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NMR observation of Tau in *Xenopus* oocytes

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ABSTRACT

The observation by NMR spectroscopy of microinjected ^{15}N-labelled proteins into *Xenopus laevis* oocytes might open the way to link structural and cellular biology. We show here that embedding the oocytes into a 20% Ficoll solution maintains their structural integrity over extended periods of time, allowing for the detection of nearly physiological protein concentrations. We use these novel conditions to study the neuronal Tau protein inside the oocytes. Spectral reproducibility and careful comparison of the spectra of Tau before and after cell homogenization is presented. When injecting Tau protein into immature oocytes, we show that both its microtubule association and different phosphorylation events can be detected.

1. Introduction

The possibility to observe biological macromolecules within the living cell by NMR spectroscopy might be able to bridge the gap between structural and cell biology. When we first observed the osmoregulated periplasmic glucans (OPGs) of the *Burkholderia solanacearum bacteria* by in-cell NMR [1], the absence of a cryoprobe at that moment and the concomitant problems of magnetic susceptibility when working at higher cell density forced us to apply High Resolution Magic Angle Spinning (HRMAS) NMR to record a reproducible high quality NMR spectrum [2]. Simultaneously, the group of V. Dötch combined bacterial protein overexpression with a cryoprobe to observe the first proteins in the cell [3]. However, contrary to our experimental system where synthesis of the endogeneous OPG molecules is physiologically high when the bacteria are grown in a low osmolar medium, the overexpression of proteins inside bacteria invariably leads to non-physiological concentrations, and the cell mostly represents a medium of realistic viscosity and crowding. Although a number of studies have recently succeeded to obtain novel biological insights through in-cell NMR [4,5], the bacterial cell and the requirement of significant overexpression remain a stringent limit on the establishment of the link between structural and cell biology. Very recently, two groups have shown that microinjection of small model proteins (GB1 and ubiquitin) in *Xenopus* oocytes could be combined with solution NMR to obtain workable spectra within an eucaryotic cell model [6,7]. With 200 oocytes inside the NMR active volume, and typical injection volumes of 50 nl for the estimated 1 μl volume of the oocyte, the millimolar stock solutions of the model protein gave rise to protein concentrations that could be observed on a time scale of 1 h. A decrease in amount of protein injected would evidently approach the experimental set-up to a more realistic *in vivo* situation, but requires measuring times that scale as the square of the inverse protein concentration.

Our goal was to explore different sample preparations to monitor lower protein concentrations inside the oocytes by NMR spectroscopy. We are particularly interested in the behaviour of Tau, a neuronal protein involved in microtubule stabilization but equally the major part of the intracellular tangles that form inside the neurons of Alzheimer diseased (AD) patients. Oocytes indeed have been previously used as a model system to evaluate the effect of specific Tau mutations on the meiotic spindle formation and M-phase entry following progesterone stimulation [8]. Moreover, *Xenopus* oocytes contain many of the kinases that are potentially involved in the generation of pathological Tau phosphorylation patterns, and the AT100 Alzheimer specific epitope can be generated upon maturation with progesterone [9]. In the latter experi-

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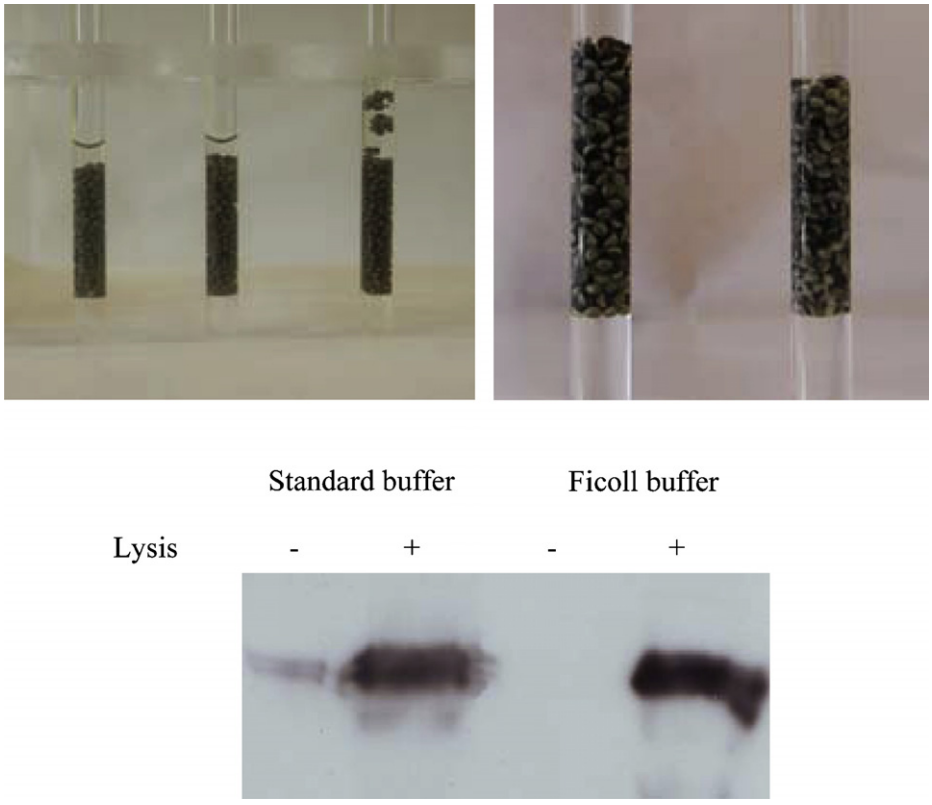

Fig. 1. (Top, left) initial samples of 200 oocytes in, from left to right, 0%, 10% and 20% Ficoll solution. The settling of the oocytes is slow in the latter condition. (Top, right) samples after overnight NMR measurement, in 20% Ficoll (left tube) or standard buffer conditions (right). (Bottom) Western blot of Tau in the supernatants after 14 h (lanes 1 and 3) of measurement. Oocytes were then lysed by adding the lysis buffer and mechanical crushing, and Tau was again dosed. Tau leaks from the oocytes in the standard buffer, whereas it remains in the intact oocytes when these are embedded in Ficoll.

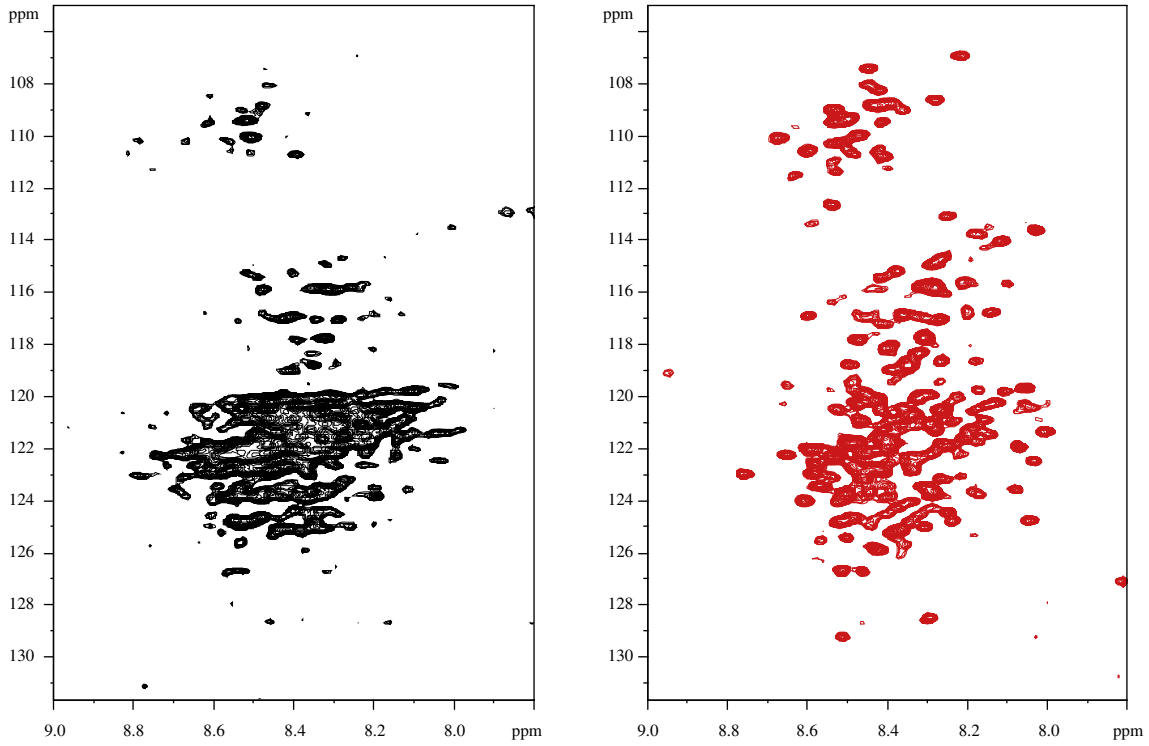

Fig. 2. Spectrum of the same oocyte sample (200 oocytes injected with 50 nl of a 100 μM ^{15}N-labelled Tau solution, in 20% Ficoll) before (left, black) and after (red, right) mechanical homogenization. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ments, though, the maximal amount of Tau injected led to a 5 μM intracellular concentration, which is nearly one order of magnitude lower than the one used in the previous NMR studies, and hence would require a measurement over 100 h to obtain a comparable signal to noise (S/N) ratio. Tau moreover is not exactly a model protein, but is with its 441 amino acids a challenge even for solution NMR. Its intrinsically disordered nature spectrum moreover leads to a badly resolved spectrum, even at high field strengths [10,11], further adding to the challenge of its observation in the cellular context of oocytes. Finally, we expect such small amounts of Tau to interact tightly with the microtubular network of the oocytes [12,13], and have hence the opportunity to evaluate the feasibility of NMR studies on a far from ideal protein inside the crowded intracellular environment of the oocyte.

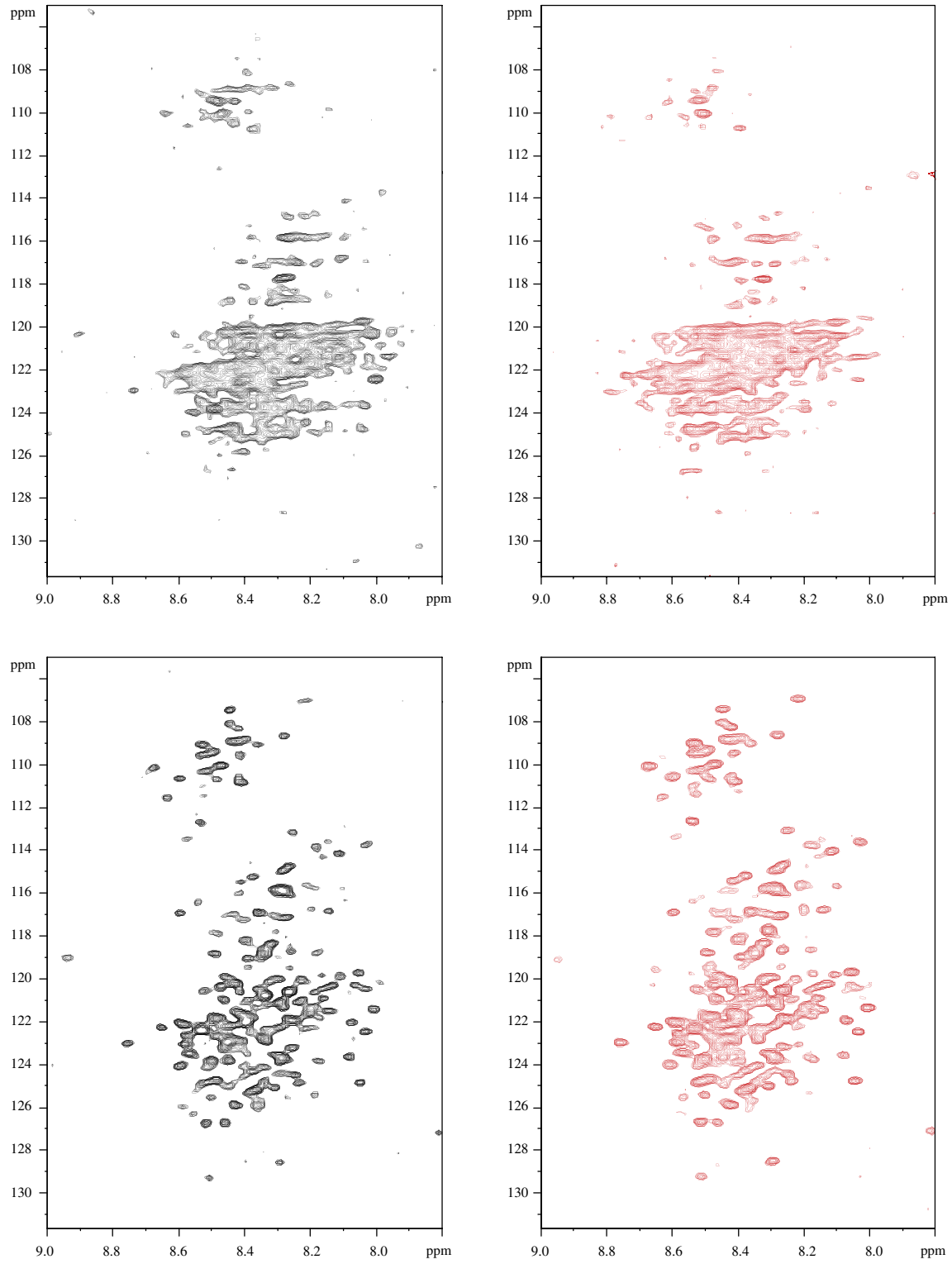

Fig. 3. Spectra of 200 oocytes injected with 50 nl of a 100 μM ^{15}N-labelled Tau solution, and embedded in a 20% Ficoll solution. Both spectra are separated by a 6 months time interval. The top panels show the spectra of the intact oocytes before mechanical homogenization, the bottom ones after lysis.

2. Experimental

^{15}N-labelled Tau protein was produced by bacterial overexpression and purified as described previously [10,11]. We made a 100 μM stock solution in a deuterated Tris–d_{11} 25 mM buffer, NaCl 25 mM, 300 μM DTT, pH 6.8, that was subsequently used for injection. DTT-containing buffer solutions were observed to exert no toxic effect on oocytes, as previously reported [14].

Full-grown stage VI oocytes were obtained by defolliculation after a 45–60 min treatment with collagenase A (2% collagenase A, 1% SBTI), and kept at 14 °C until injection. A positive displacement digital pipette (Nichyrio) was used to inject 50 nl of a 100 μM ^{15}N-labelled Tau solution in every oocyte, leading to a 5 μM intracellular concentration. The 200 oocytes were brought in the Ficoll solution, and introduced delicately into a Shigemi tube (without the plunger), in order for them to occupy the active volume of the proton coil, i.e. roughly 300 μl. For the HRMAS rotor, we filled to rotor with the Ficoll solution, and introduced the oocytes manually one by one.

Mechanical homogenization of the oocyte sample was obtained by adding 300 μl of lysis buffer (60 mM β-glycerophosphate, 15 mM paranitrophenylphosphate, 25 mM MOPS, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF and proteases inhibitors, pH 7.2) to the oocyte sample (200 oocytes in the Ficoll solution, for a total volume of 300 μl), manually crushing the oocytes by a plunger, and centrifuging the cell lysate for 10 min in an Eppendorf bench centrifuge at 14,500 rpm. The pH of the supernatant was readjusted to 6.8 before recording the NMR spectrum.

NMR spectroscopy was performed on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic probe head for the liquid state experiments, and on a Bruker Avance 800 MHz spectrometer with a ^{1}H/^{13}C/^{15}N HRMAS probe for the HRMAS experiments. HSQC spectra were recorded with a standard Bruker pulse sequence, with 1k × 128 complex points. Spectra were Fourier transformed to 2k × 1k points, after zero filling and multiplication with a π/4 or π/2 shifted square sine bell function. After measurement, 12 μl of the supernatant was loaded on a SDS gel, and the amount of Tau released in the supernatant was monitored by Western blotting with a Tau polyclonal antibody. As a control, we injected the same 200 × 50 nl = 10 μl of Tau solution in 300 μl of buffer, and did a serial dilution of this sample to estimate on the Western the amount of released Tau.

3. Results and discussion

We first wanted to evaluate HRMAS NMR to assess the feasibility of detecting the protein at these concentrations in the cells. Oocytes were manually injected with 50 nl of the ^{15}N-labelled Tau solution. With an estimated volume of 1 μl per oocyte (from the mean diameter of 1.2 mm), this leads to a final concentration of 5 μM of ^{15}N-labelled Tau within the oocyte. Allowing for the residual space between the oocytes, our final protein concentration in the NMR sample is thus 3–4 μM, and this remains the same for all further samples.

Attempts to record HRMAS spectra of Tau injected into oocytes failed because the cells did not survive. Based on previous use of Ficoll-containing solutions to maintain oocyte integrity [15], this approach was found to prolong oocyte survival in HRMAS experiments, as they migrate towards their density in the gradient and are not crushed. Nevertheless, the amount of injected protein proved too little for detection in these experiments.

In this report, we show that the use of a similar Ficoll solution allows the prolonged survival of the oocytes in the liquid state NMR tube, and hence allows detection of lower Tau concentrations in the oocyte. Tau-containing cells were transferred to a 20% Ficoll solution in the NMR tube, where they settled slowly to the bottom of the tube (Fig. 1, left). Despite the similar initial volume of samples when brought in the buffer or Ficoll solutions, after 14 h of NMR measurement, those in buffer occupied a significantly reduced volume compared to those in the Ficoll solution (Fig. 1, right). Furthermore, their supernatant has acquired a grey colour, probably because of cell lysis, whereas the supernatant in the Ficoll solution remains clear. When we dosed by Western blotting the amount of Tau released in the supernatant, we found that the supernatant of the Ficoll embedded oocytes contained no detectable amount of Tau, whereas some Tau was readily detectable in the supernatant of the oocytes embedded in the standard buffer conditions (Fig. 1).The cells in a 10% Ficoll solution still sedimented rapidly and showed a crushed aspect the next morning, whereas a 40% or 60% Ficoll solution required centrifugation for the oocytes to settle. In the latter case, because of increased osmolarity, the eggs did shrink significantly through the expulsion of water. In conclusion, a sample of oocytes embedded in a 20% Ficoll solution allows overnight NMR recording, without affecting seriously the morphology of the cells.

Because the oocytes remain structurally intact over the night in this Ficoll solution, we recorded and HSQC spectrum with 256 scans per increment, leading to a total measurement time of 20 h. The ^{15}N-labelling of the injected Tau should allow unambiguous distinction of the microinjected Tau from all other cellular proteins, and as oocytes do not express endogeneous Tau protein, we expect no molecular competition with non-labelled protein. However, oocytes do contain tubulin, and we expect most of Tau to react with the microtubules and hence yield an NMR spectrum similar to the one observed *in vitro* [16].

The resulting spectrum (Fig. 2, left) shows an envelope with a reduced proton dimension, as previously observed in the solution HSQC spectrum of Tau [10,11]. Despite this similarity, many reso-

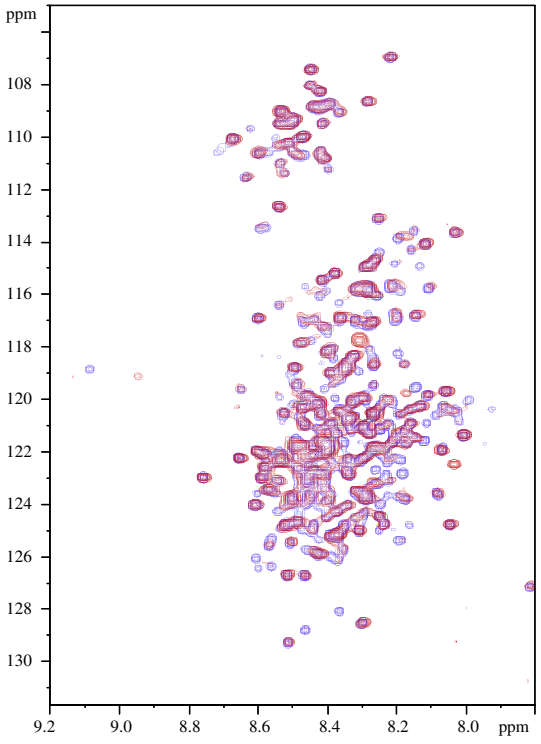

Fig. 4. Spectra of Tau phosphorylated for 15 min by PKA *in vitro* (blue, see [16] for experimental details), and the spectrum of the homogenized oocytes after injection with Tau (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nances observable in the solution spectrum of free Tau are missing. Of special interest is the comparison with the spectrum of the same sample after mechanical homogenization. Cross-peaks clearly become better defined after lysis (Fig. 2, right), pointing to intracellular viscosity or interaction with cellular partners as possible broadening mechanisms. Still, even the spectrum after homogenization does still not show all cross-peaks previously described for the full-length Tau protein, but rather resembles the spectrum of Tau bound to tubulin [16] (Fig. S1). When considering that oocytes have a cellular tubulin concentration of about 20 μM but are devoid of endogeneous Tau [17], and with a 1:3 stoichiometry of Tau to preformed microtubules (MTs) [18], we can consider that indeed all injected Tau becomes MT associated. However, on the basis of the in-cell spectrum, we conclude that other cellular factors equally compete for the same Tau. From these spectra, we thus conclude that observation of Tau at an intracellular concentration of 5 μM is feasible, although the intracellular environment leads to a severe but selective broadening of a number of resonances.

One important issue when working with living cells is the reproducibility of the spectra. In the perspective of recording multiple spectra on different samples, in order to reconstruct triple resonance spectra or perform intracellular relaxation measurements, this is of crucial importance. We thus recorded spectra on different samples at different periods of the year, because we did not want to exclude seasonal variations leading to subtle differences in oocyte structure and organization. We found that careful manual injection of the same ^{15}N-labelled Tau solution at always the same position in the equatorial unpigmented ring under the pigmented animal hemisphere of the oocyte, and subsequent selection of the "best" oocytes (in terms of minimal visual deformation and

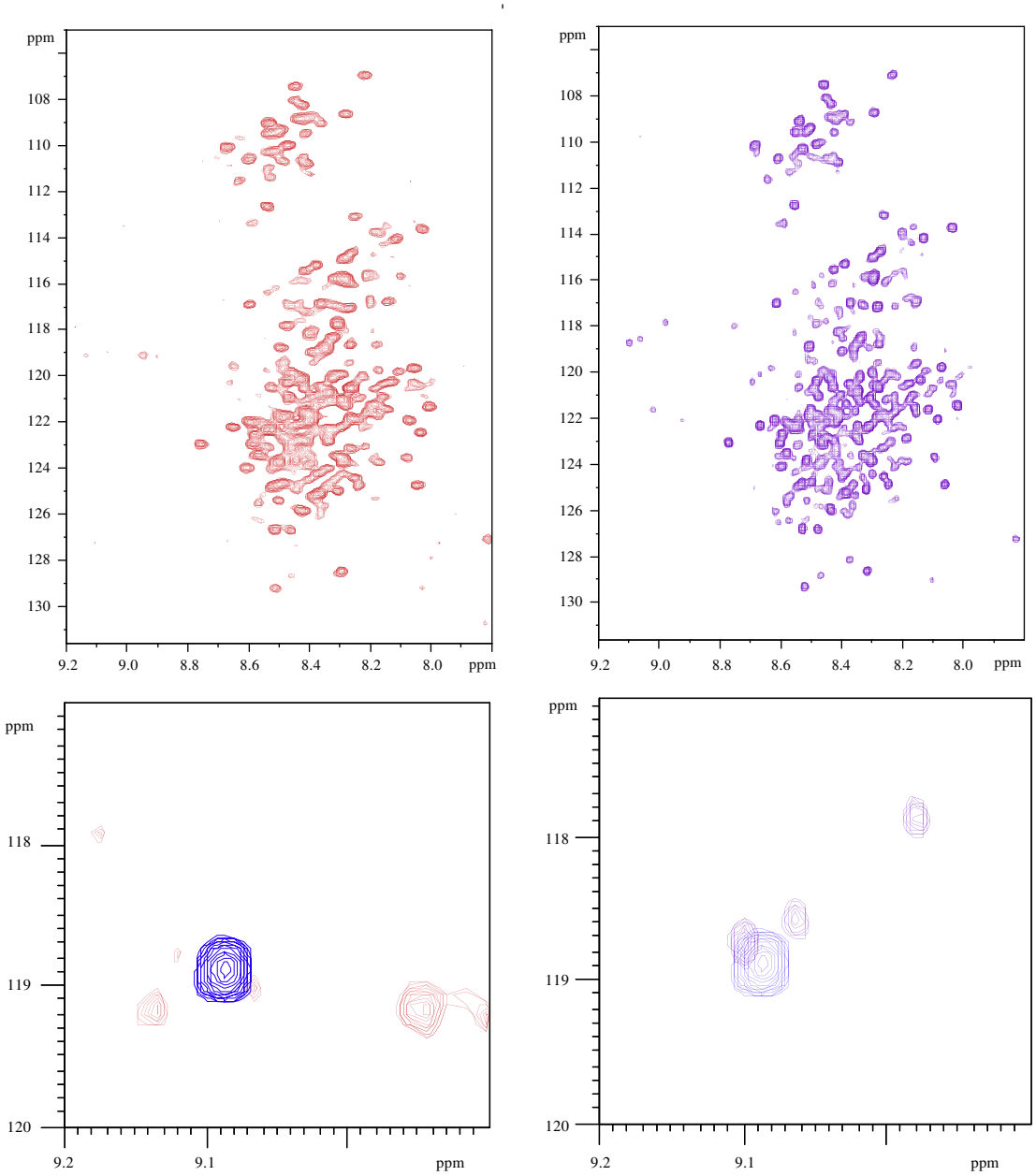

Fig. 5. (Top) comparison of the spectrum of the oocyte cell lysate (left, red; the threshold is twofold lower than in Fig. 3) and the spectrum of a Tau sample *in vitro* phosphorylated by PKA/Gsk3β (right, purple). (Bottom) zoom of the region corresponding to the phosphorylated residues. The spectrum of Tau phosphorylated for 5 min by PKA shows the pSer214 resonance (blue), that becomes split by unidentified but nearby phosphorylation events in the oocyte cell interior (red, left) as it is after the *in vitro* action of Gsk3β (purple, right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cytoplasm leak) led to the best results. Still, whereas the reproducibility of the overall aspect of the spectrum improved by this procedure (Fig. 3), it was not sufficient to exploit the cross-peak intensity in a quantitative way. As a concrete example, the cross-peak at [8.9 ppm, 120.3 ppm] that we assigned to a phosphorylation event (see below) is clearly visible in one spectrum, whereas it was below the noise level in the second one. After mechanical homogenization of both oocyte samples, though, the reproducibility of the spectra improved, and a similar cross-peak at a slightly different nitrogen frequency became visible in both spectra. We thus conclude that the recording of a series of spectra on different samples with variation of one parameter (relaxation delay, ^{13}C evolution, ...) will not be straightforward. Variation not only comes from the protein, but might equally stem from variations in the physicochemical nature of the intracellular environment.

In the in-cell spectrum, we did detect some novel signals that we assign to phosphorylated residues of Tau. Assignment of the phosphorylation sites by triple resonance spectroscopy on oocytes injected with $^{13}C/^{15}N$ Tau proved below the limit of sensibility. Mechanical homogenization of the cells led to better quality spectra (Fig. 3), with a slight change in the resonance frequency of the phosphor-peak, but the amount of protein in 200 oocytes still was too low for 3D triple resonance spectroscopy. Comparison with the spectra of Tau phosphorylated *in vitro* by PKA [19] showed that the major site observed on the *in vivo* spectrum before or after homogenization (Fig. 2, peak at [8.9 ppm, 120.3 ppm]) was not generated by this kinase (Fig. 4).

Another enzyme that was reported as active in the immature oocyte is Gsk3β [20]. We obtained a clone of this kinase, expressed and purified it. The latter was used as a second candidate kinase to obtain a standard for the identification of the *in vivo* phosphorylation pattern (A.L. and G.L., manuscript in preparation) Because Gsk3β needs a priming to be efficient, we used our PKA phosphorylated sample as a substrate for subsequent phosphorylation by PKA. When we compare the spectrum of PKA/Gsk3β phosphorylated Tau with the one of the oocyte cell lysate (Fig. 5), it is clear that the major *in vivo* peak does not overlap with any of the peaks in the *in vitro* generated spectrum. However, when we lower the threshold of the *in vivo* spectrum, and especially when we look at the spectrum of the cell lysate, we observe a second less intensive peak at 9.14 ppm, 119.1 ppm (Fig. 5). A zoom of this peak, and comparison with the PKA pSer214 correlation, suggests that *in vivo*, PKA indeed does phosphorylate the Ser214 position, but that other unidentified phosphorylation events influence this resonance position in a similar way as Gsk3β.

We conclude from the present study that the embedding of the oocytes in a 20% Ficoll solution prior to NMR recording significantly enhances the time span over which the cells remain their intact morphology, such that spectral recording on physiologically relevant protein concentrations becomes feasible. Evidently, the natively unfolded nature of Tau leads to sharper lines than for a folded protein of the same or even smaller molecular weight, and the same minimal concentration of 5 μM might therefore not be attainable with every protein.

For the case of the neuronal Tau protein that we here have studied, NMR allows the detection of its interaction with tubulin and other cellular factors, but equally of novel phosphorylation events. The reproducibility of the spectra does depend on the precise physiological state of the oocytes, and is thus not easily assessed. However, mechanical homogenization and subsequent cytoskeleton disruption improves the situation, and might thus be the method of choice to evaluate post-translational modifications of microinjected proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmr.2008.03.006.

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