The Relationship between Cobalt Binding to Tubulin and the Stimulation of Assembly*

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Co(II), which induces the assembly of tubulin in the absence of associated proteins, binds to multiple sites on the protein in a low ionic strength medium. However, in the presence of concentrations of buffer and GTP used in assembly reactions, the number of sites occupied by Co(II) is drastically reduced. At GTP and Co(II) concentrations where the major polymerized products are broad sheets of protofilaments, about 3 Co(II) atoms are bound/tubulin dimer. Increasing the GTP concentration lowers the amount of assembly, increases the proportion of microtubules to sheets, and decreases the amount of Co(II) bound. It is concluded that the binding of free Co(II) is necessary to stimulate assembly and the less Co(II) bound, the more likely is the formation of microtubules.

Tubulin containing 1 tightly bound Co(II) can be isolated by passing tubulin preincubated with an excess of Co(II) through phosphocellulose. Only 50% of the tight Co(II) binding can be prevented by Mg(II) or Mn(II), but 100% is prevented by Zn(II), Cd(II), and Cu(I). Moreover, Mg(II) can only displace a fraction of the bound Co(II). The Mg(II) content of tubulin is reduced after the cobalt is bound. Tubulin containing 1 tightly bound cobalt can assemble into microtubules and sheets if either more cobalt or taxol is added. The Mg(II) and Co(II) contents of cold-soluble assembled products are approximately 1 atom/tubulin dimer, respectively, suggesting that the Co(II) is not situated at the Mg(II) site and that only tubulin containing 1 Mg(II)/dimer assembles into protofilamentous structures.

Experimental procedures
Preparation of Tubulin—Tubulin from fresh bovine brain was prepared by a modification (13) of the recycylation procedure of Shelanski et al. (14). The protein was stored at -70 °C in 1-ml portions at a concentration of 10-15 mg/ml in buffer containing 25 mM 2-(N-morpholino)ethanesulfonic acid, 70 mM NaCl, 0.5 mM MgCl₂, 1 mM ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid, and 2 mM glycerol, pH 6.5. On the day of the experiment further purification was done to obtain tubulin devoid of other proteins. The protein was polymerized in 0.4 M Pipes, pH 6.9 (2) and the resuspended pellet passed through a column (4 × 1.2 cm) of phosphocellulose (Whatman P-11) which was layered on top of a column (25 × 1.2 cm) of Sephadex G-75. The protein was eluted with 20 mM Pipes, pH 6.9. Kontes Chromaflow extenders columns are ideal for this. The gel filtration removes excess nucleotide and other buffer components which pass through the phosphocellulose with the tubulin fraction (3).

Self-assembly Reaction—The polymerization of tubulin was monitored by turbidity measurements in a Gilford 2000 spectrophotometer. The reaction was carried out in 0.1 M Pipes, pH 6.9, at 37 °C. Other components such as MgCl₂, CoCl₂, MeSO₄, and GTP were added at concentrations given in the text.

Co(II) Binding—Equilibrium binding studies were performed using "CoCl₂ by the gel filtration technique of Hummel and Dreyer (15). Columns of Sephadex G-75 (28 × 0.7 cm) were equilibrated at room temperature with various concentrations of "CoCl₂ in buffers described in the text. The protein solution (2-5 mg/ml) was adjusted to the same "CoCl₂ concentration as in the equilibrium buffer and 0.5 ml of this solution was applied to the column. Elution was with the equilibrating buffer and 0.5-ml fractions were collected. Samples were taken for protein determination and counted either directly in a Packard model scintillation counter or, in some cases, in a scintillation counter using Aqualos 2 (New England Nuclear) as the scintillation solution.

Tight Co(II) binding was measured by passing a preincubated solution containing "CoCl₂ and tubulin through a column of phosphocellulose. Usually 1 ml of solution containing 1.5-3 mg of protein was passed through a Pasteur pipette containing about 0.5 ml of packed phosphocellulose. Control studies showed that the ion 

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1 The abbreviations used are: Pipes, 1,4-piperazinediethanesulfonic acid; MeSO₄, dimethyl sulfoxide.
Cobalt Binding to Tubulin

Table 1

<table>
<thead>
<tr>
<th>CoCl₂ (total)</th>
<th>Bound Co(II)/tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>Buffer A*</td>
</tr>
<tr>
<td>50</td>
<td>5.5</td>
</tr>
<tr>
<td>100</td>
<td>8.4</td>
</tr>
<tr>
<td>200</td>
<td>10.2</td>
</tr>
<tr>
<td>500</td>
<td>15.3</td>
</tr>
<tr>
<td>1000</td>
<td>21.2</td>
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</tbody>
</table>

*B* Buffer A is 20 mM Pipes, pH 6.9. Buffer B is 0.1 mM Pipes, pH 6.9, containing 0.5 mM GTP.

Results

Binding of Co(II) under Equilibrium Conditions—Binding was measured by gel filtration using buffers containing various concentrations of 56CoCl₂. The protein fraction contained an amount of Co(II) equivalent to the sum of the concentrations of free and bound metal. A trough followed the peak as predicted by the Hummel-Dreyer method. Curve 1 in Fig. 1 is a Scatchard plot of the binding data obtained in 20 mM GTP. CO(Z)B is bound Co(Z), CO(Z)F is free Co(Z), and CO(Z)S is bound Co(Z) and CO(Z)F is the total amount of Co(Z) equivalent to the sum of the concentrations of 60CoCl₂. The protein fraction contained an amount of Co(Z) equivalent to the sum of the concentrations of 60CoCl₂. The amount of free Co(Z) was measured by gel filtration using buffers containing various concentrations of 60CoCl₂.

Materials—GTP, EGTA, Pipes, and 2-(N-morpholino)ethanesulfonic acid were purchased from Sigma. 56CoCl₂ was a product of New England Nuclear. Taxol was a gift from Dr. J. Alejandro Donoso, University of Kansas Medical Center. Buffers and GTP solutions used in most of the experiments were passed through phosphocelulose columns to remove divalent cations.

Electron Microscopy—Negative staining was done by staining samples on carbon-coated grids with 2% uranyl acetate. Samples were viewed in a Phillips 300 electron microscope. Some samples were fixed with 2.5% glutaraldehyde and stained with tannic acid, after which they were fixed with osmium tetroxide, embedded in Araldite, sectioned, and stained again with methanolic uranyl acetate and lead citrate as described previously.

Materials—GTP, EGTA, Pipes, and 2-(N-morpholino)ethanesulfonic acid were purchased from Sigma. 56CoCl₂ was a product of New England Nuclear. Taxol was a gift from Dr. J. Alejandro Donoso, University of Kansas Medical Center. Buffers and GTP solutions used in most of the experiments were passed through phosphocelulose columns to remove divalent cations.

Fig. 1. Scatchard plot of Co(II) binding. The binding experiments were performed as described under "Experimental Procedures." Curve 1, in 20 mM Pipes, pH 6.9. Curve 2, in 0.1 mM Pipes, pH 6.9, containing 0.5 mM GTP. CO(Z)B is bound Co(Z) and CO(Z)F is free Co(Z). The dashed lines represent extensions of the straight line portions of the curves drawn to estimate Kd values.
mol of Co(II). As mentioned above we did not always observe a large decrease in Mg(II) content upon the incorporation of 1 Co(II). In these cases there was also little decrease in the nucleotide content.

**Competition by Other Cations for Tight Binding**—A number of cations at various concentrations were included in the incubation mixtures to determine whether they could compete with Co(II) for the binding site. The results show that Zn(II), Cd(II), and Cu(I) are very effective competitors (Fig. 2). Much higher concentrations of Mg(II) and Mn(II) are required to inhibit Co(II) binding, with Mn(II) being the more effective of the two. Moreover these two cations only prevent 50% of the binding (Fig. 2). Ca(II), at the concentrations used, was totally unable to prevent Co(II) binding.

**Removal of Tightly Bound Co(II)**—To determine whether the tightly bound cobalt could be removed by other agents, a Co-labeled tubulin, isolated from phosphocellulose, was incubated with other divalent cations or GTP and rechromatographed on phosphocellulose. Surprisingly, divalent cations, including Co(II), removed only 30-42% of the tightly bound cobalt. As mentioned above we did not always observe a large decrease in Mg(II) content upon the incorporation of 1 Co(II). Because we showed that the Mg(II) content of tubulin is decreased after incubation with Co(II), it was of interest to determine the Mg(II) content of protein assembled

**TABLE II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Co(II)/tubulin</th>
</tr>
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<tr>
<td>None</td>
<td>0.92</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.64</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.59</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>0.59</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.53</td>
</tr>
<tr>
<td>GTP</td>
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**Removal of tightly bound Co(II) by dialysis**

Co-tubulin was prepared as described in the legend to Table II and additional 50-ml samples containing 23 µM tubulin were dialyzed for 16 h at room temperature against 100 ml of the buffer. The dialysis buffer was 0.5 mM in pH 6.9, and where noted was supplemented with 8 M urea, 1 mM EDTA, or 10 mM 2-mercaptoethanol.

<table>
<thead>
<tr>
<th>Dialysis buffer</th>
<th>Co(II)/tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.35</td>
</tr>
<tr>
<td>Pipes</td>
<td>0.51</td>
</tr>
<tr>
<td>Pipes-urea</td>
<td>0.44</td>
</tr>
<tr>
<td>Pipes-EDTA</td>
<td>0.21</td>
</tr>
<tr>
<td>Pipes-urea-EDTA</td>
<td>0.15</td>
</tr>
<tr>
<td>Pipes-urea-2-mercaptoethanol</td>
<td>0.70</td>
</tr>
</tbody>
</table>

**Fig. 2. Competition by cations for Co(II) tight binding.**

[C2O(II)Cl₂ (200 µM) was incubated with 15 µM tubulin in 20 mM Pipes, pH 6.9, and the chloride salts of the cations shown, in a 1-ml volume. The C2O(II)Cl₂ was added last. After 30 min at room temperature the solutions were passed through phosphocellulose and the eluate examined for [C2O(II)] and protein contents. The curves have been normalized by setting the value of the control in the absence of cation at 100%.

**Fig. 3. Assembly of tubulin stimulated by taxol.**

Five ml of a 50 µM tubulin solution in 20 mM Pipes, pH 6.9, was incubated for 30 min at room temperature with 0.5 mM [C2O(II)Cl₂. The solution was then passed through a column (0.2 × 2 cm) of phosphocellulose. The eluted protein contained 1 Co(II)/dimer. The Co-tubulin at a concentration of 31 µM was incubated in 0.1 mM Pipes, pH 6.9, and 0.5 mM GTP in a 4-ml volume (B). At the arrow a 4 µl of a 12.5 mM taxol solution (in Me₂SO) was added. Upon completion of the reaction, some samples were negatively stained and others were fixed for thin sectioning. The remaining solution was centrifuged to collect the polymerized tubulin for Co(II) and Mg(II) determinations. A represents an experiment done with Mg-tubulin at the same concentrations of protein and taxol.

**Tight Co(II) Binding to Preformed Microtubules**—Prior to polymerization was incubated in the presence of 10% Me₂SO and 50 µM MgCl₂. After completion of the assembly reaction, the solution was made 0.5 mM in (C2O(II)Cl₂. A duplicate experiment was done in the absence of Me₂SO, conditions where the tubulin remains unpolymerized. In the former case, the polymerized tubulin was isolated by centrifugation and redissolved in cold buffer. The dimeric tubulin from the control, the resuspended polymerized fraction and the unpolymerized supernatant fraction were passed through phosphocellulose and the Co(II)/tubulin content was found to be 1.07, 1.05, and 1.08, respectively.

**Assembly of Tubulin Containing Tightly Bound Co(II)—**

We found that tubulin containing 1 tightly bound cobalt will not assemble at protein concentrations up to 2 mg/ml in the absence of an assembly-stimulating agent unless more Co(II) is added. This indicates that occupation of sites other than the one with the highest affinity is required to promote assembly. Because we showed that the Mg(II) content of tubulin is decreased after incubation with Co(II), it was of interest to determine the Mg(II) content of protein assembled...
FIG. 4. Tannic acid stained sections of Mg-tubulin and Co-tubulin polymerized by taxol. Samples (0.5 ml) from the experiment described in Fig. 3 were fixed, stained, and sectioned as described under "Experimental Procedures." A, Mg-tubulin; B, Co-tubulin. Bar line = 75 nm.
with Co(II). Tubulin isolated from phosphocellulose after incubation with a 15-fold excess of CoCl₂, and containing 1.52 and 0.54 atoms of Co(II) and Mg(II), respectively, was assembled in the presence of 1.0 mM CoCl₂ and 0.5 mM GTP. The assembly mixture was centrifuged at 55,000 × g and 37 °C for 30 min, and the pellet was dissolved in cold 20 mM Pipes. The protein in this dissolved pellet contained 1.45 Co(II) and 0.9 Mg(II)/tubulin dimer after being passed through phosphocellulose. The supernatant of the 55,000 × g centrifugation was then centrifuged at 150,000 × g and 37 °C for 30 min. Another pellet was obtained which was not soluble in cold buffer but after dissolving in a 0.1 N NaOH was found to contain 0.76 Co(II) and 0.1 Mg(II)/dimer. The protein in the supernatant from the second centrifugation contained 0.8 Co(II) and 1.02 Mg(II)/dimer. These results indicate that upon binding cobalt some of the tubulin loses Mg(II) and is changed into a state which forms a cold-insoluble product under assembly conditions. Protein which assembles into a cold-soluble form (sheets and microtubules) still contains 1 Mg(II) as well as the bound Co(II).

It was also of interest to know whether the assembly products formed from tubulin containing the 1 tightly bound Co(II) but lacking weakly bound Co(II) are similar to those formed from tubulin containing only tightly bound Mg(II). Such an experiment is possible only if additional metal ion does not have to be added. This can be achieved by using taxol to stimulate assembly. Taxol, an antitumor compound which promotes assembly of tubulin (20), does so in the absence of divalent cations (21). The assembly of tubulin in the presence of 12.5 μM taxol with no added cation did not appear to be affected by the presence of tightly bound Co(II) (Fig. 3). Electron microscopic examination also failed to show any noticeable differences (Fig. 4). Microtubules, sheets (C-, or S-shaped and more complex), and some double microtubules were present in both samples (Fig. 4). The very broad sheets and macrotubular structures observed when excess Co(II) is added (10) were not observed, indicating again that the formation of these products results from cobalt binding at lower affinity sites. The Co(II) and Mg(II) contents of the polymerized material were found to be 1.0 mol/mol of tubulin.

Effect of GTP and EDTA on Co(II)-Induced Assembly—If cobalt-induced assembly of tubulin and the production of large sheets are due to binding of the cation to low affinity sites, compounds which are effective chelators of Co(II) should affect the assembly process. As indicated in Table I, the presence of GTP reduces the amount of Co(II) binding to low affinity sites. The effect of increasing the GTP concentration at a constant Co(II) concentration on the assembly reaction was examined. At 0.5 mM CoCl₂, both the rate of increase and final extent of turbidity decreased as the GTP concentration was increased from 0.5 to 2 mM (Fig. 5). The decrease in turbidity could possibly be caused only by a change in the structure of the polymerized product, i.e. microtubules rather than sheets. To determine whether a decrease in assembly actually occurred, samples were centrifuged and protein in the pellet determined. In an experiment similar to that shown in Fig. 5 the amount of cold-soluble pelleted material assembled in the presence of 0.5, 1.0, and 2.0 mM GTP was 0.67, 0.49, and 0.35 mg, respectively. The concentration of GTP also affected the structure of the polymerized product. Negative stained samples showed that in 0.5 mM GTP only sheets of protofilaments were formed, whereas in 2 mM GTP microtubules were seen (Fig. 6). The molar ratio of Co(II) bound, determined under equilibrium conditions, was 3.4 in the presence of 0.5 mM GTP but fell to 1.6 in the presence of 2.5 mM GTP. One Co(II) is undoubtedly the tightly bound Co(II) since the presence of GTP does not prevent the tight binding. Separation of the polymerized from the unpolymerized tubu-

![Fig. 5. Effect of GTP concentration on the assembly of tubulin in Co(II). Tubulin (15 μM) was assembled in 0.1 M Pipes, pH 6.9, 0.5 mM CoCl₂, and the GTP concentrations shown, in 0.5 ml at 37 °C.](image-url)
lin in these experiments by centrifugation, followed by passage of the two fractions through phosphocellulose, showed that both fractions of tubulin contained 1 atom of bound Co(II)/dimer.

The inclusion of EDTA in excess over CoCl₂ in binding experiments prevented the tight, as well as low affinity, binding and in assembly experiments, totally inhibited polymerization. The addition of EDTA to tubulin assembled in the presence of Co(II) caused rapid disassembly (Fig. 7). Depolymerization was also seen with the addition of excess GTP. Tubulin which had been polymerized in CoCl₂ and depolymerized by cold treatment was found to contain 1.06 atoms of Co(II)/tubulin dimer. For assembled tubulin which was depolymerized by EDTA (Fig. 7), the value was 0.53. (To analyze for bound Co(II) in the presence of EDTA, gel filtration was used because phosphocellulose does not remove Co(II) from the Co-EDTA complex.)

**DISCUSSION**

Assembly of the tubulin dimer is stimulated by a variety of agents including associated proteins, polycations, and some organic solvents. These agents have the effect of reducing the critical protein concentration necessary for assembly. Mg(II) has a similar effect at concentrations of 10–15 mM. It is possible that at high concentrations, Mg(II) acts in a fashion similar to polycations and associated proteins and certain data support this view. For example high Mg(II) concentrations can simulate the effects of polylysine, or associated proteins, in the production of vincristine-induced tubulin protofilament spirals (22). Mg(II) binds to a large number of multiple weak sites on tubulin (11) in addition to one high affinity site. The high concentrations of Mg(II) required to induce assembly suggest that the weak binding is responsible for promoting assembly, possibly by altering the charge distribution on the protein.

Co(II) also binds to multiple sites with low affinity as well as to higher affinity sites, but in contrast to Mg(II), can induce assembly in the concentration range of 0.1 to 0.5 mM and at low protein concentrations. Moreover, in the presence of 0.5 mM GTP and 0.1 mM Pipes, conditions used in an assembly reaction, the number of weak sites occupied by Co(II) is drastically reduced. This implies that only a few divalent cations need be bound to stimulate assembly. The requirement for weak binding by Co(II) is also shown by the fact that the addition of EDTA or GTP, both of which reduce the amount of weak binding, in excess over Co(II) both inhibits assembly and causes rapid depolymerization of assembled tubulin. Although EDTA can partially remove the tightly bound cobalt, GTP cannot.

The difference in the concentration requirements for stimulation of assembly by Co(II) and Mg(II) probably reflects differences in the strength of binding at the same sites, but also may reflect binding to different sites. It should be pointed out that differences in the metal ion concentration requirement depend on the protein concentration used; i.e., the cation affects the critical protein concentration. For example, 0.5 mM Mg(II) will promote assembly but only if the protein concentration is several times higher than that used in these studies (2).

Whether microtubules or sheet structures containing many protofilaments are formed in Co(II)-induced assembly seems to depend on the number of weak sites occupied. A shift from microtubule to sheet formation upon increasing the Co(II) concentration was noted earlier (10), and in this report we show that increasing the GTP concentration, which reduces the amount of Co(II) binding, causes a shift from sheet to microtubule formation.

The tightly bound Co(II) (that which is not removed by cation exchangers) does not appear to be directly involved in the assembly reaction since either additional divalent cation or taxol is needed to induce polymerization. The nature of the one tightly bound Co(II) is not clear. Although the Mg(II) content of tubulin was usually found to be reduced after Co(II) binding, assembled tubulin which is depolymerized by cold treatment contained both 1 Co(II) and 1 Mg(II). Moreover, a portion of the protein polymerized in the presence of cobalt into a cold-insoluble form which contained very little Mg(II). The amount of cold-insoluble material was quite variable as was the amount of Mg(II) lost from the dimer after treatment with cobalt. The variability of these results suggests that a certain portion of the dimers become denatured upon binding Co(II) and release Mg(II), resulting in a form which assembles into cold-insoluble aggregates. The decrease in nucleotide content after Co(II) binding is also consistent with denaturation. The results do not indicate that Co(II) replaces Mg(II). The tightly bound Co(II) does not appear to interfere with the assembly of tubulin as long as the dimer still contains Mg(II). The fact that assembled tubulin also binds Co(II) at a high affinity site suggests that this site is easily accessible in the protofilament structure.

The results presented in Fig. 2 are difficult to explain. The presence of Mg(II) or Mn(II) causes a 50% reduction in the amount of high affinity binding, i.e., giving rise to tubulin containing 0.5 Co(II)/dimer. This implies that there may be subpopulations of tubulin in solution which differ in their tight divalent cation binding properties, perhaps representing different conformational states of the dimer. At any rate more work is required to explain the nature of the cobalt high affinity site.

In general, our conclusions concerning the requirement for cation binding directly to tubulin in order to induce assembly are in agreement with those of Gaskin (5). Gaskin did not measure cation binding, but used CrGTP or taxol to stimulate assembly in the absence of added GTP. With CrGTP, a stable metal-nucleotide complex, added Mg(II) was still required to promote optimal assembly into microtubules. Moreover, Zn(II) caused sheets to be formed in the presence of CrGTP or taxol. Gaskin concluded that direct binding of the cations to tubulin, and not just in the form of a cation-GTP complex,
was responsible for their actions. The fact that excess GTP, which complexes free Co(II), inhibits Co(II)-induced assembly, and promotes depolymerization, supports this view. Hence, the cation as well as cation-GTP are required for assembly.

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