Alternative Conformations of the Tau Repeat Domain in Complex with an Engineered Binding Protein*

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Background: Aggregates of the protein Tau are associated with Alzheimer disease and other neurodegenerative diseases.

Results: The engineered binding protein TP4, targeting the Tau repeat domain, was obtained from a novel β-wrapin protein library.

Conclusion: TP4 interacts with two alternative conformations of Tau, thereby inhibiting Tau aggregation.

Significance: Binding of aggregation-prone sequence stretches is an approach to interfere with Tau aggregation.

The aggregation of Tau into paired helical filaments is involved in the pathogenesis of several neurodegenerative diseases, including Alzheimer disease. The aggregation reaction is characterized by conformational conversion of the repeat domain, which partially adopts a cross-β-structure in the resulting amyloid-like fibrils. Here, we report the selection and characterization of an engineered binding protein, β-wrapin TP4, targeting the Tau repeat domain. TP4 was obtained by phage display using the four-repeat Tau construct K18ΔK280 as a target. TP4 binds K18ΔK280 as well as the longest isoform of human Tau, hTau40, with nanomolar affinity. NMR spectroscopy identified two alternative TP4-binding sites in the four-repeat domain, each including two hexapeptide motifs with high β-sheet propensity. Both binding sites contain the aggregation-determining PHF6 hexapeptide within repeat 3. In addition, one binding site includes the PHF6* hexapeptide within repeat 2, whereas the other includes the corresponding hexapeptide Tau(337–342) within repeat 4, denoted PHF6**. Comparison of TP4-binding with Tau aggregation reveals that the same regions of Tau are involved in both processes. TP4 inhibits Tau aggregation at substoichiometric concentration, demonstrating that it interferes with aggregation nucleation. This study provides residue-level insight into the interaction of Tau with an aggregation inhibitor and highlights the structural flexibility of Tau.

The microtubule-binding protein Tau has recently gained increased scientific attention due to its involvement in neurodegenerative diseases. The suggested physiological functions of Tau relate mostly to microtubule binding, which occurs through an imperfect repeat domain in the C-terminal part of the protein (1–4). The repeat domain consists of three (3R) or four (4R) repeats depending on alternative splicing. Although Tau is a highly soluble, intrinsically disordered protein (5), it can aggregate into straight or paired helical filaments (PHFs) 3, which further combine to form neurofibrillary tangles, the hallmark of several neurodegenerative diseases such as Alzheimer disease and frontotemporal dementia (6, 7).

Tau aggregation is a multi-step reaction characterized by conformational conversion of the repeat domain. Aggregation-promoting conditions such as heparin addition or introduction of the proaggregant ΔK280 mutation are associated with increased formation of intramolecular interactions within the repeat domain, accompanied by its compaction (8, 9). Tau aggregation progresses through dimerization and oligomerization (10–13). Two motifs with high β-sheet propensity in the repeats R3 and R2, called PHF6 (306VQIVYK311) and PHF6* (275VQIINV280), respectively, are involved in the initiation of the oligomerization process (14–18). PHFs, the products of the aggregation reaction, are characterized by a rigid cross-β-sheet core (19, 20), built up by the imperfect repeats (21–23), and an outer fuzzy coat formed by the N- and C-terminal parts of the protein (24). Due to its importance for the aggregation process, the repeat domain constitutes an interesting target for interference with Tau assembly. For example, methylene blue inhibits Tau aggregation by acting on the repeat domain (25).

In this study, we generated an engineered binding protein, termed TP4, to the Tau repeat domain and characterized its mode of binding and impact on Tau aggregation. TP4 belongs to the class of β-wrapins (β-wrap proteins), which are selected from phage display libraries based on the ZAβ scaffold (26). ZAβ is a disulfide-linked homodimeric protein derived from the Z domain of protein A (27, 28). ZAβ binds the amyloid-β peptide (Aβ) with nanomolar affinity, stabilizing a β-hairpin conformation of Aβ and inhibiting Aβ aggregation (28). Evi-

The abbreviations used are: PHF, paired helical filament; Aβ, amyloid-β peptide; ITC, isothermal titration calorimetry; K18 4R, four-repeat-domain Tau (residues 244–368) with deletion of Lys-280 (K18ΔK280); K18 4R,AA, as construct K18 4R with additional C291A and C322A exchanges; SEC, size exclusion chromatography; ThT, thioflavin T; hTau, human Tau.

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dence obtained by protein engineering, NMR spectroscopy, molecular dynamics simulations, and FTIR spectroscopy suggests that the β-hairpin conformation observed in complex with the binding protein is also transiently populated in free Aβ monomers and constitutes a component of Aβ oligomers and protofibrils (29–32). The β-wrapin AS69 selected to bind the protein α-synuclein involved in Parkinson disease likewise stabilizes a β-hairpin of the target protein and inhibits α-synuclein aggregation at substoichiometric concentration (26).

This work presents the β-wrapin TP4, which targets the repeat domain of Tau. NMR spectroscopy of the complex of TP4 with the repeat domain of the four-repeat Tau isoform reveals two alternative conformations of bound Tau. TP4 is able to inhibit aggregation of Tau at a substoichiometric concentration.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Tau Constructs—The amino acid numbering of all constructs is that of the isoform hTau40 (full-length human Tau) containing 441 residues. The K18 constructs used in this study comprised exactly the four repeat domains as originally defined (1), i.e. residues hTau(244–368), but not the C-terminal residues. 369KKIE372. K18 constructs including the deletion of Lys-280 (i.e. K18ΔK280) are denoted here by K18AΔK. K18ΔK construct including the C291A and C322A exchanges are denoted by K18ΔKA (see Fig. 2 for an overview of the Tau constructs used in this study). Codon-optimized Tau K18 DNA sequences were cloned into pET24a vector (Merck Millipore) using NdeI and BamHI (Thermo Fisher) restriction sites. K18ΔKA proline mutants were prepared by site-directed mutagenesis using phosphorylated primers, one of them containing the mutation at its 5’ end (33). The vector was amplified with the high-fidelity polymerase Velocity (Bio-line) and religated. Full-length Tau in the pET28 vector was a kind gift of Susanne Aileen Funke (Research Centre Jülich). For expression, the constructs were transformed into Escherichia coli BL21(D3) cells. Cells were grown either in lysogeny broth or, for labeled protein, in minimal medium M9 containing 1 g/liter 15N ammonium chloride and/or 2 g/liter 13C glucose until an 

expression was then induced with 0.8 was reached. Expression was then induced with 1 mol of biotin per mol of Tau K18ΔK monomer.

Cloning of the Library—A new β-wrapin library was constructed by overlapping PCR using primers encoding AQH-DEA peptide derived from the region E of protein A followed by the randomized ZAβ gene. The NNK degenerate codon (where N stands for any of four nucleotides and K indicates G or T) was employed for generating diversity at amino acid positions 16–19, 30, 34, and 45 of ZAβ3, whereas the DTS degenerate codon (where D indicates A, G, T and S indicates C, G) was used for amino acid interchange at positions 27 and 31. The primers used were as follows: L1, gcgaacagaggaagccgtataaa-aaatgaggatcaggtggca; L2, gactcggttaatgtagatg-NNMN-NNMNMMNNNntcccccacgggctctgcaattttgctcttcc; L3, cctacttttaa-cccgtatatattgaatgcgccnnkdtssatagtnkcatgatgacccaagca-acc; and L4, tttccggcgcggctacctgcatctactgctgctgtctgctcgcagtttggctgttgcactc. The gene pool was cloned between sacI and XhoI of the pComb3HSS vector (provided by C. F. Barbas, The Scripps Research Institute). In addition to the library insert, the pComb3HSS vector contained the coding sequence for c-Myc tag and albumin-binding domain from streptococcal protein G directly cloned between XhoI and SpeI. The library was transformed into electrocompetent E. coli XL1-Blue cells (Stratagene) resulting in 2.3 × 109 transformants.

Phage Display Selection—The phage library was produced by superinfection of bacteria harboring the library with M13KO7 phage (New England Biolabs) and precipitation by PEG/NaCl. The library was subjected to four successive rounds of panning against biotinylated Tau K18ΔK with decreasing target concentrations (500, 300, 50, and 1 mg/mL, respectively) and increasing

protein concentration was measured by UV absorption at 280 nm using a calculated extinction coefficient of 1490 M⁻¹ cm⁻¹. Full-length Tau protein was purified essentially in the same way. Cells were resuspended in 0.5 mM NaCl, 20 mM Pipes, pH 6.5, and 2 mM dithiothreitol (DTT), containing protease inhibitor tablets (Roche Diagnostics). After cell disruption and centrifugation, the solution was heated up to 80 °C for 15 min. The denatured proteins were removed by centrifugation (as described above), and the solution was dialyzed overnight against 50 mM NaCl, 20 mM Pipes, pH 6.5, and 2 mM DTT. The same buffer containing 1 mM NaCl was used to elute full-length Tau protein from the cation exchange column. For SEC, 20 mM sodium phosphate (NaPi), 50 mM NaCl, pH 7.4, was used as running buffer, and the purified protein was stored at −20°C.

Biotinylation of Tau K18ΔK—For phage display selection, the Tau construct K18ΔK was biotinylated using NHS-LC-Biotin (Thermo Scientific). A lyophilized Tau K18ΔK aliquot was resuspended in 100 mM NaPi, pH 7.0, and subjected to SEC using a Superdex 75 10/300 column with the same buffer as running buffer to ensure the monomeric state of the Tau construct. Freshly prepared NHS-Biotin solution was added to a 5-fold molar excess, and the reaction was incubated for 16 h at 4°C in the presence of 5 mM DTT. The reaction was stopped by removal of the biotinylation reagent using a HiTrap desalting column (GE Healthcare). The biotinylated protein was separated by an avidin agarose column (Thermo Scientific), and the biotinylation state was determined by a 4′-hydroxyazo-benzene-2-carboxylic acid assay (Thermo Scientific) to be 2 mol of biotin per mol of Tau K18ΔK monomer.

Purification of Tau Constructs—Cell pellets were resuspended in 50 mM Tris-HCl, pH 8.2, containing protease inhibitor tablets (Roche Diagnostics), 1 mM MgCl₂, and 50 units/ml Benzonase (Merck). Cells were disrupted using a high pressure-based system (2.9 kbar, Constant Systems). After clearing the solution from cell debris (40,000 × g, 40 min, 4°C), a first purification step was performed by cation exchange chromatography using a HiTrap SP FF 5-ml column (GE Healthcare) connected to an Akta Purifier system (GE Healthcare) with a high salt buffer to elute bound protein in a linear gradient (50 mM Tris-HCl, pH 8.2, 1 mM NaCl). Further purification was achieved by size exclusion chromatography (SEC) using a HiLoad Superdex 75 16/60 column (GE Healthcare) with 50 mM NH₄CO₃ as running buffer to enable subsequent lyophilization of the purified proteins. Protein aliquots were stored at −80°C. Protein concentration was measured by UV absorption at 280 nm using a calculated extinction coefficient of 1490 M⁻¹ cm⁻¹. Full-length Tau protein was purified essentially in the same way. Cells were resuspended in 0.5 mM NaCl, 20 mM Pipes, pH 6.5, and 2 mM dithiothreitol (DTT), containing protease inhibitor tablets (Roche Diagnostics). After cell disruption and centrifugation, the solution was heated up to 80°C for 15 min. The denatured proteins were removed by centrifugation (as described above), and the solution was dialyzed overnight against 50 mM NaCl, 20 mM Pipes, pH 6.5, and 2 mM DTT. The same buffer containing 1 mM NaCl was used to elute full-length Tau protein from the cation exchange column. For SEC, 20 mM sodium phosphate (NaPi), 50 mM NaCl, pH 7.4, was used as running buffer, and the purified protein was stored at −20°C.

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Washing stringency (2, 5, 10, and 20× with PBST-BSA (PBS, 0.1% (w/v) Tween 20, 3% (w/v) BSA), respectively, and once with PBS). To remove the streptavidin-binding phage, a negative selection in which the phage preparation was incubated with streptavidin magnetic beads at room temperature for 1 h preceded each selection round. In the first panning round, the incubation was carried out overnight at 4 °C, whereas the subsequent selection rounds were done for 1 h at room temperature. The phage-target complexes were captured on streptavdin magnetic beads, and following washing, bound phages were eluted by lowering the pH to 2.0. After neutralization with 1 M Tris-HCl, pH 8.0, the eluted phages were amplified in E. coli XL1-Blue cells and subjected to the following panning round. After the fourth selection round, the DNA pool was subcloned into pET302/NT-His vector (Invitrogen), and DNA from 91 single colonies was sequenced.

Purification of TP4—TP4 containing an N-terminal His8 tag was expressed from pET302/NT-His vector in E. coli BL21(DE3) cells. Cells were grown in lysogeny broth, and protein expression was induced with 1 mM isopropyl β-D-galactopyranoside at A600 of 0.6—0.8 for 5 h at 37 °C. Following centrifugation at 4000 × g, the cell pellet was resuspended in 50 mM NaPi, pH 8.0, 500 mM NaCl, containing EDTA-free protease inhibitor (Roche Applied Science) and lysed using a high pressure-based system (2.9 kbar, Constant Systems). After clearing the solution from cell debris (40,000 × g, 40 min, 4 °C), a first purification step was performed by immobilized metal ion affinity chromatography (HisTrap FF column, GE Healthcare). The dimeric fraction of TP4 was collected from a HiLoad Superdex 75 16/60 column (GE Healthcare) in 20 mM NaPi, 50 mM NaCl, pH 7.4. To facilitate concentration determination of TP4, a tryptophan fluorescence was recorded every 30 min at 480 nm (excitation, 440 nm; bandwidth, 5 nm).

Isothermal Titration Calorimetry (ITC)—ITC was performed on a Microcal iTC200 calorimeter (GE Healthcare) at 10 °C. Binding parameters were obtained from a nonlinear least-squares fit to a 1:1 binding model. K18 variants were used at a concentration of ~70 μM as titrant in the cell and TP4 at ~10-fold higher concentration as titrant in the syringe, except for K18AKAA I277P/V339P, which was used at a concentration of ~560 μM as titrant in the syringe and titrated into 31 μM TP4. Binding of K18AKAA to ZAβ3 was tested by using ZAβ3 at a concentration of 70 μM as titrant in the cell and K18AKAA at ~10-fold higher concentration as titrant in the syringe. Binding of Aβ to TP4 was tested by using recombinantly produced Aβ(1–40) with an N-terminal methionine (34) at a concentration of 33 μM as titrant in the cell and TP4 at ~10-fold higher concentration as titrant in the syringe. The heat of the reaction was measured on a JASCO J-815 spectropolarimeter using protein samples at a concentration of 30 μM in 1-mm Suprasil Quartz cuvettes (Hellma). The buffer consisted of 20 mM NaPi, 50 mM NaCl, pH 7.4. To obtain melting curves, spectra were recorded from 5 to 60 °C in 5 °C intervals.

Circular Dichroism Spectroscopy—Far-UV CD spectra were measured on a JASCO J-815 spectropolarimeter using protein samples at a concentration of 10 μM in 1-mm Suprasil Quartz cuvettes (Hellma). The buffer consisted of 20 mM NaPi, 50 mM NaCl, pH 7.4. To obtain melting curves, spectra were recorded from 5 to 60 °C in 5 °C intervals.

Size Exclusion Chromatography—SEC runs were performed on a Superdex 75 PC 3.2/30 column connected to an ÄktaMicro System (GE Healthcare) in 20 mM NaPi, 50 mM NaCl, pH 7.4, at 5 °C. The concentration of both TP4 and K18AKAA was 45 μM, except for the experiment performed at 1:3 K18AKAA:TP4 ratio, in which 22.5 μM K18AA and 67.5 μM TP4 were applied.

Aggregation Assay—The aggregation of K18AKAA was monitored with the dye thioflavin T (ThT). Samples were prepared in 20 mM NaPi, 50 mM NaCl, pH 7.4, with or without addition of 10 μM heparin. Lyophilized and resuspended Tau was subjected to SEC using a Superdex 75 10/300 column to ensure the monomeric state of the Tau construct. The final reaction contained 50 μM of K18AKAA, 60 μM ThT, 0.05% NaN3, and the indicated concentration of TP4. Aggregation was performed in 96-well round bottom plates (Nunc), containing one 2-mm glass bead per well and sealed with a polyethylene tape (Nunc), incubated in an Infinite M1000 plate reader (Tecan) at 25 °C, with orbital shaking applied for ~10% of the incubation time. ThT fluorescence was recorded every 30 min at 480 nm (excitation, 440 nm; bandwidth, 5 nm).

Transmission Electron Microscopy—Samples from the aggregation assay were diluted to a K18AKAA concentration of 4 μM, and 20 μl were applied to formvar/carbon coated copper grids (S162, Planoc), followed by incubation for 3 min. The grids were washed three times with H2O and once with 2% aqueous uranyl acetate, followed by 1 min incubation with 2% aqueous uranyl acetate for negative staining. The grids were dried overnight. The samples were examined with a Libra 120 electron microscope (Zeiss) operating at 120 kV.

RESULTS

Selection of a β-Wrapin to Tau by Phage Display—For this study, a new β-wrapin library was generated by site-directed mutagenesis of the ZAβ3 gene. In the library, the residues of ZAβ3 that constitute the binding surface in the complex with AB were randomized (Fig. 1). Although most of these residues were replaced by all 20 amino acids, residues 27 and 31 in both subunits were substituted only by hydrophobic amino acids to preserve the hydrophobic interface between the subunits (Fig. 1). The library size was 2.3 × 106 transformants.

As target for phage display selections, the Tau construct K18, including the deletion ΔK280, denoted here by K18AK, was used (Fig. 2). The deletion of Lys-280 found in frontotemporal dementia with parkinsonism–17 patients was included as it increases the population of aggregation-prone conformations of the repeat domain (9, 17), which might serve as primary
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molecular recognition features of the intrinsically disordered Tau. In total, four selection rounds on biotinylated K18ΔK were performed. After selection, 91 clones were sequenced unveiling one dominant sequence with 84% occurrence, corresponding to β-wrapin TP4 (Fig. 1B). The hydrophobic nature of the randomized residues in the α-helix was conserved in TP4. Apart from the randomized positions, TP4 contained one additional mutation, namely N52S. TP4 was purified as a disulfide-linked homodimer after recombinant expression. The affinity of TP4 for different Tau constructs was determined by ITC (Table 1 and Fig. 3). To prevent the formation of oxidation products of Tau K18ΔK during the biophysical characterization, Cys-291 and Cys-322 were replaced by alanine residues. The resulting construct, referred to as K18ΔKAA (Fig. 2), exhibited an affinity of 260 nm for TP4 (Table 1). Submicromolar affinity to TP4 was also observed for full-length hTau40 without the ΔK280 deletion (Fig. 3). In contrast, ITC did not detect any heats of binding when ZAβ3 was titrated with K18ΔKAA, demonstrating that the affinity for Tau was generated by the phage display selection. TP4 did not exhibit ITC-detectable affinity for Aβ, further emphasizing the specificity of the K18ΔKAA-TP4 interaction.

Partially Folded Conformation in the K18ΔK-TP4 Complex—The free and bound states of K18ΔKAA and TP4 were characterized by CD spectroscopy (Fig. 4). In contrast to the CD spectrum of the original ZAβ3 scaffold, which features minima at 208 nm and 221 nm characteristic for the α-helical secondary structure, CD of TP4 exhibited a minimum at 201 nm indicative of largely disordered conformation (Fig. 4A). This might be a consequence of the selection of residues with lower β-sheet propensity and lower hydrophobicity in the β-strand region of the scaffold. K18ΔKAA has an ellipticity minimum at 197 nm in agreement with the intrinsically disordered nature of K18 (Fig.

FIGURE 1. Library design and selected β-wrapin TP4. A, ribbon schematic of the ZAβ3 scaffold (gray) in complex with Aβ(1−40) (red) (Protein Data Bank code 2OTK). The side chains of ZAβ3 residues that were randomized in the library are shown as cyan (exchanged to all 20 amino acids) or dark blue (exchanged to hydrophobic residues only). Spheres. The disulfide bond connecting the two subunits ZAβ3 and ZAβ3′ is depicted in yellow. B, amino acid sequence of the selected code 2OTK). The side chains of ZAβ3 residues that were randomized in the library are shown as cyan (exchanged to all 20 amino acids) or dark blue (exchanged to hydrophobic residues only). Spheres. The disulfide bond connecting the two subunits ZAβ3 and ZAβ3′ is depicted in yellow.

FIGURE 2. Tau isoform and Tau constructs used in this study. The domain organization of the Tau isoform hTau40 and of the construct K18 is displayed. Deletions and mutations in the constructs K18ΔK, K18ΔKAA, and in the K18ΔKAA proline mutants are indicated in red. hTau40 is the longest human Tau isoform. hTau40 and K18 contain four imperfect repeats designated R1 to R4. The positions of the hexapeptide motifs PHF6, PHF6′, and PHF6′′ are highlighted in yellow.

TABLE 1

| Variant          | Kd  | n   | ΔH  |
|------------------|-----|-----|-----|
| K18ΔKAA          | 0.26±0.03 | 1.00±0.01 | −36.7±0.3 |
| K18ΔKAA I308P    | n.d.  |     |     |
| K18ΔKAA I277P    | 0.74±0.07 | 1.20±0.01 | −27.3±0.2 |
| K18ΔKAA V339P    | 0.50±0.05 | 0.95±0.01 | −26.0±0.2 |
| K18ΔKAA I277P V339P | 9.6±1.1 | 1.26±0.04 | −4.7±0.2 |
| K18ΔK oxidized   | 11.0±0.6 | 1.15±0.01 | −25.1±0.3 |

FIGURE 3. Affinity of TP4 for hTau40 determined by ITC. Titration of 680 μM TP4 into 68 μM hTau40 monitored by ITC at 10 °C, yielding an affinity of Kd = 580 ± 20 nm.
The CD spectrum of the K18/H9004 KAA/H18528 TP4 complex differs from the sum of the CD spectra of the free components. Upon binding, the ellipticity decreases in the wavelength range 220 nm, whereas it increases in the wavelength range 200 nm, indicative of partially folded conformation in the K18/H9004 KAA/H18528 TP4 complex (Fig. 4B). Thermal melting experiments demonstrated low thermostability of the K18/H9004 KAA/H18528 TP4 complex with thermal denaturation occurring in the temperature range from 10 to 40 °C at 10 μM protein concentration (Fig. 4C).

**Alternative Conformations of the Tau Repeat Domain**

Two Alternative Conformations of K18 in Complex with TP4—We used NMR spectroscopy to determine the binding site of K18 interacting with TP4. Several resonance signals in the 1H-15N HSQC spectrum of [U-15N]K18ΔKAA disappeared when [NA]TP4 was added at 1:1 molar ratio, indicative of an epitope that features intermediate exchange in the bound state (Fig. 5). The disappearing resonances were assigned to the region Tau(300–317), whose center corresponds to the PHF6 motif and in which residual -structure was detected by NMR of the free K18 monomer (15) (Fig. 6a). A 1H-15N HSQC titration experiment confirmed the 1:1 stoichiometry of the K18ΔKAA-TP4 interaction (Fig. 6a). The titration experiment moreover revealed that the regions N- and C-terminal of Tau(300–317) were also affected by the binding of TP4. These regions spanned approximately the sequence stretches Tau(268–299) and Tau(318–349), respectively. Similar to Tau(300–317), both Tau(268–299) and Tau(318–349) contain a stretch of ~10 amino acids shown previously to exhibit residual -structure in the free K18 monomer (15). In the case of Tau(268–299), this stretch includes the PHF6* motif, whereas Tau(318–349) contains a corresponding hexapeptide motif, 337VEVKSE342, which we refer to as PHF6** in this study. The 1H-15N HSQC peak heights in the regions Tau(268–299) and Tau(318–349) decreased by ~50% upon addition of an equimolar amount of TP4 (Fig. 6a). Further addition of TP4 resulted only in a minor further decrease of the peak heights. The titration experiments thus demonstrated that the region Tau(300–317) invariably underwent a conformational change upon binding, whereas each of the regions Tau(268–299) and...
Tau(318–349) did so in only about half of the K18\(^{\Delta K}\)AA-TP4 complexes, remaining in a disordered free state-like conformation in the rest of the complexes.

A plausible explanation for this observation is that partial folding of K18\(^{\Delta K}\)AA in each individual complex with TP4 involves one of two alternative binding sites, either Tau(268–317) or Tau(300–349) (see Fig. 9a). Partially folded K18\(^{\Delta K}\)AA might be stabilized by contacts between Tau(300–317) and the involved adjacent region, potentially established through the sites exhibiting residual \(\beta\)-structure in the free state (see Fig. 9b). To test this hypothesis, we decreased the \(\beta\)-sheet propensity of the critical regions by generating proline mutants. Proline mutations were introduced at the third position of the hexapeptide motifs PHF6\(^*\) (in R2), PHF6 (in R3), and PHF6\(^{**}\) (in R4) (Fig. 2). In total, four proline mutants of K18\(^{\Delta K}\)AA were generated: I277P, I308P, V339P, and the double mutant I277P/V339P. The I308P mutation decreases the \(\beta\)-sheet propensity of PHF6. Introduction of the I308P mutation into K18\(^{\Delta K}\)AA eliminated the affinity for TP4 as measured by ITC (Table 1). Consequently, the decrease in the peak heights in the \(^1\)H\(^{-1}\)N HSQC spectrum of K18\(^{\Delta K}\)AA I308P upon equimolar addition of TP4 was small compared with K18\(^{\Delta K}\)AA (Fig. 6b). These findings underscore the importance of the PHF6 motif for the K18\(^{\Delta K}\)AA-TP4 interaction. The mutations placed in PHF6\(^*\) and PHF6\(^{**}\), I277P and V339P, respectively, resulted in only a small decrease in the affinity to TP4 when introduced individually (Table 1). In bound K18\(^{\Delta K}\)AA I277P, an increased fraction of the Tau(268–299) region and a decreased fraction of the Tau(318–349) region remained in the disordered free state-like conformation compared with bound K18\(^{\Delta K}\)AA (Fig. 6c). This agrees with preferential binding of TP4 to the binding site Tau(268–299) of the I277P mutant (see Fig. 9a). Conversely, bound K18\(^{\Delta K}\)AA V339P exhibited a decreased fraction of the Tau(268–299) region and an increased fraction of the Tau(318–349) region in the disordered free state-like conformation compared with bound K18\(^{\Delta K}\)AA (Fig. 6c), in agreement with preferential binding of TP4 to the binding site Tau(268–317) of the V339P mutant (see Fig. 9a). When the I277P and V339P mutations were introduced together, binding to TP4 was strongly impaired (Table 1). Both regions Tau(268–299) and Tau(318–349) remained largely in the disordered free state-like conformation upon binding of the I277P/V339P double mutant to TP4 (Fig. 6d). Taken together, the analysis of the proline mutants demonstrated that submicromolar binding of TP4 requires the integrity of the PHF6 motif and of one of the motifs PHF6\(^*\) or PHF6\(^{**}\), consistent with the existence of two alternative conformations of bound K18\(^{\Delta K}\)AA as depicted schematically in Fig. 9b.

Long-range interactions between PHF6 and PHF6\(^*\) or PHF6\(^{**}\) established in the conformations postulated in Fig. 9b.

**FIGURE 6.** Identification of two alternative binding sites by NMR. Changes in the peak height in the \(^1\)H\(^{-1}\)N HSQC NMR spectrum of U-\(^1\)N-labeled K18\(^{\Delta K}\)AA and its proline mutants I277P, I308P, V339P, and I277P/V339P, upon addition of [\(^{15}\)N]TP4, recorded at 5 °C. a, titration of K18\(^{\Delta K}\)AA with TP4. The repeat domain organization is shown on top of the diagram. The positions of the hexapeptide motifs PHF6\(^*\), PHF6, and PHF6\(^{**}\) are indicated by striped boxes. Previously detected stretches of residual \(\beta\)-structure in the free K18 monomer (15), containing the hexapeptide motifs, are highlighted in yellow. b–d, comparison of the TP4 complexes of K18\(^{\Delta K}\)AA and the K18\(^{\Delta K}\)AA proline mutants. The complexes were prepared from equimolar amounts of TP4 and the respective Tau construct.
would entail a compaction of the Tau repeat domain. Analytical SEC was performed to compare the hydrodynamic volume of free and TP4-bound K18\(^{3\text{-K}}\)AA. The K18\(^{3\text{-K}}\)AA-TP4 complex, containing a total of 263 amino acids, eluted at roughly the same volume as free K18\(^{3\text{-K}}\)AA with only 125 amino acids, confirming significant compaction of the repeat domain upon binding (Fig. 7).

The conformations diagrammed in Fig. 9b are incompatible with an intramolecular disulfide bond between the two cysteine residues in the repeat domain, Cys-291 and Cys-322. In line with this, preoxidation of K18\(^{3\text{-K}}\) by prolonged incubation in the absence of DTT, which would lead to “compact monomers” incompetent for assembly (10, 39), strongly decreased the binding affinity of TP4 (Table 1).

**TP4 Inhibits K18 Aggregation**—The fluorescence of the dye ThT increases with the formation of Tau fibrils (40). To evaluate the effect of TP4 binding on the aggregation of K18\(^{3\text{-K}}\)AA, ThT fluorescence was monitored during incubation of K18\(^{3\text{-K}}\)AA in the absence and presence of TP4. In incubations without addition of the aggregation promoter heparin, K18\(^{3\text{-K}}\)AA fibrillation was potently inhibited both at a 1:1 and a 1:10 molar ratio of TP4 (Fig. 8a). The absence of aggregates during the prolonged lag time of TP4-containing aggregation reactions was confirmed by electron microscopy (Fig. 8, b and c). In the presence of heparin, K18\(^{3\text{-K}}\)AA aggregated without a discernible lag phase when TP4 was absent or present at 1:10 molar ratio (Fig. 8a). Addition of TP4 at equimolar ratio, however, led to the emergence of a distinct lag phase.

**DISCUSSION**

The present study reports the selection of the engineered binding protein TP4 targeting the repeat domain of Tau. TP4 was obtained from a new \(\beta\)-wrapin phage display library generated by site-directed mutagenesis of the scaffold protein \(ZA\beta_\gamma\), a binder to a \(\beta\)-hairpin motif of the A\(\beta\) peptide. TP4 binds K18 and full-length hTau40 with nanomolar affinity. The structural analysis of the K18\(^{3\text{-K}}\)AA-TP4 interaction revealed the presence of two alternative binding sites in K18, one involving the region Tau(268–317), the other the region Tau(300–349) (Fig. 9a). The two binding sites are bound with approximately the same frequency by TP4 according to the \(^{1}H-^{15}N\) HSQC titration data (Fig. 6). The determination of the high-resolution NMR structures of the K18\(^{3\text{-K}}\)AA-TP4 complexes was precluded by the absence of resonance signals of the bound state due to intermediate exchange. However, the presence of two sequence stretches with high \(\beta\)-structure propensity in each of the binding regions suggests the presence of \(\beta\)-sheet structure in bound K18, possibly similar to the \(\beta\)-hairpin motifs of \(\alpha\)-\(\beta\)-scaffolding protein ZA\(\beta_\gamma\) (28) and of \(\alpha\)-synerguscin bound to the \(\beta\)-wrapin scaffold protein ZA\(\beta_\gamma\) (29) and of \(\alpha\)-synerguscin bound to the \(\beta\)-wrapin scaffold protein ZA\(\beta_\gamma\) (28).

For the binding site Tau(268–317), the regions around PHF6* and PHF6 could contribute \(\beta\)-strands to a \(\beta\)-sheet, whereas the regions around PHF6 and PHF6** could do so for the binding site Tau(300–349) (Fig. 9). In line with this, a decrease in the \(\beta\)-sheet propensity of any of the hexapeptide motifs by amino acid exchanges to proline resulted in a reduced binding affinity of TP4 to the affected binding site(s).

The binding of Tau to TP4 features striking similarities to Tau self-assembly. For both Tau aggregation and TP4 binding, the PHF6 hexapeptide is of utmost importance. PHF6 is a mandatory element of the TP4 binding sites as well as of the Tau fibril core (14, 18, 20, 41, 42). Replacement of Ile-308 in PHF6 by...
more suggest that aggregation-prone conformations, previously proposed to trigger Tau assembly (8, 45), might share structural characteristics, e.g. certain intramolecular tertiary contacts, with TP4-bound Tau. Aggregation-prone conformations have been detected for example by single-molecule force spectroscopy and by single molecule Förster resonance energy transfer (8, 9). The relationship between the β-sheet conformations of TP4-bound Tau suggested in this study and the conformation of Tau within filaments is illustrated in Fig. 8c. The TP4-bound β-sheet features intramolecular backbone hydrogen bonds between the β-strands, whereas the β-sheets of Tau filaments are intermolecular, with parallel, in-register orientation (20, 42, 46). However, due to the absence of NMR resonance signals the number, lengths, and relative orientations of β-strands of TP4-bound Tau could not be determined.

Tau can adopt two alternative conformations in complex with TP4. A similar conformational heterogeneity of Tau aggregation intermediates would have consequences for the later steps of the aggregation reaction. For example, it could be crucial for the occurrence of aggregation-based seeding barriers that have been observed for different Tau isoforms (47, 48). Structural heterogeneity has also been observed in Tau oligomers (16).

The inhibition of aggregation of the repeat domain by stoichiometric TP4 concentrations (Fig. 8) can be explained by the sequestration of the aggregation-prone regions of Tau by TP4. The aggregation inhibition also at substoichiometric concentrations demonstrates that TP4 moreover interferes with the nucleation steps of the aggregation reaction. The mechanism of substoichiometric inhibition is not provided by the present data. It is, however, tempting to speculate that TP4 might interact with high affinity with Tau conformers in oligomeric aggregation intermediates, which are similar in structure to TP4-bound Tau monomers. The aggregation promoter heparin counteracts the substoichiometric inhibition by TP4 (Fig. 7). Heparin is known to greatly accelerate the nucleation step of Tau aggregation (49, 50). Under these conditions, the capacity of substoichiometric TP4 concentrations to interfere with the formation and/or growth of nuclei might be exceeded.

This study provides residue-level insight into structural properties of Tau interacting with an aggregation inhibitor, revealing two alternative conformations of bound Tau. The obtained data elucidates how aggregation can be halted at the stage of monomeric Tau.

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