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Evidence that the tightly bound magnesium in tubulin is associated with the N-site GTP

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In an attempt to determine whether the tightly bound Mg^{2+} found in purified tubulin in associated with the N-site GTP or the E-site GDP or GTP, we removed the E-site nucleotide by several means: (i) alkaline phosphatase treatment; (ii) displacement using excess GMPPCP; and (iii) polymerizing tubulin in the presence of alkaline phosphatase and non-hydrolyzable analogues. The Mg^{2+} content remained equal to about 1 mol/mol tubulin under conditions where denaturation did not occur. Moreover, the Mg/GTP ratio always remained equal to 1. These results indicate that the Mg^{2+} is associated with the N-site GTP.

Tubulin; Magnesium; GTP

1. INTRODUCTION

It has long been known that the tubulin dimer contains one tightly bound Mg^{2+} [1,2] which can be removed only by denaturing the protein, although it can be displaced by Mn^{2+} [3,4]. Additional Mg^{2+} in solution is required for efficient assembly of tubulin into microtubules [1,2]. Tubulin also contains two guanine nucleotide binding sites, one exchangeable with nucleotide in solution (E-site) and a non-exchangeable site (N-site). GTP is found bound to the N-site while GTP or GDP bind to the E-site with high affinity. It is the E-site GTP which is hydrolyzed during the assembly process [5,6].

A fundamental question concerning the chemistry of tubulin is the location of the tightly bound Mg^{2+} and its relationship to the bound nucleotides. By studying Mn^{2+} binding to tubulin in which the E-site was partially depleted of nucleotide, Jemiolo and Grisham concluded that the high affinity binding was to the E-site nucleotide and that the N-site nucleotide did not contain bound cation [7]. In the interpretation of results from NMR studies designed to determine the distance between the fluorine in GTP(γF) and the tightly bound divalent metal, it was also assumed that the cation was situated at the E-site [8]. On the other hand, Correia et al. presented data indicating that the slowly exchanging divalent cation is located at the N-site [4]. In this report we provide additional evidence in support of the latter hypothesis. We find that upon removal of E-site nucleotide with alkaline phosphatase or by displacement with the non-hydrolyzable analogues GMPPCP and GMPPNP, the Mg/GTP ratio remains equal to 1, suggesting that the Mg^{2+} is associated with the N-site GTP.

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Abbreviations: GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GMPPCP, guanylyl (β,γ-methylene) diphosphonate; GMPPNP, guanylylimidodiphosphonate; AP, alkaline phosphatase; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetracetic acid

2. MATERIALS AND METHODS

2.1. *Tubulin isolation*

Bovine brain microtubule protein (MTP) was prepared by two cycles of assembly-disassembly [9]. Tubulin was purified by polymerizing the MTP in 0.4 M Pipes/10% DMSO and passing the cold redissolved pellet in 0.1 M PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9) through Mg^{2+}-saturated phosphocellulose and Bio Gel P-10 [10]. Tubulin was eluted with 0.1 M PEM buffer, pH 6.9, drop frozen in liquid nitrogen and stored at −70°C. The concentration of tubulin was determined by the Bradford assay [11] using bovine serum albumin as the standard and UV absorbance at 275 nm using an ϵ value of 1.13 $ml \cdot mg^{-1} \cdot cm^{-1}$.

2.2. *Removal or displacement of E-site nucleotide*

Depletion of E-site nucleotide was done by incubation of tubulin with alkaline phosphatase (4 U/mg tubulin) in 0.1 M PEM buffer, pH 6.9, at room temperature, for 40 min. The resulting solution was gel-filtered by column centrifugation [12] into 0.1 M Pipes, pH 6.9, at 4°C. In some cases the alkaline phosphatase treatment was done in Mg^{2+}-free buffer by first exchanging tubulin into 0.1 M Pipes, pH 6.9, by gel-filtration. Alkaline phosphatase treatment was then done either at room temperature or 4°C.

Displacement of E-site nucleotide with the GTP analogues, GMPPCP and GMPPNP, was undertaken by three protocols. The first protocol involved incubation of tubulin with 1 mM GMPPCP (in 0.1 M PEM buffer) for 20 min followed by gel-filtration or alkaline phosphatase treatment as described above. In other cases tubulin containing GTP at the E-site, obtained by incubation with GTP followed by gel-filtration, was utilized because in the absence of Mg^{2+} the binding affinity for GTP is lowered by a factor of 10^3 [13]. In this case

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tubulin was incubated with a 130-fold excess of GMPPCP in the absence of Mg^{2+}. The third protocol involved incubation of tubulin with 1 mM GMPPCP or GMPPNP in 0.1 M PEM buffer at 4°C for 10 min after which the solution was treated with alkaline phosphatase at room temperature and then polymerized at 37°C. Microtubules thus formed were isolated by centrifugation at 100 000 × *g* and 37°C for 90 min through 40% sucrose and 10% DMSO in 0.1 M Pipes, pH 6.9. Some pellets were cold-depolymerized in 0.1 M Pipes and the resulting solution assayed for protein, nucleotide and Mg^{2+}. Other pellets were rinsed in PEM buffer, cold-depolymerized in the same buffer and column-centrifuged through Sephadex G-50 into 0.1 M Pipes for subsequent nucleotide and Mg^{2+} analysis.

2.3. *Nucleotide and magnesium analysis of tubulin*

For nucleotide analysis of protein solutions, the protein was denatured with 2.5% perchloric acid and the perchlorate precipitated with a 4 M CH_3COOH/10 M KOH mixture to bring the pH to about 4.5. The supernatant was injected on a 4.6 × 250 mm Whatman Partisil-10 SAX ion exchange column and eluted with a gradient of 0.2 M NaH_2PO_4/0.2 M NaCl to 0.75 M NaH_2PO_4/0.2 M NaCl, pH 4.3, at 1 ml/min. Nucleotides were quantified by peak area measurement using the Sigma Scan software (Jandell Scientific) and compared to standards.

Magnesium was analyzed by a Perkin Elmer atomic absorption spectrometer (Model 305B) using 0.1 M Pipes, pH 6.9 as a blank.

3. RESULTS AND DISCUSSION

The rationale for the following experiments is that, if the one Mg^{2+} tightly bound to tubulin is complexed through the E-site nucleotide, removal of this nucleotide should result in removal of the Mg^{2+}. If, on the other hand, the Mg^{2+} is complexed to the N-site GTP, a constant ratio of one Mg^{2+} per GTP should be found in tubulin lacking E-site nucleotide. The E-site nucleotide was removed by alkaline phosphatase treatment [14], by displacement with non-hydrolyzable nucleotide analogues (which in turn are easily removed by gel-filtration) and by a combination of both methods.

To demonstrate that the procedures of E-site nucleotide removal do not adversely affect the tubulin structure we used the self-assembly assay, a sensitive assay for tubulin native conformation. As shown in Fig. 1, tubulin retained its ability to assemble into microtubules after alkaline phosphatase treatment in the presence of Mg^{2+}. Gil-filtration of such tubulin into Mg^{2+}-free buffer, however, resulted in some dimunition of the rate and extent of assembly (Fig. 1). The extent of loss in assembly competence in the latter case varied with the time E-site depleted tubulin remained in the absence of Mg^{2+}.

Our phosphocellulose-purified tubulin typically contained about 0.7 mol GDP and 1.3 mol of GTP respectively, per mol tubulin (Table I), indicating that approximately 30% of the dimers contained GTP at the E-site. Table I shows the results of Mg^{2+} and nucleotide assays of tubulin treated with alkaline phosphatase in the presence or absence of Mg^{2+}, followed by gel filtration. The results show a large decrease in GDP content from the E-site and a 1:1 relationship between the bound Mg^{2+} and GTP. Although the GDP and GTP from the

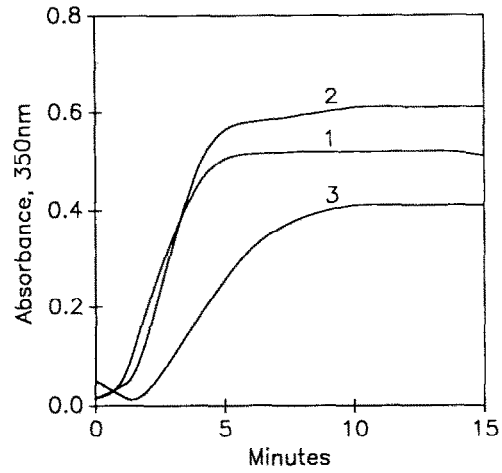

Fig. 1. Tubulin assembly in 0.1 M PEM buffer, pH 6.9, in the presence of 1 mM GTP and 10% DMSO at 37°C. (Curve 1) 15 μM phosphocellulose-purified tubulin. (Curve 2) 20 μM alkaline phosphatase-treated tubulin. (Curve 3) 28 μM alkaline phosphatase-treated tubulin followed by gel-filtration into Mg^{2+}-free buffer.

E-site were partially or completely removed there was no reduction in bound Mg^{2+}. When alkaline phosphatase treatment was done in the absence of Mg^{2+}, some denaturation of tubulin occurred, as evidenced by a loss of assembly competence and reduction of the N-site GTP to 0.5, but the Mg/GTP ratio remained at 1.

Treatment of tubulin with 1 mM GMPPCP (a 16.7-fold molar excess over tubulin) was not effective in displacing nucleotide from either the E-site or N-site; however, in the presence of alkaline phosphatase the E-site nucleotide was removed (Table II). This result is consistent with previous results [15] and reflects the 1000-fold higher affinity of tubulin for GDP or GTP than for the analogue. When a 130-fold excess of GMPPCP over tubulin (containing GTP at the E-site) was used in the absence of Mg^{2+} most of the E-site nucleotide was also displaced (Table II). Because GMPPCP binding is fairly weak [15], it does not remain bound to the protein during gel filtration. The results in Table II demonstrate that depletion of E-site nucleotide with analogue was not accompanied by a decrease in Mg^{2+} content and the Mg/GTP ratio remained equal to about 1.

Table I

Nucleotide and Mg^{2+} contents of tubulin after treatment with alkaline phosphatase in the presence and absence of Mg^{2+}

Treatment	Mg^{2+}	GDP	GTP	Mg/GTP
None	0.90	0.70	1.34	0.67
Ap, Mg present, 24°C	1.06	0.00	0.97	1.09
Ap, Mg present, 4°C	0.97	0.21	0.97	1.00
Ap, Mg absent, 4°C	0.51	0.00	0.49	1.04

Mg^{2+} and nucleotide values are given as mol/mol tubulin.

Table II

Nucleotide and Mg^{2+} contents of tubulin after treatment with GMPPCP[a]

Treatment	Mg^{2+}	GDP	GMPPCP	GTP	Total nucleotide	Mg/GTP
1 mM GMPPCP[b]	0.89	0.51	0.00	1.36	1.87	0.65
1 mM GMPPCP + AP[c]	0.80	0.16	0.00	0.72	0.88	1.11
5 mM GMPPCP[d]	0.73	0.11	0.04	0.75	0.90	0.97

[a]Values for Mg^{2+} and nucleotide are expressed as mol/mol tubulin
[b]Tubulin, 6 mg/ml, incubated with 1 mM GMPPCP at 4°C for 20 min and gel-filtered
[c]Tubulin incubated with 1 mM GMPPCP at 4°C for 10 min, then with alkaline phosphatase at room temperature for 20 min and gel-filtered
[d]Tubulin with GTP at the E-site, 4 mg/ml, incubated with 5 mM GMPPCP at room temperature for 30 min in the absence of exogenous Mg^{2+} and gel-filtered

Table III

Mg^{2+} and nucleotide contents of alkaline phosphatase treated tubulin assembled in the presence of GMPPCP or GMPPNP[a]

Assembly condition	Mg^{2+}	GDP	GMPPCP	GMPPNP	GTP	Total nucleotide	Mg/GTP
GMPPCP + AP[b]	1.0	0.18	0.72	-	1.1	2.00	0.91
GMPPCP + AP[c]	0.84	0.16	0.00	-	0.91	1.07	0.92
GMPPNP + AP[b]	1.04	0.37	-	0.63	1.06	2.06	0.98
GMPPNP + AP[c]	0.88	0.33	-	0.06	1.01	1.4	0.87

[a]Mg^{2+} and nucleotide values are expressed as mol/mol tubulin
[b]Isolated microtubules were cold-depolymerized in 0.1 M PEM
[c]The cold-depolymerized microtubules were passed through a Sephadex G-50 centrifuge column

Mg^{2+} and nucleotide contents in tubulin polymerized in the presence of alkaline phosphatase and GMPPCP or GMPPNP were also measured. Previous results demonstrated that this resulted in the incorporation of analogue into the microtubule at the expense of GDP [15]. Recovered microtubules showed good incorporation of the analogues, 0.63–0.72 mol/mol tubulin, which was easily removed by subsequent gel filtration (Table III) but the Mg^{2+} content of tubulin was still close to 1 mol/mol, indicating that it was probably not bound to the E-site nucleotide analogue.

The results of the experiments presented in this report show a clear correlation between the tightly bound Mg^{2+} and the one GTP which is situated at the N-site. There is no correlation between the Mg^{2+} and the E-site nucleotide. Such a correlation suggests that the Mg^{2+} may be complexed to the GTP at the N-site. In a study of Mn^{2+} binding to tubulin Correia et al. found two high affinity sites when GTP occupied the E-site indicating metal binding to both sites [4]. Our results are consistent with this finding which indicates that, under assembly conditions, GTP at the E-site is also complexed to the divalent cation. In a recent examination of the effect of Mg^{2+} on the kinetics of assembly induced by GMPPCP it was also concluded that 2 Mg^{2+}/tubulin can be bound under some solution conditions during the assembly reaction [16]. In addition to nucleotide complexation at the N- and E-sites Mg^{2+} (or Mn^{2+}) also binds to multiple weak sites [3,4,17]. These sites, as suggested by Correia et al. [4], may be situated in the highly acidic C-terminal regions of the α- and β-subunits and may be important for stimulating the assembly reaction.

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