Pseudophosphorylation and Glycation of Tau Protein Enhance but Do Not Trigger Fibrillization in Vitro*

Mihaela Nuclea‡ and Jeff Kuret¶

From the ‡Biophysics Program and the ¶Department of Molecular and Cellular Biochemistry, The Ohio State University College of Medicine and Public Health, Columbus, Ohio 43210

Alzheimer’s disease is defined in part by the intraneuronal aggregation of tau protein into filamentous lesions. The pathway is accompanied by posttranslational modifications including phosphorylation and glycation, each of which has been shown to promote tau fibrillization in vitro when present at high stoichiometry. To clarify the site-specific impact of posttranslational modification on tau fibrillization, the ability of recombinant full-length four repeat tau protein (htau40) and 11 pseudophosphorylation mutants to fibrillize in the presence of anionic inducer was assayed in vitro using transmission electron microscopy and laser light scattering assays. Tau glycated with D-glucose was examined as well. Both glycated tau and pseudophosphorylation mutants S199E, T212E, S214E, double mutant T212E/S214E, and triple mutant S199E/S202E/T205E yielded increased filament mass at equilibrium relative to wild-type tau. Increases in filament mass correlated strongly with decreases in critical concentration, indicating that both pseudophosphorylation and glycation promoted fibrillization by shifting equilibrium toward the fibrillized state. Analysis of reaction time courses further revealed that increases in filament mass were not associated with reduced lag times, indicating that these posttranslational modifications did not promote filament nucleation. The results suggest that site-specific posttranslational modifications can stabilize filaments once they nucleate, and thereby support their accumulation at low intracellular tau concentrations.

Especially dramatic among these is phosphorylation, which achieves significantly greater stoichiometries in filamentous tau (averaging 7–8 mol/mol relative to normal tau (averaging 2–3 mol/mol) (8, 9). The phosphate is distributed into ~30 sites, most of which are clustered into two regions flanking the microtubule-binding domain (10, 11). Because few of these sites are unique to filamentous tau, hyperphosphorylation mostly reflects increased occupancy of phosphorylation sites found in normal tau (12, 13). At least 15 of these sites fill in hierarchical fashion as neurofibrillary lesions mature, suggesting a sequential correlation between site occupancy and neuritic lesion development (14, 15). In addition to serving as a marker for diseased neurons, hyperphosphorylation neutralizes the microtubule binding activity of tau, yielding a cytosolic population available for aggregation (16–19). Phosphorylation may also directly promote fibrillization, but in vitro this reaction requires stoichiometries of phosphorylation well above average levels observed in filamentous tau (20, 21). Part of the experimental challenge stems from the diversity of tau phosphorylation sites, each of which may enhance or depress fibrillization (22), and the difficulty of recapitulating the precise pattern found in disease. To overcome these limitations, and to investigate the contribution of individual sites, phosphorylation has been mimicked by mutation of hydroxyamino acids into negatively charged Asp or Glu residues. The resultant “pseudophosphorylation” mutants imitate phosphorylation-induced changes in the structure and microtubule-binding function of tau (23–26), react with many phosphorytipe-selective antibodies (27), and reportedly can promote or inhibit tau fibrillation depending on the sequence context investigated (24, 25, 27, 28). Nonetheless, the mechanisms through which phosphorylation or phosphorylation mimicry modulates fibrillation have not been explored.

In addition to being phosphorylated, AD-derived tau protein is glycated (5, 29–31). As with phosphorylation, glycation has been implicated in events that may contribute to tau fibrillization and neuronal death (32, 33). In vitro glycation of tau can modify at least 13 sites (34), decrease its affinity for tubulin (5, 30, 35), and promote fibrillation (5, 30, 31). Again the mechanisms underlying the effect on tau aggregation have not been investigated.

Fibrillization of purified recombinant tau preparations is slow and essentially undetectable when assayed over a period of days (38, 39), but can be greatly accelerated by the addition of exogenous inducers. Among these, anionic surfactants (including fatty acids) have emerged as especially useful agents because of their efficacy with full-length tau protein under near-physiological conditions of temperature, pH, ionic strength, reducing environment, and tau concentration (38, 40). Anionic surfactants act in micellar form to stabilize assembly competent intermediates on their surfaces, which then accumulate to sufficient concentrations so that the barrier to

* This work was supported by National Institutes of Health Grant AG14452 (to J. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: OSU Center for Biotechnology, 1060 Carmack Rd., Columbus, OH 43210. Tel.: 614-688-5899; Fax: 614-392-5379; E-mail: kuret.3@osu.edu

‡ The abbreviations used are: AD, Alzheimer’s disease; AA, arachidon acid; I(90°), net light scattering intensity corrected for micellization; LLS, laser light scattering.

This paper is available on line at http://www.jbc.org
filament nucleation is overcome (41). Using this approach, differences in fibrillation efficiency among individual tau isoforms can be quantified in as short as 5 h (39).

Here we extend the approach by investigating the fibrillation properties of glycated tau and pseudophosphorylation of tau mutants that mimic occupancy of sites known to be filled in disease (14) using quantitative transmission EM and static light scattering assays. The results show that glycation and phosphorylation mimicry influence tau aggregation through mechanisms that include filament stabilization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Stocks of alkyl sulfate detergent C_{18}H_{37}SO_{4}Na (Research Plus, Bayonne, NJ) were prepared in 1:1 H_{2}O/isopropanol (AA, Wilmad, Buena Vista, NJ). 2Kg was dissolved in ethanol (333 mM) and stored at -80 °C until used. Dithiothreitol was from Sigma, d-glucose from Fisher Chemicals (Fair Lawn, NJ), and uranyl acetate, glutaraldehyde, and 300-mesh copper formvar/carbon-coated grids were from EM Sciences (Fort Washington, PA, WA).

**Oligonucleotide-directed Mutagenesis**—Vector pITC-htau40 served as a template for synthesis of all mutants using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (42). S199E, 5'-gtcaagcagcgaagcgctcctgccacagc; S199E/S202E/T205E, 5'-gtcaagcagcgaagcgctcctgccacagc; T212E, 5'-gcttttcagcgctctctcagctc; S214E, 5'-gccgtaagcagcagcagcagc; T212E/S214E, 5'-gcttttcagcgctctctcagctc; T212E/S214E/S218E, 5'-gcttttcagcgctctctcagctc; S262E, 5'-gccgtaagcagcagcagcagc; T212E/S214E/S218E, 5'-gcttttcagcgctctctcagctc; S262E, 5'-gccgtaagcagcagcagcagc; T212E/S214E/S218E/S262E, 5'-gcttttcagcgctctctcagctc; S409E, 5'-gcttttcagcgctctctcagctc; and S422E, 5'-gcttttcagcgctctctcagctc; the mutations are underlined. Double mutants were from EM Sciences (Fort Washington, PA).

**Materials**—Stocks of alkyl sulfate detergent C_{18}H_{37}SO_{4}Na (Research Plus, Bayonne, NJ) were prepared in 1:1 H_{2}O/isopropyl alcohol. AA (Cayman Chemicals, Ann Arbor, MI) was dissolved in ethanol (333 mM) and stored at -80 °C until used. Dithiothreitol was from Sigma, d-glucose was from Fisher Chemicals (Fair Lawn, NJ), and uranyl acetate, glutaraldehyde, and 300-mesh copper formvar/carbon-coated grids were from EM Sciences (Fort Washington, PA, WA).

**Assembly-critical Concentration Determination**—Tau samples (2–4.5 μM, final concentrations) were added from serially diluted stocks to assembly buffer at room temperature and incubated for 26 h in the presence of 50 μM anionic surfactant C_{18}H_{37}SO_{4}Na and in the absence of agitation. Samples were analyzed by laser light scattering as described above. The net intensity of scattered light corresponding to filbrillization was plotted against tau concentration. Critical concentration for assembly was estimated from the abscissa intercept after least squares linear regression and expressed as ± S.E. of the estimate.

**Analytical Methods—Signalmation reaction progress curves were fit to the Gompertz function (49),

\[ y = a e^{-e^{\frac{t}{b}} (1 - e^{-c})} \]  

where \( y \) is total filament length (50 nm cutoff) or light scattering intensity measured at time \( t \), \( a \) is the inflection point corresponding to the time of maximum growth rate, \( b \) is the maximum total filament length or light scattering at equilibrium, and \( c \) is the proportional growth rate of the filament population in units of time^{-1} (49). Lag times, defined as the time when the tangent to the point of maximum polymerization rate intersects the abscissa of the sigmoidal curve (50), were calculated as \( a - b \) (49).

Filament length distributions calculated as described previously (41, 51) were fit to the exponential function,

\[ y = a e^{\frac{x}{b}} \]  

where \( y \) is the percentage of all filaments filling a bin of length interval \( x \), and \( b \) is a constant (slope parameter) with units of length^{-1}. All parameters estimated by linear or non-linear regression are reported ± S.E.

**RESULTS**

**Impact of Phosphorylation Mimicry on Tau Fibrillation**—The distribution of phosphorylation sites on authentic AD-derived tau protein reported from mass spectrosopic methods (10, 11) is summarized in Fig. 1. Because of the large number of individual sites, and the even larger number of potential combinations, only a subset of these were modeled in a full-length, htau40 isofrom background by pseudophosphorylation mutagenesis and kinetic analysis (Table I). These 12 sites, which spanned the proline-rich, microtubule-binding, and C-terminal tau domains where phosphorylation sites cluster (Fig. 1), were selected because alone or in combination they compose phosphophopites that have been studied extensively by immunodiffraction reactions were diluted to 4 times with 1 ml of 10 mM d-glucose, 15% trichloroacetic acid, then harvested by centrifugation (15,800 × g for 10 min at 4 °C), washed five times with 5 ml of 10 mM d-glucose, 15% trichloroacetic acid, then resuspended in 1M NaOH and subjected to scintillation spectroscopy.
TABLE 1

Pseudophosphorylation mutants examined in this work

| Site | Probea | AD stageb | Tauopathyb |
|------|---------|-----------|------------|
| Ser209, Ser202, Thr205 | AT8 | Late | All |
| Thr212, Ser214 | AT100 | Late | All |
| Thr231, Ser235 | AT180; TG3 | Early | Late |
| Ser262 | 12E5 | Early | Most |
| Ser266, Ser404 | AD2; PHF1 | Late | All |
| Ser409 | AP422 | Mid | All |

a Site-selective immunological probes of phosphorylation occupancy (AT8, AT100, etc.) are reviewed in Ref. 1.

b Refs. 14 and 15.

nohistochemistry and shown to be occupied in nearly all tauopathies investigated to date (1). Moreover, the temporal correlation between their occupancy and neuritic pathogenesis has been quantified (14, 15). Therefore 11 mutant proteins containing selected single, double, and triple pseudophosphorylation mutations in 12 positions were prepared along with wild-type htau40 and characterized for fibrillization activity.

To determine whether low-stoichiometry site-specific pseudophosphorylation could replicate the effects of nonspecific high-stoichiometry phosphorylation, the ability of each of the mutant constructs to fibrillize under standard, near-physiological conditions of pH, ionic strength, and bulk tau concentration (4 μM) was determined relative to wild-type recombinant htau40 in the presence of AA inducer. All preparations fibrillized with twisted morphology as described previously (41), with no morphological differences apparent among samples except mutant 235, which formed a mixture of typical untwisted filaments and small (<50 nm in length) amorphous aggregates (data not shown). Despite similarities in morphology, significant differences in extents of fibrillization were apparent when reaction products were assayed by quantitative EM. Greatest differences were observed with mutants 212, 214, 212/214, and 199/202/205, which accumulated 2–3-fold as much filamentous tau as did wild-type htau40 and mutants 231, 262, 396/404, 409, and 422 (Fig. 2A).

To confirm these findings, the amount of filamentous pseudophosphorylated tau at equilibrium relative to wild-type htau40 was estimated using a second inducer (C18H37SO4Na). Results showed that pseudophosphorylation mutants differentially fibrillized in response to C18H37SO4Na inducer as well. Indeed, when the equilibrium levels of fibrillization attained with AA and C18H37SO4Na inducers were compared, a linear relationship was found with nearly identical rank orders of mutant fibrillation levels (Fig. 2B). When equilibrium levels of fibrillation attained with C18H37SO4Na were compared by EM and corrected LLS (a solution-based assay method), again a consistent rank order of fibrillation levels was observed (Fig. 2C). The only exception was mutant 235, which formed both filamentous and nonfilamentous aggregates. Because the EM assay only measures filaments, whereas LLS detects all aggregates in solution, the LLS assay overestimated filament mass for this mutant. Together these results suggested that low-stoichiometry pseudophosphorylation could recapitulate the effects of nonspecific phosphorylation on tau fibrillization levels at equilibrium, and that they were additive with the action of anionic inducers.

The equilibrium phase of tau fibrillization is attained when the rate of protein addition to filament ends equals the rate of protomer dissociation. It is characterized by a “critical” concentration corresponding to the maximal solubility of protein above which all additional assembly competent protein enters the polymeric phase (52). It is the highest concentration of protein that does not support fibrillization, and therefore can be estimated from the dependence of fibrillization on bulk protein concentration (53). To determine whether pseudophosphorylation modulated this equilibrium, the critical concentration of each mutant was estimated using corrected LLS and compared with values for wild-type htau40. The value for wild-type htau40 in the presence of the C18H37SO4Na inducer was 2.00 ± 0.07 μM (shown in Fig. 3 and summarized in Table II).

Critical concentrations for pseudophosphorylation mutants ranged from this value to as low as 680 ± 130 nM for mutant 212 (Fig. 3; Table II). The relationship between critical concentration and equilibrium levels of fibrillization (both assayed with C18H37SO4Na inducer) was linear, with lowest critical concentrations yielding the greatest amounts of fibrillation at equilibrium (Fig. 4). These data suggest that the increased fibrillization at equilibrium produced by pseudophosphorylation resulted from increased affinity of filament ends for assembly competent tau.

Anionic surfactants trigger protein fibrillization in micellar form by shifting prenuclear equilibria from assembly incompetent to assembly competent conformations (43), which then serve as substrates for nucleation reactions on the micelle surface (41, 51). The enhanced nucleation rate shifts sigmoidal reaction progress curves toward shorter lag times with increasing inducer concentrations (43, 54). Because the increase in filament mass induced by high-stoichiometry phosphorylation has been attributed to creation of “nucleation centers” (55), a similar shift in reaction progress curves should accompany increased fibrillization. To test this hypothesis using pseudophosphorylation mutants, the total length of tau filaments formed from each mutant as a function of time in response to anionic surfactant inducer C18H37SO4Na was quantified using the EM assay. All resultant reaction progress curves were sigmoidal with clear lag, exponential growth, and equilibrium phases (Fig. 5). Kinetic parameters of lag time (a measure of nucleation rate; Ref. 50) and kapp (the proportional growth rate) were derived from fitting these data to a 3-parameter Gompertz growth function as described under “Experimental Procedures.” The Gompertz function was used because it fit time course data better than the 3- or 4-parameter logistic functions we have used in the past (51, 54). Under these conditions, wild-type htau40 fibrillized with a lag time of 42.8 ± 0.8 min and a kapp, of 1.33 ± 0.07 h⁻¹, whereas pseudophosphorylation mutants fibrillized with lag times ranging from 38.9 to 61.6 min and kapp values ranging from 1.00 to 1.40 h⁻¹ (Table II). The nucleation rate as reflected by lag time was directly proportional to fibrillization at equilibrium, with longest lag times correlating with the greatest fibrillization at equilibrium (Fig. 6A). In contrast, no correlation between kapp (which incorporates nucleation, elongation, and equilibrium terms) with fibrillization at equilibrium was apparent (Fig. 6B). These data suggest that pseudophosphorylation had statistically significant effects on tau fibrillation kinetics, but did not increase filament nucleation rates.

Despite having a critical concentration (~2 μM) low enough to support fibrillation under assay conditions (4 μM), the wild-type htau40 preparation used here does not assemble spontaneously (i.e. in the absence of exogenous inducer) over time courses as long as 7 days at 37 °C in the absence of nucleation inducers (38, 39). To determine whether the assembly enhancing effect of pseudophosphorylation could promote spontaneous fibrillation, mutant 212 also was incubated (4 μM) for 7 days at 37 °C under assembly conditions. As with wild-type htau40, no spontaneous fibrillization was apparent over this period (data not shown). These data suggest that simply having a lower critical concentration for assembly competent conformations is insufficient to promote spontaneous
fibrillization at physiological tau concentrations, and that low stoichiometry pseudophosphorylation alone cannot overcome the energy barrier for nucleation over the time periods studied. The nucleation and elongation phases of protein aggregation reactions compete for assembly competent species, resulting in their time-dependent depletion until the critical concentration is reached (56). In the case of tau aggregation, the exponential distribution of tau filament lengths observed at all time points indicates that the rate of elongation exceeds that of nucleation, with both occurring throughout the reaction time course (41, 57). Under these conditions, lower critical concentrations could lead to decreased nucleation rates at the latter stages of the time course by consuming assembly competent tau. The resultant increase in filament elongation relative to nucleation should be detectable as shifts in filament length distributions toward longer lengths. For mutants with low critical concentrations, such as 212, the change in distribution was apparent on visual inspection of electron micrographs (Fig. 7). To test the normalized fibrillization lengths/field achieved in the presence of C_{18}H_{37}SO_{4}Na (Γ_f C_{18}H_{37}SO_{4}Na) relative to AA (Γ_f AA) inducer, whereas the line represents linear regression analysis of the data points (symbol key is located in panel C). The data show that the effect of mutations on tau fibrillization could be detected in the presence of both fatty acid and alkyl sulfate inducers.

FIG. 3. Pseudophosphorylation modulates the critical concentration for assembly. The fibrillization of wild-type htau40 and pseudophosphorylation mutants incubated (room temperature for 26 h) with C_{18}H_{37}SO_{4}Na inducer (50 μM) in assembly buffer was assayed as a function of tau concentration (1–4.5 μM) by static LLS. Each data point represents the net intensity of scattered light corresponding to fibrillation, I_f (90°), at a specific tau concentration, whereas each solid line represents linear regression analysis of the data. The critical concentration of assembly, which was estimated from the abscissa intercept of each regression line (summarized in Table II), was modulated by site-specific pseudophosphorylation. Although measured in quadruplicate, error bars on data points have been omitted for clarity.

FIG. 2. Site-specific modulation of tau fibrillization equilibria by pseudophosphorylation. A, wild-type and mutant htau40 preparations (4 μM) were incubated (4 h at room temperature) in assembly buffer containing 75 μM AA and then examined by transmission EM. The total lengths/field of all filaments ≥50 nm in length adsorbed onto grids (Γ_f) were measured from the resultant digitized images. All data are presented as percentages of normalized values ± S.D. (n = 3), with wild-type control htau40 defined as 100%. Significance at p < 0.05 (*) and p < 0.01 (**) was estimated by t test. Significant differences in filament mass were detectable within 4 h in the presence of AA inducer. B, fibrillation assays were performed as described above except that 50 μM C_{18}H_{37}SO_{4}Na served as the aggregation inducer as described above except that fibrillation was quantified after 26 h by the static light scattering assay. Each point represents the net intensity of scattered light corresponding to fibrillation, I_f (90°), normalized to wild-type tau relative to total filament lengths/field determined in panel B, whereas the solid line represents linear regression of the data points (excluding mutant 235, which produced amorphous aggregates as well as filaments). These data show that the effects of pseudophosphorylation on fibrillization equilibria were consistent by two distinct assay methods.
presence of up to 2 mM glucose in the assay had no significant effect on fibrillization levels at equilibrium (Fig. 9). After 7 days incubation at 37 °C with 10 mM glucose, however, statistically significant increases in fibrillization at equilibrium appeared (Fig. 9). On the basis of parallel reactions containing 14C-labeled glucose, these conditions led to the incorporation of ~1 mol/mol radiolabel. Desalting of the preparation prior to assay did not significantly diminish the stimulatory effect, confirming that the modification was stable and that carryover of unincorporated glucose was not a contributory factor (Fig. 9).

To determine the mechanism underlying these effects, the full reaction progress curves for both glycated- and non-glycated C291A/C322A were estimated using the corrected LLS assay and C18H37SO4Na as a second fibrillization inducer. All glycated samples were desalted prior to assay in these studies. Double mutant C291A/C322A was used for these experiments because it lacked Cys residues that could potentially react under glycation conditions and complicate tau aggregation kinetics (58).

Although pseudophosphorylation had only minor effects on fibrillization kinetics, the in-vitro kinetic and thermodynamic parameters of pseudophosphorylation mutants (Table II) and estimated using the corrected LLS assay and C18H37SO4Na as a second fibrillization inducer. All glycated samples were desalted prior to assay in these studies. Double mutant C291A/C322A was used for these experiments because it lacked Cys residues that could potentially react under glycation conditions and complicate tau aggregation kinetics (58).

On the basis of EM and fitted to a three-parameter Gompertz growth function as described under "Experimental Procedures" (shown in Fig. 5). Parameters are expressed as mean ± S.E. Determined by static laser-light scattering in the presence of 2–4.5 mM tau and 50 μM C18H37SO4Na (shown in Fig. 3) and expressed as mean ± S.D. Tau Glycation Promotes Tau Filament Formation—The effects of glycation on tau assembly were examined by incubating tau with d-glucose for up to 7 days (31), after which time samples were analyzed by EM- or LLS-based assays. Double mutant C291A/C322A was used for these experiments because it lacked Cys residues that could potentially react under glycating conditions and complicate tau aggregation kinetics (58).

The prediction, equilibrium length distributions were estimated after fibrillization of all tau constructs in the presence of AA, with the polydispersity of each population quantified by the slope parameter (b). Results (Fig. 8) confirmed that the increases in filament mass at equilibrium induced by pseudophosphorylation correlated with decreases in the slope parameter (i.e. shifts to longer length distributions). Together these data suggest that the principal effect of pseudophosphorylation on tau fibrillization is to reduce critical concentration, which leads to a shift in the length distribution to longer lengths, and a more stable filament population at low bulk tau concentrations.

 Tau Glycation Promotes Tau Filament Formation—The effects of glycation on tau assembly were examined by incubating tau with d-glucose for up to 7 days (31), after which time samples were analyzed by EM- or LLS-based assays. Double mutant C291A/C322A was used for these experiments because it lacked Cys residues that could potentially react under glycating conditions and complicate tau aggregation kinetics (58).

On the basis of EM assays using 75 μM AA as inducer, the presence of up to 2 mM glucose in the assay had no significant effect on fibrillization levels at equilibrium (Fig. 9). After 7 days incubation at 37 °C with 10 mM glucose, however, statistically significant increases in fibrillization at equilibrium appeared (Fig. 9). On the basis of parallel reactions containing 14C-labeled glucose, these conditions led to the incorporation of ~1 mol/mol radiolabel. Desalting of the preparation prior to assay did not significantly diminish the stimulatory effect, confirming that the modification was stable and that carryover of unincorporated glucose was not a contributory factor (Fig. 9).

To determine the mechanism underlying these effects, the full reaction progress curves for both glycated- and non-glycated C291A/C322A were estimated using the corrected LLS assay and C18H37SO4Na as a second fibrillization inducer. All glycated samples were desalted prior to assay in these studies. Double mutant C291A/C322A incubated at 37 °C for 7 days in the absence of glucose assembled with similar
kinetics as the non-glycated sample (lag time = 30.9 ± 2.6 min; $k_{\text{app}} = 1.86 ± 0.09$ h$^{-1}$), the amount of fibrillar tau at equilibrium was 1.8 ± 0.1-fold greater (Fig. 10A). Filament accumulation was accompanied by a shift in length distribution to longer lengths as indicated by a decrease in the slope parameter to 76 ± 14% of control values (data not shown). To clarify the source of increased filament mass at equilibrium, critical concentrations of both glycated and non-glycated samples were determined by LLS and found to be 1.16 ± 0.09 and 1.86 ± 0.14 M, respectively (Fig. 10B). These characteristics resemble the effects of pseudophosphorylation on tau assembly, and indicate that low-stoichiometry glycation enhances fibrillization by lowering critical concentration and stabilizing filaments.

**DISCUSSION**

**Phosphorylation and the Tau Aggregation Pathway**—Site-specific phosphorylation is an early and ongoing event in neurofibrillary lesion formation, suggesting a potential role in the process (14, 15, 59). Although phosphorylation could potentially modulate multiple aspects of tau biology (e.g., turnover, localization, etc.), it has been hypothesized that at least one major function in disease is to directly promote the fibrillization reaction (1). One established mechanism through which it may do so is the neutralization of the microtubule-binding activity of tau, thereby fostering an accumulation of freely soluble tau in the cytoplasm (16, 19). This reaction, which may represent the first step in the fibrillization pathway, has been replicated both in situ and in vitro using various phosphotransferases and also with pseudophosphorylation mutations (16–19, 23–26). But whether it represents a commitment toward fibrillization is not clear. In vivo phosphorylation of fetal tau at similar sites (12, 13) and nearly the same stoichiometry (60) as PHF-tau does not trigger fibrillization. In situ, tau hyperphosphorylation leads to formation of thioflavin S-reactive tau species with primarily reticular macroscopic morphology (61), consistent with the formation of thioflavin S-reactive intermediates but only modest amounts of filaments (51).

In contrast to these findings, experimentation in vitro suggests that nonspecific, high stoichiometry phosphorylation can directly promote tau aggregation, leading to increased amounts of amorphous and fibrillar aggregates at equilibrium (20, 21, 55). This phenomenon has been proposed to stem from incorporation of negative charge, which may neutralize the net positive charge of native tau protein and lower the energy barrier for self-association (62, 63). Thus the incorporation of negative charge accompanying phosphorylation has been proposed to create nucleation centers that directly trigger tau fibrillization (55).

Although the pseudophosphorylation mutants studied here recapitulated the effects of hyperphosphorylation with respect to increased filament mass at equilibrium, neither spontaneous nucleation nor increased nucleation rate in the presence of
small molecules carried over into the assay. Significance at a stable modification of tau protein and not from free glucose or other gel filtration prior to dilution and assay confirming that it resulted from nearly 2-fold increase in the amount of filamentous material at equilibrium.

The fibrillization of htau40C291A/C322A (4 μM) were incubated (4 h at room temperature) in assembly buffer containing 75 μM AA and then examined by transmission EM. The total lengths/field of all filaments ≥50 nm in length adsorbed onto grids (Γf) were measured from the resultant digitized images. Distributions of lengths that segregated into consecutive intervals (50-nm bins) were also calculated and quantified by a slope parameter as described under “Experimental Procedures.” All data points are presented as percentages of normalized values, with wild-type control htau40 defined as 100% on both axes. The solidline represents the best fit of the data points to an exponential function. Increases in filament mass at equilibrium produced by pseudophosphorylation were accompanied by decreases in slope.

Increasing concentrations of anionic inducers lead to decreases in slope, i.e., creating a supersaturated solution is insufficient to trigger fibrillation over incubation periods spanning 1 week. Moreover, supersaturated solutions of soluble recombinant tau protein do not efficiently polymerize onto exogenous filamentous seeds in the absence of anionic inducers (38, 62), and only small amounts of filaments form in situ even when tau is expressed at supraphysiological levels (67). These data suggest that while recombinant tau is natively unfolded (68), the equilibrium conformation is not necessarily assembly competent. Thus, despite being energetically favorable, filament nucleation proceeds slowly if at all until assembly competent conformations are attained. Under these conditions, protein turnover rates may well exceed tau nucleation rates, and the increased cytosolic levels of supersaturated tau protein engendered by phosphorylation may not lead to fibrillation.

These considerations suggest that assembly competent conformations must be populated before fibrillation can proceed efficiently. In vitro, the barriers to conformational change and nucleation can be overcome by anionic condensation agents such as anionic surfactants or polymers (40, 41, 51, 69, 70). But as shown here and previously by others (27, 38, 39, 65, 66), simply raising tau high above its critical concentration for assembly (i.e., creating a supersaturated solution) is insufficient to trigger fibrillation over incubation periods spanning 1 week. Moreover, supersaturated solutions of soluble recombinant tau protein do not efficiently polymerize onto exogenous filamentous seeds in the absence of anionic inducers (38, 62), and only small amounts of filaments form in situ even when tau is expressed at supraphysiological levels (67). These data suggest that while recombinant tau is natively unfolded (68), the equilibrium conformation is not necessarily assembly competent. Thus, despite being energetically favorable, filament nucleation proceeds slowly if at all until assembly competent conformations are attained. Under these conditions, protein turnover rates may well exceed tau nucleation rates, and the increased cytosolic levels of supersaturated tau protein engendered by phosphorylation may not lead to fibrillation.

These considerations suggest that assembly competent conformations must be populated before fibrillation can proceed efficiently. In vitro, the barriers to conformational change and nucleation can be overcome by anionic condensation agents such as anionic surfactants or polymers (40, 41, 51, 69, 70). Increasing concentrations of anionic inducers lead to decreasing lag times (i.e., increasing nucleation rates) and increasing...
quantities of filamentous tau at equilibrium without changes in critical concentration (43). Similar data has been obtained with micelle-mediated fibrillation of α-synuclein (54). The ability of micellar inducers to increase levels of filamentous tau at equilibrium appears to derive in part from changes in pre-nuclear equilibria, including the conversion of assembly incompetent to assembly competent conformations (43).

On the basis of these considerations, we propose two general classes of tau assembly modulators. Those agents or posttranslational modifications that modulate pre-nuclear equilibria by stabilizing assembly competent conformations can act to trigger fibrillation. Examples include anionic membranes (41), which because of their association of tau filaments in biopsy specimens of AD tissue (71), may be pathophysiologically relevant sources of nucleating activity. The second category includes modifications that affect postnuclear equilibria such as critical concentration. These enhance fibrillation by stabilizing filaments and increasing the driving force for nucleation without necessarily triggering it or increasing its rate. The negative charges introduced by pseudophosphorylation exemplify this second category. Because of their different underlying mechanisms, anionic inducers and pseudophosphorylation act synergistically in vitro. Similarly, multiple fibrillation triggers and enhancers may combine to promote tau fibrillation in disease.

**Molecular Mechanism**—The extent of fibrillation of tau and pseudophosphorylation mutants at equilibrium was found linearly related to the critical concentration, which is defined as (53),

\[
a_{\text{crit}} = \frac{k_{-}}{k_{+}} \tag{Eq. 3}
\]

where \(a_{\text{crit}}\) is the critical concentration, and \(k_{-}\) and \(k_{+}\) are the rate constants for protein monomer dissociation from and association with filament ends, respectively. Lowering of critical concentration implies a decrease in \(k_{-}\), an increase in \(k_{+}\), or a combination of both. Although pseudophosphorylation clearly modulates filament elongation relative to nucleation rate, we could not directly test whether elongation rates (i.e. \(k_{+}\)) change in response to pseudophosphorylation because of the inability to seed fibrillation reactions in the absence of condensing agents (38, 62).

The relationship between critical concentration and filament nucleation in nucleation-elongation reactions has been derived as (72),

\[
k_{\text{crit}} = \frac{C}{a_{\text{crit}}^{2}} \tag{Eq. 4}
\]

where \(k_{\text{crit}}\) represents the operational nucleation rate from \(n\) monomers (i.e. \(n = \text{nucleus size}\)) and \(C\) is a constant. Thus, decreases in critical concentration can associate with decreases in \(k_{-}\), increases in \(k_{+}\), or decreases in nucleation molecularity (\(n\)). It has been shown in other systems that these parameters can change in combination in response to decreases in critical concentration (73). In the case of tau pseudophosphorylation, however, the absence of increased nucleation rates suggests that increases in \(k_{\text{crit}}\) can be ruled out.

The relationship between critical concentration and length distribution has been derived as (72),

\[
x_{j} = C \left( \frac{a}{a_{\text{crit}}} \right)^{j} \tag{Eq. 5}
\]

where \(a\) is the concentration of free monomer and \(x_{j}\) is the concentration of polymers composed of \(j\) protomers. Thus, decreases in critical concentration are associated with lengthening of filament length distributions at equilibrium. This effect was observed for all pseudophosphorylation mutants that decreased critical concentration.

These data suggest that the kinetic and equilibrium effects of pseudophosphorylation can be rationalized from changes in critical concentration, and that in addition to destabilizing tau-tubulin interactions, a second effect of pseudophosphorylation and potentially phosphorylation is to stabilize filaments once nucleated. This mechanism could be significant at early stages of disease by supporting filament accumulation at submicromolar intracellular concentrations.

**Comparison with Previous Work**—The effects of pseudophosphorylation on tau fibrillization have been contentious with many conflicting studies. Much of this stems from differences in the methodology used to assay fibrillization. Previously we showed a strong increase in fibrillization tendency with the pseudophosphorylation mutant 396/404 (28). However, these results were based on an LLS assay that was not corrected for inducer micellization, which makes a major contribution to scattering intensity (43). Eliminating the micelle background as described here brings the corrected LLS assay into harmony with the EM assay, and reveals that the increases in fibrillization at equilibrium accompanying pseudophosphorylation at 396/404 are statistically significant but modest. Others have assayed pseudophosphorylation mutants using fluorescent reporter probes such as thioflavin S (27). But this reagent fluoresces in the presence of tau intermediates as well as filaments, and therefore is not specific for fibrillization when employed under the conditions used here (41). Similarly, centrifugation-based assays pellet both fibrillar and non-fibrillar tau aggregates, potentially masking fibrillation-specific effects. This limitation is exemplified by mutant 235, which formed both filamentous and amorphous aggregates, and therefore yielded higher signals in LLS assays compared with EM assays. Finally, results are highly dependent on tau concentration. The effects described here, which are readily apparent at near physiological levels of tau protein, would be difficult to detect at the higher tau concentrations (40 to 100 µM) used in other assembly paradigms (22, 27).

An example of these considerations in the context of the surfactant-induced tau aggregation system employed here is illustrated in Fig. 4. The relationship between critical concentration and total fibrillization at equilibrium was found to be linear with an extrapolated ordinate intercept of 2.8 ± 0.1 µM htau40. These data indicate that tau preparations with critical concentrations above this level cannot fibrillize under our standard bulk tau concentrations (4 µM), and may explain why certain tau isoforms fail to assemble in the presence of AA (39) but readily do so at high concentration (40–100 µM) in response to heparin (66). Similarly, the abscissa intercept of this plot shows that the maximum increase in fibrillization theoretically detectable at “zero” critical concentration and 4 µM bulk htau40 concentration is ~3.6-fold. Relative increases will appear smaller at higher bulk tau concentrations, illustrating the importance of working at physiological tau concentration for these studies.

Non-specific, high-stoichiometry phosphorylation of recombinant tau in the presence of crude extracts has been found to enhance aggregate formation with both amorphous and filamentous morphologies (20, 55). The requirement for high stoichiometry (i.e. ≥10 mol/mol, or ~1.5 S.D. above the mean stoichiometry observed in autopsy-derived filamentous tau) may result from non-specific incorporation of anionic charge into multiple sites, each of which may differ in the ability to enhance fibrillization (as found here). Although these data have been interpreted as evidence for the phosphorylation-dependent creation of nucleation centers, nonphosphorylated re-
combinant full-length htau40 also aggregated spontaneously in these studies (20). As discussed above, the latter result is highly unusual, and suggests the presence of exogenous aggregation inducers that are absent from most in vitro fibrillation experiments. Quantitative experiments with purified components will be required to establish whether high stoichiometry phosphorylation can also directly accelerate nucleation rate and promote spontaneous assembly.

**Sites of Pseudophosphorylation**—On the basis of immunohistochemical studies, tau phosphorylation sites fill hierarchically during the course of AD (14, 15). Early modifications predominating in the pre-tangle stage include sites 231, 235, 262, and 409. Pseudophosphorylation mutants corresponding to single residue changes at these positions did not differ significantly from wild-type htau40 with respect to assembly kinetics or final equilibrium position. Sites more fully occupied in classic intracellular NFTs, such as those detected by monoclonal antibodies AT100 (212/214) and AT8 (199/202/205), however, were associated with the largest changes in fibrillation behavior. Although both 212 and 214 single mutants greatly favored fibrillization characteristics, the effects of these closely spaced mutations were not additive in the 212/214 double mutant. Mutants corresponding to sites enriched in late-stage disease, including those identified by PHF1 (396/404) and site 422, were not substantially different from wild-type tau. Conclusions with lesion stage must be interpreted with caution, however, because staging data depends on the differential reactivity of monoclonal antibodies and is potentially biased toward sites that are stable to the proteolysis (74) and changes in tau conformation (75) that accompany lesion maturation. Thus, rather than maturation stage, the rank order of pseudophosphorylation efficacy may more closely reflect their location in the polypeptide chain, with activating mutations clustering in residues 199–214 of the proline-rich domain. This region flanks the microtubule-binding domain and is required for tau-induced tubulin nucleation in vitro (76). It also promotes interaction of tau with the microtubules (37). The ability of tau to induce microtubule nucleation is lost upon phosphorylation of this region (36). Together, these observations support the hypothesis that phosphorylation within the proline-rich domain critically affects the physiological function and pathological aggregation of tau.

**Role of Glycation**—As shown previously (5, 30, 31), and confirmed here, in vitro glycation can increase the equilibrium levels of tau fibrillation at constant bulk tau concentration. The reaction must proceed for several days to yield these differences, however. Like pseudophosphorylation mutants, glycation depressed critical concentration, indicating that filament stabilization is one mechanism underlying the phenomenon. Despite its ability to enhance tau fibrillation, glycated tau did not fibrillize spontaneously or increase the nucleation rate, again suggesting that the glycation conditions used here (i.e. yielding low-stoichiometry modification) did not promote the accumulation of assembly competent conformations.

Together these data are consistent with at least two key roles for tau modifications in promoting tau fibrillation in early stage disease. The first is to modulate tau/microtubule equilibrium to regulate intracellular concentrations of free tau. The second is to lower the critical concentration for assembly thereby stabilizing filaments and increasing the driving force for nucleation. The approach outlined here will be useful in characterizing the ability of diverse tau modifications to modulate tau aggregation kinetics.

**Acknowledgments**—We thank Drs. Aida Abraha (Chicago State University) and Lester I. Binder (Northwestern University Medical School) for providing pseudophosphorylation mutant 396/404.
Posttranslational Modification and Tau Assembly

49703

51. Chirita, C. N., and Kuret, J. (2004) Biochemistry 43, 1704–1714
52. Harper, J. D., and Lansbury, P. T., Jr. (1997) Annu. Rev. Biochem. 66, 385–407
53. Timasheff, S. N. (1981) in Protein-Protein Interactions (Frieden, C., and Nichol, L. W., eds) pp. 315–336, John Wiley and Sons, New York
54. Necula, M., Chirita, C. N., and Kuret, J. (2003) J. Biol. Chem. 278, 46674–46680
55. Alonso, A. C., Grundke-Iqbal, I., and Iqbal, K. (1996) Nat. Med. 2, 783–787
56. Fesce, R., Benfenati, F., Greengard, P., and Valtorta, F. (1992) J. Biol. Chem. 267, 11289–11299
57. Wilson, D. M., and Binder, L. I. (1995) J. Biol. Chem. 270, 24306–24314
58. Thorpe, S. R., and Baynes, J. W. (2003) Amino Acids 25, 275–281
59. Bancher, C., Brunner, C., Laemmle, M., Budka, H., Jellinger, K., Wiche, G., Seitelberger, F., Grundke-Iqbal, I., Iqbal, K., and Wisniewski, H. M. (1989) Brain Res. 477, 90–99
60. Keressey, A., and Yen, S. H. (1993) Brain Res. 629, 40–46
61. Sato, S., Tatebayashi, Y., Akagi, T., Chui, D. H., Murayama, M., Miyasaka, T., Planel, E., Tanemura, K., Sun, X., Hashikawa, T., Yoshikawa, K., Ishiguro, K., and Takashima, A. (2002) J. Biol. Chem. 277, 42060–42065
62. Friedhoff, P., von Bergen, M., Mandelkow, E. M., Davies, P., and Mandelkow, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15712–15717
63. Alonso, A., Mederlyova, A., Novak, M., Grundke-Iqbal, I., and Iqbal, K. (2004) J. Biol. Chem. 279, 34873–34881
64. Kashchiev, D. (2000) Nucleation: Basic Theory with Applications, Butterworth-Heinemann, Oxford
65. Crowther, R. A., Olsen, O. F., Smith, M. J., Jakes, R., and Goedert, M. (1994) FEBS Lett. 337, 135–138
66. Friedhoff, P., Schneider, A., Mandelkow, E. M., and Mandelkow, E. (1998) Biochemistry 37, 10223–10230
67. Ko, L. W., DeTure, M., Sahara, N., Chihab, R., and Yen, S. H. (2002) J. Mol. Neurosci. 19, 311–316
68. Schweers, O., Schonbrunn-Hanbeck, E., Marx, A., and Mandelkow, E. (1994) J. Biol. Chem. 269, 24290–24297
69. Perez, M., Valpuesta, J. M., Medina, M., Montejo de Garcia, E., and Avila, J. (1996) J. Neurochem. 67, 1183–1199
70. Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) Nature 383, 550–553
71. Gray, K. G., Paula-Barbosa, M., and Roher, A. (1987) Neuropathol. Appl. Neurobiol. 13, 91–110
72. Edelstein-Keshet, L., and Ermentrout, G. B. (1998) Bull. Math. Biol. 60, 449–475
73. Bubb, M. R., Secretary, I., Beyer, B. B., and Fosan, K. M. (2000) J. Biol. Chem. 275, 5163–5170
74. Braak, E., Braak, H., and Mandelkow, E. M. (1994) Acta Neuropathol. 87, 554–567
75. Garcia-Sierra, F., Ghoshal, N., Quinn, B., Berry, R. W., and Binder, L. I. (2003) J. Alzheimers Dis. 5, 65–77
76. Brandt, R., and Lee, G. (1993) J. Biol. Chem. 268, 3414–3419