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Facilitated Folding of Actins and Tubulins Occurs via a Nucleotide-Dependent Interaction between Cytoplasmic Chaperonin and Distinctive Folding Intermediates

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In the cytoplasm of eukaryotes, the folding of actins and tubulins is facilitated via interaction with a heteromeric toroidal complex (cytoplasmic chaperonin). The folding reaction consists of the formation of a binary complex between the unfolded target protein and the chaperonin, followed by the ultimate release of the native polypeptide in an ATP-dependent reaction. Here we show that the mitochondrial chaperonin (cpn60) and the cytoplasmic chaperonin both recognize a range of target proteins with different relative affinities; however, the cytoplasmic chaperonin shows the highest affinity for intermediates derived from unfolded tubulins and actins. These high-affinity actin and tubulin folding intermediates are distinct from the "molten globule" intermediates formed by noncytoskeletal target proteins in that they form relatively slowly. We show that the interaction between cytoplasmic chaperonin and unfolded target proteins depends on the chaperonin being in its ADP-bound state and that the release of the target protein occurs after a transition of the chaperonin to the ATP-bound state. Our data suggest a model in which ATP hydrolysis acts as a switch between conformational forms of the cytoplasmic chaperonin that interact either strongly or weakly with unfolded substrates.

The biological function of most, if not all, proteins depends critically upon their three-dimensional structure. Although the information contained in the linear sequence of amino acids is thought to be sufficient to specify this structure (2, 24), the correct folding of many proteins is not a spontaneous process under physiological conditions; rather, there is a requirement for interaction with a class of proteins or protein complexes known as molecular chaperones (reviewed in references 14, 18, and 39). In particular, one class of chaperones (typified by GroEL in prokaryotes) consist of multisubunit toroidal ring assemblies and are believed to function by providing a sequestered environment in which aberrant folding and aggregation are prevented; the correctly folded polypeptide is ultimately discharged in an Mg-ATP-dependent reaction. These toroidal structures are termed chaperonins (6, 10, 19, 28, 34, 35, 46).

Actin and tubulin are the major soluble cytoplasmic proteins in eukaryotic cells and are the subunits from which actin filaments and microtubules are assembled. The folding of actins, tubulins, and actin- and tubulin-related proteins is facilitated by a chaperonin (15–17, 31, 49) that differs from its prokaryotic and mitochondrial homologs in that it is heteromeric: the multisubunit toroidal structure is assembled from eight different (though related) polypeptides (15, 38), one of which is the t-complex polypeptide TCP-1 (27, 42). As is the case for other chaperonins, the cytoplasmic chaperonin forms a stable binary complex with unfolded target polypeptides in the absence of Mg-ATP, and the hydrolysis of Mg-ATP is absolutely required for the generation of folded proteins (15–17). However, an important difference exists between the mechanisms of actin and α- and β-tubulin folding: actin is discharged from the chaperonin as a native polypeptide in the presence of Mg-ATP alone (16), while the generation of native α- or β-tubulin requires the presence of two additional protein cofactors as well as Mg-GTP (17, 38).

The mechanisms by which chaperonins recognize their target proteins and facilitate their folding are essentially unknown. In the case of GroEL, there is evidence that the chaperonin is involved in the facilitated folding of a very wide range of *Escherichia coli* target proteins, accounting for about half of all the proteins in the cell (22, 45); in many cases, the release of correctly folded protein occurs in conjunction with the cochaperonin GroES (19, 32), which functions at least in part by modulating the rate of hydrolysis of ATP (7, 20, 44).

We examined the events that lead to binary complex formation between cytoplasmic chaperonin and potential target proteins. We show that the chaperonin recognizes intermediates derived from unfolded α- and β-tubulin with a higher affinity than corresponding intermediates derived from a number of unfolded noncytoskeletal target proteins. We present evidence that the cytoplasmic chaperonin in its ADP-bound state recognizes and binds target proteins and that release of the folded polypeptide is controlled by the relatively low affinity of target protein for chaperonin in its ATP-bound state. Our data suggest a model in which ATP hydrolysis acts as a switch between conformational forms of the cytoplasmic chaperonin that interact either strongly or weakly with potential target proteins.

MATERIALS AND METHODS

Purification of chaperonins and in vitro folding assays. Cytoplasmic chaperonin was purified from bovine testis by the method of Frydman et al. (15), with the following modifications: 0.4 to 0.5 kg of testis tissue was macerated in a Waring blender in 0.8 volume of buffer A and centrifuged, and the remaining cell debris was removed first by treatment with cell debris remover (Whatman Inc.) as recommended by the manufacturer and then by filtration through a 0.4-μm Millipak

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filtration unit (Millipore, Inc.). The filtrate, which contained about 8 g of protein, was applied to a 400-ml column of Q-Sepharose HR (Pharmacia, Inc.). Gradient elution, determination of protein concentration, sucrose gradient fractionation, and affinity chromatography on an agarose-bound ATP column were all performed as described by Frydman et al. (15). Protein (10 to 20 mg) eluting from the affinity column with Mg-ATP was applied to a column (2.5 by 100 cm) of Superose 6 (Pharmacia, Inc.) equilibrated and run in 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.9]–1 mM $MgCl_2$–1 mM ethylene glycol tetraacetic acid (EGTA)–1 mM dithiothreitol (folding buffer). The symmetrical peak with an apparent molecular mass of 700 to 800 kDa that emerged was concentrated by ultrafiltration to 1 mg/ml, flash-frozen in liquid N_2, and stored at −70°C. All chaperonin preparations were tested for their biochemical homogeneity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis; each preparation was also tested for its capacity to fold labeled, denatured β-actin as described by Gao et al. (16). Folding assays and the analysis of the reaction products of these assays by native polyacrylamide gel electrophoresis were performed as described previously (16, 17).

Cytoplasmic chaperonin in its ADP-bound state was prepared by incubating the chaperonin at 4°C for 1 h in folding buffer supplemented with 2 mM ADP and then separating the chaperonin from free nucleotide by gel filtration on a column of Sephadex G25 equilibrated in folding buffer. The nucleotide content of the chaperonin thus prepared was assayed by high-pressure liquid chromatography (HPLC) after $HClO_4$ extraction as described previously (29).

GroEL and recombinant mitochondrial cpn60 were prepared as described previously (44, 46).

Preparation of unfolded target proteins. Cloned cDNAs encoding the entire coding regions for β-actin (11), actin–vertebrate actin-related protein (RPV) (26), α- (47), β- (48), and γ-tubulin (50), TCP-1 (1), cyclin B (36), cap-binding protein (40), c-Myc (37), and $p21^{ras}$ (H-Ras) (25) were expressed as labeled polypeptides in *E. coli* BL21(DE3) by using pET vectors (43), and the labeled denatured proteins were purified from insoluble inclusion bodies as described before (16, 17, 31). The radiochemical and biochemical purity of each target protein was determined by analysis on SDS-polyacrylamide gels; this allowed an accurate estimation of specific radioactivity. Unlabeled target proteins were prepared as described by Gao et al. (17).

Measurement of relative affinities. Labeled, unfolded target proteins were diluted at three different concentrations (0.1, 0.2, and 0.5 μM) in folding buffer containing a constant amount (0.1 μM) of cytoplasmic chaperonin, and the reaction products were analyzed on a nondenaturing polyacrylamide gel after incubation at 30°C for 30 min. The yield of binary complex was quantitated with a phosphorimager. The affinity of a given target protein for cytoplasmic chaperonin relative to the affinity of α-tubulin for the chaperonin was calculated by dividing the ratio of the intensity of each binary complex band (relative to that of α-tubulin) by the ratio of specific radioactivities.

Kinetic analyses. Aliquots were withdrawn from in vitro folding reaction mixes at various times after the initial dilution of target protein. Glycerol was added to 20% (final concentration), and the reactions were terminated by plunging the mixtures in a dry-ice bath. Processing of reactions in this manner allowed us to examine time intervals as short as 10 s after reaction initiation. At the end of each kinetic experiment, the frozen samples were thawed and immediately applied to a native polyacrylamide gel (16), with the voltage applied so as to minimize the opportunity for further reaction before entry into the gel.

Measurement of ATP hydrolysis. The rate of ATP hydrolysis was measured at 30°C in reaction mixes containing cytoplasmic chaperonin (0.125 μM) either on its own in folding buffer or after the addition of denatured β-actin or α- or β-tubulin. The latter was accomplished by a 100-fold dilution of target protein (25 μM) contained in 7.5 M urea. In either case, the yield of acid-labile P_i was measured as described by Melki et al. (30).

The beryllium fluoride complexes (which are structural analogs of P_i [4, 5]) were used to probe the ADP-P_i state of the cytoplasmic chaperonin essentially as described previously for similar analyses of a number of other ATP- and GTP-binding proteins (5, 9, 12, 33): chaperonin was incubated in folding buffer containing 5 mM NaF and 0.5 mM $BeSO_4$ for 20 min at 30°C before addition of unfolded target protein.

RESULTS

Kinetics of actin and tubulin binary complex formation. The association of target proteins with GroEL is thought to occur via the formation of a molten globule intermediate that forms rapidly (less than 1 s) when a target protein is suddenly diluted from the denaturant (28). This intermediate can then form a binary complex with the chaperonin in a reaction that does not depend on the presence of ATP. To determine whether cytoplasmic chaperonin recognizes a similar kind of molten globule intermediate, we monitored the kinetics of binary complex formation between cytoplasmic chaperonin (present at a fourfold molar excess) and denatured β-actin and compared the results with those for parallel reactions in which the same target protein was presented to GroEL or its mitochondrial homolog cpn60. Labeled, denatured β-actin was diluted 100-fold into a buffer containing either cytoplasmic chaperonin, GroEL, or cpn60, and the reaction products were analyzed on a nondenaturing polyacrylamide gel. In the case of cytoplasmic chaperonin, the formation of a binary complex is relatively slow, rising to a plateau with a $t_{1/2}$ of 4 min (Fig. 1A and B). In contrast, in reactions done under identical conditions with either GroEL (Fig. 1C) or cpn60 (Fig. 1D) a saturating amount of binary complex formed rapidly, so that the reaction was complete within 15 s or less; 15 s is the shortest time interval that we explored in these experiments. These data suggest either that cytoplasmic chaperonin and GroEL (or cpn60) have very different affinities for nonnative β-actin or that they recognize different kinds of folding intermediates that form upon dilution from denaturant, or both.

If the recognition of β-actin by cytoplasmic chaperonin indeed requires the generation of folding intermediates that form relatively slowly after dilution of the target protein from denaturant, we might expect the rate of β-actin/cytoplasmic chaperonin binary complex formation to be relatively enhanced when such intermediates are formed before the addition of chaperonin to the reaction mixture. The data shown in Fig. 1E show that this is indeed the case. In this experiment, denatured β-actin was diluted into buffer alone and incubated for 3 min; cytoplasmic chaperonin was then added, and the incubation was continued for various times before the reaction products were analyzed on a nondenaturing polyacrylamide gel. Under these conditions, the β-actin/cytoplasmic chaperonin binary complex forms with a $t_{1/2}$ of about 1 min (Fig. 1F). This result is consistent with the acquisition by about half of the β-actin molecules of a structure that is recognizable by cytoplasmic chaperonin within 4 min after dilution from the denaturant.

Are these relatively slow forming β-actin intermediates

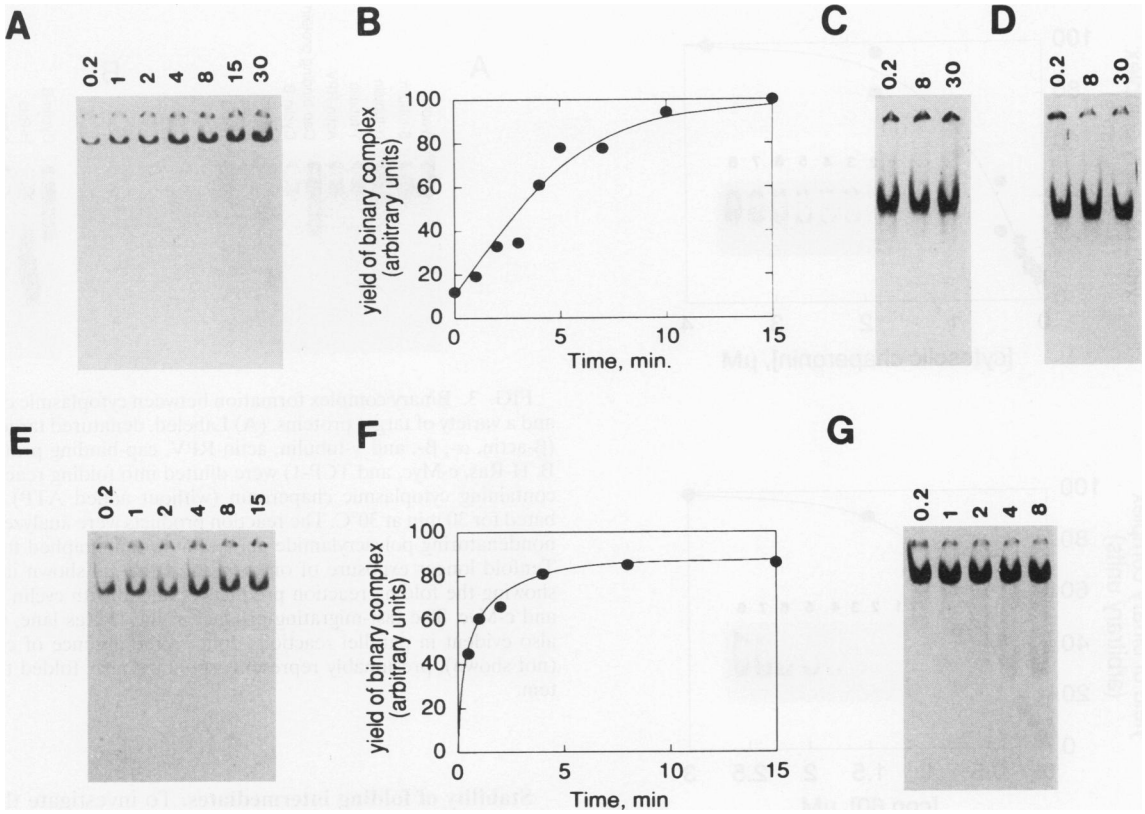

FIG. 1. Kinetics of binary complex formation between unfolded β-actin and chaperonins. (A, C, D) Labeled, unfolded β-actin was diluted into folding buffer containing either cytoplasmic chaperonin (A), GroEL (C), or the mitochondrial chaperonin cpn60 (D). In each case, the chaperonin was present at a fourfold molar excess over the target protein. The reaction mixes were incubated at 30°C. At the times shown (in minutes), aliquots were withdrawn, and the products were analyzed on a 4% nondenaturing polyacrylamide gel. (B) Quantitation of data obtained from experiments similar to that shown in panel A. (E) Labeled, unfolded β-actin was diluted into folding buffer alone and incubated for 3 min at 30°C before the addition of cytoplasmic chaperonin. The incubation was continued for the times shown (in minutes), and the reaction products were analyzed on a 4% nondenaturing gel. (F) Quantitation of the data shown in panel E. (G) Labeled, unfolded β-actin was diluted into a folding reaction mix containing a 20-fold molar excess of cytoplasmic chaperonin. Aliquots were withdrawn from the reaction at the times shown (in minutes), and the products were analyzed on a 4% nondenaturing polyacrylamide gel.

uniquely recognized by the cytoplasmic chaperonin, or can the chaperonin also recognize other, more rapidly generated intermediates, albeit with lower affinity? To address this question, we compared the rate of β-actin/chaperonin binary complex formation over a range of ratios (from equimolar to a 20-fold excess) of chaperonin to denatured target protein. In reactions done with cpn60, there is no observable change in the amount of binary complex generated beyond the shortest time point (10 s after dilution from the denaturant) irrespective of the amount of chaperonin present in the reaction mix (data not shown). In contrast, parallel reactions done with the cytoplasmic chaperonin present at up to a 10-fold molar excess display observable kinetics of binary complex formation with a $t_{1/2}$ of 4 min, similar to the curve shown in Fig. 1B; at higher chaperonin concentrations, however, the rate of binary complex formation becomes very fast, indistinguishable from that in reactions done with cpn60 under any conditions (Fig. 1G).

We also explored the minimum concentration of cytoplasmic chaperonin and cpn60 that is capable of forming a binary complex with β-actin diluted from the denaturant. The results of this experiment show that cytoplasmic chaperonin and cpn60 have similar overall affinities for the same target protein (Fig. 2). We conclude that the cytoplasmic chaperonin recognizes slowly generated actin and tubulin folding intermediates with a higher relative affinity than molten globule intermediates which form within seconds of dilution from the denaturant, while cpn60 appears to have a relatively high affinity for both these kinds of folding intermediate.

Binary complex formation with noncytoskeletal target proteins. We found that a number of cytoskeletal proteins (actin-RPV and α-, β-, and γ-tubulins) behaved like β-actin in kinetic reactions done with cytoplasmic chaperonin (data not shown). To extend these experiments, we tested the ability of cytoplasmic chaperonin to interact with five arbitrarily chosen, labeled, denatured target proteins of noncytoskeletal origin: cyclin B, H-Ras, c-Myc, TCP-1, and cap-binding protein. Although all of these target proteins are capable of forming a binary complex with biochemically homogeneous chaperonin, they do so with low efficiency compared with actins or tubulins (Fig. 3). Moreover, when we examined the kinetics of binary complex formation between a fourfold molar excess of cytoplasmic chaperonin and any of the noncytoskeletal target proteins, we found that, in contrast to parallel experiments done with β-actin or α- or β-tubulin probes (Fig. 1A and B) or, indeed, with actin- or tubulin-like proteins such as actin-RPV and γ-tubulin, binary complex formation was very fast, occurring in a manner indistinguishable from that in similar reactions done with cpn60 in place of cytoplasmic chaperonin. These data

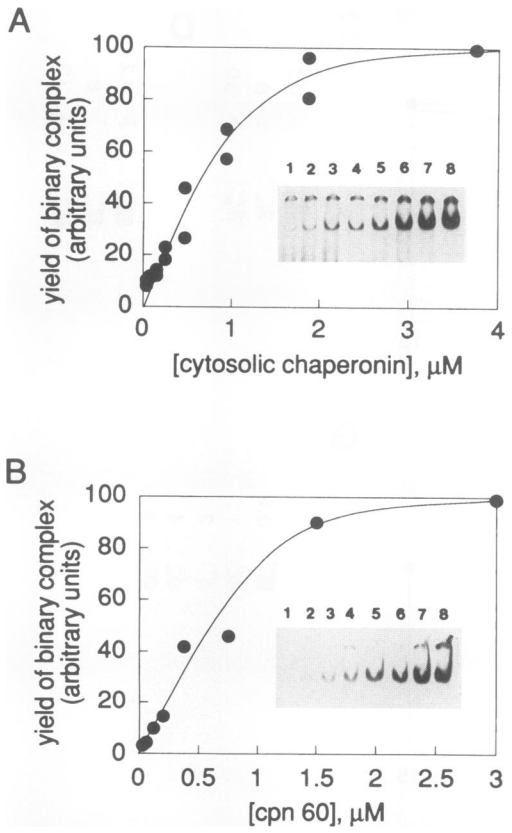

FIG. 2. Relative affinity of unfolded β-actin for cytoplasmic chaperonin and mitochondrial chaperonin. Labeled, unfolded β-actin was diluted into a folding reaction mix (without added ATP) containing different amounts of either cytoplasmic chaperonin (A) or the mitochondrial chaperonin cpn60 (B). The reaction mixes were incubated at 30°C for 30 min, and the reaction products were analyzed on a 4% nondenaturing polyacrylamide gel. Quantitation of the data presented in the insets is shown in the graphs. Tracks 1 to 8 show reactions done with 0.03, 0.07, 0.15, 0.25, 0.47, 0.94, 1.88, and 3.75 μM cytoplasmic chaperonin (A) and 0.03, 0.06, 0.12, 0.20, 0.38, 0.75, 1.50, and 3.00 μM mitochondrial cpn60 (B).

raise the possibility that there is a significant difference between actin and tubulin folding intermediates and the folding intermediates formed by the noncytoskeletal proteins used in our experiments.

In view of this unexpected difference between the behavior of actins or tubulins on the one hand and certain noncytoskeletal target proteins on the other, we measured the relative affinities of all of these target proteins for association with either cpn60 or cytoplasmic chaperonin. To do this, 5 to 20 pmol of each of several different labeled, denatured target proteins of known specific radioactivity were diluted into reaction mixes containing 2.5 pmol of either cpn60 or cytoplasmic chaperonin, and the relative yields of binary complex formed were quantitated after resolution of the reaction products on a nondenaturing gel. The results of these experiments show that in the case of cpn60, the target proteins tested fall within a range of relative affinities that differ by no more than a factor of about 8 (Table 1). In contrast, the relative affinities of the target proteins for cytoplasmic chaperonin cover a much wider range: tubulins and actins interact most strongly, while the noncytoskeletal proteins c-Myc and H-Ras have about a 35-fold-lower relative affinity for the cytoplasmic chaperonin (Table 1).

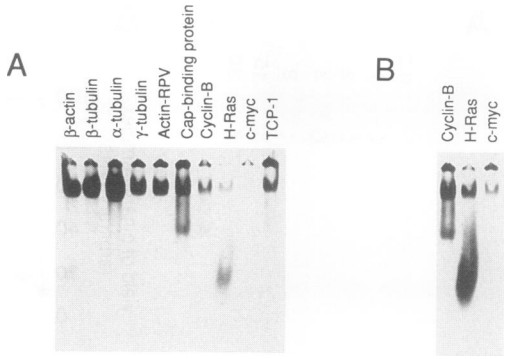

FIG. 3. Binary complex formation between cytoplasmic chaperonin and a variety of target proteins. (A) Labeled, denatured target proteins (β-actin, α-, β-, and γ-tubulin, actin-RPV, cap-binding protein, cyclin B, H-Ras, c-Myc, and TCP-1) were diluted into folding reaction mixes containing cytoplasmic chaperonin (without added ATP) and incubated for 30 min at 30°C. The reaction products were analyzed on a 4% nondenaturing polyacrylamide gel and autoradiographed for 2 h. (B) Tenfold longer exposure of one region of the gel shown in panel A, showing the folding reaction products obtained with cyclin B, H-Ras, and c-Myc. The fast-migrating product in the H-Ras lane, which was also evident in parallel reactions done in the absence of chaperonin (not shown), presumably represents spontaneously folded target protein.

Stability of folding intermediates. To investigate the generation and stability of actin and tubulin intermediates that can be recognized by chaperonins, we diluted β-actin from the denaturant into buffer alone and added either GroEL, mitochondrial cpn60, or cytoplasmic chaperonin at different times after the initial dilution. Aliquots withdrawn from the dilution reaction mix were analyzed on a nondenaturing polyacrylamide gel (Fig. 4). The data show that at short times (i.e., up to about 5 min), the majority of the target molecules are captured by chaperonin to form a binary complex. However, with progressively longer times of incubation in buffer alone, the amount of target protein that is capable of interaction with chaperonin declines, with a concomitant increase in the amount of large aggregates that remain at the origin of the nondenaturing gel. Indeed, after about 1 h of incubation of unfolded β-actin in buffer alone, no material remains that is capable of capture by chaperonin. This pattern is observed

TABLE 1. Relative affinities of a range of unfolded target proteins for mitochondrial cpn60 and cytoplasmic chaperonin

Target protein	Relative affinity[a]	
	cpn60	Cytoplasmic chaperonin
α-Tubulin	1.00	1.00
β-Tubulin	1.00	0.67
γ-Tubulin	0.67	0.40
β-Actin	0.67	0.30
Actin-RPV	0.25	0.33
Cap-binding protein	0.33	0.10
Cyclin B	0.13	0.05
H-Ras	0.25	0.05
c-Myc	0.20	0.03
TCP-1	0.40	0.15

[a] Relative to the affinity of α-tubulin.

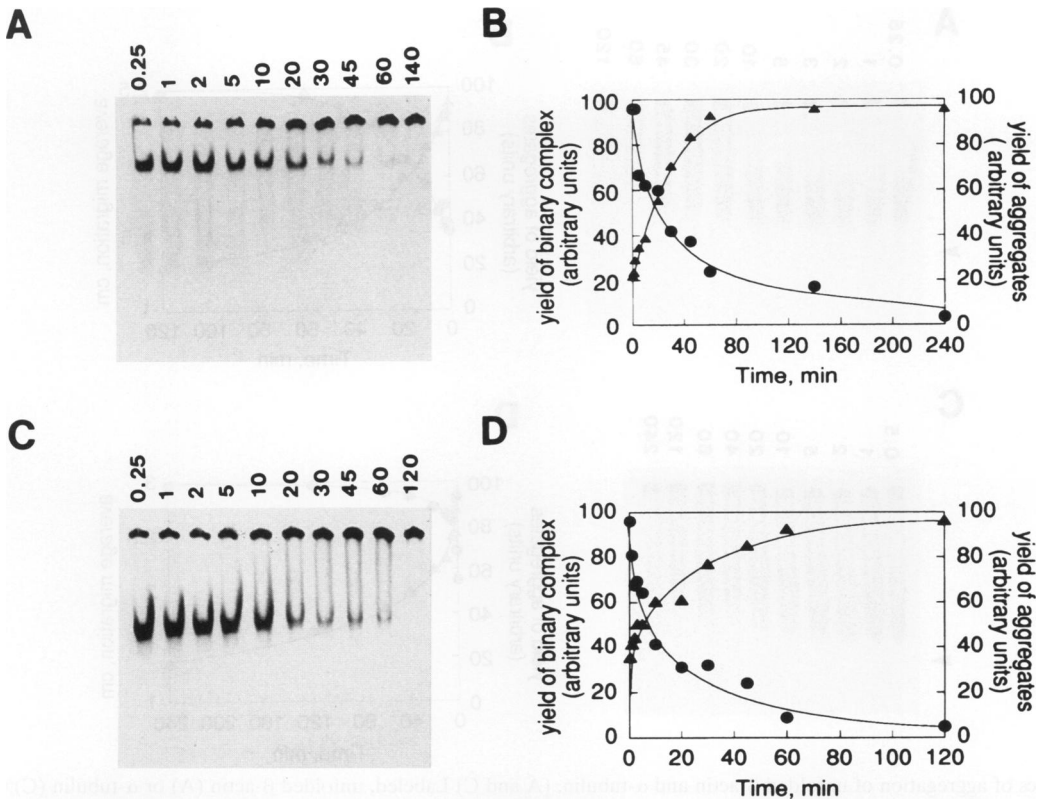

FIG. 4. Stability of β-actin folding intermediates. (A and C) Labeled, denatured β-actin was diluted into folding buffer and incubated at 30°C for the times shown (in minutes). Cytoplasmic chaperonin (A) or mitochondrial cpn60 (C) was added, the incubation was continued for another 10 min, and the reaction products were analyzed on a 4% nondenaturing polyacrylamide gel. (B and D) Quantitation of data averaged from several experiments such as those shown in panels A and C, respectively. ●, yield of chaperonin/β-actin binary complex; ▲, yield of aggregated β-actin at the origin of the polyacrylamide gel.

irrespective of the chaperonin (cytoplasmic chaperonin, GroEL, or mitochondrial cpn60) used in the experiment.

To determine whether the aggregates generated in these experiments were capable of dissociation into species that could be recognized by chaperonins, we isolated high-molecular-mass aggregates from a reaction mix in which denatured β-actin was diluted into buffer and incubated for 4 h. This material was then incubated with chaperonin, and the products were analyzed on a nondenaturing gel. Only a trace of the input radioactivity was found complexed with chaperonin (data not shown). From these data, we conclude that dilution of β-actin from the denaturant results in the formation of short-lived species that are recognizable by chaperonin and that subsequently generated aggregates are not in equilibrium with these species.

To further characterize the species that can interact with chaperonin, we monitored the kinetics of β-actin and α-tubulin aggregation upon dilution from the denaturant under conditions that were identical to those used in our folding assays (Fig. 5). At early times (from 15 s to 2 min), the bulk of the radioactivity migrates as a broad band with a slightly slower mobility than monomeric native actin or tubulin dimers run under identical conditions. After about 3 min, however, there is a progressive decline in the mobility of the radioactive species, with a corresponding increase in the amount of material trapped at the origin of the gel. Indeed, after 2 h, virtually all of the input radioactivity is found at the origin. This behavior is in sharp contrast to that of all the noncytoskeletal target proteins: under the same assay conditions, no significant aggregation was observed, nor was there any significant decline in the yield of binary complex formed after dilution into buffer alone and incubation at 30°C for different times before the addition of chaperonin. We conclude that under the conditions used in our folding assays, actin and tubulin species differ from other potential target proteins in that they rapidly and irreversibly aggregate into elements that cannot be recognized by chaperonin.

Role of ATP hydrolysis in facilitated folding by cytoplasmic chaperonin. To investigate the role of ATP hydrolysis in the facilitated folding of β-actin and α- and β-tubulin, we measured the rates of ATP hydrolysis of chaperonin both alone and when associated with β-actin or α- or β-tubulin target proteins. Cytoplasmic chaperonin on its own hydrolyzes ATP at a linear rate of 1.75 to 2.00 min^{-1} (Fig. 6). This rate is unaffected by the addition of at least 140 mM urea, twice the concentration of urea introduced as a consequence of the addition of unfolded target proteins in our folding reactions. Under conditions of target protein and ATP excess, when β-actin or α- or β-tubulin is in the process of being discharged from the cytoplasmic chaperonin, there is a fourfold increase in the ATP hydrolysis rate relative to the steady-state rate. After the initial 30 to 60 min, the rate of ATP hydrolysis reverts to a linear rate comparable to the steady-state hydrolysis rate of cytoplasmic chaperonin on its own. The decline in the rate of ATP hydrolysis after the initial burst is due to exhaustion of competent substrate, since adding more denatured target

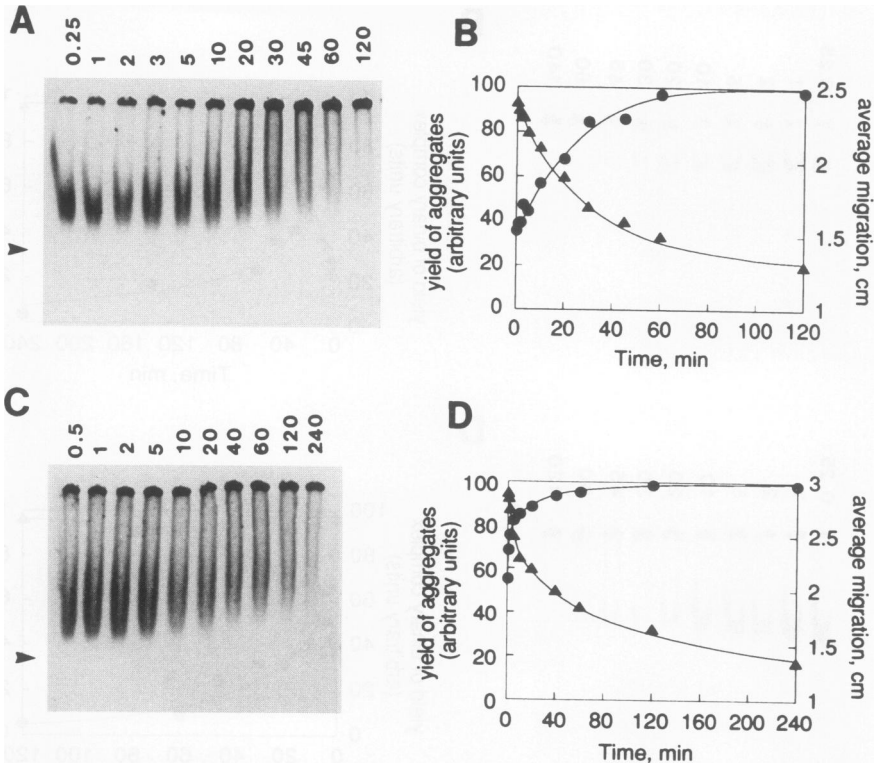

FIG. 5. Kinetics of aggregation of unfolded β-actin and α-tubulin. (A and C) Labeled, unfolded β-actin (A) or α-tubulin (C) was diluted into folding buffer and incubated at 30°C for the times shown (in minutes), and the reaction products were analyzed on a 4% nondenaturing polyacrylamide gel. Arrowheads indicate the location of native monomeric brain β-actin (A) and dimeric brain tubulin (C) run on the same gel and detected by staining with Coomassie blue. (B and D) Quantitation of data from experiments such as those shown in panels A and C, respectively. ▲, distance from the origin of the gel of the mid-point of the radioactive species that entered the nondenaturing gel; ●, yield of high-molecular-mass aggregates at the origin of the gel.

protein to the reaction mix results in a second burst of ATP hydrolysis with the same initial slope as that observed after the initial dilution reaction (Fig. 6). We conclude that the hydrolysis of ATP is coupled to the folding and release of chaperonin-bound target proteins.

In the experiments described above, ATP hydrolysis is occurring concomitant with two other reactions: the binding to cytoplasmic chaperonin of unfolded target proteins and their discharge as folded polypeptides. To determine the form of cytoplasmic chaperonin which captures target proteins, we measured the yield of β-actin binary complex and native β-actin generated in folding reaction mixes containing cytoplasmic chaperonin in its ADP-bound form and different ratios of ATP and ADP. In these experiments, free nucleotide was removed from the chaperonin by gel filtration immediately before its use in the dilution reactions. Analysis of nucleotide extracted from this material by HPLC showed that it contained essentially only ADP (data not shown). In the presence of ATP alone, about 50% of the input radioactivity is converted to native monomeric β-actin (Fig. 7A and B). With increasing ADP/ATP ratios, however, there is a progressive decline in the yield of native product, with a corresponding increase in the amount of radioactivity contained in the binary complex; at an ADP/ATP ratio of 3:1, no discernable native β-actin is released and virtually all of the input radioactivity is contained in the binary complex (Fig. 7C). Thus, in the presence of a large molar excess of ADP, the chaperonin efficiently forms a binary complex with the target protein but is unable to discharge it even in the presence of a substantial molar excess of ATP.

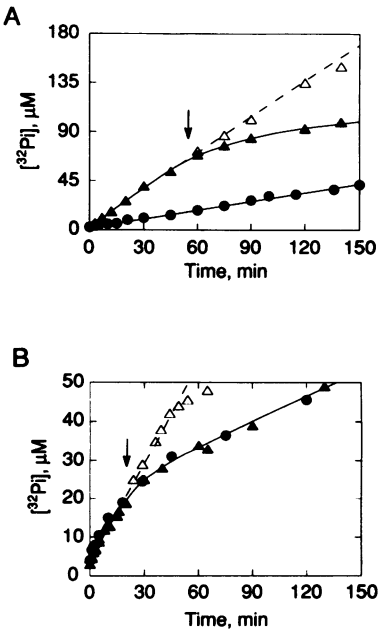

FIG. 6. Kinetics of ATP hydrolysis by cytoplasmic chaperonin in the presence and absence of target proteins. (A and B) Kinetics of γ-phosphate cleavage by cytoplasmic chaperonin alone (●), or in the presence of unfolded β-actin (▲; A) or unfolded α- or β-tubulin (● and ▲, respectively; B). Arrows show the time of readdition of target protein to part of the reaction mixture; △, data points for samples taken after this readdition.

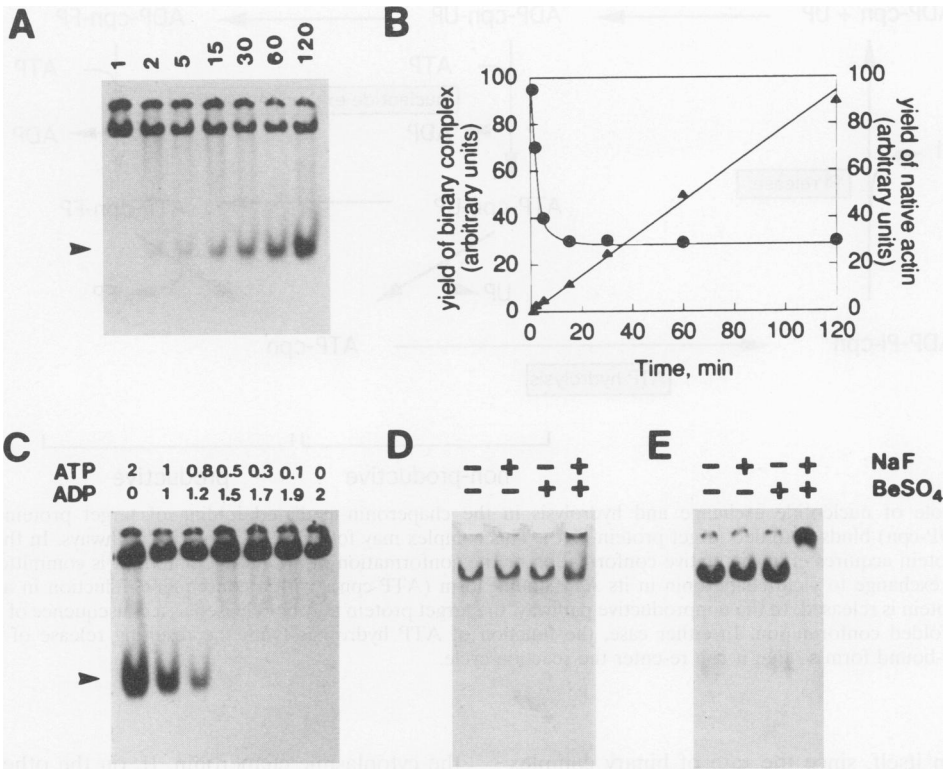

FIG. 7. Chaperonin-bound nucleotide and its influence on the capacity of chaperonin to recognize unfolded target protein. (A) Labeled, unfolded β-actin was diluted in a folding reaction mix containing cytoplasmic chaperonin in its ADP-bound form (see Materials and Methods) and incubated at 30°C for 10 min to allow binary complex formation. ATP was then added to 1 mM, the incubation was continued for the times (in minutes) shown, and the reaction products were analyzed on a 4% nondenaturing polyacrylamide gel. Arrowheads in panels A and C show the locations of native monomeric brain actin run on the same gels and detected by staining with Coomassie blue. (B) Quantitation of the data shown in panel A. ●, yield of β-actin/chaperonin binary complex; ▲, yield of native β-actin. (C) β-Actin folding reactions done with cytoplasmic chaperonin in the presence of different concentrations (millimolar) of ATP and ADP, as shown. (D and E) β-Actin folding reactions done in the presence of cytoplasmic chaperonin with and without preincubation with either NaF or $BeSO_4$ (see Materials and Methods). Reaction products were analyzed on a 4% nondenaturing polyacrylamide gel. (D) Reaction products visualized by Coomassie blue staining; (E) autoradiograph of the same gel shown in panel D.

These data imply that cytoplasmic chaperonin has a higher affinity for target protein when it is in an ADP-bound form. Indeed, when β-actin is complexed with cytoplasmic chaperonin in its ADP-bound form, it cannot be displaced by incubation with mitochondrial cpn60 (data not shown), even though the latter chaperonin can form a tight binary complex with free unfolded β-actin (see Fig. 1D). Furthermore, when cytoplasmic chaperonin is converted to an ADP-P_i form by incubation with a structural analog of P_i (i.e., beryllium fluoride complexes [BeF_3] [12]), no detectable binary complex is formed (Fig. 7D and E). This failure to observe the formation of binary complex is not a consequence of denaturation of the chaperonin by BeF_3, since most of the BeF_3-treated chaperonin comigrates with untreated chaperonin as a discrete sharp band on a nondenaturing gel. We conclude that the chaperonin recognizes target protein only in its ADP-bound form, that the binding of target protein to ADP-bound cytoplasmic chaperonin is essentially irreversible, and that ATP exchange and hydrolysis are obligatory steps for the generation of native polypeptides.

DISCUSSION

The two major classes of soluble protein in the eukaryotic cytosol, actins and tubulins, both undergo facilitated folding in vitro via interaction with cytoplasmic chaperonin. The chaperonin-bound proteins are released in forms that are destined to assume their native state by incubation with Mg-ATP and/or Mg-GTP (15–17). Here we show that five randomly chosen noncytoskeletal proteins also form binary complexes with cytoplasmic chaperonin when presented as denatured targets, although they bind to the chaperonin with significantly lower relative affinities than tubulins and actins (Table 1). To examine the nature of chaperonin-target protein interactions and the role of ATP hydrolysis in the generation of folded molecules, we compared the kinetics of binary complex formation using different target proteins and either the bacterial, mitochondrial, or cytoplasmic chaperonin. We also determined the form of cytoplasmic chaperonin that interacts with unfolded target proteins as well as the rate of ATP hydrolysis in the presence and absence of substrate proteins.

Our data demonstrate a significant difference in the mechanism by which GroEL (or its mitochondrial homolog cpn60) and cytoplasmic chaperonin interact with unfolded target proteins. In the case of GroEL and cpn60, the formation of a chaperonin-target protein binary complex is in all cases very rapid, occurring in less than about 15 s. In contrast, in reaction mixes containing the same concentration of cytoplasmic chaperonin, the formation of a binary complex with unfolded actins or tubulins is relatively slow (Fig. 1). This behavior is a function

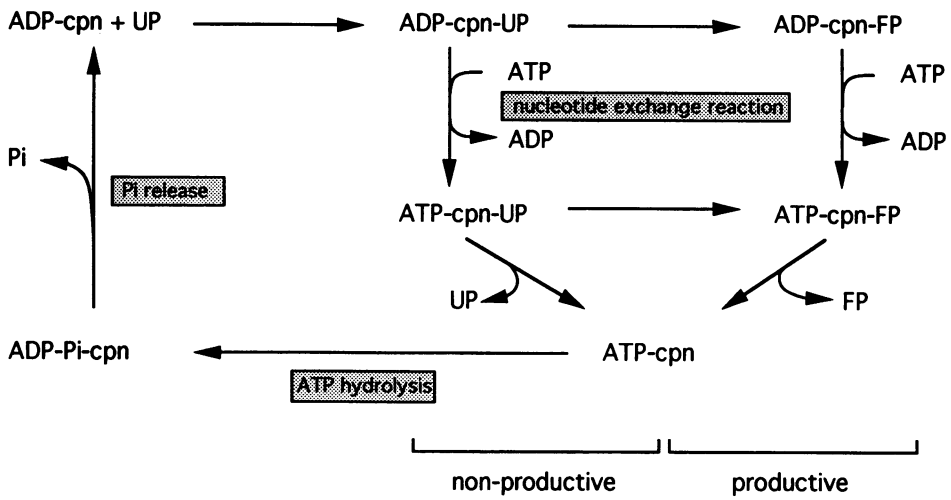

FIG. 8. Proposed role of nucleotide exchange and hydrolysis in the chaperonin-mediated folding of target protein. Chaperonin in its ADP-bound form (ADP-cpn) binds unfolded target protein (UP). This complex may follow two alternative pathways. In the productive folding pathway, the target protein acquires either its native conformation or the conformation of an intermediate that is committed to correct folding (FP); after nucleotide exchange to yield chaperonin in its ATP-bound form (ATP-cpn) (with a consequent reduction in affinity for the target protein), the folded protein is released. In the nonproductive pathway, the target protein may be released as a consequence of nucleotide exchange without acquiring its folded conformation. In either case, the function of ATP hydrolysis (with the resulting release of P_i) is to regenerate chaperonin in its ADP-bound form so that it can re-enter the reaction cycle.

of the target protein itself, since the rate of binary complex formation in reaction mixes in which a variety of noncytoskeletal target proteins are presented to cytoplasmic chaperonin is indistinguishable from the rate of binary complex formation in parallel reactions done with cpn60. Moreover, cytoplasmic chaperonin recognizes unfolded actin, tubulin, and actin- and tubulin-like proteins with a much higher affinity than it does several noncytoskeletal proteins, while a much less dramatic distinction was detected in experiments with the same target proteins and cpn60 (Table 1). Finally, under the experimental conditions used in our in vitro folding assays, actins and tubulins aggregate irreversibly upon dilution from the denaturant into species that are no longer recognizable by chaperonins, while none of the noncytoskeletal target proteins that we assayed aggregate to any significant extent. These data imply that, upon dilution from the denaturant, actins and tubulins slowly and spontaneously form folding intermediates that have some common structural characteristics that favor interaction with the cytoplasmic chaperonin. Although the nature of these characteristics is uncertain, it seems likely that they include a high proportion of exposed hydrophobic residues that can interact with the chaperonin surface. The exposure of a high proportion of hydrophobic regions is consistent with the pronounced tendency of unfolded actins and tubulins to aggregate irreversibly (Fig. 4). Such exposed hydrophobic surfaces may persist to some extent in properly folded actins and α- and β-tubulin and reflect their ability to coassemble into long polymeric structures. X-ray diffraction analysis suggests that intersubunit interactions between neighboring actin monomers in the actin filament depend essentially on hydrophobic interactions (21).

The biological significance of the cytoplasmic chaperonin's high relative affinity for intermediates derived from unfolded actin, tubulin, or actin- and tubulin-like proteins is unclear. If a given tissue contains an excess of cytoplasmic chaperonin compared with the concentration of folding intermediates generated by de novo protein synthesis, it is conceivable that most, if not all, newly synthesized proteins may interact with the cytoplasmic chaperonin. If, on the other hand, there is a substoichiometric amount of chaperonin, this would imply that actins and tubulins interact preferentially. A careful analysis of the intracellular concentrations of chaperonin and folding intermediates will be required to resolve this issue.

The hydrolysis of ATP seems to be an integral part of the mechanism by which chaperonins facilitate folding. Our data show that the cytoplasmic chaperonin on its own hydrolyzes ATP at a linear steady-state rate of 1.75 to 2.00 min^{-1}. This rate increases substantially upon presentation of unfolded target protein (Fig. 6); in the case of β-actin, the increase is fourfold and remains linear for at least 1 h under our experimental conditions. In the case of α- or β-tubulin, the initial increase is similar to that with β-actin but declines after 30 min because of exhaustion of available substrate target molecules (Fig. 6). Our value for the steady-state rate of ATP hydrolysis by the cytoplasmic chaperonin differs significantly from that reported by Frydman et al. (15), possibly because of our introduction of an additional purification step (see Materials and Methods), differences in experimental conditions, or both.

We used two approaches to dissect the role of ATP hydrolysis in the chaperonin-mediated folding reaction and to determine which nucleotide-bound form of the cytoplasmic chaperonin binds unfolded target protein. First, we prepared cytoplasmic chaperonin in its ADP-bound form and showed that it was fully competent for binary complex formation. Second, we prepared cytoplasmic chaperonin in an ADP-P_i form by using beryllium fluoride complexes as a structural analog of P_i (4, 5); this form of chaperonin, which mimicks the transitional (ADP-P_i) state that is equivalent to the ATP-bound form, completely failed to bind unfolded target protein (Fig. 7). These experiments show that target proteins bind strongly to chaperonin only when the latter is in the ADP-bound state. Our data support the notion that the role of ATP hydrolysis is to act as a switch between two conformational states (3, 23): one (the ADP-bound form) has the ability to bind unfolded target proteins with high affinity (i.e., less than 0.1 μM), while the other (the ATP-bound form) cannot.

Structural differences between chaperonin forms have already been noted from electron microscopic analyses (16, 41).

Our concept of the role of ATP hydrolysis in the chaperonin-mediated folding reaction is summarized in Fig. 8. This scheme, which parallels the mechanism of actin-myosin interaction (13) and of G-protein interactions (8), implies that exchange of ATP for ADP results in a decline in the chaperonin's affinity for the bound protein. As a consequence, multiple cycles that alternate between weak and strong interactions between the target protein and the chaperonin may be required for the ultimate generation of the correctly folded product. Spontaneous folding might then occur during the phase of the cycle when interaction with the chaperonin is weak.

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