Incorporation of tubulin subunits into dimers requires GTP hydrolysis

Ana Fontalba1, Rosanna Paciucci1, Jesus Avila2 and Juan C. Zabala1,+*

1Departamento de Biologia Molecular, Facultad de Medicina, Universidad de Cantabria e Instituto de Estudios Avanzados en Fisica Modena y Biologia Molecular, CSIC, Spain
2Centro de Biologia Molecular, Facultad de Ciencias, Universidad Autonoma de Madrid, (CSIC-UAM), Spain
*Author for correspondence

SUMMARY

A toroid multisubunit complex of 800-900 kDa has been implicated in assisting protein folding of at least two cytoplasmic proteins, actin and tubulin. This process is dependent on the presence of magnesium ions and ATP hydrolysis. In vitro translation of cDNAs encoding different α- and β-tubulin isotypes also gives rise to the formation of complexes of about 300 kDa. These complexes have been functionally implicated in the incorporation of tubulin monomers within the tubulin heterodimer. This work shows that, in addition to ATP hydrolysis, the incorporation of newly synthesized tubulin subunits into functional heterodimers requires GTP hydrolysis in the presence of magnesium ions. A two-step process is suggested, a first ATP-dependent step in which the 900 kDa complexes are implicated in a similar way to the step taking place in actin folding, and a second ATP-dependent step in which the 300 kDa complexes are involved in the assembly of the heterodimer.

Key words: GTP hydrolysis, native electrophoresis, tubulin, dimerization, magnesium, protein assembly

INTRODUCTION

Protein assembly often involves interactions of unfolded proteins with preexisting proteins that function as molecular chaperones. Different groups of molecular chaperones have been involved in assisting protein folding, including the heat-shock protein 70 group, and the chaperonins (Langer et al., 1992; Ellis and van der Vies, 1991). By means of non-denaturing electrophoresis and anion-exchange chromatography it has been shown that newly synthesized β-tubulin interacts with cellular factors forming at least two different complexes (the high molecular mass form C900 and the low molecular mass form C300) before it can incorporates into dimers (Yaffe et al., 1988, 1992; Zabala and Cowan, 1992). A similar behaviour has been observed for α-tubulin subunits, although the kinetics of appearance and proportion of the different molecular forms are different from those of β-tubulin (Zabala and Cowan, 1992).

A toroid multisubunit particle of about 800-900 kDa is present in the cytosol of different animal cells. This particle contains TCP-1, a protein highly related in sequence to TF55, which has ATPase activity and is involved in protein folding (Trent et al., 1991) with four to six other unidentified proteins and two Hsp70 heat-shock proteins (Lewis et al., 1992). These oligomeric particles have been implicated in folding of completely denatured actin and α- and β-tubulin (Yaffe et al., 1992; Gao et al., 1992) constituting, therefore, a chaperone complex (C900). The chaperone complex binds actin or tubulin polypeptides, giving rise to a binary complex. These complexes formed between purified particles and denatured actin polypeptides are able to release functional actin monomers in the presence of Mg-ATP (Gao et al., 1992). The complexes formed between the C900 and tubulins are also able to release functional tubulin in the presence of Mg-ATP (Yaffe et al., 1992; Fyrdman et al., 1992; Gao et al., 1993).

In vitro translation in reticulocyte extracts of tubulin polypeptides gives rise to the formation of complexes of about 180-300 kDa termed C300. The molecular composition of these complexes that tubulin forms with reticulocyte proteins needs to be further investigated, although they have been functionally implicated in tubulin dimerization (Yaffe et al., 1988; Zabala and Cowan, 1992). The interaction of β-tubulin with the 900 kDa complex appears to be a fast initial step in the tubulin folding and assembly process: prolonged incubation at 37°C of in vitro translation reactions reduced the amount of C900 complexes compared to the amount of C300 complexes (Yaffe et al., 1992). Kinetic analysis of tubulin dimer formation has also shown that, in a slower fashion, the proportion of C300 complexes, monomers and dimers changed: the amount of dimers formed was related to the disappearance of C300 complexes and monomers (Zabala and Cowan, 1992). We have investigated the process by which newly synthesized β-tubulin monomers and C300 complexes give rise to the formation of tubulin dimers. We present evidence that heterodimers can form only after GTP is hydrolyzed and that this process is dependent on the presence of Mg2+. Moreover, the incorporation of tubulin subunits from C300...
and/or monomers into dimers is independent of ATP hydrolysis. These data are discussed in the context of a two-step mechanism implicated in the tubulin folding and assembly process.

MATERIALS AND METHODS

Materials

Reticulocyte extracts were obtained from Promega, [35S]methionine (>1,000 Ci/mmol) from Amersham and GTPγS and ATPγS from Boehringer Mannheim.

In vitro transcription and translation of β3-tubulin

Full-length cDNA encoding wild-type β3-tubulin (Wang et al., 1986) cloned into pGEM-2 vector was used as template for in vitro transcription (Melton et al., 1984). Transcribed mRNA was translated in a rabbit reticulocyte cell-free system (Pelham and Jackson, 1976) in the presence of [35S]methionine (Zabala and Cowan, 1992). In vitro synthesized products were centrifuged at 100,000 g for 30 min at 4°C in a Beckman TLX centrifuge and extensively dialyzed against 50 mM MES, pH 6.7.

Partial purification of C300 β3-tubulin complexes

In vitro translation reactions were carried out at 1 hour for 30°C and centrifuged at 100,000 g for 30 minutes at 4°C. C300 complexes were partially purified by a modification of the method previously described (Zabala and Cowan, 1992). Aliquots of in vitro translation reactions were loaded onto a (Pharmacia) Superose 6 column equilibrated with buffer (100 mM MES, pH 6.7) and run at a flow rate of 0.25 ml min-1. Fractions of 200 µl were collected and C300 complexes eluted in ml 13.4.

Preparation of native brain tubulin and reticulocyte extracts

Purified brain tubulin was prepared as described (Weingarten et al., 1974; Shelanski et al., 1973), by consecutive passages through phosphocellulose, cation-exchange FPLC (Zabala and Cowan, 1992), and G-25 equilibrated in 150 mM MES, pH 6.7. Reticulocyte extracts were centrifuged at 100,000 g for 30 minutes at 4°C and depleted of low molecular mass components by passage through G-25 equilibrated in 150 mM MES, pH 6.7.

Native gel electrophoresis

Aliquots from reactions carried out as described in the legend of the figures were diluted one-fifth with 25% sucrose (final concentration of 5%) in 50 mM MES, pH 6.7 and immediately loaded onto a 7% non-denaturing polyacrylamide gel, containing ME buffer (100 mM MES, pH 6.7, 1 mM EGTA) with 0.1 mM GTP and 1 mM MgCl2, 0.1 mM GTPγS and 1 mM MgCl2, or 0.1 mM GTP. Following electrophoresis, gels were fluorographed, dried and exposed to film. The radioactive bands were excised and quantified by scintillation counting.

Protein blotting

Protein samples were electrophoresed through 4.5% and 7% non-denaturing polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes in transfer buffer without methanol. The membranes were stained with Ponceau S (Sigma), and after several washes blocked with 5% dried milk in Tris-buffered saline with 0.05% Tween-20 (Sigma).

Monoclonal anti-β-tubulin antibodies were purchased from Amersham and the rat mAb 91A anti-mouse TCP-1 was generously provided by K. Willison. Primary antibodies were incubated with the blot for 1 hour. These primary antibodies were detected with horseradish peroxidase-conjugated donkey anti-mouse or anti-rat secondary antibody, followed by chemiluminiscent processing using Amersham ECL reagents. Exposure to Amersham ECL films was carried out for one second to several minutes.

RESULTS

Incorporation of newly synthesized tubulin into dimers is dependent on the amount of exogenous tubulin added in the presence of GTP and magnesium ions

The C300 multimolecular complexes that α- and β-tubulin form with unknown factors present in reticulocyte extracts might represent intermediates in the biosynthesis of tubulin monomers; the extent of monomer incorporation into dimers depends on the amount of exogenous brain tubulin added (Zabala and Cowan, 1992). To investigate the requirements for tubulin dimer formation, the fate of the newly synthesized β-tubulin was followed after addition of exogenous purified brain tubulin under different conditions (Fig. 1). The proportion of C300 complexes, monomers (M) and dimers (D) was analyzed by non-denaturing gel electrophoresis under conditions where the amount of C300 complexes was reduced compared with the amount of C300 complexes (i.e. prolonged incubation at 37°C of in vitro translation reactions). The proportion of newly synthesized β-tubulin in these different molecular forms (C300, D and M) was calculated by measuring the associated radioactivity after the bands were excised and quantified by scintillation counting, since the densitometric analysis did not give a linear response. Increased incorporation of tubulin subunits into dimers is observed in the presence of GTP and Mg2+ (compare Fig. 1A and B), suggesting that GTP or Mg2+ or both are implicated in the formation of the heterodimer.

To analyze the role of Mg2+, the experiments were repeated in the presence of 0.3 mg ml-1 of brain tubulin, GTP, and increasing concentrations of Mg2+. The incorporation of newly synthesized β-tubulin within the heterodimer was found to be a function of the concentration of Mg2+ (Fig. 2). Magnesium is probably required for the binding of the guanine nucleotide to tubulin (Correia et al., 1987).

To establish the role of GTP in the formation of the heterodimer, newly synthesized β-tubulin was incubated with 0.3 mg ml-1 of brain tubulin, in the presence of increasing concentrations of GTP (Fig. 3A). In this case, no differences in the relative amounts of monomers, dimers, and C300 multimolecular complexes were detected: in the presence of 0.3 mg ml-1 tubulin, interconversion between these forms does not change (see Fig. 1). The high amount of GTP present in reticulocyte lysates and the difficulty of dialyzing them efficiently might explain the apparent inefficiency of GTP in this reaction. To address this point we repeated the experiment in the presence of GTPγS, a non-hydrolyzable GTP analog, which can substitute for GTP during microtubule assembly (Kirsh and Yarbrough, 1981). When GTPγS was used instead of GTP, incorporation of newly synthesized tubulin into dimers was prevented in a concentration-dependent manner (Fig. 3B). To confirm that
Tubulin dimer formation requires GTP hydrolysis

629

The effect was due to GTPγS itself (and not to a possible contamination with ATPγS), the experiments were repeated in the presence of ATP and GTPγS. Similar results to those shown in Fig. 3B were obtained. Thus, the incorporation of newly synthesized β-tubulin within the heterodimer is dependent on GTP hydrolysis.

Heterodimer formation from C300 complexes is dependent on GTP hydrolysis but independent of ATP hydrolysis

The overall process of tubulin dimer formation requires among others, ATP and magnesium ions. The proportion of C900 complexes increases when ATPγS or EDTA were added to in vitro translation reactions, indicating the importance of ATP hydrolysis and magnesium ions in the reaction involving the C900 complexes. To investigate the significance of GTP and ATP hydrolysis in the reaction step concerning the C300 complexes, we carried out the following experiment (Fig. 4): aliquots from partially purified C300 complexes were incubated at 37°C for 30 minutes in the presence of Mg2+ for control reactions (track 1), or with reticulocyte extracts (Materials and Methods) and purified native brain tubulin and either added with GTP (track 2), ATP and GTPγS (track 3), and GTP plus ATPγS (track 4). The results confirm that the release of tubulin monomers from C300 complexes and incorporation into dimers requires the presence of unknown factors in the reticulocyte extracts (Release Factors) and GTP hydrolysis. This process is independent of ATP hydrolysis.

TCP-1 is present in C900 complexes but not in C300 complexes

Native electrophoresis through 7% gels of in vitro-synthesized β-tubulin isotypes resulted, in addition to substantial quantities of radioactive material that remained at or close to the origin, in a number of bands, which have been assigned as monomers (M), dimers (D), and C300 multimolecular complexes (Zabala and Cowan, 1992; Fig. 5A). These different molecular forms apparent upon native gel electrophoresis of in vitro β-tubulin translation reactions were examined and compared to the endogenous forms present in reticulocyte lysates by using specific antibodies. Immunodetection using monoclonal anti-β-tubulin antibodies showed that reticulocyte extracts contained all these different molecular forms of β-tubulin. The pattern obtained...
using anti-β-tubulin antibodies was indistinguishable from the pattern obtained after fluorography of the native gel containing 35S-labelled in vitro translation products, indicating the close relationship existing between the in vitro synthesized products and the in vivo endogenous forms of β-tubulin (Fig. 5A, compare tracks 1 and 3).

When in vitro translation reactions were subjected to 4.5% polyacrylamide gel electrophoresis under the same conditions, most of the material that remains at or close to the origin under analysis on 7% non-denaturing gels, generated two slowly migrating bands. These bands contained both β-tubulin and TCP-1 peptides as shown by immunodetection using specific monoclonal antibodies suggesting that both represent multimolecular complexes identified as C900 binary complexes (Fig. 5B). Anti-TCP1 monoclonal antibodies react with the C900 but not with the C300 complexes (Fig. 5A and B) suggesting that the C300 complexes are devoid of TCP-1 peptides.

**DISCUSSION**

The different molecular forms present when α- or β-tubulins are translated in vitro (complexes among tubulin and reticulocyte proteins (C900 and C300), dimers and monomers) represent progressive stages of the folding pathway of tubulin proteins. We have shown here that the final step of this folding reaction, the formation of the heterodimer, is dependent on tubulin concentration in the presence of magnesium ions and GTP hydrolysis and does not require ATP.

The equilibrium between the tubulin αβ dimer and the dissociated α and β monomers has been studied widely. The reported dissociation constant for this reaction ranged from $10^{-6}$ M to $10^{-7}$ M (Detrich and Williams, 1978; Sackett et al., 1989; Sackett and Lippoldt, 1991; Mejillano and Himes, 1989; Panda et al., 1992). Kinetic studies of β-tubulin exchange, synthesized in vitro, led Yaffe et al. (1989) to the conclusion that β-tubulin exchange is a first-order process rather than a pseudo-first-order process. The measured exchange rates were independent of tubulin concentration. We have found that the increase in tubulin dimer formation is correlated with the amount of exogenous tubulin added (Fig. 1), as also reported previously (Zabala and Cowan, 1992). We measured the amount of tubulin present in reticulocyte extracts by semi-quantitative western blots using β-tubulin antibodies. Reticulocyte extracts contain 0.1 mg ml$^{-1}$ tubulin (A.F. and J.C.Z., unpublished results). Therefore, endogenous tubulin is present in the assays of Figs 1, 2 and 3 at 0.01 mg ml$^{-1}$ (10$^{-7}$ M). Under these conditions, where the amount of endogenous tubulin is much greater than the amount of radiolabeled β-tubulin synthesized in vitro) tubulin dimer concentration is about 40-50% (Fig. 1B). Thus, in our experiments, this distribution of monomers and dimers is comparable to the expected theoretical distribution according to the dissociation constant (i.e. 10$^{-7}$ M). The theoreti-
Tubulin dimer formation requires GTP hydrolysis

Fig. 5. Reticulocyte extracts probed with anti-β-tubulin and anti-TCP-1 antibodies. Protein samples from reticulocyte extracts were electrophoresed through 7% (A) and 4.5% (B) non-denaturing polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Beta-tubulin and TCP-1 proteins were detected by using specific monoclonal antibodies (see Materials and Methods). Tracks 1, aliquots of 35S-labelled in vitro translated β3-tubulin isotype used as a marker for the presence of monomers (M), dimers (D), C300 and C900 multimolecular complexes. 35S-labelled proteins were electrophoresed through 7% (A) and 4.5% (B) non-denaturing polyacrylamide gels. Following electrophoresis gels were fluorographed, dried and exposed to film. Tracks 2, purified brain tubulin used as a marker of native tubulin heterodimers probed with anti-β tubulin antibodies (see Materials and Methods); tracks 3 and 4, aliquots of reticulocyte extracts probed with anti-β tubulin and anti-TCP-1 antibodies, respectively.

Theoretical distribution can be calculated from the equilibrium reaction: $D \rightleftharpoons M\alpha + M\beta$ where $M\alpha + M\beta = M$. This reaction has a monomer-dimer equilibrium constant:

$$K_D = \frac{[M]^2}{[D]} = 10^{-7} \text{ M}.$$ 

The total concentration of tubulin is:

$$C_T = [M] + [D] = [M] + 2[M_D],$$

where $[M]$ is the free monomer concentration and $[M_D]$ the monomer concentration in dimeric form. Thus, the theoretical distribution of tubulin at different tubulin concentration in monomeric and dimeric form could be calculated. Comparison of the theoretical data with the experimental distribution suggests that the concentration of tubulin in dimeric form correlates with the concentration of newly synthesized tubulin that has the proper conformation to assemble into dimers. However, a more direct experiment should be done in order to test for that correlation.

On the other hand, in the absence of reticulocytes, Mg2+ or GTP the equilibrium does not take place as predicted (see Figs 1, 2 or 3). Monomers partially purified through Superose-12 (according to the method of Zabala and Cowan, 1992) are not incorporated into preformed dimers in the absence of reticulocyte extracts (data not shown). Therefore, we consider the monomers observed by gel permeation and non-denaturing electrophoresis as incompletely folded monomers. Similar results were obtained by Gao et al. (1993) who showed that the monomeric product is not competent for exchange or coassembly with added carrier microtubules and suggested that correctly folded monomeric β-tubulin is unstable unless it can exchange with available tubulin heterodimers.

In the tubulin folding pathway, the accumulation of C900 complexes when ATPγS was added to in vitro translation reactions suggests the need for ATP hydrolysis at least in the first part of the process (Yaffe et al., 1992). Here we present evidence that incorporation of monomeric β-tubulin into dimers in reactions containing partially purified C300 complexes requires GTP hydrolysis, but is independent of ATP (Fig. 4). The use of prolonged incubations at 37°C of the translation reactions reduced the amount of C900 compared to C300 complexes (Yaffe et al., 1992 and results not shown); under these conditions the residual C900 complexes are not participating in the formation of the heterodimer as is also suggested by the fact that the addition of ATPγS to the translation reactions did not preclude the formation of the dimer (A.F. and J.C.Z. unpublished results). Furthermore, TCP-1 could not be detected in C300 complexes as assayed by immunoblotting with an anti-TCP-1 monoclonal antibody; since TCP-1 is an ATP-dependent protein this result might substan our finding. Although further characterization of the release factors and the composition of the C300 complexes will facilitate a better understanding of the process of tubulin folding and dimer formation, the presently available data are compatible with a two-step mechanism. A first step involves the interaction of newly synthesized polypeptides with the C900 complex, its dissociation dependent on ATP (Yaffe et al., 1992). A second step would include the transition from the large complex to the C300 complex and monomers and, finally, the assembly of the heterodimer. This second step requires GTP binding and hydrolysis. GTP also might be required for tubulin to adopt the active conformation. Although tubulin is a GTP-binding protein (Jacobs et al., 1974; Weisenberg et al., 1976), the involvement of another GTP-binding protein in the folding process is conceivable. From preliminary results we have indications that GTP hydrolysis is also necessary for the incorporation of α-tubulin into the tubulin dimer. Since α-tubulin is a GTP-binding protein and probably does not hydrolyze GTP (Carlier, 1982), it would suggest that a protein different from tubulin is responsible for the hydrolysis of GTP. This putative factor might correspond to one

$$\text{tubulin dimer formation requires GTP hydrolysis}$$
Fig. 6. A general pathway for tubulin folding and dimer assembly. Newly synthesized tubulin forms a complex with chaperone particles containing TCP-1 (C\textsubscript{900}). The complex releases tubulin in the presence of ATP-Mg. Released tubulin from chaperone particles forms complexes of about 300 kDa (C\textsubscript{300}) as intermediates in the assembly of the heterodimer. The acquisition of the quaternary structure is dependent on unknown release factors, GTP hydrolysis and magnesium ions. UT, unfolded tubulin; T, tubulin monomers; RF, release factors; D, dimer. Interactions between complexes and tubulin are boxed to indicate a possible oversimplification of the model.

of the two protein cofactors required for the proper folding of tubulin, as recently reported (Gao et al., 1993). In addition, multiprotein complexes have been recently isolated from nocodazole-arrested CHO cells, which may be involved in mitotic spindle nucleation. They have been shown to contain α, β, and γ-tubulin, HSP70, and a 50 kDa protein identified as elongation factor 1α, a GTP-binding protein (Marchesi and Ngo, 1993).

In summary, comparison of the proposed folding mechanisms for actin, and α- and β-tubulin suggests a common or similar first ATP-dependent step, and a second step in which GTP hydrolysis is required for the proper conformation of tubulin subunits and the assembly of the heterodimer (Fig. 6).

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Tubulin dimer formation requires GTP hydrolysis

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