Interaction between Chromium GTP and Tubulin

STEREOCHEMISTRY OF GTP BINDING, GTP HYDROLYSIS, AND MICROTUBULE STABILIZATION*

(Received for publication, January 22, 1991)

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Chromium GTP (CrGTP) has been used to probe the stereochemistry of metal-GTP binding to exchangeable site of tubulin and to examine the fate and role of nucleotide-bound metal ion in GTP hydrolysis associated with microtubule assembly. The absolute stereo-configuration of the two pairs of diastereomers of β,γ-bidentate CrGTP has been determined by comparison of their visible circular dichroism spectra with those of the β,γ-CrATP isomers whose configurations have been established (Lin, I., and Dunaway-Mariano, D. (1988) J. Am. Chem. Soc. 110, 950–956). Tubulin binds metal-GTP preferentially in the Δ pseudoaxial configuration. CrGTP-tubulin shows a high propensity to undergo tubulin-tubulin interactions with associated hydrolysis of CrGTP. Hydrolysis of CrGTP in microtubule assembly develops in three consecutive steps: cleavage of the γ-phosphate followed by release of P, and chromium. In contrast to other NTPases (acting, hexokinase) tubulin appears able to catalyze the dissociation of the stable chromium-phosphate bonds, which implies a highly nucleophilic environment of the binding site of the metal-triphosphate moiety of GTP. Microtubules assembled from CrGTP-tubulin are made of 90% GDP subunits, and their stability is linked to a 10% proportion of CrGDP-P, subunits, scattered along the microtubule, from which P, does not dissociate. The possibility is evoked that some tubulin variants do not catalyze release of P, and metal ion efficiently, and their presence could affect microtubule dynamics.

In the presence of GTP, two molecules of MgGTP are tightly bound to tubulin. One, on the α-subunit is structurally bound and nonexchangeable (N-site); the other, on the β-subunit, exchanges freely with medium GTP (E-site) and is hydrolyzed into GDP during microtubule assembly (1, 2). Mg&sup+; bound to the N-site is very slowly exchangeable (3). In the presence of GDP, unchelated GDP is bound to the E-site because of the much lower affinity of Mg&sup+; for GDP-tubulin than for GTP-tubulin (3), and MgGTP is structurally bound to the N-site (2–4). These results raise the problem of the role and fate of GTP-bound metal ion in GTP hydrolysis in microtubule assembly. It has been shown recently that P, release follows cleavage of the γ-phosphate of GTP on microtubules (5). Therefore the following events take place at the tubulin E-site in a cycle of assembly.

\[
\begin{align*}
\text{T-GTP} & \rightarrow \text{T-GDP} \\
\text{(T-GTP)MT} & \rightarrow \text{(TGDP-P)MT} \\
\text{P,} & \downarrow \\
\text{(TGDP-P)MT} & \rightarrow \text{(TGDP)MT}\end{align*}
\]

The release of P, is linked to a destabilization of tubulintubulin interactions in the microtubule, thus potentializing its rapid depolymerization (6). In the above scheme MgGTP is known to bind to dimeric tubulin in state I whereas unchelated GDP is bound in state V. Therefore Mg&sup+; is released from tubulin at some unknown step of the assembly cycle. To understand fully the role of Mg&sup+; in GTP hydrolysis and possibly in the control of the important step of P, release it is necessary to know the nature of the magnesium-nucleotide complex bound to tubulin in the different steps of the polymerization scheme. The coordination of the phosphate oxygens of GTP to Mg&sup+; can be studied either using thiophosphoryl guanine nucleotides of known absolute configuration (7) or the exchange-inert Cr&sup+; complexes of GTP which exchange metal ion 10^4-fold more slowly than MgGTP (8). The stereoselectivity of GTP binding to tubulin has been studied recently by Roychowdhury and Gaskin (9) who concluded that the GTP&sup+; (A or Sp) isomer was preferred by tubulin in the absence of microtubule-associated proteins whereas GTP&sup+; (B or Rp) isomer was preferred in the presence of microtubule-associated proteins. Also GTP&sup+; (B) was essentially nonhydrolyzed on microtubules from pure tubulin, but GTP&sup+; (A) was hydrolyzed. Similarly, CrGTP was found able to promote microtubule assembly and was partially hydrolyzed on microtubules (10, 11); however, the stereochemistry of CrGTP binding to tubulin was not examined in these works. We have separated the four isomers of β,γ-bidentate CrGTP, and we show that metal-GTP binds to tubulin E-site in the Δ pseudoaxial configuration. We show that CrGTP is efficiently hydrolyzed by tubulin, with release of both P, and Cr&sup+; in the medium, and that the high stability of microtubules assembled in the presence of CrGTP is caused by a low proportion (10%) CrGDP-P, remaining bound to microtubules.  

MATERIALS AND METHODS

Chemicals—MES was from Calbiochem; GTP disodium salt was from Boehringer Mannheim. Chromium(III) chloride hexahydrate (CrCl2, 6H2O) was from Merck. All other reagents were analytical grade. [γ&sup-32]P]GTP came from Amersham Corp.; \(^{11}\)Cr (in CrCl2 form) and \(^{19}\)H)GTP came from Du Pont-New England Nuclear.

CrGTP Synthesis—β,γ-Bidentate CrGTP was synthesized using the method described for synthesis of CrATP (8). Briefly a solution (50 ml) of 10 mM each Na2GTP and CrCl2 was brought to 80°C for

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* This work was supported in part by the Ligue Nationale Française contre le Cancer and by the Association Française contre les Myopathies. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
12 min. Separation of unreacted CrCl₃ and of the major part of GTP from the mixture was achieved on a 1.5 x 10 cm column of Dowex 50W-X2, the hexaqua chromium ion remained tightly bound to the resin whereas the unchelated GTP eluted with H₂O slightly ahead of CrGTP. Contaminating GTP was then separated efficiently from Cr-GTP by DEAE cellulose chromatography using a 0.8 x 2 cm column. CrGTP was eluted by 18 mM HCl.

When γ⁻²P-labeled or Cr-labeled CrGTP was synthesized, the amounts of reactivating CrGTP and GTP were reduced 5-10-fold, and the sizes of the Dowex 50 and DEAE columns were reduced accordingly. The specification of purity of the radiolabeled CrGTP was currently 5 mCi of γ⁻²P/mmol and 20 μCi of ⁵¹Cr/mmol. The purity of CrGTP was estimated by anion exchange HPLC, spectrophotometric determination of total chromium in the sample after H₂O oxidation in the presence of NaOH (8), spectrophotometric determination of nucleotide at 580 nm (ε = 12,400 M⁻¹ cm⁻¹ at pH 3) and of β,γ chromium diphosphate at 425 and 605 nm (ε = 20 M⁻¹ cm⁻¹ Ref. 8). The solutions of β⁺CrGTP were kept on ice at 2-10 °C, pH 3, for up to 4-6 weeks without deterioration.

Separation of the Diastereomers of CrGTP—Separation of the two pairs of diastereomers of β,γ-CrGTP was carried out by ion-pairing reversed phase HPLC using a Chropak LC-18-DB, 0.46 x 15-cm column (Supelco) and isotropic elution by 10 mM methanesulfonic acid, pH 2.5, as described for CrATP (12, 13). Identification of the nucleotide bound to tubulin polymerized in the presence of CrGTP was carried out by anion exchange HPLC of the nucleotide perchlorate extract after adjustment of the pH to 5.5 by KCH₃CO₂. A Synchropak AX-300 column (Synchrom) was used. Separation of CrGTP, CrGDP-P, and GDP was achieved by 0.25 M KH₂PO₄, pH 3.5, containing 0.6 M NaCl as eluting buffer, with a flow rate of 1 ml/min. The elution of nucleotide was monitored at 254 nm (13). When tubulin polymerization was carried out in the presence of ³⁵Cr- or ²⁵P-labeled CrGTP, the nature of the eluted species was further by collecting fractions of 0.35 ml and assigning these fractions for the presence of ⁵¹Cr or ³²P using a liquid scintillation spectrometer (Packard CA2000). β⁺-CrATP was prepared as described (13). The four isomers of both β⁺-CrATP and β,γ-CrATP were isolated by reversed phase HPLC as previously described (13). Briefly, as repeating the ion-pairing HPLC separation procedure a dozen times using a 100-μl loading loop, 0.5-1 μmol of each isomer could be obtained at a concentration of 200-700 μM. The purity of each isomer was checked by HPLC ion-pair chromatography. No reequilibration occurred when β⁺-CrGTP isomers were kept on ice in 10 mM methanesulfonic acid, pH 2.5, for at least a week. In P buffer (50 mM MES, pH 6.8, containing 0.5 mM EDTA, 0.25 mM MgCl₂, and 4 mM glycerol), however, interconversion of the isomers of β⁺-CrGTP could be detected. Kinetic measurements of the reequilibrium of each isomer in polymerization buffer containing 6 mM MgCl₂ at 37 °C showed that within 5 min the isomer had undergone isomerization, and total equilibration was reached in 20 min. Isomer 1 interconverted into isomer 3 preferentially, and isomer 2 into isomer 4, in agreement with the view (13) that the pseudooxidial = pseudoequatorial transition between ring conformers is energetically favored versus the A = A transition, where Δ and Λ refer to optical isomers having the right-handed screw sense and left-handed sense, respectively. Using anion exchange HPLC, the rate of dissociation of CrGTP into Cr⁺ + GTP was examined. It was found that 5-10% of the CrGTP was converted into GTP upon 15-min incubation at 37 °C in polymerization buffer. On the other hand, no detectable interconversion was occurring at 0 °C for 15 min under similar conditions. In view of these results all polymerization experiments carried out in the presence of CrGTP could be considered valid if polymerization developed within less than 15 min.

**Protein—** Tubulin was purified from fresh pig brain by three cycles of polymerization according to Shelanski et al. (14) followed by phosphocellulose chromatography (15) and was stored at -80 °C in P buffer supplemented with 0.1 mM GTP. GDP-tubulin 1:1 complex was prepared as follows. PC-tubulin at 70 μM was polymerized in the presence of 6 mM MgCl₂ and 500 μM GTP at 37 °C; the microtubules were resuspended at 400,000 g for 15 min at 37 °C on a 30% sucrose cushion in the TL100 ultracentrifuge (Beckman) and resuspended in ice-cold buffer P. The solution then was clarified by centrifugation at 100,000 g, 0 °C, for 15 min and used as such. Tubulin concentration was determined by UV spectrophotometry using an extinction coefficient of α at 278 nm.

**Circular Dichroism Measurements—** Circular dichroism spectra of the four isomers of CrGTP and CrATP at pH 2.5 were recorded in the range of 400-700 nm at 1 nm/s (three cycles) with a MacV Jouan dichrograph using 1-cm-square quartz cuvettes. Each isomer was 0.2-0.7 mM.

**Polymerization Measurements—** Tubulin polymerization was monitored turbidimetrically at 360 nm using a Kontron Uvikon spectrophotometer and a 1-cm light path cell thermostated at 37 °C. Polymerization buffer was P buffer supplemented with CrGTP or GTP at the indicated concentration and with 6 mM MgCl₂. The mass concentration of polymerized tubulin was derived from the comparison of the extent of turbidity change with the amount of sedimented material determined by protein assay. In the low tubulin concentration range (<15 μM) used throughout this work a linear correlation was observed between the increase in turbidity and the amount of polymerized tubulin. GTPase measurements were carried out on the same sample during the assembly process as described (17).

**Hydrolysis of CrGTP by Tubulin—** ³²P, liberated in solution after hydrolysis of γ⁻²P-P-CrGTP was determined by extraction of acid-labile ³²P, as described (17). However, this method can detect only free P, because the cleavage of the γ-phosphoester bond of β⁺-γ-CrGTP yields β⁺γ-CrGDP-P., in which the chromium ion is chelated to the β-phosphate of GDP and P, via acid-stable bonds (13); these bonds can be cleaved irreversibly by alkaline treatment, thus liberating P, which then can be extracted by ammonium molybdate in acid solution. Therefore, use of the conventional method for measurement of acid-labile P, leads to the determination of free P, whereas alkaline treatment (2 N NaOH) of the samples prior to reacidification and phosphate extraction leads to the determination of the sum of CrGDP-P, and P, as demonstrated previously (13). Both measurements carried out simultaneously on the same sample allow to derive, by difference, the time courses of chemical cleavage of the γ-phosphate and of liberation of P, (that is, cleavage of the CrGDP-P bond).

**RESULTS**

**Assignment of the Stereoconfigurations of the Four Diastereomers of β⁺-γ-CrGTP—** Synthesis of β⁺-γ-CrGTP as described under “Materials and Methods” yielded a mixture of four isomers (two pairs of diastereomers) of β⁺-γ-CrGTP as described under “Materials and Methods” yielded a mixture of four isomers (two pairs of diastereomers) that could be separated by ion-pairing HPLC, as had been first described for β⁺-γ-CrATP (12). Fig. 1 shows the elution patterns of CrGTP and CrATP isomers in ion pair chromatography. These isomers can be designated 1, 2, 3, and 4 based on their order of elution from reversed phase HPLC. It has been shown that isomers 1 and 4 of β⁺-γ-CrATP have the Δ (or S) β⁻P configuration (i.e. show a negative Cotton effect at 575 nm and have a right-handed screw sense configuration), but the isomers 2 and 3 have the Δ (or R) β⁻P configuration, i.e. show a negative Cotton effect at 575 nm and have a left-handed screw sense configuration (12). Recent experiments (18) have led further
to the identification of the absolute configurations of the four isomers of \( \beta,\gamma\)-CrATP; isomers 1 and 2 are in the pseudoaxial configuration, and isomers 3 and 4 are in the pseudoequatorial configuration, which leads to the conclusion that 1 = \( \Delta_0 \); 2 = \( \Lambda_0 \); 3 = \( \Lambda_\alpha \); 4 = \( \Delta_\alpha \). Accordingly, the visible CD spectra of 1 and 2 and of 3 and 4, respectively, are mirror images.

On the basis of these data and by comparison of the CD spectra of \( \beta,\gamma\)-CrATP and \( \beta,\gamma\)-CrGTP isomers we have tried to identify the stereoconfiguration of the four isomers of \( \beta,\gamma\)-CrGTP. Fig. 2 shows the visible CD spectra of all isomers. It is apparent that the spectra of isomers 1 and 2 of CrGTP are mirror images and are identical in shape to the spectra of isomers 1 and 2, respectively, of CrATP. On the other hand, the spectra of isomers 3 and 4 of CrGTP are also mirror images and match the spectra of isomers 4 and 3, respectively, of CrATP. That is, isomers 1, 2, 3, 4 of \( \beta,\gamma\)-CrGTP, match isomers 1, 2, 4, and 3 of \( \beta,\gamma\)-CrATP. This piece of data allows us to attribute the following stereoreconfigurations to \( \beta,\gamma\)-CrGTP diastereomers: 1 = \( \Delta_\text{axial} \); 2 = \( \Lambda_\text{axial} \); 3 = \( \Delta_\text{equatorial} \); 4 = \( \Delta_\text{equatorial} \).

Bulk solutions of all four isomers of \( \beta,\gamma\)-CrATP or \( \beta,\gamma\)-CrGTP exhibited a doublet elution pattern when submitted to HPLC anion exchange chromatography (Fig. 1). It was observed that the enantiomers 3 and 4 (in equatorial configuration) of CrATP eluted in peak 1 on the anion exchanger; enantiomers 1 and 2 (in axial configuration), in peak II. On the other hand, diastereomers 2 and 4 of CrGTP (both \( \Delta \)) eluted in peak I, and diastereomers 1 and 3 (both \( \Lambda \)), in peak II. As illustrated in Fig. 3, in the pseudoequatorial configuration one oxygen of the \( \beta \)-phosphate makes a hydrogen bond with a water molecule of the coordination sphere of chromium whereas in the pseudoaxial configuration this hydrogen bond is made with an oxygen of the \( \alpha \)-phosphate, leaving the more nucleophilic oxygen of the \( \beta \)-phosphate nonliganded. We would therefore expect the pseudoaxial isomers to have a slightly more negative charge than their pseudoequatorial counterparts. This prediction seems verified in the case of CrATP but not in the case of CrGTP. The reason for this surprisingly different behavior may lie in the presence of \( \text{NH}_2 \) groups on the guanine base which may also contribute to the charge of the complexes and affect their separation on an anion exchanger; or there may be another unknown reason.

**Stereospecificity of CrGTP Binding to Tubulin**—The ability of each of the four isomers of CrGTP to bind to tubulin was assayed using the Hummel and Dreyer method (19). \(^{[3]H}\)GDP-tubulin 1:1 complex was prepared as described under “Materials and Methods” at 6 \( \mu \)M. To 1 ml of the tubulin solution was added 20 \( \mu \)M of each isomer, and the samples were chromatographed immediately over Sephadex G-25 columns (PD-10) equilibrated at 4 °C in P buffer containing a 20 \( \mu \)M concentration of each isomer. The elution was monitored both at 280 nm for protein determination and by measuring \(^{[3]H}\)GDP radioactivity. The extent of displacement of bound \(^{[3]H}\)GDP by each isomer, \( d \), was derived from the amount of \(^{[3]H}\)GDP bound to tubulin coming off the column according to the following equation

\[
d = 1 - \frac{[^{[3]H}]GDP_{\text{bound to tubulin}}}{[^{[3]H}]GDP_{\text{bound}}}
\]

Tubulin was eluted from the column within at most 10 min, a period of time short enough to minimize interconversion of the isomers at 4 °C. Table I shows that each isomer of CrGTP was able to compete for bound GDP to some extent; however, isomer 1 showed a higher affinity for tubulin.

The ability of each isomer to support tubulin polymerization was then assayed. The GDP-tubulin 1:1 complex (10 \( \mu \)M) was incubated on ice with a 150 \( \mu \)M concentration of each isomer for 15 min before being supplemented with 6 mM MgCl\( _2 \) and polymerized at 37 °C. Fig. 4 shows that, in agreement with the binding data, isomer 1 was able to support tubulin polymerization much more efficiently than the three other isomers. The slow polymerization observed with isomers 2 and 3 after 10 min is most likely due in part to the interconversion of the isomers, giving more and more isomer 1 with time. However, we cannot eliminate the possibility that tubulin can actually catalyze isomerization of CrGTP into the \( \Delta_\text{axial} \) configuration. Isomer 4 repeatedly showed a peculiar polymerization curve, with a lag of 7 min followed by

**Table I**

| Isomer | GDP displaced from tubulin (%) |
|-------|-------------------------------|
| 1     | 43                            |
| 2     | 15                            |
| 3     | 23                            |
| 4     | 17                            |

FIG. 2. Identification of the stereo configuration of the four diastereomers of \( \beta,\gamma\)-CrGTP by comparison of their circular dichroism spectra with those of \( \beta,\gamma\)-CrATP diastereomers. The diastereomers of \( \beta,\gamma\)-CrGTP were isolated at 200–700 \( \mu \)M by using ion pairing HPLC as described under “Materials and Methods.” The visible circular dichroism spectra of isomers 1 and 2 (top panels) and 3 and 4 (bottom panels) of CrGTP (left panels) and CrATP (right panels) were recorded (see “Materials and Methods”) at 1 nm/s (three cycles). Isomer 2 of both CrGTP and CrATP was contaminated by the \( \gamma \)-monodentate complex which has no visible circular dichroism spectra.

FIG. 3. Structure of the \( \Delta \) pseudoaxial (left) and pseudoequatorial (right) diastereomers of CrGTP (derived from Ref. 8).
be disregarded that during the lag, GTP accumulates in tubules assembled in the presence of CrGTP did not depolymerize at all when CrGTP is bound in this configuration. These experiments demonstrate that the metal-GTP complex binds to tubulin E-site preferentially in the inclusion, these experiments indicate that the metal-GTP isomers of CrGTP. It has been reported (10, 11) that microtubules were present. The dependence of tubulin polymerization on Mg2+ ion concentration was found to be the same whether MgGTP or CrGTP was bound to the E-site (data not shown). In the presence of 0.25 mM MgCl2 (originally present in buffer P) and 0.3 mM CrGTP, tubulin at 10 μM did not polymerize into microtubules unless millimolar amounts of Mg2+ ion, known to neutralize repulsive negative charges and to allow tubulin-tubulin interactions, were present. The dependence of tubulin polymerization on Mg2+ ion concentration was found to be the same whether MgGTP or CrGTP was bound to the E-site (data not shown). In the presence of 0.25 mM MgCl2 (originally present in buffer P) and 0.3 mM CrGTP, tubulin at 10 μM did not polymerize into microtubules. However, as shown in Fig. 6, CrGTP was actively hydrolyzed by tubulin in a steady-state fashion with liberation of free (acid-labile) P3. In the presence of 6 mM MgCl2 added to buffer P, tubulin polymerized, and CrGTP was hydrolyzed at a higher rate and in a manner that appeared uncoupled from the polymerization process, i.e. hydrolysis started well before microtubule assembly; the rate of hydrolysis grew slower as tubulin was incorporated in microtubules. These data are quite surprising because in most enzymatic systems, chromium nucleotides are hydrolyzed at a much slower rate than magnesium or calcium nucleotides and act as dead-end inhibitors. In addition, since CrGTP had been reported to be only partially hydrolyzed on microtubules, which are stable (10, 11), one would have expected no hydrolysis of CrGTP during tubulin polymerization. To understand better by which mechanism CrGTP is hydrolyzed on tubulin the following experiments have been carried out. GDP-tubulin at different concentrations was incubated at 37 °C with 300 μM [γ-32P]CrGTP and MgCl2 in a range of low concentrations (<3 mM) at which it was found that no polymerization occurred. The initial steady-state rate of hydrolysis of CrGTP was measured. The dependence of the rate of CrGTP hydrolysis on tubulin and MgCl2 concentration (in the absence of any microtubule formation) is displayed in Fig. 7. The rate of hydrolysis, at a given concentration of tubulin, increased with the concentration of Mg2+ and also increased cooperatively with tubulin concentration at all concentrations of MgCl2 (Fig. 7A), indicating that hydrolysis was linked to tubulin-tubulin interactions involved in the formation of oligomers that may be precursors of microtubules. Accordingly, the initial rate of hydrolysis of CrGTP varied linearly with the square of the concentration of tubulin at each concentration of MgCl2 (Fig. 7B). In this context, the data indicate that oligomer formation is more favorable when CrGTP, rather than MgGTP, is bound to tubulin E-site. In the experiments shown in Figs. 6 and 7, the production of acid-labile P3 from CrGTP had been measured. As developed under "Materials and Methods," the acid-labile P3 as a result of hydrolysis of CrGTP results from two reactions: cleavage of the γ-phosphoester bond, leading to CrGDP-P; and subsequent cleavage of the acid-stable chromium-phosphate bonds, leading to Cr + GDP + P3. The production of CrGDP-P in solution (in which chromium ions make stable bonds with the β-phosphate of GDP and P3) was assayed, as described under "Materials and Methods," by alkaline treatment of aliquots
Properties of CrGTP-Tubulin

12365

Fig. 7. Hydrolysis of CrGTP in relation with tubulin-tubulin interactions. GDP-tubulin 1:1 complex at the indicated concentrations was incubated in the presence of 300 μM [γ-32P]CrGTP and brought to 37°C in P buffer supplemented with 0 (●, ○), 2 (∆, ◦), and 3 (▲) mM MgCl2. The initial rate of CrGTP hydrolysis was measured in each sample. It was checked turbidimetrically that no polymerization occurred at least during the 5 min necessary for GTPase measurements. The initial rate of CrGTP hydrolysis is plotted versus the concentration of GDP-tubulin (panel A) and versus the square of the concentration of GDP-tubulin (panel B).

Fig. 8. Cleavage of the γ-phosphoester and of chromium-phosphate bonds during tubulin polymerization. GDP-tubulin (10 µM) was polymerized in the presence of 300 μM γ-32P-labeled CrGTP. The assembly process was monitored turbidimetrically (thick line). The liberation of P3 in solution was assayed by the measurement of acid-labile P3 (●) as described under “Materials and Methods.” The sum of the reactions of cleavage of the γ-phosphate (leading to CrGDP-P3) and cleavage of chromium-nucleotide bonds (leading to free P3) was assayed by alkaline treatment followed by extraction of P3 as above (∆). The difference between the two time courses (dashed line) represents the formation of CrGDP-P3.

withdrawn from the solution at time intervals, prior to reacification and extraction of acid-labile P3. Comparison of the time courses of accumulation of P3 and of (CrGDP-P3 + P3), displayed in Fig. 8, showed that in the early stages of the polymerization process, appreciable amounts of CrGDP-P3 accumulated transiently in solution whereas in the late stages of assembly and at steady state the time courses of production of P3 and CrGDP-P3 + P3 were superimposable, i.e. CrGDP-P3 was not present in solution. The concentration of CrGDP-P3 reached a maximum of 6–7 μM at the onset of microtubule assembly and declined in parallel to the polymerization process. The same observation was made in several experiments. The rate of hydrolysis of CrGTP was maximum at time zero and decreased as tubulin was incorporated in microtubules. These results indicate that in the overall process of hydrolysis of β, γ-CrGTP associated to tubulin-tubulin interactions, P3 release is slower than cleavage of the γ-phosphoester bond, leading to CrGDP-P3 as an intermediate species. Since CrGDP-P3, per se is stable in solution, the release of P3 must be catalyzed by tubulin after its incorporation into microtubules or into oligomeric precursors of microtubules. Assuming that all CrGDP-P3 was bound to tubulin, the data indicate that at the onset of microtubule assembly a large proportion of the tubulin had CrGDP-P3 as bound nucleotide, and release of P3 occurred during its incorporation into microtubules. These results lead us to anticipate the puzzling conclusion that upon microtubule assembly from CrGTP-tubulin, GTP hydrolysis and P3 release occur essentially as efficiently as upon polymerization of MgGTP-tubulin.

Analysis of Nucleotide Bound to Microtubules Assembled from CrGTP-Tubulin—The nature of nucleotide bound to steady-state microtubules was analyzed by anion exchange HPLC of the perchloric extract of the sedimented material. This experiment was performed on pellets of microtubules polymerized with either [52Cr] or [γ-32P]CrGTP as described under “Materials and Methods.” The average of 10 experiments led to the conclusion that 85–90% of the nucleotide bound to the E-site on microtubules was GDP. Of the remaining 10–15%, 50 ± 10% was CrGDP-P3, which is characterized by its elution from HPLC at a position intermediate between CrGTP and GDP. 25 ± 5% was CrGTP (apparently isomer 1 since it eluted at the position of peak II of bulk CrGTP), and 25 ± 5% was CrGDP, which elutes early, ahead of CrGTP. The elution patterns of nucleotide bound to tubulin polymerized in the presence of [52Cr]- and [γ-32P]CrGTP are shown in Fig. 9.

The conclusion of these experiments is that upon microtubule assembly in the presence of metal-GTP, tubulin catalyzes the release of both P3 and tightly bound metal ion, leading to unchelated GDP as the main nucleotide species bound to polymerized tubulin. Microtubules assembled from CrGTP-tubulin appear to be 90% identical in their nucleotide content to microtubules assembled from MgGTP-tubulin. The high stability of microtubules polymerized in the presence of CrGTP appears to be caused by a small proportion (≤10%) of CrGDP-P3, and CrGTP subunits. To decide whether these subunits are scattered all along the polymer or clustered into a steady-state terminal cap at the ends of microtubules, the following experiment, displayed in Fig. 10, was carried out. GDP-tubulin 1:1 complex (10 µM) was polymerized in the presence of 300 μM CrGTP. The stability of microtubules was assayed by monitoring the cold-induced depolymerization process turbidimetrically. For this purpose the solution was cooled to 4°C before and at different times after the addition of 250–500 μM GTP. Upon addition of GTP, which binds to tubulin, in the presence of Mg2+, with a higher affinity than CrGTP, the turnover of microtubules via incorporation of CrGTP-tubulin is arrested, hence maintenance of a putative steady-state CrGDP-P3 cap can no longer be exerted. It was observed that microtubules assembled from CrGTP-tubulin depolymerized at the same very slow rate when the solution was cooled either before or 20–30 min after the addition of GTP. This period of time is 5-fold longer than the half-life of CrGDP-P3, on tubulin, as estimated from Fig. 8, and is certainly sufficient for total disappearance of a putative cap of CrGTP/CrGDP-P3 subunits clustered at the ends of microtubules at steady state in the presence of CrGTP. Therefore,
before or, in parallel samples, at different times after the addition of was shifted to bulus assembled from CrGTP-tubulin does not seem to decrease with time under conditions in which the steady-state of GTP.

Accordingly, the same proportion of CrGDP-Pi subunits was found in pellets of microtubules assembled in the presence of CrGTP and sedimented at time 10 min or 30 min after reaching steady state. Two possible interpretations can account for the inability of these polymerized tubulin subunits to release Pi. Either these subunits represent a subclass of tubulin isotypes which have an altered GTP site conformation and the relative proportion of such tubulins in microtubules could regulate microtubule stability; or 10% of the tubulin molecules bind a stereomer of β,γ-CrGTP different from the preferred one, and binding of GTP in a stereocorefiguration different from the Δ pseudoaxial does not allow the release of P i. The latter interpretation compares favorably with the report of Roychowdhury and Gaskin report (9) that GTPpS (B), which has the configuration of Λ CrGTP, is not hydrolyzed on microtubules. On the other hand, the latter interpretation is not supported by two observations. First, the low proportion of CrGTP remaining bound to microtubules elutes from the anion exchange HPLC column at the position of peak II, consistent with its being isomer 1 (the preferred one) or isomer 3 (Δ pseudoequatorial, which binds poorly). Second, microtubules assembled from pure isomer 1 of β,γ-CrGTP were as cold stable as those assembled from the mixture of all four isomers, indicating that some CrGDP-Pi, also remains bound to microtubules after hydrolysis of isomer 1. Therefore the former hypothesis, which stipulates that some tubulin variants may have a lower ability than others to release Pi, and metal ion, seems more attractive. However, it is difficult at this point to propose a definite conclusion, and the present data only encourage the design of new experiments to discriminate between the above possibilities. In any case, the fact that a low proportion of GDP-Pi subunits in microtubules provides extensive stability to the polymer is in agreement with previous results obtained using BeF 2, H 2 O as a structural analogue of P i. (20), The rate of depolymerization of microtubules was shown to vary with the proportion of GDP-Pi-like subunits in microtubules according to the following equation

\[ k_{-}/k^{\text{exo}} = \bar{Y}(R - 1) + 1 \]  

in which \( k^{\text{exo}} \) is the measured rate of depolymerization of a microtubule containing a proportion \( \bar{Y} \) of GDP-Pi subunits; \( k_\text{-} \) is the rate of depolymerization of a GDP-microtubule, and \( k_\text{+}/R \), the rate of depolymerization of a GDP-Pi microtubule. The present data indicate that with \( \bar{Y} = 0.1, k_\text{-}/k^{\text{exo}} = 100 \) at 4 °C, which leads to a value of 10^4 for R. Within this model, GDP-Pi subunits would dissociate from microtubule ends at a 10-fold slower rate than GDP-subunits. This figure is reasonably consistent with previous estimates of the relative rates of dissociation of terminal and internal subunits at 37 °C in a regime of growth (21-23), therefore confirming the validity of the GTP/GDP-Pi, cap model of microtubule dynamic instability (5, 6, 20).

DISCUSSION

The inert complexes of Cr(III) with GTP have been useful to probe both the stereocorefiguration of the metal-GTP bound to tubulin and the role of the metal ion in GTP hydrolysis during microtubule assembly. The absolute configuration of the two pairs of diastereomers of β,γ-CrGTP could be identified by comparison of their CD spectra with those of the stereomers of β,γ-CrATP whose configuration is known (18). The data led to the conclusion that the β,γ-metal-GTP complex is bound to tubulin E-site in the Δ pseudoaxial configuration. Accordingly, tubulin should bind preferentially the S 2 (or A) isomer of GTP/PS, a result that was actually obtained for pure tubulin (while GTP/PS (B, or Rb) bound preferentially...
to tubulin in the presence of microtubule-associated proteins Ref. 9). Comparison can be made with the G-protein ras p21, which also binds MgGTP in the ∆ configuration (24), and with transducin, which also binds the Sp (A) isomer of GTP/PS more tightly than the Rb (B) conformer (25). Although tubulin does not possess the consensus sequences characteristic of G-proteins (26, 27) it is possible that it shares with eukaryotic GTPases sterical relationships of the binding site of the triphosphate-metal moiety of MgGTP. Such a kinship, based on function, may be more relevant than sequence homology. Similarly, the stereospecificities of pyridine nucleotide dehydrogenases are highly conserved (28). More extensive comparative studies of the structure of the GTP site on tubulin and other G-proteins are however necessary to test this possible evolutionary kinship.

CrGTP-tubulin shows more propensity to undergo tubulin-tubulin interactions, with associated hydrolysis of GTP, than MgGTP-tubulin. Incidentally, actin was also found to nucleate more actively in the presence of CrATP (13). In agreement with a previous report that GDP-P, microtubules are kinetic intermediates in the polymerization of MgGTP-tubulin (5), we find that CrGDP-P, is transiently bound to tubulin during polymerization of CrGTP-tubulin. The half-life of the CrGDP-P, transient is about 5 min compared with 25 s for MgGDP-P, which is consistent with the higher stability of chromium-phosphate bonds. A quite peculiar and unique feature of CrGTP hydrolysis by tubulin is the nature of the products released. Chromium nucleotides are generally used as dead end inhibitors in enzyme-catalyzed phosphoryl transfer reactions. The main reason for the very slow catalytic rate exhibited by these enzymes upon utilizing chromium nucleotides resides in the chemical stability of chromium-phosphate bonds. The fact that chromium-phosphate bonds are more stable than magnesium-phosphate bonds prevents the efficient dissociation of the products P or acceptor-P so that the product of the reaction is either the CrNDP-P, compound as in the case of mitochondrial F1-ATPase (29) or actin (13), or a CrNDP-acceptor-P compound in which the acceptor is glycolate in the case of pyruvate kinase (30) or glucose in the case of hexokinase (31). This product generally remains tightly bound to the enzyme which, for this reason, catalyzes a single turnover reaction. In contrast to the above picture, tubulin appears able to catalyze the cleavage of both the γ-phosphoester and the chromium-phosphate bonds during microtubule assembly so that unchelated GDP is the main nucleotide species remaining bound to polymerized tubulin, and both Cr+ and P are liberated in the medium. Since highly alkaline conditions are required to dissociate chromium-phosphate bonds of chromium-nucleotides when free in solution, we have to conclude that strongly nucleophilic residues exist in the GTP site in close proximity of the metal-phosphate bond. These nucleophilic residues catalyze the dissociation of chromium-phosphate bonds, which eventually leads to the release of metal ion and P. It is more likely that upon polymerization of MgGTP-tubulin, Mg++ is released even more easily than Cr++ since the magnesium-phosphate bonds are less stable than the chromium-phosphate bonds. The GTPase site of tubulin therefore appears to be chemically different from the ATPase site of F-actin (13, 32, 33) or the GTPase site of ras p21 (24), on which the hydrolysis of metal-NTP leads to the release of P, in solution, while NDP-Mg remains bound to the protein. It has been shown that the change in coordination of Mg++ triggers structural changes affecting the interaction of ras protein with the effector GAP (24). The Mg++ dependence of GTP affinity for tubulin (2, 3) indicates that Mg++ also interacts with nucleophilic residues in the site; we may therefore expect that important rearrangements occur upon liberation of P, and Mg++. We may, for example, expect large displacements of charged residues to compensate for the release of Mg++ and P; a similar hypothesis, based on conservative sequence analysis, has been proposed recently (34).

Our experiments do not provide any information about the relative rate constants for metal ion and P, dissociation; however, the fact that the amount of bound CrGDP-P, is larger than the amount of bound CrGDP suggests that the dissociation of metal ion follows the dissociation of P, closely. A measurable constant proportion of CrGDP-P, remains bound to microtubules assembled from CrGTP-tubulin even when monomer-polymer exchange reactions taking place at steady state in the presence of CrGTP are inhibited. This result suggests that on a few tubulin subunits, P, and metal ion release is catalyzed less efficiently than on others. Whether this is caused by binding of CrGTP in a configuration less adequate for product release than the ∆ pseudooxial or to the lower ability of some tubulin variants to release P, and metal ion or to a combination of both is not clear yet. However, we may expect that since MgGTP is in rapid equilibrium between all four stereoisomers, the same phenomenon occurs during polymerization of MgGTP-tubulin, although to a lesser extent because cleavage of magnesium-phosphate bonds is energetically more favorable than cleavage of chromium phosphate. The putative presence of a few MgGDP-P, subunits scattered on the microtubule (much less than the 10% amount observed in the presence of CrGTP) will have a stabilizing effect on the polymer structure and may generate the "stops" and "pauses" in the depolymerization process which have been observed in video microscopy of individual microtubules (35). More systematic experiments will be designed to test this hypothesis.

Acknowledgments—We thank Drs. Michel Desmatdri and Jeanine Yon for kindly providing access to the dichrograph, and Micheline Terriee and Gérard Charly for technical assistance in tubulin purification.

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Properties of CrGTP-Tubulin

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