Supplementary data

Phosphorylation of human Tau protein by the microtubule affinity regulating kinase 2

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EXPERIMENTAL PROCEDURES

Protein preparation

The longest 441-residue splice-isoform of human Tau protein, 2N4R Tau, was recombinantly expressed and purified in ^{15}N and ^{13}C,^{15}N-labeled form as described previously.[1,2] Purification of wild-type (MARK2cat) and the constitutively active T208E mutant of the catalytic domain of MARK2 (MARK2cat-T208E) was described elsewhere.[3,4]

Peptide synthesis

Tau(254-284) peptides, roughly equivalent to the second half of R1 plus the first half of R2 and containing either a non-phosphorylated or phosphorylated Ser-262 residue, were produced by standard Fmoc-solid-phase peptide synthesis using an ABI 433A synthesizer (Applied Biosystems). Peptides were synthesized with acetyl- and amide protection groups at the N- and C-termini, respectively. Peptides were further purified by reversed-phase HPLC and the pure product was lyophilized.

Sequential resonance assignment of 2N4R Tau

As described previously,[5] assignment was performed in an automated way using automated projection spectroscopy[6] and the assignment software MARS.[7] 5D APSY-HNCOCACB[8] and 7D HNCO(CA)CBCANH[9] spectra were acquired on a Bruker 1.7 mm cryogenic, triple resonance probe at 800 MHz. The sample contained 280 µM MARK2cat-T208E-phosphorylated ^{13}C,^{15}N-labeled 2N4R Tau in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, 10% ($^{v}/_{v}$) D_2O. The sweep widths for the 5D and 7D APSY were 8.0 kHz (^1H, F5/F7 direct dimension), 1.4 kHz (^1H, F1 dimension in 7D), 2.2 kHz (^{15}N), 2.0 kHz (C') and 14.0 kHz (C^{α} and C^{β}). The offset in the direct

dimension was set to the resonance of water, to 8.2 ppm (^{1}H, F1 dimension in 7D), 117.5 or 118 ppm (^{15}N, 5D or 7D), 173 or 174 ppm (C'), and 42 ppm (C^{α} and C^{β}). The projections were acquired with 16 transients (24 for 7D), 1024 x 320 total points (1024 x 260 for 7D). For GAPRO processing of the 5D APSY spectrum, 28 projections were analyzed using the parameters: R_{min} =4.0, $\Delta\nu_{min}$ = 5.0 Hz, r_{min} = 15 Hz, and $S_{min1/2}$ =6. Fifty-six projections from the 7D spectrum were analyzed with R_{min} =3.3, $\Delta\nu_{min}$ = 6.0 Hz, r_{min} = 13 Hz, and $S_{min1/2}$ =10. Due to sensitivity problems, minor phosphorylation sites (less than 50% phosphorylation for a particular serine) were manually assigned using 3D (HA)CANNH[10] and 3D HNN[11] spectra. Both spectra were acquired on a sample containing 100 µM MARK2cat-T208E-phosphorylated ^{13}C,^{15}N-labeled 2N4R Tau in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, 10% ($^{v}/_{v}$) D_2O. The 3D (HA)CANN was recorded at 800 MHz with 8 transients, sweep widths of 9.0 x 2.1 x 5.0 kHz (^{1}H x ^{15}N x ^{13}C) and 1024 x 192 x 80 total points (^{1}H x ^{15}N x ^{13}C). The 3D HNN was acquired at 900 MHz and used 12 transients, sweep widths of 9.0 x 2.1 x 2.1 kHz (^{1}H x ^{15}N x ^{15}N) and 1024 x 120 x 120 total points (^{1}H x ^{15}N x ^{15}N). Spectra were processed using Topspin (Bruker Biospin, Rheinstetten, Germany) or NMRPipe,[12] and analyzed using CcpNmr.[13] Normalized weighted average chemical shift differences for the amide proton and nitrogen were calculated according to $\Delta\delta\ (HN) = [\Delta\delta_H^2 + (0.2*\Delta\delta_N)^2]^{1/2}$.

Paramagnetic relaxation enhancement (PRE)

^{15}N-labeled 2N4R Tau was labeled with MTSL (S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methane-sulfonothioate) at the native cysteines Cys-291 and Cys-322 as described previously [14]. ^{1}H,^{15}N-HSQC spectra of MARK2cat-phosphorylated 2N4R Tau labeled with MTSL were acquired at a concentration of

15 µM in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, 5% D_2O. To acquire spectra in the diamagnetic state, the MTSL label was cleaved off from 2N4R Tau through the addition of 4 mM DTT to the same sample and subsequent incubation at 45 ºC for 15 min. Spectra were acquired at field strengths of 800 MHz with 128 transients, sweep widths of 8.0 x 2.0 kHz (^1H x ^{15}N) and 1024 x 600 total points (^1H x ^{15}N). Carrier frequencies were set to the water resonance in the ^1H and to 118 ppm in the ^{15}N dimension. PRE ratios were calculated from the ratio of the peak intensities in the paramagnetic and diamagnetic state. PRE ratios are reported as three residue averages.

Peptide assignment

Tau(254-284) peptides with and without phosphorylation at Ser-262 were assigned at 5 ºC using two-dimensional ^1H,^1H-TOCSY and ^1H,^1H-NOESY spectra. Samples typically had a concentration of 3 mM and the buffer contained 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, 5% ($^v/_v$) D_2O. Acquisition parameters were commonly set to 32 transients, sweep widths of 7.0 x 7.0 kHz (F2 x F1), 2048 x 512 total points (F2 x F1), and mixing times of 40 ms (TOCSY) or 150 ms (NOESY). Carbon and nitrogen chemical shifts were derived from natural abundance ^1H,^{13}C-HSQC and ^1H,^{15}N-SOFAST-HMQC[15] spectra. Secondary chemical shifts for $^{13}C^{\alpha}$ were calculated in reference to the neighbor corrected IDP chemical shift library.[16] No corrections for the phosphorylated serine were applied.

Isotropic couplings were determined from the same samples used for assignment. Weakly aligned samples were prepared at peptide concentrations of 4 mM dissolved in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8, 10% ($^v/_v$) D_2O and 5% ($^w/_v$) pentaethyleneglycolmonooctylether (C8E5)/n-octanol.[17] One bond $^1D_{NH}$ RDCs

(residual dipolar couplings) were determined from BSD-IPAP-HSQC spectra[18] at 5 ºC and 600 MHz with 256 transients, sweep widths of 6.0 x 1.3 kHz (^{1}H x ^{15}N) and 2048 x 512 total points (^{1}H x ^{15}N). Carrier frequencies were set to the water resonance in the ^{1}H and to 117.5 ppm in the ^{15}N dimension. Band-selective ^{1}H decoupling pulses were centered at 2.4 ppm and covered a bandwith of 3.4 kHz.

One bond $^1D_{CaHa}$ RDCs were determined from J-modulated CT-HSQC[19] spectra. To this end, samples with 2 mM peptide were prepared in D_2O. The peptides were first dissolved in 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.8 and then twice lyophilized and dissolved in 99.9% D_2O. After dissolution in D_2O the pD was measured and if necessary adjusted to 6.8. Weakly aligned samples were obtained by adding 5% ($^w/_v$) C8E5/n-octanol. Spectra were acquired at 5 ºC and 700 MHz with 48 transients, sweep widths of 6.0 x 5.0 kHz (^{1}H x ^{13}C) and 2048 x 270 total points (^{1}H x ^{13}C). Carrier frequencies were set to the water resonance in the ^{1}H and to 47.5 ppm in the ^{13}C dimension. $^1J_{CH}$ evolution delays were set to 23.48, 23.80, 24.12, 24.82, 25.18, 25.92, 26.20 and 27.992 ms.

Ensemble Description

Ensemble descriptions of the phosphorylated and non-phosphorylated Tau(254-284) peptides were generated using a recently described protocol combining experimental NMR data, Monte-Carlo-based statistical coil sampling and ensemble selection.[20] The approach is briefly outlined below. 20000 conformers of phosphorylated and non-phosphorylated Tau(254-284) peptides that broadly sample the conformational space defined by amino acid-specific potential wells, using the statistical coil model proposed by *flexible-meccano.* Chemical shifts were calculated for each member of the ensemble using the program SPARTA as previously described.[21,22] RDCs were

calculated for each member of the ensemble by calculating the alignment tensor of each member of the ensemble. Optimal ensemble size was estimated using cross-validation of experimental data not included in the analysis to be approximately 200. This number was used for all ensembles shown. The specifically designed genetic algorithm ASTEROIDS,[23] was then used to select conformational sub-ensembles in agreement with the experimental data ($^{13}C^{\alpha}$, $^{13}C^{\beta}$, $^{1}H^{N}$ and ^{15}N chemical shifts and $^{1}D_{NH}$ and $^{1}D_{CaHa}$ RDCs) within the limits of estimated experimental uncertainty. Secondary shifts were calculated using random coil values taken from RefDB.[24] Chemical shifts from Tau(254-284) peptides were shifted to match measured chemical shifts from the K18 domain of Tau at 25 °C, as the chemical shift prediction algorithm is calibrated against proteins measured at this temperature. Chemical shifts from the phosphorylated amino acid were not included in the selection approach.

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