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REVERSIBLE β-PLEATED SHEET FORMATION OF A PHOSPHORYLATED SYNTHETIC τ PEPTIDE

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Serine^{416} of human τ protein is believed to be phosphorylated in Alzheimer neurofibrillary tangles. We synthesized a fragment of τ, consisting of amino acids 408-421 in both non-phosphorylated and serine^{416}-phosphorylated forms. Circular dichroism in a trifluoroethanol-water mixture indicated a β-turn → β-pleated sheet conformational transition upon phosphorylation. The β-structure formation is intermolecular and can be inhibited by addition of Ca^{2+} ions or a phosphorylated tripeptide, but not with its non-phosphorylated analog. The presence of the phosphorylated τ peptide did not facilitate the formation of β-pleated sheets of a phosphorylated neurofilament fragment. Multivalent cations induced a conformational transition of this phosphorylated neurofilament peptide, but the effect was less specific than the transition induced in the τ fragment, and it could also be reversed with the competing phosphorylated tripeptide.

The brains of Alzheimer's disease (AD) patients are characterized by abundant fibrous lesions, i.e. senile plaques (SPs), neurofibrillary tangles (NFTs) and neurophil threads (1-3). Although not restricted to AD, the burden of NFTs and SPs correlates well with the severity of AD (4-5). Tangles represent dense accumulations of ultrastructurally distinct paired helical filaments (PHFs) (6-7). Recently it was shown that PHFs are comprised of abnormally phosphorylated forms of τ, a group of low molecular weight microtubule-associated proteins. Specifically, we showed that Ser^{396} may be abnormally phosphorylated in normal τ leading to the generation of an altered form of τ (i.e. A68), which is the major subunit of PHF (8). Another phosphate acceptor site at Ser^{416} in τ was shown to be the target of Ca^{2+} calmodulin-dependent kinase (CaMk) and the reduced electrophoretic mobility of τ phosphorylated at this site resembles that of PHF τ and this may reflect a conformational change (9). It is important to understand how an elastic molecule like normal τ (10) folds into the β-pleated sheets characteristic of PHFs in NFTs.

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CaMk phosphorylates only Ser^{416} (9) and it was shown that phosphorylation of τ by CaMk has a dramatic effect on the secondary structure of the protein by making it long and stiff (11). It is worth mentioning that Ser^{416} lies close to the microtubule binding domain of τ and it affects the structure of the microtubule binding region in an indirect manner (9). Since it appears that the secondary structural change of τ can be modelled with the sequence around Ser^{416}, we prepared synthetic peptides corresponding to amino acids 408-421 (H-Leu-Ser-Asn-Val-Ser-Ser-Thr-Gly-Ser-Ile-Asp-Met-Val-Asp-NH_2) in a non-phosphorylated peptide (τcamk) and in a Ser^{416}-phosphorylated peptide (τ-camkPh) to determine if the conformational transition of τ might be due to phosphorylation at this site. Since motifs in other neuronal cytoskeletal proteins, such as the multiphosphorylation domain of neurofilament (NF) proteins, may also be present in NFTs, we also determined if the change in the conformation of τ facilitates the conformational transition of the multiphosphorylation repeat of NF proteins (12). Finally, we investigated whether these conformational transitions can be reversed or inhibited by competitive binding with charged molecules.

MATERIALS AND METHODS

Peptide τ-camk was synthesized on solid-phase using Fmoc-amino acid Pfp esters as coupling reagents (13). Ser^9 (Ser^{416} on the full protein) was incorporated side-chain unprotected and part of the peptide-resin was phosphorylated after the peptide chain assembly was completed by two different methods, described previously (14,15). Peptides were cleaved off the resin with trifluoroacetic acid (TFA) and purified by reversed-phase HPLC using a 0.1% aqueous TFA-acetonitrile gradient system. τ-camkPh was eluted 1.9 min (2.5% CH_3CN) earlier than τ-camk. The integrity of both peptides was verified by amino acid analysis and Edman-sequencing. A similar protocol was used for the syntheses of Ac-Pro-Lys-Ser-NH_2 (PKS) and its serine-phosphorylated analog (PKSPh). The presence of organic phosphate on τ-camPh and PKSPh was also demonstrated by phosphate analysis (16). The preparation of the double phosphorylated neurofilament peptide (HNFM 1-17 PhPh; H-Glu-Glu-Lys-Gly-Lys-Ser-Pro-Val-Pro-Lys-Ser-Pro-Val-Glu-Glu-Lys-Gly-NH_2) was published earlier (17).

Circular dichroism (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 trifluoroethanol (TFE) were used as solvents. Peptide concentration was 0.25-0.35 mg/ml, except the dilution study, where the concentration of τ-camkPh was 0.06 mg/ml. Mean residue ellipticity ($[\theta]_{MR}$) is expressed in deg cm^2/dmole by using a mean residue weight of 110. When the conformational steering effect of a "helper" peptide to a "target" peptide was studied, the CD curve of the peptide mixture was baseline corrected, and the previously detected CD of the individual helper peptide was digitally subtracted. $[\theta]_{MR}$ was finally calculated based on the concentration of the target peptide. CD curve smoothing was done using software supplied by Jasco.

Secondary structural prediction was made using a modified Chou-Fasman algorithm (18).

RESULTS

Secondary structural prediction of the τ-camk peptide suggests a high propensity of β-turn formation at the N-terminal and middle section of the sequence and helical structure at the C-terminus. Based on the prediction, β-pleated sheet formation all over the molecule cannot be excluded either, but the turn-helical system probably overrides it. This is fully supported by CD studies in TFE-water=9:1 (v/v). τ-camk exhibits a CD spectrum (Fig. 1, curve a) which can be most accurately described as a mixture of type C and D spectra, according to the classification of Woody (19). Type C spectra are characteristic for type I (III) β-turns (20), type D spectra are a sign of distorted turn structure. We found type C spectra earlier for a non-phosphorylated peptide based on the human middle molecular weight NF protein (HNFM) and assigned the conformation as a series of type I β-turns or a 3_{10}-helix (21). After phosphorylation the same peptide exhibited type D spectrum, and we interpreted the secondary structure featuring a turn with long-range interactions (22). Based on this, τ-camk probably exists as a mixture of different β-turns. The most dominant band in the CD spectrum of τ-camkPh is the negative ellipticity one at 217 nm together with a red-shift of the positive ellipticity band compared to the non-phosphorylated analog (Fig. 1, curve b) as a clear indication of the appearance of a considerable amount of conformer featuring β-pleated sheet structure (22). Recently we found similar turn → β-sheet transition for phosphorylated peptide HNFM 1-17 upon addition of Ca^{2+} and Al^{3+} ions (23). The β-pleated sheets of τ-camkPh are intermolecular, since, after dilution the peptide exhibits a type C-D spectrum very similar to the non-phosphorylated parent peptide

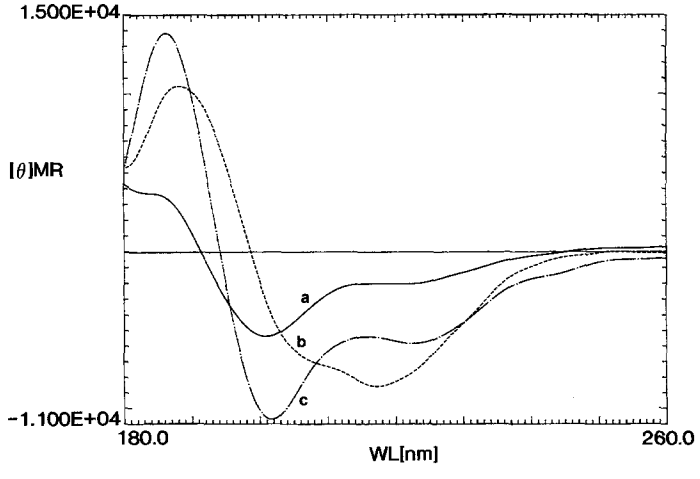

Figure 1. CD spectra of τ-camk and τ-camkPh peptide in 90% TFE. Curve **a** (———) is the non-phosphorylated peptide; curve **b** (- - - -) is the phosphorylated peptide at a concentration of 0.3 mg/ml; curve **c** (-- · -- · --) is the phosphorylated peptide at a concentration of 0.06 mg/ml.

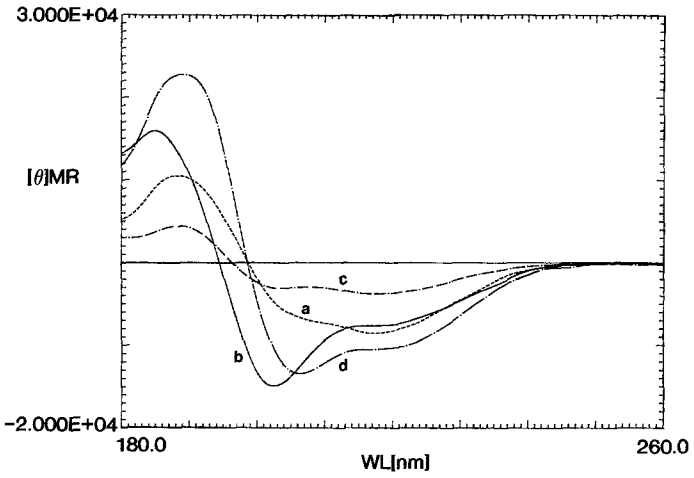

Figure 2. CD spectra of τ-camkPh peptide in different environmental conditions in 90% TFE. Curve **a** (- - - -) is the peptide alone; the additives are as follows: curve **b** (———), a 5 molar excess of Ca^{2+} ions; curve **c** (- · - · -), a 10 molar excess of peptide PKS; curve **d** (-- · -- · --), the same peptide phosphorylated, PKSPh.

(Fig. 1, curve c). The addition of a 5 molar excess of Ca^{2+} ions also restored the C-D spectrum (Fig. 2, curve b), but Na^{+} ions did not alter the β-structure (data not shown). However, after the addition of a 10 molar excess of non-phosphorylated tripeptide PKS the type of the spectrum of τ-camkPh remained unchanged β (Fig. 2, curve c), while after the addition of the same peptide phosphorylated, PKSPh, a type C spectrum was detected (Fig. 2, curve d).

The type C-D spectrum of HNFM 1-17 PhPh in TFE (Fig. 3, curve a) is converted to a CD spectrum featuring a considerable amount of β-pleated sheets

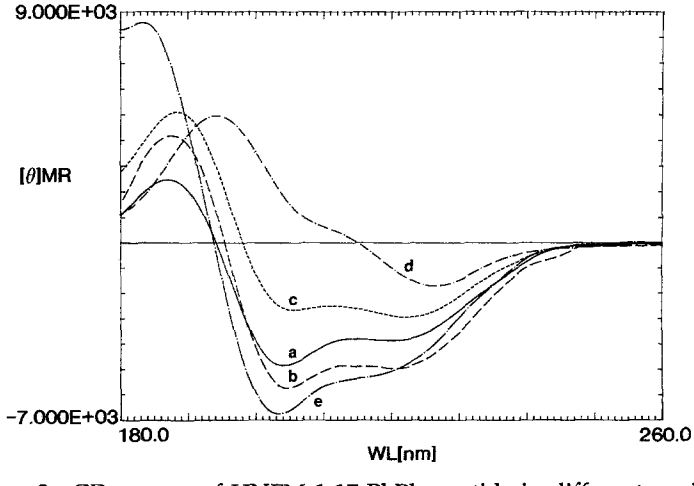

Figure 3. CD spectra of HNFM 1-17 PhPh peptide in different environmental conditions in 100% TFE. Curve **a** (———) is the peptide alone; the additives are as follows: curve **b** (— — —), an equivalent amount of τ-camkPh; curve **c** (- - - -), a 10 molar excess of Ca^{2+} ions; curve **d** (- · - · -), a 10 molar excess of peptide PKS; curve **e** (-- · -- · --), the same peptide phosphorylated, PKSPh.

after addition of Ca^{2+} ions (Fig. 3, curve c) (23). Similar to the τ-camkPh peptide, PKS did not restore the original C-D spectrum (Fig. 3, curve d), only its phosphorylated form, PKSPh (Fig. 3, curve e). When τ-camkPh in β-pleated sheets was added to HNFM 1-17 PhPh without Ca^{2+} ions, no conformational transition of the neurofilament peptide was detected (Fig. 3, curve b).

DISCUSSION

In this study we characterize the turn → intermolecular β-pleated sheet conformational transition of a synthetic peptide corresponding to the Ca^{2+}-calmodulin-dependent kinase site of human τ protein after *in vitro* phosphorylation. Earlier we observed a phosphate loss of synthetic phosphorylated NF and τ peptides during workup or storage. This phosphate loss of τ-camkPh is the fastest among the sequences studied; it occurs even after lyophilization or few days of storage at room temperature as detected by HPLC and Edman-sequencing. We tried both methods of selective phosphopeptide synthesis as described in the Methods section, but similar unstable phosphopeptides were obtained. The unusual instability of τ-CamkPh may also reflect unique physical-chemical properties. Nevertheless, the short period of time required for CD studies made the secondary structural analysis possible. The physical properties of τ-camk and τ-camkPh are different from the other peptides examined; the non-phosphorylated peptide is not soluble in 100% TFE, while the phosphorylated is, and this is exactly the opposite of our previous experience. TFE was found to be very useful to prevent function-determining conformations of biological systems (24), and to identify the effect of addition of cations on the conformation of biologically active peptides (25). Although excellent computer algorithms are available for analysis of CD curves (26,27), we have decided not to use them since, as a result of mixing of the peptide component solutions and additives as well as the mathematical manipulations used in this study, we expect consistent curve shapes, but variable band intensities.

Both τ-camk and HNFM 1-17 have a tendency to aggregate after phosphorylation. This is more specific for τcamkPh since the conformational transition is detected without additives. The intermolecular β-pleated sheets of τcamkPh are most probably formed through salt bridges between the N-terminus and the phosphoserine residue. This mimics the natural sequence, where the next amino acid to the N-terminus is the similarly positively charged histidine. (We expect even more synthetic difficulties if attempts at preparation of the His containing peptide were to be made). Similar conformational transition of HNFM 1-17 PhPh requires addition of multivalent cations and its non-phosphorylated and selectively phosphorylated analogs give the same transition upon addition of Ca^{2+} ions, but to a different degree (23). Moreover, the conformational transition of τ-camkPh takes place in a wide TFE concentration range, even in the presence of salt,

while the multivalent cation-induced β-pleated sheet formation of HNFM 1-17 peptides is detected only in 100% TFE. In contrast to the NF peptides, the β-pleated sheet formation of τcamkPh can be reversed by addition of Ca^{2+} ions, most probably through complexing with the phosphoserine (23). This complex formation is important since the β-structure of τcamkPh was unmodified when 0.9% aqueous NaCl was used instead of water in the CD solvent mixture. The β-pleated sheet formation can be prevented (or the original structure restored) by addition of a slight excess of a phosphorylated tripeptide for both τcamkPh and HNFM 1-17 PhPh. The parent, non-phosphorylated tripeptide did not alter the β-structures, suggesting a mechanism of prevention, in which the initial step is a specific binding of the phosphate group to the salt bridge-forming positive charge. We found that the presence of τcamkPh did not force HNFM 1-17 PhPh to β-pleated sheets. If the multiphosphorylation repeat of NF proteins participates in the formation of NFT, it must go through by a mechanism other than co-aggregation with the CaMk site of τ. The studies presented here give insights into modification of normal τ and its deposition of τ in NFT. Understanding this process fully may lead to the development of measures to prevent or remove the formation of NFTs.

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