Proline-directed Pseudo-phosphorylation at AT8 and PHF1 Epitopes Induces a Compaction of the Paperclip Folding of Tau and Generates a Pathological (MC-1) Conformation*5

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Tau, a neuronal microtubule-associated protein that aggregates in Alzheimer disease is a natively unfolded protein. In solution, Tau adopts a “paperclip” conformation, whereby the N- and C-terminal domains approach each other and the repeat domain (Jeganathan, S., von Bergen, M., Brutzlach, H., Steinhoff, H. J., and Mandelkow, E. (2006) Biochemistry 45, 2283–2293). In AD, Tau is in a hyperphosphorylated state. The consequences for microtubule binding or aggregation are a matter of debate. We therefore tested whether phosphorylation alters the conformation of Tau. To avoid the ambiguities of heterogeneous phosphorylation we cloned “pseudo-phosphorylation” mutants of Tau where combinations of Ser or Thr residues were converted into Glu. These mutations were combined with FRET pairs inserted in different locations to allow distance measurements. The results show that the paperclip conformation becomes tighter or looser, depending on the pseudo-phosphorylation state. In particular, pseudo-phosphorylation at the epitope of the diagnostic antibody AT8* (S199E + S202E + T205E) moves the N-terminal domain away from the C-terminal domain. Pseudo-phosphorylation at the PHF1 epitope (S396E + S404E) moves the C-terminal domain away from the repeat domain in both cases the paperclip conformation is opened up. By contrast, the combination of AT8* and PHF1 sites leads to compaction of the paperclip, such that the N-terminus approaches the repeat domain. The compaction becomes even stronger by combining pseudo-phosphorylated AT8*, AT100, and PHF1 epitopes. This is accompanied by a strong increase in the reaction with conformation-dependent antibody MC1, suggesting the generation of a pathological conformation characteristic for Tau in AD. Furthermore, the compact paperclip conformation enhances the aggregation to paired helical filaments but has little influence on microtubule interactions. The data provide a framework for the global folding of Tau dependent on proline-directed phosphorylation in the domains flanking the repeats and the consequences for pathological properties of Tau.

Microtubules that serve as the tracks for motor proteins are important for the intracellular transport of vesicles, organelles, and protein complexes by motor proteins (2, 3). Microtubule dynamics are modulated by microtubule-associated proteins that bind to the surface of microtubules; among these, Tau protein is one of the major microtubule-associated proteins in neurons (4, 5). Its expression is strongly up-regulated during neuronal development to promote the generation of cell processes and to establish cell polarity (6). During this phase, Tau becomes sorted into the axon, and it diversifies into 6 different isoforms by alternative splicing (7, 8). In Alzheimer disease, Tau becomes hyperphosphorylated, missorted into the somatodendritic compartment, and aggregates into neurofibrillary tangles (9).

The numerous phosphorylation sites of Tau (10) can be broadly subdivided into three classes: (i) SP/TP motifs in the flanking regions of the repeat domain are targets of proline-directed kinases such as glycogen synthase kinase3β (11, 12), cyclin-dependent kinase 5 (CDK5) (13), or mitogen-activated kinase and its relatives (14). (ii) KXGS motifs in repeats are targets of non-proline directed kinases, such as MARK (15), SAD kinase (16), or PKA (17). (iii) Tyrosine residues at Tyr-18 and Tyr-394 are targets of Src family kinases such as fyn and c-Abl (18, 19). Phosphorylation at SP/TP motifs has only a moderate influence on Tau-microtubule interactions but is up-regulated in AD3 and other tauopathies (3, 20). This characteristic feature can be recognized by various diagnostic antibodies (3, 21). Phosphorylation by certain non-proline directed kinases (e.g. by MARK or SADK at the KXGS motifs of the repeat domain, or Ser-214 by PKA) results in a strong reduction of the ability of Tau to bind to microtubules (15, 22–24) and inhibits the formation of PHFs (25). The region of Tau responsible for microtubule binding comprises the repeat domains (R1–R4) and the proline-rich flanking regions (Fig. 1). The repeat domain is also responsible for forming the core of the
PHFs (26). The flanking domain upstream of the repeats contributes to MT binding, but can also bind to other proteins, e.g. Pin-1 (27) or protein phosphatase 2A (28). The N-terminal domain of Tau (~200 residues) projects away from the microtubule surface (29) and may serve as an anchor for other cell components such as kinases, membranes, or motor components (30).

In solution, Tau behaves as a “natively unfolded” or “intrinsically disordered” protein (31). NMR spectroscopy confirmed the paucity of secondary structural elements, but there are motifs in R2 and R3 showing inherent β-structure propensity that coincide with the hexapeptide motifs that nucleate PHF aggregation (26, 32). Several observations suggest that Tau cannot simply be a “random coil” in the strict sense. Hints for special conformational states come from the reactivities of antibodies such as Alz50, MC1, Tau-66, MN423, and SM134 that have discontinuous epitopes on Tau. Antibodies Alz50 and MC1 recognize conformations of Tau in brain tissue that occur at an early stage of AD. Their epitopes comprise residues near the N terminus and in the third repeat and this conformation is called “pathological conformation of Tau” as it precedes aggregation (33, 34). Similarly, Tau-66 reactivity depends on the elements upstream of the repeat domain and residues in repeat R3 (35), SM134 reacts to a folded state of Tau wherein the repeat domain and one of the KSP motifs upstream or downstream from the repeats are required (36) and antibody MN423 requires a truncation site downstream of the repeats (at Glu-391) and the residues within the repeat domain (37). We recently characterized this globally folded state of Tau in solution by generating Tau variants containing FRET pairs at different positions and measuring their distance by the fluorescence energy transfer from the donor (tryptophan) to the acceptor (cysteine carrying a dansyl group). This study revealed a double hairpin or “paperclip” conformation where the C terminus was folded near the repeat domain, and the N terminus was folded back near the C terminus (1).

Given the results, the next question was whether phosphorylation at critical sites would have an influence on this global conformation of Tau and could induce the pathological state seen by the MC1 antibody. Ideally, it would be desirable to phosphorylate Tau by predetermined sites with 100% efficiency. This cannot be achieved due to the open structure of Tau, which makes many sites accessible to various kinases. Therefore, phosphorylation reactions generally result in a heterogeneous mixture of Tau molecules phosphorylated at different sites and to different extents. To circumvent this ambiguity we generated Glu mutants where phosphorylatable serine or threonine residues were replaced by glutamate. Although glutamate is not a perfect substitute for phosphorylation, it is a reasonable approximation (38), and the extent of “pseudo-phosphorylation” is by definition specific and specific. The effect of pseudo-phosphorylation on the aggregation and microtubule binding of Tau has also been studied (39, 40). But it has not been studied whether there are changes in the global folding of Tau that could be linked to its properties. We have therefore combined phospho-mutants with FRET donor/acceptor mutants to measure intramolecular distances. Here we report that phosphorylation at critical sites upstream or downstream of the repeats indeed modifies the global conformation of Tau so that the paperclip structure is either opened up or tightened. In the latter case the N-terminal domain approaches the repeats, which is reminiscent of the conformation recognized by conformation-dependent antibody MC1. This has consequences for the aggregation behavior of Tau.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Proteins**—Chemicals of biochemical grade such as GdnHCl were purchased from Sigma. The fluorescent label 1,5-IAEDANS was obtained from Invitrogen. Point mutations were made in the htau40 coding sequence carried by plasmid pNG2 by site-directed mutagenesis using the QuickChange kit (Stratagene). The plasmids were sequenced on both strands to confirm the mutations. A number of mutants of the full-length Tau isoform htau40 (4R/2N) that carry mutations for FRET pairs, i.e. tryptophan (donor) and cysteine linked to IAEDANS (acceptor) at different positions, was used in the previous study (1). For the current study, the mutant for mapping the proximities between the N terminus and repeats (N-R) were chosen with tryptophan at residue 310 and cysteine at 17; the mutant for mapping the distance between repeats and the C terminus (R-C) were chosen with tryptophan at residue 432 and cysteine at either 291 or 322; the mutant for mapping the proximities between the N and C terminus (N-C) were chosen with tryptophan at residue 432 and cysteine at 17. These FRET pair mutations were combined with phosphomimetic mutations (glutamic acid) (Fig. 1B), for example, phosphorylated epitopes of antibodies: AT8* (Ser-199, Ser-202, Thr-205) (41, 42), AT100 (Thr-212, Ser-214) (24, 43), and PHF1 (Ser-396, Ser-404) (44, 45). Thus to analyze the effect of mimicking phosphatepoptes (AT8*, AT100, and PHF1), constructs were made carrying Glu mutations and FRET pairs at different positions (e.g. N-R, R-C, and N-C). The expression of phosphomimetic mutants were done using the BL21(DE3) Escherichia coli strain. The purification of Tau was usually done by making use of a heating step as described earlier (46). But FRET mutants of Tau were purified with modifications involving a stepwise ammonium sulfate precipitation but no heating step. Briefly, the cell lysate was initially brought to 25% (NH4)2SO4 and then centrifuged for 45 min at 127,000 × g to clear the supernatant. The supernatant was then adjusted to 55% saturated (NH4)2SO4 to precipitate Tau protein and centrifuged for 45 min at 127,000 × g to collect the pellet that was then dissolved and dialyzed against buffer. Further purification was carried out using the ion exchange column SP Sepharose, followed by a gel filtration column G200 (Amersham Biosciences). The purity of the proteins was analyzed by SDS-PAGE. As an example of terminology, Tau/N-CAT8* + AT100 + PHF1 will be used to denote the htau40 mutant carrying a combination of Glu mutations for epitopes of AT8*, AT100, and PHF1 with tryptophan at 432 and cysteine at 17 (N-C).

**Labeling of Proteins**—Protein in 4 M GdnHCl, phosphate-buffered saline buffer (~100 μM) was incubated with 10 M excess DTT for 10 min at 37 °C. DTT was then removed by size exclusion chromatography (Fast Desalting column, Amersham Biosciences) and the eluted protein was immediately supple-
ment with ~20 mM excess IAEDANS (dissolved in N,N-di-methylformamide). The labeling reaction was allowed to proceed at room temperature for 2 h, or alternatively “solid state-based labeling” was used (47), which is achieved by reducing the protein with DTT, then precipitating with 70% (NH₄)₂SO₄, followed by dissolving the protein pellet with buffer containing IAEDANS. The solution was then dialyzed against phosphate-buffered saline and residual IAEDANS was removed by size exclusion chromatography. The concentration of protein was determined by absorption at 280 nm using the molar extinction coefficient ε_{Tau} = 11,460 to 12,950 M⁻¹ cm⁻¹, depending on the Tau mutants. The amount of bound IAEDANS was determined by the absorption at 336 nm (ε_{IAEDANS} = 6,100 M⁻¹ cm⁻¹) (48). The protein concentration was corrected for the contribution of the IAEDANS at 280 nm and the stoichiometry was calculated. Typically the labeling stoichiometry was 0.7–0.9. The distances calculated with or without correction for the fractional labeling ratio are within a difference of 10–15% (see below).

**Fluorescence Spectroscopy**—All steady state fluorescence measurements were performed with a Spex Fluoromax spectrophotometer (Polytect, Waldbronn, Germany), using 3 × 3-mm quartz microcuvettes from Hellma (Mühlheim, Germany) with 20 μl sample volumes. Protein was irradiated at 290 nm to excite tryptophan but not tyrosine. In all cases, the experimental parameters were as follows: scan range = 300–550 nm, excitation slit width = 4 nm, emission slit width = 6 nm, integration time = 0.25 s, and photomultiplier voltage = 950 V. Each time 3 spectra were scanned and averaged. A protein concentration of 4 μM was used and checked by SDS-PAGE as a control. In denaturation experiments with GdnHCl, the efficiency was calculated from emission intensities of labeled protein and unlabeled protein at the same GdnHCl concentration. The influence of various GdnHCl concentrations on the fluorimetric properties of tryptophan and IAEDANS was controlled with free dyes alone and in combination. The effects due to GdnHCl as a solvent were minor (<10%) in comparison to the FRET effects and the spectra were corrected for it. The FRET efficiency was measured by the energy transfer,

\[ E_{\text{FRET}} = \frac{1 - D_A/D}{1/f_A} \quad (\text{Eq. 1}) \]

where \( D_A \) is the fluorescence intensity of the donor in the presence of the acceptor and \( D \) is the fluorescence intensity of donor in the absence of acceptor. The apparent efficiencies were normalized by \( f_A \), the fractional labeling with acceptor, as shown in Equation 1. The distance \( R \) between donor and acceptor was calculated by the Förster equation,

\[ E_{\text{FRET}} = \left[ 1 + (R/R_0)^6 \right]^{-1} \quad (\text{Eq. 2}) \]

where the Förster radius \( R_0 \) is 22 Å for the Trp-IAEDANS pair (49). In the case of the tryptophan-IAEDANS pair, a small error in the labeling ratio would give distance values that are within acceptable error range even without a correction factor, due to the dependence of the efficiency on the 6th power of the distance. For example, if the measured efficiency is 0.5 with 100% labeling, then \( r = 22 \) Å. On the other hand, if the measured FRET efficiency is 0.5 with only 80% labeling (\( f_A = 0.8 \)) and the correction for fractional labeling is applied, then \( r = 20.2 \) Å. Note that in unfolded proteins the distance between a given FRET pair shows a wider distribution and the apparent FRET reflects this heterogeneity (50).

**CD Spectroscopy**—All measurements were carried out with a Jasco J-810 CD spectrometer (Jasco, Groß-Untmstadt, Germany) in a cuvette with a path length of 0.1 cm. The scanning speed was 100 nm/min, bandwidth 0.1 nm, and a response time of 4 s. In each experiment, measurements were done at 20 °C and 4 spectra were summed and averaged. The CD spectra were normalized for the concentration at 214 nm using bovine serum albumin as a standard.

**PHF Assembly**—Aggregation was induced by incubating soluble Tau typically in the range of 50 μM in volumes of 20 μl at 37 °C in 20 mM BES, pH 7.4, plus 25 mM NaCl buffer with the anionic cofactor heparin 6000 (molar ratio of Tau to heparin = 4:1). The formation of aggregates was monitored by ThS fluorescence and confirmed by electron microscopy. For ThS fluorescence, 5 μl of 50 μM assembly reaction was diluted to 50 μl with NH₄Ac, pH 7.0, containing 20 μM Ths. Fluorescence measurements were done at 25 °C in a Tecan spectrofluorimeter (Crautscheim, Germany) with an excitation wavelength of 440 nm and an emission wavelength of 521 nm (slit width 7.5 nm each) in a black microtitre plate with 384 round wells (ThermoLabsystems, Dreieich, Germany). The background fluorescence was subtracted when needed. For electron microscopy, protein solutions were diluted to 1–10 μM and placed on 600 mesh carbon-coated copper grids for 45 s, washed twice with H₂O₂, and negatively stained with 2% uranyl acetate for 45 s. The samples were examined with a Philips CM12 electron microscope at 100 kV.

**Microtubule Polymerization Assay**—Microtubule assembly was monitored by UV light scattering at an angle of 90° at a wavelength of 350 nm in a black microtitrator plate with 384 round wells (ThermoLabsystems) in a Tecan spectrofluorimeter in the presence and absence of Tau. 5 μM Tau was mixed with 30 μM tubulin dimer at 4 °C in microtubule assembly buffer (100 mM Na-PIPES, pH 6.9, 1 mM EGTA, 1 mM MgSO₄, 1 mM GTP, 1 mM DTT) in a final volume of 40 μl. The reaction was started by raising the temperature to 37 °C.

**Western Blotting**—The protein samples were added to SDS sample buffer and boiled at 95 °C for 5 min. Equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels for subsequent electrophoresis. The proteins were transferred to a nitrocellulose membrane at 100 V for 40 min. After the transfer, the blot membrane was blocked with 5% milk and incubated overnight at 4 °C with the primary antibody (MC1 at 1:1000 dilution). After washing the unbound primary antibody, the secondary antibody (goat anti-mouse IgM conjugated to horseradish peroxidase at 1:1000 dilution) was incubated with the blot. The blot was then stripped of MC1 antibody and incubated with the pan-Tau antibody K9JA at 1:8000 dilutions (A0024, DAKO, Glostrup Denmark). Protein bands were visualized using chemiluminescence (ECL, Amersham Biosciences).
Phosphorylation and Global Conformation of Tau

RESULTS

Proteins and Phosphomimic Mutations—Tau is a natively unfolded protein that does not contain a significant amount of secondary structure (31). However, it is possible that there are global conformations defined by interactions between the different domains of Tau, as suggested by certain antibodies (e.g. Alz50, MC1) that are diagnostic of a pathological conformation and react with a discontinuous epitope comprising residues near the N terminus and the third repeat (33, 34). Indeed, as shown previously, Tau in solution adopts preferred long range interactions between the repeat domain and the C terminus and between the N and C terminus (paperclip conformation), as judged by FRET (1). For mapping FRET distances we inserted Trp as a donor and Cys-dansyl as an acceptor near the N terminus (e.g. residue Y18W or T17C-dansyl), near the C terminus (e.g. V432W), or within the repeats (Cys391-dansyl in R2, Cys322-dansyl in R3 or Y310W in R3). In solution, the C-terminal domain of Tau is unexpectedly close (19–23 Å) to the repeat domain and therefore causes a pronounced FRET signal, whereas the N-terminal domain (residue 17) is not within the FRET range of the repeat domain, but close to the C-terminal tail where it causes FRET (21–24 Å, Fig. 1A).

Because the functions of Tau are regulated by phosphorylation we were interested whether this would influence the global conformation. In particular, we wanted to know whether phosphorylation could induce a state reminiscent of the pathological conformation. To mimic the phosphorylation in vitro, we used pseudo-phosphorylation mutants by substituting Glu at the phosphorylatable residues. A series of Tau mutants based on the full-length isoform hTau40 were generated with certain pseudo-phosphorylation sites alone or in combination. The choice of phosphorylation sites was based on the epitopes of antibodies that are characteristically elevated in AD (Fig. 1B). For example, the triple mutation S199E + S202E + T205E generates pseudo-phosphorylation at the epitope recognized by antibody AT8 (41, 42, 51). For brevity, we will refer to this mutant as the “AT8” mutant (note: phosphorylated Ser202 + Thr205 suffice for the reaction with antibody AT8, but Ser199 is usually also phosphorylated in brain tissue and can be detected by the antibody, hence we chose the triple mutation and denote it as AT8*). Similarly, the “PHF1” mutant contains mutations S396E + S404E because phosphorylation at these sites generates the epitope for antibody PHF1 (44, 45) and the “AT100” mutant contains mutations T212E + S214E (24, 43). Together with the Glu mutations, FRET pairs were introduced into Tau to allow mapping of the distances between the N terminus and repeats (N-R), C terminus and repeats (R-C), or N and C terminus (N-C), for the different states of (pseudo-) phosphorylation.

Site-specific Pseudo-phosphorylation at Epitopes AT8* or PHF1 but Not AT100 Alters Long Range Interactions of Tau—We first describe the effects of “single arm” pseudo-phosphorylation, either upstream of the R-domain (sites AT8* or AT100) or downstream (site PHF1). The phosphorylation of Tau at the AT100 epitope has been shown to reduce microtubule binding both in vitro and in vivo (22–24). To analyze the effect of pseudo-phosphorylation at the epitope AT100 on the global folding of Tau, we mutated Thr212 + Ser214 into Glu and combined this with FRET pair mutations in the N, R, and C-terminal domains. For the unlabeled mutant Tau/N-RAT100 (single tryptophan at 310, single cysteine at position 17, mutations T212E + S214E, and intrinsic Cys residues 291 and 322 mutated to Ala, no dansyl label), the tryptophan fluorescence emission has a maximum around 350 nm, indicating that the tryptophan residue is solvent-exposed (supplemental Fig. S1A, black curve) (52). The labeled mutant Tau/N-RAT100 (as above, but Cys32-labeled with IAEDANS) resulted in a FRET efficiency of only 0.15 (supplemental Fig. S1A, red curve), showing that pseudo-phosphorylation at the AT100 epitope does not cause a change in the distance between the N and R domains, compared with the unphosphorylated Tau mutant (E = 0.19) (1). Similarly, no significant change in FRET efficiency was observed for Tau/R-CAT100 or Tau/N-CAT100, showing that the phosphomimic Glu212 + Glu214 does not alter the paperclip folding of Tau (Table 1 and supplemental Fig. S1, A–D).

A different picture emerged with the mutant Tau/N-CAT8 (mimicking phosphorylation at Ser199 + Ser202 + Thr205, epitope AT8*), which resulted in a ~4-fold drop of the FRET efficiency between the N- and C-domains from 0.6 in the control to 0.14 (Fig. 2C). This indicates that the single-arm pseudo-phosphorylation at the AT8* epitope causes the N-domain to swing away from the C-domain, thus loosening the paperclip structure (Fig. 2D). However, the FRET between R- and C-domains (mutant Tau/R-CAT8, E = 0.44) did not change significantly from the unphosphorylated mutant showing that the C-domain remained close to the repeats, whereas the N-domain remained outside the FRET distance of the repeats (Fig. 2, A and B). Likewise, Glu mutations at Ser396 and Ser404 (to mimic the PHF1 epitope in the arm downstream of the repeats)
caused an increase in the distance seen by FRET between the R-domain and C-domain, but at the same time a decrease in the distance between the C terminus to the N terminus (Fig. 2, E–G). Thus the FRET results show that phosphorylation mimic at the PHF1 site causes the C terminus to swing away from the repeats toward the N terminus, whereas the N terminus still remains outside the FRET distance from the repeats (Fig. 2H). This indicates that phosphorylations at sites recognized by AT8 or PHF1 epitopes, when present alone, open up the N- and C-terminal domains, respectively. As a result, the repeat domain would become more exposed and might facilitate aggregation into paired helical filaments.

**Combination of Pseudo-phosphorylation at AT8*, AT100, and PHF1 Epitopes Confers Pathological Folding to Tau**—We next asked whether the conformation of Tau is affected by a combination of pseudo-phosphorylated epitopes. We tested the combination of Glu mutations at the AT100 plus PHF1 sites. Surprisingly, the “double arm” phosphorylation causes a compaction of the Tau molecule, in contrast to the single arm mutations described above that loosened up the paperclip con-

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**TABLE 1**

Summary of results

| Tau constructs (Antibody mimic) | Sites of Glu mutations | Folding | FRET pairs | FRET measured | Effect on the folding |
|---------------------------------|------------------------|---------|------------|---------------|----------------------|
| Tau40 | 212+214 | N-R | 0.15 | 28.4 | → |
| Tau40/EAT100 | 212+214 | N-R | 0.16 | 28.9 | → |
| Tau40/EAT8* | 199+202+205 | N-R | 0.18 | 28.5 | → |
| Tau40/EAT100-PHF1 | 212+214+396+404 | N-R | 0.34 | 24.6 | ↑ |
| Tau40/EAT8*+PHF1 | 199+202+205+396+404 | N-R | 0.41 | 23.4 | ↑ |
| Tau40/ EAT8*+AT100+PHF1 | 199+202+205+214+396+404 | N-R | 0.46 | 22.6 | ↑ |
| Tau40/DCT | 199+202+205+214+396+404 | N-R | 0.39 | 23.7 | ↑ |

*W, donor (tryptophan); DANS, acceptor (IAEDANS linked to cysteine); Glu mutations given.*

(E–G) Thus the FRET results show that phosphorylation mimic at the PHF1 site causes the C terminus to swing away from the repeats toward the N terminus, whereas the N terminus still remains outside the FRET distance from the repeats (Fig. 2H). This indicates that phosphorylations at sites recognized by AT8 or PHF1 epitopes, when present alone, open up the N- and C-terminal domains, respectively. As a result, the repeat domain would become more exposed and might facilitate aggregation into paired helical filaments.

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Phosphorylation and Global Conformation of Tau

formation. The FRET efficiencies for Tau/R-C_{AT100+PHF1} (E = 0.31) and Tau/N-C_{AT100+PHF1} (E = 0.56) were unchanged, but Tau/N-R_{AT100+PHF1} showed a notable increase of efficiency from 0.14 to 0.34, indicating that the N-domain comes closer to the repeats (Fig. S2, A–D). To test whether this effect is epitope specific, we created mutants carrying other combinations of phosphomimics in both flanking regions of the repeats. Indeed, the combination of AT8* + PHF1 phosphomimics shows an even stronger effect of AT100 + PHF1 on the compaction of Tau by swinging the N-domain very close to the repeats, E = 0.41 for Tau/N-R_{AT8*+PHF1} (Table 1 and supplemental Fig. S3, A–D). This corresponds to the pathological conformation detected by diagnostic antibodies MC1 or Alz50, reacting with a discontinuous epitope and the R-domain.

In the triple epitope mutant that bears double arm Glu mutations to mimic the epitopes of AT8*, AT100, and PHF1, the C terminus approaches the repeats very closely (with a high FRET efficiency of E = 0.6), whereas the N-terminal domain also approaches the repeats (E = 0.46, Fig. 3, E and F). At the same time the N-domain is still close to the C-domain, albeit with a decreased efficiency (E = 0.28; Fig. 3G). Thus there appears to be a joint swinging of both termini toward the repeat domain. Overall, the triple epitope pseudo-phosphorylation of both arms at AT8*, AT100, and PHF1 causes an observable compaction of the molecule (Fig. 3H).

Cleavage of the C-terminal Domain Is Not a Requisite for the Folding of the N Terminus Close to the Repeats—The cleavage of the C-terminal tail by caspases is believed to favor Tau aggregation (53). To analyze if the deletion of the C-terminal tail allows the N-domain to approach the repeats (similar to the compaction by double-arm phosphorylation, see above), we created a Tau mutant lacking amino acids 422–441 with Trp at residue 310 and Cys-IAEDANS at residue 17 (denoted as TauΔCT/N-R). However, the FRET efficiency of TauΔCT/N-R remained low (E = 0.13, Fig. 4A), comparable with the full-length htau40 mutant. Thus the deletion of the C-terminal tail did not change the distance between the N- and R-domains. We then combined deletion of the C-terminal tail with single arm phosphorylation mutants (AT8* or AT100 epitopes). The mutants TauΔCT/N-R_{AT8*}, TauΔCT/N-R_{AT100}, or TauΔCT/N-R_{AT8*+AT100} all resulted in efficiencies less than 20% showing that pseudo-phosphorylation upstream of the repeats combined with C-terminal deletion are not sufficient to swing the N-domain close to the repeats (Table 1 and supplemental Fig. S3, B, D). However, the triple mutant TauΔCT/N-R_{AT8*+AT100+PHF1} resulted in a higher efficiency (E = 0.39), indicating a compaction of the N-domain toward the repeats (Fig. 4, B and C).

GdnHCl Denaturation Decreases FRET Efficiencies—Some of the phosphomimic mutants of Tau, particularly the double arm combination of AT8* + PHF1 or AT100 + PHF1 or AT8* + AT100 + PHF1, showed a remarkably high FRET efficiency between Tau domains compared with unphosphorylated Tau, indicating a compaction of the Tau molecule. We therefore investigated the stability of these mutants and measured the change of FRET efficiency with increasing GdnHCl concentration. The efficiency of unphosphorylated Tau (Tau/C-R) decreased already at low GdnHCl concentration (<1.0 M), showing that the paperclip conformation is labile and can be easily perturbed (Fig. 5A). The same was true for the phosphomimic mutants Tau/N-R_{AT8*+PHF1} and Tau/N-R_{AT8*+AT100+PHF1} (Fig. 5B), revealing that Glu mutations did...
we performed titration experiments. To 4 μM FRET mutant protein Tau/N-R\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} or Tau/N-R\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} increasing concentrations of non-fluorescent hTau40wt or Tau\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} (lack tryptophan, but has 5 tyrosine residues) were added up to 20 μM. The efficiency of the mutant proteins remained largely unchanged (supplemental Fig. S4D). Moreover, the addition of unlabeled mutant protein to labeled mutant protein only increased the tryptophan emission but not the IAEDANS emission (data not shown). These results showed that the FRET effects observed for the phosphomimics of Tau arise from intramolecular interactions.

Increased Aggregation Propensity and MC1 Antibody Reactivity Induced by Pseudo-phosphorylation—To relate the global conformational change of Tau induced by pseudo-phosphorylation to the functions of Tau we tested the propensity for aggregation, the ability to promote microtubule polymerization, and the reaction with antibody MC1, which is characteristic of a pathological conformation of Tau in AD. These properties were compared for wild-type Tau and the triple pseudo-phosphorylated mutant Tau\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} which shows the tightest compaction of the papierclip conformation. In the aggregation assay, Tau\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} showed a moderate but significant increase of ThS fluorescence (indicative of a higher extent of β-structure and aggregation) compared with Tau\textsubscript{wt} (Fig. 6A). This result suggests that the compaction due to pseudo-phosphorylation at both arms (AT8\textsuperscript{*} + AT100 + PHF1) raises the tendency for aggregation (beyond the effect of polyanions alone). In contrast, the microtubule polymerizing ability of Tau\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} showed no difference in final extent and only a small retardation on assembly rate (Fig. 6B). This is consistent with the notion that the AD-like phosphorylation in the flanking domains has no or only a small effect on Tau-microtubule interactions (39, 54, 55). Third, we tested the reactivity of the Tau mutant proteins with antibody MC1 (Fig. 6C). There was a clear increase in signal for the triple-site mutant Tau/N-R\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1}. Remarkably, the increase became very pronounced with the C-terminal truncated version of the protein Tau\textsubscript{ΔCT}/N-R\textsubscript{AT8}\textsuperscript{*}+AT100\textsuperscript{+PHF1} compared with the unphosphorylated protein Tau\textsubscript{ΔCT}/N-R. Both of these observations suggest that the compaction of the molecule observed by FRET resembles the “pathological conformation” detected by the MC1 antibody.
Phosphorylation and Global Conformation of Tau

DISCUSSION

Tau is a target of several kinases, it can be phosphorylated at multiple sites that lead to a decrease of Tau-microtubule binding and to altered microtubule dynamics. In AD, Tau is abnormally phosphorylated at many sites, mostly within the repeat domain (KXGS motifs) and in the flanking domains (mainly SP or TP motifs). The latter are of diagnostic value because certain antibodies against Alzheimer Tau recognize these motifs, including antibodies AT8, PHF1, and AT100 (24, 41, 45). Early clues to the conformation of Tau came from EM studies that showed Tau as an elongated rod (56, 57). Subsequent spectroscopic and x-ray studies revealed Tau as a natively unfolded protein (31). However, several observations suggested that there is a global conformation that is somehow related to Tau pathology. One evidence came from studies of PHF assembly showing that the repeat domain aggregated more readily than full-length Tau, consistent with a model whereby the domains outside the repeat domain protect the repeats from interacting with other molecules (5, 57). Another evidence came from monoclonal antibodies diagnostic of early stages of AD, which recognized a pathological folded conformation because the epitope was discontinuous (e.g. Alz50, MCI, SM134 (33, 34, 36, 58)). We therefore investigated the conformation by the FRET method and found that Tau in solution adopts a conformation reminiscent of a paperclip (1). This prompted the question whether the paperclip conformation was influenced by the state of phosphorylation. One could approach this issue by generating FRET pair mutants and phosphorylating Tau at the desired sites. However, because of the large number of phosphorylation sites and the incomplete extent of phosphorylation by kinases, this approach does not yield quantitative results. The alternative is to generate phospho-mimicking Ser/Thr to Glu mutations, which were combined with FRET pairs in the N-, R-, and C-domains (Table 1). For mutations, we chose epitopes of 3 well known antibodies known to become hyperphosphorylated early in AD (AT8, PHF1, and AT100).

The results can be summarized by stating that single arm pseudo-phosphorylation, either upstream of the repeats at the AT8* epitope or downstream at the PHF1 epitope, loosens up the paperclip conformation, whereas double arm pseudo-phosphorylation (AT8* + PHF1) tightens the paperclip (Fig. 7). In
other words, the AT8\* phosphomimic moves the N-terminal domain away from the C-terminal domain so that the FRET between the N and C terminus is decreased; the PHF1 phosphomimic moves the C-terminal domain away from the repeats and closer to the N-terminal domain; but the combination of both AT8\* and PHF1 moves N-, R-, and C-domains closer to one another and causes a compaction of the paperclip fold so that even FRET between the N- and R-domains can now take place, which is normally not visible, and is a sign of the pathological conformation seen by the MC1 antibody. The double site AT100 (T212E\*/S214E) has no effect on the paperclip conformation as judged by FRET, even though phospho-Ser214 strongly reduces microtubule binding. However, AT100 in combination with AT8\* and PHF1 accentuates the compaction of the paperclip, as seen by the strong FRET between the C-domain and the repeats, as well as between the N-domain and the repeats. Finally, the wide separation between N- and R-domains is not dependent on the C-terminal tail, because it is present even when the C-terminal tail of Tau is cleaved off (Fig. 4).

Altogether, the data support the view of a global conformation of Tau, which is regulated by phosphorylation at proline-directed sites in the microtubule-binding regions flanking the repeats. Nevertheless, all of the mutants show the spectroscopic hallmarks of natively unfolded proteins (e.g. minimum of CD spectra at 200 nm, supplemental Fig. S4). This is not a contradiction because CD spectroscopy reports on the local environment of residues, not on long-range interactions. In addition, denaturation studies show that the paperclip folding can be destroyed by GdnHCl, consistent with the view that certain (limited) stretches of secondary structure are responsible for generating the global folding, even though the protein is rather flexible on the level of residues. Such pockets of secondary structure have indeed been observed by NMR spectroscopy (32, 59).

If one models Tau as a random Gaussian coil, the mean distances between donor and acceptor would be expected to be \( \sim 88 \text{ Å} \) for Tau/R-C (Cys\(^{322}\)–Trp\(^{432}\) in R- and C-domains) and \( \sim 143 \text{ Å} \) for Tau/N-R (Cys\(^{17}\)–Trp\(^{310}\) in N- and R-domains), using the relation \( L_m = 8.3 \sqrt{N} \text{ Å} \), where \( N \) represents the intervening chain length (60). These values would be far outside the observable FRET distance, and therefore we can conclude that there must be some specific folding (i.e. paperclip) that brings the residues within FRET range. Conversely, the fact that FRET disappears upon exposure to GdnHCl supports the view that the polypeptide chain approaches a more random configuration, as expected for a denatured protein. The comparison illustrates the distinction between a “random” denatured protein and a natively unfolded protein with “global folding.”

Perhaps the most provocative aspect of the results is the relationship between Alzheimer-like phosphorylation (at AT8\*, AT100, and PHF1) epitopes, the compaction of the paperclip conformation, and the reactivity of Tau with antibodies that report on the pathological conformation of Tau in early stages of AD, such as MC1, Alz50, and others. This reaction is best observed in brain tissue and has been difficult to reproduce with Tau \textit{in vitro}. However, the fact that the most compact conformations are also the ones showing the highest reactivity with MC1 (Fig. 6C) argues that the compaction of the paperclip conformation reflects the pathological state. The increase in MC1 reactivity becomes more pronounced when the C-terminal tail is absent, which argues that this tail normally opposes a close approach between the N terminus and the repeats (consistent with the paperclip model). As a caveat, we note that antibody Alz50 shows only a very weak reaction, both for wild-type soluble Tau and the phosphomimic mutants, even though the epitope comprises similar residues of Tau as antibody MC1.
Phosphorylation and Global Conformation of Tau

In the case of neurons, we speculate that multiple phosphorylation plus stabilization by other interactions may lead to the enhanced visibility of the pathological conformation seen in brain tissue. Whether or not this conformation promotes aggregation directly or some other intermediate state (e.g. oligomers of Tau) remains open at present. From a structural perspective, the folding of the N- and C-domains over the repeat domain would be expected to protect against aggregation, and indeed Tau forms aggregates more readily when the non-repeat domains are cleaved off (5, 26, 57, 61, 62). This is reminiscent of other amyloid aggregation processes that are enabled only when the protective surroundings are removed, e.g. by cleavage or unfolding (63, 64). Contrary to these principles, we find that the compact pseudo-phosphorylated conformation forms aggregates somewhat more readily, whereas microtubule interactions show little change. A possible explanation is that the compactions of the paperclip takes place such that the amyloid-forming hexapeptide motifs in the repeat domain become exposed, which would then promote further aggregation. These issues must await further structural analysis, for example, by environment-sensitive tags.

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Phosphorylation and Global Conformation of Tau

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