Cofactor A Is a Molecular Chaperone Required for β-Tubulin Folding: Functional and Structural Characterization†

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Received April 2, 1996; Revised Manuscript Received May 28, 1996⊗

ABSTRACT: Actin and tubulin polypeptide chains acquire their native conformation in the presence of the chaperonin containing TCP-1 (CCT) and, in the case of α- and β-tubulin additional protein cofactors. We recently identified one of these cofactors, termed cofactor A, that is required for the proper folding of the β-tubulin chain [Gao *et al*. (1994) *J. Cell. Biol. 125*, 989−996]. We show here that cofactor A, a monomeric protein that has no measurable affinity for nucleotides, is a highly conserved protein among vertebrates. Its NH_2-terminal region is essential for the structural integrity of the protein and consequently for its activity. We demonstrate that cofactor A does not interact with CCT nor does it affect the intrinsic ATPase activity of CCT, alone or in the presence of different target proteins. Thus, unlike GroES, cofactor A does not modulate or coordinate ATP hydrolysis. It does not act as a nucleotide exchange factor or a catalyst in tubulin folding. Rather, we demonstrate that cofactor A participates in the tubulin folding process by interacting with a folding intermediate of β-tubulin that is released from CCT. Our data imply that cofactor A is a chaperone involved in tubulin folding.

In many cases proteins attain their biologically active conformation by interacting, during or after translation, with accessory proteins known as molecular chaperones [reviewed by Rothman (1989), Ellis and van der Vies (1991), and Gething and Sambrook (1992)]. A class of molecular chaperones, termed chaperonins, are multisubunit toroidal complexes that facilitate protein folding (Bochkareva *et al*., 1988; Cheng *et al*., 1989; Martin *et al*., 1991; Goloubinoff *et al*., 1989; Ostermann *et al*., 1989; Viitanen *et al*., 1992a; Phipps *et al*., 1993). Two groups of chaperonins are distinguished on the basis of their primary structure identity (Kubota *et al*., 1995). Group I comprises the bacterial chaperonin GroEL, its mitochondrial counterpart Hsp60, and the Rubisco-subunit-binding protein (RBP) from chloroplasts. They all show 7-fold rotational symmetry and are composed of one (GroEL, Hsp60) or two (RBP) types of subunits. Members of group II have so far only been found in Archaebacteria and in the eukaryotic cytosol. The all show 8- or 9-fold rotational symmetry. The archaebacterial chaperonins TF55 and the thermosome are composed of only two subunit species, while the cytosolic chaperonin contains eight distinct but related polypeptides (Frydman *et al*., 1992; Rommelaere *et al*., 1993; Kubota *et al*., 1994), one of which is the t-complex polypeptide TCP-1 (Lewis *et al*., 1992; Silver *et al*., 1979). This chaperonin is therefore usually referred to as chaperonin containing TCP-1 (CCT;[1] other names found in the literature are TRiC and eukaryotic cytosolic chaperonin).

Interaction of unfolded proteins with chaperonins occurs in two stages. The first stage is the binding of target protein to the ADP-form of chaperonin (Badcoe *et al*., 1991; Jackson *et al*., 1993; Melki & Cowan, 1994), the former possessing some elements of secondary structure (Martin *et al*., 1991). The second stage involves nucleotide exchange concomitant with a conformational change in the chaperonin (Gao *et al*., 1992; Saibil *et al*., 1993; Chen *et al*., 1994) and release of the bound protein (Melki & Cowan, 1994). The bound ATP is hydrolyzed after this stage to regenerate chaperonin in its ADP-bound form which can re-enter an interaction cycle with a target protein.

GroEL facilitates the folding of a range of proteins (Viitanen *et al*., 1992b; Horwich *et al*., 1993). Protein release from GroEL often requires, or is enhanced by, interaction with the co-chaperonin GroES (Hemmingsen *et al*., 1988; Goloubinoff *et al*., 1989; Lubben *et al*., 1990; Mendoza *et al*., 1991; Bochkareva *et al*., 1992). GroES also exists in the form of a heptameric ring (Chandrasekhar *et al*., 1986; Georgopoulos & Ang, 1990) and functions by interacting with the ends of the GroEL cylinder (Llorca *et al*., 1994; Schmidt *et al*., 1994, Engel *et al*., 1995), thereby modulating and coordinating the hydrolysis of ATP by GroEL (Gray & Fersht, 1991; Langer *et al*., 1992; Todd *et al*., 1993, 1994).

CCT forms a binary complex with a number of unfolded cytoplasmic polypeptides *in vitro* and facilitates their folding (Gao *et al*., 1992; Frydman *et al*., 1992; Yaffe *et al*., 1992; Melki & Cowan, 1994). Correctly folded actin, actin-RPV, and γ-tubulin can be generated when the corresponding

† This work was supported by the Centre National de la Recherche Scientifique, the Association pour la Recherche sur le Cancer, the Ligue Nationale Française pour la Recherche sur le Cancer, and NFWO Grants G 3.0008.94 to C.A. and G.0060.96 and GOA-91/96-3 to J.V. H.R. is a predoctoral fellow, and C.A. is a research associate of the Belgian National Fund for Scientific Research (NFWO).

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: CCT, chaperonin containing TCP-1; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; folding buffer, 80 mM MES, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 1 mM GTP; HPLC, high-performance liquid chromatography.

S0006-2960(96)00788-X CCC: $12.00

unfolded polypeptides are incubated in the presence of CCT and Mg-ATP (Gao *et al*., 1992; Melki *et al*., 1993). In contrast, the generation of correctly folded α- and β-tubulin was shown to require Mg-ATP, Mg-GTP, and two additional lysate fractions containing cofactors A and B (Gao *et al*., 1993; Rommelaere *et al*., 1993). Recently bovine cofactor A was purified and shown to cause a 4-fold increase in the steady state rate at which CCT hydrolyzes ATP, which was interpreted as resulting from a direct interaction between cofactor A and the chaperonin (Gao *et al*., 1994). The binding of GroES to GroEL reduces the rate of ATP hydrolysis by the latter (Todd *et al*., 1993). Thus, cofactor A and GroES appear to have opposing effects on chaperonin-mediated ATP hydrolysis. Furthermore, the amino acid sequence of cofactor A, deduced from a cloned mouse cDNA, shares no significant homology to GroES (Gao *et al*. 1994).

In this study we further characterize cofactor A structurally and functionally and present the complete primary structure of bovine, rabbit, and chicken cofactor A. We also demonstrate that cofactor A does not interact with CCT nor does it affect the intrinsic ATPase activity of CCT. Thus, cofactor A does not act on the chaperonin as a catalyst in tubulin folding. We present data showing that cofactor A acts as a monomeric protein in the tubulin folding process by interacting with a β-tubulin folding intermediate(s) following its release from β-tubulin−CCT binary complex. This implies that cofactor A is a chaperone involved in tubulin folding.

MATERIALS AND METHODS

(A) Chemicals. 2-(*N*-Morpholino)ethanesulfonic acid (MES) was purchased from Calbiochem. ATP-agarose (ATP coupled through C8), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1-N^6-ethenoadenosine 5′-triphosphate (ϵ-ATP), and sodium dodecyl sulfate (SDS) were from Sigma. 1-Ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) came from Pierce. Adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), and 1,4-dithiothreitol (DTT) were from Boehringer. [^{35}S]-L-Methionine, [^{3}H]-ATP, and [γ-^{32}P]ATP came from Amersham. 2,2,2-Trifluoroethanol (TFE) came from Aldrich. All other chemicals were analytical grade.

(B) Purification of the Chaperonin Containing TCP-1 and in Vitro Folding Reactions. CCT was purified from either rabbit reticulocyte lysate (Gao *et al*., 1992) or bovine testis following the procedure of Frydman *et al*. (1992) as later modified (Melki & Cowan, 1994). ^{35}S-labeled unfolded β-tubulin and β-actin target proteins were generated and purified as described by Gao *et al*. (1992, 1993). Denatured unlabeled TCP-1, γ-tubulin, CAP binding protein, and cyclin B were obtained as described in Melki and Cowan (1994). *In vitro* folding assays and analysis of the reaction products on non-denaturing polyacrylamide gels were performed as described previously (Gao *et al*., 1993). Following staining and destaining, the gels were fluorographed and dried. The yield of various products identified on the gel was quantified using a phosphorimager.

(C) Cloning of Bovine and Murine Cofactor A into pET11d. Adult mouse or bovine testis poly(A)$^+$ mRNA were reverse transcribed using Superscript (Bethesda Research Laboratories) and oligo(dT)15 primers (Boehringer). An aliquot (10 ng) of the resulting cDNA was used as template in a PCR reaction containing Vent DNA polymerase (New England Biolabs) and the following cofactor A oligonucleotide primers: 5′-AGGGACCATGGCCGATCCTC-3′ and 5′-CCGGATCCCATACAGAAAATGTC-3′. The primers correspond to the 5′ end of murine cofactor A cDNA (Gao *et al*., 1994) surrounding the initiation codon (*Nco*I site) and to the reverse complement of cofactor A cDNA downstream of the stop codon, with a *Bam*HI site engineered onto the end. The PCR product was digested with *Nco*I and *Bam*HI and cloned into pET11d (Studier *et al*., 1990) that had been cut with the same enzymes. The nucleotide sequences of both the murine and bovine cofactor A cDNA cloned into pET11d were determined by didioxy sequencing (Sanger *et al*., 1977). Expression of these cDNAs results in a truncated form due to internal initiation at Met25 (murine) or Met24 and 25 (Bovine) (see Results). To circumvent this problem we performed a site-directed mutagenesis of Met25 to Leu25 of murine cofactor A using two additional oligonucleotide primers: 5′-TCTTTTTCGTACAGCACTCTTTCTTT-3′ and 5′-AAAGAAAGAGTGCTGTACGAAAAAGA-3′.

(D) In Vitro Translation of Cofactor A. Recombinant pET-cofactor A plasmid (see above) was added to a TNT reticulocyte T7 coupled transcription translation system (Promega). The reaction was performed according to the manufacturer's recommendations in the presence of [^{35}S]-methionine. Translation products were analyzed either by SDS−PAGE followed by autoradiography or by gel filtration followed by scintillation counting using a Packard CA 2000 scintillation spectrometer.

(E) Purification of Native Cofactor A from Different Species and of Murine Recombinant Cofactor A. Cofactor A was purified from bovine testis and rabbit and chicken reticulocyte lysate as described previously (Gao *et al*., 1994). Recombinant cofactor A forms (unlabeled or ^{35}S-labeled) were expressed in *Escherichia coli* BL21 (DE3) following IPTG induction in the presence of rifampicin (Studier *et al*., 1990). Recombinant cofactor A was found to be in the supernatant fraction following lysis in 20 mM Tris, pH 7.2, 20 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT (lysis buffer) and centrifugation at 20 000*g*. Tracer ^{35}S-labeled cofactor A was added to the centrifuged lysate. The lysate was supplemented with $MgCl_2$ to 20 mM, treated with cell debris remover (CDR, Whatman), filtered by passage through a 0.2 μm Millipore filter, and applied to a Mono Q HR 16/10 column (Pharmacia) equilibrated in lysis buffer containing 20 mM $MgCl_2$. After being washed with 50 mL of the same buffer, the column was developed with a 120 mL linear gradient of 20−250 mM $MgCl_2$. Fractions containing labeled cofactor A were pooled, adjusted to 20 mM potassium phosphate, pH 6.8, 20 mM KCl, 2 mM $MgCl_2$, 1 mM DTT by passage through a Sephadex G25 column and applied to a 20 mL hydroxylapatite column (Pentax, American International Chemical). The column was washed with 30 mL of the same buffer and developed with a 50 mL linear gradient of 20−250 mM potassium phosphate, pH 6.8. Fractions containing labeled cofactor A were analyzed for their protein content by SDS−PAGE (20%) (Laemmli, 1970). Aliquots (1 μL) of each fraction were also assayed for cofactor A activity in 25 μL β-tubulin folding reactions containing purified CCT. The reaction products were analyzed on a 4.5% non-denaturing polyacrylamide gel (Gao *et al*., 1993).

For sequence and mass analysis we further purified cofactor A by C18 reversed phase high-pressure liquid

chromatography (HPLC) (Rommelaere *et al*., 1993). Cofactor A elutes as a single peak at 31% CH_3CN.

(F) Molecular Weight Determination and Peptide Sequence Analysis of Cofactor A from Different Species. To determine the exact molecular weight, aliquots of purified bovine, rabbit, chicken, recombinant murine, and truncated murine and bovine cofactor A in 0.1% TFA/31% CH_3CN were loop-injected (0.005 mL/min) on an electrospray mass spectrometer (VG Platform, Fisons Instruments, Manchester, U.K.), scanning from 500 to 1500 *m*/*z* (bovine), 650 to 1100 *m*/*z* (rabbit), and 600 to 1500 *m*/*z* (murine and chicken) during 6 s. Native bovine, chicken, and rabbit cofactor A were digested with endoproteinase Lys-C in 25 mM Tris-HCl, pH 8.5, 1 mM EDTA, at 37 °C overnight. We also digested rabbit and chicken cofactor A with endoproteinase Asp-N in 50 mM Na_3PO_4, pH 7.5, at 37 °C overnight (enzymes from Boehringer Mannheim Biochemicals, Indianapolis, IN). We purified the resulting peptides by C18 reversed phase HPLC. A fraction of the material ($^1/_5$) was deviated to the on-line electrospray mass spectrometer (scanning between 300 and 1500 *m*/*z* during 6 s for bovine cofactor A, between 400 and 1500 *m*/*z* during 6 s for rabbit cofactor A, and between 400 and 1600 *m*/*z* during 7 s for chicken cofactor A), and the remainder was collected for automated sequencing (model 470A or 477A, Applied Biosystems, Inc., Foster City, CA, equipped with an on-line phenylthiohydantoin amino acid analysis system, model 120A). We subdigested the NH_2-terminal peptides with endoproteinase Asp-N in 50 mM Na_3PO_4, pH 7.5, at 37 °C overnight and identified them by sequencing and/or by matrix-assisted laser desorption time of flight mass-analysis (MALDI-TOF, Bruker, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid as a matrix.

Equilibrium sedimentation was performed at 20 °C on a Beckman Optima XLA ultracentrifuge equipped with an AN 60Ti four-hole rotor and cells with two-channel 12 mm path length centerpieces. Sample volumes of 100 μL were centrifuged at 40 000 rpm. Radial scans of absorbance at 278 nm were taken at 2 h intervals; we concluded that equilibrium was attained when the plots of average molecular weight (M_w) versus centrifugation time gave a constant M_w. Equilibrium was reached after 10 h of centrifugation. The data were analyzed using the programs XLAEQ and EQASSOC supplied by Beckman. The partial specific volume was calculated to be 0.738 $cm^3\ g^{-1}$, and the solvent density was 1.00 $g\ cm^{-3}$.

(G) Circular Dichroism. We recorded circular dichroism (CD) spectra of conventional or HPLC-purified proteins and peptides, dissolved in 10 mM Na_3PO_4, pH 7.4, or in 10 mM Na_3PO_4, pH 7.4/TFE (4/6 v/v), in cylindrical quartz cells (1 mm) in a Jasco-710 spectropolarimeter and a wavelength scan from 260 to 184 nm with a step size of 0.5 nm. We averaged nine scans of each sample. We did not observe differences between native and HPLC-purified proteins except that the noise in the low-UV region was reduced in the latter samples. The amount of the different secondary structure features (α-helix, anti-parallel and parallel β-sheets, β-turn, and other structures) of the proteins and peptides was calculated using the algorithm described by Manavalan and Johnson (1985).

(H) Chemical Cross-Linking. We redissolved 6 μg of HPLC-purified and dried cofactor A in 10 mM Na_3PO_4, pH 7.4, at a concentration of 0.25 mg/mL. We added EDC and NHS, both freshly prepared as 100 mM stock solutions in water, to a final concentration of 4 mM (Staros *et al*., 1986). After 1 h we quenched the reaction by adding glycine to a final concentration of 5 mM. We immediately analyzed the reaction products by SDS−PAGE (17.5%).

(I) Nucleotide Exchange Kinetics on Chaperonin. [^{3}H]-ATP−CCT was prepared by incubating the chaperonin at 4 °C for 2 h in folding buffer containing 1 mM [^{3}H]ATP (0.31 Ci/mmol) followed by separation of the chaperonin from the free nucleotide by gel filtration on a Sephadex G 25 column equilibrated in folding buffer. ϵ-ATP−CCT was prepared using the same procedure except that CCT was initially incubated with ϵ-ATP instead of [^{3}H]ATP. The rate of bound [^{3}H]ATP dissociation from [^{3}H]ATP−CCT complex was monitored at 0 °C by means of a filtration assay on nitrocellulose. In this assay, 100 μL aliquots were filtered at time intervals following addition of 1 mM ATP to a solution of 0.15 μM [^{3}H]ATP−chaperonin. [^{3}H] radioactivity on the filter was quantified by liquid scintillation counting. The dissociation of ϵ-ATP from ϵ-ATP−CCT complex was followed using the change of ϵ-ATP fluorescence in its bound and free states at excitation and emission wavelengths of 340 and 410 nm, respectively. Kinetic measurements were performed with a stopped-flow apparatus (Applied Photophysics, DX-17 MV) thermostated at 5 °C and equipped with a WG 380 cutoff filter (Schott) in the emission pathway. The excitation slit was 0.5 mm, corresponding to a 2.5 nm bandwidth. Syringe A contained ϵ-ATP−CCT at 0.15 μM in folding buffer, and syringe B contained folding buffer either alone or containing ATP or both ATP and purified cofactor A. The dead time was 1 ms.

(J) ATPase Activity Measurements. We measured ATP hydrolysis at 30 °C in folding buffer containing either CCT alone, CCT with cofactor A, CCT with denatured target protein, or CCT with both, by extraction of the [^{32}P] phosphomolybdate complex formed in 1 N HCl as described previously (Melki *et al*., 1990).

(K) Additional Methods. We chemically synthesized the NH_2-terminal peptide (residues 2−24) from murine cofactor A (Npep-coA):Ac-ADPRVRQIKIKTGVVRRLVKERV using solid-phase Fmoc chemistry on an Applied Biosystems model 431A automatic peptide synthesizer. Protein concentrations were determined by either the Lowry or the Bradford method. Molecular weights of 800 000 and 12 700 were employed for the chaperonin containing TCP-1 and cofactor A, respectively.

We used the Chou and Fasman algorithm in the GCG-Wisconsin software package to predict the secondary structure content of cofactor A. Alignments (gap weight 3.0, gap length weight 0.1) and distance matrix calculations (threshold of comparison of 1, denominator length of shorter sequence without gaps) were done with the same software package.

RESULTS

(A) Expression of Wild Type Recombinant Cofactor A cDNA Results in Truncated Forms. The generation of assembly competent αβ-tubulin heterodimers requires the interaction of unfolded α- and β-tubulin target polypeptides with CCT in the presence of Mg-GTP and Mg-ATP and of partially purified fractions containing at least two protein cofactors (cofactors A and B) (Gao *et al*., 1993). We recently purified bovine cofactor A to homogeneity from bovine testis by assaying its capacity to generate monomeric β-tubulin in folding assays containing CCT, Mg-ATP, and

Mg-GTP (Gao *et al*., 1994). Peptide sequence information allowed us to clone the murine homologue (Gao *et al*., 1994). Using a similar strategy we then cloned bovine cofactor A (Figure 1A).

When we expressed bovine and murine cofactor A cDNAs in *E. coli*, a soluble form of cofactor A accumulated in the bacterial cells. This form, however, had a slightly higher mobility on SDS−PAGE compared to native cofactor A, and, unlike the latter, the purified recombinant cofactor A was not capable of generating monomeric β-tubulin in *in vitro* folding reactions containing denatured β-tubulin, CCT, Mg-GTP, and Mg-ATP (Figure 1B).

Molecular weight determination and automated Edman degradation of these purified recombinant murine and bovine forms of cofactor A demonstrated an internal initiation of translation occurring at methionine 25 for murine recombinant cofactor A and methionines 24 and 25 for bovine recombinant cofactor A, resulting in truncated proteins that have molecular masses of 9928 (Figure 1C), 9907, and 10 030 Da (data not shown), respectively. This is due to a Shine Delgarno-like sequence within the part of the gene encoding for the first 24 amino acids of the protein. We conclude from these observations that the truncated cofactor A is not functional and that the 24 NH_2-terminal amino acids missing in the truncated cofactor A are essential for its function.

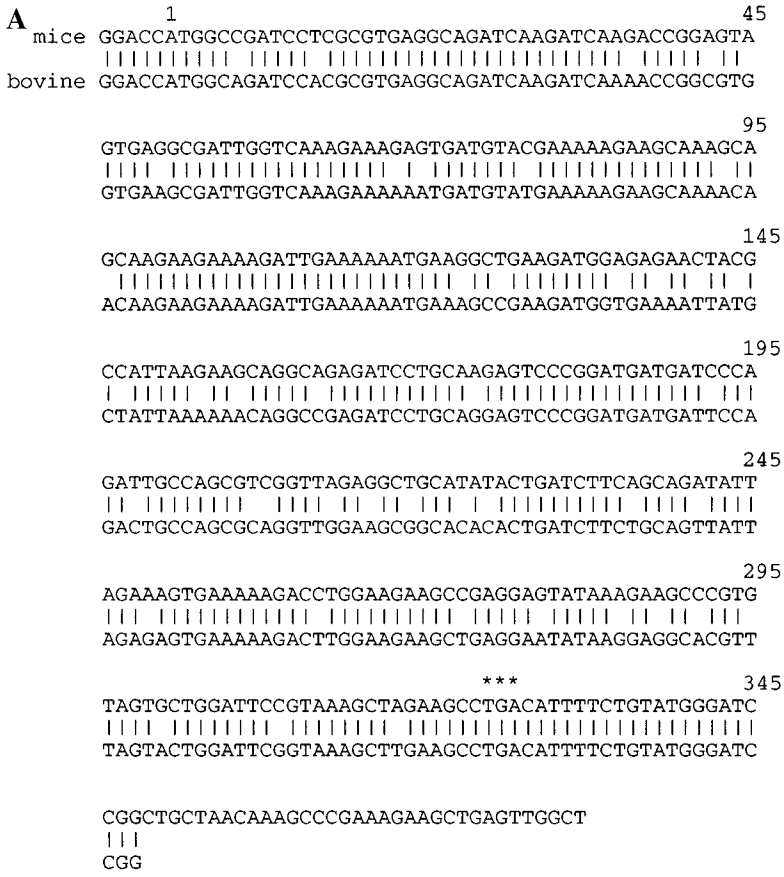
To prevent internal initiation of translation at position 25, we mutated the AUG codon (encoding Met) in the murine cofactor A into CUG (encoding Leu). Expression of this mutated murine cofactor A resulted in an active cofactor A (Figure 1B), indistinguishable from the native bovine cofactor A on the basis of its mobility in a 20% SDS−PAGE (data not shown).

(B) Posttranslational Modifications of Cofactor A and Primary Structure of Rabbit and Chicken Cofactor A. In a previous paper we reported the full-length sequence of murine cofactor A (based on cDNA sequencing) and the partial protein sequence of the bovine isoform (Gao *et al*., 1994). We report here the full-length sequence of bovine cofactor A based on cDNA sequencing (Figure 1A). The calculated (12 706.8 Da) and the observed (12 618.8 Da) masses of bovine cofactor A (Figure 1D) are different. This discrepancy prompted us to search for posttranslational modifications of cofactor A. Authentic bovine cofactor A is refractory to Edman degradation, indicating that the NH_2-terminus of the protein is blocked. Therefore we treated bovine cofactor A with endoproteinase Lys-C and determined the masses of the different peptides generated by this proteolysis (data not shown). One of these peptides is blocked at its NH_2-terminus and has a mass of 1124.7 Da. Subdigestion of this peptide with endoproteinase Asp-N and sequence determination of one of the fragments confirmed the identity of the NH_2-terminal peptide (residues 3−10) (Figure 1E). This fragment has a molecular mass of 1011.2 Da. The presence of a blocking group and the mass difference (113.5 Da) between the original and the derived peptide are consistent with the acetylation of the alanine residue at position 2. The calculated mass based on the complete sequence including the NH_2-terminal acetyl group (12 617.6 Da) fits, within experimental error, the observed molecular mass (12 618.8 Da). We conclude from the comparison of the sequences derived from the bovine cofactor A cDNA and that obtained from peptide sequencing that the initiator methionine is cleaved following translation of cofactor A mRNA and that the alanine residue at position two is consecutively acetylated (Figure 1E). This is the only posttranslational modification of cofactor A we observed.

To assess the degree of conservation of cofactor A among vertebrates, we determined the full-length sequences of cofactor A purified from rabbit and chicken reticulocyte lysate by combining sequence information and mass data from proteolytic fragments obtained by endoproteinase Lys-C and endoproteinase Asp-N digestion (data not shown). The sequences we obtained could be readily aligned to those of murine and bovine cofactor A (Figure 1E). The initiator methionine of cofactor A was systematically found missing, and the following alanine was acetylated. The measured molecular masses of rabbit and chicken cofactor A, respectively 12 593.0 and 12 620.6 Da, are in good agreement with the theoretical molecular masses, respectively 12 594.5 and 12 617.6 Da.

We used the Chou and Fasman algorithm to predict the secondary structure of the vertebrate cofactor A. The secondary structure predictions for all mammalian cofactors A are very similar to each other (Figure 1E shows only the prediction for bovine cofactor A). The secondary structure prediction for chicken cofactor A follows the same pattern except that the COOH-terminal part is predicted to be entirely α-helical. Thus the Chou and Fasman algorithm predicts that most of the protein is in an α-helical conformation.

(C) Cofactor A Secondary Structure Determination; the Truncated Cofactor A Is Not Structured in Aqueous Solution. We recorded the CD spectra of cofactor A from different sources in aqueous solution, in the low-UV range, to determine the secondary structure content of this protein. We also measured the elipticity of both the truncated cofactor A and its NH_2-terminal moiety (Npep-coA, i.e., amino acid residues 2−24) in aqueous solution and in 60% TFE. The data obtained for bovine, chicken, and the full-length recombinant murine cofactor A in aqueous solution are presented in Figure 2A. The three spectra are very similar, suggesting that the amino acid exchanges do not influence the structure to a great extent. The calculated α-helical content is, respectively, 25% and 32% for the chicken and the murine recombinant cofactor A. The total score for β-strands (parallel and anti-parallel) is 22% and 19%, respectively, and the score for turns is 15% for both. This would correspond to 27−34 amino acids out of 107 being in an α-helical structure, 18−21 in β-strands, and 14 in β-turns. In contrast, the CD spectrum obtained for the truncated cofactor A in aqueous solution is that of a typical random coil (Figure 2B). The CD spectra of the truncated and the full-length recombinant murine cofactor A were also recorded in the presence of 60% TFE, a solvent known to stabilize α-helices (Figure 2B). No significant change in the secondary structure content of the full-length cofactor A was detected (data not shown), whereas we observed a marked difference for the truncated factor A: its CD spectrum became very similar to that of the full-length cofactor A in aqueous solution (Figure 2B). The calculated α-helical content of the truncated recombinant murine cofactor A in 60% TFE is 23%, and the scores for β-strands and for turns are respectively 22% and 16%. This corresponds to approximately 19 amino acids being in an α-helical structure in the truncated cofactor A and strongly suggests that Npep-coA somehow stabilizes the overall structure of the protein. This suggestion prompted us to determine the conformation of Npep-coA. Figure 2 shows that Npep-coA



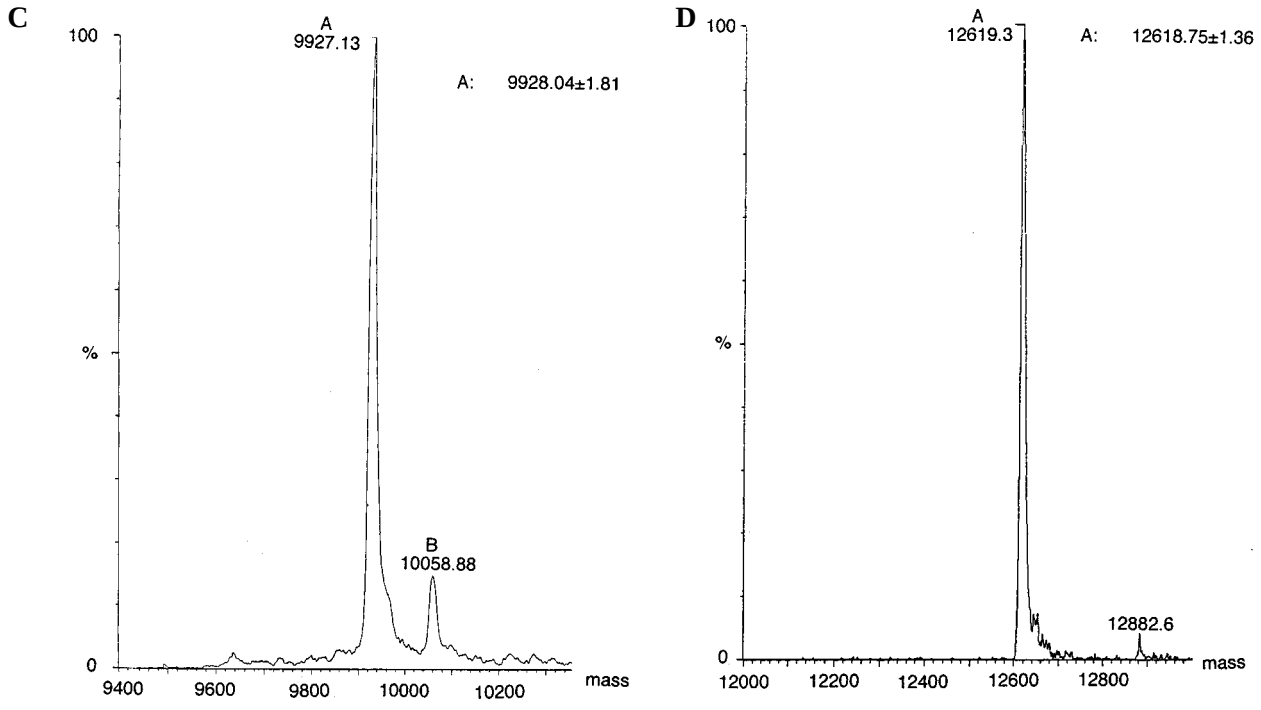
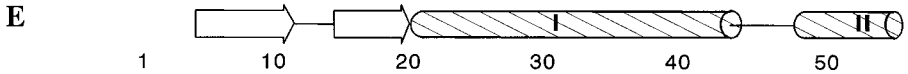


FIGURE 1: Characterization of authentic and recombinant cofactor A from different species. (A) Sequence alignment of bovine and murine cofactor A cDNAs. Bovine cDNA sequence data is available from EMBL nucleotide sequence database under accession number X97224. (B) *In vitro* β-tubulin folding reactions containing purified CCT, labeled denatured β-tubulin, Mg-ATP, Mg-GTP, and native bovine cofactor A (lane 1), truncated recombinant mouse cofactor A (lane 2), and full-length recombinant mouse cofactor A (lane 3) were incubated at 30 °C for 2 h, and the reaction products were analyzed on a 4% non-denaturing gel and autoradiographed overnight. Upper and lower arrowheads indicate the location of β-tubulin−CCT binary complex and soluble β-tubulin, respectively. (C) Mass spectrum of truncated recombinant mouse cofactor A. (D) Mass spectrum of bovine cofactor A. (E) Alignment of the amino acid sequences of cofactor A from different species. Bovine, rabbit, and chicken cofactor A sequences were determined in this work. Rabbit and chicken sequence data are available from SWISS-PROT protein sequence database under accession numbers P80584 and P80585, respectively. The murine cofactor A sequence

E

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Murine     MADPRVRQIKIKTGVVRRLVKERVMYEKEAKQQEEKIEKMKAEDGEN.YAIKKQAE
Rabbit   Ac-ADPRVRQIKIKTGVVKRLVKEKVMYEKEAKQQEEKIEKMRAEDGEN.YAIKKQAE
Chicken  Ac-ADPRLRQIKIKTGVVKRLAKEKVMYEKEAKQQEEKIEKMKAEACDD.YGIKKQAE
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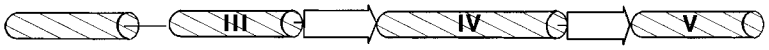


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          60        70        80        90       100
Bovine     ILQESRMMIPDCQRRLEAAHTDLLQLLESEKDLEEAEEYKEARLVLDSVK..LEA
Murine     ILQESRMMIPDCQRRLEAAYTDLQQILESEKDLEEAEEYKEARVVLDSVK..LEA
Rabbit     ILQESRMMIPDCQRRLEAACTDLQQILESEKDLEEAEEYKEARLVLDSVK..LEA
Chicken    ILQESRMMIPDCQRRLEIAHADLTQLLENEKELEEAEEYKEARSILESVK..LEA
Yeast      VLDDTKRLLPTLYEKIREFKEDLEQFL...KTYQGTEDVSDARSAITSAQELLDSK
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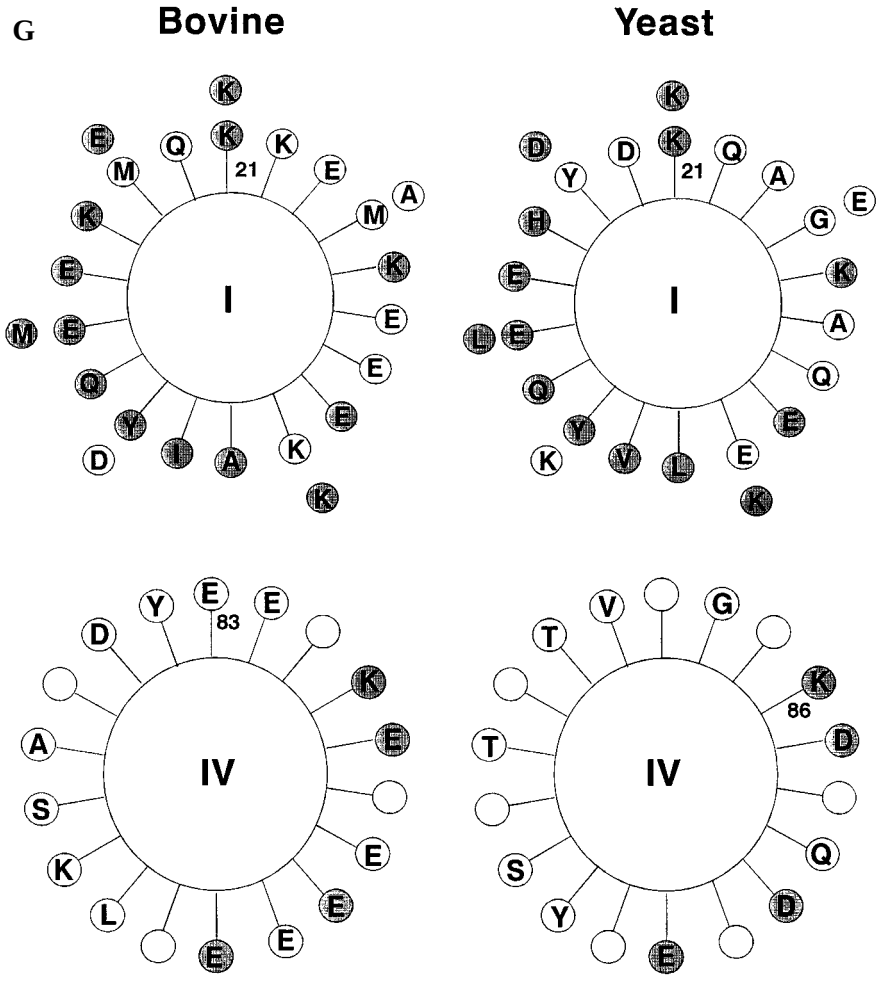
F

	murine	rabbit	bovine	chicken	yeast
murine	1.0000	0.9626	0.9346	0.8505	0.3868
rabbit		1.0000	0.9533	0.8598	0.3868
bovine			1.0000	0.8785	0.4057
chicken				1.0000	0.4151
yeast					1.0000

G



was determined by Gao *et al*. (1994), and the yeast cofactor A sequence was determined by Archer *et al*. (1995). The residue numbering is according to the murine sequence. Positions of deletions are indicated with a point. Ac- represents the NH_2-terminal acetylation. The secondary structure predicted for the bovine cofactor A by the Chou and Fasman algorithm is shown on top of the sequences. β-Strands are shown as arrows, turns and loops as lines, and α-helices as cylinders. α-Helices are numbered with roman numbers. (F) Distance matrix. (G) Helical wheel model of the first helix (I) of bovine (left) and yeast (right) cofactor A and the fourth helix (IV) of bovine (left) and yeast (right) cofactor A. Helix positions are defined in panel E. The first residue of each helix is indicated by its residue number in the primary structure (see Figure 1E). Amino acid residues are represented by their one-letter code inside circles. Dark circles denote conserved residues between bovine and yeast cofactor A.

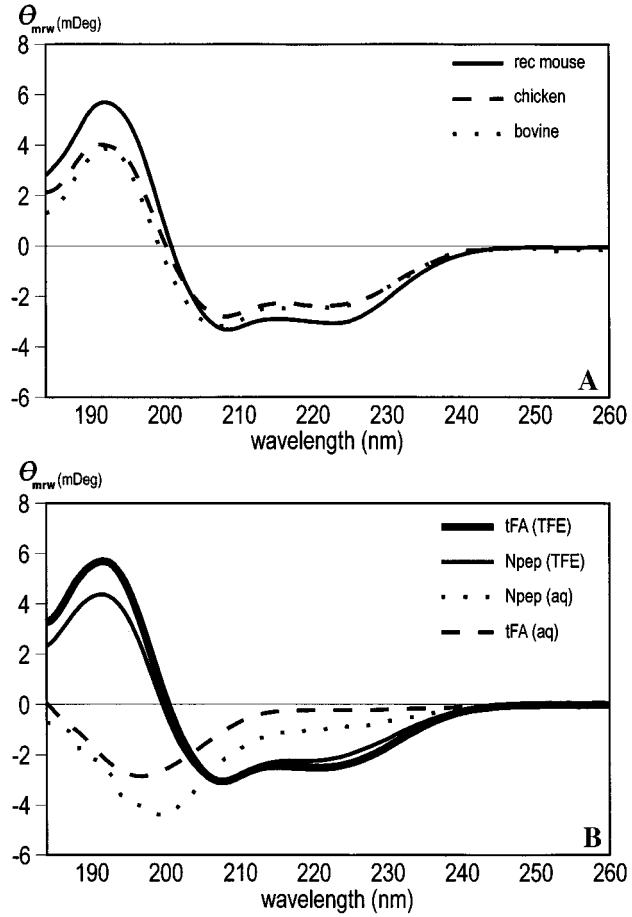


FIGURE 2: Structural characterization of authentic and truncated cofactor A. (A) CD spectra of bovine, chicken, and recombinant mouse (rec mouse) cofactor A in aqueous solution. (B) CD spectra of truncated mouse cofactor A (tFA) and the NH_2-terminal peptide of cofactor A (Npep) in aqueous solution (aq) and in 60% TFE (TFE). The data are plotted as mean residue weight elipticity (θ_{mrw}) versus wavelength.

has a random coil conformation in aqueous solution. Upon addition of TFE it adopts a structure that is predominantly α-helical (43%, β-strands 6%, turns 23%) (Figure 2B). The data obtained from the truncated cofactor A Npep-coA taken together suggest that about 29 amino acids participate in α-helical structures, in agreement with the data obtained for the full-length cofactor A.

(D) Addition of the NH_2-Terminal Peptide Partially Restores the Activity of Truncated Cofactor A. To determine whether the NH_2-terminal part of cofactor A, which is missing in the truncated cofactor A, is required for the generation of monomeric β-tubulin we incubated CCT, denatured β-tubulin, Mg-ATP, and Mg-GTP with increasing amounts of the synthetic peptide (up to 14.5 mM) representing the 24 NH_2-terminal amino acid residues of cofactor A (Npep-coA). No monomeric β-tubulin could be detected in these *in vitro* folding reactions after analysis of the reaction products on non-denaturing polyacrylamide gels (Figure 3, lane 3). When the same reaction was repeated in the presence of 16 μM truncated cofactor A, trace amounts of monomeric β-tubulin representing approximately 4% of the amount of monomeric β-tubulin obtained in folding reactions containing equimolar amounts of native cofactor A (Figure 3, lane 1) were generated (Figure 3, lanes 4−6). We conclude from this experiment that both NH_2- and COOH-parts of cofactor A are required for the generation of monomeric β-tubulin. The fact that cofactor A activity is

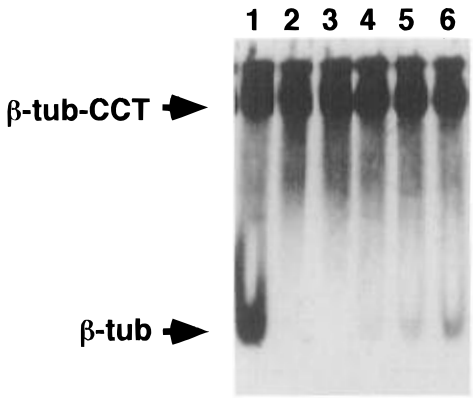


FIGURE 3: NH_2-terminal part of cofactor A is essential for its function. Analysis on a 4% native polyacrylamide gel of *in vitro* β-tubulin folding reactions incubated at 30 °C for 2 h. All contain CCT, Mg-ATP, and Mg-GTP. Full-length recombinant murine cofactor A (lane 1), truncated murine cofactor A, 16 μM (amino acid residues 25−108) (lane 2), the synthetic peptide: Ac-ADPRVRQIKIKTGVVRRLVKERV, that reproduces cofactor A NH_2-terminus, 14.5 mM (lane 3), or both truncated cofactor A (16 μM) and its synthetic NH_2-terminal moiety at the following concentrations: 3.6 mM (lane 4), 7.25 mM (lane 5), and 14.5 mM (lane 6).

restored when its NH_2- and COOH-terminal moieties are mixed together may suggest that these two parts of the molecule interact with each other to stabilize a highly structured state of cofactor A.

(E) Cofactor A Is a Monomer in Solution. It was recently suggested that cofactor A acts as a dimer because it elutes from a size exclusion column as a single peak with an apparent molecular weight of 28 000, while purified bovine cofactor A has an apparent molecular weight of 15 000 on a denaturing polyacrylamide gel (Gao *et al.*, 1994) in approximate agreement with our mass spectrometry measurements (12 618.8 Da). To investigate whether the elution behavior of cofactor A from a size exclusion column is due to its dimerization or to its behavior as a non-globular protein we determined the molecular mass of native bovine cofactor A by equilibrium centrifugation and performed cross-linking experiments.

The molecular weight of pure native bovine cofactor A (158 μM) in aqueous solution measured by equilibrium sedimentation is 13 000 (Figure 4A), consistent with its behavior as a monomeric protein.

To further strengthen this conclusion, we performed cross-linking experiments. EDC is a zero-length cross-linker connecting COOH-groups with NH_2-groups into an isopeptide linkage only when the participating residues are in close contact with each other. If cofactor A acts as a dimer, cross-linking may generate a covalently linked product of approximately 30 kDa. In contrast, when pure native bovine cofactor A is treated with EDC, the cross-linked product shifts toward a lower molecular weight on a 17.5% denaturing gel (Figure 4B). This result suggests that the molecule becomes more compact and is indicative of internal cross-linking.

(F) Functional Role of Cofactor A. Recently, Gao *et al.* (1994) measured an increase in the rate of ATP-hydrolysis by CCT in the presence of purified cofactor A. This result was interpreted as the consequence of a physical interaction between the chaperonin and cofactor A in a manner distinct from the interaction of potential target protein with cytoplasmic chaperonin, given that no binary complex is formed between the two molecules. Although no direct evidence

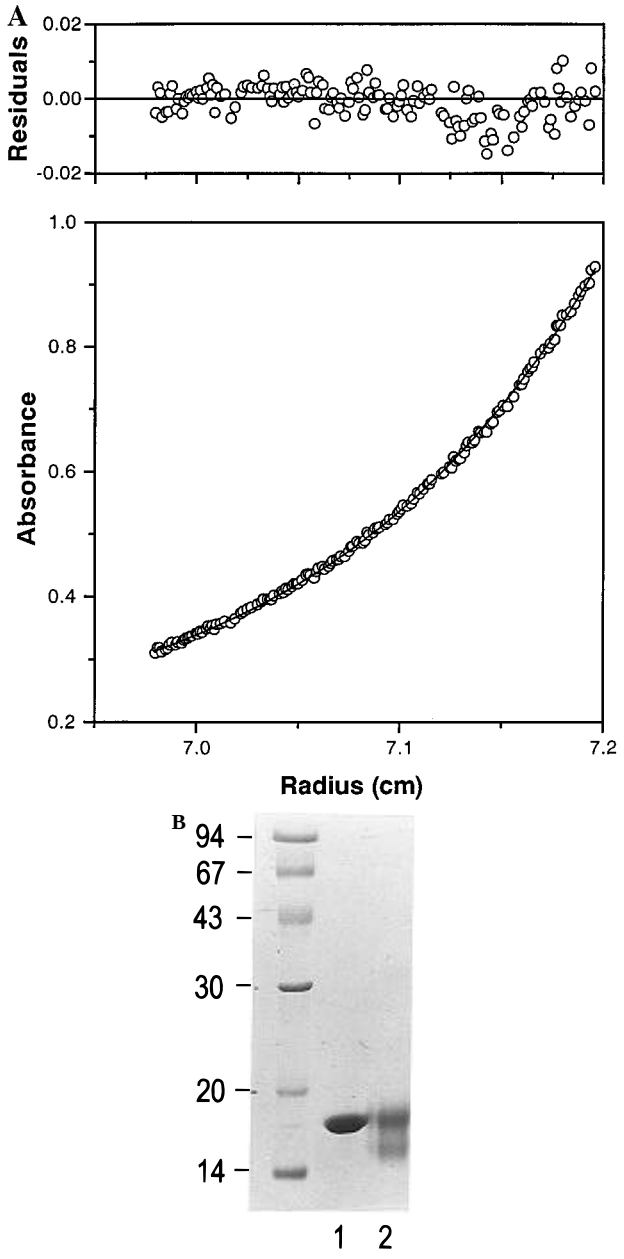


FIGURE 4: Cofactor A is a monomeric protein. (A) Measurement of the molecular weight of cofactor A by equilibrium ultracentrifugation. Equilibrium sedimentation was performed as described in Materials and Methods. The data (symbols), obtained at 20 °C and 40 000 rpm, at an initial cofactor A concentration of 2 mg/mL were fitted using a monomer model (solid line). The inset shows the deviation of the data from the fitted curve. (B) Analysis on a 17.5% SDS−polyacrylamide gel of native cofactor A (lane 1) and of the EDC-cross-linked cofactor A (lane 2). Protein molecular weight markers with their respective molecular masses in kDa are shown on the left side.

for such an interaction was presented, the data were interpreted to mean that cofactor A might increase the rate at which the chaperonin-bound nucleotide is exchanged with nucleotide free in solution. Similar results may be accounted for by considering that cofactor A interacts with a tubulin folding intermediate which by consequence is stabilized when released in the medium after interacting with the chaperonin. To test this hypothesis, we first examined the effect of adding pure recombinant cofactor A on the rate at which CCT hydrolyzes ATP. Purified cytoplasmic chaperonin hydrolyzes ATP at a steady-state rate of 0.83 min^{-1} in the absence or presence of 0.5−3 molar equiv of pure cofactor A. The rates at which CCT hydrolyzes ATP in the absence or

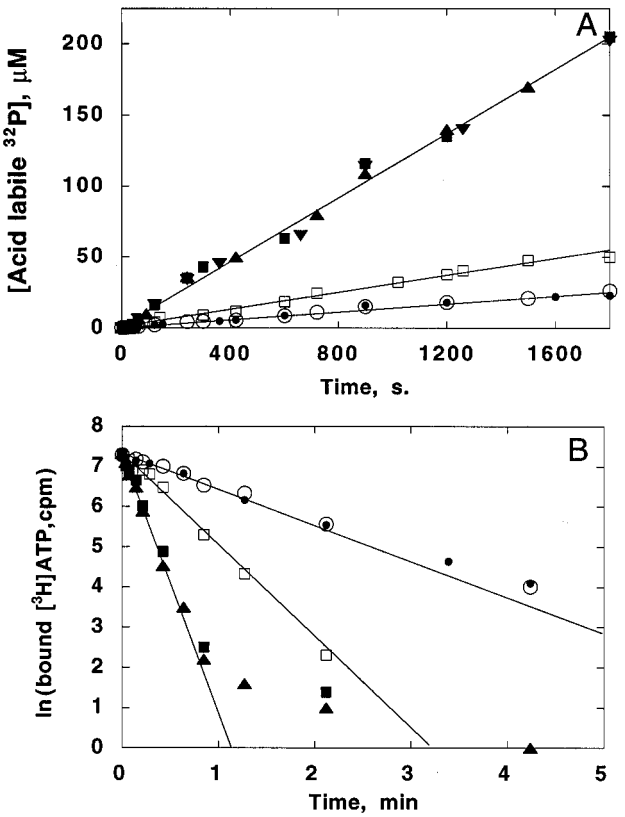


FIGURE 5: Kinetics of ATP hydrolysis and nucleotide exchange on the chaperonin containing TCP-1 in the presence or absence of target proteins. (A) Kinetics of cleavage of ATP γ-phosphate by CCT (0.25 μM) in folding buffer (80 mM MES, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 1 mM [γ-^{32}P]ATP, 50 μM GTP), alone (○), or in the presence of pure recombinant cofactor A (7 μM) (●) or unfolded target proteins (0.2 mg/mL): β-tubulin (□), β-actin (▲), or both pure recombinant cofactor A and unfolded β-actin (▼) or β-tubulin (■). (B) Kinetics of [^{3}H]ATP dissociation from [^{3}H]ATP−CCT 1:1 complex in folding buffer in the presence of unlabeled ATP alone (○), or in the presence of pure recombinant cofactor A (●) or unfolded target proteins: β-tubulin (□), β-actin (▲), or both cofactor A and β-tubulin (■).

Table 1: Rate Constants for ATP Hydrolysis and Nucleotide Exchange on CCT in the Absence or Presence of Different Target Proteins and Cofactor A

	rate of ATP hydrolysis (min^{-1})		rate of nucleotide exchange (min^{-1})	
target protein	− cofactor A	+ cofactor A	− cofactor A	+ cofactor A
none	0.83	0.83	0.80	0.80
β-actin	6.70	6.70	6.60	6.60
α-tubulin	6.40			
β-tubulin	1.82	6.87	2.19	6.60
γ-tubulin	6.75			
TCP-1	6.83			
CAP-binding protein	6.98			
cyclin B	6.58			

presence of cofactor A and the presence of a molar excess of denatured β-actin, α-, β-, or γ-tubulin, TCP-1, cap-binding protein, or cyclin B are shown in Table 1. In all cases, we observed linear kinetics during at least 30 min (Figure 5A). When β-tubulin was presented to the chaperonin as a denatured target protein, the rate of ATP hydrolysis is equal to 1.82 min^{-1}. This rate is approximately 4-fold slower than the rate at which the chaperonin hydrolyzes ATP in the presence of other target proteins (Figure 5A). This reduced rate is not due to a lower concentration of competent substrate, since further addition of denatured β-tubulin did

not result in an increase of chaperonin ATPase activity. When cofactor A was added to β-tubulin folding reactions, the initial rate of ATP hydrolysis increased approximately 4-fold and became comparable to the rates observed in the presence of the other denatured target proteins used in this study.

The data presented above strongly suggest that cofactor A affects chaperonin ATPase activity only when denatured β-tubulin is presented to the chaperonin. The discrepancy between our observations and those Gao *et al*. (1994) is probably due to the fact that cofactor A preparations used in previous studies were partially purified. Thus, the burst in the ATPase activity of the chaperonin may have been the consequence of an interaction between contaminating proteins present in cofactor A preparations and CCT, similar to that of other target proteins with the chaperonin.

In the experiment described above, the measured ATPase activity is a consequence of ATP hydrolysis and nucleotide exchange on the chaperonin. To further demonstrate that cofactor A does not affect nucleotide exchange on CCT, the time course of dissociation of ^{3}H-labeled ATP or ϵ-ATP from the chaperonin was monitored at 4 °C using a filter assay or a change of the fluorescence of ϵ-ATP in its bound and free states. The use of a 1:1 ATP−CCT complex in this experiment allows us to examine a single cycle of nucleotide exchange and subsequent hydrolysis. The rate of nucleotide exchange on the chaperonin in the absence of cofactor A or target proteins is 0.78 min^{-1}. This rate does not markedly change in the presence of equimolar to 5-fold molar excess of pure cofactor A alone (Figure 5B), but it does increase by a factor of 8.5 when denatured target proteins (β-actin, γ-tubulin, TCP-1, or cap-binding protein) are presented to the chaperonin (Figure 5B). Strikingly, when denatured β-tubulin is presented to CCT in the absence of cofactor A, the rate of nucleotide exchange on the chaperonin increases less than 3-fold while in its presence, this rate became comparable with those measured when other target polypeptides are presented to CCT in the absence or presence of cofactor A (Figure 5B). Comparable results were obtained when the time course of dissociation of ϵ-ATP from the chaperonin was monitored (data not shown).

We conclude from these experiments that nucleotide exchange on CCT increases upon presentation of a target protein to the chaperonin. The extent of the increase in the rate of nucleotide exchange on the chaperonin appears to be constant except when denatured β-tubulin is presented to CCT. The fact that the rate of nucleotide exchange is not affected by the presence of cofactor A except when denatured β-tubulin is presented to the chaperonin indicates that cofactor A is not a nucleotide exchange factor for CCT but specifically affects, probably indirectly, nucleotide exchange on the chaperonin in β-tubulin folding reactions.

(G) Cofactor A Interacts with β-Tubulin Monomer. When the products of a β-tubulin *in vitro* folding reaction containing purified CCT, cofactor A, labeled denatured β-tubulin, Mg-ATP, and Mg-GTP are subjected to size exclusion chromatography, two radioactive peaks with apparent molecular masses of 900 and 65 kDa emerge from the column. Two radioactive peaks are also observed when the products of a β-tubulin *in vitro* folding reaction containing purified CCT, labeled recombinant cofactor A, unlabeled denatured β-tubulin, Mg-ATP, and Mg-GTP are subjected to size exclusion chromatography. These products have an apparent molecular mass of 65 and 28 kDa (Figure 6A). The material that has a molecular mass of 28 kDa is authentic cofactor A

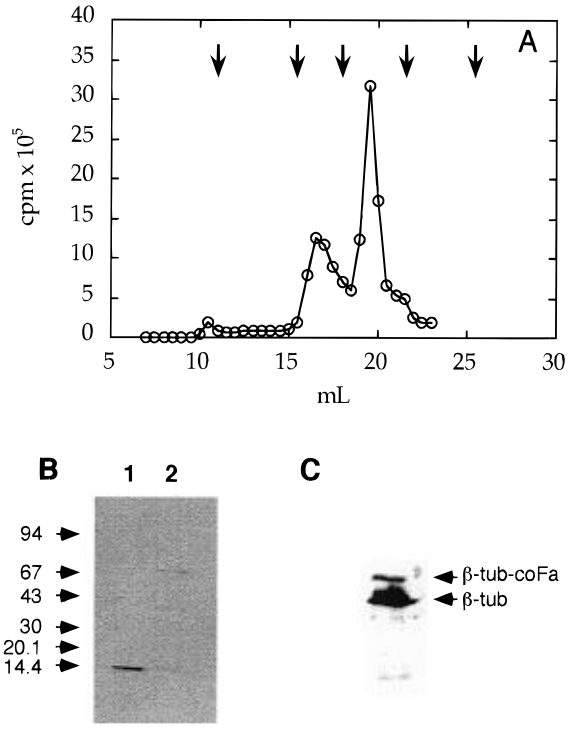


FIGURE 6: Cofactor A interacts with β-tubulin monomer. (A) Elution profile of a β-tubulin *in vitro* folding reaction containing ^{35}S-labeled full-length cofactor A on a Superose 6 gel-filtration column. Arrows show the location of molecular size markers (thyroglobulin, 670 kDa; immunoglobulin G, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa, and vitamin B12, 1.35 kDa) run under identical conditions on the same column. (B) Analysis on a 10% SDS−polyacrylamide gel of the products that emerges from the superose 6 column with an apparent molecular mass of 65 kDa following EDC treatment. Lane 1, autoradiogram of a control reaction incubated for 1 h at room temperature in the absence of EDC; lane 2, autoradiogram of the same sample incubated in the presence of 4 mM EDC and sulfo-NHS and quenched after 1 h by 5 mM glycine. Locations of molecular weight markers (94, 67, 43, 30, 20.1, and 14.4 kDa) are shown at the left. (C) Immunoblot detection of β-tubulin following chemical cross-linking by EDC. Proteins that emerge from the sizing column with an apparent molecular mass of 65 kDa were separated on a 10% SDS−polyacrylamide gel following an hour of incubation with EDC and sulfo-NHS and transferred to a nitrocellulose membrane. β-Tubulin was revealed using the DM1B monoclonal anti-β-tubulin antibody from Amersham (Breitling & Little, 1986).

(Gao *et al*., 1994), and the material eluting around 65 kDa is likely to be cofactor A bound to β-tubulin. To demonstrate such an interaction, we incubated the material emerging from the size exclusion column with an apparent molecular weight of 65 000 with EDC and sulfo-NHS and analyzed the products of the cross-linking reaction on a 10% denaturing polyacrylamide gel followed by western blotting. The presence of β-tubulin on the blot was detected by the use of a monoclonal anti-β-tubulin antibody and that of labeled cofactor A by autoradiography. Figure 6C clearly shows that radioactivity is located in two bands that have apparent molecular masses of 65 and 14 kDa and that two bands that have apparent molecular masses of 65 and 50 kDa are revealed using the anti-β-tubulin antibody. These bands correspond to the cofactor A−β-tubulin cross-link (65 kDa) to free tubulin (50 kDa) and cofactor A (14 kDa). We conclude from this experiment that the material emerging from the sizing column with an apparent molecular weight of 65 000 corresponds to the cofactor A−β-tubulin complex.

In order to document the role of cofactor A in chaperonin-assisted β-tubulin folding, labeled, denatured β-tubulin was

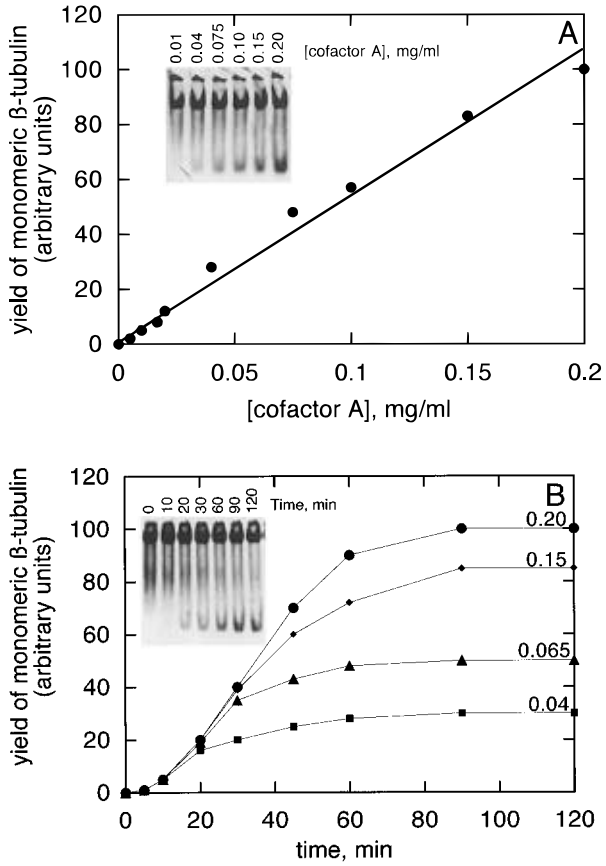


FIGURE 7: Accumulation of soluble β-tubulin in CCT mediated folding reactions as a function of cofactor A concentration. (A) The yield of soluble β-tubulin generated at 30 °C from *in vitro* folding reactions containing a constant amount of CCT (0.2 mg/mL), Mg-ATP and Mg-GTP (1 mM each), labeled denatured β-tubulin (0.2 mg/mL), and increasing amounts of cofactor A (0−0.2 mg/mL) was quantitated using a phosphorimager after separation of the reaction products on a 4% non-denaturing polyacrylamide gel presented in the inset. (B) Kinetics of β-tubulin generation at various cofactor A concentrations. Aliquots from a β-tubulin *in vitro* folding reactions incubated at 30 °C, containing CCT (0.2 mg/mL), Mg-ATP and Mg-GTP (1 mM each), labeled denatured β-tubulin (0.2 mg/mL), and 0.2, 0.15, 0.065, and 0.04 mg of cofactor A/mL, were withdrawn at the times indicated on the graph, and the reaction products analyzed on a 4% non-denaturing polyacrylamide gels, one of which is presented in the inset. The amount of soluble β-tubulin was quantitated using a phosphorimager.

diluted 100-fold into folding buffer containing CCT, Mg-ATP, Mg-GTP, and various concentrations of cofactor A. The folding reaction was carried out at 30 °C for 2 h, and the reaction products were analyzed on a nondenaturing polyacrylamide gel. Figure 7A show that the yield of β-tubulin increases with the amount of added cofactor A.

To further characterize the specific requirement for cofactor A in tubulin folding reactions, we monitored the kinetics of generation of β-tubulin in folding reactions (containing CCT, Mg-ATP, Mg-GTP) in the presence or absence of cofactor A and compared them with the kinetics of folding of β-actin. Following dilution of the target protein from denaturant in the absence of cofactor A, labeled, denatured β-tubulin forms a binary complex with CCT. The amount of binary complex decreases slightly during the incubation, with a corresponding increase in the amount of high molecular weight aggregates trapped at the top of the gel. No β-tubulin is generated under these conditions (Figure 7B, lanes 1 and 2). Under the same conditions native β-actin is generated with a half-time of 30 min (Gao *et al*., 1994; Melki & Cowan, 1994). In the presence of cofactor A, β-tubulin is generated with a half-time of 30 min (Figure 7B, lanes 3−7). When the molar ratio of cofactor A to CCT present in *in vitro* β-tubulin folding reactions was decreased from 30:1 to 1.5:1, the yield of β-tubulin dramatically decreased (Figure 7A). Further analysis of the evolution with time of the amount of β-tubulin generated in *in vitro* folding reactions containing increasing amounts of cofactor A allowed us to demonstrate that β-tubulin is generated at a constant rate at various cofactor A concentrations. We conclude from these experiments that the amount of soluble β-tubulin generated in β-tubulin *in vitro* folding reactions depends on the amount of cofactor A present in the reaction mix. Given that the rate of β-tubulin generation corresponds exactly to the rate at which β-tubulin is released from the β-tubulin−chaperonin binary complex in the presence of Mg-ATP (Gao *et al*., 1994), we conclude that the observed rate of β-tubulin generation reflects its rates of folding and release.

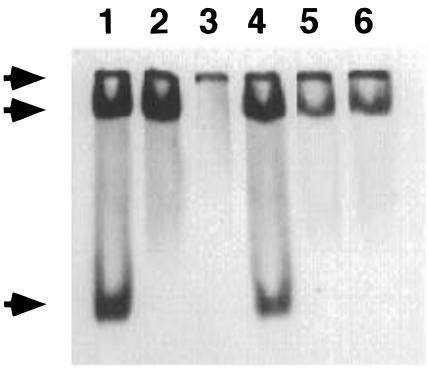


FIGURE 8: Cofactor A binds native or quasinative β-tubulin. Labeled denatured β-tubulin was diluted into folding buffer containing Mg-ATP and Mg-GTP (1 mM each) and either CCT alone or cofactor A alone, and the reaction mixes were incubated at 30 °C for 60 min. Each sample was split into two identical aliquots. The incubation was continued for one-half of each reaction (lanes 2 and 3, respectively), while cofactor A was added to the folding reaction containing CCT alone (lane 4) and CCT was added to the folding reaction containing cofactor A alone (lane 5) and the incubation continued for 30 min. Lane 1, control β-tubulin folding reaction, incubated for 90 min at 30 °C, containing CCT and cofactor A; lane 6, labeled denatured β-tubulin incubated for 60 min in folding buffer containing Mg-ATP and Mg-GTP prior to addition of CCT and further incubation for 30 min at 30 °C. The reaction products were analyzed on a 4% nondenaturing polyacrylamide gel and autoradiographed. Upper, middle, and lower arrows show the location of the aggregated β-tubulin, the β-tubulin−CCT binary complex, and soluble β-tubulin, respectively.

(H) Cofactor A Interacts with Native or Quasinative β-Tubulin. To further document the role of cofactor A in the β-tubulin folding pathway and probe the state of the β-tubulin folding intermediate that interacts with cofactor A, we diluted labeled denatured β-tubulin in folding buffer containing Mg-ATP, Mg-GTP, and either CCT alone or cofactor A alone and incubated these reaction mixes at 30 °C. Each sample was split into two identical aliquots after 60 min. One-half of each reaction mix was maintained unchanged. Cofactor A was added to the other half of the reaction mix containing labeled denatured β-tubulin and CCT, while CCT was added to the other half of the reaction mix containing labeled denatured β-tubulin and cofactor A. In all cases, the incubation at 30 °C was continued for an additional 30 min. A control folding reaction containing labeled denatured β-tubulin, Mg-ATP, Mg-GTP, CCT, and cofactor A was incubated for the same time at 30 °C. The

reaction products were then analyzed on a non-denaturing gel (Figure 8). β-Tubulin−CCT binary complex is formed, and soluble β-tubulin is generated in the control reaction (Figure 8, lane 1). In the presence of CCT alone, β-tubulin−CCT binary complex is formed but no soluble β-tubulin is generated (Figure 8, lane 2), while in the presence of cofactor A alone, β-tubulin aggregates into high molecular weight products that are trapped at the origin of the gel in a manner similar to that observed upon dilution of β-tubulin from denaturant into folding buffer (Melki & Cowan, 1994) (Figure 8, lane 3). Soluble β-tubulin is generated when cofactor A is added to the reaction mix containing CCT and labeled β-tubulin (Figure 8, lane 4). The yield of folded β-tubulin that is generated from such a reaction is equivalent to one-third of that of an *in vitro* folding reaction that was incubated three times longer at 30 °C (compare lanes 1 and 4 in Figure 8), demonstrating that folding and/or release of quasinative β-tubulin that binds to cofactor A are slow processes.

Finally, no soluble β-tubulin is generated upon addition of CCT to the reaction mixes containing cofactor A and labeled denatured β-tubulin or β-tubulin alone, that were incubated 60 min at 30 °C prior to addition of CCT and the yield of binary complex that is generated in both cases are identical (compare lanes 5 and 6 in Figure 8), and correspond to one-fifth of the amount of binary complex formed when labeled denatured β-tubulin is diluted from denaturant in the presence of CCT (compare lanes 5 and 6 to lane 2 in Figure 8). This indicates that β-tubulin aggregates following dilution from denaturant either in the presence or in the absence of cofactor A. We conclude from these experiments that cofactor A stabilizes specifically a quasinative conformational state of β-tubulin following its release from CCT−β-tubulin binary complex and that the generation of this folding intermediate is the rate-limiting reaction in the chaperonin-assisted folding process.

DISCUSSION

The folding of actins and tubulins is facilitated both *in vivo* and *in vitro* by the chaperonin containing TCP-1 (CCT) [reviewed in Kubota *et al*. (1995)]. In the case of α- and β-tubulin polypeptide chains other protein cofactors are required for the generation of a functional tubulin dimer (Rommelaere *et al*., 1993; Gao *et al*., 1993). We recently identified one of these protein cofactors, termed cofactor A, which is required for the correct folding of the β-tubulin chain (Gao *et al*., 1994). In this paper we further document the structure and function of cofactor A.

Alignment of the primary structure of bovine, rabbit, chicken (this paper), and murine (Gao *et al*., 1994) cofactor A shows that this protein is highly conserved among vertebrates (Figure 1G). The mutations are not evenly distributed along the sequence but are grouped in four clusters (amino acids 17−24, 41−49, 73−84, and 99−102, see Figure 1E). Recently Archer and co-workers (1995) published the sequence of a yeast homologue of cofactor A (RB12p), and, although less obvious because the sequence is clearly more divergent, the amino acids in the homologous regions of these clusters are also less conserved. The calculated evolutionary distance between yeast and vertebrate cofactor A is only 39%−41%. It is much lower than the degree of conservation between, for instance, yeast and murine β-tubulin (80%). This is somewhat surprising since overexpression of murine cofactor A rescues (to some extent) the lethal phenotype of β-tubulin overexpression in yeast (Archer *et al*., 1995).

CD data suggest that a number of amino acid residues in cofactor A are in α-helical structures (about 30%). Secondary structure prediction according to Chou and Fasman also suggests the presence of α-helices, although the predicted total content appears much higher (65%). Indeed, the protein is predicted to adopt α-helical structures interrupted by two turns (amino acids 45−48 and 65−67) and two short β-strands (amino acids 78−82 and 98−101). It is noteworthy that some of these interrupting regions and flanking sequences seem to correspond to the clusters with a high mutational variance identified above. Thus, it appears that the core of each α-helix is well conserved between vertebrate cofactors A.

If we assume that the regions predicted to be α-helical by the Chou and Fasman algorithm are correctly assigned (e.g., the core of an α-helix is correctly predicted), the inspection of the helices of mammalian cofactor A, plotted on a helical wheel, reveals a striking pattern for the first and the fourth helices. Both contain a patch of charged residues on one side (right side of the helical wheel in Figure 1F). The functional significance of this observation is unclear. The presence of five glutamic acid residues on one side of the fourth helix may serve to anchor this helix to the rather basic NH_2-terminal part of the molecule. This hypothesis is supported by the observation that deletion of the latter part destabilizes the native structure of cofactor A. Furthermore, we were able to restore cofactor A activity when we mixed its NH_2- and COOH-terminal moieties together, which suggests that these two parts of the molecule interact with each other and stabilize the structured and active state of cofactor A.

The other sides of the first and the second α-helices are hydrophobic in vertebrates as well as in yeast (left side of the helical wheel in Figure 1F and data not shown). One of these conserved features may serve as a template for interaction with β-tubulin, alternatively it may be essential for the proper scaffolding of cofactor A. Finally if the first α-helix is 25 residues long (as predicted by the Chou and Fasman algorithm, its length would be approximately 37 Å and possibly give the molecule an elongated shape, in agreement with our finding that cofactor A is a monomeric protein with an aberrant elution behavior on size exclusion columns (Gao *et al*., 1994).

In a previous report (Gao *et al*., 1994), the stimulation of CCT ATPase activity by cofactor A was considered to be the consequence of a direct interaction between cofactor A and CCT, with cofactor A acting on CCT either as a nucleotide exchange factor or as a ligand that induces a conformational change required for the folding of β-tubulin. Here we demonstrate that cofactor A has no effect on the rate at which CCT subunits hydrolyze ATP nor does it influence nucleotide exchange on CCT. Cofactor A addition specifically increases CCT ATPase activity only in β-tubulin folding reactions to a level which becomes comparable to the rates measured in the presence of other target proteins. This effect probably reflects the acceleration of β-tubulin polypeptide turnover on the chaperonin concomitant with nucleotide exchange and ATP hydrolysis due to the stabilization of β-tubulin folding intermediates by cofactor A.

Our data clearly show that the initial rate of β-tubulin generation at variable cofactor A concentrations is constant

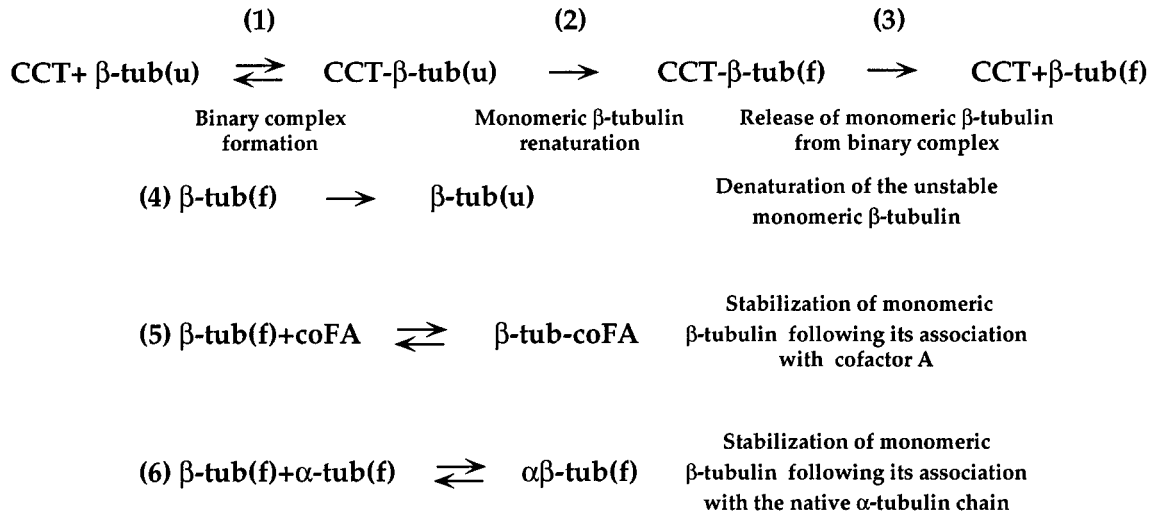


FIGURE 9: Theoretical model for CCT-assisted β-tubulin folding. Unfolded or misfolded β-tubulin binds strongly to CCT in its ADP form and can be released following nucleotide exchange from the ATP form of CCT (Melki & Cowan, 1994). Following folding monomeric β-tubulin is released into solution and may have several fates thereafter: (i) β-tubulin is intrinsically unstable (Gao *et al*., 1994), unfolds (pathway 4), and may rebind to CCT starting a new folding cycle; (ii) in the presence of cofactor A, a stable β-tubulin−cofactor A binary complex may form allowing the storage of native or quasinative monomeric β-tubulin (pathway 5); (iii) when a native α-tubulin molecule is available, a stable $\alpha\beta$-tubulin heterodimer may form (pathway 6). Note that a crude fraction containing an activity termed cofactor B (Gao *et al*., 1993) is required for the generation of an $\alpha\beta$-tubulin heterodimer (Rommelaere *et al*., 1993).

and that it corresponds to the rate at which β-tubulin is released from β-tubulin−CCT binary complex in the presence of Mg-ATP. These facts indicate that folding or release, or both reactions, constitute the kinetic barrier in the process of β-tubulin facilitated folding. Furthermore, the fact that the yield of "monomeric" β-tubulin depends on the amount of cofactor A present in the folding reaction implies that a cofactor A-associated form of β-tubulin accumulates following its release from β-tubulin−CCT binary complex. We conclude from these observations that cofactor A does not act as a catalyst in β-tubulin folding but that it is a chaperone required for the stabilization of native or quasinative β-tubulin monomer.

Our concept of the role of cofactor A in chaperonin-mediated β-tubulin folding is summarized in Figure 9.

When a polypeptide is diluted from denaturant, it is thought to adopt a conformation in less than 1 s, termed molten globule, that contains some elements of secondary structure (Martin *et al*., 1991). This intermediate is competent for binary complex formation with a chaperonin. In the case of actins and tubulins, the formation of folding intermediates that are recognizable by CCT is slow (Melki & Cowan, 1994). Furthermore, these short-lived intermediates aggregate irreversibly into elements that cannot be recognized by CCT (Melki & Cowan, 1994). Addition of cofactor A to these short-lived β-tubulin folding intermediates does not influence the irreversible aggregation of denatured β-tubulin in folding buffer. We conclude that cofactor A is incapable of interacting with unfolded β-tubulin but that it interacts with a β-tubulin folding intermediate that is released from β-tubulin−CCT binary complex. This function of cofactor A is consistent with the observation that yeast cofactor A (Rblp2) is an effective and specific suppressor of *in vivo* toxicity associated with β-tubulin overexpression in yeast (Archer *et al*., 1995). Thus, cofactor A distinguishes between a β-tubulin folding intermediate closely related to the native state and other folding intermediates. In this view, cofactor A may interact with native or quasinative β-tubulin in a manner similar to the interaction of β-tubulin and α-tubulin chain in the $\alpha\beta$-tubulin dimer. If this is indeed the case, then the association of folded β-tubulin with cofactor A must be reversible in order to allow the association of native α- and β-tubulin chains with each other, which is the ultimate stage in the generation of a functional $\alpha\beta$-tubulin dimer.

The fact that Rbl2p is as efficient in suppressing the toxicity due to β-tubulin overexpression in yeast as α-tubulin overexpression (Archer *et al*., 1995) strongly suggests that the yeast homologue of cofactor A may act as a buffering capacity for β-tubulin in its folding pathway before assembly in the $\alpha\beta$-tubulin dimer. Knockout of *RBL2* is not lethal under normal cell growth conditions (Archer *et al*., 1995), probably because the levels of α- and β-tubulin polypeptide chains are not dramatically different. Alternatively, Rbl2p may not be the only cellular protein that stabilizes β-tubulin folding intermediates that can associate with native α-tubulin polypeptide chain to form native $\alpha\beta$-tubulin dimers.

In the presence of both cofactor A and B, native $\alpha\beta$-tubulin dimer is generated from *in vitro* folding reactions containing CCT, α- and β-tubulin target proteins, Mg-ATP, and Mg-GTP (Gao *et al*., 1993; Rommelaere *et al*., 1993). The elucidation of the role of cofactor A in β-tubulin generation and the strict requirement of a crude protein fraction, termed cofactor B, suggests that the latter protein fraction contains chaperone activity(ies) that fulfils in the folding process of α-tubulin polypeptide chain a function similar to that of cofactor A in β-tubulin folding process, i.e., an activity required for the stabilization of native or quasinative α-tubulin monomer.

ACKNOWLEDGMENT

We thank Mark Goethals and Jozef Van Damme for technical support, Gérard Batelier for running the analytical ultracentrifuge, Jean-Pierre Le Caer for his help in mass spectrometry measurements, Prof. M. Rosseneu for use of the CD photospectrometer, Prof. M. Van Montagu for use of the GCG software, Drs. Marie-France Carlier, Dominique Pantaloni, and Paul Walden for helpful discussions, and Prof. Robley C. Williams, Jr., for critical reading of the manuscript.

We acknowledge Prof. N. Cowan for an initial gift of bovine and truncated murine cofactor A.

NOTE ADDED IN PROOF

After the completion of the manuscript, we found a partial human cDNA sequence that is 82% identical to murine cofactor A in the EMBL/GenBank/DDBJ databases under accession number Z30197. Translation of this cDNA yields the following peptide: ^{31}KPPEEKIEKMRAEDGENY-DIKKQAEILQESRMMIPDCQRRLEAAYLD-LQRILENEKDLEEAEEYKEARLVLDSVKLEA^{108}, which corresponds to the C-terminal part of human cofactor A. Human cofactor A is thus 88%, 86%, 90%, and 75% identical to murine, bovine, rabbit, and chicken cofactor A, respectively. Non-conservative amino acid substitutions are localized at positions 32, 33, 49, 76, 80, and 84 of murine cofactor A.

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BI960788R