A Novel Cochaperonin That Modulates the ATPase Activity of Cytoplasmic Chaperonin

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Abstract. The folding of α- and β-tubulin requires three proteins: the heteromeric TCP-1–containing cytoplasmic chaperonin and two additional protein cofactors (A and B). We show that these cofactors participate in the folding process and do not merely trigger release, since in the presence of Mg-ATP alone, α- and β-tubulin target proteins are discharged from cytoplasmic chaperonin in a nonnative form. Like the prokaryotic cochaperonin GroES, which interacts with the prototypical *Escherichia coli* chaperonin GroEL and regulates its ATPase activity, cofactor A modulates the ATPase activity of its cognate chaperonin. However, the sequence of cofactor A derived from a cloned cDNA defines a 13-kD polypeptide with no significant homology to other known proteins. Moreover, while GroES functions as a heptameric ring, cofactor A behaves as a dimer. Thus, cofactor A is a novel cochaperonin that is structurally unrelated to GroES.

CHAPERONINS are a class of toroidal, multisubunit proteins with which many (and perhaps most) proteins must interact in order to acquire their native conformation under physiological conditions (Bochkareva et al., 1988, 1992; Goloubinoff et al., 1989; Ostermann et al., 1989; Martin et al., 1991; Gething and Sambrook, 1992; Viitanen et al., 1992*b*; Phipps et al., 1993). Such chaperonin-mediated folding reactions depend upon the hydrolysis of ATP, and are thought to prevent aberrant folding and aggregation by providing a favorable environment in which correct folding can occur. The reaction of an unfolded target protein with chaperonin occurs in two stages. In the first stage, a folding intermediate that contains some elements of secondary structure (Martin et al., 1991) forms a tight binary complex with the chaperonin; this reaction does not depend on the presence of ATP. The location of the target protein in the binary complex is thought to be within the cavity that lies in the center of the chaperonin rings, where it is sequestered from the surrounding cytoplasm (Langer et al., 1992; Braig et al., 1993). In the second stage, ATP hydrolysis occurs, the chaperonin changes its conformation (Gao et al., 1992; Saibil et al., 1993) and the target protein is released, but in many cases this release is dependent on interaction with a cochaperonin. For example, the prokaryotic chaperonin GroEL facilitates the folding of a range of proteins in *Escherichia coli* (Viitanen et al., 1992*b*; Horwich et al., 1993), often in conjunction with the cochaperonin GroES (Hemmingsen et al., 1988; Goloubinoff et al., 1989; Lubben et al., 1990; Mendoza et al., 1991; Bochkareva et al., 1992; Schmidt et al., 1994). There is evidence that GroES, which is itself a heptameric ring (Chandrasekhar et al., 1986; Georgopoulos and Ang, 1990), functions at least in part by interacting with the ends of the GroEL cylinder, such that it modulates and coordinates the hydrolysis of ATP by GroEL (Gray and Fersht, 1991; Langer et al., 1992; Martin et al., 1993; Todd et al., 1993).

Recent studies have identified a distantly related homologue of GroEL in the cytoplasm of eukaryotes (Frydman et al., 1992; Gao et al., 1992; Lewis et al., 1992; Yaffe et al., 1992). The cytoplasmic chaperonin folds actin and tubulin in vitro, but no homologue of GroES has been discovered. In the case of actin, the hydrolysis of ATP is the only requirement for the release of the correctly folded polypeptide from cytoplasmic chaperonin (Gao et al., 1992). In contrast, the generation of correctly folded α- and β-tubulin requires two additional protein cofactors (cofactors A and B) as well as the presence of ATP and GTP (Gao et al., 1993; Rommelaere et al., 1993). The function of both these cofactors was hitherto unknown, because they were identified only as crude fractions that contained activities required for the generation of properly folded tubulin in in vitro folding assays. Here we describe the purification of cofactor A, and show that it causes a fourfold increase in the steady state rate at which the cytoplasmic chaperonin hydrolyzes ATP. These

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data demonstrate a direct interaction between the two molecules. The amino acid sequence of cofactor A derived from a cloned cDNA defines a 13-kD polypeptide with no significant homology to other known proteins. Moreover, while GroES functions as a heptameric toroid, we show that cofactor A behaves in its native state as a dimer. Thus, cofactor A is a novel cochaperonin that is structurally unrelated to the prokaryotic cochaperonin GroES.

Materials and Methods

In Vitro Folding Assays

Chaperonin purification, the generation of labeled, unfolded α- and β-tubulin target proteins, the use of these target proteins in in vitro folding reactions, and the analysis of the products of in vitro folding reactions on nondenaturing polyacrylamide gels were performed as described previously (Gao et al., 1992, 1993).

Purification of Cofactor A

All procedures were done at 4°. Bovine testis tissue (~500 g; Max Cohen, Inc., Livingston, NJ) was homogenized in 0.9 vol of homogenization buffer (20 mM Tris, pH 7.3, 20 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and aprotinin [10 μg/ml]) in a Waring (New Hartford, CT) blender for 90 s at maximum speed. The homogenate was centrifuged for 15 min at 22,000 *g* in a Beckman JA14 rotor, and the supernatant further centrifuged for 1 h at 100,000 *g* in a Beckman Ti65 rotor (Beckman Instrs., Inc., Fullerton, CA). This final supernatant, which contained ~8 g of protein, was treated with cell debris remover (CDR; Whatman Inc., Clifton, NJ) and filtered by passage through a series of filters (AP20, AP15, Millipak [0.4 μ]; Millipore Corp., Bedford, MA). The filtrate was applied to a 300-ml column of Q-Sepharose HP (Pharmacia LKB, Piscataway, NJ) equilibrated in homogenization buffer. The column was washed with 800 ml of equilibration buffer and developed with a 1.7-l linear gradient of 5–250 mM $MgCl_2$ in the same buffer. In this and all subsequent dimensions, 3-μl aliquots of fractions were assayed for cofactor A activity in 25 μl β-tubulin folding reactions containing purified cytoplasmic chaperonin (5 pmols), and the reaction products analyzed on a 4.5% nondenaturing polyacrylamide gel as described previously (Gao et al., 1992, 1993). Fractions containing cofactor A activity were pooled and readjusted to the original starting conditions (except that the $MgCl_2$ concentration was increased to 20 mM) by passage through a column of Sephadex G25. This material was applied to a MonoQ HR 10/10 column (Pharmacia LKB). The column was washed with 20 ml of equilibration buffer and developed with a 45-ml linear gradient of 20–250 mM $MgCl_2$ in the same buffer. Fractions containing cofactor A activity were pooled and adjusted to 20 mM triethanolamine-HCl, pH 8.2, 20 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT by passage through a column of Sephadex G25 equilibrated in this buffer. The resulting material was reapplied to the MonoQ HR10/10 column, which was washed with 20 ml and developed with a linear gradient of 20–500 mM KCl in the same buffer. Fractions containing cofactor A activity were pooled, adjusted to 20 mM potassium phosphate buffer, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, and 1 mM DTT by passage through a Sephadex G25 column, and applied to a 10-ml column of hydroxylapatite (Pentax; American International Chemical, Inc., Natick, MA) equilibrated in this buffer. The column was washed with 20 ml of the equilibration buffer and developed with a linear gradient of 20–250 mM potassium phosphate buffer. Fractions containing cofactor A activity were pooled, concentrated by ultrafiltration on Centricon 10 (Amicon, Beverly, MA) and applied to a 60-cm gel filtration column (TSK 3000; TosoHAAS, Philadelphia, PA) equilibrated and run in 20 mM MES, pH 6.9, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT. The TSK 3000 column was calibrated with molecular size markers (IgG [158 kD], ovalbumin [44 kD], myoglobin [17 kD], and vitamin B_{12} [1.3 kD]). Fractions containing cofactor A activity were analyzed for their protein content on a 12% tricine-SDS-polyacrylamide gel (Schagger and Von Jagow, 1987) (Integrated Separation Systems, Hyde Park, MA). In some experiments, cofactor A was further purified by C18 reverse phase high pressure liquid chromatography (HPLC) (Rommelaere et al., 1993); cofactor A eluted as a single peak at 31% CH_3CN.

Peptide Sequence Analysis and cDNA Cloning

Bovine cofactor A purified as described above was cleaved with either endoproteinase Lys-C (in 25 mM Tris-HCl, pH 8.5) or endoproteinase Asp-N (in 100 mM Tris-HCl, pH 8.5) (both enzymes from Boehringer Mannheim Biochemicals, Indianapolis, IN) for 3 h at 37°. Peptides were purified by C18 reverse phase HPLC and most of the peptides were sequenced using a sequencer (model 470A or 477A; Applied Biosystems, Inc., Foster City, CA) equipped with an on-line phenylthiohydantoin amino acid analysis system (model 120A; Applied Biosystems, Inc.). Overlapping sequences were aligned where possible. Degenerate oligonucleotides based on the peptide sequence data were used as primers in PCR reactions using mouse testis cDNA as template. DNA sequence analysis of a major band of 164 bp produced in one such PCR reaction showed it to contain an encoded internal cofactor A peptide. This PCR product was subcloned and used as a labeled probe to screen a mouse testis cDNA library. Overlapping clones encoding the 5′ end of cofactor A were obtained by the RACE procedure (Frohman et al., 1988).

RNA Blot Transfer Analysis

Blot transfer analysis of RNA from adult mouse tissues was done as described by Villasante et al. (1986), using 10 μg of total RNA per lane. The blot was probed with the 164-bp fragment of cloned cofactor A (see above) labeled by nick-translation.

Preparation of Labeled Native Tubulin

HeLa cells were labeled at 70% confluence by incubation for 3 h in Dulbecco's modified Eagle's medium lacking methionine and containing [^{35}S]methionine (0.2 mCi/ml). Cells were harvested and lysed in a glass Dounce homogenizer in 0.1 M MES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.05% Triton X-100. The suspension was centrifuged at 100,000 *g* for 20 min at 4°. Labeled tubulin in the supernatant was recovered by copolymerization with added unlabeled bovine brain tubulin purified as described (Shelanski et al., 1973; Weingarten et al., 1975). The specific radioactivity of the final product was 5×10^7 cpm/mg.

Isolation of Tubulin/Chaperonin Binary Complexes and Their Discharge in the Presence of Mg-ATP and/or Mg-GTP

Labeled, denatured α- and β-tubulin target proteins were diluted in folding reactions (Gao et al., 1992, 1993) containing cytoplasmic chaperonin (0.1 μmol) and incubated at 30° for 15 min to allow binary complex formation. The reaction products were applied to a Superose 6 column (Pharmacia LKB) equilibrated and run in 20 mM MES, pH 6.9, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and the radioactive peak emerging at 700 kD concentrated on Centricon 30 (Amicon). This material was used in reactions containing a twofold molar excess of mitochondrial cpn60 (Viitanen et al., 1992*a*) and 1 mM each of Mg-ATP and GTP; at various times, aliquots were withdrawn from the reaction and analyzed on a nondenaturing polyacrylamide gel as described previously (Gao et al., 1992, 1993).

Kinetics of ATP Hydrolysis

Rates of ATP hydrolysis were measured at 30° in reactions containing cytoplasmic chaperonin (Gao et al., 1992, 1993) (0.125 μM) either on its own in folding buffer, or together with a threefold molar excess of cofactor A, or in the presence of denatured β-tubulin target protein (Gao et al., 1993) added by 100-fold dilution from 7.5 M urea to a final concentration of 0.25 μM, or in the presence of both cofactor A and β-tubulin target protein. The yield of acid-labile P_i was measured as described (Melki et al., 1990).

Results

The tubulin heterodimer is the subunit from which microtubules are assembled, and consists of one α- and one β-tubulin polypeptide. Both α- and β-tubulin are GTP-binding proteins, and both form binary complexes when presented to

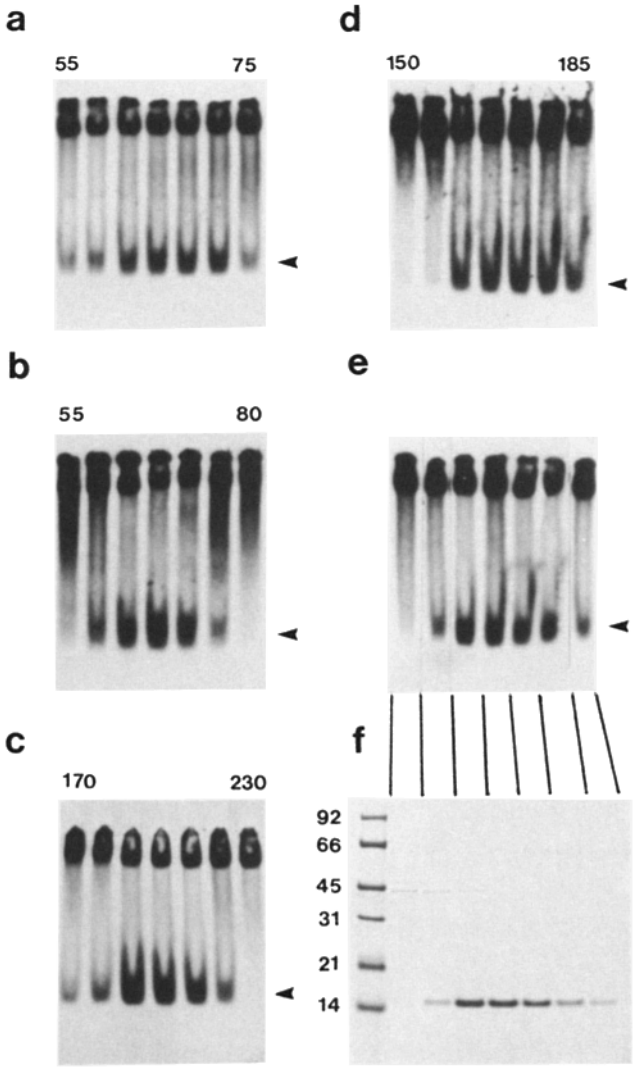

Figure 1. Purification of cofactor A. Cofactor A was purified from a crude extract of bovine testis tissue by ion exchange and size exclusion chromatography (see Materials and Methods). The location of cofactor A emerging from each column was determined by assaying its ability to generate monomeric β-tubulin (Gao et al., 1993) in in vitro folding reactions (Gao et al., 1992, 1993) containing purified cytoplasmic chaperonin (Frydman et al., 1992; Gao et al., 1992) Mg-ATP and Mg-GTP. The products of these folding reactions were then analyzed on nondenaturing polyacrylamide gels (Gao et al., 1992, 1993). (*a–e*) Folding assays done on fractions in those regions of each successive dimension that showed cofactor A activity, together with the corresponding elution conditions (shown as the salt concentration in mM in *a–d*). Arrows show the location of monomeric β-tubulin. (*a* and *b*) Ion exchange chromatography on Q-Sepharose and MonoQ, respectively, (both at pH 7.2 in Tris-HCl buffer and developed with $MgCl_2$); (*c*) ion exchange chromatography on MonoQ (at pH 8.2 in triethanolamine-HCl buffer, developed with KCl); (*d*) ion exchange chromatography on hydroxylapatite, developed with potassium phosphate; (*e*) size exclusion chromatography on TSK 3000; and (*f*) analysis on a 12% tricine-SDS-polyacrylamide gel (Schagger and Von Jagow, 1987) of purified cofactor A emerging from the size exclusion column. Molecular mass markers (in kD) are shown at the left.

cytoplasmic chaperonin as unfolded target proteins; however, folding reactions done in the presence of ATP and/or GTP fail to result in the release of α- or β-tubulin in an assembly-competent form. Rather, two protein cofactors (cofactors A and B) are required for the generation of correctly folded α/β tubulin heterodimers (Gao et al., 1993). Nonetheless, β-tubulin folding reactions done in the presence of cytoplasmic chaperonin, ATP, GTP, and crude fractions containing cofactor A (but not cofactor B) result in the release of the target protein as a monomeric polypeptide that is presumably incompletely or wrongly folded, since it fails to exchange with native α/β-tubulin heterodimers (Gao et al., 1993). We purified cofactor A to homogeneity by assaying its capacity to generate such monomeric β-tubulin in folding assays containing purified chaperonin, Mg-ATP and Mg-GTP; the monomeric β-tubulin is readily detected as a fast-migrating band upon nondenaturing polyacrylamide gel electrophoresis of the reaction products (Fig. 1, *a–e*). After several dimensions, the purified cofactor A behaved on a gel filtration column as a single peak with a molecular mass of ~28 kD (data not shown). Upon SDS-polyacrylamide gel analysis, this material migrated as a single band with an apparent molecular mass of ~15 kD (Fig. 1 *f*); cofactor A probably therefore exists as a dimer in its native state. Cofactor A thus purified supported the generation of native α- and β-tubulin in in vitro folding reactions containing cytoplasmic chaperonin, Mg-ATP, Mg-GTP, carrier bovine brain microtubules, and a crude fraction containing cofactor B (Y. Gao and N. J. Cowan, unpublished observations).

We used peptide sequence information derived from purified cofactor A to obtain a cloned cDNA encoding the entire cofactor A polypeptide (Fig. 2 *a*). The cofactor has a calculated molecular mass of 12,757 Daltons, consistent with the behavior of the monomeric subunit on an SDS-polyacrylamide gel. The amino acid sequence shows no significant similarity to any other proteins whose sequence is known. The pattern of expression, determined in a blot transfer experiment using RNA from a variety of adult mouse tissues, parallels that of TCP-1 (Silver and White, 1982): the cofactor is widely expressed, but most abundantly in testis (Fig. 2, *b–d*). The relatively high level of cofactor A synthesis in testis probably reflects a requirement for the facilitated folding of large quantities of tubulin destined for incorporation into flagellae and the spermatid manchette during spermatogenesis.

We considered the possibility that cofactor A might function as a cochaperonin in a manner akin to GroES. This prokaryotic cochaperonin promotes the release from GroEL of many target proteins in their native conformation, and enhances the cooperativity of ATP binding and hydrolysis of the chaperonin (Hemmingsen et al., 1988; Goloubinoff et al., 1989; Lubben et al., 1990; Mendoza et al., 1991; Bochkareva et al., 1992; Langer et al., 1992; Schmidt et al., 1994). Since cofactors A and B are absolutely required for the generation of α- and β-tubulin (Gao et al., 1993), it seemed possible that the release of tubulin target proteins from cytoplasmic chaperonin might be dependent on one or both of these cofactors. To test this idea, we purified α- and β-tubulin/cytoplasmic chaperonin binary complexes and incubated them with Mg-ATP and Mg-GTP alone. Control

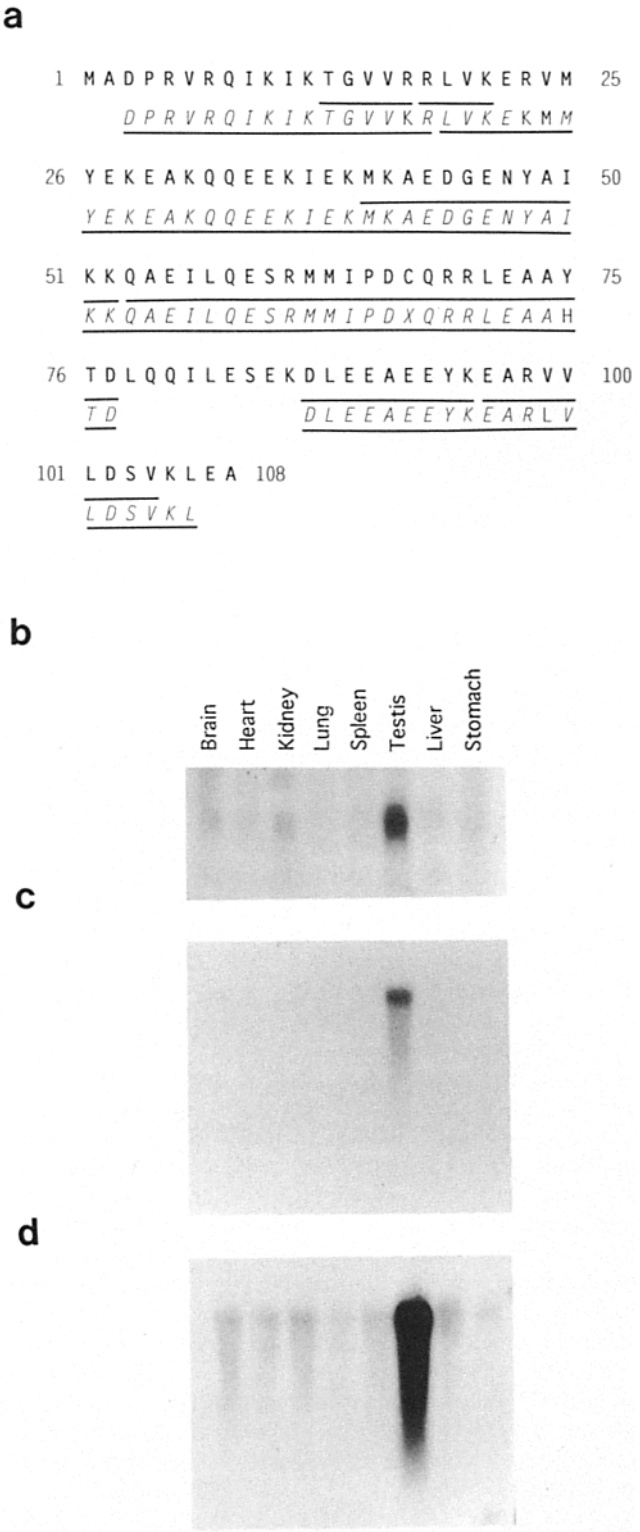

Figure 2. (*a*) Encoded amino acid sequence of a cloned cDNA-encoding mouse cofactor A. These sequence data are available from EMBL/GenBank under accession number UO5333. Corresponding amino acid sequences of peptides derived from purified bovine cofactor A are shown below; identical residues are in italics. Sequences from endoproteinase-Lys C or endoproteinase Asp-N–derived cofactor A peptides are underlined and overlined, respectively. (*b–d*) RNA blot transfer analysis of cofactor A (*b*) and TCP-1 (*c* and *d*) expression in adult mouse tissues; *d* is identical to *c*, except that the blot has been overexposed to show the expression of low levels of TCP-1 mRNA in tissues other than testis.

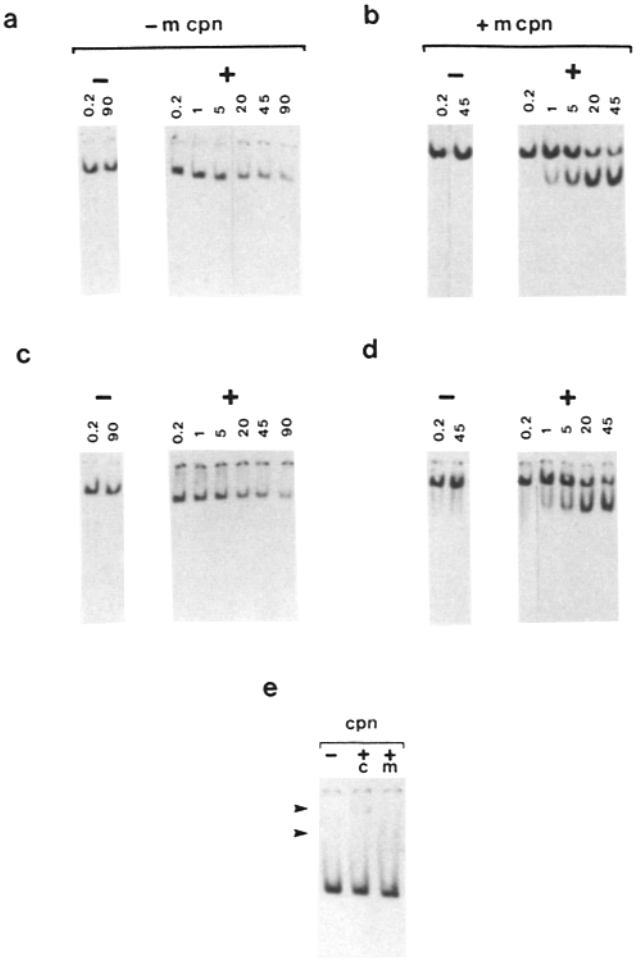

Figure 3. Release of tubulin target proteins from cytoplasmic chaperonin in the presence of nucleotide. (*a–d*) Analysis on 4.5% nondenaturing polyacrylamide gels of reactions in which isolated binary complexes of cytoplasmic chaperonin and α- (*a* and *b*) or β- (*c* and *d*) target proteins (see Materials and Methods) were incubated for the times (in min) shown in the figure either without (−m cpn) (*a* and *c*) or with (+m cpn) (*b* and *d*) mitochondrial chaperonin in the absence (−) or presence (+) of Mg-ATP and Mg-GTP. (*e*) Nondenaturing polyacrylamide gel analysis of reactions in which native, ^{35}S-labeled tubulin was incubated for up to 1 h either alone (−) or in the presence of a 10-fold molar excess of either cytoplasmic (+c) or mitochondrial (+m) chaperonin. Upper and lower arrows indicate the position of cytoplasmic chaperonin and mitochondrial cpn60, respectively.

reactions done in the absence of nucleotide showed that the binary complexes were stable for the duration of the experiment (Fig. 3, *a* and *c*). In contrast, in the presence of nucleotide, there was a progressive decline in the radioactivity contained in binary complex, suggesting that release of tubulin target proteins can indeed take place in the absence of cofactors. Curiously, however, we were unable to determine the fate of material released from tubulin binary complexes upon incubation with Mg-ATP and Mg-GTP. We therefore repeated these experiments in the presence of added mitochondrial cpn60 (Viitanen et al., 1992*a*), included to act as a capturing agent of nonnative material that might otherwise be difficult or impossible to detect under these conditions. When tubulin/cytoplasmic chaperonin binary complexes

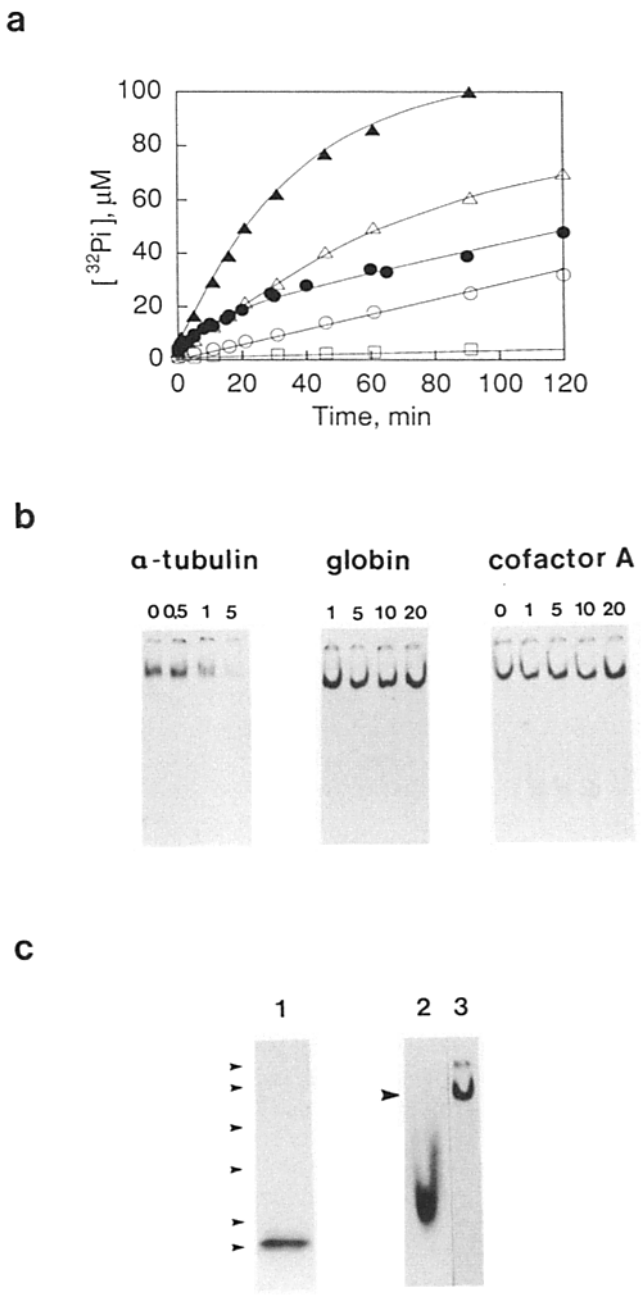

Figure 4. Cofactor A enhances the rate at which cytoplasmic chaperonin hydrolyzes ATP, but is not itself a potential target protein for cytoplasmic chaperonin. (*a*) Kinetics of ATP hydrolysis by cofactor A alone (□), cytoplasmic chaperonin alone (○), by chaperonin in the presence of cofactor A (●), by chaperonin in the presence of β-tubulin target protein (△), and by chaperonin in the presence of both cofactor A and β-tubulin target protein (▲). (*b*) Nondenaturing polyacrylamide gel analysis of cytoplasmic chaperonin-mediated β-actin folding reactions done without Mg-ATP (so as to allow binary complex formation without discharge of native protein [Gao et al., 1993; Melki and Cowan, 1994]) and containing increasing amounts (shown as the fold molar excess over labeled β-actin) of either unlabeled α-tubulin (which competes efficiently for binary complex formation [Melki et al., 1993]), globin (which fails to compete for binary complex formation [Gao et al., 1993; Melki et al., 1993]) or purified cofactor A. (*c*) Lane *1*, analysis on a 15% SDS-polyacrylamide gel of cofactor A expressed as a ^{35}S-labeled polypeptide in *E. coli* under conditions where host protein synthesis is suppressed by rifampicin (Studier et al., 1990); location of molecular size markers (97, 66, 45, 31, 21, and 14 kD) is shown at the left. Lane *2*, nondenaturing polyacrylamide gel analysis of the products of an in vitro folding reaction in which labeled, unfolded cofactor A was presented to cytoplasmic chaperonin in the absence of Mg-ATP so as to promote binary complex formation (Gao et al., 1992; Melki and Cowan, 1994); lane *3*, control folding reaction using unfolded β-actin as target protein. Arrow marks the location of the chaperonin/target protein binary complex.

were incubated with cpn60 in the absence of nucleotide, no transfer of radioactive material to the mitochondrial chaperonin was observed. However, the progressive decrease in the amount of α- and β-tubulin/cytoplasmic chaperonin complexes together with a corresponding increase in the amount of α- and β-tubulin/cpn60 binary complexes during the course of experiments done with Mg-ATP and Mg-GTP clearly demonstrates the release of tubulin target proteins from the cytoplasmic chaperonin under these conditions, presumably in a partially or completely unfolded form that is still recognizable by cpn60 (Fig. 3, *b* and *d*). Parallel experiments with Mg-ATP alone gave essentially identical results (data not shown). Finally, a control experiment showed that native ^{35}S-labeled tubulin does not bind to either mitochondrial cpn60 or to cytoplasmic chaperonin on its own (Fig. 3 *e*). We conclude that cofactors are not required for the release per se of α- and β-tubulin target proteins from the cytoplasmic chaperonin, though in the absence of cofactors, the released polypeptides are in a nonnative form.

To study the role of cofactor A in the folding of α- and β-tubulin, we examined the effect of cofactor A on the rate at which the cytoplasmic chaperonin hydrolyzes ATP (Fig. 4). In the absence of target protein, cytoplasmic chaperonin hydrolyzes ATP at a steady state rate of 2.1 min^{-1}, while cofactor A on its own does not hydrolyze ATP to any measurable extent. When cofactor A is incubated with chaperonin, however, there is a significant increase in the rate of ATP hydrolysis. The fourfold enhancement in the rate of cytoplasmic chaperonin-mediated ATP hydrolysis upon addition of cofactor A is observed with an equimolar amount of the cofactor, and does not vary upon addition of further amounts of the cofactor. The rate of ATP hydrolysis upon addition of cofactor A is even greater in folding reactions containing tubulin target proteins: in such reactions, the rate of ATP hydrolysis is 8 min^{-1} in the absence of cofactor A; this rate increases 2.5-fold in the presence of cofactor A.

We considered the possibility that the enhanced rate of ATP hydrolysis by cytoplasmic chaperonin in the presence of cofactor A might be a result of the cofactor itself acting as a target protein. If this were the case, then unlabeled cofactor A should compete for binary complex formation with labeled, unfolded β-actin in a manner similar to other bona fide target proteins such as α-, β-, or γ-tubulin or actin-RPV (Gao et al., 1993; Melki et al., 1993). Under experimental conditions where these target proteins compete efficiently for binary complex formation with cytoplasmic chaperonin, no detectable competition was observed in in vitro folding reactions in which β-actin was presented to cytoplasmic chaperonin in the presence of increasing concentrations of cofactor A (Fig. 4 *b*). Furthermore, in contrast to a large number of cytoplasmic proteins (Melki and Cowan, 1994), cofactor A itself fails to form a detectable binary complex with cytoplasmic chaperonin when presented

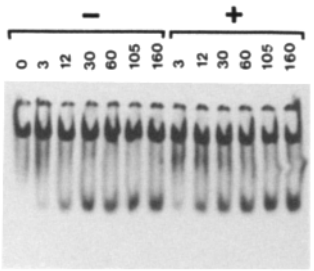

Figure 5. Cofactor A does not affect the kinetics of β-actin folding in vitro. Cytoplasmic chaperonin-mediated β-actin folding reactions were performed in vitro in the absence (−) or presence (+) of a threefold molar excess of purified cofactor A for the times (in min) shown in the figure. The reaction products were analyzed on a 4.5% nondenaturing polyacrylamide gel (Gao et al., 1992).

as a radioactive denatured target protein (Fig. 4 *c*); rather, the labeled material forms a fast-moving species upon native gel electrophoresis, presumably as a consequence of spontaneous folding. We conclude that cofactor A interacts physically with the cytoplasmic chaperonin in a manner distinct from potential target proteins.

The addition of an equimolar amount of cofactor A causes a burst of chaperonin-mediated ATP hydrolysis (corresponding to a fourfold increase) which then reverts to the basal rate (Fig. 4 *a*). Addition of greater quantities of cofactor A does not affect the kinetics, demonstrating a saturating effect. This observation reinforces our conclusion that cofactor A interacts with the cytoplasmic chaperonin in a way that is functionally distinct from target proteins, since the addition of increased quantities of a bona fide target protein would result in enhanced rates of hydrolysis of longer duration. We conclude that cofactor A acts as a stimulator of chaperonin-mediated ATP hydrolysis, and that this enhanced rate is essential as part of the process whereby properly folded tubulin polypeptides are ultimately generated. Note, however, that interaction of α- or β-tubulin/chaperonin binary complexes with cofactor A is not on its own sufficient to generate correctly folded tubulin polypeptides: the presence of cofactor B is also required (Gao et al., 1993), although the contribution of this latter cofactor to the overall tubulin-folding pathway remains to be established.

Our observation that cofactor A enhances the rate of chaperonin-mediated ATP hydrolysis led us to test whether the presence of this cofactor might increase the rate at which β-actin is discharged; this target protein is released from cytoplasmic chaperonin in its native conformation in the presence of Mg-ATP alone (Gao et al., 1992). To do this, we measured the rate of formation of native β-actin in in vitro folding reactions in the absence or presence of cofactor A. No discernable difference in the kinetics of β-actin folding was observed (Fig. 5). Thus, although the enhanced rate of chaperonin-mediated ATP hydrolysis that results as a consequence of interaction with cofactor A is essential for the generation of properly folded α- and β-tubulin, this interaction does not affect the folding of β-actin.

Discussion

Actins and tubulins are the two most abundant proteins in the eukaryotic cytosol. Both β-actin and α-, β-, and γ-tubulin are folded via interaction with a heteromeric, TCP-1-containing chaperonin that, like its prokaryotic, mitochondrial, and chloroplastic homologues, hydrolyzes ATP as part of the process whereby properly folded polypeptides are ultimately released (Frydman et al., 1992; Gao et al., 1992, 1993; Lewis et al., 1992; Yaffe et al., 1992; Melki et al., 1993; Rommelaere et al., 1993; Sternlicht et al., 1993). In the case of β-actin and γ-tubulin, the presence of cytoplasmic chaperonin and Mg-ATP (or Mg-ATP and Mg-GTP) is sufficient to yield correctly folded products (Gao et al., 1992; Melki et al., 1993). However, two protein cofactors (A and B) are required, in addition to cytoplasmic chaperonin, for the generation of correctly folded α- and β-tubulin polypeptides (Gao et al., 1993; Rommelaere et al., 1993). On the other hand, incubation of α- or β-tubulin/cytoplasmic chaperonin binary complexes with nucleotide in the absence of cofactors results in the discharge of target polypeptides, but in a nonnative form. It follows that cofactors A and B participate in the folding of α- and β-tubulin, and do not merely trigger the release of folded molecules from the chaperonin.

When a prototypical polypeptide is suddenly diluted from denaturant, it is thought to form a 'molten globule' intermediate that is more compact than the unfolded protein and contains some elements of secondary structure (Martin et al., 1991). This intermediate forms very rapidly, i.e., in less than one second, and is competent for binary complex formation with chaperonin. In the case of β-actin and α- and β-tubulin, however, the formation of intermediates that are competent for binary complex formation with the cytoplasmic chaperonin occurs much more slowly, with a half time of about 4 min at 30° (Melki and Cowan, 1994). These relatively slow kinetics are indistinguishable for actins and tubulins, in spite of their different cofactor requirements. We conclude that cofactor A is not involved in the capture of folding intermediates by cytoplasmic chaperonin, nor does it interact with potential target proteins prior to binary complex formation.

In experiments in which α- or β-tubulin/cytoplasmic chaperonin binary complexes are incubated with Mg-ATP in the absence of added cofactors, the target protein is released, but in a nonnative form that is competent for binary complex formation with mitochondrial cpn60 (Fig. 3). No such transfer of target protein from cytoplasmic chaperonin to cpn60 occurs in the absence of added nucleotide. These data imply that, in the absence of Mg-ATP, the binding of α- and β-tubulin target proteins to cytoplasmic chaperonin is essentially irreversible, while binding of Mg-ATP to the chaperonin significantly weakens the interaction such that unfolded or partially folded target protein can be released. Under native conditions and in the absence of a capturing agent such as mitochondrial cpn60, this released material cannot be detected on a nondenaturing gel, presumably because it adheres to the walls of reaction vessels and/or smears throughout the gel. On the other hand, when cpn60 is included in the reaction, the protein released from cytoplasmic chaperonin is captured and retained as a stable binary complex with cpn60.

Our data demonstrate that cofactor A stimulates the ATPase activity of cytoplasmic chaperonin (Fig. 4). Given that no trace of properly folded tubulin is detectable in folding reactions done in the absence of cofactor A (Gao et al., 1993; Rommelaere et al., 1993), the cofactor is not merely acting to speed up the folding reaction by accelerating the rate of ATP hydrolysis. The interaction of cofactor A could

cause a conformational change in the chaperonin that is required for the correct folding of tubulin; this conformational change could then result in an enhanced rate of ATP hydrolysis. Alternatively, cofactor A might increase the rate at which some or all of the chaperonin subunits hydrolyze ATP by increasing the rate of exchange of ATP for ADP. Proteins that perform this function are known: for example, the actin-binding protein profilin functions as an enhancer of ATP/ADP exchange (Mockrin and Korn, 1980). In the case of chaperonin, an enhanced rate of nucleotide exchange would result in a larger proportion of chaperonin subunits being in an ATP-bound state at any given time. The consequent loosening of chaperonin/substrate interactions (Badcoe et al., 1991; Mizobata et al., 1992; Jackson et al., 1993) might then give the protein the freedom it needs to fold. There is evidence that the cochaperonin GroES acts by coordinating ATP hydrolysis in GroEL subunits (Martin et al., 1993; Todd et al., 1993); thus GroES and cofactor A may both function by increasing the proportion of chaperonin subunits in the ATP-bound state, albeit via different mechanisms.

Though the action of cofactor A alone on cytoplasmic chaperonin does not lead to the generation of native tubulin polypeptides, this cofactor is absolutely required (in conjunction with cofactor B) for the facilitated folding of α- and β-tubulin (Gao et al., 1993). Cofactor A stimulates the rate of chaperonin-mediated ATP hydrolysis (Fig. 4 *a*), but it is incapable of interacting with cytoplasmic chaperonin in a manner akin to bona fide target proteins (Fig. 4, *b* and *c*). It follows, therefore, that cofactor A interacts with the chaperonin and stimulates chaperonin-mediated ATP hydrolysis via a mechanism distinct from target protein interactions. The requirement for this essential interaction in the chaperonin-mediated folding of α- and β-tubulin define cofactor A as a cochaperonin.

The inclusion of cofactor A in β-actin folding reactions has no detectable effect on the kinetics of production of properly folded product under the conditions of our in vitro assay (Fig. 5). This result contrasts with the effect of the prokaryotic cochaperonin GroES on GroEL: the facilitated folding of a number of proteins by GroEL requires interaction with GroES only under nonpermissive conditions, but under permissive conditions, GroES accelerates the rate of ATP-dependent target protein release (Schmidt et al., 1994). It is conceivable that conditions exist in which facilitated folding of β-actin by the cytoplasmic chaperonin might require interaction with cofactor A or B (or both). Alternatively, the requirement for cofactors in addition to cytoplasmic chaperonin in α- and β-tubulin folding reactions may reflect some special properties of α- and β-tubulin polypeptides. These properties might be shared by a number of other proteins such that their facilitated folding by cytoplasmic chaperonin also requires interaction with cofactors A and B.

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