Vol. 188, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1992 Pages 162-169

A SERINE → PROLINE CHANGE IN THE ALZHEIMER'S DISEASE-ASSOCIATED EPITOPE TAU 2 RESULTS IN ALTERED SECONDARY STRUCTURE, BUT PHOSPHORYLATION OVERCOMES THE CONFORMATIONAL GAP

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Received August 27, 1992

Monoclonal antibody Tau 2 was raised against bovine τ protein, was reported to recognize a conformational epitope, and stained τ was found in neurofibrillary tangles of Alzheimer's disease, but not normal human τ. We synthesized tetradeka peptides corresponding to the original bovine sequence, its serine → proline substituted analog, the genuine human sequence of this region, and the bovine epitope phosphorylated on the crucial serine. The secondary structure of the peptides was determined by circular dichroism. It was found that only the original bovine epitope showed a tendency to form the β-pleated sheets characteristic of the neurofibrillary tangles. The spectra of the human peptide, its analog, and the phosphorylated bovine sequence were very similar, featuring a weak, helical β-turn character. Eventual phosphorylation of epitopes of this otherwise heavily phosphorylated protein may overcome inter-species conformational gaps. © 1992 Academic Press, Inc.

The brains of AD patients are characterized by abundant fibrous lesions, i.e. senile plaques, NFTs, and neurophil threads (1-3). Tangles represent dense accumulations of ultrastructurally distinct PHFs (4,5). The major component of PHFs is the low molecular weight, microtubule-associated protein τ (6,7), most probably in an abnormally phosphorylated form (8-10). Enzymatically (11) and chemically phosphorylated (12) synthetic peptides were used to identify and characterize the immunological and conformational properties of epitopes that distinguish τ found in PHFs from normal τ. Synthetic peptides were also used to demonstrate how single phosphorylation of τ may convert this elastic protein into the β-pleated sheets characteristic of PHFs in NFTs (13). Remarkably, only the phosphorylated peptide showed a tendency to form β-pleated sheets in TFE and water-TFE mixtures; the non-phosphorylated form did not, as determined by CD

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The abbreviations used are: AD, Alzheimer's disease; NFTs, neurofibrillary tangles, PHFs, paired helical filaments; TFE, trifluoroethanol; CD, circular dichroism; and mAb, monoclonal antibody.

0006-291X/92 $4.00

spectroscopy. CD spectroscopy in TFE was also employed to analyze other τ and neurofilament epitopes (12-14) that were suggested to be involved in NFTs (2).

Tau 2, an mAb, was raised against bovine τ (15), and was reported to stain axons, neuronal somata, dendrites, and astrocytes. It was also shown that Tau 2 very intensely stains tangles, plaque neurites, and curly fibers in the AD tissue section, but not in the control (16). The epitope for the antibody was reported to be comprised of the sequence AGIGDTSNLEDQAA (16). This synthetic peptide is reported to compete with the full protein for Tau 2 binding. A peptide analog, in which Ser^7 is replaced by a proline, failed to compete with bovine τ. The serine → proline substitution is made since Tau 2 binds very weakly to human, mouse, and rat τ, and human, mouse, and rat τ contain a proline in this amino acid position (besides Asn^8 → Ser and Gln^{12} → Glu specific substitutions in the human sequence). The difference in antibody recognition was rationalized by a difference in the conformation of the two peptides ("Ser peptide-like conformation" versus "Pro peptide-like conformation") featuring a more extended structure for the peptide containing the serine residue. This is not obvious from the secondary structural prediction (17), since both serines and prolines are suggested to participate in β-turns, rather than in β-pleated sheets. In this paper, we undertook to characterize the secondary structure of the serine-containing and the proline-containing peptides, and the peptide of human τ corresponding to the same region (amino acids 106-119, numbering according to ref. 18). We also wanted to see whether phosphorylation of the bovine epitope results in a secondary structure closer to the human peptide(s), thereby allowing the possibility of crossing conformational inter-species gaps and obtaining antigenic variants that bind to antibodies across species after phosphorylation. This post-translational modification is abundant in the neuronal cytoskelatal protein family, including τ.

MATERIALS AND METHODS

Peptide synthesis, purification, and analysis - Peptides were made on a Milligen 9050 automated synthesizer. Fmoc-amino acid pentafluorophenyl esters (19) were used for peptide chain assembly with a continous-flow method on a polystyrene-polyepoxy graft copolymer resin (20). Completion of coupling and deprotection cycles were monitored by ultraviolet adsorbance at 365 nm. Phosphorylation was done on the resin by the phosphoramidite method (21). The yield of the phosphorylation reaction was around 50%. A trifluoroacetic acid-thioanisole mixture (95:5, v/v) was used to detach the peptides from the solid support. After cleavage, the peptides were amides at the C-termini and had free N-termini. Peptides were purified (and the phosphopeptide was separated from its contaminating non-phosphorylated analog) by reverse-phase high performance liquid chromatography using a C18 column and a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. Fast atom bombardment mass spectroscopy was done in the positive ion mode in the Department of Chemistry at the University of Pennsylvania and M-Scan, Inc. Plasma desorption mass spectroscopy was done by

Multiple Peptide Systems, Inc. Amino acid analysis verified the expected composition of the peptides within 5% of the expected values. The presence of the phosphate group was also demonstrated by bound phosphate analysis (22).

CD spectroscopy - CD spectra were taken on a Jasco J720 instrument at room temperature in a 0.2 mm path-length cell. Double-distilled water and spectroscopy-grade TFE were used as solvents. Excepting the dilution study (0.05 mg/ml), peptide concentration was 0.2-0.4 mg. The CD curves on Figs. 1 and 2 are baseline-corrected and smoothed using the algorithm provided by Jasco. Mean residue ellipticity ($[\theta]_{MR}$) is expressed in deg cm^2/dmole using a mean residue weight of 110.

Enzyme-linked immunoadsorbent assay and dot blot - Binding of 0.04-10.00 μg amounts of the synthetic peptides was tested with 1:100, 1:500, and 1:1000 dilutions of mAb Tau 2 (Sigma) on Linbro plates by enzyme-linked immunosorbent assay and on Westran paper by dot blot. Bovine τ was used as a positive control. A 1:1000 dilution of goat anti-mouse horseradish peroxidase conjugate was used as a secondary antibody. Color development was made with o-phenyl-diamine, and was measured at 450 nm for the enzyme-linked immunosorbent assay, and with 3,3-diamino-benzidine for the dot blot.

RESULTS AND DISCUSSION

The sequence of the synthetic peptides and their analysis is outlined in Table 1. Excepting purification of the phosphorylated peptide, the synthesis proceeded without any difficulties. Phosphorylation usually reduces the retention times on reverse-phase high performance liquid chromatography considerbly, and this can be correlated with their less ordered secondary structure compared with the non-phosphorylated parent peptides (23). The elution time of peptide Tau 2BSPh, however, was very close to that of peptide Tau 2BS, indicating a possible secondary structural orientation on the surface of the bonded phase, and consequently anticipating a phosphopeptide conformation other than unordered.

Secondary structural prediction (17) of all three non-modified peptides reveals a high propensity to form β-turns at the N-terminal halves of the molecules and α-helices at the C-terminal ends. Considering that a type III (I) β-turn can be regarded as a single unit of a 3_{10}-helix (24), it is conceivable that the peptides assume

Table 1. Synthetic Peptides and Methods Used for Their Analysis

Peptide	Sequence	Origin	Reverse-phase high performance liquid chromatography (min)	Mass spectroscop
Tau 2BS	AGIGDTSNLEDQAA	Bovine τ 92-108	23.0	M+H = 1361
Tau 2P	AGIGDTPNLEDQAA	-	24.0	M+H = 1371
Tau 2H	AGIGDTPSLEDEAA	Human τ 106-119	24.7	M+H = 1345
Tau 2BSPh	AGIGDTS(Ph)[1]NLEDQAA	-	22.8	M = 1441

[1]Ph, phospho

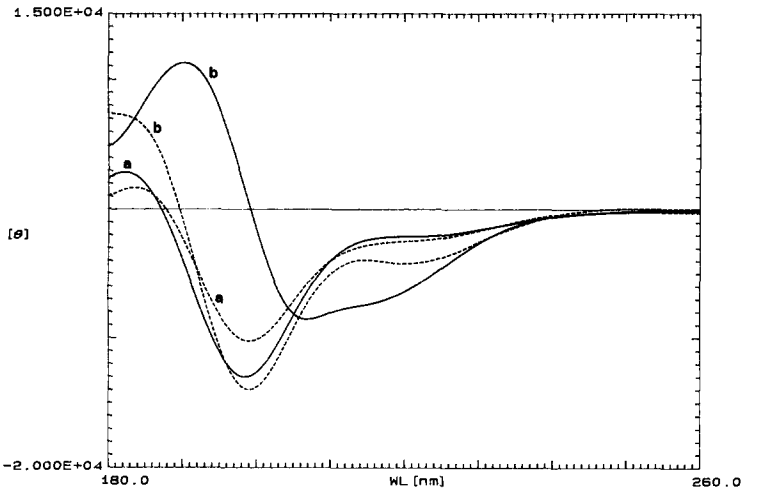

Fig. 1. CD spectra of peptides Tau 2BS (solid lines) and Tau 2P (dotted lines) in water alone (curves a) and in TFE after addition of 10 molar excess of Ca^{2+} ions (curves b).

a continous helix conformation in which the geometry of the helix varies through the chain. A slight propensity of formation of β-pleated sheets is predicted at the N-termini of the molecules, but the turn-helical system probably overrides it.

In water, all peptides exhibit CD spectra characteristic for a mainly unordered conformer population featuring a single intense negative ellipticity band between 198 and 200 nm (25) (Fig. 1). This is consistent with our earlier experience on the CD of 10-20 residue fragments of τ and neurofilament proteins (12-14).

TFE is widely used to preserve function-determining conformations of biological systems (26). It is noted that TFE does not induce ordered structure in new regions of peptides, but merely stabilizes the nascent ones (27). CD measurements in TFE fully support the secondary structural prediction for peptides Tau 2P (the bovine peptide containing the proline) and Tau 2H (the genuine human sequence), but not for Tau 2BS (the original bovine peptide). Peptides Tau 2P, Tau 2H, and Tau 2BSPh (the phosphorylated bovine sequence) exhibit type C spectra according to the classification of Woody (28), featuring negative ellipticity bands at 204-206 nm and 223 nm, and a positive band at 188 nm (Fig. 2). Type C spectra are similar to those of an α-helix (25), except that their band intensities are significantly lower. Type C spectra were measured for β-turns with established type I (III) character (29,30). The partial α-helical character is apparent from the slightly increased intensity of the curves. Application of the estimation of α-helicity introduced by Greenfield and Fasman (25) reveals 10, 20, and 19% α-helix content for peptides Tau 2P, Tau 2H, and Tau 2BSPh, respectively. The small redshift of the negative split of the $\pi\pi^*$ band of phosphorylated bovine peptide Tau 2BSPh indicates a somewhat stabilized conformation, but basically its spectrum is very close to the non-phosphorylated human analog (Tau 2H) and the supposedly conformationally similar bovine "Pro

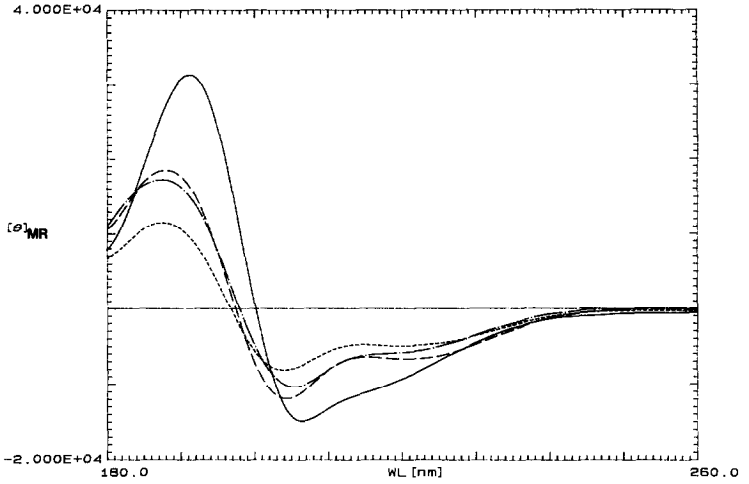

Fig. 2. CD spectra of Tau 2 peptides in TFE. Tau 2BS (solid lines); Tau 2P (dotted lines); Tau 2H (dashes); Tau 2BSPh (dots and dashes).

peptide" (Tau 2P) (16), but does not resemble that of its non-phosphorylated parent peptide Tau 2BS. The detected very high secondary structural similarity of the real human peptide and the proline-containing bovine peptide analog proves the presumption made by Watanabe *et al.* (16), and justifies the use of peptide Tau 2P to mimic the local conformation found in the human protein.

Surprisingly, the CD spectrum of bovine peptide Tau 2BS differs from the other 3 spectra. It is characterized with a positive band at 201 nm and a broad negative band which can be resolved into two bands: one at 206 nm and one around 217 nm (Fig. 2). The location and the increased intensity of the $\pi\pi^*$ bands indicate a stronger α-helix (34% helix content) with a considerable amount of β-pleated sheet conformer population. The characteristic features of β-pleated sheets are a negative band at 216 nm and a positive band between 195 and 200 nm (31) with antiparallel orientation toward the shorter wavelengths of the range. The shape of the CD curves of peptides representing type II β-turns is similar to that of β-pleated sheets, but the bands are located at higher wavelengths and their intensity is considerably smaller; consequently, a type II β-turn cannot be regarded as a contributor to the conformation of peptide Tau 2BS. The peptide exhibits unchanged spectrum after dilution by fivefold, indicating an intramolecular arrangement of the β-pleated sheets. We discarded the possibility of computer analysis of the CD curves, since the newest version of these algorithms (32) (used successfully for other τ peptides [12]) revealed an unacceptably high error rate for the estimation.

It appears that the "hidden" extended structure, proposed by the secondary structural prediction, is manifested only in the unchanged bovine sequence. mAb Tau 2 is reported to bind this peptide (but not the human analogs), and also to stain NFTs (16). Since the NFTs are organized in β-pleated sheets, the selective

recognition and staining pattern of mAb Tau 2 is in perfect accordance with the CD studies of the synthetic peptides and indicates that mAb Tau 2 indeed recognizes a tangles-related conformational epitope. Furthermore, our CD studies substantiated the existence of a "Ser conformation" and its difference from a "Pro conformation" of these peptides proposed by Watanabe *et al.* (16).

Addition of Ca^{2+} ions to synthetic peptides and phosphopeptides corresponding to neuronal cytoskeletal proteins was reported to indicate the conformational stability either by destabilizing ordered conformations (12,13), or by the opposite, increasing β-pleated sheet potentials (14). The CD behavior of the Tau 2 peptides after addition of $Ca(ClO_4)_2$ in TFE concur with both effects, and further prove the structural differences. As Fig. 1 shows, peptide Tau 2P undergoes a conformational transition resulting in mostly unordered secondary structure with some residual helix-turn contribution. Peptides Tau 2H and Tau 2BSPh behaved similarly, but these peptides retain more of their original character (data not shown). Most of the starting structure is retained in peptide Tau 2BSPh, in accordance with its slightly higher conformational stability without the presence of Ca^{2+} ions. Addition of Ca^{2+} ions to peptide Tau 2BS increased the contribution of the β-pleated sheets in the conformational equilibrium by slightly decreasing the α-helix amount (16%), but not affecting the β-pleated sheet content (Fig. 1).

Many serines of the τ protein can be phosphorylated by several kinases, like calcium/calmodulin-dependent kinase (33), protein kinases A and C (33,34), and another recently identified kinase family (mitogen-activated kinases [35]). It is reported that mitogen-activated kinases transform τ directly into an AD-like state (35). The conformational change upon phosphorylation of a single serine can be detected equally on the full τ protein (36) and on its representative synthetic peptide fragments (12,13). Phosphorylation appears to play a major role in defining the secondary structure of τ. Phosphorylation of the bovine Tau 2 peptide resulted in a conformation very similar to that of the non-phosphorylated human peptide. It appears that phosphorylation can overcome inter-species conformational barriers. If the Tau 2 site is indeed a conformation-dependent epitope as the data indicate, the phoshporylated bovine sequence should no longer be recognized by mAb Tau 2. Rather, it might be recognized by a specific, yet to be developed, anti-human Tau 2 mAb, and will also bind to antibodies across species.

None of the synthetic peptides was recognized, however, by anti-bovine mAb Tau 2 in our hands by either enzyme-linked immunosorbent assay or dot blot. Our binding studies were attempted in conditions identical to our earlier successful indentification of immuno-dominant τ (2,8,12) and neurofilament (37,38) epitopes, both in non-phosphorylated and phosphorylated forms. The positive control bovine τ protein was strongly recognized. One of the explanations for the discrepancy between our results and those of Watanabe *et al.* (16) is the fact that we

used solid-phase binding assays and applied the peptides in the μg range in contrast with the other study where binding of the synthetic peptides to mAb Tau 2 was found in solution, using mg and g amounts of peptides (16). These amounts of synthetic peptides in purified forms are not available to us. The other explanation is that although this particular tetradeka peptide is part of the epitope, it does not represent the entire epitope, which would be optimal for recognition by mAb Tau 2. This idea is strongly supported by a report which locates the epitope for mAb Tau 2 further from the amino terminus (39), between Val^{117} and Ala^{157} of bovine τ. A third report places a 28 amino acid-long major epitope of human τ in the area of the studied tetradeka peptide, but elongates it at both termini (40).

In summary, we have demonstrated that the conformation-dependent epitope for mAb Tau 2 binds to the antibody and stains NFTs only in an AD-specific β-pleated sheet secondary structure. We also documented how phosphorylation of epitopic regions can overcome inter-species conformational gaps. The approach used here may be useful for identifying new AD-specific epitopes of τ, and may lead to the development of measures to prevent or remove the formation of NFTs.

Acknowledgments - The authors thank Dr. Hildegund C.J. Ertl for her critical reading of the manuscript. This work was supported by research grants from the National Institutes of Health (AG 10670) and from the American Health Assistance Foundation.

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