Quasi-native Chaperonin-bound Intermediates in Facilitated Protein Folding*

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Chaperonins are known to facilitate protein folding, but their mechanism of action is not well understood. The fact that target proteins are released from and rebind to different chaperonin molecules ("cycling") during a folding reaction suggests that chaperonin function by unfolding aberrantly folded molecules, allowing them multiple opportunities to reach the native state in bulk solution. Here we show that the cycling of α-tubulin by cytosolic chaperonin (c-cpn) can be uncoupled from the action of cofactors required to complete the folding reaction. This results in the accumulation of folding intermediates which are chaperonin-bound, stable, and quasi-native in that they bind GTP non-exchangeably. We present evidence that these intermediates can be generated without the target protein leaving c-cpn. These data show that, in contrast to prevailing models, target proteins can maintain, and possibly acquire, significant native-like structure while chaperonin-bound.

The final stage in the flow of genetic information from DNA to expressed proteins is the folding of each protein into the three-dimensional structure that specifies its biological activity. In principle, such folding reactions can occur spontaneously, since all the necessary information required to determine the final folded structure is contained within the primary sequence of amino acids. However, under physiological conditions, constraints of temperature and the tendency of unfolded proteins to aggregate are such that many proteins must undergo facilitated folding via interaction with protein complexes termed chaperonins (1–10). These protein complexes take the form of toroidal structures that facilitate protein folding in an ATP-dependent manner. For example, the prokaryotic chaperonin GroEL facilitates the folding of a range of proteins in E. coli (6, 27), often in conjunction with the cochaperonin GroES (1, 4). There is evidence that GroES, which is itself a heptameric ring (12), functions at least in part by interacting with the ends of the GroEL cylinder, such that it modulates and coordinates the hydrolysis of ATP by GroEL (11, 13–15).

Many models depict facilitated folding occurring within the central cavity that is present in all chaperonins, thereby protecting the target protein from interaction with other proteins in the general milieu; release then occurs following acquisition of the native structure (3, 8, 9, 10, 16). Recently, however, this concept has been challenged by evidence that target proteins can jump between different chaperonin molecules during a folding reaction ("cycling") (11, 17). Thus, the function of chaperonins might be to unfold and release proteins that have misfolded. In this view, protein folding occurs spontaneously in solution, while the function of the chaperonin is to "recycle" aberrantly folded molecules so that they can return to a potentially productive pathway. Accordingly, a given target molecule might require multiple rounds of interaction with different chaperonin molecules before partitioning to the native state.

To understand the mechanism of chaperonin-mediated folding, it is essential to know the states of folding intermediates during the cycling process. However, such intermediates are usually difficult to study because of their heterogeneity, transient existence, and pronounced tendency to aggregate. To examine the states of folding intermediates produced during chaperonin-mediated folding, we took advantage of the observation that the facilitated folding of α-tubulin by cytosolic chaperonin (c-cpn) cannot proceed to the native state in the absence of specific protein cofactors (18, 19). This system allowed us to establish the existence of a novel class of chaperonin-bound quasi-folded intermediates that are generated during ATP-dependent facilitated folding.

EXPERIMENTAL PROCEDURES

Materials—[^35S]Methionine and α-[32P]GTP were from DuPont NEN. c-cpn, mt-cpn, and cofactors required for the productive folding of α-tubulin were purified as described previously (18, 20). Hexokinase was from Boehringer Mannheim.

Folding Reactions—α-tubulin folding reactions were done at 30 °C in 20 μl of folding buffer (22) containing c-cpn (3 pmol), 1 mM each of ATP and GTP, native calf brain tubulin (0.2 mg/ml), and cofactors required for the productive folding of α-tubulin (18). In some experiments, ATP was quenched by the addition of glucose (to 10 mM) and hexokinase (2.5 units). The yield of various products identified on fixed, dried nondenaturing polyacrylamide gels run as described (18, 20) was quantitated using a PhosphorImager.

Sucrose Gradients—Sucrose gradients were prepared in thick-walled 1-ml Beckman polycarbonate ultracentrifuge tubes by overlaying successive layers (each of 0.13 ml) of 0.1% in 20 mM Tris-HCl, 0.6 mg/ml native calf brain tubulin (0.2 mg/ml, respectively, and the mixture was fractionated on a 1-ml Mono Q anion exchange column as described (24). The radioactive peak containing tubulin heterodimers and eluting at 0.62 M NaCl was concentrated using a microcon 30 ultrafiltration device. Native tubulin generated in vitro translation or target protein-c-cpn binary com-

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1 The abbreviations used are: c-cpn, cytosolic chaperonin; mt-cpn, mitochondrial chaperonin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
accumulated as a result of incubation with ATP and GTP. The action of cofactors act accumulate slowly during ATP exchange and hydrolysis. α-Tubulin-c-cpn binary complexes were incubated at 30°C for the times shown in the presence of ATP and GTP, and the amount of native tubulin quantitated to 100%. c, quantitative analysis of the data shown in b (averaged from four experiments and with the plateau level of native tubulin set at 100%). d, cofactors act rapidly on intermediates accumulated as a result of incubation with ATP and GTP. α-Tubulin-c-cpn binary complexes were incubated in the presence of ATP and GTP for 45 min at 30°C. The reaction was quenched with hexokinase and glucose, supplemented with cofactors and carrier native tubulin, and aliquots were withdrawn from the reaction at the times shown, e, half-life of accumulated α-tubulin folding intermediates. α-Tubulin folding reactions were incubated for 45 min at 30°C in the presence of ATP and GTP. The reactions were quenched with hexokinase, and the incubation continued at 30°C. At the times shown, aliquots were withdrawn, supplemented with cofactors and carrier native tubulin, and incubated for an additional 2 min. f, semi-log plot of the data shown in e (averaged from four experiments and with the initial yield of native tubulin set at 100%); arrow shows t1/2. Upper and lower arrows in a, b, d, and e show the location of the α-tubulin-c-cpn binary complex and native tubulin, respectively.

RESULTS AND DISCUSSION

The facilitated folding of β-actin requires ATP-dependent interaction with c-cpn, the eukaryotic cytosolic homolog of GroEL (20). In contrast, the facilitated folding of α- and β-tubulin requires interaction with both c-cpn and additional protein cofactors (18, 19). To see whether it might be possible to decouple the ATP-driven c-cpn-mediated reaction from the action of these cofactors, an α-tubulin folding reaction was done in which α-tubulin-c-cpn binary complexes were incubated with ATP and GTP alone. After 45 min, the ATP-dependent reaction was quenched by the addition of hexokinase and glucose. Cofactors and carrier native tubulin were then added, and the reaction was allowed to continue at 30°C. We found that the ATP-driven reaction is almost as efficient when uncoupled from the action of cofactors, since the amount of native tubulin product was very similar in a parallel reaction that contained cofactors at the outset of the incubation with ATP and GTP (Fig. 1a, lanes 1 and 2). A control folding reaction in which glucose and hexokinase were included at the outset resulted in no detectable product, demonstrating the effectiveness of the ATP quench in arresting c-cpn-mediated folding (Fig. 1a, lane 3). The ATP-dependent reaction that generates the species upon which cofactors act is slow, requiring about 45 min to reach equilibrium (Fig. 1, b and c), while the action of cofactors on this intermediate seems to be very fast, requiring at most only a few seconds at 30°C (Fig. 1d).

To determine the stability of the subset of ATP-generated intermediates that can be converted to native α-tubulin molecules by the action of cofactors, we incubated α-tubulin-c-cpn binary complexes with ATP and GTP for 45 min to generate these intermediates, quenched the ATP-dependent reaction with hexokinase and glucose, and continued the incubation at 30°C for increasing times before completing the reaction by adding cofactors and native carrier tubulin. We found that the subset of intermediates that can be converted to native molecules by the action of cofactors is quite stable, with a half-life of about 50 min at 30°C (Fig. 1, e and f). We define this subpopu-
To investigate the extent of native-like structure in α-tubulin target molecules acquired as a consequence of their interaction with c-cpn, we compared the extent of target protein protease resistance in c-cpn-bound c-cpn binary complexes that had been incubated with or without ATP and GTP. As controls, we first established the resistance to proteolysis of urea-unfolded α-tubulin diluted into buffer alone, as well as the resistance of native tubulin synthesized by in vitro translation. Under the conditions used in our experiments, no intact tubulin survived beyond the initial addition of protease when the target protein was diluted into buffer (Fig. 3a). In contrast, there was no significant loss of intact α-tubulin in a parallel experiment done with native tubulin (Fig. 3b). In experiments to measure the proteolytic sensitivity of target protein-c-cpn binary complexes, we found that resistance to proteolysis of c-cpn-bound α-tubulin increased significantly upon incubation with ATP and more so upon incubation with both ATP and GTP (Fig. 3, c–e). These data imply that α-tubulin end states generated as a result of ATP-dependent cycling with c-cpn are more extensively folded than molecules that have not been cycled.

The αβ-tubulin heterodimer binds two molecules of GTP, one of which is exchangeable and is located on the β-subunit.
(the E-site), and a second that is nonexchangeable, located on
the α-subunit (the N-site) (25). Our observation that α-tubulin-c-cpn
binary complexes become more resistant to proteolysis upon incubation with both ATP and GTP (compared to incubation with ATP alone) (Fig. 3, d and e) implies that at least one function of GTP binding is to stabilize tubulin mole-
cules during their facilitated folding. To probe the state of α-tubulin
I0 intermediates in terms of their nonexchangeable (N-site) GTP
binding properties (25), we performed c-cpn-medi-
ated folding reactions in the presence of ATP and α-[32P]GTP,
using unlabeled unfolded α-tubulin as target protein. The in-
corporation of bound, labeled GTP into c-cpn-α-tubulin binary
complex is ATP- and target protein-dependent (Fig. 4a). When
these binary complexes were incubated with cofactors and na-
tive carrier tubulin in the presence of excess unlabeled GTP, we
found nonexchangeable label in association with both c-cpn and
native tubulin (Fig. 4b, lanes 1 and 2). This GTP-labeled tubu-
lin was native as shown by its ability to copolymerize with
authentic brain tubulin. These experiments demonstrate that
labeled native tubulin (Fig. 4b), as well as to the N-site of other inter-
mediates are so extensively native-like that they contain
the GTP binding pocket.

When c-cpn is diluted sufficiently to preclude efficient cycling and
in the presence of a mitochondrial chaperonin (mt-cpn)
trap for the capture of released non-native target protein (22),
labeled GTP can still become incorporated into α-tubulin inter-
mediates (Fig. 4b, lanes 4–6). These data suggest that the
nonexchangeable GTP binding site can form while the target
protein is chaperonin-bound. GTP is not acquired before binary
complex formation, since the complex is formed in the absence
of GTP. Nor is GTP (labeled or unlabeled) acquired after the
addition of cofactors: when folding reactions containing
α-[32P]GTP and unfolded [35S]methionine-labeled α-tubulin were
quenched with unlabeled GTP before the addition of cofac-
tors, each mole of labeled native tubulin produced contained
1 mol of nonexchangeable bound labeled GTP.

Like α-tubulin, the GroEL-mediated folding of rhodanese (3)
and the c-cpn-mediated folding of β-tubulin2 also proceed via
the formation of chaperonin-bound protease-resistant inter-
mediates. These data suggest that chaperonin-bound quasi-native
intermediates are an important feature of the mechanism of
facilitated folding (although there is normally no accumulation
of such intermediates). The existence of such highly structured
c-cpn-bound intermediates is surprising in view of current
models of GroEL-mediated protein folding (11, 17), but is con-
sistent with our observation that different chaperonins produce
distinct spectra of folding intermediates (22). Furthermore, our
evidence that α-tubulin can acquire its GTP binding pocket via
a single cycle of interaction with c-cpn suggests that, in con-
trast to the prevailing view, some target protein folding occurs
either on the chaperonin surface or in its central cavity, rather
than in bulk solution.

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