Hyperphosphorylation induces structural modification of tau-protein

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Abstract The effect of hyperphosphorylation on the structural properties and conformational stability of bovine tau-protein was studied by means of circular dichroism and fluorescence lifetime techniques. Normal protein contains unusual secondary structure elements: extended left-handed helices. The structure of this protein was assumed to be of a 'tadpole' type – a globular C-terminal part with a long and rigid tail included in the extended left-handed helix. Either a decrease or an increase of pH induced only minor changes of the normal tau-protein surface. Hyperphosphorylation affected the extended part of the protein molecule; the decrease of pH in this case induced considerable structural rearrangements, and the conformation of the C-terminal part of the protein molecule was transformed into a molten globule-like state.

Key words: Tau-protein; Molten globule; Protein structure and stability; Conformational change

1. Introduction

Cytoplasmic microtubules are found in all eukaryotic cells. They play an important role in the maintenance of cell shape, cell division, axonal transport, secretion, and receptor activity [1]. The structure of microtubules, being determined by the polymerisation of tubulin dimers, is very dynamic. These filaments are stabilised by a number of accessory proteins (known as MAPs, microtubule-associated proteins). Microtubules are considerably more abundant in nerve cells, where they are involved in intraneuronal transport. Among the factors that distinguish neuronal microtubules from non-neuronal filaments is the presence of a set of neuronal-specific MAPs [2]. Tau-proteins are potential representatives of this collection [3]. The presence of tau-protein is essential for both nucleation and elongation of the filaments [4,5]. It was shown that tau-proteins bind microtubules by repetitive sequences in their C-terminal area [6]. The importance of phosphorylation for regulation of the catalytic activity of tau-protein was suggested. It was shown that hypophosphorylated tau-proteins are capable of tubulin polymerisation, whereas hyperphosphorylated forms catalyse the depolymerisation of microtubules into tubulin [7].

The interest in tau-proteins has been dramatically increased by the discovery of MAP aggregation in neuronal cells in the progress of Alzheimer's disease and various other neurodegenerative disorders. In these cases specific tau-protein-containing neurofibrillary tangles were shown to be formed [7].

The tau-protein isolated from brain microtubules represents a family of proteins which migrate during SDS gel electrophoresis as close bands of 55–62 kDa molecular weight [8]. Heterogeneity is explained in part by alternative mRNA splicing leading to the appearance of one, two, three or four repeats in the C-terminal region of a protein molecule [9]. Post-translational phosphorylation of tau-protein should be considered an additional source of microheterogeneity in SDS gel electrophoretic experiments [8,10]. The phosphorylation-dephosphorylation cycle plays an important role in regulation of tau-protein activity [10]. Analysis of the paracrystalline structure of the tau-protein suggested that the protein molecule became longer and more rigid with increasing degree of phosphorylation [11]. Increased phosphorylation was shown to be a common characteristic of a pathological tau-protein [12–15]. An important role of proline-directed MAP kinases (recognising sequences of -X-(Ser/Thr)-Pro-X-) in tau-protein phosphorylation has been demonstrated [14]. Interestingly, the largest isoform of human tau-protein contains at least 14 potential sites for phosphorylation by such kinases.

As shown by analytical ultracentrifugation and sedimentation studies, the native tau-protein isoform with a molecular weight of 57 kDa has a sedimentation coefficient of 2.6 S. It favours a highly asymmetric structure with an axial ratio (calculated on the basis of an assumed prolate ellipsoid model) of 20:1 [16].

Although biological and biochemical aspects of tau-protein functioning and its involvement in the development of various neurodegenerative disorders have been extensively studied, only little is known about conformational changes induced by phosphorylation. The aim of this work was to study structural changes of the tau-protein molecule resulting from hyperphosphorylation.

2. Materials and methods

2.1. Materials

We used bovine tau-protein from Sigma-Aldrich GmbH (Deisenhofen, Germany; Lot #107H4011); activated proline-directed MAP kinase from Stratagene GmbH (Heidelberg, Germany; Lot #206115); [γ-^{32}P]ATP was from ICN Biomedicals GmbH (Eschwege, Germany).

Protein concentrations of 5 and 0.5 μM were kept throughout all the circular dichroism (CD) and fluorescence experiments, respectively. 8-Anilino-1-naphthalene sulphonate (8-ANS) concentration was 5 μM. Measurements were performed at 23°C.

2.2. Instrumentation

Circular dichroism measurements were carried out by means of a JASCO-700 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan), using a cuvette with a light path of 0.1 mm. Protein concentration was kept at 0.1 mg/ml throughout all experiments. Steady-state measurements of 8-ANS were performed using a SFM-25 spectrofluorimeter (Tegimenta AG, Switzerland). Decay times of ANS

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fluorescence were measured by means of the time domain fluorescence lifetime method. The frequency-tripled output of a titanium sapphire laser Tsunami (Spectra Physics, Mountain View, CA, USA) was used for the excitation of fluorescence. The laser was run in mode-locked operation at 82 MHz producing pulses of 250 fs pulse width with an averaged energy of 42 mW at 263 nm wavelength. The fluorescence decay was recorded by a streak camera C4334 (Hamamatsu, Japan) in the 10 ns time window. The fluorescence emission was spectrally resolved by a polychromator in front of the streak camera.

2.3. Phosphorylation of tau-protein with activated proline-directed MAP kinase

Tau-protein (5 μM) was phosphorylated by activated MAP kinase under standard conditions (25 mM HEPES, 10 mM magnesium acetate, 100 μM ATP (or [γ-^{32}P]ATP), pH 7.5) using 0.01 μM of activated MAP kinase. The reaction mixture was incubated for 24 h at 30°C. The final volume of the reaction mixture was 200 μl in the case of ATP and 20 μl in the case of [γ-^{32}P]ATP. In the latter case the sample was mixed with SDS sample buffer and analysed on a 10% SDS polyacrylamide gel [17]. The gel was stained with Coomassie brilliant blue R, washed off, dried and analysed by autoradiography. It has been established that practically all γ-^{32}P was bound by the tau-protein at the given conditions. It allows us to estimate the degree of tau-protein phosphorylation to be approximately 20.

3. Results

3.1. Tau-protein molecule contains an extended left-handed helix as an element of secondary structure

As seen from Fig. 1, the far-UV CD spectrum of the normal bovine tau-protein differs significantly from the far-UV CD spectra common for globular proteins. This suggests that tau-protein contains *specific* (unusual) secondary structure elements, e.g. stretched left-handed helical structures of the poly-L-proline type [18]. This is supported, first, by the extended highly asymmetric shape of the tau-protein molecule [16]; second, by the extremely high content of proline residues

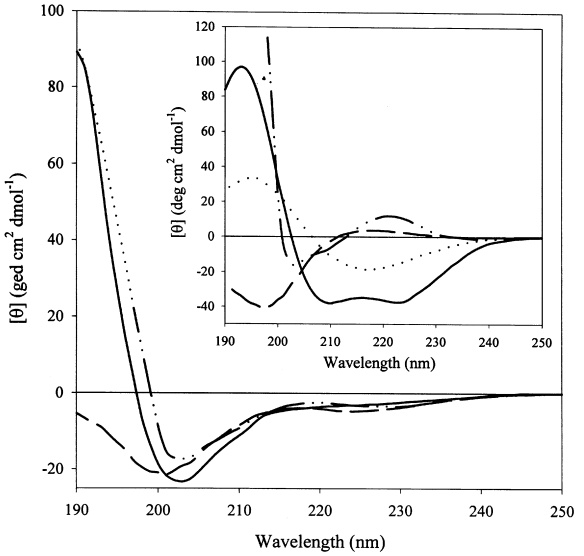

Fig. 1. Far-UV CD spectrum of bovine tau-protein (bold line) and spectra resulting from the composition of model spectra of elementary structures: 15% α-helix+10% β-structure+75% random coil (dashed line); 15% α-helix+10% β-structure+25% extended left-handed helix+50% random coil (dash-dotted line). Inset represents model spectra for α-helix [19], β-structure [19], random coil [19] and extended left-handed helix [18] (bold, dotted, dashed and dash-dotted lines, respectively).

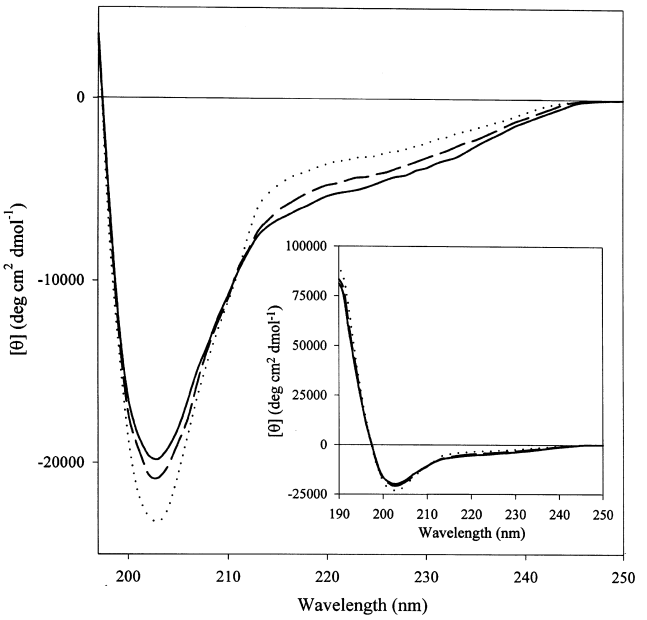

Fig. 2. Far-UV CD spectra in the limited wavelength range measured for normal tau-protein at different pH values: pH 2.5, 7.5 and 10.0 (solid, dotted and dashed lines, respectively). Inset represents the full-scale spectra measured at the same conditions.

mainly concentrated in the middle part of the protein molecule.

The presence of extended left-handed helical elements in the tau-protein molecule was confirmed by further analysis of its far-UV CD spectrum. The content of common secondary structure elements (α-helix and β-structure) in the tau-protein molecule was estimated from the amino acid sequence, using the ALB software [20]. As shown by this analysis, tau-protein has a relatively low content of ordered secondary structures (15% α-helix and 10% β-structure). The reconstructed spectrum comprising 15% α-helix, 10% β-structure and 75% random coil did not fit the experimentally obtained spectrum, especially in the short-wave region (Fig. 1). All our attempts to obtain a spectrum resembling that of the tau-protein have failed for the content of α-helical and β-structural elements between 5 and 45%. This discrepancy can be explained by the presence of unusual structural elements not taken into account in the modelling approach. Let us assume that approximately 25% of protein amino acid residues are involved in an extended left-handed helical structure. Since the spectrum of the left-handed helix was determined earlier [18], we tried to restore the far-UV CD spectrum of tau-protein as a sum of spectra of elementary structures with weight coefficients of 15, 10, 25 and 50 for α-helix, β-structure, extended left-handed helix and random coil, respectively (Fig. 1). The restored spectrum was rather close to the measured one. Therefore, the extended left-handed helical structure appears to be one of the structural elements of the normal tau-protein.

3.2. Effect of pH on normal tau-protein structure

As seen from Fig. 2, far-UV CD spectra of bovine tau-protein demonstrate no significant alterations in the pH range from 2.5 to 10.0. Some additional information of the pH effect on structural properties of tau-protein was obtained by analysing the interaction of this protein with the hydrophobic

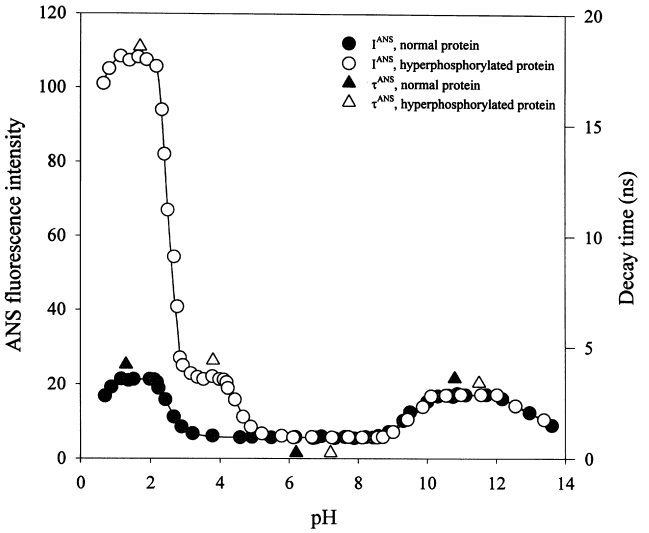

Fig. 3. Effect of pH on the fluorescence parameters of the hydrophobic fluorescent probe 8-ANS in the presence of normal and hyperphosphorylated tau-protein (grey and open symbols, respectively). Arrow indicates the level of free 8-ANS fluorescence.

fluorescent probe 8-ANS. It is known that the fluorescence intensity of 8-ANS increases considerably upon interaction with large water-inaccessible hydrophobic clusters of the protein molecules [21,22]. Non-native conformations of globular proteins (like the molten globule intermediate state) were shown to have a higher affinity for 8-ANS than native and unfolded molecules [22]. It was also established that the decay time of free 8-ANS in aqueous solution is about 0.3 ns, whereas the formation of the probe-protein complexes yields a 10–60-fold increase in decay time [23]. The existence of at least two different types of 8-ANS-protein complexes was described. Upon the formation of complexes of the first type, the 8-ANS molecule interacts with the surface of the protein molecule and is relatively well accessible to the solvent. These complexes are characterised by a decay time of 2–5 ns. Being involved in the formation of complexes of the second type, the 8-ANS molecule is embedded into the protein molecule and practically inaccessible to water. The characteristic lifetime of 8-ANS fluorescence in this case will be ~10 ns for the native proteins and ~15–20 ns for the molten globule proteins [23].

We have used these observations to analyse the pH-induced structural transformations of a tau-protein molecule. The results of these investigations are summarised in Fig. 3. It can be seen that in the pH range between 3.0 and 9.0 there is no 8-ANS-protein interaction. Fig. 3 also shows a considerable change in both fluorescence parameters (intensity and lifetime) beyond this pH range, probably due to structural changes of the protein molecule and to the formation of 8-ANS-protein complexes. As in these cases the lifetime of 8-ANS fluorescence is about 4 ns, we may conclude that the formation of complexes of the first type took place. In other words, the pH-induced structural transformation of the tau-protein is only small and the major part of the protein molecule remains practically immutable and preserves a relatively rigid structure.

3.3. *Effect of hyperphosphorylation on tau-protein structure and stability*

Far-UV CD spectra of normal and hyperphosphorylated forms of bovine tau-protein at different pH are presented in Fig. 4. The left-hand part of Fig. 4 shows that at neutral pH the minimum at 202 nm, characteristic for the normal tau-protein, is practically absent in the spectrum of the hyperphosphorylated protein. This tempted us to suppose that major hyperphosphorylation-induced changes of the tau-protein can be expected in the extended part of the protein molecule.

As follows from the right-hand part of Fig. 4, the far-UV CD spectrum of hyperphosphorylated protein at pH 4.0 is very close to that of the normal protein at pH 2.0. A further pH decrease leads to additional spectral changes, e.g. a 2-nm shift is observed of the minimum to longer wavelengths, accompanied by a considerable increase in the corresponding

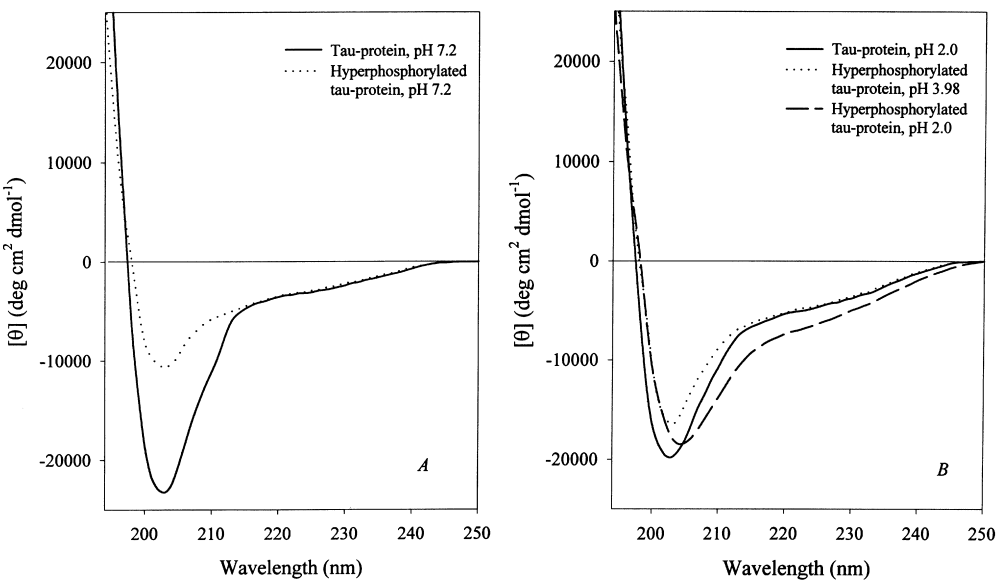

Fig. 4. Far-UV CD spectra of normal and hyperphosphorylated forms of bovine tau-protein measured at different pH values. Left, neutral pH; right, acidic pH.

negative ellipticity ($[\theta]_{222} = -4800$ and -7000 deg cm^2/dmol at pH 4.0 and 2.0, respectively).

8-ANS fluorescence parameters, determined for normal and hyperphosphorylated tau-proteins, behave quite differently in their response to decreasing pH (see Fig. 3). It was already noted that both a decrease and an increase in pH affect the structure of normal tau-protein equally, resulting only in some changes of the protein molecule surface. It can be seen that a comparable structural rearrangement can be expected for the hyperphosphorylated tau-protein at high ($pH > 9.0$) or moderately low (pH between 4.0 and 3.0) pH values, while a further decrease of the pH value leads to an additional structural transformation of this form of protein (Fig. 3). This means that the decrease in pH value induces two consecutive conformational transitions in the hyperphosphorylated tau-protein: to the end of first (pH 4.0) and second transition (pH 2.0) the intensity of 8-ANS fluorescence increases 3- and 20-fold, respectively (Fig. 3). Interestingly, 8-ANS fluorescence lifetimes were 0.3, 4.4 and 18.5 ns at pH 7.5, 3.8 and 1.7, respectively. Fluorescence lifetimes longer than 10 ns correspond to the protein-embedded 8-ANS molecules [23]. When exceeding 15 ns, the 8-ANS molecules are placed in a highly flexible hydrophobic environment (like in a non-polar protic solvent). Such a situation was assumed to occur upon the transformation of a protein molecule into the molten globule state [23].

4. Discussion

Our data show that upon a decrease of the pH value the hyperphosphorylated tau-protein undergoes a two-step structural transformation. At the first step (pH 4.0) the solvent-accessible hydrophobic clusters appear on the surface of the relatively rigid protein molecule. At the second step (pH 2.0) the protein molecule is transformed into a highly flexible conformation with a pronounced secondary structure. The nature of this conformation (molten globule-like intermediate) would be obvious if we had worked with a usual globular protein. However, with tau-protein the situation is more complicated. Indeed, it was already noted that the tau-protein molecule is highly asymmetric [16]. This gives rise to several important questions. Can we expect the appearance of the structural transformations described within a single protein molecule or is the hydrophobic environment in which 8-ANS becomes embedded created by the formation of protein associates? If, nevertheless, such structural transformations take place within a protein molecule, which part of it can be affected?

The well-known feature of the hyperphosphorylated tau-protein is its trend to form neurofibrillary tangles responsible for the development of several neurodegenerative disorders [7]. Paired helical filaments (PHF) are the major component of these neurofibrillary deposits [12]. It is a widespread belief that hyperphosphorylation of the tau-proteins is just the process preceding formation of the PHF [24]. Quite recently it was established that the appearance of PHF in vitro required the presence of sulphated glycosaminoglycans (heparin). Moreover, in this case PHF was found to be formed both from normal and from hyperphosphorylated forms of tau-protein with nearly equal efficiency. On the other hand, in the absence of heparin, filament appearance was not observed even in the case of hyperphosphorylated tau-protein [25]. This observation is in a good agreement with our data – we did not see any substantial light scattering from the solutions of either normal or hyperphosphorylated tau-protein. This fact favours the absence of large protein associates in both cases. Consequently, there are some hints to assume that decreasing pH induces considerable intramolecular conformational transformations in the molecule of hyperphosphorylated tau-protein.

In which part of an extended protein molecule can we expect the development of such structural changes? To answer this question the analysis of secondary structure element localisation within the amino acid sequence was applied. Results of such a prediction from the program ALB [20] have shown that the distribution of the secondary structure within the sequence of tau-protein is extremely uneven. The highest probability to be involved in the formation of a regular secondary structure is characteristic for the C-terminal part of a protein molecule, known as a microtubule assembly domain, containing several highly conserved 18-acid repeats. It is difficult to exclude that the normal tau-protein molecule has the 'tadpole' shape, i.e. it includes the globular C-terminal part with up to 50% of amino acid residues and a relatively long and rigid tail, the main part of which is a constituent of the extended left-handed helix.

Hyperphosphorylation leads to some perturbation within the extended part of a protein molecule, whereas its C-terminal part remains practically unchanged. With decreasing pH the hyperphosphorylated protein undergoes considerable structural rearrangements and the C-terminal part of a molecule is transformed into the molten globule-like conformation. If this is true, it is quite reasonable to suggest that just the C-terminal part of hyperphosphorylated tau-protein is responsible for the association of protein molecules. Indeed, it is known that the capability to associate (or aggregate) is the specific property of partially folded conformations of globular proteins [26–34]. Considerable structural transformations and an increase in conformational stability of a protein molecule can be observed as the results of the association [32–34].

Taking all this into account, we assume that at definite cell conditions (e.g. near the membrane surface) hyperphosphorylated tau-protein molecules undergo structural changes, which transform the C-terminal part of a protein molecule into the molten globule-like state. This may cause association of the tau-protein molecules and formation of the paired helical filaments. If so, tau-protein molecules within PHF should be stacked 'head-to-head', ensuring the best contact of their hydrophobic surfaces. Interestingly, it was shown by immunoelectron microscopy that monoclonal antibodies against different parts of tau-protein differ in their ability to interact with the paired helical filaments. In particular, PHF was effectively decorated by antibodies directed against the amino-terminus of tau-protein, but not by the antibody against the microtubule-binding repeat region. It was concluded that in filaments the repeat region of tau-protein is inaccessible to the antibodies [25,35].

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