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Nascent structure in the kinase anchoring domain of microtubule-associated protein 2☆

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Abstract

Biological processes are often viewed as highly ordered interactions between well-folded protein domains. The specific interactions exhibited by certain highly abundant neuronal proteins such as microtubule-associated protein 2 (MAP2) and tau stand in stark contrast because these proteins do not show evidence of structure by standard biophysical assays, yet they do bind to specific targets. It is conceivable that there are regions of MAP2 and tau with propensity to form structural domains upon binding a target. To search for evidence of such regions, limited proteolysis experiments were carried out on MAP2c, the smallest MAP2 isoform. Increased protease resistance was observed around the binding site for the RII subunit of cAMP-dependent protein kinase. Protein constructs spanning this region were produced based on the long-lived tryptic fragments Ser44–Arg93 and Ile94–Arg182, and were probed for structure using spectroscopic methods. The results support the existence of regions of nascent structure in the N-terminal region of MAP2c, which are believed to contribute to its regulatory function.

Keywords: Circular dichroism; Limited proteolysis; MAP2; Nascent structure; NMR; Non-globular protein

Understanding the signal transduction processes that convert extracellular signals into morphological information for the neuron is a key to understanding the structural plasticity of the nervous system. Microtubule-associated protein 2 (MAP2) is one of the most abundant proteins in the brain. It regulates and organizes microtubules (MTs) and may function as a scaffolding protein to bring different neuromodulatory activities together. For example, MAP2 was the first protein identified as a member of a growing class of molecules now known as 'AKAPs'—A-kinase anchoring proteins, whose function is to target cAMP-dependent protein kinase to specific subcellular domains [1]. In dendrites, the loss of MAP2 leads to a 15–20-fold reduction in the MT-bound cAMP-dependent protein kinase[2]. The specific binding site for the RII subunit of cAMP-dependent protein kinase has been mapped to the N-terminal region of MAP2 [3–5].

Little is known about the structure of MAP2 and its homologues. Isolated MAP2 molecules imaged by metal shadowing electron microscopy show that the overall structure is elongated and rather rod-like [6]. Molecular biological and biochemical studies have mapped different MAP2 activities to specific locations in the sequence [7]. In addition to the RII binding site in the N-terminal region [4,5], it has been shown that MT binding occurs through three semi-homologous MT-binding repeats near the C-terminus [8,9] (Fig. 1A). Other molecules capable of interacting with MAP2 include calmodulin [10] and SH3 domains of the non-receptor protein tyrosine kinases Src and Fyn as well as the adaptor protein Grb2 [11,12]. The region between the N- and C-terminal binding regions has been thought of as a flexible spacer

☆ *Abbreviations:* AKAP, A-kinase anchoring protein; CD, circular dichroism; MALDI, matrix-assisted laser desorption ionization, MAP2, microtubule-associated protein 2; MT, microtubule; NMR, nuclear magnetic resonance; RII, regulatory domain of cAMP-dependent protein kinase type II; TMAO, trimethylamine *N*-oxide; TFE, trifluoroethanol.

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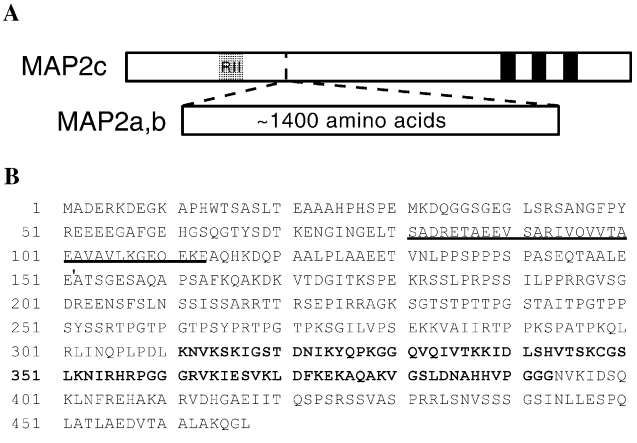

Fig. 1. MAP2 topology and sequence. (A) Topology diagram. The RII binding site is indicated with a shaded box and the MT-binding repeats with black boxes. (B) MAP2c sequence. The RII binding site is underlined and the MT-binding domain is in boldface. The splice site for the extra ~1400 amino acids in HMW-MAP2, located between Glu151 and Ala152 is indicated with an apostrophe (′).

element [13], but may also play a scaffolding role too, bringing together effector molecules, such as those mentioned above, with their relevant targets. MAP2 is phosphorylated by a number of kinases and its interaction with MTs and certain other targets depends on its phosphorylation state [7]. Its abundance and many phosphorylation-dependent interactions make it likely to be a key factor in development and maintenance of neuronal structure.

Given this reasonably well-characterized set of binding interactions, it was initially anticipated that MAP2 would contain a number of specific structural domains. However, MAP2 and the related protein tau have been the subject of several studies by traditional biophysical approaches, yet they do not appear to contain any appreciable folded domains or regions containing extensive amounts of well-formed secondary structure. A low level of regular secondary structure and a flexible and/or extended conformation have been reported on the basis of CD and sedimentation equilibrium experiments [14]. In an NMR study, Woody et al. [15] identified flexible portions of high molecular weight (HMW) MAP2 by sharp ^{1}H resonances both in isolation and in assembled microtubules, and these were assigned to residues in the N-terminal projection domain based on proteolytic digestion experiments. These observations contrast with the above-noted interactions between particular regions of MAP2 and its binding partners. The apparent discrepancy between specificity of target interactions and lack of structure led us to suspect that regions of MAP2 exist that may in fact be structured in the in vivo context, but are only partially folded in vitro in the absence of their normal binding partners. This study reports a series of experiments to search for such regions.

Experimental procedures

Protein expression and purification. Full-length rat MAP2c was expressed as described previously [16]. The lysate was boiled for 10 min, swirling periodically [17], and cleared by centrifugation for 20 min at 10,000*g*. MAP2c was further purified on a 1 ml Pharmacia HiTrap SP cation exchange column using a NaCl gradient (0–500 mM) in 50 mM NaAc buffer at pH 7.0 containing 1 mM EDTA and 1 mM EGTA.

The genes for the N-terminal MAP2c fragments were amplified by PCR from the full-length rat MAP2c gene and cloned into a modified p-RSETa expression vector (Invitrogen) including an N-terminal 6-His tag. Protein was expressed in BL21 pLysS (DE3) cells grown in LB-media in the presence of ampicillin and chloramphenicol. Protein expression was induced at OD_{600} ~0.5–0.6 by addition of 0.5 mM IPTG and cells were harvested after 3 h. The proteins were purified using Ni–NTA resin from Qiagen.

Proteolytic digestions. Full-length MAP2c (1–467) (20 μM) was digested with 0.2, 2, and 20 mg/L trypsin or chymotrypsin for 5, 15, and 60 min in 150 mM KCl, 40 mM Tris/HCl, pH 7.5 [18,19]. The reaction was stopped with PMSF and monitored on SDS–PAGE gels. The fragments were separated the by reverse phase HPLC and their sequence was identified using MALDI-TOF mass spectrometry [18].

CD spectroscopy. CD experiments were conducted using a Jasco J-720 spectropolarimeter in 10 mm quartz cuvettes. Solutions were 5.5, 2.1, 4.1, and 5.7 μM, for $MAP2_{1–187}$, $MAP2_{1–159}$, $MAP2_{38–187}$, and $MAP2_{38–93}$, respectively, in 1 mM Tris/HCl, 100 mM KCl, and pH 7.1. Additional experiments on $MAP2_{1–187}$ were performed involving addition of TFE, TMAO, and urea. Spectra were collected in three scans from 170 to 300 nm with a step size of 1 nm, an average time of 2 s for each point, and background correction against a buffer blank.

^{1}H NMR spectroscopy. The NMR experiments were conducted using a Bruker DRX 500 spectrometer on 0.1 mM $MAP2_{1–187}$, $MAP2_{1–159}$, $MAP2_{38–187}$, and full-length MAP2c, and 0.03 mM $MAP2_{38–93}$, in 40 mM Tris/HCl, 100 mM KCl, 10% D_2O, and pH 7.1. The $MAP2_{38–93}$ sample was used for acquisition of 2D homonuclear COSY [20] and TOCSY [21,22] spectra.

Fluorescence spectroscopy. Tryptophan fluorescence was measured on a Spex 1681 Fluorolog spectrofluorimeter in 10 mm quartz cuvettes on a 1 μM $MAP2_{1–187}$ sample in 1 mM Tris/HCl, 100 mM KCl, pH 7.1. The excitation wavelength was 295 nm and emission spectra were collected from 300 to 400 nm with a step size of 0.5 nm and background correction against a buffer blank.

Results

Limited proteolysis

To search for the presence of specific folded units in MAP2, the smallest MAP2 isoform (MAP2c, 467 amino acids; Fig. 1) was subjected to limited tryptic and chymotryptic digestions, respectively (Fig. 2). Two specific fragments that were more resistant to tryptic digestion than the rest of the protein were identified (Figs. 2 and 3A). After separation by HPLC, MALDI-TOF mass spectrometry showed these to be consecutive fragments Ser44–Arg93 and Ile94–Arg182 (Figs. 2B and C). The observation of a common cleavage site for these two fragments suggested that the two fragments form a single unit of structure, and presumably with a more flexible loop containing Arg93/Ile94, a fact that is further emphasized by the presence of longer and shorter

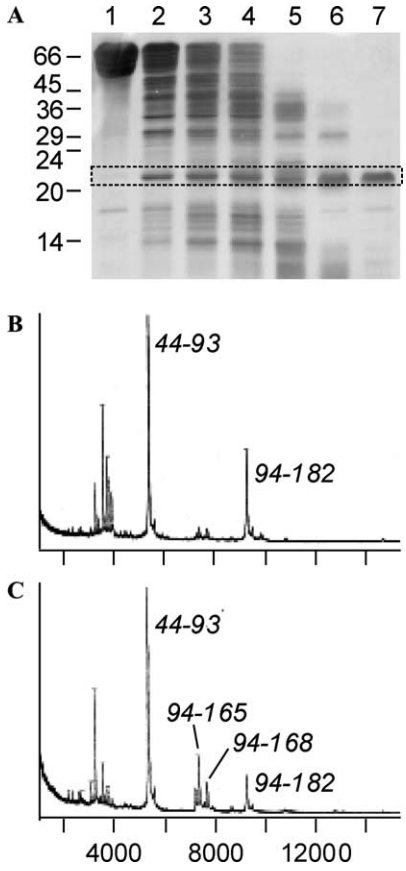

Fig. 2. Proteolytic digestion of MAP2c by trypsin. (A) SDS–PAGE gel showing the full-length protein (lane 1) and proteolytic digests with 0.2 mg/L protease for 5, 15, and 60 min (lanes 2–4), and similarly for 2 mg/L (lanes 5–7). The bands withing the dotted rectangle correspond to fragments 94–165, 94–168, and 94–182. (B,C) MALDI-TOF mass spectrum of proteolytic digests with 2 mg/L protease for (B) 15 and (C) 60 min. The identity of the proteolytic fragments is indicated. In (A) reference molecular weights in kilodaltons are indicated on the left.

C-terminal extensions of the Ile94–Arg182 fragment (i.e. Arg196, Lys168, and Lys165) at earlier and later time points, respectively (Fig. 2). The chymotryptic digests similarly indicate increased stability in this region, showing a number of fragments with resistance to proteolysis around a core consisting of residues Ser68–Leu106. The fact that the protected region encompasses the known RII binding region (Asp83–Glu113) of MAP2c [4,5] motivated more detailed analysis.

Secondary structural prediction

The sequence-based local α-helix prediction algorithm Agadir (http://www.embl-heidelberg.de/Services/serrano/agadir/agadir-start. html) [23–26] was applied to the MAP2c sequence, along with additional analyses using PSIPRED [27] and Target 99 [28]. Only a few regions are predicted to have regular secondary structural elements. The regions predicted to have the highest propensity are located in the middle of and after the

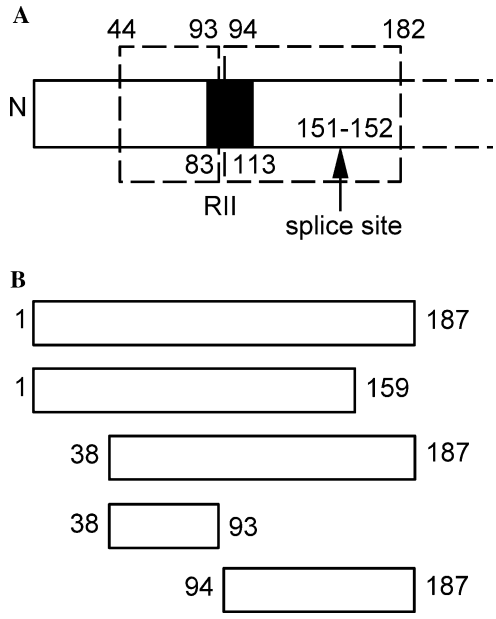

Fig. 3. Identification and production of MAP2c N-terminal fragments. (A) The schematic diagram of fragments with enhanced resistance to tryptic digestion. (B) The topology diagrams of the five constructs prepared.

MT-binding repeats near the C-terminus, in particular, Asp371–Lys379 and Val458–Lys464. However, these are rather short stretches and would not be expected to form a stably folded structural unit on their own. In contrast, the RII binding region near the N-terminus of MAP2c has a much longer stretch of amino acids (Asp81–Gln115) that is predicted to form helical elements, although the value of the scoring function is lower. Two additional segments with some helical propensity are Ser18–His25 and Ser143–Glu151.

MAP2 fragments

To further explore the characteristics of the RII binding region of the protein, five partially overlapping constructs (Fig. 3B) were subcloned into a vector with an N-terminal 6-His tag, overexpressed, and purified. These constructs span the N-terminal protected fragment ($MAP2_{38–93}$), the C-terminal protected fragment ($MAP2_{94–187}$), both protected fragments ($MAP2_{38–187}$), the N-terminus plus the two protected fragments ($MAP2_{1–187}$), and the N-terminus plus the part of the protected fragments that is N-terminal of the MAP2ab/MAP2c splice site ($MAP2_{1–159}$). Four of the five constructs expressed well after transformation, but $MAP2_{94–187}$ did not.

Biophysical characterization

In an effort to determine if there exists a propensity to form folded and/or globular regions in the N-terminal region of MAP2, the four overlapping MAP2 constructs

that could be expressed and purified were analyzed by CD and 1D ^{1}H NMR spectroscopy. CD spectra acquired in standard aqueous buffers for all four constructs revealed a relatively modest amount of α-helix (Fig. 4). The fluorescence spectrum arising from the sole tryptophan in $MAP2_{1–187}$, Trp14, has a high intrinsic intensity and emission wavelength consistent with a high degree of solvent exposure. 1DNMR spectra acquired on samples in the same buffer are poorly dispersed, indicating a lack of stable tertiary structure. The spectra for the three longer constructs, $MAP2_{1–187}$, $MAP2_{1–159}$, and $MAP2_{38–187}$, are very similar, while the shorter $MAP2_{38–93}$ has somewhat narrower lines. However, all four constructs have NMR signal linewidths that are significantly larger than anticipated for strictly unfolded molecules of their respective molecular weights (Figs. 5B and C; $MAP2_{1–159}$ and $MAP2_{38–187}$ are not shown). The linewidths are also larger than anticipated for proteins comprising a stably folded globular domain, and are rather consistent with aggregation and/or exchange between different conformational sub-states. Surprisingly, the 1D spectrum of intact MAP2c looks very similar to the spectrum of $MAP2_{1–187}$ (Fig. 5D).

A 2D homonuclear TOCSY spectrum was acquired for the smallest fragment, $MAP2_{38–93}$ at pH 7.1. Even at this relatively high pH, a full complement of H^N–H^α peaks (~50; Fig. 5E) was observed. However, the cross-peaks were not dispersed in the manner of a well-folded globular protein. Together, these observations suggest significant populations of more than one structural sub-state rather than a single well-defined tertiary structure.

The combination of the specificity of RII binding interactions, resistance to proteolysis, and the observation of a complete set of amide resonances at pH 7.1 led us to suspect that the N-terminal region of MAP2c has a

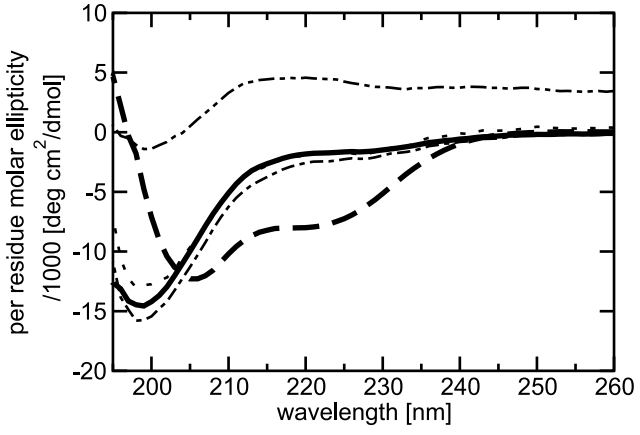

Fig. 4. CD spectra of $MAP2_{1–187}$ (thick solid line), $MAP2_{1–159}$ (thin dotted line), $MAP2_{38–187}$ (thin dotted and dashed line), and $MAP2_{38–93}$ (thin double-dotted and dashed line), respectively. Data were acquired in a buffer containing 1 mM Tris/HCl, 100 mM KCl, and pH 7.1. In a separate experiment TFE was added to 30% v/v to the $MAP2_{1–187}$ sample (thick dashed line).

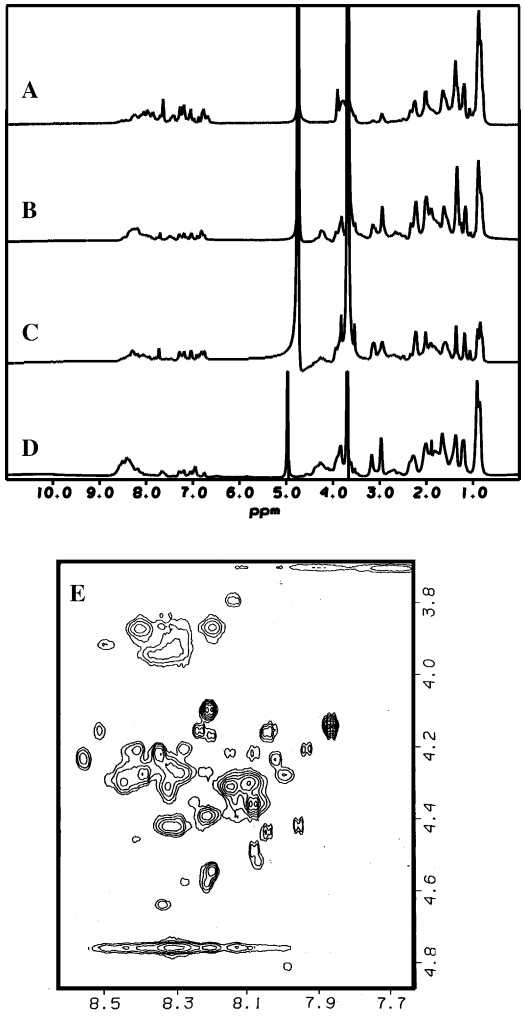

Fig. 5. 1D ^{1}H NMR spectra of MAP2c N-terminal fragments. The 500 MHz spectra of $MAP2_{1–187}$ in (A) 30% TFE and (B) aqueous solution, (C) $MAP2_{38–93}$ in aqueous solution and (D) full-length MAP2c in aqueous solution, and (E) the finger print region of a 2D TOCSY spectrum of $MAP2_{38–93}$ in aqueous solution.

propensity to fold, but not to the point of forming a single stably folded structural domain on its own. Moreover, isolated MAP2c fragments in standard aqueous buffer may not accurately reflect the native milieu for the protein. A biophysical characterization of MAP2 in the presence of its natural binding partners represents an exceptionally difficult experimental challenge that is beyond the scope of this analysis. To obtain some preliminary insights, the characterization of the MAP2c N-terminal fragments was extended in an effort to trap one or more of the conformational sub-states. All of these experiments were carried out on the largest N-terminal fragment, $MAP2_{1–187}$.

The initial step involved far-UV CD experiments and assays of ionic strength (0–500 mM KCl), addition of the detergent CHAPS, and addition of the known helix inducer trifluoroethanol (TFE; 0–45%) were tried. TFE

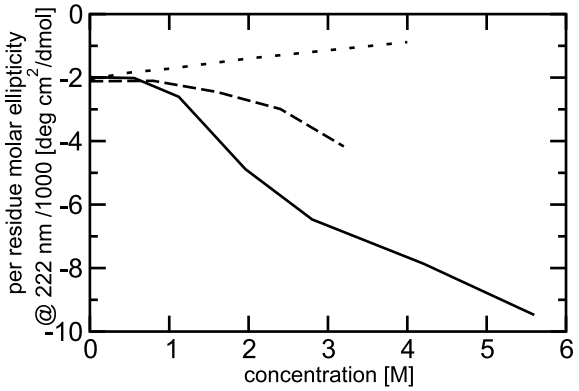

Fig. 6. Change in helical structure of $MAP2_{1–187}$ upon addition of co-solvents. Plot of the change in CD ellipticity at 222 nm versus addition of TFE (solid line), TMAO (dashed line), and urea (dotted line). The data were acquired at room temperature and for solutions starting in aqueous buffer at pH 7.1.

was found to induce a significant amount of helix in $MAP2_{1–187}$ (Figs. 5 and 6). To rule out that the level of secondary structure dependent on aggregation, a series of additional CD experiments were performed over the concentration range 30–160 μM $MAP2_{1–187}$ in the presence of 30% TFE. In this concentration range, no such effect was observed. Addition of the natural osmolyte trimethylamine *N*-oxide (TMAO) [29] up to 3.2 M also induces a significant amount of helix, but not to the same degree as TFE (Fig. 6). Given the helical propensity in $MAP2_{1–187}$, a urea denaturation experiment was carried out in standard aqueous buffer (Fig. 6). There is a slight decline in helical content of $MAP2_{1–187}$ upon addition of urea, which is consistent with "denaturation" of the equivalent of 5–20 helical residues.

A series of 1D ^{1}H NMR experiments were performed in parallel to the CD measurements. In the temperature range 278–328 K, and the pH range 5.2–7.2, the sharpest and most well-dispersed proton signals were obtained at 278 K and pH 7.2. As in the CD experiments, ionic strength (0–100 mM KCl) and protein concentration (12–300 μM) have a very limited effect on the NMR spectra. The similarity of the secondary structure effects of TFE and TMAO as monitored by CD encouraged us to acquire NMR spectra in the presence of TFE. These spectra are slightly more dispersed in the amide region, indicating a tendency towards formation of specific secondary structure elements. However, the increase in secondary structure does not appear to correlate with the formation of a specific tertiary structure, as there is little change in the aliphatic region of the spectrum.

Discussion

The current consensus view of biological processes involves interactions between well-folded proteins or protein domains in a highly ordered manner. In contrast, over the past few years a growing number of studies of physiologically active proteins show that they are "unfolded" in vitro, e.g., [30]. Many of these proteins are not completely unfolded in the traditional sense of a random coil, but rather exist in dynamic equilibrium between a set of preferred conformations [31].

The characterization of unfolded protein conformations represents a significant technological challenge. Recently great progress has been made in the use of NMR for conformational studies of partially and fully unfolded proteins [35–38]. The common findings in these studies and others are an unfolded state with an average structure that is in some cases very similar to the folded state [35,36], and native and non-native long-range hydrophobic interactions that stabilize the unfolded state [37,38]. Molecular dynamics simulations indicate that even though the average structure of the unfolded state is very similar to the folded state, none of the individual structures exhibit native-like features [39].

The NMR studies referred to above [35–38] all describe unfolded states of proteins where structure of the folded active state is known. Does a protein which appears to be largely unfolded, at least under aqueous in vitro conditions, behave similarly with segments with a well-defined compact average structure stabilized by long-range hydrophobic interactions? In the case of MAP2, structure stabilization through long-range interactions might be limited by the relatively low occurrence of large hydrophobic residues. However, long-range hydrophobic and electrostatic interactions between the sidechains of MAP2, as well as sequence dependent local sidechain–backbone interactions [40], might severely restrict the number of conformations populated by certain regions of the protein.

Observation of broad lines in the NMR spectra of the N-terminal MAP2 fragments, which do not narrow at lower protein concentrations, indicates that the N-terminal region of MAP2c is subject to conformational exchange on the intermediate chemical shift timescale (microseconds to milliseconds). Since low temperature lowers the rate of exchange between sub-states and decreases the population of the higher energy states, the fact that the sharpest lines are observed at lower temperature suggests that the exchange process occurs on the slower side of the chemical shift timescale and/or that the ensemble of sub-states contains a dominant lower energy conformation. Since the spectra show a chemical shift dispersion typical for a random coil peptide, the significant line broadening suggests that the other states have significantly different chemical shifts, shifts that require the complexity of a fold. It should be noted that while Woody et al. [15] found the ^{1}H linewidths surprisingly narrow for the 1500 amino acid projection domain of HMW MAP2, the linewidths for the 55–187 amino acid N-terminal MAP2 constructs in

this study are surprisingly broad considering their molecular size. The close similarity between a spectrum of the $MAP2_{1–187}$ fragment and that of intact MAP2c either indicates that a similar range of residues are visible in both spectra, or that all (visible) residues in MAP2c have the same spectral features. The former agrees well with the estimate of Woody et al. [15] to see approximately 10% of the HMW MAP2 projection domain in their spectra.

A promising technique to study the folded conformations of "unfolded" proteins is the use of natural osmolytes like TMAO [41]. These osmolytes are expressed to maintain protein structure in the presence of denaturing stresses [41], and in contrast to organic solutes like TFE that forces many proteins or peptides into unnatural helical conformations [42], they stabilize the folded active form of the protein [29]. Interestingly enough, the structure inducing effect of TMAO and unfolding effect of urea are exactly counteractive on a molar basis [43]. An example of the value of TMAO studies is the refolding of destabilized variants of the $\alpha + \beta$ proteins ribonuclease T and staphylococcal nuclease [29].

The induction of a significant amount of α-helical structure in $MAP2_{1–187}$ by TMAO indicates potentiated or nascent structure [29], and the urea-induced decrease in helical signal indicates the presence of residual helical structure also in the absence of the osmolyte [43]. It is important to note that in both these cases, the induced change in helical content does not necessarily represent folding or unfolding of a structural element but rather a stabilization or destabilization of specific conformational sub-states. While the absence of a specific tertiary structure, with or without structure inducing agents, suggests that MAP2c does not form a stable globular domains in vitro, it may well be part of one when bound to a target protein.

Molecular biological studies previously mapped the RII binding domain of MAP2 to amino acid residues Asp83–Glu113 [5]. Within this region, an 18 amino acid stretch, spanning residues 86–103, contains a consensus sequence for RII binding [32]. This region of MAP2 is predicted to have a propensity for amphipathic helix formation, which is common to analogous sites in other AKAPs [1], and in fact, amphipathic helical structure was recently observed in NMR structures of two different AKAP peptides complexed with the docking and dimerization domain of RII [33]. Here the presence of some degree of structure in the region Ser44–Arg182 of MAP2 was initially indicated by the observed protection from proteolytic digestion. Although specific or random self-association could conceivably contribute to protection against proteolysis, the N-terminus of MAP2 has been reported to lack high affinity dimerization activity [34].

The data presented here show that the RII binding domain of MAP2 has a propensity to occupy helical sub-states. We do not yet know if the N-terminal domain of MAP2 contains domains with well-defined average structures and if so what they look like. However, the present data do suggest that these domains span specific conformational sub-states, populated to a degree that gives significant protection against proteolysis, perturbs NMR linewidths, and allows natural osmolytes to stabilize potentiated structure. In parallel to a growing number of studies showing the importance of disorder to order transactions for fine-tuning binding free energies (e.g., [31]), we propose these conformational sub-states and the dynamic processes connecting them may be a characteristic that is important for the function of MAP2 and related proteins.

Acknowledgments

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