Site-specific pseudophosphorylation modulates the rate of tau filament dissociation

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Abstract Hyperphosphorylation of tau is of fundamental importance for neurofibrillary lesion development in Alzheimer's disease, but the mechanisms through which it acts are not clear. Experiments with pseudophosphorylation mutants of full-length tau protein indicate that incorporation of negative charge into specific sites can modulate the aggregation reaction, and that this occurs by altering the critical concentration of assembly. Here, the kinetic origin of this effect was determined using quantitative electron microscopy methods and pseudophosphorylation mutant T212E in a full-length four-repeat tau background. On the basis of disaggregation rates, decreases in critical concentration resulted primarily from decreases in the dissociation rate constant. The results suggest a mechanism through which site-specific posttranslational modifications can modulate filament accumulation at low free intracellular tau concentrations.

Keywords: Amyloid; Tau; Fibrillization; Kinetics; Phosphorylation; Charge effect

1. Introduction

Alzheimer's disease (AD) and other tauopathic neurodegenerative diseases are characterized in part by the intraneuronal accumulation of filaments composed of the microtubule-associated protein tau [1]. Filament formation correlates with neurodegeneration and cognitive decline, and thus is closely linked with clinical endpoints of disease [2,3]. Because of this linkage, there is great interest in identifying key factors regulating the tau aggregation pathway. Posttranslational modifications are leading candidates for carrying out this role. In brain tissue undergoing tauopathic neurodegeneration, a portion of tau becomes hyperphosphorylated, with filamentous tau containing 3–4-fold higher stoichiometries of phosphate than tau isolated from normal brains [4,5]. Phosphorylation on certain sites decreases the affinity of tau for microtubules, fostering the accumulation of a cytosolic pool of tau potentially capable of aggregation [6,7]. Thus, phosphorylation has been postulated to be a key initiating event in the tau aggregation pathway. With respect to the aggregation reaction itself, however, the effects of phosphorylation are contentious. In vivo, phosphorylation of fetal tau at similar sites [8,9] and nearly the same stoichiometry [10] as filamentous-tau does not trigger fibrillization. In vitro, phosphorylating conditions can promote [11] or inhibit [12] tau aggregation depending on experimental conditions, sample purity, assay format, sites modified, and source of phosphorylating activity. To overcome the ambiguity associated with phosphorylation reactions, and to extend resolution to the level of individual phosphorylation sites, the incorporation of negative charge inherent in phosphorylation has been mimicked by mutation of hydroxyamino acids into negatively charged Asp or Glu residues. These imitate phosphorylation-induced changes in the structure and microtubule-binding function of tau [13–16] and react with many phosphoepitope-selective antibodies [17]. At physiological bulk tau concentrations (1–10 μM, [18]), full-length recombinant tau pseudophosphorylated at certain sites (e.g., residue 212) aggregates more efficiently than control samples, yielding greater filament mass at equilibrium, and lower critical concentrations [19].

The critical concentration reflects post-nuclear equilibria (i.e., the extension reaction, where protein molecules equilibrate with filament ends), and is a characteristic feature of nucleation–elongation reactions. It is defined as [20]

$$K_{\mathrm{crit}} = k_{-}/k_{+}, \tag{1}$$

where K_{crit} is the critical concentration, and k_{-} and k_{+} are the rate constants for protein monomer dissociation from and association with filament ends, respectively, assuming a simple single step binding model. The ability of pseudophosphorylation to lower critical concentration implies that it decreases k_{-}, reflecting filament stabilization, or increases k_{+}, reflecting a more efficient association reaction.

Here, we test this hypothesis using a pharmacological approach and length distribution measurements. Results indicate that pseudophosphorylation acts primarily to stabilize filaments by slowing rates of dissociation.

2. Materials and methods

2.1. Materials

Recombinant His-tagged wild-type htau40 and pseudophosphorylation mutant T212E were prepared as described previously [19,21]. Stock solutions of alkyl sulfate detergent sodium octadecyl sulfate

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Abbreviations: AD, Alzheimer's disease; $C_{18}H_{37}SO_4Na$, sodium octadecyl sulfate; DMSO, dimethylsulfoxide; N744, 3-(2-hydroxyethyl)-2-[2-[[3-(2-hydroxyethyl)-5-methoxy-2-benzothiazolylidene]methyl]-1-butenyl]-5-methoxybenzothiazolium; PHF, paired-helical filament; TEM, transmission electron microscopy

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($C_{18}H_{37}SO_4Na$) (Research Plus, Bayonne, NJ), 3-(2-hydroxyethyl)-2-[2-[[3-(2-hydroxyethyl)-5-methoxy-2-benzothiazolylidene]methyl]-1-butenyl]-5-methoxybenzothiazolium (N744) (Neuronautics, Evanston, IL), and ThS (Sigma, St. Louis, MO) were prepared in 1:1 H_2O/isopropanol, dimethylsulfoxide (DMSO), and water, respectively.

2.2. Tau aggregation assay

Tau preparations were incubated without agitation in assembly buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM dithiothreitol) at 37 °C for up to 24 h in the presence or absence of $C_{18}H_{37}SO_4Na$ inducer. For transmission electron microscopy (TEM) analysis, aliquots were removed, treated with 2% glutaraldehyde (final concentration), mounted on formvar/carbon-coated 300 mesh grids, and negatively stained with 2% uranyl acetate as described previously [22,23].

2.3. Thioflavin S-fluorescence assay

Tau was aggregated at 37 °C for 24 h as described above except that the reactions contained varying concentrations of ThS probe (2–40 μM) and inhibitor N744 (0–5 μM). Equilibrium fluorescence (λ_{ex} = 440 nm; λ_{em} = 495 nm) was then measured in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA) operated at sensitivity 10, high PMT using black 96-well plates sealed with transparent foil. Fluorescence data were analyzed in Hill plot format

$$\log\frac{Y}{1-Y} = -n\log[\mathrm{N744}] + \log \mathrm{IC}_{50}, \tag{2}$$

where Y is the fluorescence in the presence of N744 expressed as a fraction of the fluorescence recorded in the absence of N744 and n is the Hill coefficient. IC_{50} values were determined from interpolated values of N744 concentration at an ordinate value of zero after fitting data to a linear regression.

2.4. Tau disaggregation assay

Tau preparations (4 μM) were polymerized under standard conditions as described above for 24 h, then divided into two separate tubes. One tube received N744 at a final concentration of 4 μM, whereas the second tube received DMSO vehicle alone. Aliquots were removed from each sample after 0, 1, 3, 5, 9, and 12 h incubation and subjected to the TEM assay described above.

2.5. Analytical methods

Dissociation rate constants (k_-) were determined as described for exponential length distributions [24,25]. Briefly, disaggregation data derived from TEM length measurements were fit to an exponential decay function [26]

$$y = y_0 e^{-k_{app}t}, \tag{3}$$

where y is the filament length at time t, y_0 is filament length at time zero, and k_{app} is the pseudo-first order rate constant for the process. After solving for k_{app}, the initial velocity of disaggregation (dy/dt) was determined from the first derivative of the exponential decay function

$$dy/dt = -y_0 k_{app}. \tag{4}$$

Dissociation rate constants were extracted from initial velocities by converting length into tau protomer units (assuming 1.5 tau molecules/nm of straight filament, [27]) and then dividing by the number of filaments measured at time zero (i.e., it was assumed that dissociation proceeded from only one end of each filament). Association rates were then estimated from established critical concentration values [19] and Eq. (1) assuming a two state model (i.e., all tau was either monomeric or incorporated into filaments).

3. Results and discussion

3.1. Experimental approach

The strategy for estimating elementary rate constants associated with the extension reaction rests on five principal considerations. First is the use of purified recombinant full-length tau isoform htau40, which aggregates most efficiently of the six naturally occurring full-length isoforms under near-physiological conditions [28]. Within the htau40 background, pseudophosphorylation mutant htau40^{T212E} was chosen for analysis because it returns the largest change in critical concentration of any phosphorylation mimicry mutant studied to date [19], thereby assuring that differences in aggregation rates would be large enough to measure. Second is the use of an anionic surfactant to accelerate the aggregation reaction. In the presence of alkyl sulfate detergent $C_{18}H_{37}SO_4Na$, htau40 filaments reach equilibrium and adopt an exponential distribution of lengths well within 24 h [23]. In addition, the length distributions induced by $C_{18}H_{37}SO_4Na$ are long relative to other inducers [23], thereby simplifying and increasing the precision of counting methods. Third is the use of TEM methods to assess fibrillization time course. TEM-based measurements are linear with filament concentration over the range used here, and yield estimates of both total filament lengths and numbers that are required for kinetic analysis [29]. Fourth is the use of small-molecule inhibitor N744 to perturb the final tau aggregation equilibrium [26]. Because N744 raises the critical concentration of assembly, its addition to an equilibrium population of filaments results in immediate initiation of a time-dependent, endwise disaggregation [26] that at early times depends on k_- and the concentration of filament ends. Finally, because endwise disaggregation from exponential filament length distributions follows pseudo-first order kinetics [24], it is possible to measure apparent rate constants and relate them to elementary rate constant k_-. An estimate of association rate constant k_+ is then obtained from the measured critical concentration and Eq. (1). Because tau filaments remain associated with $C_{18}H_{37}SO_4Na$ inducer through one end over the time periods examined here [18], the analysis assumes all extension/disaggregation events occur at the remaining free filament end.

3.2. N744 interacts similarly with both mutant and wild-type tau preparations

Successful application of the strategy requires that N744 interact similarly with both mutant and wild-type tau. Therefore, the binding affinity of N744 for both wild-type htau40 and htau40^{T212E} was estimated from ThS competition assays (ThS is a fluorescent probe for β-sheet structure). Increasing concentrations of N744 inhibited the fluorescence associated with ThS binding. As found previously for inhibition of fibrillization, inhibition of ThS binding was cooperative with respect to N744 concentration for both htau40 (Hill coefficient = 1.18 ± 0.03) and htau40^{T212E} (Hill coefficient = 1.26 ± 0.31). Increasing concentrations of ThS probe produced a family of parallel lines shifted toward higher IC_{50} values (Fig. 1). The overall pattern for both htau40 and htau40^{T212E} was consistent with competitive inhibition. Similar results were obtained for initial rates (data not shown) and final equilibrium (Fig. 1). In competitive binding reactions, the relationship between inhibitor IC_{50} and its K_i is directly proportional to the concentration of probe (ThS) relative to its EC_{50} [30]. Therefore, K_i values were estimated from the abscissa intercepts of IC_{50} vs. ThS concentration replots (Fig. 2). Values for htau40^{T212E} mutant (162 ± 17 nM) and WT (156 ± 18) were not significantly different at $p < 0.01$. These data indicate that N744 interacts similarly with both mutant and wild-type tau constructs.