Partial Occlusion of Both Cavities of the Eukaryotic Chaperonin with Antibody Has No Effect upon the Rates of β-Actin or α-Tubulin Folding*

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The eukaryotic chaperonin containing T-complex polypeptide 1 (CCT) is required in vivo for the production of native actin and tubulin. It is a 900-kDa oligomer formed from two back-to-back rings, each containing eight different subunits surrounding a central cavity in which interactions with substrates are thought to occur. Here, we show that a monoclonal antibody recognizing the C terminus of the CCTa subunit can bind inside, and partially occlude, both cavities of apo-CCT. Rabbit reticulocyte lysate was programmed to synthesize β-actin and α-tubulin in the presence and absence of anti-CCTa antibody. The binding of the antibody inside the cavity and its occupancy of a large part of it does not prevent the folding of β-actin and α-tubulin by CCT, despite the fact that all the CCT in the in vitro translation reactions was continuously bound by two antibody molecules. Furthermore, no differences in the protease susceptibility of actin bound to CCT in the presence and absence of the monoclonal antibody were detected, indicating that the antibody molecules do not perturb the conformation of actin folding intermediates substantially. These data indicate that complete sequestration of substrate by CCT may not be required for productive folding, suggesting that there are differences in its folding mechanism compared with the Group I chaperonins.

Chaperonins are ATP-dependent protein folding machines composed of two back-to-back rings, each containing seven, eight, or nine polypeptides of ~60 kDa. Each ring encloses a cavity that is capped by the co-chaperonin ring of Group I chaperonins, such as GroES in the case of GroEL (1), or access to the cavity may be closed off by an in-built lid, which is the helical protrusion of the thermosome, a Group II chaperonin (2, 3). Two extreme views can be taken for the existence of cavities in chaperonins. Either they act primarily as folding cages, which sequester folding polypeptide chains, or they exist as a consequence of the allosteric mechanism of action of chaperonin rings, which may require the specific positioning of subunits in relation to one another. The rings of the Group II chaperonin containing T-complex polypeptide 1 (CCT)1 are composed of eight different subunit species (4), with one copy of each subunit occupying a fixed position in each ring (5).

CCT subunits are divergent from each other in their apical, putative substrate binding domains (6). Unlike the Group I chaperonins, which fold a broad range of proteins, the folding substrates of CCT are predominantly limited to actins and tubulins (7), although G-protein α-transducin has recently been identified as a substrate for CCT in vivo (8). The complex arrangement of divergent CCT subunits and the limited number of CCT substrates are highly suggestive of specific, sequence-dependent interactions occurring between CCT and its substrates, rather than the more general interactions that presumably occur between GroEL (which contains only one type of subunit) and non-native proteins (5).

In this study we have utilized a monoclonal antibody (mAb), 23C, which recognizes the extreme C terminus of the CCTa subunit (9, 10). Analysis of the crystal structure of the Group I chaperonin, GroEL (11), and of the Group II chaperonin, the thermosome (3), has shown that the C termini lie within the central cavity. Therefore, an antibody such as 23C would be expected to occupy at least part of the central cavity of CCT. This has been confirmed here by using electron microscopy to analyze negatively stained CCT-23C complexes. The images also support the biochemical model in which CCT contains a single copy of CCTa in each ring, although they do not prove formally that this is the case. The ability of CCT to produce native actin and tubulin when bound by two molecules of the 23C antibody has been investigated in functional in vitro translational experiments using rabbit reticulocyte lysate programmed with both β-actin and α-tubulin.

EXPERIMENTAL PROCEDURES

Electron Microscopy and Image Processing—CCT-23C complexes were obtained by incubation of CCT with excess 23C, which yields a mixture of single bound and double bound CCT-23C complexes. Lower antibody/CCT ratios were used for electron microscopy compared to the biochemical experiments to reduce the background on the grid due to unbound free antibody. Afterward, CCT-23C complexes were incubated with 10 mM MgCl₂ and 5 mM ATP to induce the appearance of a larger percentage of side views and stained with 1% uranyl acetate. Front and side views of the CCT-23C complexes were directly recorded using a GATAN ssCCD camera attached to a JEOL 1200EX II microscope. The particles were centered using a synthetic mask and aligned using a free-pattern algorithm (12, 13). The average images of the front views of the CCT-23C complexes and of the side views of CCT bound to one or two 23C antibodies were generated with 423, 371, and 71 particles, respectively.

In Vitro Translation—In vitro translations of β-actin and α-tubulin (10) were carried out by priming TNT® rabbit reticulocyte lysate (Promega, Madison, WI) with Bluescript SKI+ plasmids encoding full-

polyepptide 1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

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1 The abbreviations used are: CCT, chaperonin containing T-complex polypeptide 1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; mAb, monoclonal antibody.
length human β-actin and α-tubulin cDNAs cloned in T7 orientation for transcription. Co-translations were carried out with a 1.325-μg input DNA template in 50-μl reaction volumes containing 40 μCi of [35S]methionine (in vitro cell-labeling grade, Amersham Pharmacia Biotech) at 30 °C with the β-actin and α-tubulin DNA template ratio optimized to produce similar levels of native proteins. Time course samples (4 μl) were taken and further translation was stopped by addition of EDTA (pH 8.0) to 6 μl. Samples were stored on ice prior to loading on a 6% native polyacrylamide gel. Native polyacrylamide gel electrophoresis (PAGE) was carried out according to Liou and Willison (5). Upon co-translation, to quantitate more accurately the [35S]-protein bands corresponding to native β-actin and α-tubulin monomers by phosphor imaging, monoclonal antibodies to β-actin and α-tubulin were added to samples of the reticulocyte mixture after translation had been stopped by the addition of EDTA. The formation of actin- or tubulin-antibody complexes results in a less diffuse migration of the [35S]β-actin and [35S]α-tubulin polypeptides during electrophoresis but still allows for discrimination of the respective proteins. Samples of reticulocyte lysate reaction (2 μl) were mixed with 6 μl of phosphate-buffered saline (PBS), 1.1 μg of anti-β-actin mAb (A5441, Sigma), and 1.5 μg of anti-α-tubulin mAb (TS168, Sigma) and incubated on ice for 30 min prior to analysis by native PAGE and autoradiography.

RESULTS

One objective of these experiments was to address the question of the degree of homogeneity of CCT. Is “core” CCT composed of two identical rings containing one copy of each of the eight CCT subunits as previously proposed (5)? Biochemical analysis of the CCT/antibody interaction showed that the migration of rabbit CCT in native polyacrylamide gel bandsift assays can be substantially retarded by addition of mAb 23C, which binds specifically to the CCTα subunit, and that replacement of one CCTα subunit by a mutant CCTα subunit unable to bind mAb 23C produced an intermediated migrating complex (10). Liou et al.’s interpretation of these results was that the CCT 16-mer contains two copies of the CCTα subunit, one in each ring. Furthermore, because all CCT complexes were shifted by mAb 23C in these experiments, Liou et al. (10) suggested that all CCT complexes contain two copies of the CCTα subunit.

Now we have examined CCT-mAb 23C complexes by negative-stain electron microscopy and image processing. CCT-antibody complexes are composed of CCT and either one (Fig. 1B) or two (Fig. 1C) antibody molecules. Side views of double antibody-bound CCT only showed one antibody bound to each ring (Fig. 1C), which is consistent with there being only one CCTα subunit present in each ring. However, if two CCTα subunits were directly adjacent in each of the rings, it is possible that steric hindrance would result in only one antibody molecule binding. The disposition of antibody in the double bound complexes also shows that the CCTα subunits do not contact each other across the rings; in fact, the two CCTα subunits are opposite, or nearly opposite, each other in the opposing rings. Clearly, the antibody binds within the central cavity of CCT and partially occludes it, suggesting that the C terminus of CCTα is located within the cavity. Although the C termini of the chaperonin subunits in the structures of GroEL (11) and the thermosomes (3) are disordered, the structures show that they are also located within the central cavity. A further indication that 23C is fairly deeply embedded in the cavity of CCT is that, although 23C is a bivalent IgG antibody, it is not able to cross-link multiple CCT complexes (10). Nevertheless, if an anti-rat secondary antibody is added to the CCT-23C complex, all the CCT becomes cross-linked and trapped in the well of a native gel (data not shown), demonstrating that the Fc portion of the 23C IgG is protruding, as can be seen clearly in the side view images of 23C-decorated complexes (Fig. 1, B and C).

We have already shown that 23C can bind CCT complexes that contain bound substrates such as α-tubulin (10). Therefore, we wondered whether 23C might interfere with processing of substrates by CCT in rabbit reticulocyte lysate in vitro translation reactions, and we have analyzed β-actin and α-tubulin folding in this system. Various experiments were carried out to optimize the amount of 23C to avoid inhibitory effects of the addition of exogenous protein to this biochemically rather labile system. A final concentration of 60 μg ml⁻¹ 23C was well tolerated (data not shown), and this concentration was in excess for complete CCT binding. An example of a stacking gel is shown (Fig. 2A) to confirm that little protein denaturation/aggregation is occurring in this system. Individual translation reactions were programmed with both β-actin and α-tubulin to act as internal controls for each other for any possible effects of 23C on folding of substrates. The results are clear and slightly surprising; there are no inhibitory effects of 23C on the rate of production or yield of either β-actin or α-tubulin over a 90-min time course (Fig. 2, A–G). The lag phase seen during the production of α-tubulin monomers may be a result of the co-factor binding of α-tubulin after release from CCT (14) and has been observed previously in our native gel system (Fig. 2) (10). Although the total level of native actin produced in the presence and absence of 23C appears unchanged, it would appear that some difference occurs between the distribution of actin in...
changes in protease sensitivity when b internal methionines during translation (Fig. 4, smaller fragments, known to be the products of initiations at

Upon trypsin treatment a further three complexes. The amount of 23C added, in this proteolysis

3 E. A. McCormack and K. R. Willison, unpublished results.

FIG. 2. Analysis of co-translations of b-actin and a-tubulin in rabbit reticulocyte lysate in the presence and absence of the monoclonal anti-CCTa antibody 23C. A, a-tubulin cDNA was translated for 45 min at 30°C in rabbit reticulocyte lysate in the presence and absence of 60 µg ml⁻¹ 23C mAb. The production of native a-tubulin and the 23C-induced shift of CCT were analyzed by native PAGE and autoradiography. CCT(u), non-antibody-bound CCT (unshifted); CCT(s), CCT bound by two molecules of 23C (shifted) as previously demonstrated by Liou et al. (10). The diffuse a-tubulin monomers are shown not having been bound with anti-a-tubulin antibody just prior to electrophoresis to allow comparison with the tightly migrating antibody-bound a-tubulin species in B–D. (Fig. 3A), showing that the position of thyroglobulin molecular mass marker (669 kDa) is indicated. B–D, co-translation of b-actin and a-tubulin in rabbit reticulocyte lysate was carried out over a 90-min time course at 30°C and monoclonal antibodies to b-actin and a-tubulin added prior to analysis by native PAGE. Autoradiograms of native PAGE analysis of in vitro translation time courses in the absence of 23C (B) and presence of 60 µg ml⁻¹ 23C (C) are shown. D, the 90-min time point lanes taken from each native gel in B and C and aligned to show clearly the unshifted (CCT(u)) and antibody-shifted (CCT(s)) CCT and a-tubulin complexes. E–G, the radioactive signals of the two gels shown in B and C were quantitated by phosphor imaging using a Molecular Dynamics Storm 860 system. Band intensities were expressed as a percentage of total counts per lane, although little variation was observed between the total counts per lane at corresponding time points from different translations. E, the percentage of counts on CCT (corresponding to both b-actin and a-tubulin) in the absence (●) and in the presence of 23C (●). F, yield of native b-actin in the absence (●) and presence of 23C (●). G, yield of native a-tubulin in the absence (●) and presence of 23C (●).
In principle there could be room to accommodate a folding actin or tubulin molecule and 23C in the folding cavity. A recent study on the maximum size of proteins able to occupy and complete folding in the GroEL cavity underneath GroES showed that a 54-kDa fused dimer of green fluorescence protein could fold, but an 82-kDa green fluorescence protein trimer could not (16). CCT seems to function normally with its cavity occupied at the upper limit of the GroEL substrate size range, because $\beta$-actin and $\alpha$-tubulin have a combined molecular mass of 70- and 80-kDa, respectively. Furthermore, Weissman et al. (17) showed that the substrate is protease-resistant in preformed polypeptide-GroEL-GroES complexes, although we found $\beta$-actin bound to CCT to be highly sensitive to proteolysis.

It can be seen from the negatively stained side views that the CCT rings bound by 23C are distorted; this may reflect the flexibility of the helical protrusions of the apical domains. Perhaps, because of this inherent flexibility (2, 3), the apical domains are still able to undergo the movements required (15) to close off the CCT cavity in the presence of antibody without perturbing the folding cycle. Apart from being smaller, the antibody-occluded folding cavity could reasonably be expected to have a different structure, with some of its internal surfaces completely obscured. If all the eight apical domains have equivalent functions, one would predict the folding rates of the two substrates would be slowed down by the presence of 23C. Therefore, as suggested by Liu and Willison (5), perhaps not all the subunits are involved in substrate interactions. Nevertheless, both actin and tubulin fold normally in such smaller cavities, and these experiments point to differences between the folding mechanisms of CCT and GroEL.

**Fig. 3.** Analysis of the integrity of $\beta$-actin produced in rabbit reticulocyte lysate in the presence and absence of the 23C mAb. A, human $\beta$-actin was translated in rabbit reticulocyte lysate in the presence and absence of 60 $\mu$g ml$^{-1}$ 23C mAb. Translation was stopped after 90 min by the addition of EDTA to 6 mcat. Samples (4 $\mu$l) were prepared for SDS-PAGE and resolved on a 12% polyacrylamide gel, and the production of $^{35}$S-labeled $\beta$-actin was analyzed by autoradiography. The positions of molecular standards and their sizes (in kDa) are indicated on the left. B, 1 $\mu$l of stopped lysate was incubated in the presence and absence of 10 $\mu$g of DNase I with PBS added to 10 $\mu$l for 30 min at 4 °C. 1.5 $\mu$l of loading buffer was added, and the entire sample was resolved on a 6% polyacrylamide native gel followed by autoradiography. The positions of CCT, native $\beta$-actin, $\beta$-actin/DNase I complex, and a $\beta$-actin complex formed during later stages of translation (but disrupted in the presence of DNase I) are indicated.

**Fig. 4.** Limited proteolysis of $\beta$-actin bound to CCT and CCT-23C complexes. Rabbit reticulocyte lysate (50 $\mu$l) was programmed with 0.9 $\mu$g of cDNA encoding full-length human $\beta$-actin in the presence and absence of the 23C mAb. After translation for 20 min at 30 °C, 1-$\mu$l samples were incubated in the presence and absence of 10 ng of trypsin for 15 min at room temperature. 6 $\mu$l of PBS and 1 $\mu$l of sample buffer were added, and the entire sample was resolved on a 6% polyacrylamide native gel. A, $\beta$-actin translated in the absence of 23C; B, $\beta$-actin translated in the presence of 23C; C, $\beta$-actin translated in the absence of 23C followed by trypsin treatment; D, $\beta$-actin translated in the presence of 23C with subsequent trypsin treatment. The positions of unshifted (CCT(u)) and antibody-shifted (CCT(s)) CCT and the position of native $\beta$-actin in the first gel dimension are indicated.
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