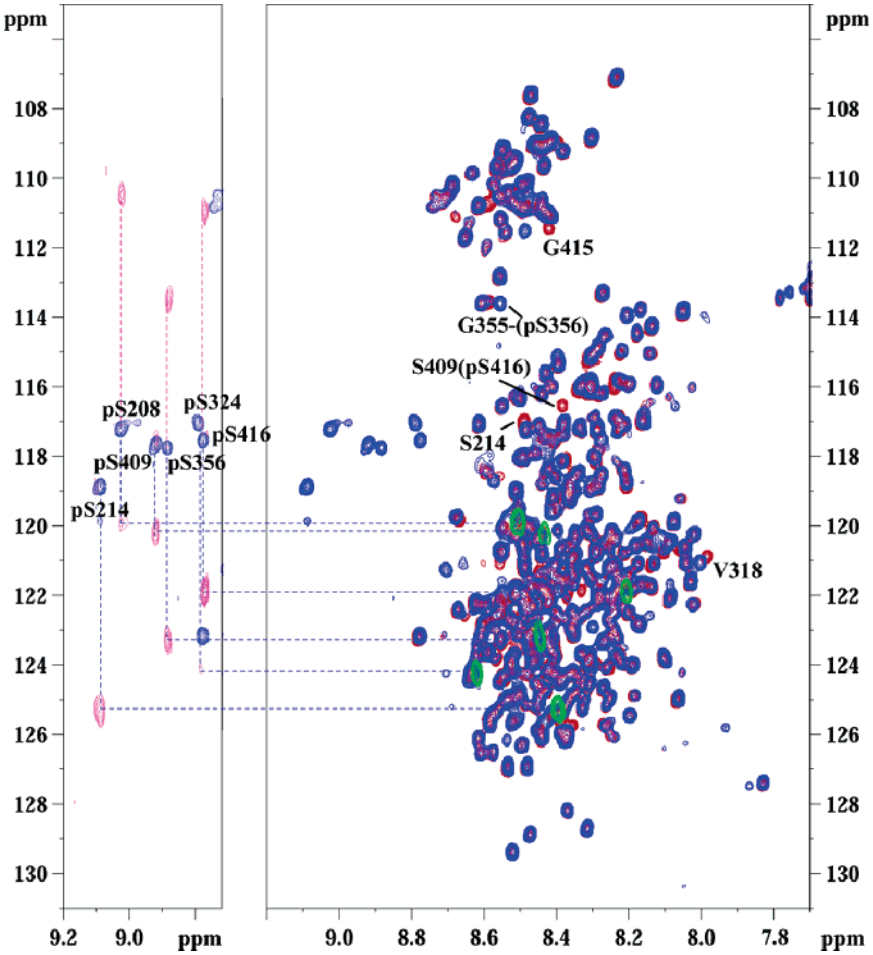

Figure 4. Superposed HSQC spectra of PKA-phosphorylated ^{15}N,^{13}C Tau (in blue) and ^{15}N Tau (in red). The labeled residues correspond to phosphorylated Ser and those affected by their phosphorylation. The green resonances superposed on the HSQC spectrum correspond to the resulting $^{1}H-^{15}N$ sum plane extracted from the 3D CBCAcoNH for Cβ values at 66 $\pm$ 0.5 ppm. On the left part: zoom of the phospho-Ser resonances, and the connections made by the HNN spectrum to the ^{15}N resonance of the (i + 1) residue. In some cases, the phospho-Ser also connected to the preceding Gly residue in this 3D pulse sequence. Phospho-Ser208, phospho-Ser356 and phospho-Ser416 are connected to both their $^{15}N(i + 1)$ and $^{15}N(i - 1)$ nitrogen frequencies located in the glycine region of the HSQC spectrum.

Table 1. Assignment of the Amino Acid Triplet around the Phospho-Serines

	residue $i-1$				residue i				residue $i+1$	
	^{1}H	^{15}N	CA	CB	^{1}H	^{15}N	CA	CB	^{1}H	^{15}N
Gly_{207}-pS_{208}-Arg_{209}		110.5	45.2	X	9.03	116.9	58.1	66.1	8.53	119.8
Pro_{213}-pS_{214}-Leu_{215}	X	X	63.1	32.1	9.09	118.6	57.6	66.0	8.42	125.3
Gly_{323}-pS_{324}-Leu_{325}			45.2	X	8.80	116.7	57.6	66.2	8.65	124.2
Gly_{355}-pS_{356}-Leu_{357}	8.50	113.5	44.9	X	8.89	117.6	58.0	66.2	8.47	123.0
Leu_{408}-pS_{409}-Asn_{410}			55.4	42.4	8.92	117.4	57.8	65.8	8.46	120.2
Gly_{415}-pS_{416}-Ile_{417}		111.0	45.0	X	8.78	117.3	57.8	66.1	8.23	121.8

Real-Time Spectroscopy of the Phosphorylation Reaction. An alternative to sampling the phosphorylation kinetics by removing samples at different time points from the reaction mixture would be to monitor the enzymatic reaction as it proceeds in the NMR tube. Although the reaction mixture is complex, with enzyme, ATP, and substrate, ^{15}N labeling allows for efficient filtering of all signals except for those of ^{15}N-Tau amide protons. Sequential 1D HSQC spectra are one possibility to quantify by NMR the reaction kinetics, if spectral overlap of different amide protons does not interfere with the quantification. In the case of overlap, rapid 2D HSQC spectra give a solution, but the salt concentrations in the reaction buffer and the concomitant high power dissipation prevented the use of rapid versions of this experiment.[24] We decided to extract kinetic data from the line shape of the correlation peaks in a regular HSQC spectrum recorded while the reaction is taking place in the NMR tube. Akin to the in situ monitoring at the level of individual amino acids of protein folding after a pH pulse,[33] one expects to see the growing population of phosphorylated residues through their amide correlation in a regular HSQC. As the signal increase translates into a slow exponential increase representing the enzymatic modification of the residue, superimposed on a damped sinusoide wave characterizing the transverse relaxing ^{15}N signal of the fully phosphorylated residue, fitting the ^{15}N interferogram should immediately give the relevant time constant.

(33) Balbach, J.; Forge, V.; Lau, W. S.; van Nuland, N. A.; Brew, K.; Dobson, C. M. *Science* **1996**, *274*, 1161.

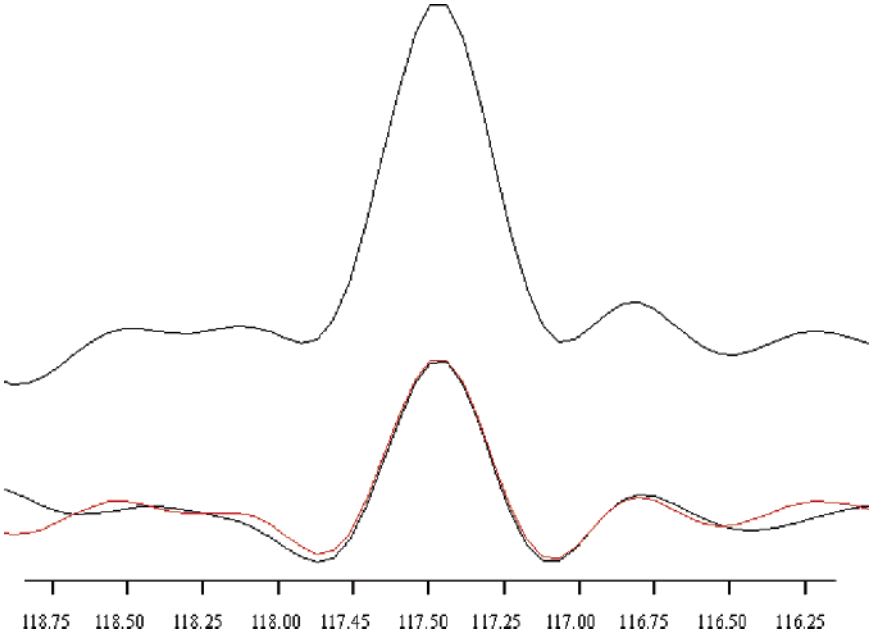

Figure 5. Experimental (black) and simulated (red) ^{15}N traces through the phospho-Ser208 resonance in the final spectrum after heating (top) or in the real-time phosphorylation experiment (bottom). For the simulation of the line shape of the latter, the interferogram of the reference spectrum was multiplied with an exponentially increasing function with a time constant of 5 h (see text).

The experiment was performed at 20 °C using a sample of 10 μM ^{15}N labeled Tau with 100 nM of PKA. After addition of ATP, the HSQC was started in a regular fashion. At the end of the spectral recording, the sample was heated for 15 min to 70 °C, to stop all enzymatic activity, and a second HSQC was recorded to obtain a reference spectrum. For Ser214, its trace in both spectra perfectly superimposes, confirming that phosphorylation at this site is very rapid and happens within the dead time of the experiment. For the Ser208 site, whose phosphorylation sets in at a later time, the comparison of the ^{15}N trace at the relevant proton frequency indeed shows the characteristic line shape expected for the difference of two lines with differential line width (Figure 5). When we convoluted the corresponding trace in the final spectrum (after heating) with an exponentially increasing function, we obtained an excellent fit, with $k = (5\ \mathrm{h})^{-1}$ for Ser208 and a similar value for Ser324. The other sites proved too slow to build up on the time scale of the experiment to allow a reliable fitting.

Evaluation of Inhibitors of the Phosphorylation Reaction. The direct measurement on the reaction mixture, without any sample preparation step, and the detection of the one or several phospho-Ser through their amide resonance in an isolated region of the spectrum open the possibility for screening potential inhibitors of the kinase involved in the enzymatic reaction. Various kinase inhibitors were thus assayed by NMR spectroscopy for their capacity to block the enzymatic activity.

A single phosphorylation peak at 9.13 ppm, corresponding to phospho-Ser214, was detected in the control ^{15}N Tau 1D-HSQC spectrum acquired on the reaction mixture containing a 10 μM sample of ^{15}N-Tau after an overnight incubation with 10 nM PKA (Figure 6). The peak integral is proportional to the extent of phosphate incorporation on this site, allowing the quantitative evaluation of the kinase inhibition by candidate inhibitory molecules. However, in our initial saturating conditions of 5 mM ATP, Staurosporine, a well-known competitive binder of the ATP binding site, was not able to successfully inhibit the enzymatic reaction despite its reported inhibition constant of 7 nM.[29] (data not shown). After controlling that PKA with only 30 μM ATP still gave constitutive phosphate integration at the Ser214 position after an overnight reaction (Figure 6, trace A), we tested both Staurosporine and H-89 in these novel low ATP conditions. Both molecules, with respective inhibition constants of 7 and 48 nM, indeed led to a complete disappearance of the phospho-Ser214 amide proton resonance

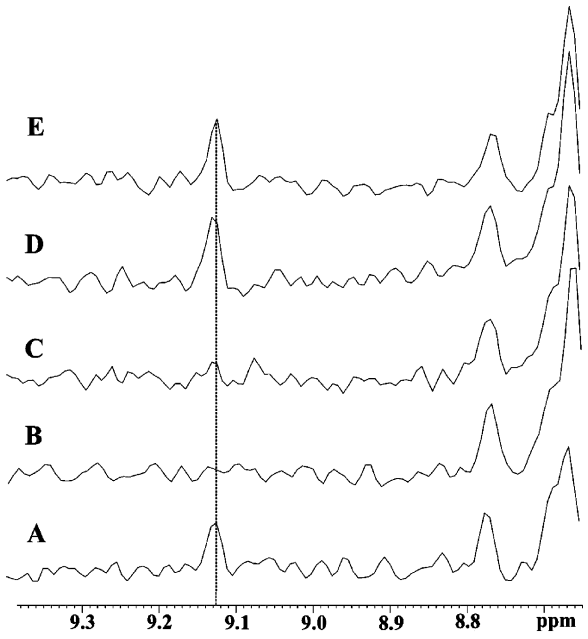

Figure 6. 1D ^{1}H,^{15}N HSQC NMR spectrum of overnight PKA-treated ^{15}N-Tau with 30 μM pf ATP and in the presence of 2 μM kinase inhibitors. The control phosphorylation reaction (bottom 1D spectrum) in the absence of inhibitor leads to the appearance of the phospho-Ser214 peak at 9.3 ppm. Enzymatic reactions in the presence of the following, from bottom to top spectrum: (A) no inhibitor (control), (B) Staurosporine, (C) H-89, (D) HA-1004, and (E) LiCl. No preprocessing of the crude samples after the enzymatic reaction was required.

when added in a 2 μM concentration to the reaction mixture (Figure 6, traces B and C). HA-1004 was not able to inhibit the enzymatic reaction, although its reported inhibition constant of 2.3 μM is of the same order as the dose used in this experiment (Figure 6, trace D). We finally used LiCl as a negative control as this compound affects the kinase activity of GSK3 but not of PKA. Comparison of the 1D NMR spectrum in the absence or presence of LiCl shows them to indeed be identical, confirming the selectivity of this compound (Figure 6, trace E).

Discussion

The covalent attachment of a phosphate group to the side chain of Ser, Thr, or Tyr residues is a reversible process governed by the balance between kinases and phosphatases. One-third of all the eukaryotic proteins are estimated to undergo reversible phosphorylation at a given time,[34] and signal transduction heavily relies on this modification. Because phosphorylation is dynamic, signaling events also depend critically on a quantitative parameter, in terms of both the number of phosphorylation sites and the level of modification of any individual site.[35]

Not surprisingly, the kinases and/or phosphatases involved in diverse regulation processes are implicated in various human diseases and hence have become attractive drug targets. A well-known example is the cyclin dependent kinases, controlling the cell cycle progression, that can be inactivated by competitive inhibitors of the ATP binding site like roscovitine or olomoucine, thereby affecting directly uncontrolled cell proliferation in cancer.[36−38] Alzheimer's disease is another field where phosphorylation plays a pivotal role,[14,15] as phosphorylation tends to detach the neuronal Tau protein from the microtubules, and hyperphosphorylation is characteristic for Tau aggregated into neurofibrillary tangles (NFTs).[7−9] The occupation level of individual sites is moreover of crucial importance, as, except for a few sites specific to the disease, the difference between the AD Tau and the normal Tau is indeed more a matter of quantity than of phosphorylation pattern.[11] Up to 25 sites can be phosphorylated by different kinases in a combinatorial fashion, leading to samples whose characterization remains a serious analytical challenge. Therefore, our knowledge about the precise function of these different phosphorylation states other than that they alter the binding kinetics of Tau to microtubules remains limited, and the same is true for their precise role in the pathological aggregation mechanisms. The lack of satisfactory analytical means to assess rapidly the phosporylation pattern on many Tau samples is clearly one of the underlying reasons that the phosphorylation code leading to Tau (dys)function is still not fully elucidated.

The use of an in vitro assay with one or several recombinant kinases avoids the potential regulation of the kinases in a cellular context and lowers the level of complexity brought in by other in vivo post-translational modifications. However, even in this simplified context of Tau phosphorylation, present analytical methods do not always agree, and further methods development to address the quantitative and qualitative aspect of phosphorylation is still needed. We present here a novel application of heteronuclear NMR spectroscopy toward this question of Tau phosphorylation and show that NMR spectroscopy can successfully monitor the modifications in a qualitative and quantitative manner. We demonstrate the approach for the case of Tau phosphorylation by PKA, an enzyme previously described to modify Tau in vitro, allowing a detailed comparison of our novel approach with the more conventional methods of immunochemistry or protein digestion followed by mass spectrometry identification of the peptides.

Immunodetection is a first method of choice to detect phosphorylation sites and has proven valuable in the identification of phosphorylation patterns on Tau molecules isolated from many given cellular contexts. The PHF-1 monoclonal antibody,[39] recognizing phospho-Ser396 and phospho-Ser404 on Tau, or the AT-8 antibody[40] with a specificity for both phospho-Ser199/phospho-Ser202 and phospho-Thr205, have been intensively used in both research and clinical settings. However, despite its technical advantages of simplicity and sensitivity, immunodetection can only be performed against those sites for which antibodies are available and is not necessarily optimal to quantify the occupation level of a given site, because fully phosphorylated standard samples often are lacking. The detection of phospho-Ser262 after phosphorylation with PKA isolated from beef heart[41] through the use of the phospho-Ser262 and phospho-Ser356 specific antibody 12E8 is an example of this. The HNN spectrum (Figure 4, green) unambiguously shows that we have no Thr residue in the (i + 1) position of any of the major phospho-Ser detected in our samples, thereby excluding Ser262 as a next neighbor of Thr263. The 12E8 antibody, however, because of its extremely high sensitivity might well have detected the phospho-Ser262 at such a low level that it goes undetected in our NMR analysis. A later study using the same 12E8 antibody confirmed that PKA has significantly poorer enzymatic activity toward this site than other kinases such as CaMKII.[42] Finally, immunochemical analysis of Tau with phosphorylation dependent monoclonal antibodies cannot even reliably reflect the phosphorylation state of specific sites in a background of multiple phosphorylations, as was shown in a correlation study between 2D-gel electrophoresis with ^{32}P-labeled PK40^{erk2}-phosphorylated Tau and immunochemical analysis with several monoclonal antibodies of the same samples.[43]

Mass spectrometry (MS) is the other method of choice to determine phosphorylation sites of Tau.[17−19] We confirm five phosphorylations mapped onto the Tau shortest foetal isoform (Ser214, 324, 356, 409, and 416) by protease digestion of a PKA phosphorylated Tau352 sample, peptide purification by reversed-phase HPLC, and further analyis by liquid secondary-ion mass spectrometry and N-terminal sequencing of the resulting peptides.[44] Two interesting observations emerge from the comparison with our results. First, we detect an additional phosphorylation site at Ser208, but subtle differences in tem-

(34) Zolnierowicz, S.; Bollen, M. *EMBO J.* **2000**, *19*, 483.
(35) Deshaies, R. J.; Ferrell, J. E., Jr. *Cell* **2001**, *107*, 819.
(36) Gray, N.; Détivaud, L.; Doerig, C.; Meijer, L. *Curr. Med. Chem.* **1999**, *6*, 859.
(37) Fischer, P. M.; Lane, D. P. *Curr. Med. Chem.* **2000**, *7*, 1213.
(38) Sielecki, T. M.; Boylan, J. F.; Benfield, P. A.; Trainor, G. L. *J. Med. Chem.* **2000**, *43*, 1.
(39) Otvos, L., Jr.; Feiner, L.; Lang, E.; Szendrei, G. I.; Goedert, M.; Lee, V. M. *J. Neurosci. Res.* **1994**, *39*, 669.
(40) Goedert, M.; Jakes, R.; Vanmechelen, E. *Neurosci. Lett.* **1995**, *189*, 167.
(41) Litersky, J. M.; Johnson, G. V.; Jakes, R.; Goedert, M.; Lee, M.; Seubert, P. *Biochem. J.* **1996**, *316*, 655.
(42) Sironi, J. J.; Yen, S. H.; Gondal, J. A.; Wu, Q.; Grundke-Iqbal, I.; Iqbal, K. *FEBS Lett.* **1998**, *436*.
(43) Roder, H. M.; Fracasso, R. P.; Hoffman, F. J.; Witowsky, J. A.; Davis, G.; Pellegrino, C. B. *J. Biol. Chem.* **1997**, *272*, 4509.

perature and enzymatic activity might explain this. A second more important difference resides in the quantification. Quantification of the level of phosphate integration in the previous study relied on radioactivity measurement on the same peptides after using [γ-^{32}P] ATP as enzyme substrate and indicated substoechiometric phosphorylation of some sites with only an average 2.6 mol of phosphate per mol of Tau after 6 h of enzymatic reaction. Without using radioactivity, we confirm that only the Ser214 site becomes readily phosphorylated, but we can equally determine the final phosphate incorporation for the other sites from the same experiment, as well as a comparative time scale for the enzymatic reaction (Figure 3). Real-time kinetic measurement of the phosphorylation reaction directly in the tube furthermore allows setting the same time scale on the basis of a single experiment, without any requirement for purification or further sample handling such as digestion, desalting, ..., thereby giving a competitive advantage to our method. Illenberg et al.,[45] while using a similar methodology based on [γ-^{32}P] ATP incorporation, digestion, and identification of phosphopeptides by mass spectrometry, identified additionally Ser262 (amidst 10 other sites) after extended incubation with PKA. Although we cannot exclude that the use of higher enzyme concentrations or longer incubation times eventually would lead to phosphate incorporation at this Ser262 site, we did prefer not to go beyond the conditions where phosphorylation at certain sites levels off (Figure 3), because we fear that the kinase eventually will lose specificity.

MS clearly is highly sensitive, making it the method of choice for analysis of biological material isolated from tissue, and the technique is able to define the location of phosphorylation sites. However, because of ionization problems in MALDI-TOF spectrometry, phosphopeptides are generally not observed as intense peaks. The negative charges of phosphopeptides further hinder ionization by electrospray when performed as usual in the positive mode, and ion suppression in the presence of unphosphorylated peptides can further reduce their intensity. The method requires furthermore preliminary enzymatic cleavage of the protein, with subsequent purification by RP-HPLC, thin-layer chromatography (TLC), or 2D-PAGE before measurement. Phosphorylation and especially clusters of phosphorylated residues can also reduce the cleavage efficiency. Given the numerous proximal phosphorylation sites found on Tau, peptide mass is moreover often not sufficient to identify the precise phosphorylated residue, requiring further analysis based on Edman degradation or LC−MS−MS sequencing. Finally, MS is used as a qualitative technique, and without a standard for every phosphorylated peptide, the intensity of the ion signals does not accurately measure the phosphorylation level on each site. These considerations demostrate how mass spectrometry in the field of phosphorylation mapping is still not a routine activity in most laboratories.[17−19]

One of the obvious drawbacks of our heteronuclear NMR methodology is the need to obtain the assignment of the phosphorylated residues. After identification of these residues as serines, based on the Cα/Cβ chemical shifts extracted from a CBCANH spectrum, our initial assignment was only based on the two residues surrounding the phospho-Ser. Resonances of phosphorylated residue indeed are conveniently shifted toward a spectral region devoid of resonances in the HSQC spectrum of the natively unfolded Tau, giving us a limited and highly simplified spectral region to consider. The CBCA(CO)NH spectrum then allows ready identification of the nature of the preceding residue, specifically easy in the case of a natively unstructured protein such as Tau where random coil carbon chemical shift values are good estimates of the true values.[32] Moreover, when extracting the carbon plane from the same spectrum centered around 66 ppm, a value highly specific for a phospho-Ser, we could identify the residues following the six phospho-Ser residues. Connecting those (i + 1) neighbors to their corresponding phospho-Ser was possible through the HNN experiment (Figure 4). As a last step, we used our recently achieved NMR data of Tau [31,32] to verify several phospho-Ser assignments, thereby confirming the pairwise assignment strategy.

Another obvious aspect to consider, clearly to the disadvantage of our methodology over mass spectrometry, is the limited sensitivity of the NMR experiment. Despite recent hardware developments with increased field strengths and cryogenic probes,[46] and pulse sequence development leading to faster data accumulation, [24] the Boltzmann factor determining the spin polarization makes that NMR sensibility will never compare to that of MS. However, the absence of extensive sample preparation steps, mainly through the efficient ^{15}N filtering of all but Tau signals, is a factor in favor of NMR, especially when we compare the extensive pretreatment required by MS identification of phosphorylation patterns. Our in vitro kinetics experiment, with a single overnight measurement on a 10 μM sample, yields indeed kinetic data for the modification of the different sites. For the NMR evaluation of PKA inhibitors, sensitivity becomes even more of an issue, as it determines whether the method can be applied in a high throughput mode or not. Using the SOFAST-HMQC sequence[24] with a cryogenic probe head on a spectrometer operating at 600 MHz, we now obtain a satisfactory signal-to-noise ratio of 5 for the isolated phospho-Ser214 signal within 10 min. Considering that a mixture of potential inhibitors can be simultaneously tested and that this assay could be easily adapted to flow-through NMR equipment,[47] a reasonable screening rate can be easily attained. Although this rate cannot compare with those other sensitive assays based on antibody detection,[16] the present NMR based assay does not depend on the preliminary biotinylation of the sample or any other pretreatment. Moreover, adapting it to another enzyme is straightforward, with only the requirement of gaining once an approximate idea of the enzymatic efficiency to obtain detectable phosphorylation. A simple IEF experiment, as we used in this study, can set the correct time scale (Figure 1). Testing the selectivity of compounds against different kinases in a secondary screen might indeed be an ideal application for our NMR assay, as it does not require the lengthy step of setting up an immunochemistry assay for each phosphorylation site.

Finally, we have not exploited here the structural information that NMR spectroscopy can bring in. Disorder-to-order or reversely order-to-disorder transitions might happen upon phos-

(44) Scott, C. W.; Spreen, R. C.; Herman, J. L.; Chow, F. P.; Davison, M. D.; Young, J.; Caputo, C. B. *J. Biol. Chem.* **1993**, *268*, 1166.
(45) Illenberger, S.; Zheng-Fischhofer, Q.; Preuss, U.; Stamer, K.; Baumann, K.; Trinczek, B.; Biernat, J.; Godemann, R.; Mandelkow, E. M.; Mandelkow, E. *Mol. Biol. Cell* **1998**, *9*, 1495.
(46) Styles, P.; Soffe, N. F.; Scott, C. A.; Cragg, D. A.; Row: F.; White, D. J.; White, P. C. J. *J. Magn. Res.* **1984**, *60*, 397.
(47) Ross, A.; Schlotterbeck, G.; Klaus, W.; Senn, H. *J. Biomol. NMR* **2000**, *16*, 139.

phorylation and could also conveniently be monitored by mere chemical shift changes of residues other than the direct neighbors of the phosphorylated residues. In the present case study with PKA, the $^{1}H-^{15}N$ HSQC spectrum of the phosphorylated Tau protein indicated no such global conformational change upon phosphorylation, as chemical shift changes are limited to the pair of residues preceding or following the phospho-Ser. We presently are investigating with the use of residual dipolar couplings the structural consequences of the chemical shift changes detected for Lys317 and Val318 upon phosphorylation of Ser324. Even more, for other kinases or combinations of kinases, that are capable of generating specific antibody epitopes,[48] it will be most interesting to see whether the addition of phosphates mediates structural changes that could explain the transition to the aggregated state.

(48) Zheng-Fischhofer, Q.; Biernat, J.; Mandelkow, E. M.; Illenberger, S.; Godemann, R.; Mandelkow, E. *Eur. J. Biochem.* **1998**, *252*, 542.

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