Role of Phosphorylation in the Conformation of τ Peptides Implicated in Alzheimer's Disease†

Norelle L. Daly,‡ Ralf Hoffmann,§ Laszlo Otvos, Jr,|| and David J. Craik*,‡

Institute for Molecular Bioscience, Centre for Drug Design and Development, University of Queensland, Brisbane, 4072 QLD, Australia, Biologisch-Medizinisches Forschungszentrum (BMFZ), Heinrich-Heine Universität, Postfach 101007, 40001 Düsseldorf, and The Wistar Institute, 3601 Spruce St, Philadephia, Pennsylvania 19104

Received March 2, 2000; Revised Manuscript Received May 1, 2000

ABSTRACT: A series of peptides corresponding to isolated regions of Tau (τ) protein have been synthesized and their conformations determined by ^{1}H NMR spectroscopy. Immunodominant peptides corresponding to τ(224−240) and a bisphosphorylated derivative in which a single Thr and a single Ser are phosphorylated at positions 231 and 235 respectively, and which are recognized by an Alzheimer's disease-specific monoclonal antibody, were the main focus of the study. The nonphosphorylated peptide adopts essentially a random coil conformation in aqueous solution, but becomes slightly more ordered into β-type structure as the hydrophobicity of the solvent is increased by adding up to 50% trifluoroethanol (TFE). Similar trends are observed for the bisphosphorylated peptide, with a somewhat stronger tendency to form an extended structure. There is tentative NMR evidence for a small population of species containing a turn at residues 229−231 in the phosphorylated peptide, and this is strongly supported by CD spectroscopy. A proposal that the selection of a bioactive conformation from a disordered solution ensemble may be an important step (in either tubulin binding or in the formation of PHF) is supported by kinetic data on Pro isomerization. A recent study showed that Thr231 phosphorylation affected the rate of prolyl isomerization and abolished tubulin binding. This binding was restored by the action of the prolyl isomerase Pin1. In the current study, we find evidence for the existence of both trans and cis forms of τ peptides in solution but no difference in the equilibrium distribution of cis−trans isomers upon phosphorylation. Increasing hydrophobicity decreases the prevalence of cis forms and increases the major trans conformation of each of the prolines present in these molecules. We also synthesized mutant peptides containing Tyr substitutions preceding the Pro residues and found that phosphorylation of Tyr appears to have an effect on the equilibrium ratio of cis−trans isomerization and decreases the cis content.

Alzheimer's disease (AD)[1] is the most common and best investigated neurodegenerative disorder associated with Tau (τ) pathology. Other tauogenic neurodegenerative disorders include Pick's disease (PiD), corticobasal degeneration (CBD), and progressive supranuclear palsy (PSP). The major histopathological abnormalities that characterize the brains of patients with AD include excess of neurofibrillary tangles (NFT), composed of paired helical filaments (PHF) (*1*, *2*). Both PHF in AD and Pick bodies in PiD are composed of hyperphosphorylated forms of the low molecular weight microtubule-associated τ protein, known as PHF-τ (*3*−*7*).

Although hyperphosphorylation of τ was recognized early as a major feature of AD, it is still one of the most controversial areas of current AD research. The weakness of the PHF-τ hypothesis, which attributes the deficiency of microtubule-binding of PHF-τ to hyperphosphorylation, is the lack of consistent evidence at the molecular level, e.g., a specific deregulated kinase or phosphatase which acts on PHF-τ. Although hyperphosphorylation has been attributed to aberrantly activated mitotic or apoptotic events (*8*, *9*) and altered kinase or phosphatase activities affected by Apolipoprotein E (ApoE), Presenilin 1, phospholipase C-γ, and Aβ peptides (*10*−*14*), attempts to identify AD-specific phosphorylation sites on τ have not yet yielded conclusive results. The most probable candidates are Ser262 and the region around Thr231 and Ser235.

Ser262, once considered as selectively phosphorylated in PHF-τ (*15*) but phosphorylation of which was later shown to be developmentally regulated (*16*), is one of the likely abnormal phosphorylation sites. Phosphorylation of Ser262 strongly inhibits the binding of τ to microtubules (*17*). This site can be phosphorylated by a number of kinases (*18*), including GSK-3 (*19*). In addition to Ser262, which is located in the first microtubule-binding domain, phosphorylation of

† D.J.C. is an Australian Research Council Senior Fellow. The institute for Molecular Bioscience is a Special Research Centre of the Australian Research Council.

* To whom correspondence should be addressed. Phone: 61-7-3365 4945. Fax: 61-7-3365 2487. E-mail: d.craik@mailbox.uq.edu.au.

‡ Institute for Molecular Bioscience.

§ Biologisch-Medizinisches Forschungszentrum.

|| The Wistar Institute.

[1] Abbreviations: AD, Alzheimer's disease; PiD, Pick's disease; CBD, corticobasal degeneration; CD, circular dichroism; PHF, paired helical filaments; ApoE, Apolipoprotein E; NOE, nuclear Overhauser enhancement; DSS, 4,4-dimethyl-4-silapentane-1-sulfonate; TOCSY, total correlated spectroscopy; DQF-COSY, double quantum filtered correlated spectroscopy; NOESY, NOE spectroscopy; TFE, trifluoroethanol.

10.1021/bi0004807 CCC: $19.00
Published on Web 07/06/2000

Table 1: Synthetic τ Peptides[a]

peptide	224					229						234					239
τ(224−240)	K	K	V	A	V	V	R	T	P	P	K	S	P	S	S	A	K
[Thr^{P231},Ser^{P235}] τ(224−240)	-	-	-	-	-	-	-	T^{P}	-	-	-	S^{P}	-	-	-	-	-
[Tyr^{231}] τ(224−240)	-	-	-	-	-	-	-	Y	-	-	-	-	-	-	-	-	-
[Tyr^{P231}] τ(224−240)	-	-	-	-	-	-	-	Y^{P}	-	-	-	-	-	-	-	-	-
[Tyr^{235}] τ(224−240)	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	-
[Tyr^{P235}] τ($^{224-240}$)	-	-	-	-	-	-	-	-	-	-	-	Y^{P}	-	-	-	-	-

[a] The native sequence is shown at the top of the table. In the subsequent peptides the dashes represent the residue in the native peptide and the changes are marked. The superscripted P stands for phosphorylation.

Thr231, just upstream a few residues, is also required for maximal inhibition of τ binding to microtubules (*20*). Our group was the first to identify bisphosphorylation of adjacent phosphorylation sites Thr231 and Ser235 as a specific feature of PHF-τ and a valid biochemical marker for AD diagnosis (*21*). Two recent studies confirm that this τ region may be a major functional site in τ that is regulated by kinases and phosphatases via different pathways. First, Lee and co-workers reported that τ interacts in vitro and in vivo with src-family nonreceptor tyrosine kinases (*22*). The SH3 binding PXXP motif in τ is located between prolines 233 and 236, close to phosphorylation sites Thr231 and Ser235. Phosphorylation at one or both positions may regulate τ binding to the SH3 domain. Association of τ and fyn, a brain specific isoform of the src-family, now directly links signal transduction pathways in neuronal cells to the microtubule cytoskeleton and suggests a mechanism for coupling extracellular signals to the cytoskeletal system.

Second, Lu and co-workers showed that Pin1, a prolyl isomerase that specifically binds to phosphorylated Ser/Thr-Pro motifs, alters the Pro imide bond configuration and regulates the function of mitotic phosphoproteins (*23*). They showed that Pin1 binds to the phosphorylated Thr231 region and restores the ability of PHF-τ to bind microtubules and promote microtubule assembly in vitro. The authors concluded that in AD hyperphosphorylation of proline-directed sites in τ creates more binding sites for Pin1, which in turn reduces the Pin1 action on Thr231. The hyperphosphorylated τ cannot bind microtubules, and subsequently forms PHF, affecting neuronal function.

Despite the importance of hyperphosphorylated τ, there is a lack of experimental information on the conformation of the key binding region. In the current study, we have used NMR spectroscopy to determine the solution structure of τ-(224−240) and various phosphorylated derivatives (Table 1). The combination of sequence substitutions (Tyr for Ser/Thr), phosphorylation, and variations in solution conditions allowed the role of both intrinsic and extrinsic effects on conformations to be examined.

EXPERIMENTAL PROCEDURES

Peptide Synthesis. Peptides were synthesized on a 433A (PE Biosystems, Weiterstadt, Germany) or a Milligen 9050 (Millipore, Framingham) peptide synthesizer using standard Fmoc/^{t}Bu chemistry and *O*-benzotriazol-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazol (HBTU/HOBt) (*24*). Phosphoserine and phosphothreonine were incorporated as Fmoc-Ser(PO_3HBzl)-OH and Fmoc-Thr(PO_3-HBzl)-OH (*25*). Both tyrosine-containing peptide analogues were synthesized with side-chain unprotected tyrosine (Fmoc-Tyr-OH). An aliquot was phosphorylated globally after completion of the synthesis using dibenzyl-*N*,*N*-diisopropyl-lphosphoramidite (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) (*26*). After TFA-cleavage, peptides were purified by reversed-phase (RP) HPLC and characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

NMR Spectroscopy. Samples for ^{1}H NMR measurements contained ∼1 mM peptide in either 90% H_2O/10% $^{2}H_2O$ (v/v) or various concentrations of aqueous trifluoroethanol (TFE) up to 50% TFE/40% H_2O/10% $^{2}H_2O$ (v/v). Spectra were recorded at 290 K on a Bruker ARX-500 or AVANCE 750 MHz spectrometer equipped with a shielded gradient unit. Low-temperature studies employed a temperature-controlled stream of cooled air using a Bruker BCU refrigeration unit and a B-VT 2000 control unit. 2D NMR spectra were recorded in phase-sensitive mode using time-proportional phase incrementation for quadrature detection in the F_1 dimension (*27*). The 2D experiments included DQF-COSY (*28*), TOCSY (*29*) using a MLEV-17 spin lock sequence (*30*) with a mixing time of 80 ms, and NOESY (*31*) with mixing times of 300 and 400 ms. For DQF-COSY experiments, solvent suppression was achieved using selective low-power irradiation of the water resonance during a relaxation delay of 1.8 s. Solvent suppression for NOESY and TOCSY experiments was achieved using a modified WATERGATE sequence (*32*). Spectra were routinely acquired with 4096 complex data points in F_2 and 512 increments in the F_1 dimension, with 32 scans/increment (64 for NOESY).

Spectra were processed on a Silicon Graphics Indigo workstation using XWINNMR (Bruker) software. The F_1 dimension was zero-filled to 2048 real data points, and 90° phase-shifted sine bell window functions were applied prior to Fourier transformation. Chemical shifts were referenced to DSS at 0.00 ppm.

CD Spectroscopy. CD spectra were taken on a Jasco J720 instrument at room temperature in a 0.2 mm path-length cell. Doubly distilled water and spectroscopy-grade trifluoroethanol were used as solvents. The peptide concentrations were 0.4 mg/mL (approximately 0.2 mM), determined by quantitative RP-HPLC (*33*). The accuracy of this concentration-determination for peptides is usually over 95%. Curves were smoothed by the algorithm provided by Jasco. Mean residue ellipticity ($[Q]_{MR}$) is expressed in degrees squared centimeters per decimole (°C cm^2/dmol) by using a mean residue mass of 106.

RESULTS

A series of τ peptides, corresponding to τ(224−240), its bisphosphorylated derivative, [Thr^{P231},Ser^{P235}] τ(224−240),

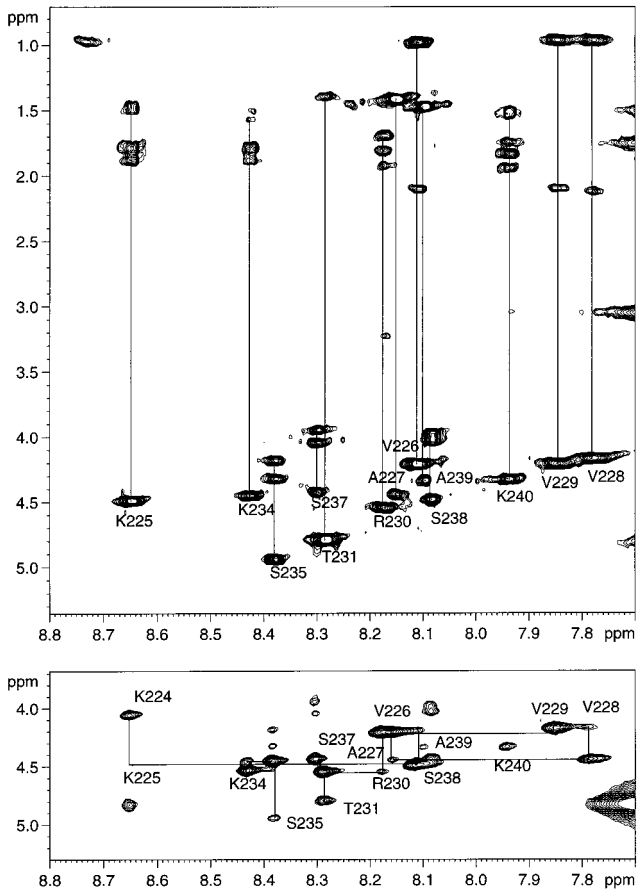

FIGURE 1: ^{1}H NMR spectra of [Thr^{P231},Ser^{P235}] τ(224−240) recorded on a Bruker 750 MHz spectrometer at 290 K. The peptide was dissolved in 50% TFE (v/v) in aqueous solution. The top panel is the fingerprint region of the TOCSY spectrum with the resonance assignments labeled and the spin systems highlighted with vertical lines. The lower panel is the αH−NH region of the NOESY spectrum recorded with a mixing time of 400 ms. The resonance assignments are labeled and the sequential connectivities are indicated.

and four peptides in which either Thr231 or Ser235 is replaced with Tyr or Tyr^P, [Tyr^{231}] τ(224−240), [Tyr^{P231}] τ(224−240), [Tyr^{235}] τ(224−240), or [Tyr^{P235}] τ(224−240) were synthesized using solid-phase methods (Table 1). The ^{1}H NMR spectra of τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240) were examined in both aqueous solution and in various concentrations of aqueous TFE. A range of different solution conditions was examined because preliminary spectra in aqueous solution suggested a degree of conformational heterogeneity, and it was of interest to determine the role of solution environment on the stabilization of individual conformers. The spectra of both peptides in 50% aqueous TFE had a significantly greater dispersion of amide signals (∼0.9 ppm) than those in aqueous solution (∼0.5 ppm) and were used in the first instance to make the resonance assignments. Spin systems were readily identified from TOCSY spectra and were sequentially assigned using NOESY spectra. For example, the sequential assignment for the bisphosphorylated peptide, [Thr^{P231},Ser^{P235}] τ(224−240), in 50% aqueous TFE is summarized in Figure 1. Chemical shift assignments were also determined in water for this peptide and for the nonphosphorylated peptide, τ(224−240), in both water and TFE, and are supplied as Supporting Information.

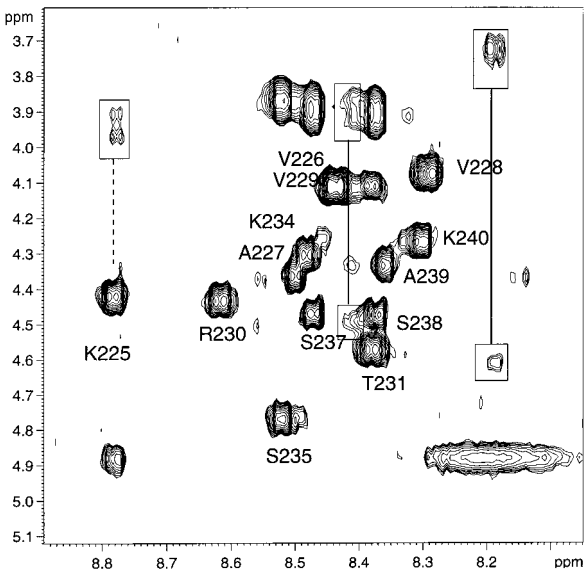

FIGURE 2: The αH−NH region of the TOCSY spectrum of τ(224−240) recorded on a Bruker 750 MHz spectrometer at 290 K in 90% H_2O/10% ^2H_2O. The resonance assignments of the major conformation are labeled and the boxed peaks represent the additional serine resonances of the minor conformation.

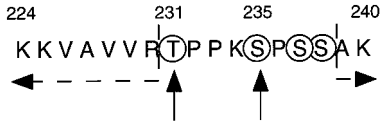

FIGURE 3: The residues which appear to be most perturbed in the minor conformation(s) of τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240) are highlighted. All doubled residues (circled) are in close proximity to one of the Pro residues (232, 233, or 236). All residues which are not doubled (enclosed in dotted arrow) are distant from Pro residues. The phosphorylation sites are arrowed (231 and 235).

The NMR spectra in water suggested the presence, in addition to the major conformer, of one or more minor conformations for both τ(224−240) and [Thr^{P231},Ser^{P235}] τ-(224−240), evidenced by the appearance of additional resonances of lower intensity than those in the major conformer. On the basis of the TOCSY patterns of these additional peaks, it appears that there are three extra sets of Ser resonances, as illustrated for the amide region of the spectrum of τ(224−240) in Figure 2. In the aliphatic region of the spectrum, there is also evidence for additional Thr resonances of lower intensity than the major conformer. Similar results were observed for [Thr^{P231},Ser^{P235}] τ(224−240). The NMR data thus suggest that the three Ser and the single Thr residue in the peptides each have an alternative conformation. The most likely cause of this second conformation is isomerization around one or more of the peptide bonds preceding the three Pro residues. Figure 3 summarizes the residues which are "doubled" and highlights the positions of the Pro residues with respect to the doubled resonances. Given that the single Thr precedes Pro232 and all three Ser residues appear to have an alternative conformation it is likely that both Pro232 and Pro236 are involved in cis−trans isomerization, although the possibility that Pro233 is also involved cannot be excluded.

In the major solution conformer, all three Pro residues are in the trans conformation, as judged by the observation of $\alpha H_i-\delta H_{i+1}$NOEs between each of the Pro residues and the sequentially preceding residue. Although many additional

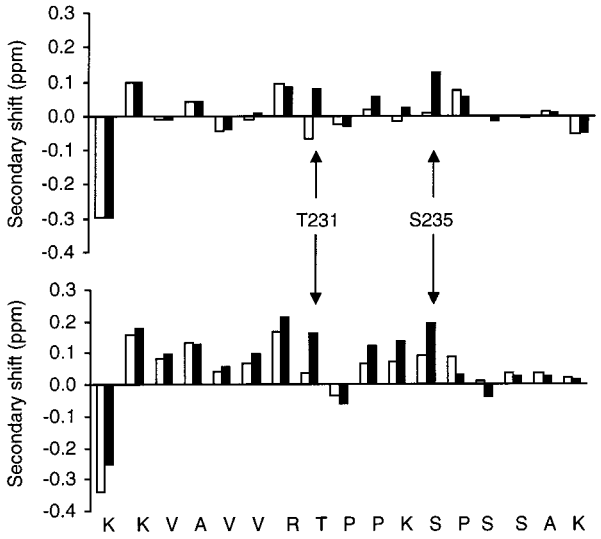

FIGURE 4: Secondary αH shifts [i.e., differences between αH shifts in τ peptides and random coil shifts (*49*)] for each residue in τ(224−240) (white) and [Thr^{P231},Ser^{P235}] τ(224−240) (black). The upper panel (a) compares the chemical shifts in H_2O and the lower panel (b) in 50% TFE. The phosphorylated residues are arrowed.

peaks were observed in the spectra corresponding to minor forms, it was not possible to unambiguously assign them all, particularly those near Pro residues. Therefore, it could not unequivocally be established that the minor isomer(s) were due to cis−Pro bonds although this seems highly likely. The proportion of these additional resonances appears to be modulated by the solution conditions and in particular by the relative concentration of added TFE. In water, the proportion of the additional peaks ranges from 3 to 9% of the major conformation for τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240). However, at increasing concentrations of TFE the intensity of these peaks decreases and they are not observed in 50% TFE. It thus appears that the major (trans) conformation is stabilized by a more hydrophobic environment, to the extent that the minor form is not detectable in 50% TFE/50% H_2O. Stabilization of the major form is also reflected by the increased amide chemical shift dispersion seen as TFE is titrated into aqueous solutions of the peptides.

We return later to a discussion of the implications of cis−trans Pro isomerization in τ peptides but turn now to a determination of the solution conformation of the major (trans) isomer. In particular, it was of interest to determine the conformation of the nonphosphorylated peptide, τ(224−240), and to see how phosphorylation and/or solution conditions changed this. αH chemical shifts provide a rapid and reliable indicator of secondary structure elements and in particular the difference between αH shifts and random coil values, referred to as secondary shifts, are diagnostic of local elements of secondary structure. A comparison of the secondary shifts of τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240) in aqueous solution is given in the top panel of Figure 4. The secondary shifts for the two peptides are very similar, with the major differences, not surprisingly, being at the residues which are phosphorylated. In general the secondary shifts are <0.1 ppm for all residues excluding Lys224 and Ser235, indicating a random coil conformation for both peptides. The slightly larger secondary shifts observed for Lys224 and Ser235 are most likely due to charge effects from the N-terminus and phosphate groups, respectively. In the lower panel, of Figure 4 the secondary shifts of τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240) in 50% aqueous TFE are presented. The secondary shifts are larger than those observed in aqueous solution and generally positive in sign for residues 225−236, suggesting that TFE stabilizes β-type structure in this region. The enhancement of β-type structure appears to be more pronounced for [Thr^{P231},Ser^{P235}] τ(224−240) compared to τ(224−240). The C-terminal region (237−240) is unaffected by the change in solution environment, showing that the secondary shift changes reflect a specific conformational effect rather than a nonspecific dielectric or other effect of TFE.

The conclusion to emerge from the chemical shift analysis is that both peptides are predominantly unstructured in aqueous solution but that addition of TFE promotes the formation of β-type structure, particularly in the bisphosphorylated peptide.

Figure 5 shows a summary of short- and medium-range NOE data for both peptides in water and in TFE. The pattern of strong αH−NH$_{i+1}$ and weak NH−NH$_{i+1}$ NOEs, together with a relative lack of medium- and long-range NOEs confirms, that both peptides exist predominantly in random coil forms in both solvents. The only significant difference between the two peptides is a single αN$_{(i,i+2)}$ NOE detected between residues Val229 and Thr231 in [Thr^{P231},Ser^{P235}] τ(224−240). This provides tentative evidence for a small population of peptide containing a turn at this point. The existence of a similar turn in τ(224−240) cannot be excluded because of overlap in this region of the spectrum of the nonphosphorylated peptide. Overall, the NOE data are consistent with the chemical shift analysis although the latter is more sensitive in identifying a slight increase in β-structure in the τ peptides in hydrophobic solution and the former in identifying a small population containing a turn between Val229 and Thr231.

CD spectroscopy verified the turn structure of the bisphosphorylated peptide. CD spectra of the two peptides were taken in water and 50% TFE. In water, the nonphosphorylated version exhibited a strong negative band at 195 nm and a small positive band around 218 nm (Figure 6), characteristic for peptides without conformational preferences (*34*). Addition of TFE reduced the intensity of the unordered band, indicating the presence of some ordered conformers, but no additional spectral changes were observed. In contrast, the bisphosphorylated peptide exhibited a transitional spectrum between types U and C in water, reflecting an equilibrium between random structures and type I (III) β-turn (*35*). This spectrum featured a small, but obvious red-shift of the negative band and the disappearance of the positive band. The presence of β-turns became beyond doubt in 50% TFE, when the peptide exhibited a classical type C spectrum. In this spectrum, the negative band was red-shifted to 200 nm and its intensity was decreased, accompanied by the appearance of a weak negative band at 222 nm. Peptides with type C CD spectra assume clean type I (III) β-turns or mixtures of type I and type II turns (*36*).

Returning to the question of cis−trans isomerization in the τ peptides, the 2D TOCSY spectra recorded in aqueous solution were volume integrated to determine the relative amounts of the two configurational isomers and the results

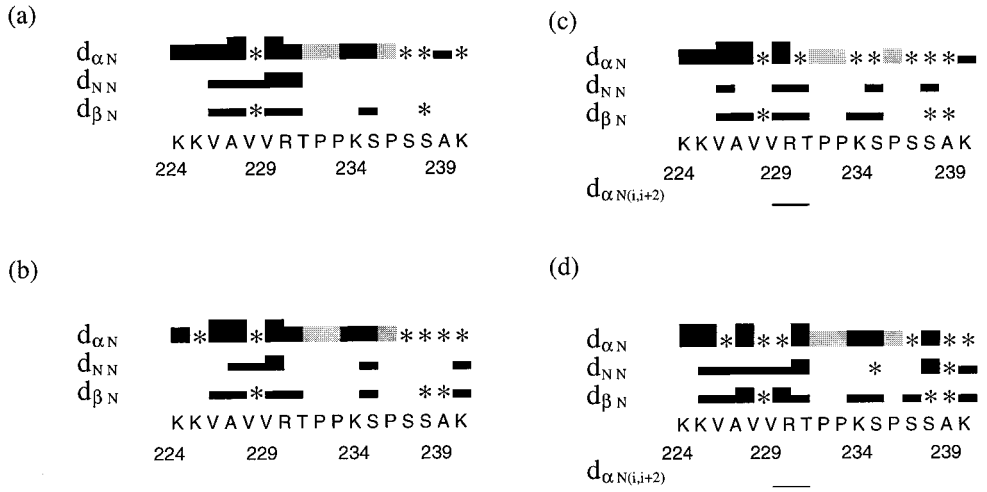

FIGURE 5: Summary of the sequential and medium-range NOE connectivities observed for τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240). The connectivities of τ(224−240) in (a) aqueous solution, and in (b) 50% aqueous TFE are indicated as are the connectivities of [Thr^{P231},Ser^{P235}] τ(224−240) in (c) aqueous solution and in (d) 50% aqueous TFE. Filled bars indicate sequential connectivities observed in 400 ms NOESY spectra. Shaded bars correspond to sequential $\alpha H_i - H\delta_{i+1}$ and $NH_i - H\delta_{i+1}$ connectivities for Pro residues. The height of the bar indicates the strength of the NOE. Overlapping NOEs are indicated by an asterisk (*).

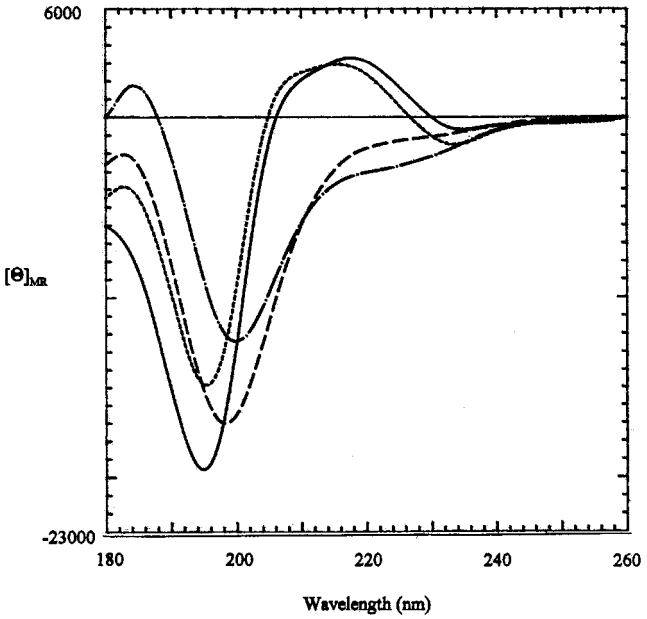

FIGURE 6: CD spectra of τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240). Spectra were recorded on a Jasco J720 spectrometer at room temperature, a peptide concentration of 0.4 mg/mL and in aqueous solution or 50% aqueous TFE. The τ(224−240) spectrum recorded in aqueous solution is shown as a solid line and as a dotted line for 50% aqueous TFE. The [Thr^{P231},Ser^{P235}] τ(224−240) spectrum recorded in aqueous solution is shown as a dashed line and as a dashed and dotted line for 50% aqueous TFE.

are shown in Table 2. The cis−trans ratio for both peptides in aqueous solution is approximately 9% at the Pro236 site and 4−5% at Pro232/233 in both phosphorylated and nonphosphorylated peptides. Thus, there is a small, but significant difference in the equilibrium ratios for the two sites; however, phosphorylation does not affect the ratio at a given site.

Because we observed cis−trans Pro isomerization in aqueous solution but not under more hydrophobic conditions and because of the putative importance of cis−trans isomerization in τ peptides (*23*), it was of interest to further explore the factors determining this equilibrium. Additional NMR data were thus recorded on the four peptides, [Tyr^{231}] τ(224−240), [Tyr^{P231}] τ(224−240), [Tyr^{235}] τ(224−240), and [Tyr^{P235}] τ(224−240), in which a tyrosine residue is substituted for Ser or Thr preceding individual Pro sites. The 2D spectra in 50% TFE/50% H_2O (data not shown) for three of these peptides clearly indicate the presence of additional Tyr resonances, the likely explanation again being cis−trans isomerization of Pro residues adjacent to the Tyr. The cis content was estimated by volume integration of the peaks and is given in Table 2. The cis content for the nonphosphorylated peptides, [Tyr^{231}] τ(224−240) and [Tyr^{235}] τ(224−240), is significantly higher than that observed for the respective phosphorylated derivatives and then that observed for τ(224−240) and [Thr^{P231},Ser^{P235}] τ(224−240). For the latter two peptides in 50% TFE/50% H_2O, no cis conformers were detected. Replacement of Ser/Thr by Tyr clearly enhances the population of cis forms, supporting the idea of strong sequence dependency of the Pro cis−trans isomerization, but indicating that this feature is not particularly dominant in AD PHF. Aromatic residues have previously been shown to have a marked propensity to precede a cis−Pro because of favorable interactions between the aromatic ring and the Pro residue (*37*). Overall, the data show that both intrinsic (sequence) and extrinsic (solution environment) effects are able to modulate cis−trans ratios.

Table 2: Percentage of cis Isomers in τ Peptides[a]

	Pro232/3		Pro236	
	H_2O	TFE	H_2O	TFE
τ(224−240)	4%	0%[b]	9%	0%
[Thr^{P231},Ser^{P235}] τ(224−240)	3%	0%	9%	0%
[Tyr^{231}] τ(224−240)	nd	20%		
[Tyr^{P231}] τ(224−240)	nd	10%		
[Tyr^{235}] τ(224−240)			nd	25%
[Tyr^{P235}] τ(224−240)			nd	10%

[a] nd, not determined. [b] No cis peptide was observed at an estimated detection threshold of 0.5%.

DISCUSSION

τ stabilizes the internal microtubular structure of neurons. In AD, τ is hyperphosphorylated and in this state is not able

to bind to microtubules (*38*−*42*). It was therefore of interest to determine whether a conformational change in the AD-specific midsequence τ domain is associated with phosphorylation of τ and if so whether this might indicate an allosteric mechanism by which binding to microtubules is inhibited. Binding of the proline isomerase Pin1 restores the ability of phosphorylated τ to bind to microtubules in vitro (*23*). This enzyme alters the rate of isomerization of the peptide bond between phosphorylated Ser or Thr and an adjacent Pro (pSer/Thr-Pro bonds) in τ (*43*). It was therefore of further interest to determine the cis−trans states of Ser/Thr-Pro bonds in τ to see how they are influenced by phosphorylation and by the solution environment.

It was clear from an analysis of NMR secondary shifts that the peptide corresponding to the region 224−240 of τ, which incorporates residues known to be phosphorylated and associated with both microtubule and Pin1 binding, adopted an essentially random coil conformation in water and that no structure is induced by phosphorylation. This suggests the possibility that binding of τ to microtubules may involve a process in which a preferred binding conformation is selected from an ensemble of disordered conformers in solution. If this is the case then the phosphate groups appear not to shift the solution equilibrium away from a preferred conformation but either must alter the kinetics associated with production of a preferred bound conformation or interact unfavorably with the tubulin binding site in that bound conformation. It should be cautioned that this interpretation assumes that the isolated τ(224−240) peptide provides a good model for the corresponding region of the intact protein. The possibility that this region adopts some more ordered structure in the presence of tertiary interactions from the remainder of the protein cannot be excluded at this stage.

The data recorded in the more hydrophobic environment provided by 50% TFE showed that part of the τ sequence undergoes structural ordering in response to solution conditions. Although small, the effect is specific to residues incorporating Ser/Thr-Pro phosphorylation sites. Residues C-terminal to this region were unchanged relative to those in water, providing an internal control which confirms the specificity of the conformational change. The increased secondary shifts are associated with a more extended (β-type) secondary structure, and the ordering effect induced by the hydrophobic solvent is slightly greater in the phosphorylated peptide. The shift from random coil to extended structure shows that τ is amenable to conformational changes in response to the solution environment, supporting the suggestion that it may undergo a conformational change on binding to tubulin. While there are insufficient data at this stage to identify the bound conformation, it appears more likely to be in an extended (β-type) rather than helical conformation. Alternatively to a structural change upon tubulin binding, in a hydrophobic environment phosphorylated τ may undergo a self-association process, via β-pleated sheet formation. It has been a puzzle for some time as to how τ, an elastic protein (*44*), becomes aggregated to β-sheets when assembled to PHF (*45*), but the data reported here on a susceptibility to conformational changes with hydrophobicity provide some clues. In a cellular environment, increased hydrophobicity may result from a number of factors, including increased concentration of proteins, for example apolipoproteins.

The NMR and CD data from the current study also identified a β-turn-forming ability of the bisphosphorylated τ(224−240) peptide around phosphorylated Thr231 when such turns were absent from the spectra of the nonphosphorylated variant. The turn-forming potential was especially visible in TFE, and this finding corroborates earlier immunological observations. The 224−240 region of τ is an immunodominant domain and carries the recognition site of two of the three currently known AD PHF-specific monoclonal antibodies, PHF-27 and TG-3. TG-3 recognizes this peptide fragment phosphorylated on Thr231 (*46*), and PHF-27 also recognizes the Thr231-monophosphorylated peptide to some degree (*21*). The antigen recognition of both monoclonal antibodies is markedly increased when the Thr231-monophosphorylated peptide is plated down to the ELISA assay from TFE (*21*, *46*), indicating that these PHF-specific antibodies not only recognize phosphorylated Thr231, but also the conformation around this amino acid residue. This is especially interesting because some additional immunological evidence suggests that the conformationally sensitive antibodies ALZ-50 and MC-1 recognize an intramolecular association between the extreme N-terminus and the third microtubule-binding repeat domain of τ (*47*), and the bend of τ was suggested to occur in the vicinity of phosphorylated Thr231 (*46*).

The NMR data recorded in aqueous solution clearly show the presence of extra conformations in τ associated with cis−trans isomerization about at least two of the three Pro residues. The Ser235−Pro236 bond is present mainly in the trans configuration, but is cis in approximately 9% of the solution conformers. Similarly, the Thr231−Pro232 (or Pro232−Pro233) bond has a majority trans configuration but is cis for approximately 5% of conformers. The current data suggest that the recently reported selectivity of Pin1 for the pThr−Pro232 motif compared to pSer−Pro236 (*23*) does not appear to be related to the intrinsic equilibrium position of cis−trans isomers for the two sites. While the probability of a cis peptide bond at the latter site is about twice that of the former, the binding selectivity for the pThr−Pro site is 10-fold that of any other site in the molecule. The cis−trans ratios are clearly affected by the solution environment, with decreasing stability of the cis forms being associated with increasing hydrophobicity of the solution.

The possible implications of the cis−trans conformational interchange on τ binding interactions are summarized in Figure 7. The upper left of the figure represents an ensemble of random coil conformers, most of which are trans but with a minority of cis forms. If the tubulin binding form is selected from the cis subpopulation, then any decrease in the rate of cis−trans equilibration, as is the case for the phosphorylated derivatives, could decrease the availability of bound forms. Studies of model peptides have clearly established a decreased rate of cis−trans interconversion on phosphorylation and decreased tubulin binding on phosphorylation. Addition of Pin1 enhances the rate of cis−trans isomerization and restores tubulin binding (bottom right of figure). This model also explains increased PHF formation on phosphorylation. With a decreased rate of tubulin binding, the free phosphorylated peptide becomes more available for PHF formation, as indicated at the lower left of the figure. In AD, hyperphosphorylation leads to increased PHF, increased sequester-

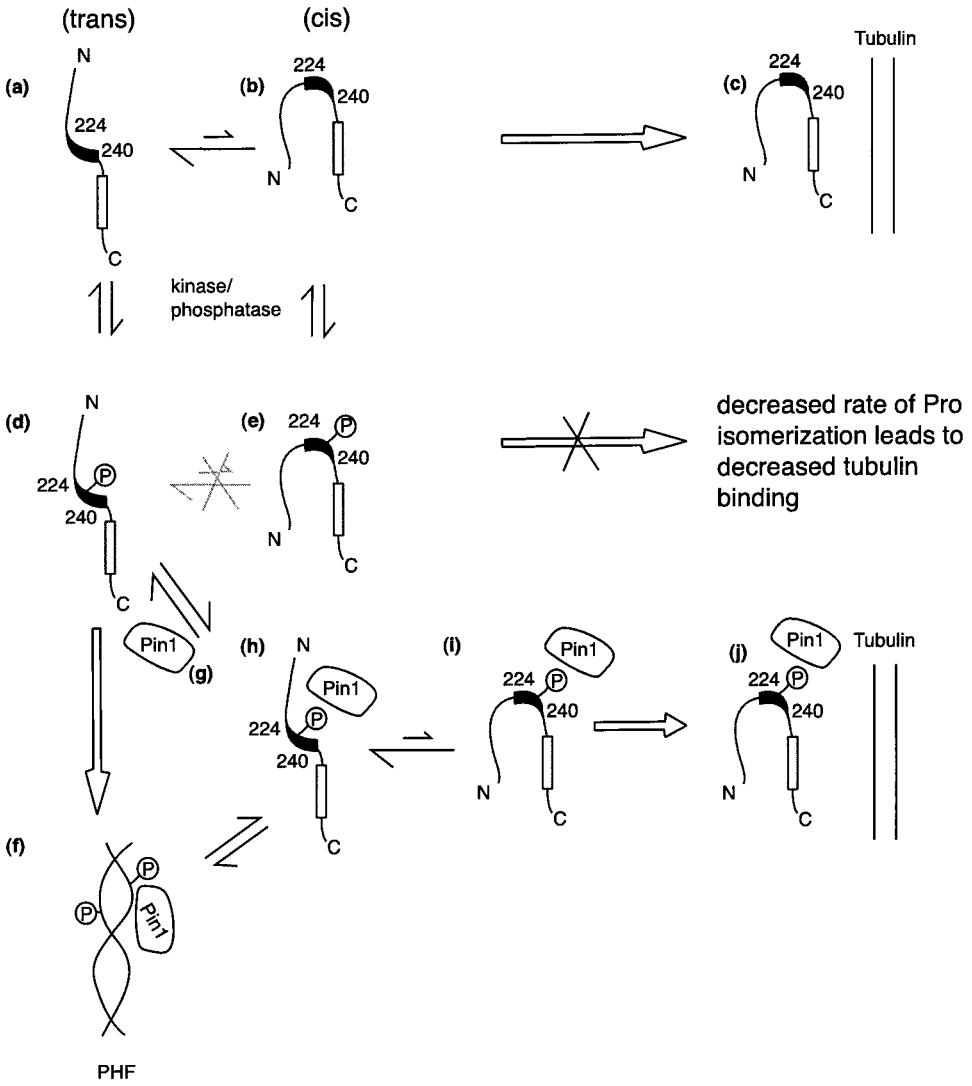

FIGURE 7: Schematic representation of possible conformational and binding interactions of τ. The τ protein is drawn with the residues 224−240 highlighted and the tubulin binding domain as a boxed region. In solution τ(224−240) exists as a random coil ensemble, some conformers of which contain trans Pro [(a) represented as an extended conformation, >90%] and others cis Pro [(b) represented as a folded conformation, <10%]. A very small fraction of conformers, perhaps accessible via cis Pro intermediates, adopts a conformation suitable for binding to tubulin (c). On phosphorylation (d), the cis−trans equilibrium is slowed in rate (but not extent) leading to a reduced rate of supply of scheme e. The conformational rearrangements necessary for selection of a binding conformer are thus slowed sufficiently that binding to tubulin is reduced. The free phosphorylated peptide (d) thus becomes more available for PHF formation (f). Free Pin1 (g) is able to bind either to phosphorylated PHF (f) or phosphorylated soluble τ (h). In the latter case, it speeds up prolyl isomerization to the cis form (i), thereby leading to enhanced selection of binding conformations (j), explaining how Pin1 can reverse the lack of binding seen for phosphorylated τ. In AD hyperphosphorylation leads to increased PHF (f), increased sequestering of free Pin1 and hence reduced soluble Pin1 for facilitating prolyl isomerization (h to i), also resulting in reduced tubulin binding by τ (j). For clarity, the figure does not show some of the other factors influencing this complex interplay of conformations and binding interactions. For example, the cis−trans ratios of τ(224−240) are affected by the degree of hydrophobicity and under hydrophobic conditions phosphorylation produces some ordering of the conformational ensemble and induces turn formation.

ing of free Pin1, and hence reduced soluble Pin1 for facilitating prolyl isomerization, also resulting in reduced tubulin binding by τ.

We also examined a number of mutant peptides in which a tyrosine precedes the isomerizing proline residues and found that there is a larger cis content relative to the wild type (Ser/Thr mutants). These mutant peptides were examined in 50% aqueous TFE and were still found to contain at least one minor conformation, most likely from cis−trans isomerization. These results are consistent with a previous study by Schutkowski et al. (*48*) which examined a series of peptides that contained phosphorylated or nonphosphorylated Ser/Thr/Tyr-Pro bonds and showed ~30% cis content for Tyr-Pro containing peptides and generally much lower values for Ser/Thr-Pro containing peptides. However, in the present study, phosphorylation appears to have an effect on the equilibrium ratio of cis−trans isomerization in these Tyr derivatives and decreases the cis content. This is in contrast to the previous study which found that phosphorylated Tyr did not affect prolyl isomerization (*48*). Apparently this process is highly sequence dependent. In this respect, the extent, as well as the kinetics of cis−trans Pro isomerization, may play a role in the development of AD, although further work is necessary to define it.

ACKNOWLEDGMENT

We thank Carsten Boenke (BMFZ) for synthesis of the tyrosine-containing peptides.

SUPPORTING INFORMATION AVAILABLE

Chemical shifts for nonphosphorylated and phosphorylated peptides in water and TFE. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Lee, V. M., and Trojanowski, J. Q. (1992) *Curr. Opin. Neurobiol. 2*, 653−656.
2. Kosik, K. S. (1992) *Science 256*, 780−783.
3. Selkoe, D. J. (1993) *Trends Neurosci. 16*, 403−409.
4. Trojanowski, J. Q., Schmidt, M. L., Shin, R.-W., Bramblett, G. T., Goedert, M., and Lee, V. M.-Y. (1994) *Clin. Neurosci. 1*, 184−191.
5. Braak, H., and Braak, E. (1998) *Exp. Neurol. 153*, 351−365.
6. Delacourte, A., Sergeant, N., Wattez, A., Gauvreau, D., and Robitaille, Y. (1998) *Ann. Neurol. 43*, 193−204.
7. Mailliot, C., Sergeant, N., Bussiere, T., Caillet-Boudin, M. L., Delacourte, A., and Buee, L. (1998) *FEBS Lett. 433*, 201−204.
8. Nuydens, R., Dispersyn, G., de Jong, M., van den Kieboom, G., Borgers, M., and Geerts, H. (1997) *Biochem. Biophys. Res. Commun. 240*, 687−691.
9. Vincent, I., Jicha, G., Rosado, M., and Dickson, D. W. (1997) *J. Neurosci. 17*, 3588−3598.
10. Takashima, A., Murayama, M., Murayama, O., Kohno, T., Honda, T., Yasutake, K., Nihonmatsu, N., Mercken, M., Yamaguchi, H., Sugihara, S., and Wolozin, B. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9637−9641.
11. Wang, X., Luebbe, P., Gruenstein, E., and Zemlan, F. (1998) *J. Neurosci. Res. 51*, 658−665.
12. Shea, T. B., Dergay, A. N., and Ekinci, F. J. (1998) *Neurosci. Res. Commun. 22*, 45−48.
13. Takashima, A., Honda, T., Yasutake, K., Michel, G., Murayama, O., Murayama, M., Ishiguro, K., and Yamaguchi, H. (1998) *Neurosci. Res. 31*, 317−323.
14. Jenkins, S. M., and Johnson, G. V. (1998) *Neuroreport 9*, 67−71.
15. Watanabe, A., Hasegawa, M., Suzuki, M., Takio, K., Morishima-Kawashima, M., Titani, K., Arai, T., Kosik, K. S., and Ihara, Y. (1993) *J. Biol. Chem. 268*, 25712−25717.
16. Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G. V., Litersky, J. M., Schenk, D., Lieberburg, I., and Trojanowski, J. Q., et al. (1995) *J. Biol. Chem. 270*, 18917−18922.
17. Biernat, J., Gustke, N., Drewes, G., Mandelkow, E. M., and Mandelkow, E. (1993) *Neuron 11*, 153−63.
18. Litersky, J. M., Johnson, G. V., Jakes, R., Goedert, M., Lee, M., and Seubert, P. (1996) *Biochem. J. 316*, 655−660.
19. Wang, J. Z., Wu, Q., Smith, A., Grundke-Iqbal, I., and Iqbal, K. (1998) *FEBS Lett. 436*, 28−34.
20. Sengupta, A., Kabat, J., Novak, M., Wu, Q., Grundke-Iqbal, I., and Iqbal, K. (1998) *Arch. Biochem. Biophys. 357*, 299−309.
21. Hoffmann, R., Lee, V. M. Y., Leight, S., Varga, I., and Otvos, L., Jr. (1997) *Biochemistry 36*, 8114−8124.
22. Lee, G., Newman, S. T., Gard, D. L., Band, H., and Panchamoorthy, G. (1998) *J. Cell. Sci. 111*, 3167−3177.
23. Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) *Nature 399*, 784−788.
24. Fields, G. B., and Noble, R. L. (1990) *Int. J. Pept. Protein Res. 35*, 161−214.
25. Wakamiya, T., Saruta, K., Yasuoka, J., and Kusumuto, S. (1994) *Chem. Lett.* 1099−1102.
26. Hoffmann, R., Wachs, W. O., Berger, R. G., Kalbitzer, H. R., Waidelich, D., Bayer, E., Wagner-Redeker, W., and Zeppezauer, M. (1995) *Int. J. Pept. Protein Res. 45*, 26−34.
27. Marion, D., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967−974.
28. Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−485.
29. Braunschweiler, L., and Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521−528.
30. Bax, A., and Davis, D. G. (1985) *J. Magn. Reson. 65*, 355−360.
31. Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546−4553.
32. Piotto, M., Saudek, V., and Sklenar, V. (1992) *J. Biomol. NMR 2*, 661−665.
33. Szendrei, G. I., Fabian, H., Mantsch, H. H., Lovas, S., Nyeki, O., Schon, I., and Otvos, L., Jr. (1994) *Eur. J. Biochem. 226*, 917−924.
34. Woody, R. W. (1985) in *The peptides* (Hruby, V. J., Ed.) pp 15−114, Academic Press, Orlando FL.
35. Smith, J. A., and Pease, L. G. (1980) *CRC Crit. Rev. Biochem. 8*, 315−399.
36. Perczel, A., Hollosi, M., Sandor, P., and Fasman, G. D. (1993) *Int. J. Pept. Protein Res. 41*, 223−236.
37. Wu, W. J., and Raleigh, D. P. (1998) *Biopolymers 45*, 381−394.
38. Lee, V. M., Balin, B. J., Otvos, L., Jr., and Trojanowski, J. Q. (1991) *Science 251*, 675−678.
39. Goedert, M., Spillantini, M. G., Cairns, N. J., and Crowther, R. A. (1992) *Neuron 8*, 159−168.
40. Greenberg, S. G., Davies, P., Schein, J. D., and Binder, L. I. (1992) *J. Biol. Chem. 267*, 564−569.
41. Bramblett, G. T., Goedert, M., Jakes, R., Merrick, S. E., Trojanowski, J. Q., and Lee, V. M. (1993) *Neuron 10*, 1089−1099.
42. Yoshida, H., and Ihara, Y. (1993) *J. Neurochem. 61*, 1183−1186.
43. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J. U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) *Science 278*, 1957−1960.
44. Cleveland, D. W., Hwo, S. Y., and Kirschner, M. W. (1977) *J. Mol. Biol. 116*, 227−247.
45. Kirschner, D. A., Abraham, C., and Selkoe, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 503−507.
46. Jicha, G. A., Lane, E., Vincent, I., Otvos, L., Jr., Hoffmann, R., and Davies, P. (1997) *J. Neurochem. 69*, 2087−2095.
47. Jicha, G. A., Bowser, R., Kazam, I. G., and Davies, P. (1997) *J. Neurosci. Res. 48*, 128−132.
48. Schutkowski, M., Bernhardt, A., Zhou, X. Z., Shen, M., Reimer, U., Rahfeld, J. U., Lu, K. P., and Fischer, G. (1998) *Biochemistry 37*, 5566−5575.
49. Wishart, D. S., Bigam, C. G., Holm, A., Hodges, R. S., and Sykes, B. D. (1995) *J. Biomol. NMR 5*, 67−81.

BI0004807