Journal of the Korean Magnetic Resonance Society **2011**, *15*, 104-114

DOI : 10.6564/JKMRS.2011.15.2.104

The Status of Guanine Nucleotides in Taxol-Stabilized Microtubules Probed by ^{31}P CPMAS NMR Spectroscopy

Taslima Ferdous[1,+], Sang Hak Lee[1], Kwon Joo Yeo[2], and Younkee Paik[3,*]

[1] *Department of Chemistry, Kyungpook National University, Daegu 702-701, Korea*
[2] *Ochang Center, Korea Basic Science Institute, Ochang 363-883, Korea*
[3] *Daegu Center, Korea Basic Science Institute, Daegu 702-701, Korea*
(Received Nov 5, 2011; revised November 28, 2011 ; accepted December 13, 2011)

Abstract: Rapid exchange and hydrolysis of the tubulin-bound guanine nucleotides have been known to govern the dynamics of microtubules. However, the instability and low concentration have made it difficult for the microtubule-bound GTP to be observed directly. In this study, we circumvent these problems by lyophilization and using cross-polarization techniques. ^{31}P NMR signals were detected from the tubulin-bound GTP in microtubules for the first time. Analysis of the ^{31}P CPMAS NMR spectrum indicates that GTP hydrolysis was delayed by the presence of taxol.

Keywords : Microtubules, GTP, Taxol, ^{31}P CPMAS NMR, Lyophilization

INTRODUCTION

The highly organized arrangement of microtubules is essential for intracellular trafficking of vesicles and organelles, cellular motility and mitotic chromosome segregation.[1-3] The functional roles of microtubules in living cells are associated with various small molecules such as microtubule-associated proteins (MAPs), plus-end-tracking proteins (+TIPs), and Patronins.[4-6]

Microtubules are hollow cylinders consisting normally of 12 to 15 protofilaments which are the

* To whom correspondence should be addressed. E-mail : ykpaik@kbsi.re.kr
+ Current address : Applied Chemistry and Chemical Technology, University of Dhaka, Dhaka 1000, Bangladesh

linear assemblies of α,β-tubulin heterodimers.[7-9] A tubulin protein incorporates two guanine nucleotides, one in each subunit. The GTP in α-tubulin is non-exchangeable and non-hydrolysable (N site), while the nucleotide in β-tubulin is both exchangeable and hydrolysable (E site) (Fig. 1).[1]

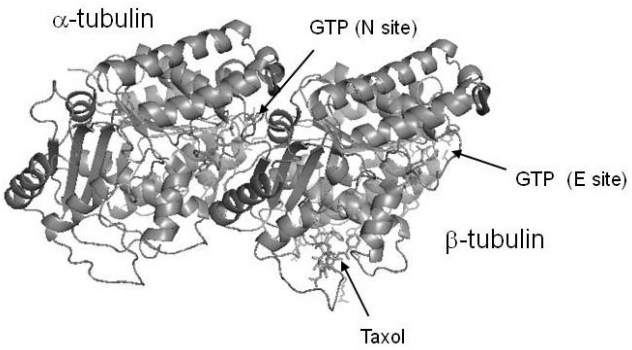

Figure. 1. The structure of α,β-tubulin dimer (PDB code: 1JFF). The ligand sites for GTP and Taxol are indicated by arrow.

According to the GTP-cap model, the status of guanine nucleotides in β-tubulin located at both ends of microtubules are very important as it determines whether the microtubules continue to grow or shrink.[10,11] Nonetheless, the fate of the guanine nucleotides in tubulin after they were polymerized into microtubules, *i.e.*, the hydrolysis mechanisms of GTP in β-tubulin, are still remained unclear.[12] ^{31}P NMR spectroscopy has been used for elucidating the switching mechanisms of small GTP-binding proteins Ras and its mutated family.[13,14] Here, we apply ^{31}P cross-polarization magic-angle spinning (CPMAS) NMR spectroscopy to probe the status of nucleotides in microtubules freshly

prepared with a polymerizing agent taxol. In order to keep the guanine nucleotides from being further hydrolyzed during the NMR data collection period, the microtubules were lyophilized right after the polymerization.

EXPERIMENTAL

Tubulin polymerization with taxol

Purified bovine brain tubulin was purchased from Cytoskeleton, Inc. All other chemicals including taxol and GTP were purchased from Sigma-Aldrich, Inc. The tubulin assembly buffer (BRB80) was freshly prepared to contain 80 mM PIPES, 1mM $MgCl_2$, and 1 mM EGTA at pH 6.8 adjusted with KOH. Tubulin was incubated at 37 ^{o}C in BRB80 supplemented with 1mM DTT and 1mM GTP. A tubulin concentration of 2 mg/ml was used. Taxol was dissolved in DMSO and sequentially added to the tubulin to the taxol concentration of 0.2, 2.0, and 20 μM at 5 min intervals. The final solution was incubated further for 15 min at 37 ^{o}C to obtain a suspension of taxol-stabilized microtubules. High polymerization yield (>95%) was confirmed by SDS-PAGE analysis.

Lyophilization of microtubules

The microtubule suspension was centrifuged on 30% glycerol cushion buffer using a TL-100 Beckman ultracentrifuge with a TLA 100.2 rotor for 30 min at 50,000 rpm at 25 ^{o}C. The supernatant was discarded. The pellet was rinsed five times with BRB80, dissolved in a lyophilizing buffer, and

snap-frozen in liquid nitrogen. The lyophilizing buffer was 5% sucrose in DI water. Lyophilization of the frozen sample was conducted on Bondiro system (IlsinBioBase Co., Ltd., Korea) for 6 hours at -50 ^{o}C and 10^{-5} mmHg.

SDS-PAGE analysis

Polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE) was performed using 12% acrylamide in the separating gel. Low molecular weight calibration kit (97, 66, 45, 30, 20.1, and 14.4 kDa) was purchased from GE Healthcare and used for standards. Gels were stained with Coomassie brilliant Blue.

Electron microscopy

Lyophilized microtubules were resuspended in BRB80 supplemented to 20 μM taxol and 1 mM DTT. The microtubule suspension was adsorbed onto 200 mesh, Formvar carbon-coated copper grids and analyzed by a Technai G2 Spirit Twin tunneling electron microscope (TEM) operated at 120 kV.

^{31}P NMR spectroscopy

Three sets of samples were investigated by ^{31}P NMR spectroscopy. The first set is the lyophilized, taxol-stabilized microtubules. The second set consists of two control samples: GTP and GDP were dissolved in BRB80 to 20 mM and analyzed in the liquid state. The third set is another two control samples: GTP and GDP were dissolved in BRB80, lyophilized, and analyzed in the solid state.

^{31}P CPMAS NMR experiments were performed on a Varian Unity Plus 200 MHz spectrometer using a double-resonance MAS probe equipped with a 2-mm rotor spinning module. About 10 mg of solid samples were packed into sample rotors and spun at 10 kHz. $^{1}H \rightarrow {}^{31}P$ cross-polarization match was employed at 73 kHz for 2.5 ms, with ^{1}H 90^{o} pulse of 2.7 μs and 55 kHz dipolar decoupling with TPPM phase modulation. The pulse repetition delay was 2.5 sec. The liquid samples were examined by direct polarization with ^{31}P 90^{o} pulse of 2.7 μs without sample spinning. The pulse repetition delay was 0.9 sec. All the NMR experiments were performed at ambient temperature. The ^{31}P NMR spectra were referenced to 85% H_3PO_4 at 0 ppm.

RESULTS AND DISCUSSION

Figure 2 shows the TEM images of the taxol-stabilized microtubules: as-prepared (Fig. 2a) and resuspended after lyophilization (Fig. 2b). The lyophilized microtubules were stored in a capped tube at room temperature with humidity below 15% for one week before the TEM observation. Most microtubules show lengths longer than 1 μm before and after the lyophilization, which supported that the microtubules remained intact during the lyophilizing processes and the preservation period at room temperature.

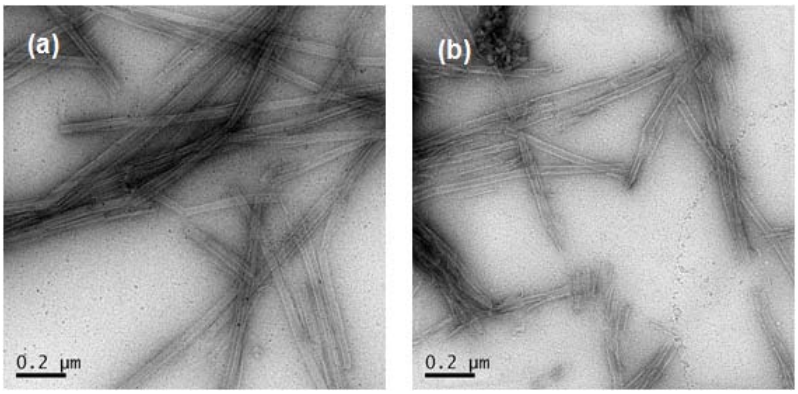

Figure 2. The TEM images of taxol-stabilized microtubules: (a) before and (b) after the lyophilization. The lyophilized microtubules were stored at room temperature for one week before being resuspended for the examination.

Figure 3 shows the ^{31}P NMR spectra of the taxol-stabilized microtubules and of the control samples. The ^{31}P NMR resonances from the two control samples (Fig. 3a and 3b) were assigned following the literature:[13,15] the peaks at -8.2, -11.2, -22.3 ppm (Fig. 3a) and at -4.3, -9.0, -19.4 ppm (Fig. 3b), respectively, are assigned to γ-, α-, and β-phosphates of GTP. The assignments of the ^{31}P resonances from GDP of the two control samples (Fig. 3d and 3e) are following: the peaks at -6.6, -9.7 ppm (Fig. 3d) and at -8.0, -10.8 ppm (Fig. 3e), respectively, are assigned to β- and α-phosphates of GDP. As cross-polarization from proton to phosphorus was used in acquiring the ^{31}P NMR spectra of solid samples (Fig. 3b-d), the peak intensities in these spectra should be compared carefully.

The phosphorus signals obtained from the taxol-stabilized microtubules were very weak even after 262,144 scans (Fig. 3c). However, this spectrum is very similar to the ^{31}P NMR spectrum of a control sample that contains ca. 0.1 mg of free GTP (Fig. 3b). When the broad peak was deconvoluted into three resonances, at -0.3, -8.7, and -18.6 ppm, the relative ratios among these peak intensities were very similar to those observed in Fig. 3(b). Due to the distinctive location, the resonance at -18.6 ppm must be assigned to the β-phosphates of the tubulin-bound GTP.[13,15] Then, the other two signals, -0.3 and -8.7 ppm, can be easily assigned to the γ- and α-phosphates of the tubulin-bound GTP. A difference is obvious in the ^{31}P resonances of the γ-phosphates between the taxol-stabilized microtubules (-0.3 ppm; Fig. 3c) and the control sample (-4.3 ppm; Fig. 3b). The size of this difference (4 ppm) corresponds to the value observed between the Ras-bound and free form of guanosine nucleotides.[13,15] The result suggests that the γ-phosphates of the microtubule-bound GTP bind directly to Mg^{2+} ions in the binding pocket, which is consistent with a recent crystallography study.[16] The above observation is a supportive evidence to rule out the possibility that there may remain a significant amount of GTP on the microtubule surfaces which is the remnant of the original tubulin polymerizing buffer that might have not completely removed by the centrifugation and several times of washing of the pellets.[17,18] Any surface-adsorbed GTP would yield a ^{31}P resonance close to the value observed in Fig. 3(b). Therefore, it is concluded that the dominant form of nucleotides in our lyophilized microtubules is GTP. At the given signal-to-noise ratio of 7.5 even after one week of data acquisition, analysis for quantitative information of ^{31}P NMR signals from the

hydrolyzed nucleotide (GDP) was not possible which must be below 15% of the total nucleotides at best.

It is interesting that the ^{31}P resonances from the α-phosphates of guanine nucleotides in solid samples (Fig. 3b-d) are located very closely from each other, *i.e.*, they fall within 1-ppm range between -8.7 and -9.7 ppm. These are close to the shifts observed in liquid samples (Fig. 3a and 3e), which suggests that the α-phosphates bind rather weakly to the metal ions than other phosphate groups.

An atomic force microscopy (AFM) study on freshly prepared, taxol-stabilized protofilaments have reported structural changes from straight to curved protofilaments as a function of aging time.[19] Most of the protofilaments were straight when observed within 4 hours, while ring formation was dominant after 24 hours. Our ^{31}P CPMAS NMR results are consistent with the above AFM observation. Since the microtubules were snap-frozen and lyophilized within an hour of preparation, it is likely that most of the GTP was locked in the E-sites of β-tubulin before they were hydrolyzed into GDP and P_i. It seems that the presence of taxol have delayed the GTP hydrolysis.[10] Further optimization and higher field experiments would yield ^{31}P NMR spectra with high resolution enough to determine quantitatively how much of each guanine nucleotides are present in the polymerized tubulins of various origins.

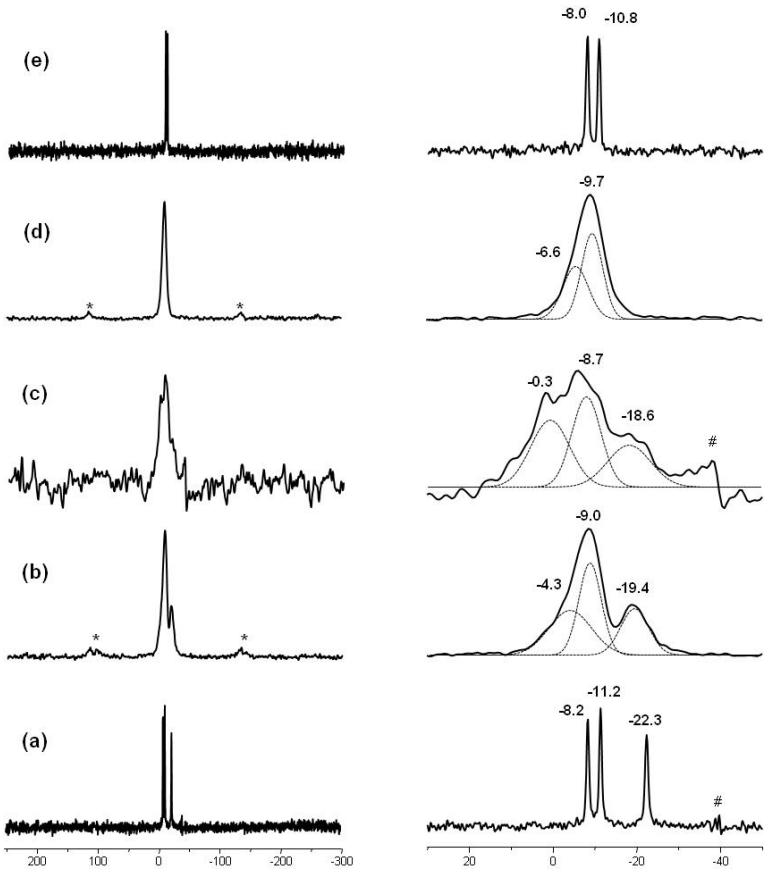

Figure. 3. The ^{31}P NMR spectra of taxol-stabilized microtubules and of control samples: (a) 20 mM GTP in BRB80 (liquid), (b) 4 mM GTP in BRB80 (lyophilized solid), (c) taxol-stabilized microtubules (lyophilized solid), (d) 4 mM GDP in BRB80 (lyophilized solid), (e) 20 mM GDP in BRB80 (liquid). The expansions and deconvolutions of the spectra are shown on the right. Sample spinning sidebands (*) and spikes of the applied RF (#) are marked. A total of 262,144 scans were acquired to obtain the spectrum (c).

CONCLUSIONS

It is shown that taxol-stabilized microtubules can be lyophilized with sucrose and stored at room temperature. ^{31}P NMR spectrum was acquired from the tubulin-bound guanine nucleotides in microtubules for the first time. Analysis of the ^{31}P CPMAS NMR spectrum indicates that the dominant form of guanine nucleotides in the freshly-prepared microtubules is GTP. The ^{31}P resonance of the γ-phosphates is shifted by 4 ppm to higher frequency compared with the control solid sample, which suggests that the γ-phosphates bind directly to Mg^{2+} ions. Our study will lead to a better understanding of the mechanisms of GTP hydrolysis in microtubules.

Acknowledgment

This work was supported by KBSI grant (F30602) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0012663).

REFERENCES

1. A. Desai, T. J. Michison, *Annu. Rev. Cell Dev. Biol.* **13**, 83, (1997).
2. R. D. Vale, R. A. Milligan, *Science* **288**, 88, (2000).
3. C. A. Athale, A. Dinarina, M. Mora-Coral, C. Pugieux, F. Nedelec, E. Karsenti, *Science* **322**, 1243, (2008).
4. A. Akhmanova, C. C. Hoogenraad, *Curr. Opin. Cell Biol.* **17**, 47, (2005).
5. R. Dixit, J. L. Ross, Y. E. Goldman, L. F. Holzbaur, *Science* **319**, 1086, (2008).
6. S. S. Goodwin, R. D. Vale, *Cell.* **143**, 263, (2010).

7. L. A. Amos, T. S. Baker, *Nature* **279**, 607, (1979).

8. E. Nogales, S. G. Wolf, K. H. Downing, *Nature* **391**, 199, (1998).

9. E. Nogales, M. Whittaker, R. A. Milligan, K. H. Downing, *Cell.* **96**, 79, (1999).

10. H. P. Erickson, E. T. O'Brien, *Annu. Rev. Biophys. Biomol. Struct.* **21**, 145, (1992).

11. A. Dimitrov, M. Quesnoit, S. Moutel, I. Cantaloube, C. Pous, F. Perez, *Science* **322**, 1353, (2008).

12. H. Y. Kueh, T. J. Mitchison, *Science* **325**, 960, (2009).

13. M. Geyer, T. Schweins, C. Hermann, T. Prisner, A. Wittinghofer, H. R. Kalbitzer, *Biochem.* **35**, 10308, (1996).

14. M. Spoemer, T. Prisner, A. E. Bennett, M. M. Hertel, N. Weiden, T. Schweins, H. R. Kalbitzer, *Magn. Reson. Chem.* **43**, S74, (2005).

15. P. Rosch, A. Wittinghofer, J. Tucker, G. Sczakiel, R. Leberman, I. Schlichting, *Biochem. Biophys. Res. Commun.* **135**, 549, (1986).

16. A. Nawrotek, M. Knossow, B. Gigant, *J. Mol. Biol.* **412**, 35, (2011).

17. G.-A. Gang, S.-S. Choi, T.-J. Park, Y. Kim, *J. Kor. Magn. Reson. Soc.* **14**, 88, (2010).

18. T.-J. Park, M.-H. Lee, S.-S. Choi, Y. Kim, *J. Kor. Magn. Reson. Soc.* **15**, 25, (2011).

19. C. Elie-Caille, F. Severin, J. Helenius, J. Howard, D. J. Muller, A. A. Hyman, *Curr. Biol.* **17**, 1765, (2007).