NMR spectroscopy

International Edition: DOI: 10.1002/anie.201501714
German Edition: DOI: 10.1002/ange.201501714

Folding of the Tau Protein on Microtubules**

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Abstract: *Microtubules are regulated by microtubule-associated proteins. However, little is known about the structure of microtubule-associated proteins in complex with microtubules. Herein we show that the microtubule-associated protein Tau, which is intrinsically disordered in solution, locally folds into a stable structure upon binding to microtubules. While Tau is highly flexible in solution and adopts a β-sheet structure in amyloid fibrils, in complex with microtubules the conserved hexapeptides at the beginning of the Tau repeats two and three convert into a hairpin conformation. Thus, binding to microtubules stabilizes a unique conformation in Tau.*

Microtubules (MTs) play crucial roles in cell organization and function.[1] The formation and dynamics of MTs are modulated by accessory proteins termed microtubule-associated proteins.[2] An important microtubule-associated protein is the protein Tau, which is detached from MTs during the course of Alzheimer's disease.[3] Microtubule-associated proteins contain several imperfect repeats in their carboxy terminal half.[4] Biochemical studies have shown that the repeat domain and the neighboring basic proline-rich regions contribute strongly to MT binding.[5] Moreover, regions outside of the MT-binding domain may influence the spacing between MTs.[6] In addition, a variety of binding models of the Tau–MT complex have been proposed.[7]

Tau belongs to the class of intrinsically disordered proteins.[8] In solution, it does not fold into a well-defined structure but populates a dynamic ensemble of conformations.[9] During aggregation, part of the repeat domain folds into a rigid β-sheet structure and forms the core of neurofibrillary tangles.[10] However, little is known about the three-dimensional structure of Tau bound to MTs, because of the high-molecular weight of MTs and the dynamic nature of the interaction. Using NMR spectroscopy we provide herein insight into the conformation of the repeat domain of Tau in complex with MTs.

MTs were assembled from unpolymerized tubulin following established methods (Figure 1a).[9a] The MTs were then added to a sample of ^{15}N-labelled 441-residue Tau. Addition of MTs changed the position and intensity of cross peaks in a two-dimensional ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectrum (Figure 1b),[7e,9a,11] consistent with a low-micromolar affinity of Tau for tubulin and MTs.[5a,12] On the basis of the assignment of resonance signals of the Tau backbone,[9a,13] the perturbation was analyzed in a sequence-specific manner (Figure 1c). In agreement with previous results,[9a,11] the strongest signal broadening was observed for Tau residues 168–185, 224–237, 245–253, 269–284, 300–313, and 375–398.

The first two of these broadened residue stretches map to the proline-rich region, while residues 245–253, 269–284, and 300–313 are located in the repeat domain. Residues 375–398 belong to a region near the C-terminus, which retains some similarity to the four pseudorepeats in 441-residue Tau. In contrast to the central part of Tau, the N-terminal domain up to residue 70 experienced little signal perturbation, suggesting that it interacts at best weakly with MTs.

The MT-induced broadening profile of the NMR signals suggested that six short regions of Tau are important for binding to MTs. Indeed, several studies employing Tau peptides and fragments indicated that Tau uses an array of weak interactions involving by small groups of amino acids to bind to MTs.[24] Moreover, because short Tau peptides contain only a single or a subset of these MT-interacting groups of amino acids, their affinity to MTs is lower. For example, for a 23-residue Tau peptide from repeat 1 or a Tau fragment, which only contains repeat 2 and 3, K_d values of approximately 200 μM were reported.[5a,14] To further support the presence of localized MT-binding motifs, we synthesized the 46-residue fragment Tau(267–312), which overlaps with the two broadened regions in repeats two and three. In addition, Tau(265–290) and Tau(296–321) were prepared, each covering only a single MT-binding hot spot. Tau(267–312) is particularly interesting as it contains the two conserved hexapeptides at the beginning of repeats two and three, which are converted into a β-sheet structure upon the aggregation of Tau into amyloid fibrils.[10b] In addition, Tau(267–312) forms the core of Tau fragments that are generated by endogenous

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[**] This work was supported by the MPG consortium Toxic Protein Conformation (to E.M.), the Wellcome Trust (to E.M), the Foundation for Polish Science START and Ventures Programme (to M.J. and L.J.) co-financed by the EU European Regional Development Fund and the Deutsche Forschungsgemeinschaft through project ZW 71/8-1 (to M.Z.). We thank Christian Griesinger and Eva Mandelkow for discussions, Sabrina Hübschmann for sample preparation, and Kerstin Overkamp for peptide synthesis.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201501714.

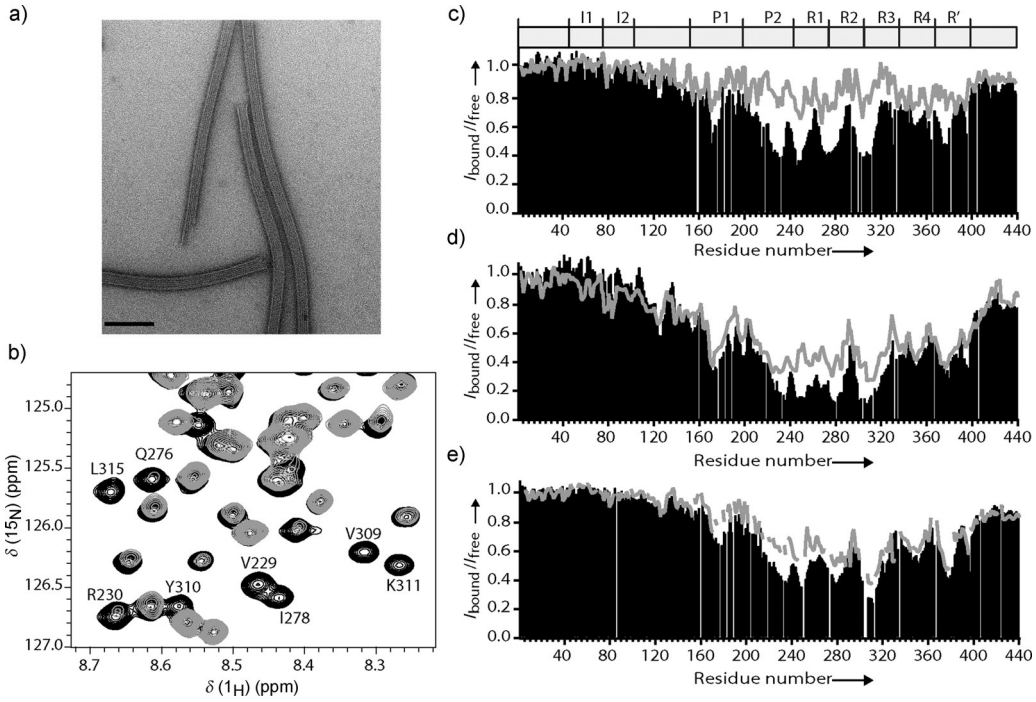

Figure 1. Single-residue analysis of Tau binding to MTs. a) Electron micrograph of paclitaxel-stabilized MTs in the presence of Tau. The black scale bar represents 100 nm. b) Selected region of ^{1}H–^{15}N HSQC spectra of 441-residue Tau in the absence (black) and presence (gray) of MTs (Tau:tubulin heterodimer ratio of 2:1). c)–e) Competition between different Tau peptides and full-length Tau for binding to MTs. Black bars show the ratio of ^{1}H–^{15}N NMR signal intensities of Tau bound to MTs (I_{bound}) and free Tau (I_{free}). The estimated error in I_{bound}/I_{free} was on average 0.08 based on the to the signal-to-noise ratio in the spectra. Upon addition of a 15-fold excess of Tau(267–312 (c), Tau(265–290) (d) and Tau(296–321) (e) the signal intensities increased (gray line). Tau's domain organization is shown at the top: R repeat, I insert, P proline-rich region. Differences in NMR intensity ratios in (c–e) prior to peptide addition are due to variations in tubulin preparation, which is obtained from pig brain.

proteases in neuronal cells and start N-terminally between residue 257/258 and end around residues 353–364.[15] The region is also important for binding of Tau to phospholipid membranes as well as a variety of endogenous and engineered binding proteins.[16]

The Tau fragments were then used in competition experiments with full-length Tau. To this end a 15-fold molar excess of the peptides was added to a solution containing ^{15}N-labelled Tau and MTs and the changes in NMR signal intensity of Tau residues were monitored. All three peptides did compete with full-length Tau for binding to MTs (Figure 1 c–e). In contrast, the peptide Tau(52–69), which maps to a Tau region that was broadened very little in the presence of MTs, was not able to compete (Figure S1 in the Supporting Information). The data suggest that the Tau fragments Tau(267–312), Tau(265–290), and Tau(296–321) bind to a similar site on MTs as full-length Tau. This hypothesis is consistent with the ability of shorter Tau fragments from the repeat domain to stimulate MT assembly and suppress MT dynamics in a similar way to full-length Tau.[5a,17]

To directly probe the binding of Tau(267–312) to MTs, we recorded two-dimensional Nuclear Overhauser Effect (NOE) spectra in the absence and presence of MTs (Figure 2). When a peptide or ligand exchanges sufficiently fast between the free and the MT-bound state, NOE contact information from the MT-bound conformation of the peptide will be transferred to the free state. This so-called transferred NOE effect has been widely used to determine the structure of ligands in complex with high-molecular weight binding partners.[18] Figure 2 a shows a superposition of NOE spectra of Tau(267–312) in the absence and presence of MTs. Upon addition of MTs the overall NOE signal intensity increased and new cross peaks appeared (Figure 2 a, red). Analysis of NOE signal intensities as a function of NOE mixing time showed that spin diffusion, which might occur owing to the large molecular weight of MTs, does not significantly contribute to NOE intensities at mixing times up to at least 100 ms (Figure S2). In contrast to Tau(267–312), no transfer NOE effect was observed for the control peptides Tau(283–300; Figure 2 b) and Tau(327–353; Figure S3). Taken together the data demonstrate that 1) the exchange is sufficiently fast that magnetization is transferred from the MT-bound to the free state and 2) Tau(267–312) folds upon binding to MTs into a conformation that is characterized by defined inter-residue contacts.

The observation of transferred NOE contacts opened the possibility to determine the 3D structure of Tau(267–312) in complex with MTs. To this end, the NOE spectrum of Tau(267–312) was sequence-specifically assigned using a combination of two-dimensional NOE and TOCSY spectra (Tables S1–S3 and Figure S4). Two-dimensional NOE and TOCSY spectra were also recorded and sequence-specifically assigned for the shorter Tau peptides Tau(265–290) and Tau(296–321), in which signal overlap was strongly reduced (Tables S1–S3 and Figure S4). Subsequently, the cross peaks in the transferred NOE spectra of Tau(267–312) were analyzed (Figure S5 and Figure 3 a). Most medium- and long-range contacts were observed for residues 268–282 and 300–312 (Figure 3 a), in agreement with the strong MT-induced broadening of these residues in full-length Tau (Figure 1). On the basis of the experimentally derived contacts (Table S4), structure calculations for Tau(267–312) were performed. To minimize contributions to the NOE

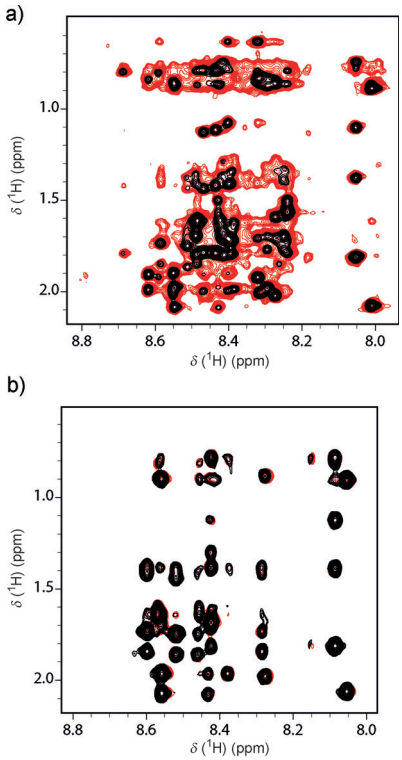

Figure 2. 2D NOE spectra of Tau(267–312) (a) and Tau(283–300) (b) in the absence (black) and presence (red) of MTs. The NOE mixing time was 100 ms.

intensities from the free peptide, only experimentally observed medium- and long-range contacts were used. In addition, for all medium- and long-range contacts the lower limit was set to 1.8 Å and the upper limit to 6.0 Å to avoid a potential miscalibration of NOE intensities. Thus, the only information which entered the structure calculation was the pure presence of the NOE contact in the bound state, that is, that the two protons are less than 6.0 Å apart. Note that the resulting ensemble of conformations represents the average structure that is best in agreement with the experimental NOE data and does not exclude the presence of multiple conformations of Tau in the MT-bound state. In addition, we performed the same structure calculations for Tau(267–312) in solution, that is, on the basis of the NOE spectrum in the absence of MTs (black spectrum in Figure 2a), which did not converge to one major conformer (Figure S6).

The 10 lowest energy conformations of Tau(267–312) bound to MTs are shown in Figure 3b (protein databank (PDB) code: 2MZ7). The conformers have a converged conformation for residues 269–284 and 300–310 (Figure 3c,d, Figure S7, and Table S5). Both residue stretches fold into a hairpin-like structure upon binding to MTs. The turn of the hairpin is formed by the highly conserved PGGG motif and is stabilized by long-range contacts to Pro270 and Pro301 (Figure S8). The PGGG-turn is followed by an extended structure of the hexapeptide ^{275}VQIINK^{280} in repeat 2 (R2) and ^{306}VQIVYK^{311} in R3 (Figure 3c,d). Notably, a peptide composed of residues 274–281, which are the eight residues at the center of the MT-binding motif in repeat 2 (Figure 3c), is able to kinetically stabilize steady-state MT dynamics in a manner qualitatively similar to full-length Tau.[17]

On the basis of the transferred NOE contacts, we also calculated the conformation of Tau(296–321) in complex with MTs (Figure S9). In line with the results for Tau(267–312), the calculation converged to a hairpin-like structure. In addition, we detected medium-range contacts for residues 314–321 (Figure S9). However, owing to the lack of long-range contacts the orientation of residues 314–321 relative to the hairpin structure was not well defined. No defined conformation was also observed for residues 285–299 in Tau(267–312) (Figure 3b), suggesting that the two MT-binding hot spots are flexibly linked to each other. The flexibility of these regions might enable transient contacts with MTs. Transient electrostatic interactions of positively charged Tau residues with the acidic C-terminal tail of tubulin are indeed supported by the finding that removal of the C-terminal tail of tubulin by subtilisin decreases Tau binding and increases tubulin polymerization.[19]

The best known function of Tau is to bind to MTs to promote the formation of axonal MTs.[20] Because of the biological relevance of Tau binding to MTs, there has been a long-standing interest in the three-dimensional structure of Tau bound to MTs. Previous attempts have largely failed to analyze the structure of Tau in complex with MTs. This is because of the mobility of the Tau structure and the dynamic nature of the Tau-MT interaction.[5a,8] Our study now reveals that—although Tau is largely extended when bound to MTs and does not fold into a single globular structure[7a,21]—distinct regions of Tau fold into a defined conformation upon binding to MTs. The formation of a hairpin conformation, which is formed around the PGGG motif at the end of the repeats 1 and 2 is consistent with the previously suggested importance of the PGGG motif for binding to MTs.[7b] The hairpin conformation resembles a molecular hook, which seems perfectly suited to anchor Tau to MTs through insertion into a pocket/groove, which is present in the tubulin heterodimer. The MT-induced folding of the repeat domain of Tau is in agreement with biochemical studies, which showed that these Tau domains are important for MT binding and assembly.[5] The MT-specific conformational changes are further supported by the finding that the positive charge of Tau itself is not sufficient to induce normal MT assembly.[22]

Another important aspect of our study is that the two hexapeptides at the beginning of repeats 2 and 3 are directly involved in formation of the MT-bound conformation of Tau. The two hexapeptides are the most aggregation-prone regions of Tau and form the core of Tau amyloid fibrils.[23] Our study thus shows that when Tau is bound to MTs, residues which are critical for pathogenic aggregation of Tau are stabilized in an alternative conformation, which has to be destabilized for Tau to misfold into an amyloidgenic β-sheet structure. This further suggests that stabilization of the MT-bound conformation by site-directed mutagenesis or antibodies, which specifically recognize the MT-bound hairpin conformation of Tau, might delay pathogenic aggregation and neurotoxicity of Tau.

Keywords: Alzheimer's disease · microtubules · NMR spectroscopy · structure elucidation · Tau protein

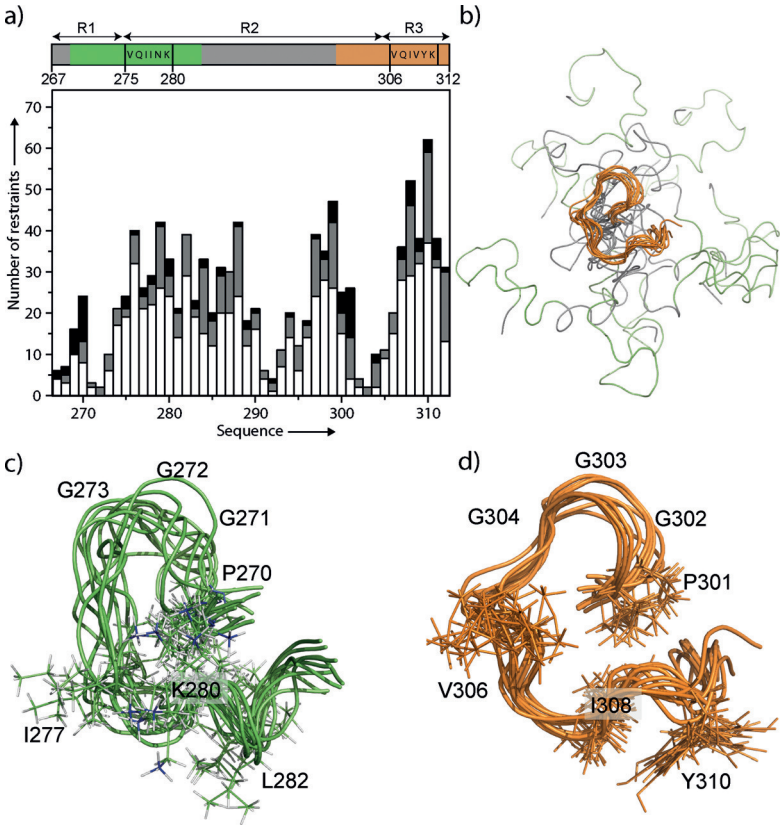

Figure 3. Structure formed in the repeat domain of Tau upon binding to MTs. a) Distribution of distance constraints as a function of residue number in Tau(267–312). Distance constraints were classified as intra-residual and sequential ($|i-j| \leq 1$; white), medium-range ($1 < |i-j| \leq 4$; gray), and long-range ($|i-j| \geq 5$; black), respectively. b) Ensemble of the 10 lowest energy conformers of Tau(267–312), determined on the basis of medium- and long-range NOEs observed in the presence of MTs. Residues 300–310 were aligned. Conformation of residues c) 269–284 and d) 300–311 of Tau(267–312). Backbone traces of the 10 lowest energy conformers are shown. Side chains of selected residues are displayed in stick representation.

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 10347–10351
Angew. Chem. **2015**, *127*, 10488–10492

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Received: February 23, 2015
Revised: April 1, 2015
Published online: June 19, 2015

