The effects of methylamine osmolytes on the cytoskeleton microtubule assembly

Huang-Chun Tseng
Iowa State University

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The effects of methylamine osmolytes on the cytoskeleton microtubule assembly

By

Huang-Chun Tseng

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

Major: Neuroscience
Major Professor: Donald J. Graves

Iowa State University
Ames, Iowa
1999

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This is to certify that the Doctoral dissertation of

Huang-Chun Tseng

has met the dissertation requirements of Iowa State University
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ABBREVIATIONS

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>cAMP</td>
<td>3', 5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PHF</td>
<td>Paired helical filament</td>
</tr>
<tr>
<td>TMAO</td>
<td>Trimethylamine N-oxide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EGTA</td>
<td>[Ethylenebis(oxyethylenenitrilo)]tetraacetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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Microtubules and Microtubule-Associated Protein Tau

Microtubules are major cytoskeletal structures found in eukaryotic cells. The main functions of microtubules include chromosome segregation in mitosis (1), intracellular transport (2), and providing cell support (3). This cytoskeleton is a hollow cylinder formed by the assembly of tubulin. Tubulin is mainly the associated product of two different subunits: α and β tubulin. γ tubulins, another tubulin, are found only in the microtubule organizing center (4). α and β tubulins are highly conserved proteins (5). Both subunits have an approximate molecular mass of 50 kDa and contain about 450 amino acid residues with some variation depending on the isoforms (5). Results of proteolysis show that tubulin consists of at least two domains: an N-terminal domain which binds GTP, and a C-terminal domain (including the acidic tail) which binds microtubule-associated proteins (6). Both α and β tubulins bind GTP but only β tubulin hydrolyzes GTP during microtubule
assembly (7). A 3-D structure for α and β tubulin revealed by electron crystallography shows that they have similar conformation (8). "Dynamic instability" is a general property of microtubules (9) and is a consequence of the assembly-disassembly of microtubules. The assembly and disassembly of tubulin into microtubules are sensitive to the concentration of tubulin, temperature, pH, ion, GTP/GDP (10), hydrostatic pressure (11), microtubule poisons (such as colchicine) (12), and microtubule-associated proteins (13).

Microtubule-associated proteins (MAPs) are the proteins co-purified with tubulin during assembly-disassembly cycle during tubulin purification (13). There are several subfamilies in microtubule-associated protein family: MAP1, MAP2 (14), MAP3 (15), MAP4 (16), and tau (13). MAP2, MAP4, and tau have a long rod-like structure (13, 16). MAP2, MAP4, and tau are grossly similar in their domain structure (16, 17), that is composed of a microtubule-binding domain and a projection domain extending from the microtubule wall. Imperfect 18-mer repeats (consensus VXSKXGSXXNIXHXPAGG) are located in the microtubule-binding domain (17). Phosphorylation of microtubule-binding repeats in tau (18)
and mutation occurring within microtubule-binding repeats (19) can reduce tau's binding affinity for microtubules.

Tau is enriched in neuronal cells (20). Tau promotes tubulin assembly, stabilizes microtubules, and nucleates microtubules in vitro (21, 22). The double immunofluorescent staining with tau antibodies (7A5 rabbit anti-tau) and tubulin antibodies (DM1a mouse anti-tubulin) in PC12 cells show that tau and tubulin are colocalized (23), suggesting that tau binds to microtubules in vivo. Introduction of tau to cells can increase cellular microtubules (24) and promote neurite outgrowth (25). The addition of tau antisense oligonucleotides can block the neurite outgrowth in rat cerebellar neurons (26). In addition, the binding of tau also increases the stiffness of microtubules (27).

In the human brain, tau exists as six isoforms and these forms are derived from a single gene in chromosome 17 by different mRNA splicing reactions (28-31). The largest brain tau containing 441 amino acids possesses two N-terminal inserts and four microtubule-binding repeats, but the smallest form possesses no N-terminal insert and has three microtubule-binding repeats instead (Fig. 1). The microtubule-binding repeats contain many basic residues and
Figure 1. Bar diagrams and domain structures of six human brain tau isoforms. 1, 2, 3, 4 represent the microtubule-binding repeats. I and II are the N-terminal inserts.
Projection Domain  MT-Binding Domain

Proline-rich Region  MT-binding Region

I441  I151  Q244  G401

M1
are believed to directly bind to the acidic C-terminal of tubulin to stabilize microtubules (6, 30). The tau structure for all isoforms is enriched with proline, glycine, and hydrophilic amino acids (30). The proline-rich region is next to the microtubule-binding repeats (Fig. 1). Results from the study using tau domain peptides show that both the proline-rich region and the microtubule-binding region contribute to the binding affinity to microtubules (32).

Tau is an extended molecule rather than a globular protein. Early biophysical studies of tau using analytical centrifugation (13), electron microscopy (33), circular dichroism (CD) (34), and X-ray solution scattering (35) suggested that tau is a highly flexible extended molecule with little secondary structure. This view was confirmed by $^1$H-NMR spectroscopy (36). This extended structure with little secondary structure may explain why tau is a heat-stable, acid-stable protein (30).
Phosphorylation and Tau

Protein phosphorylation is a post-translational modification which transfers the γ-phosphate of ATP into a serine, threonine, or tyrosine residue in proteins and can change the protein’s properties (37). Phosphorylation can change the characteristics of tau. Studies by Hagestedt et al. using paracrystal imaging showed that a change of flexibility of tau is caused by phosphorylation with Ca²⁺/calmodulin-dependent kinase (38). Tau becomes long and stiff after phosphorylation (38). Phosphorylation also reduces tau’s ability to stimulate microtubule assembly (39). Phosphorylation on the microtubule-binding domain of tau can abolish tau’s binding affinity (18) and cause microtubule disruption (40).

Tau possesses many different phosphorylation sites and at least 25 sites have been identified in paired-helical filaments (PHFs) which are mainly composed of tau (Table 1)(41, 42). PHF is one of the main hallmarks of Alzheimer’s disease brains (43, 44). All are Ser/Thr phosphorylation sites. Phosphorylation of those sites by various protein kinases (Table 1), in general, can be classified as proline-directed phosphorylation and non-proline-directed
Table 1. Phosphorylation sites of tau and acting kinases.

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Abbreviations: GSK-3 (α or β): glycogen synthase kinase-3; CDC2: cyclin dependent kinase 2; CDK5: cyclin-dependent kinase 5; MAPK: mitogen-activated protein kinase; PKA, cyclic-AMP dependent protein kinase; PKC: protein kinase C; CaMKII: Ca/calmodulin-dependent protein kinase II; MARK: microtubule-associated protein/microtubule affinity regulating kinase.
phosphorylation (42, 45). Both types of phosphorylation can affect tau’s function. For example, activation of mitogen-activated protein kinase (46), glycogen synthase kinase-3 (47), or microtubule-associated protein/microtubule regulating kinase (48) can phosphorylate intracellular tau and cause microtubule disruption. A recent study shows that tyrosine of tau can be phosphorylated by tyrosine kinase fyn (49).

In addition to kinases, hypoactive protein phosphatases are also proposed to relate to the consequence of hyperphosphorylation on tau. Hyperphosphorylation on tau occurs in Alzheimer’s disease brains (43, 44). Protein phosphatase 1 and 2A are associated with microtubules and are capable of dephosphorylating tau (50, 51). Protein phosphatase 2B is also colocalized with microtubules and regulates the phosphorylation of tau in vivo (52). Inhibitors to PP1 or PP2A, such as okadaic acid and calyculin A, can result in hyperphosphorylation of tau, which reduce binding ability to microtubules and promote microtubule disruption (50, 51). Cyclosporin A or a specific peptide, both of which inhibit PP2B activity, changes the phosphorylation state of tau and prevents axonal elongation in dissociated rat cerebellum cells (52). If
phosphorylated tau is dephosphorylated with PP1, PP2A, or PP2B, tau can recover its ability to promote tubulin assembly (39).

Organic Osmolyte Methylamines

Water-stressed cells accumulate small organic molecules to maintain cellular osmotic balance and cell morphology (53-56). Those small osmolytes are generally categorized into three groups: amino acids and their derivatives, polyols, and methylamines (Table 2). Molecules of the first two groups are "compatible osmolytes" which means cells accumulate those osmolytes to high concentrations without significantly perturbing protein functions. The third group of osmolytes is referred to as "counteracting osmolytes" for their counteracting effect on reversing the perturbations caused by urea.

Methylamine osmolytes are commonly found in many urea-rich tissues, such as the muscle of elasmobranchs (53, 54), coelacanth (53, 54), moridase and macrouridae (57), and the mammalian kidney (56). The concentrations of urea and methylamines form an approximately 2:1 ratio in many marine
Table 2. Common cellular osmolytes

<table>
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<tr>
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<tr>
<td>Alanine</td>
<td>Glycerol</td>
<td>Betaine</td>
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<tr>
<td>Proline</td>
<td>Sorbitol</td>
<td>Trimethylamine N-oxide</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Mannose</td>
<td>Glycerolphosphocholine</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>Sucrose</td>
<td>Sarcosine</td>
</tr>
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<td>Taurine</td>
<td>Myo-inositol</td>
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<td></td>
<td>Trehalose</td>
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animals (53, 54, 57). Trimethylamine N-oxide (TMAO), a methylamine osmolyte, can be found at concentrations up to 200 mM in elasmobranchs including cartilaginous fishes, such as sharks and rays (51, 52). At this concentration, TMAO may counteract denaturation caused by urea, which reaches concentrations of up to 0.4 M in elasmobranchs (53). Several enzymatic kinetics studies have shown that when urea and TMAO are present in a 2:1 ratio TMAO can offset the urea protein perturbations on creatine kinase, pyruvate kinase, argininosuccinate lyase, and lactic dehydrogenase (53). TMAO has been reported to behave as a chemical chaperon (56). TMAO can force chemically modified RNase and mutant RNase, two inactive, unfolded proteins, to fold and to become active (59). RNase T1 with its disulfide bonds reduced and chemically derivatized and staphylococcal nuclease mutant protein (T62P) exist as unfolded biologically inactive structures (59). However, in the presence of TMAO these proteins become folded and biologically active.

Possible mechanisms of action of methylamines on proteins have been extensively studied. Transition temperature (Tm) measurement for ribonuclease shows that TMAO raises the Tm but urea lowers the Tm (60). When urea
and TMAO are present in a 2:1 ratio, the effects of urea and TMAO on the Tm were found to be essentially the algebraic sum of their individual effects (60). TMAO acts as a co-solvent and causes preferential hydration on the surface of proteins (61-64). TMAO is excluded from the protein surface—this is called solvophobic effect (61-64). TMAO, therefore, changes the energy states of proteins (59, 60). Furthermore, release of bound water molecules occurring during folding or in specific association reactions (63, 64) in the presence of TMAO also contributes to the favorable energetics.

Rationale for My Research

Microtubule assembly is inhibited by urea both in vitro (65) and in vivo (66). How urea-rich cells protect their microtubules from the deleterious effects caused by urea is not yet clear. The rationale for this study is based on the properties of TMAO, which include the abilities to promote protein folding and/or aggregation and refold unfolded proteins. Tau is an extended protein and aggregates with microtubules during tubulin assembly. A
previous study done by Sackett shows that TMAO can enhance tubulin self-assembly but molar TMAO is needed in the assembly (65). TMAO should be able to promote the aggregation of tau-tubulin. In addition, taxol, which is used to stabilize microtubules, reduces the amount of TMAO needed to promote tubulin assembly (65). It is likely that TMAO at a physiological level should be able to promote tubulin assembly in the presence of tau. Therefore, I first hypothesized that TMAO at 200 mM should promote microtubule assembly in the assistance of microtubule-associated proteins. Second hypothesis is, based on the ability to correct the function of modified proteins, that TMAO could change the properties of phosphorylated tau to promote tubulin assembly. The last asked question in this dissertation is whether the increase of microtubule assembly caused by TMAO in vitro can be reproduce in cells.

Dissertation Organization

The first chapter is a general introduction of this dissertation. The next three chapters are in the formats of journal papers. Chapter 2, which has been published in
Biochemical and Biophysical Research Communications, presents the first finding about the effects caused by methylamine osmolytes on tau-microtubule assembly in vitro. Chapter 3, which has been accepted by the Proceedings of National Academy of Sciences of the USA, discusses the compensation of the functional deficit of phosphorylated tau in the event of tau-microtubule assembly, using TMAO. Chapter 4, which will be submitted to the Journal of Cell Biology for publication, describes that TMAO can enhance cellular microtubule assembly in XRL glial cells. A general summary and discussion of the entire dissertation and an outlook for further work are also offered in Chapter 5. The references cited in the general introduction and the general summary and discussion are listed in the general references.
CHAPTER 2

NATURAL METHYLAMINE OSMOLYTES, TRIMETHYLAMINE N-OXIDE AND BETAIN, INCREASE TAU-INDUCED POLYMERIZATION OF MICROTUBULES

A paper published in Biochemical and Biophysical Research Communications 250:728-730 (1998)

Huang-Chun Tseng and Donald J. Graves*

Neuroscience Program, and Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA 50011

Abstract

The natural osmolyte trimethylamine N-oxide (TMAO) at 200 mM increases the extent and the rate of formation of polymerized microtubule (MT) complex caused by tau. TMAO at this concentration has no effect on tubulin alone. Urea at
200 mM blocks tubulin assembly caused by tau, but this inhibition can be reversed by an equal amount of TMAO. Besides TMAO, betaine, another natural osmolyte, was found to have the same effects on MT as TMAO. On the contrary, glycerol (a carbohydrate osmolyte) and glycine (an amino acid osmolyte) do not increase tau-induced MT assembly. The mechanism by which TMAO and betaine enhance tau's effectiveness is not known, but physical studies suggest that the secondary structure of tau is not appreciably changed by 200 mM TMAO. This is the first report showing that natural osmolytes, TMAO and betaine, at a near physiological concentration, are able to stimulate tau-induced tubulin assembly.

Introduction

Water-stressed cells accumulate small organic molecules to maintain cellular osmotic balance and cell morphology. Those small osmolytes are generally categorized into three groups: amino acids and their derivatives, carbohydrates, and methylamines (1). Molecules of the first two groups are "compatible osmolytes" which means cells accumulate those
osmolytes to high concentrations without significantly perturbing protein functions. The third group of osmolytes is referred to “counteracting osmolytes” for their counteracting effect reversing the perturbations caused by urea.

One counteracting osmolyte is trimethylamine N-oxide (TMAO). This substance can be found at concentrations up to 0.2 M in elasmobranchs including cartilaginous fishes, such as sharks and rays (2). At this concentration, TMAO may counteract denaturation caused by urea which reaches concentrations of up to about 0.4 M in elasmobranchs. Mammalian kidneys may also accumulate urea at high concentrations. To adapt to this condition, renal medulla cells accumulate osmolytes such as betaine, sorbitol, inositol, and glycerophosphorylcholine (3). Among these, betaine, which is a methylamine compound, may counteract the denaturing effect of urea on proteins.

Urea has previously been shown to inhibit in vitro taxol-stabilized microtubule (MT) assembly (3). Interestingly, TMAO reversed this urea-induced inhibition (3). These results suggest that TMAO may help to maintain the MT cytoskeleton in urea-rich cells. But how counteracting osmolytes work is still unknown. It may be
that TMAO could force some proteins to fold and to resist protein denaturation. Previous studies showed this osmolyte could force thermodynamically unfolded ribonuclease T1 to fold. Moreover, TMAO also can restore the biological function of the chemically modified ribonuclease T1 and the mutant nuclease through forcing protein structure refolding (4).

Among several factors affecting microtubule assembly, microtubule-associated proteins (MAPs) influence the process of nucleation, polymerization and maintenance during tubulin assembly (5, 6). Tau, a MAP, is enriched in neuronal axons and assists the polymerization of microtubules. However, if tau becomes hyperphosphorylated, it will not assist MT polymerization (7). This is significant since the hyperphosphorylated form of tau is a key component in an Alzheimer’s disease hallmark, paired helical filament (8).

As mentioned above, TMAO can force proteins to fold. It is not known whether the natural osmolytes could change tau’s conformation and/or influence the strength of the interaction of tau with tubulin.

In this report, we used an in vitro system with purified porcine brain tubulin and expressed human tau protein to investigate the roles of natural osmolytes in
MAPs-tubulin assembly. We also used this system to re-evaluate the counteracting effects of natural osmolytes, including TMAO and betaine, etc., on the inhibition of assembly caused by urea.

Materials and Methods

Materials

TMAO, betaine, urea, EGTA, EDTA, MOPS, MES, GTP, and glycine were purchased from Sigma; acetonitrile, glycerol, guanidine-HCl, NaCl, and MgCl$_2$ from Fischer; dithiothreitol (DTT) from Boehringer Mannheim; protein assay reagent from Bio-Rad; and BL21 (DE3) *E.coli* cells from Novagen.

Tau Protein

A plasmid (T441-pRK172) with the longest human tau cDNA is provided by Dr. Iqbal from New York State Institutes for Basic Research in Developmental Disabilities. The plasmid is transformed into BL21(DE3) competent cells, following a procedure described in Novagen’s pET system manual. The procedure protein expression follows a method described by Huang except that the protein induction is 4 hours (9).
Cell pellet collected from 1 liter culture was suspended with 1 ml of MOPS buffer containing 25 mM MOPS, pH 6.8, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT. The cell suspension was lysed by sonication. Taking the advantage of heat stability of tau (10), the tau lysate is heated to 80°C for 5 min. Protein aggregates were removed by centrifugation at 30,000g for 30 min at 4°C. Tau was further purified by two liquid chromatographic steps. A 5-ml HiTrap SP column (Pharmacia) equilibrated with the MOPS buffer is used to isolate tau. The bound tau is eluted using a linear NaCl gradient (0-500 mM, 80 ml, in 40 min). The tau fractions are further purified with a C18 reverse-phase HPLC. A linear gradient from 0-50% of acetonitrile in water is used to bind proteins. The tau peak was collected, lyophilized and stored at -20°C.

Porcine Brain Tubulin

Fresh porcine brains were obtained from the Veterinary College, Iowa State University (ISU). Purification of porcine brain tubulin mainly followed the procedure by Williams and Lee (11). A 5-ml HiTrap SP (Pharmacia) column replaces the phosphocellulose column used by Williams and Lee. The solutions used in HiTrap chromatography are the
same as the solutions used in the Williams' method, except that β-mercaptoethanol was substituted by DTT. The flow rate in this method is 2 ml/min. Tubulins came out in the flow-through fraction. The purified tubulin proteins were quickly frozen with liquid nitrogen and stored at -70°C.

**Tubulin Assembly**

To remove tubulin aggregates, thawed tubulin was centrifuged at 15,000 g for 10 min, at 4°C. This supernatant was used for assembly. The assembly condition was 10 mM tubulin, 1 mM GTP, 1 mM MgCl₂, 1 mM EGTA, and 0.1 mM EDTA in 100 mM MES, pH 6.4 at 30°C. The concentrations of tau, osmolytes and urea are indicated in figure legends. The assembly was started by the addition of tubulin and the assembly process was monitored by following changes in absorbance at 350 nm using a Beckman DU640 spectrophotometer.

**Protein Concentration Determination**

Porcine brain tubulin was dissolved in 6 M guanidine-HCl and determined by photo-spectrometry at 275 nm. The extinction coefficient for tubulin in guanidine-HCl at 275 nm is 1.03 ml/mg/cm (12). Tau concentration of tau was
measured by the Bradford method, using an interpolation in a standard curve of tau of which concentrations are estimated with amino acid analysis. The amino acid analysis was performed at the Protein Facility of ISU.

Results

Fig. 1 shows the effect of TMAO and tau on the polymerization of tubulin. Note that when tubulin is alone or in the presence of 200 mM TMAO in the assembly buffer there is little or no change in absorbance. No apparent tubulin assembly is observed in these two conditions. However, when 0.8 mM tau is added to the solution of tubulin (10 mM) the absorbance at 350 nm changes. The absorbance change is interpreted to mean that tubulin polymerization takes place under these conditions (13). When TMAO is added to the solution containing tubulin and tau, the rate and extent of change of absorbance appears to increase suggesting that TMAO changes the tubulin assembly process.
Figure 1. The TMAO effect in tau-dependent tubulin assembly.

Tubulin is incubated with 0.8 μM tau in the assembly buffer at 30°C, without 200 mM TMAO (open circles) or with TMAO (closed circles). Tubulin without tau is incubated in the same condition, with TMAO (closed triangles) or without TMAO (closed squares).
To investigate how TMAO can affect the tau-induced polymerization of tubulin, we investigated the effects of TMAO on the extent and initial rate of tubulin polymerization at different concentrations of tau. The extent of the reaction was evaluated under the different conditions by measuring the absorbance at 350 nm after 15 min. At this time, the absorbance change levels off, suggesting that the equilibrium position has been reached.

Fig. 2A shows that in the absence of TMAO, the final change in absorbance at 350 nm can be described as a curve-linear upward line. A curvature of this type is characteristic of a cooperative process. When TMAO is added, the curve is changed. Tubulin assembly with TMAO is increased, compared with that without TMAO. When comparing the changes caused by TMAO at low concentrations of tau, we found tau at 0.2 mM has no obvious effect on MT formation, but in the presence of 200 mM TMAO, it can promote MT formation effectively. In short, TMAO-tau can stimulate more MT formation than tau.

TMAO also changes the kinetics of tau-tubulin assembly. The half time ($t_{1/2}$) of total MT formation at various tau concentrations was determined. The results show that the $t_{1/2}$ of the TMAO promoted reaction is shorter than that obtained
Figure 2. A. Effect of TMAO on the extent of tau-tubulin formation. The changes of O.D. at 350 nm were determined after 900 sec of a reaction. The square is the relationship of tau concentration and the total O.D. change which represents the amount of tau-microtubule at the equilibrium stage. The circle is the group with 200 mM TMAO.

B. Effect of TMAO on the rate of formation of microtubules. T_{1/2} was determined by measuring the time when half the amount of total microtubule was formed. The squares represent the group without TMAO, the circles, with TMAO.
without TMAO (Fig. 2B). TMAO reduces the $t_{1/2}$ of the assembly process by approximately one-half when tau is in the range of 0.4 to 0.8 mM. For example, the $t_{1/2}$ values for the reaction of 0.8 mM tau with TMAO compared to the reactions without TMAO are about 170 sec and 340 sec, respectively. Thus, the decrease of $t_{1/2}$ indicates TMAO accelerates the rate of tau-tubulin assembly.

On one hand, when comparing data in Fig. 2A and Fig. 2B, we found 0.4 mM tau in TMAO generates a similar final amount tau-MT complex as 0.85 mM tau without TMAO (Fig. 2A). Meanwhile, the $t_{1/2}$ of 0.4 mM tau with TMAO is similar to $t_{1/2}$ of 0.85 mM tau without TMAO (Fig. 2B). Above all, TMAO increases the extent of tau-induced assembly and the formation rate by increasing tau’s effectiveness. The effectiveness of tau appears to be related to the binding affinity of tau.

The mechanism by which TMAO affects tau-tubulin assembly is under investigation. TMAO has been proposed to force proteins to fold into thermodynamically favorable structures (4). We wondered whether TMAO could influence the conformation of tau. To test this, we utilized two different physical measurements, circular dichroism (CD) and photoacoustic Fourier transform infrared (FT-IR), to examine
conformational change of tau caused by TMAO. The preliminary results show no differences in signal of tau with or without TMAO (results not shown).

Next, we investigated the effect of urea on tau-induced polymerization and whether TMAO could reverse the effect of urea. Fig. 3A shows the results obtained with low concentrations of TMAO and urea on tau-induced polymerization. The controls, tubulin alone or tubulin plus TMAO again show no change in absorbance with time. Closed circles of Fig. 3A shows the effect of tau, and the closed square of Fig. 3A shows that 200 mM urea inhibits the tau-induced polymerization. With TMAO (200 mM) the largest change in absorbance is seen and that the combination of TMAO and urea gives a rate profile for what is seen for tau alone, i.e., TMAO overcomes the inhibitory effect of urea.

In addition to our studies with TMAO, we also examined the effects of betaine, glycerol, and glycine. Betaine, one natural methylamine osmolyte found in mammalian renal cells, was also found to promote the extent of assembly and reverse the urea inhibition (Fig. 3B). The effects of betaine on tau-induced assembly are similar to those seen with TMAO. It increases the extent of polymerization and also accelerates the formation of tau-tubulin assembly. Like
Figure 3. A. Effect of TMAO on the inhibition of assembly caused by urea. B. Effect of betaine on the inhibition of assembly caused by urea. The closed circles represent the tau-tubulin control; the open circles, with 200 mM osmolyte (TMAO in A, or betaine in B); the squares, with 200 mM urea. The crosses represent tau-tubulin with the combination of 200 mM osmolyte and 200 mM urea. The reverse triangles represent the tubulin-alone control; the triangles, the tubulin-osmolyte. Tau is 0.8 μM in both A and B.
Change of O.D. at 350 nm

A

B
TMAO, it does not stimulate MT formation when tau is absent. Note that the assembly with the equal combination of betaine and urea (crosses of Fig. 3B) is similar to the assembly with betaine (open circles of Fig. 3B). The rescuing effect by betaine seems more effective than that caused by TMAO when both osmolytes are at 200 mM. Other natural organic osmolytes, either 200 mM glycine, or 200 mM glycerol failed to stimulate tau-induced tubulin assembly (data not shown). In the controls for glycine and glycerol, neither of them can stimulate tubulin-alone assembly.

Discussion

Earlier, it was reported that TMAO at near physiological concentration could affect tubulin assembly but taxol, a microtubule-stabilizing agent, was needed for this effect. We observed that TMAO stimulated tau-induced tubulin assembly in the absence of taxol, but only if tau was included in the reaction mixture (Fig. 1). It has been reported that higher TMAO concentrations (> 700 mM) can stimulate tubulin-alone assembly (3). Because the assembly
stimulation needs tau, we suspect TMAO at lower concentrations could promote a tau-tubulin interaction.

In the tau-induced tubulin assembly, tau concentration determines the polymerization. In Fig 2A, the final absorbance change increases with increasing tau. The effectiveness of tau can be roughly measured from the value of absorbance change at 350 nm/tau amount which presumably represents microtubule amount/tau amount, according to the turbidity measurement theory (13). Results shows that high tau is more effective in polymerization than low tau. The effectiveness of tau is not linearly proportional to its concentrations. This suggests that tau-induced tubulin assembly is a cooperative process.

Positive cooperation could occur if the binding of tau alters the conformation of tubulin so as to promote the binding of additional tau. TMAO lessens the amount of tau needed for assembly and alters the shape of the curve (Fig. 2A). It may be that these osmolytes mimic the initial binding event of tau. This suggests that tau binding or TMAO/betaine cause a tubulin and/or tau conformational change and that this change favors tubulin assembly.
There are other possible mechanisms, which might explain how TMAO works. According to the Bolen's solvophobic model of TMAO, polypeptide backbones try to avoid contacting TMAO (4, 14). Thus, it may be that TMAO drive tau and tubulin together, i.e. increase tau affinity for tubulin. Kita proposed that TMAO may also change system entropy through affecting water surface tension on proteins (15). This entropy model could be used to explain how TMAO affects tau-induced tubulin assembly. Perturbation of water structure might happen around hydrophobic residues in either tau or tubulin. We are currently examining the relationship between TMAO, tau and tubulin.

Prior to this report, only TMAO was suggested to stimulate tubulin assembly in cartilaginous fishes (3). No molecule is known to have a TMAO-like role on microtubules in mammalian cells. We chose betaine to test because of the structural similarity of betaine and TMAO. Moreover, betaine is a natural metabolite of choline in mammalian cells. Betaine actually counteracted the effects of urea better than TMAO (Fig. 3). Furthermore, since glycerol and glycine were ineffective or in the case of glycine, inhibitory to the process of tubulin polymerization, the effects of TMAO or betaine can not be described to a simple
change in osmotic conditions. Thus, the trimethyl groups of TMAO and betaine may act to surround the hydrophobic residues of tubulin or tubulin. Since TMAO and betaine are amphipathic, these substances may also interact with hydrophilic groups or water. Although tau is not found in kidney cells, other MAPs, such as MAP2, might play the same role as tau does. Regardless of this, our report shows that betaine is capable of influencing MT assembly in mammalian cells and counteracting substances like urea.

Finally, it is known that tau loses its binding affinity to MT in Alzheimer’s disease. In the disease state, tau is hyperphosphorylated, which may contribute to microtubule disassembly and neuron death (16). Based on the effect of TMAO and betaine on our observations presented in this study, it will be important to determine the effect of TMAO or betaine on the interaction between phosphorylated tau and tubulin. These studies are currently in progress in our laboratory.
Acknowledgements

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Reference


CHAPTER 3

PHOSPHORYLATED TAU CAN PROMOTE TUBULIN ASSEMBLY

A paper accepted by Proceedings of the National Academy of Sciences of the U.S.A.

Huang-Chun Tseng*†, Quan Lu‡, Eric Henderson‡,
& Donald J. Graves*†§

*Neuroscience Program,
†Department of Zoology and Genetics,
& ‡Department of Biochemistry, Biophysics, and Molecular Biology
Iowa State University, Ames, Iowa 50011, USA

Abstract

Phosphorylation can affect the function of microtubule-associated protein tau. Here, the human brain tau with 441 amino acids was phosphorylated by cyclic-AMP-dependent
protein kinase (PKA) or glycogen synthase kinase-3β. PKA-phosphorylated tau (2.7 mol phosphates/mol) does not promote tubulin assembly as judged by spectrophotometric and atomic force microscopy measurements, unless trimethylamine N-oxide (TMAO), a natural occurring osmolyte, is included in these assays. TMAO is also found to promote tubulin assembly of glycogen synthase kinase-3β-phosphorylated tau (1.6 mol phosphates/mol). TMAO does not act by causing a chemical dephosphorylation of phosphorylated tau but it acts to overcome the functional deficit caused by phosphorylation. PKA-phosphorylated tau binds to tubulin in the presence of TMAO and lowers the critical concentration of tubulin needed for assembly. From these data we conclude that PKA-phosphorylated tau retains the ability to bind tubulin and promote tubulin assembly. TMAO is required, however, to sensitize the reaction. Possible uses of TMAO in relation to studies of tubulin assembly in vitro, in intact cells, and in relation to Alzheimer’s disease are presented in this report.
Introduction

Hyperphosphorylation or abnormal phosphorylation of tau has been proposed as one potential cause of Alzheimer's disease (1-4). It is believed that this hyperphosphorylation reduces the affinity of tau for tubulin and can contribute the self association of tau and formation of neurofibrillary tangles or paired helical filaments (PHF) (1-4), major hallmarks of Alzheimer's disease. A recent study shows, however, that some phosphorylation reactions of tau may protect it against formation of PHFs (5). At least 25 sites have been identified in the tau of paired helical filaments (PHF-tau) (6, 7). These sites can be classified as either proline-directed or non-proline-directed phosphorylation sites (6, 7). Both types of phosphorylation can affect tau's function. For instance, tau phosphorylated by either cyclic-AMP-dependent protein kinase (PKA) or glycogen synthase kinase-3β (GSK-3β) reduces its ability to stimulate the formation of microtubules (MTs) (8, 9). PKA, a non-proline-directed kinase, phosphorylates tau on Ser-214, Ser-262, Ser-316, Ser-320, Ser-356, Ser-409, Ser-416 and one of residues, Ser-235, Ser-237, or Ser-238 (5-8, 10, 12). GSK-3β, a proline-directed kinase, phosphorylates tau on
Ser-199, Thr-231, Ser-235, Ser-262, Ser-396, Ser-400, and Ser-413, most of which are in Ser/Thr-Pro motifs (6, 7, 9, 11). Activation of PKA in SH-SY5Y neuroblastoma cells can increase phosphorylation of Ser-262/Ser-356 in tau and cause the disassociation of tau from MTs (13). GSK-3β phosphorylation has been proposed to play a role in Alzheimer's disease (14, 15). A previous study showed that endogenous tau can be phosphorylated by GSK-3β in cultured human NT2N neurons (16).

Tau exists as six isoforms in the human brain and these forms are derived from a single gene by different mRNA splicing reactions (17-19). The largest brain tau containing 441 amino acids possesses two N-terminal inserts and four MT-binding repeats, but the smallest form possesses no N-terminal insert and has three MT-binding repeats instead (17, 18). The tau structure for all isoforms is enriched with proline, glycine, and hydrophilic amino acids (19). Early biophysical studies of tau using analytical centrifugation, electron microscopy, gel filtration, CD, and 1H-NMR spectroscopy suggest that tau is a highly flexible extended molecule with little secondary structure (20-25). Recent studies suggest that hyperphosphorylation with mitogen-activated protein kinase induced structure into the
C-terminal region of tau (23), and that the secondary structure of Alzheimer's disease-hyperphosphorylated tau can be changed by dephosphorylation (26). Studies by Hagestedt et al. by using paracrystal imaging showed that Ca/calmodulin protein kinase II can reduce tau's flexibility by phosphorylation (27). In addition, binding of tau to MTs can also generate some ordered structures, indicating that MTs can induce some conformational changes in tau (25).

Small molecular weight organic osmolytes found in elasmobranchs, e.g., sharks and rays, act to stabilize proteins by counteracting the denaturing effect of urea in vivo (28-31). In a recent paper, it was reported that trimethylamine N-oxide (TMAO) can force thermodynamically unfolded proteins to fold (32). RNase T1 with its disulfide bonds reduced and chemically derivatized and staphylococcal nuclease mutant protein (T62P) exist as unfolded biologically inactive structures (32). However, in the presence of TMAO these proteins become folded and biologically active. The authors state "The ability of TMAO to force thermodynamically unstable proteins to fold presents an opportunity for structure determination and functional studies of an important emerging class of proteins that have little or no structure without the
presence of TMAO" (32). A previous report of ours showed that 200 mM TMAO, a physiological concentration (28, 29), could increase the formation of tau-induced MTs and counteract inhibition of assembly caused by urea (33). On the basis of this and of the background information on tau suggesting it exists as an unfolded protein, we initiated work on the effect of TMAO on tau phosphorylated by either PKA or GSK-3β and their interactions with tubulin. The results, shown herein, provide evidence that low concentrations of TMAO can overcome the functional deficit of phosphorylation of tau by PKA or GSK-3β.

Materials and Methods

**Materials**

The plasmid of the human tau cDNA encoding 441 amino acids was provided by K. Iqbal from New York State Institutes for Basic Research in Developmental Disabilities, Staten Island, New York. PKA and GSK-3β were purchased from Sigma; *E.coli* BL21(DE3) cells from Novagen; and [γ-32P]ATP from ICN. TMAO was purchased from Sigma, recrystallized
from deionized water, dried, and stored in a desiccator at room temperature. All other reagents were reagent grade.

**Phosphorylation of Tau**

Human recombinant tau was purified as described previously (33). Pure recombinant tau (20 μM, based on a molecular mass of 45.7 kDa) was phosphorylated by kinases in the phosphorylation solution (1 mM ATP/10 mM MgCl/1 mM EDTA/0.5 μg/ml leupeptin/0.7 μg/ml pepstatin/1 μg/ml aprotinin/85 μg/ml PMSF/1 μM microcystin-LR/25 mM HEPES, pH 7.5). The concentrations of kinases in the phosphorylation reactions are 0.2 units/μl PKA for PKA phosphorylation and 0.4 units/μl GSK-3β for GSK-3β phosphorylation. The phosphorylation reactions were carried out at 30°C and terminated by heating the reaction solutions at 95°C for 10 min. The cooled phosphorylated tau was centrifuged at 10,000 X g for 10 min to remove protein aggregates, and the supernatants were ready for use in the MT assembly. Non-phosphorylated tau was treated as the same condition as phosphorylated tau, except that no ATP was added. To determine the phosphorylation stoichiometry, 1 μCi of [γ-32P]ATP was included in the
phosphorylation mixture. After incubation, a portion of reaction mixture was spotted on a ET-31 paper disc, washed with trichloroacetic acid, and analyzed by liquid scintillation counting (34). This filter paper assay was also used to evaluate dephosphorylation of phosphorylated tau by TMAO.

**Tubulin Preparation and Tubulin Assembly**

The preparation of porcine brain tubulin followed a method described by Williams and Lee (36) except that a SP-cation exchange column (high-performance SP from Amersham Pharmacia) was used rather than a phosphocellulose column (33, 36). The elution rate in the chromatography was 0.5 ml/min. The elution and collection of tubulin were the same as described previously (36). The assembly condition and the turbidity measurement were described in a previous paper (33). In brief, thawed tubulin was centrifuged at 15,000 X g for 10 min, at 4°C to remove tubulin aggregates. This supernatant was collected and used for assembly reactions. Assembly started with the addition of tubulin (8 μM, based on a molecular mass of 55 kDa) to assembly mixture which contained without/with 200 mM TMAO/with/without 0.8 μM tau/1 mM GTP/1 mM DTT/1 mM MgCl₂/1 mM EGTA/0.1 mM EDTA/100 mM MES,
pH 6.4. The assembly process was monitored by following changes in absorbance at 350 nm by using a Beckman (Beckman Dickinson) DU640 spectrophotometer. The presence of a small amount of ATP from the phosphorylation reaction did not affect tubulin assembly. The determination of tubulin-critical concentrations followed the principle described by Timasheff's laboratory (37, 38). The concentrations of tau and PKA-phosphorylated tau (2.8 mol phosphates/mol) were 0.8 μM. TMAO was 200 mM. The assembly buffer and the turbidity measurement were the same as above, except that the incubation time was 20 min. At least four different concentrations of tubulin were applied in each assembly group. A linear curve fitting method was used to obtain a line, which fits the data in the same assembly group. The x-intercepts of the fitted lines were designated to be the tubulin-critical concentrations.

Analysis of Phosphorylated Tau-Tubulin Complex

Tau was phosphorylated with PKA in the presence of [γ-\(^{32}\)P]ATP. Phosphorylated tau was incubated with tubulin or without tubulin in the assembly buffer containing 200 mM TMAO. After 15 min assembly incubation, the pellets were
collected by a centrifugation of 50,000 X g for 30 min at 25°C. The pellets were washed three times with pre-warmed (30°C) assembly buffer containing 200 mM TMAO. The washed pellets were resuspended in 50 µl cold assembly buffer (0-4°C) and analyzed for radioactivity.

**Atomic Force Microscopy (AFM)**

The assembly mixtures were incubated under the assembly condition described above, followed by a fixation with 0.8% glutaraldehyde for 30 s. Ten microliters of the fixed samples was deposited onto a disc with a freshly cleaved mica surface and incubated for 5 min, followed by gentle rinsing with 1 ml HPLC grade water. The mica discs were then dried under a lamp for 5 min, during which time the temperature of the mica surface was gradually raised to 45°C. After this, sample discs were transferred into a 55°C oven with vacuum and incubated for 24 hr. The samples were imaged in air with a NanoScope III AFM (Digital Instruments, Santa Barbara, CA) by using a Tapping Mode. The imaging conditions usually were: height and amplitude modes, Ultra levers (Park Scientific, Sunnyvale, CA), scanning rate 1.97 Hz, and < 50% relative humidity. Captured AFM images were processed and analyzed using NANOSCOPE software.
Micropipette tips used in this AFM experiment were cut off to avoid shearing.

**Protein Concentration Determination**

Porcine brain tubulin was dissolved in 6 M guanidine·HCl and its absorbance was determined by spectrophotometric measurement at 275 nm. The extinction coefficient for tubulin in guanidine·HCl at 275 nm is 1.03 ml/mg/cm (37). Tau concentration was measured by the Bradford method (39), by using tau solutions with known concentrations as standards.

**Results**

Human brain recombinant tau was phosphorylated with PKA to an extent of 2.7 ± 0.1 mol phosphates/mol. Using conditions that promote tubulin assembly, we found, like others (8), that PKA-phosphorylated tau does not promote tubulin polymerization as judged by spectrophotometric measurements (Fig. 1a). However, if 200 mM TMAO is included in the assembly mixture, turbidity increases and the rate and extent of the reaction are equivalent to that seen with
Figure 1. (a) Spectrophotometric measurement of tubulin assembly. Tubulin was incubated (30°C, 15 min) alone (solid circles) or with TMAO (solid triangles) and the assemblies were monitored by an increase in the absorbance at 350 nm. By using the same conditions, tubulin was assembled in the presence of tau (open circles), or tau and TMAO (open triangles). Finally, tubulin was assembled with PKA-phosphorylated tau in the presence (open squares) or absence (solid squares) of TMAO. The phosphorylation stoichiometry of PKA-phosphorylated tau was $2.7 \pm 0.1$ phosphates/mol. (b) SDS/PAGE analysis of tau proteins. Non-phosphorylated tau (lane 1) and PKA-phosphorylated tau with 2.8 phosphates/mol (lane 2) were analyzed in a 10% polyacrylamide SDS gel. Proteins in the gel were visualized by a Coomassie brilliant blue R staining. (c) Radioautogram of $^{32}$P-labeled tau in a SDS/PAGE analysis. Tau was phosphorylated with PKA in the presence of [$\gamma^{32}$P]ATP. Any aggregates of phosphorylated tau-TMAO (lane 1) and phosphorylated tau-tubulin-TMAO (lane 2) were collected by centrifugation and analyzed by SDS/PAGE gel.
a

![Graph showing the change of O.D. at 350 nm over time for different conditions.](image)

- **tubulin alone**
- **tau+tubulin**
- **TMAO+tubulin**
- **tau+TMAO+tubulin**
- **PKA-tau+tubulin**
- **PKA-tau+TMAO+tubulin**

b

![Western blot analysis](image)

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c

![Image of protein bands](image)
non-phosphorylated tau. The upper curve in Fig. 1a shows that stimulatory effect of TMAO by using non-phosphorylated tau, and it is similar to what was reported earlier by us (33). If the temperature is lowered in an assembly mixture, the formed MTs can disassemble (40). We found that the change in absorbance caused by the presence of mixtures of tau-tubulin or tau-tubulin-TMAO observed at 30°C could be reversed by altering the temperature to 4°C (results not shown).

SDS/gel electrophoresis profiles of tau and tau phosphorylated by PKA are shown in Fig. 1b. Non-phosphorylated tau (lane 1) migrates as a single band, and phosphorylated tau (lane 2) migrates slower as expected (8). One major band is found for phosphorylated tau. A slower moving minor band is found, indicating some heterogeneity in the phosphorylated tau preparation. However, no non-phosphorylated tau is detected by SDS/PAGE analysis. Similar results were seen with capillary iso-electric focusing electrospray ionization mass spectrometry of PKA-phosphorylated tau containing 2.6 mol phosphates/mol (12). These results strongly suggest that PKA-phosphorylated tau induces tubulin polymerization judged by the spectrophotometric measurements of Fig. 1a. Results shown
in Fig. 1a can not be explained by the presence of non-phosphorylated tau in the PKA-phosphorylated preparation.

The possibility existed that TMAO might act to cause a dephosphorylation of tau because it has been reported that TMAO can act as a nucleophile to remove phosphate from phosphorylated γ-picoline monoanion (41). To test this possibility, we generated \(^{32}\)P-labeled tau by phosphorylation with PKA and incubated phosphorylated tau with 200 mM TMAO at 30°C. After incubation of 60 min, we found no loss of radioactivity from phosphorylated tau (data not shown). Thus, the effect of TMAO on phosphorylated tau can not be explained by a chemical reaction leading to the formation of non-phosphorylated tau.

The spectrophotometric results in Fig.1a suggest that phosphorylated tau bound to tubulin in the presence of TMAO. To test this idea more directly, we incubated tubulin with \(^{32}\)P-labeled tau (2.7 ± 0.1 mol phosphates/mol) and collected the polymerized tubulin by centrifugation (Materials and Methods). This fraction contained radioactivity (30.0 picoCi) but little or no \(^{32}\)P-labeled tau sedimented in the control (0.27 picoCi). An autoradiogram of the SDS/PAGE gel of phosphorylated tau and phosphorylated tau-TMAO-tubulin is shown in Fig.1c. Only lane 2 shows detectable
radioactivity. These results suggest that PKA-phosphorylated tau associates with tubulin in the presence of TMAO, and that TMAO does not promote self aggregation of PKA-phosphorylated tau in our assembly conditions.

The change in absorbance at 350 nm shown in Fig. 1a is characteristic of the formation of MTs (37), but it is not clear what exact structures are being formed, particularly in the new reactions containing TMAO. To examine the morphology of the structures formed in the reaction mixtures, we used AFM. First, as expected from the data of Fig. 1, tubulin alone (Fig. 2a) and tubulin plus 200 mM TMAO (Fig. 2c) do not aggregate. But when tau is added, MTs are formed (Fig. 2b and d). Note there are no apparent differences in the structures of MTs formed in the absence (Fig. 2b) and presence (Fig. 2d) of TMAO. Thus, at 200 mM, TMAO has no adverse effect on the morphology of MTs. Under these conditions, the MTs are generally straight and are several-micrometers long (Fig. 2b, d, and f). In contrast, those formed by using phosphorylated tau (Fig. 2f) tend to be shorter. As expected from the spectrophotometric studies, PKA-phosphorylated tau (2.7 ± 0.1 mol phosphate/mol) plus tubulin does not make any MTs (Fig. 2e) unless 200 mM TMAO is present (Fig. 2f). The heights of all MTs in Fig. 2b, d,
Figure 2. AFM images of tubulin assembly. Tubulin (a) was assembled using standard reaction conditions (Figure 1), and fixed. The mixture was then spotted onto a mica disc, dried and scanned by AFM. Other reaction mixtures that included tau (b), TMAO (c), tau + TMAO (d), PKA-phosphorylated tau with 2.7 phosphates/mole (e), or PKA-phosphorylated tau + TMAO (f) were also scanned. The sample height is color coded as indicated by the height scale bar. Note field sizes for a, c, and e (2 μm X 2 μm) are smaller than those for b, d, and f (25 μm X 25 μm) in order to highlight features. The heights of MTs shown in b, d and f are all about 20 nm, in general.
and f are all about 20 ± 5 nm. The widths of all MTs shown above are about the same. Because of the association of $^{32}$P-labeled tau with tubulin in the presence of TMAO, we conclude that the polymerized MTs shown in Fig. 2f are caused by the combination of phosphorylated tau with tubulin.

To learn how TMAO affects MT assembly, we determined the critical concentration of tubulin needed for assembly (Fig. 3). The critical concentration of tubulin required for tubulin self assembly is about 0.8 mg/ml (Fig. 3) similar to what were reported previously (37, 38, 42). TMAO (200 mM) lowers the critical concentration of tubulin to about 0.4 mg/ml (Fig. 3), whereas that in the presence of tau is 0.1 mg/ml or TMAO plus tau is about 0.03 mg/ml (Fig. 3). Note that when PKA-phosphorylated tau is used there is no change in the critical concentration of tubulin (0.79 mg/ml), but when TMAO is added the critical concentration drops to a value of about 0.25 mg/ml (Fig. 3). This value is significantly lower than that found with TMAO and tubulin. Thus, TMAO not only lowers the concentration of tubulin needed for assembly but also sensitizes the assembly process to tau and phosphorylated tau, which was completely ineffective under these experimental conditions.
Figure 3. The determination of tubulin-critical concentrations. Six assembly groups were analyzed:

(i) tubulin alone (open circles); (ii) tubulin plus TMAO (close circles); (iii) tubulin plus tau (open triangles); (iv) tubulin, tau, plus TMAO (closed triangles); (v) tubulin plus phosphorylated tau (open squares); (vi) tubulin, phosphorylated tau, plus TMAO (closed squares).

The detailed assembly conditions are described in Materials and Methods. The lines are the results after a linear curve fitting processing for each group.
Phosphorylation of GSK-3β is known to affect tau function (9, 11, 16). In contrast to PKA, GSK-3β has a substrate preference with the sequence for Ser/Thr-Pro, which characterizes a "proline-directed" kinase (6). To examine whether TMAO can affect tubulin polymerization with proline-directed phosphorylated tau, we generated GSK-3β-phosphorylated tau and tested it with TMAO. As shown in Fig. 4a, the assembly of MTs caused by 0.8 μM GSK-3β-phosphorylated tau (1.6 mol phosphates/mol) was significantly decreased compared to non-phosphorylated tau. However, tubulin assembly with this phosphorylated tau could be promoted by the addition of 200 mM TMAO to an extent seen with non-phosphorylated tau (Fig. 4a). SDS gel electrophoresis of GSK-3β-phosphorylated tau (1.6 mol phosphates/mol) shows two major bands which migrate slower than the control non-phosphorylated tau. A small amount of non-phosphorylated tau (approximately 10%) may be present in the GSK-3β-phosphorylated sample (results not shown). On this basis and from our earlier results of the amount of non-phosphorylated tau needed for assembly (33), we conclude
Figure 4. Analysis of tubulin assembly with GSK-3β-phosphorylated tau. (a) Spectrophotometric measurement of tubulin assembly. Reaction mixtures contained tubulin alone (solid circles) or tubulin + TMAO (solid triangles). Other reactions contained tubulin in the presence of tau (open circles), tau + TMAO (open triangles), GSK-3β-phosphorylated tau (solid squares) or GSK-3β-phosphorylated tau + TMAO (open squares). The phosphorylation stoichiometry of GSK-3β-phosphorylated tau is 1.6 phosphates/mol. (b) AFM image of the reaction mixture that contained tubulin, GSK-3β-phosphorylated tau and TMAO.
a

![Graph showing change of O.D. at 350 nm over time for different conditions: tubulin alone, tau+tubulin, TMAO+tubulin, tau+TMAO+tubulin, GSK-tau+tubulin, GSK-tau+TMAO+tubulin.](image)

b

![Image showing a 25 µm scale with color gradient indicating distances from 0.0 nm to 50.0 nm.](image)
that the results of Fig. 4a can only be explained by the participation of GSK-3β-phosphorylated tau in the tubulin assembly. To correlate these data with a physical structure, we used AFM to observe the final products in assembly mixture. Again, it was found, as with PKA-phosphorylated tau, that MTs are assembled in the co-presence of GSK-3β-phosphorylated tau and TMAO (Fig. 4b). These results show that TMAO can also restore GSK-3β-phosphorylated tau-MT assembly.

Discussion

It is thought that in Alzheimer’s disease abnormal phosphorylation of tau causes tau to lose its ability to promote tubulin assembly. This functional deficit can be overcome by enzymatic dephosphorylation in vitro (1-4, 43). Yet, how might the consequence of abnormal phosphorylation be overcome in the brain in Alzheimer’s disease? Much research is being done to study which specific protein phosphorylations-dephosphorylations occur and how they are regulated in the diseased state. In this paper, we report
new findings suggesting that a small organic osmolyte TMAO can overcome the functional deficit of tau caused by specific enzymatic phosphorylation reactions. Because no dephosphorylation occurs, the effect of TMAO is unlike that caused by phosphatases acting on tau.

How might TMAO work to overcome the functional deficit of tau caused by phosphorylation? We found that in the presence of 200 mM TMAO, PKA-phosphorylated tau binds to tubulin and promotes formation of MTs (Fig. 1). TMAO can lower the critical concentration of tubulin, and the addition of PKA-phosphorylated tau lowers the concentration further (Fig. 3). No effect on critical concentration of tubulin is seen with PKA-phosphorylated tau in the absence of TMAO, suggesting that PKA-phosphorylated tau has little or no effect by itself on tubulin assembly. Studies done by Lee and Timasheff (38) showed that observed critical concentration of tubulin required for assembly is a thermodynamic indicator of assembly; the lower the critical concentration the more negative is the apparent free energy for assembly (38, 42). On this basis, a lower apparent free energy for the assembly could be expected in the presence of TMAO, PKA-phosphorylated tau, and tubulin, compared to that observed for TMAO and tubulin.
Extensive studies from Timasheff's laboratory showed that osmolytes like TMAO stabilize proteins by causing a preferential hydration of the proteins (44-47). TMAO has been found to raise the free energy of the unfolded state of proteins and to promote folding by a "solvophobic" effect on proteins (32, 47). In the presence of a cosolvent with solvophobic properties, water is preferred to surround the protein surface and the cosolvent is excluded (46). Release of bound water molecules occurring during folding or in specific association reactions (46, 47) contributes to the favorable energetics. The release of bound water was found in tubulin assembly (39) and may explain how TMAO can lower the critical concentration of tubulin for tubulin self assembly, as well as for tau-tubulin assembly.

How can TMAO change the properties of phosphorylated tau? Studies from Urry's laboratory suggest that covalently bound phosphate in elastin polypeptides can influence a conformational transition and change local interactions by influencing hydration (48, 49). Phosphate groups tend to prevent the formation of more ordered water structure around hydrophobic moieties (48). Possibly, TMAO, by influencing hydration, could alter key contacts needed for the association of phosphorylated tau with tubulin.
We found the concentration of TMAO needed for assembly is far less than that required to promote folding of unfolded proteins. Chemically derivatized RNase and a staphylococcal nuclease mutant, both of which are unfolded, requires 2-3 M concentrations of TMAO to restore structure and function of these proteins. An earlier study showed that molar concentration of TMAO affected tubulin assembly (35). In this work, we found that TMAO can act at lower concentrations (200 mM) on tau. However, TMAO at this concentration has no effect on the CD spectrum of tau (33). One possibility is that the requirement for the amount of TMAO needed to induce structure may be lower for proteins that associate with each other if one protein serves as a folding template for the other. Because tau and its phosphorylated forms are thought to be highly extended molecules with little secondary structures, tubulin may alter tau's flexibility. $^1$H-NMR studies suggest that the flexibility of tau can be changed, as shown by the fact that half of the methyl-containing residues of tau become immobilized upon tau binding to tubulin (25). Therefore, specific binding of tau with tubulin may explain why 200 mM TMAO is so effective in promoting tau-tubulin assembly.
The fact that concentrations of TMAO found in living tissues can affect tubulin assembly in vitro suggests there may be some application for studies of effects of TMAO on tubulin assembly in cells. Experiments with the XRL glial-like cell line, isolated from Xenopus retinal neuroepithelium, show that the amounts of cellular MTs are increased by adding TMAO into medium, but that they return to the basal level by removing TMAO (H.-C.T., D.S. Sakaguchi, D.B. Fulton, and D.J.G., unpublished work).

Last, both PKA-phosphorylated tau (Figs.1a and 2) and GSK-3β-phosphorylated tau (Fig. 4) can promote MT formation in the presence of TMAO and the appearances of the MTs formed in both cases are similar. Because GSK-3β is known to phosphorylate different sites than PKA in tau (6, 7), our results suggest that the effects of TMAO are not related just to one specific type of phosphorylation. Studies are in progress to define the different sites of phosphorylation with PKA and GSK-3β. Preliminary mass spectrometry studies show that Ser-214 and Ser-356 are major sites of phosphorylation in PKA-phosphorylated tau containing 2.6 moles of phosphate/mol (12). Overall, our studies suggest that TMAO may be used as a tool to study microtubule-
associated protein-induced MT assembly and MT disassembly in relation to neurodegenerative disease, such as Alzheimer’s disease, which may be caused in part by tau phosphorylation.

Footnotes

Abbreviations: MT, microtubule; PKA, cyclic-AMP-dependent protein kinase; GSK-3β, glycogen synthase kinase-3β; AFM, atomic force microscopy; TMAO, trimethylamine N-oxide.
§To whom reprint requests should be addressed. e-mail: djgraves@iastate.edu

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References


Methylamine osmolytes are enriched in urea-rich cells and can promote microtubule assembly in vitro (1). It is unclear, however, whether methylamines can promote microtubule assembly in cells. We tested trimethylamine N-
oxide (TMAO), a methylamine osmolyte, and examined its effects on the microtubule assembly of the XRL glial cell line, using immunocytochemistry. The results of immunofluorescent staining showed that an increase in microtubule assembly occurred when culture media contained 200 mM TMAO, and this increase in assembly could be reversed by removal of TMAO from media. The increase in tubulin assembly is not caused by an increase in the biosynthesis of tubulin as shown by an immunoblotting assay. The change of microtubule assembly was correlated with the concentrations of intracellular TMAO, analyzed by $^1$H-NMR spectroscopy. The intracellular concentration of TMAO reached 30-50 mM after 6-hour incubation with 200 mM TMAO. During this time period microtubule assembly increased significantly. A spectrophotometric assay for microtubule assembly in vitro showed that 50 mM TMAO affected microtubule assembly, supporting the observations in cells. TMAO did not affect F-actin fibers as judged by fluorescent staining with rhodamine-phalloidin. Glycerol, another organic osmolyte, did not increase microtubule assembly of XRL cells. These results indicate that the methylamine osmolytes, such as TMAO, can specifically enhance microtubule assembly in cells.
Introduction

Cells accumulate organic osmolytes to maintain intracellular osmotic balance in hypertonic environments. Three types of organic osmolytes are known to be accumulated by cells: polyols (such as glycerol), amino acids and amino acid derivatives, and methylamines (2). The first two types of osmolytes are called compatible osmolytes because high concentrations of these osmolytes do not alter proteins' functions (2, 3). Methylamine osmolytes can counteract the denaturation effect of urea on proteins and, therefore, are also called counteracting osmolytes (2-4).

Methylamine osmolytes are commonly found in many urea-rich tissues, such as the muscle of elasmobranchs (2, 4), coelacanth (2, 4), moridase (5), macrouridae (5), and the mammalian kidney (6). The concentrations of urea and methylamines form an approximately 2:1 ratio in many marine animals (2, 4). In elasmobranchs, the concentration of trimethylamine N-oxide (TMAO), a methylamine osmolyte, can be as high as 200 mM in which the concentration of urea is approximately 400 mM (2, 4). Several enzymatic kinetics studies have shown that when urea and TMAO are present in a 2:1 ratio, TMAO can offset the urea protein perturbations on
creatine kinase, pyruvate kinase, argininosuccinate lyase, and lactic dehydrogenase (4). TMAO has been reported to behave as a chemical chaperon (3). TMAO can force chemically modified RNase and mutant RNase, two inactive, unfolded proteins, to fold and to become active (7).

Microtubules are cytoskeletal structures that are formed by polymerizing tubulin dimers and microtubule-associated proteins (MAPs) (8). The assembly-disassembly of microtubules is sensitive to many factors, including the concentration of tubulin, temperature, pH, ions (9), hydrostatic pressure (10), mitotic poisons (such as colchicine) (11), and MAPs (8). Microtubule assembly is inhibited by urea both, in vitro (12) and in vivo (13). How urea-rich cells protect their microtubules from the deleterious effects caused by urea is not yet clear.

One earlier study shows that the osmolyte TMAO can uniquely promote tubulin assembly in vitro (14). Paclitaxel-stabilized tubulin assembly is inhibited by glycine, sarcosine, taurine, glycylcycine, trimethylamine, or betaine (14). Betaine is a methylamine osmolyte widely found in mammalian cells, such as renal cells (6). It is presently unknown how renal cells maintain microtubule structure under the condition of high urea and high
osmolytes but in the absence of TMAO. A recent report showed that TMAO and betaine exhibit a similar enhancement on the assembly of MAP tau-tubulin and counteract the inhibition of urea in vitro (1). That enhancement of tau-tubulin assembly occurs in the presence of 200 mM TMAO or betaine without any microtubule-stabilizing drug (1). These results suggest that MAPs play important roles on methylamine-enhanced microtubule assembly. MAPs are ubiquitous in cells. On these bases, we hypothesize that methylamine osmolytes, such as TMAO, at physiological concentration could promote microtubule assembly in cells. In the present report we confirm this hypothesis with observations in intact cells.

For these studies we chose the XRl cell line. These cells were originally isolated from Xenopus retinal neuroepithelium (15). XRl cells are glial in nature based on their immunoreactivities with antibodies directed against glial fibrillary acidic protein and vimentin, two glial cell markers (15). XRl cells also promote neurite outgrowth from embryonic retinal explants (15). The microtubules and F-actin of XRl cells were analyzed by immunocytochemistry and fluorescent phalloidin staining, respectively. The intracellular TMAO concentration was determined by $^1$H-NMR
spectroscopy. The specificity and the mechanism of the TMAO-induced assembly reaction are examined in this report.

Materials and Methods

Media and Solutions

XRl cells were cultured in a control medium composed of 60% L15 medium supplemented with 10% fetal bovine serum and Xenopus embryo extract [L15 media purchased from Sigma, MO; fetal bovine serum, Upstate Biotechnology Inc., NY; the preparation of embryo extract, (15)]. Phosphate-buffered saline (PBS) was comprised of 136 mM NaCl, 2.68 mM KCl, 10.4 mM Na₂PO₄, and 1.76 mM KH₂PO₄, at pH 7.4. Phosphate buffer (PB) was made of 0.1 M sodium phosphate, pH 7.4. Blocking solution was made of 5% goat serum (Sigma, MO), 0.4% bovine serum albumin (Sigma, MO) and 0.2% Triton X-100 (Fisher Scientific, NH) in PBS. Hank’s dissociation solution was made of 5.37 mM KCl, 0.44 mM KH₂PO₄, 10.4 mM Na₂HPO₄, 137.9 mM NaCl, 9.0 mM D-glucose, and 0.04 mM Phenol Red in Sigma double-processed tissue culture water, pH 7.4, supplemented with 2% fungibact (Sigma, MO), 1% penicillin/streptomycin (Sigma, MO), 526 mM EDTA (Fisher
Scientific, NH) and 0.05% trypsin (Sigma, MO), pH 7.4. TMAO (Sigma, MO) was recrystallized in deionized water, dried, and stored in a dessicator. TMAO stock solution was dissolved in a 60% L15 solution and sterilized by filtration with a 0.22 μm membrane (Fisher Scientific, NH). The glycerol stock solution was prepared by dissolving glycerol (Fisher Scientific, NH) in 60% L15 media followed by a membrane filtration. Glycine (Sigma, MO) and NaCl stock solutions were prepared similarly. All osmolyte stock solutions were freshly prepared immediately before experiments and the pHs were adjusted to 7.4 with diluted NaOH or HCl. The buffer used in nuclear magnetic resonance (NMR) spectroscopic measurement was made of 100 mM KCl, 30 mM NaCl, 5 mM NaH₂PO₄/Na₂HPO₄, and 1 mM MgSO₄ to mimic the cell cytosol (16).

Cell Culture

The XR1 cell line was cultured in the control culture medium at room temperature in air. The XR1 cells were seeded onto 12-mm glass coverslips (Fisher Scientific, NH) prior to the experiments. To seed XR1 cells, subconfluent XR1 cells were detached from culture flasks by incubating in dissociation solution for 10 min at room temperature with
gentle shaking. Detached cells were collected by centrifugation (1,000 \( \times \) g for 10 min) and resuspended in control culture media. The XRI cells were seeded onto acid-washed glass coverslips coated with ECL (Upstate Biotechnology Inc., NY) at a concentration of 10 \( \mu \)g/ml.

Experiments used confluent XRI cells. The XRI cell coverslips were transferred into control media or experimental media containing TMAO, glycerol, or NaCl to start the experiments. Incubations were carried out at room temperature in air.

**Immunocytochemistry**

After experimental treatments (as described in text and legends), XRI cells were fixed with 4\% paraformaldehyde (Fisher Scientific, NH) in PB for at least 20 min, followed by three 10-min washes with PBS. The fixed cells were subsequently incubated in blocking solution for 30 min.

Microtubule cytoskeletons of XRI cells were labeled using E7, a monoclonal antibody directed against \( \beta \)-tubulin (Developmental Studies Hybridoma Bank, Univ. of Iowa, Iowa City, IA) overnight at 4\(^\circ\)C. The working concentration of E7 was at a dilution ratio of 1:20, diluted in blocking solution. The labeling of tubulin primary antibodies was
visualized by the application of Alexa 488-conjugated goat-anti-mouse secondary antibodies (Molecular Probes, OR). The Alexa 488-conjugated secondary antibodies were diluted in blocking solution at a dilution ratio of 1:200. Finally, the actin cytoskeleton was visualized in cells previously labeled with the anti-β-tubulin antibody using rhodamine-conjugated phalloidin (1:400, Molecular Probes, OR) for 20 min, in the dark. Cells were then rinsed extensively in PBS. Stained cells were mounted onto microscope slides with Vectashield (Vector Labs, CA) and visualized by fluorescence microscopy.

**Microscopy and Digital Imaging System**

Fluorescent staining patterns in XR1 cells were visualized with a Nikon Microphot FXA (Nikon, Japan) using a 10X subjective, a 20X air objective, or a 40X oil immersion objective. Images were captured with a Kodak Megaplus 1.4 CCD camera (Kodak Corp., CA) connected to a Perceptics MegaGraber framegrabber (Perceptics Corp., TN) in a Macintosh 8100/80 AV computer (Apple Computer, Cupertino, CA) with NIH Image 1.58VDM software (Wayne Rasband, National Institutes of Health, Bethesda, MD; obtained at FTP site zippy.nimh.nih.gov). To hinder subjectivity in the
interpretation of fluorescent images, four samples were processed at each condition in blind experiments (i.e., the experimenter did not know the condition when examining the sample and taking pictures). Image enhancement was performed on a Macintosh PowerPC 7300/200 (Apple Computer Co., CA) using Adobe Photoshop 4.0 (Adobe Systems Inc., CA). The images were printed on photo paper in 1,440 X 720 dot per inch (dpi) resolution using an SP700 printer (Epson America, Inc., CA) for this publication.

NMR Spectroscopy

XR1 cells with different TMAO treatments were washed twice with 5 ml of the control media and detached from the culture flask in 10 ml of the dissociation solution. Cell pellets were collected by centrifugation of 1,000 X g for 10 min. Cells were resuspended and homogenized in the NMR buffer solution. Centrifugation of 20,000 X g for 10 min was used to separate the cytosol (the supernatant) and the membrane fraction (the pellet).

All \(^1\text{H}-\text{NMR}\) spectra were recorded on a Bruker DRX500 spectrometer (Bruker Instrument, MA) operating at 499.865 MHz and equipped with a 5-mm \(^1\text{H}/^{13}\text{C}/^{15}\text{N}\) TXI probehead. All spectra, including those of whole cells, were recorded of
0.4 ml samples in an aqueous buffer (see Media and Solutions) with 10% added D$_2$O (for the deuterium lock channel) in standard 5-mm NMR tubes (Wilmad Glass, NJ). External 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) (Sigma, MO) was used as the chemical shift reference. Spectra were recorded at 25° C using the spectrometer's temperature control unit. The solvent signal was suppressed by setting the RF frequency on resonance with water and employing a 40-Hz presaturation pulse during each relaxation delay. The acquisition parameters were sweep-width = 6000 Hz, scans = 16, relaxation delay = 2 s, excitation pulse-width = 12 microsec, and FID size = 8192 complex points. The processing parameters were exponential line broadening = 2 Hz and final spectrum size = 16384 real points.

The concentration of cytosolic TMAO was determined by quantitative $^1$H-NMR. A cytosol sample was prepared from a fixed number of cells that were treated with 200 mM TMAO for 6 hours. NMR measurements were performed using a 5-mm sample tube fitted with a stem coaxial insert (Wilmad Glass, NJ) containing 5 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in D$_2$O that served as an external intensity reference. Spectra were collected from the cell sample and a 10-mM TMAO standard solution under identical conditions. In each case the integral of the TMAO methyl signal was normalized to the
integral of the DSS methyl signal. The total cytosolic TMAO concentration was calculated from the ratio of the normalized TMAO integrals. The cell volume fraction in the NMR sample was estimated from the volume increase (20 μl) observed after the addition of buffer solution (400 μl) to the cell pellet. The intracellular TMAO concentration was estimated by dividing the total cytosolic TMAO concentration by the cell fraction.

**In Vitro Tubulin Assembly**

The expression and preparation of the recombinant human tau were described in a previous study (1). The preparation of porcine brain tubulin followed a method described by Williams and Lee (17) except that an SP-cation exchange column (high performance SP from Amersham Pharmacia) was used rather than a phosphocellulose column (1). The elution and collection of tubulin were the same as described previously (18). The assembly condition and the turbidity measurement were described in a previous paper (1). In brief, thawed tubulin was centrifuged at 15,000 × g for 10 min, at 4°C, to remove tubulin aggregates. This supernatant was collected and used for assembly reactions. Assembly was initiated by the addition of tubulin (8 μM, based on a
molecular weight of 55 kDa) to the assembly mixture without/with TMAO (50 mM, 100 mM, or 200 mM), with/without 0.8 μM tau (based on a molecular weight of 45.7 kDa), 1 mM GTP, 1 mM DTT, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA, and 100 mM MES, pH 6.4. The assembly process was monitored by following changes in absorbance at 350 nm using a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., CA).

**Immunoblot and Desitometric Analysis**

XR1 cells were homogenized and sonicated in NMR buffer solution at 4°C. The homogenates with 20 μg proteins were mixed with SDS-loading buffer, heated under 95-100°C for 5 min and loaded onto a 10% polyacrylamine SDS-PAGE gel. The resolved proteins were electroblotted onto a polyvinylidene difluoride (PVDF)(Bio-Rad, CA) membrane. The PVDF membrane was immunoblotted with the E7 monoclonal antibody directed against Xenopus β-tubulin, followed by incubation with horseradish peroxidase-conjugated anti-mouse antibodies and visualized using the Immun-Blot assay kit (Bio-Rad, CA). The color-developed PVDF membrane was dried and scanned by a GS-670 imaging densitometer (Bio-Rad, CA). The scanned images were analyzed by the Molecular Analyst version 1 (Bio-Rad, CA) software package.
Results

**Microtubule Assembly in XR1 Cells Is Increased by Bath Application of TMAO**

The XR1 glial cells were grown in TMAO-containing media to examine the effects of TMAO on microtubule assembly. XR1 cells were incubated in 100 mM or 200 mM TMAO prepared in culture media for 1, 3, or 6 hours. Cellular microtubules of XR1 cells were analyzed using immunocytochemical procedures with anti-β-tubulin antibodies followed by Alexa 488-conjugated secondary antibodies. The tubulin-labeling images are shown in Fig. 1. The visualized images are microtubule structures.

We found that the immunofluorescent intensities were stronger if cells were incubated in TMAO-containing media (Fig. 1a-c and 1e-g) compared with those in control cells (Fig. 1i-k). At the same incubation time, the relative intensities increased with an increase of TMAO in the media (Fig. 1). We interpret this intensity increase in tubulin immunofluorescence as evidence of more microtubules in the cells. The increase of microtubule assembly appeared not only in the microtubule-organizing centers in cells but also in other subcellular locations (Fig. 1c, g, and k).
Figure 1. Immunofluorescent analysis of microtubules in XRl cells. Cells in Fig. 1a-d were treated with 200 mM TMAO media; cells in Fig. 1e-h, 100 mM TMAO media; cells in Fig. 1i-l, control media. Cells in a, e, and i were incubated for 1 hour in TMAO before fixation; cells in b, f, and j, 3-hour incubation; cells in c, g, and k, 6-hour incubation. Cells in d, h and l had 6-hour incubation with TMAO or control media and were removed to fresh control media for an additional 18-hour incubation in the absence of TMAO. Calibration bar, 50 μm.
200 mM TMAO
e 100 mM TMAO
Control

1h

3h

6h

6h + 18h
Furthermore, increasing the incubation time in TMAO resulted in an increase in the relative tubulin immunofluorescent staining (Fig. 1a-c and 1e-g). The formed microtubules increased up to 6 hours, which was the longest incubation time in this study. If TMAO-treated cells were moved to a control culture medium (containing no TMAO) and incubated for another 18 hours, the labeling intensities (Fig. 1d and h) decreased to a basal level (Fig. 1l). But, the cells in Fig. 1d still had a stronger immunofluorescent staining than did the control cells (Fig. 1l). Usually, the immunofluorescent staining for cells under the conditions of Fig. 1h was the same as that in Fig. 1l. The apparently strong staining is due to more cells in Fig. 1h. These results show that TMAO treatment is able to promote cellular microtubule assembly in XRL cells.

During treatments with TMAO, we did not find any change in the cell growth rate or notable cell death. TMAO-treated cells (Fig. 1f) continued to perform cell division at the same rate as control cells. In addition, there was no obvious change in cell volume for TMAO-treated cells. Occasionally, smaller cells were found in TMAO-treated cultures. However, a difference in cell size under hypertonic solution is expected. With 400 mM TMAO in the
culture medium we found that most cells had shrunk and detached from the substrate (data not shown).

**Intracellular TMAO Determination**

As 200 mM TMAO was able to stimulate microtubule-associated protein-microtubule assembly in vitro (1), it was hypothesized that the increase of microtubule assembly shown in Fig. 1 may be due to the presence of TMAO within XR1 cells. To analyze for TMAO, we used $^1$H-NMR spectroscopy to detect the methyl group signal of TMAO. The cytosol fractions isolated from cells in Fig. 1a, c, d, and k were analyzed. But the medium in the cells in Fig. 1k, or the control cells, was replaced with a 200 mM TMAO medium and was incubated for one min before being harvested. The purpose of this one-min TMAO treatment for control cells was to rule out the possibility of TAMO contamination in the cytosol fraction during sample preparation.

We found that the proton signal of the methyl group in TMAO appeared in the cytosol sample prepared from TMAO-treated cells (Fig. 2a, b, and c), based on the methyl proton chemical shift in the TMAO reference (Fig. 2e). The TMAO signals in Fig. 2a, b, and c can not be attributed to contamination because the control cells with one-min TMAO
Figure 2. $^1$H-NMR spectra of the cytosol fractions of XR1 cells. The cytosol fractions were isolated from XR1 cells with or without 200 mM TMAO treatment. The collected cells were homogenized in an NMR buffer with pH 7.4. After centrifugation, the cytosol fractions were analyzed using $^1$H-NMR spectroscopy (see Materials and Methods for details). a. The spectrum of the cytosol sample of XR1 cells treated with 200 mM TMAO for 1 hour; b. 6 hours; c. XR1 cells incubated in fresh control medium subsequent to TMAO treatment (200 mM TMAO, 6 hours); d. the spectrum of the cytosol fraction from control cells (see text for the detail treatment); e. the reference spectrum of TMAO in an NMR buffer, pH 7.4; f. a reference spectrum of trimethylamine in an NMR buffer.
treatment did not have the TMAO signal (Fig. 2d). The TMAO signal was the strongest peak in the spectra a, b, and c, except for water (whole spectra not shown). The intracellular TMAO did not convert to trimethylamine for which the methyl proton chemical shift is 2.93 ppm (Fig. 2f). Some weak proton signals around 1.5 ppm are due to other intracellular compounds. The nearly identical strengths of those signals indicate that the cytosol samples (Fig. 2a, b, c, and d) were prepared from a similar number of cells. Therefore, changes in the signals seen for TMAO reflect changes in the concentration of TMAO in cells but not differences in the number of cells. The membrane fractions of TMAO-treated cells (6 hours) have only a very weak signal at 3.3 ppm (data not shown). That weak signal may be explained as TMAO in membrane, TMAO contamination, or other compounds. Therefore, we conclude that the observed TMAO was predominately present inside of the XR1 cells.

The intensity of the TMAO methyl signal in Fig. 2b is stronger than that in Fig. 2a. The increase in TMAO signal intensity indicates that the intracellular TMAO concentration increased with time. Meanwhile, the labeling intensity for tubulin in Fig. 1c was stronger than that in Fig. 1a. When examining the cytosol sample prepared from
cells shown in Fig. 1d, we found that there was a TMAO peak in the spectrum (Fig. 2c). These results suggest that the increase of cellular microtubules is related to the concentration of TMAO. The observation that microtubules in Fig. 1d did not return fully to the basal level can be explained by the residual TMAO in cells.

The intracellular TMAO concentration of the cells in Fig. 1c was also determined by $^1$H-NMR spectroscopy (see Materials and Methods section). We used 5-mM DSS as an external reference, which was held in a coaxial insert tube. The DSS coaxial insert tube was inserted into an NMR tube containing either the cytosol sample or a 10-mM TMAO standard. The integrals of cytosolic TMAO, TMAO reference, and DSS reference in NMR spectra (data not shown) were used to estimate the intracellular TMAO concentration. The integral ratio of the TMAO standard to DSS is 26.35. The integral ratio of the cytosol TMAO to DSS is 4.251. Therefore, the concentration of the cytosol TMAO NMR sample is 1.6 mM. That is, the preparation of the cytosol NMR sample recovered 0.68 μmoles of TMAO from 2 x $10^6$ cells. We estimate that the intracellular TMAO concentration is approximately 30-50 mM, based on the amount of recovered TMAO and the wet cell volume for 2 x $10^6$ cells (see Materials
and Methods section). The rate of increase of intracellular TMAO was estimated as 5-8 mM/hour. The rate of removal of intracellular TMAO was approximately 0.5-0.8 mM/hour.

**Concentrations of TMAO Influences the Microtubule Assembly**

Because the TMAO concentration and the increase of microtubule assembly were correlated in vivo (Figs. 1 and 2), TMAO concentrations should also affect microtubule assembly in vitro. We applied different amounts of TMAO into an in vitro assembly mixture containing purified porcine brain tubulin and expressed human tau. The microtubule assemblies were monitored by spectrophotometric measurements, shown in Fig. 3.

The polymerization of tubulin and tau was monitored by the increase of O.D. at 350 nm (Fig. 3). In our assembly conditions, tubulin alone and tubulin plus 200 mM TMAO did not polymerize unless tau was present (Fig. 3). When the tau-tubulin mixture included 50 mM TMAO, microtubule assembly increased (Fig. 3). With TMAO in the assembly mixture, more microtubules were formed (Fig. 3). That is, the TMAO concentrations affected the increase of tau-tubulin assembly in vitro. This result verifies that TMAO concentration and microtubule increase are related and
Figure 3. Spectrophotometric measurement of tubulin
assembly. Tubulin was incubated (30°C, 15 min)
alone (open circles), or with 200 mM TMAO (open
squares) or with tau (closed squares) and the
assemblies were monitored by an increase in the
absorbance at 350 nm. Using the same conditions,
tubulin plus tau were assembled in the presence of
50 mM TMAO (crosses), 100 mM TMAO (closed
triangles), or 200 mM TMAO (closed circles). The
details of the assembly conditions were described
in the Materials and Methods section.
Change of O.D. at 350 nm vs. Time (sec)

- tubulin+tau
- 200 mM TMAO+ tubulin+tau
- 100 mM TMAO+ tubulin+tau
- 50 mM TMAO+ tubulin+tau
- 200 mM TMAO+ tubulin
- tubulin alone
suggests a direct role for TMAO in the increase of cellular microtubules.

**TMAO Does Not Change Total Tubulin in Cells**

Hypertonic shock can induce intracellular alkalinization (19). Alkalinization in Xenopus oocytes results in an increase of protein synthesis (20). Tubulin synthesis might be affected by the presence of TMAO and affect microtubule assembly through this alkalinization mechanism. We examined the total β-tubulin in TMAO-treated cells (200 mM TMAO, 6 hours) with an immunoblotting assay. A fixed number of cells with or without TMAO treatment was analyzed. The results of densitometric measurements for total area intensity and peak intensity are shown in Table 1. The total and peak intensities for both blots are similar (Table 1). This immunoblotting result suggests that total cellular tubulin amounts are unchanged or slightly decrease in TMAO-treated cells. Therefore, it is unlikely that the change in microtubule assembly is due to an increase in total cellular tubulin.
Table 1. Densitometric analysis of β-tubulin immunoblots.

The proteins in cell extracts of XR1 cells with/without TMAO treatment (200 mM, 6 hours) were separated by electrophoresis and blotted onto a PVDF membrane. After color development, the PVDF membrane was scanned by a GS-670 imaging densitometer. The scanned images were analyzed using the software of the Molecular Analyst version 1.

<table>
<thead>
<tr>
<th>Cell Extracts</th>
<th>Area</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMAO-Treated Cells</td>
<td>22.7</td>
<td>0.20</td>
</tr>
<tr>
<td>Control Cells</td>
<td>24.6</td>
<td>0.21</td>
</tr>
</tbody>
</table>
**F-actin Organization Remains Intact in TMAO-Treated Cells**

Inasmuch as TMAO is capable of assisting the polymerization of microtubules, we investigated whether TMAO could act on another cytoskeletal assembly system, F-actin. Hypertonic osmotic shock induced by salts is known to disturb the F-actin cytoskeleton of C6 glial cells (21). We analyzed cellular F-actin of XR1 cells using rhodamine-conjugated phalloidin that binds F-actin.

Results showed that the F-actin fibers in TMAO-treated cells (200 mM TMAO, 6 hours) do not exhibit a significant difference in organization (Fig. 4a and b) compared with those in control cells (Fig. 4c and d). The F-actin stress fibers in both TMAO-treated cells and control cells are relatively straight. Many fibers extend from one side of the cells to the other side. We did not observe any significant decrease in the length of F-actin fibers as was reported earlier in hypertonic shocked cells (21). The distributions of stress fibers and cortical fibers was similar in both TMAO-treated cells and control cells. The diameters of all fibers appeared to be uniform. There was no obvious change in the amount of F-actin in TMAO-treated cells as assessed by rhodamine fluorescent staining although we did not analyze the relative amounts of G-actin and F-
Figure 4. Fluorescence images illustrating rhodamine-phalloidin labeling in XR1 glial cells. XR1 cells in a and b were treated with 200 mM TMAO for 6 hours. c and d are photomicrographs of control cells. Images in a and c were captured using a 20X objective. Images in b and d are at higher magnification (40X objective). The calibration bars in a and b correspond to 50 μm and 25 μm, respectively.
actin using biochemical analysis. The rhodamine-phalloidin staining results suggest that TMAO does not cause any apparent change in cellular F-actin.

Glycerol Does Not Increase Cellular Microtubule Assembly and F-actins

To determine whether the microtubule increase is specific to TMAO, we tested glycerol on XR1 cells. Glycerol is a polyol osmolyte and is well known to stabilize microtubules in vitro (22, 23). Microtubules and F-actin of glycerol-treated cells (200 mM, 6 hours) were examined, using the fluorescent staining procedures as described previously. The fluorescent staining images are shown in Fig. 5.

Immunofluorescent staining results show that there is a decrease in the β-tubulin immunostaining between glycerol-treated cells (Fig. 5a) and control cells (Fig. 5b). The actin organization in glycerol-treated cells (Fig. 5c) is similar to that in control cells (Fig. 5d). The F-actin staining in some glycerol-treated cells is somewhat weaker than that in control cells. The diameters of F-actin fibers in both cells are also similar. Nevertheless, we conclude that the osmotic shock induced by 200 mM glycerol does not
Figure 5. The immunofluorescent labeling of XR1 cells by E7 antibodies against β-tubulin (a, b) or rhodamine-conjugated phalloidin (c, d). a and c are photomicrographs of XR1 cells treated with 200 mM glycerol-medium for 6 hours; b and d, cells treated with control media. All images were captured using a 40X oil immersion objective. Calibration bar, 25 μm.
increase microtubule assembly and has no effect on actin organization in Xr1 cells.

Discussion

The results shown in the previous section support our hypothesis that TMAO at physiological concentrations can promote cellular microtubule assembly.

TMAO increases the rate and extent of microtubule formation in vitro, suggesting a thermodynamic role for TMAO (1). Lee and Timasheff proposed that tubulin-critical concentrations could be treated as thermodynamic indexes for assembly (22, 23). Determination of the tubulin-critical concentration shows that TMAO can lower the critical concentrations for tubulin self-assembly and microtubule-associated protein tau-tubulin assembly in vitro (18). This means that TMAO drives the microtubule-associated protein-microtubule assembly process forward. Here, we have demonstrated that there is an increase in cellular microtubules (Fig. 1) in the presence of intracellular TMAO (Fig. 2). These results suggest that intracellular TMAO can generate an environment that favors the formation of
microtubules by lowering the tubulin-critical concentration in cells.

The intracellular TMAO concentration of cells in Fig. 1c is estimated to be within a range of 30-50 mM (see Results section). This concentration range is well below the TMAO concentration in the culture medium. The longest incubation time with TMAO described in this report was 6 hours. Cells may accumulate additional TMAO after 6 hours. The accumulation of organic osmolytes, such as betaine, usually takes hours (6). The observed accumulation of TMAO in XR1 cells was also slow, as was the removal of intracellular TMAO. The mechanism for TMAO translocation remains unclear. Nevertheless, intracellular TMAO with 30-50 mM is clearly sufficient to promote microtubule assembly (Fig. 1c). This assembly stimulation by a low TMAO concentration can be reproduced in a tau-tubulin assembly system (Fig. 3), but not in the tubulin self-assembly system (14). These results suggest that the assembly stimulation caused by low concentrations of TMAO in cells is being assisted by MAPs, such as tau. Besides, TMAO concentration as high as 200 mM can be found in many marine animals (2). It can be expected that TMAO in those animals has a
significant effect on cellular microtubules to counteract the deleterious effect of urea.

Betaine is an important osmolyte in renal medulla (6) and is structurally similar to TMAO. Betaine can counteract the denaturing effects caused by urea (24, 25). Although betaine does not promote tubulin self-assembly (14), it does promote MAP-tubulin assembly in vitro (1). Because MAPs are ubiquitous in cells, it is possible that betaine can promote cellular microtubule assembly by a mechanism similar to TMAO.

Hypertonic shock induced by 400 mOsm NaCl can affect F-actin in C6 glial cells (21). We also found that XRL glial cells detached from culture substrates containing an additional 100 mM NaCl, implying an abnormality in F-actin (data not shown). The intact organization of F-action in Fig. 4, however, indicates that TMAO does not induce the disassembly of F-actin. Osmotic shock induced by glycerol does not increase microtubule assembly (Fig. 5a). In many cases, the amount of microtubules in glycerol-treated cells is less than that in control cells (Fig. 6a and b). The microtubule increase caused by TMAO can not be explained simply by common hypertonic shock.
Hypertonic osmotic stress with salts can activate stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (26), and p38 mitogen-activated protein kinase (MAPK) (26, 27) cascades in kidney cells. The activation of SAPK and MAPK is also seen in PC12 cells with salt hypertonic shock (28). MAPKs are believed to be involved in cytoskeleton reorganization (29). The interphase-M phase transition of microtubule dynamics can be induced by the addition of purified *Xenopus* M phase MAPK to interphase extracts (30). Microtubule-associated protein 2 is a good substrate for MAPKs (29). p38 MAPK and stress-activated kinase/c-Jun kinase can phosphorylate the MAP tau (31-33). Hyperphosphorylation or abnormal phosphorylation of tau is believed to lead to disruption of microtubules that may occur in Alzheimer’s disease (34-37). Hypertonic osmotic stress can increase phosphorylation of tau and affect microtubule dynamics in SH-SY5Y neuroblastoma cells (38). These results suggest that phosphorylation on tau may also increase in XRl cells with a hypertonic stress. Recently, Tseng et al. demonstrated that TMAO was able to compensate for the functional deficit of phosphorylated tau to promote tubulin assembly in vitro, using spectrometric and atomic force microscopy measurements (18). We propose that XRl
cells with TMAO hypertonic shock may have higher SAPK and/or MAPK activities that will result in the phosphorylation of MAPs. In addition to assembly caused by non-phosphorylated microtubule-associated protein-tubulin assembly, intracellular TMAO may also promote tubulin assembly by phosphorylated MAPs in TMAO-treated cells. But the determination of kinase activities and the phosphorylation of MAPs are needed to verify this speculation. Studies on the effects of protein phosphorylation of MAP on tubulin assembly in these cells are currently being carried out in our laboratory.

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CHAPTER 5

GENERAL SUMMARY AND DISCUSSION

Urea-rich cells contain high concentrations of methylamine osmolytes to counteract the effects of urea (53-56). Urea inhibits microtubule (MT) assembly in vivo and in vitro (65, 66). Before my research reported in this dissertation, little was known about what strategy urea-rich cells may use to maintain their microtubules in the presence of high concentrations of urea.

In this dissertation, results in Chapter 2 show that methylamine including TMAO and betaine can stimulate microtubule-associated protein (MAP) tau-tubulin assembly and counteract the effects of urea on tau-tubulin assembly. The concentration of TMAO used in the in vitro experiments is at a physiological level, 200 mM, found in elasmobranchs (53, 54). This concentration of TMAO alone did not promote tubulin assembly at a tubulin concentration of 1 mg/ml but needs the presence of tau in order to show its effect. TMAO changes the rate and extent of tau-tubulin assembly, suggesting a change in energetics of assembly. This suggestion is supported by a critical concentration determination in Chapter 3. TMAO is able to lower the
tubulin-critical concentrations for either tubulin self-assembly or tau-tubulin assembly. Free energies for tubulin assembly and tau-tubulin assembly are lowered by the presence of TMAO because the tubulin-critical concentration is related to the free energy of the assembly (shown as the following equation) (67).

\[ \Delta G = -RT \ln (Cr)^{-1} \]

\( \Delta G \) standard free energy

R: gas constant  \( T \): absolute temperature

Cr: tubulin-critical concentration

The increase of MTs caused by TMAO also occurs in cells, shown in Chapter 4. The MTs of XR1 glial cells are increased when culture media contain TMAO. The increase of MTs can be reversed by the removal of TMAO from culture media. Importantly, evidences from \(^1\)H-NMR measurement show that TMAO is present inside these cells if cells are incubated with TMAO-media. The intracellular TMAO and microtubule increase are correlated, supported by the results of tubulin-immunofluorescent labeling, \(^1\)H-NMR measurement and in vitro tubulin assembly in Chapter 4. Based on these results, I conclude that TMAO can promote cellular microtubule assembly.
The concentration of intracellular TMAO in the XR1 glial cells that are treated with 200 mM TMAO for 6 hours is estimated to be 30-50 mM. This concentration is much less than that used to enhance tubulin self-assembly (65). It is observed that cellular microtubule assembly is enhanced by this concentration of TMAO, shown in Chapter 4. In elasmobranchs, intracellular concentration of TMAO is as high as 200 mM (53). It may be expected that TMAO in elasmobranchs can enhance microtubules in order to counteract the deleterious effect caused by urea. Tau is always required in my in vitro assembly system in order to show that low concentration of TMAO can promote microtubule assembly. Glial cells, like neurons, express several microtubule-associated proteins including MAP2 and tau. It is likely that microtubule-associated proteins in XR1 cells contribute to the increase of cellular microtubules caused by TMAO.

Phosphorylation on tau can reduce tau’s ability to promote tubulin assembly (39, 43, 44). PKA-phosphorylated tau and GSK-3β-phosphorylated tau lose their stimulation of tubulin assembly. Although phosphorylation sites in both the PKA-phosphorylated tau and GSK-3β-phosphorylated tau used in my studies are not completely defined, preliminary mass
spectrometry studies show that Ser-214 and Ser-356 are major sites of phosphorylation in PKA-phosphorylated tau. This preliminary result is consistent with previous reports (Table 1, in Chapter 1). The functional deficit can be compensated by TMAO (Fig. 1). TMAO does not cause chemical dephosphorylation on tau. PKA-phosphorylated tau (with 2.6 mol phosphate/mol tau) does not change tubulin critical concentration, indicating no interaction or little interaction between this phosphorylated tau and tubulin. However, the addition of TMAO to the assembly mixture containing the PKA-phosphorylated tau significantly lowers tubulin critical concentration for assembly. These results suggest that phosphorylated tau can interact with tubulin and promote tubulin assembly. A result of microtubules incubated with $^{32}$P-labeled tau indicates that the microtubules contain phosphorylated tau. Microtubules containing PKA-phosphorylated tau (with 2.7 mol phosphate/mol tau) look like the microtubules containing non-phosphorylated tau, as judged by atomic force microscopy. If the phosphorylation stoichiometry of PKA-phosphorylated tau reaches 4.0 mol phosphate/mol, the phosphorylated tau-microtubules in the presence of TMAO appears deformed (Fig. 2). The change of the apparent
Figure 1. Model of the effect of TMAO on microtubule-associated protein tau-tubulin assembly.
GTP, 30°C

4°C, Ca²⁺

Microtubule (tau-tubulin)

GTP, 30°C

Microtubule (P-tau-tubulin)

TMAO

Phosphorylated Tau

Phosphorylated Tau
Figure 2. Atomic force microscopic (AFM) image of tau-tubulin assembly. Tubulin and PKA-phosphorylated tau with 4.0 phosphates/mole were assembled using the standard reaction condition. The fixed mixture was then spotted onto a mica disc, dried and scanned by AFM.
persistence length of microtubules indicates that some phosphorylation reactions of tau can change the characteristics of assembled microtubules containing phosphorylated tau. How TMAO might force phosphorylated tau-microtubule assembly is suggested in Chapter 3. The mechanisms for the action of TMAO on phosphorylated proteins remain unclear.

Hypertonic stress can activate stress-activated protein kinases (SAPK), and mitogen-activated protein kinases (MAPK) in cells (68-70). These two kinases can phosphorylate microtubule-associated proteins tau (46, 71-73) and result in microtubule reorganization (74, 75). This microtubule decrease is also observed in XR1 cells if cells were incubated in media containing additional 200 mM glycerol, 200 mM NaCl or 200 mM glycine (Fig.3). However, XR1 cells incubated in 200 mM TMAO medium have more microtubules. Based on results in Chapter 3, intracellular TMAO may be able to compensate the functional deficit of phosphorylated microtubule-associated proteins, and maintain microtubules in hypertonic shocked cells.

Hyperphosphorylation or abnormal phosphorylation of tau has been proposed as one potential cause of Alzheimer's disease (43, 44). In the patients' brains, paired helical
Figure 3. Immunofluorescent analysis of microtubules in XR1 cells by E7 antibodies against β-tubulin. Cells in a were treated with 200 mM glycine media for 6 hours; cells in b, control media. The immunofluorescent labeling in a and b represents the structure of cellular microtubules.

Calibration bar, 25 µm.
filaments (PHFs), one of the major hallmarks, mainly consist of tau proteins (76, 77). Tau proteins in PHFs are hyperphosphorylated. The phosphorylation stoichiometry on normal brain tau is 2-3 moles phosphate/mole of the protein but that on Alzheimer’s tau is approximately 5-9 moles phosphate/mole of the protein (43). Before research reported in Chapter 3, removing the phosphates from tau through phosphatase dephosphorylation is the only way to recovery the tau’s biological function (76). Now, TMAO is shown to be able to compensate the functional deficit of phosphorylated tau without the removal of phosphates. The compensation is not specifically limited to a certain type of phosphorylation (see Chapter 3). Therefore, TMAO may be used as a new tool to study MT disassembly in neurodegenerative diseases, such as Alzheimer’s disease, caused by abnormal phosphorylation on microtubule-associated proteins. Several single mutations, such as G272V, V279M, P301L, V337M, within microtubule-binding domain of tau was found in frontotemporal dementia patients and has been proposed to be the cause of frontotemporal dementia (78-81). The mutant tau (P301L, V337M) has less binding affinity to taxol-stabilized microtubules (80). Since TMAO can rescue the PKA-phosphorylated tau containing Ser-356
phosphorylation in microtubule-binding domain (Chapter 3), TMAO may have chances to compensate those mutants. This idea is supported by the fact that TMAO can correct the protein folding in a cystic fibrosis study (82). One application of TMAO on scrapie-infected mouse neuroblastoma (ScN2a) cells also shows that TMAO can prevent the formation of pathogenic form of prion proteins (83).
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