Disassembly of the Cytosolic Chaperonin in Mammalian Cell Extracts at Intracellular Levels of K\(^+\) and ATP* 

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The eukaryotic, cytoplasmic chaperonin, CCT, is essential for the biogenesis of actin- and tubulin-based cytoskeletal structures. CCT purifies as a doubly toroidal particle containing two eight-membered rings of ~60-kDa ATPase subunits, each encoded by an essential and highly conserved gene. However, immunofluorescence detection with subunit-specific antibodies has indicated that in cells CCT subunits do not always co-localize. We report here that CCT ATPase activity is highly dependent on K\(^+\) ion concentration and that in cell extracts, at physiological levels of K\(^+\) and ATP, there is considerable dissociation of CCT to a smaller oligomeric structure and free subunits. This dissociation is consequent to ATP hydrolysis and is readily reversed on removal of ATP. The ranking order for ease with which subunits can exit the chaperonin particle correlates well with the length of a loop structure, identified by homology modeling, in the intermediate domain of CCT subunits. K\(^+\)-ATP-induced disassembly is not an intrinsic property of purified CCT over a 40-fold concentration range and requires the presence of additional factor(s) present in cell extracts.

The chaperonins are a group of molecular chaperones characterized by their double-ringed structure of ~60-kDa ATPase subunits enclosing a central cavity (1). Within this “Anfinsen cage” (2), folding substrates may be sequestered and undergo alternating cycles of nucleotide-dependent binding and release until a structure committed to forming the native state is achieved. Our current knowledge of the structure and mechanism of action of chaperonins rests largely on numerous studies of the homo-oligomeric tetradecameric chaperonin GroEL and its cochaperonin, GroES (e.g. Refs. 3 and 4), which facilitate the correct folding of a range of Escherichia coli proteins (5). Together with other eubacterial chaperonins and eukaryotic homologues in mitochondria and chloroplasts, these constitute group I type chaperonins (6), originally defined on the basis of amino acid sequence homology. Each GroEL subunit comprises three domains; the equatorial domain containing the ATP binding pocket and most intra-ring and all inter-ring contacts, which is connected via the intermediate domain to the apical domain, which contains binding sites for unfolded proteins and GroES (7). Folding substrate binding, ATP binding, GroES binding, folding substrate release, and ATP hydrolysis by GroEL subunits is a highly co-ordinated sequence of events (8), and this, together with their forming the enclosed central folding cavity, is likely to make the double-toroidal structure formed by the GroEL subunits essential to most of the protein folding activities of this chaperonin in vivo (9). Quite recently, however, recombinant GroEL apical domain “minichaperones” proved to have molecular chaperone activities for a limited range of substrates in vitro (10). Moreover, the activity of these minichaperones in vivo suggests functionality in protein folding for single GroEL subunits (11). However, there is no evidence at present for a function-related disassembly of the group I chaperonins other than to the single ring state of mitochondrial hsp60 (12), which is also folding-competent in vivo and in vitro (13).

The chaperonin in eukaryotic cytosol, CCT\(^1\) (Ref. 14; also termed e-cpn (15) and TriC (16)), together with the archaeabacterial chaperonins, constitute class II chaperonins. Apart from being defined by sequence homology (17), they are also characterized by having eight- or nine-membered rings within the double torus (18, 19) and by a unique helix-turn-helix motif in the apical domains of their component subunits, which might function as a GroES-type lid on the chaperonin cylinder (20–22), thereby removing the requirement for a GroES-like cochaperonin in class II chaperonins. The main function of CCT in the eukaryotic cytosol, as presently understood, is to promote the correct folding of actins and tubulins (23, 24). CCT is hetero-oligomeric and is unique among chaperonins in the complexity of its subunit composition. Its eight distinct but sequence-related subunits (14), each the product of an essential gene, are highly conserved across eukaryotes. The degree of sequence relatedness between subunit orthologues across species possibly argues for unique and conserved functions for each particular subunit type. These subunits are deployed as two eight-membered rings stacked back-to-back (18, 25) and “nearest neighbor” analysis of subunit microcomplexes, containing two or three subunits, has provided evidence that within each ring a fixed order of subunits may prevail (26). The current view of CCT-mediated protein folding, based on in vitro folding studies, is that a hetero-oligomeric particle containing all eight subunits is required and mechanisms similar to those for GroEL-ES mediated folding have been proposed (e.g. Ref. 27). However, a number of observations are not consistent with the subunits of group II chaperonins functioning only as components of the oligomeric chaperonin particle. Small amounts of CCT polypeptides exist as free subunits and microcomplexes containing two or three subunits, which can be detected after

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1 The abbreviations used are: CCT, cytosolic chaperonin containing T-complex polypeptide 1; AMP-PNP, adenosine 5'-β,γ-imino-triphosphate; PAGE, polyacrylamide gel electrophoresis.
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sized-based resolutions of cell extracts (26, 28). Incorporation of newly synthesized CCT subunits into chaperonin particles occurs via a single ring “template” generated by nucleotide-dependent disassembly to single rings and microcomplexes (29). The folding cycle of an archaeobacterial chaperonin appears to involve nucleotide-dependent disassembly to free subunits (30). The functional cycle of group II chaperonins is therefore likely to be more different from that of group I chaperonins than previously supposed (27). A mechanistic difference between the two chaperonin types is also indicated by the recent electron microscopy study of Llorca et al. (31), which revealed very extensive loosening of both intra- and inter-ring contacts between subunits in just one of the two CCT rings upon binding of ATP or the non-hydrolyzable analog, AMP-PNP. No equivalent opening out occurs in GroEL upon ATP binding, but rather, large movements about hinge regions in the intermediate domain result in the movements of substrate binding sites on the apical domain up and away from the central cavity (3).

Our own data, based on immunofluorescence microscopy and various biochemical analyses, utilizing antibodies specific to each of the eight CCT subunits, have consistently indicated that substantial amounts of CCT subunits are not always components of the hetero-oligomeric chaperonin particle in the environment of the cell (28, 32, 33). Initially, we interpreted these data as indicative of chaperonin particles of variable subunit composition, but we, and others, have consistently failed to demonstrate such particles by a variety of isolation procedures. We recently reported that, although immunoprecipitations targeted at the majority of CCT subunits from cell extracts yielded the “core” hetero-oligomeric particle, immunoprecipitation in the presence of intracellular levels of ATP tended to yield only the subunit targeted for precipitation (see Fig. 8 in Ref. 28). These observations were made in cell extracts prepared in a buffer containing 90 mM KCl. We report here that in Fig. 8 in Ref. 28). These observations were made in cell extracts prepared in a buffer containing 90 mM KCl. We report here that these experiments were also carried out with 10–40% (w/v) sucrose gradients of 20–70% HEPES-NaOH, pH 7.2, plus 90 mM KCl, which revealed very extensive loosening of both intra- and inter-ring contacts between subunits in just one of the two CCT rings upon binding of ATP or the non-hydrolyzable analog, AMP-PNP. No equivalent opening out occurs in GroEL upon ATP binding, but rather, large movements about hinge regions in the intermediate domain result in the movements of substrate binding sites on the apical domain up and away from the central cavity (3).

MATERIALS AND METHODS

Cells—The mouse neuroblastoma/rat dorsal root ganglion hybrid cell line, ND/723 (34), and the P19 mouse embryonal carcinoma cell line (35), were maintained and radiolabeled with [35S]methionine/cysteine (Promix™, Amersham Pharmacia Biotech) as we have described previously (28, 32).

Antibodies—CCT subunit-specific rabbit, rat, and guinea pig polyclonal antibodies were raised against keyhole limpet hemocyanin conjugates of synthetic peptide sequences based on the extreme COOH-terminal sequences (28) for murine CCT subunits (500, 50, and 100 µg of immunogen protein per dose for rabbits, rats and guinea pigs, respectively, and 5–8 biweekly injections). A similar procedure was used to raise a rabbit polyclonal to the constitutive form of hsp70 (hsc70) based on a sequence near the COOH terminus of this protein (36). Antibodies were affinity-purified and characterized as we described previously (28). For immunoprecipitation, these were diluted 1:10 to 1:200 in PBS, 1:10–40 µg of protein) of cell extract was resolved over a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated and eluted with the appropriate extraction buffer at 0.5 ml/min, collecting 1-ml fractions. 25–µl aliquots of fractions were taken for immunoblot analysis and 100–µl aliquots, made 0.5% with respect to Triton X-100, were taken for immunoprecipitation with the washed immunoprecipitates being taken up in 100 µl of SDS-PAGE sample buffer prior to loading 30 µl for SDS-PAGE resolutions. For urea-treated CCT, purified rabbit testis CCT was first exposed to 2.5 M urea for 1 h at 4°C, then dialyzed against 20 mM HEPES-NaOH, pH 7.2, plus 90 mM KCl, 0.5% Triton X-100 and protease inhibitors as above. 20 S particles, purified by ion-exchange chromatography over Resource Q (Amersham Pharmacia Biotech), as we have described previously (32).

Immunoprecipitation—Immunoprecipitation procedures were essentially as we have described elsewhere (28). Radiolabeled cell extract protein concentrations were usually ~1 mg/ml (2000–2500 kBq/ml). Assays were done in a range of 0.01–0.125 µg/ml was examined. Puriﬁed CCT samples (0.015–0.040 mg of protein/ml) were made 0.5% (w/v) with respect to Triton X-100 prior to immunoprecipitation. Antibodies were generally incubated 2–4 h on ice with extracts or puriﬁed CCT prior to incubation with protein A beads (10–µl packed volume; P-3391, Sigma) overnight at 4°C, but incubations as short as 1 h with antibody, followed by 1 h with protein A beads, were also examined. Immune precipitates were washed in three 1-ml aliquots of the appropriate buffer prior to solubilization in 50–100 µl of Laemmli (39) SDS-PAGE sample buffer. For immunoprecipitation under denaturing conditions, final concentrations 0.5% (w/v) sodium deoxycholate and 0.05% SDS were added to cell extracts. Western blots of SDS-PAGE resolutions of proteins immunoprecipitated with rabbit antibodies were probed with antibodies raised in rats or guinea pigs and detected with the appropriate peroxidase-conjugated secondary antibody (Sigma) with ECL (Supersignal®, Pierce). Autoradiography of radiolabeled immune precipitates was with Hyperfilm MP, Amersham) and 16-h to 7-day exposures. Images were quantiﬁed using Bio-Rad Multi-Analyzer™/IP data analysis software.

ATPase Assay—Assays were based on the charcoal method described by Bais (40). Briefly, 25 µg of puriﬁed CCT, free of hsc70 as judged by immunoblot, in 200 µl of extraction buffer, containing varied concentrations of KCl or NaCl, were preincubated at 35°C for 5 min prior to addition of 4 µl of 0.1 mM ATP containing 3.7MBq/ml γ–[32P]ATP-labeled ATP (Amersham Pharmacia Biotech). At 2 and 17 min, 90 µl of reaction mixture were withdrawn and pipetted into 500 µl of ice-cold 100 µg/ml activated charcoal (Sigma) suspended in 0.33 mM HCl. After 5 min on ice, samples were centrifuged at 13,000 × g for 2.5 min and then 150 µl of supernatant taken for Cerenkov counting. Preliminary experiments showed that, under these assay conditions, ATP hydrolysis was linear for at least 40 min and calculations of specific activity took into account the very low rate of spontaneous ATP hydrolysis.

Size-exclusion Chromatography—100 µl (0.5–0.6 mg of protein) or 400 µl (2–2.4 mg of protein) of cell extract were resolved over a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated and eluted with the appropriate extraction buffer at 0.5 ml/min, collecting 1-ml fractions. 25–µl aliquots of fractions were taken for immunoblot analysis and 100–µl aliquots, made 0.5% with respect to Triton X-100, were taken for immunoprecipitation with the washed immunoprecipitates being taken up in 100 µl of SDS-PAGE sample buffer prior to loading 30 µl for SDS-PAGE resolutions. For urea-treated CCT, puriﬁed rabbit testis CCT was first exposed to 2.5 M urea for 1 h at 4°C, then dialyzed against 20 mM HEPES-KOH, pH 7.2, containing 25 mM NaCl, 1 mM diethiothreitol, and 1 mM EDTA. Following co-precipitation of the dialysate by centrifugation at 13,000 × g for 10 min, 100 µl (0.04 mg of protein) were resolved over the Superose 6 column as above.

Molecular Modeling—The amino acid sequences from all CCT subunits found in Swiss-Prot and TrEMBL were aligned using Multalin version 4 (42) to create five homology models of each subunit using the b-subunit crystal structure (21), kindly provided by Dr. S. Steinbacher, as a template. The model with the lowest objective function was used in comparisons with other subunits using Quanta ’96 (Molecular Simulations Inc.).

RESULTS

The data presented below were obtained from cell extracts and CCT prepared from the ND/723 cell line unless otherwise stated. Many of these experiments were also carried out with P19 cell line with essentially the same results.

Immunoprecipitation of CCT Subunits at Intracellular Lev-
els of $K^+$ and ATP—Throughout, antibody concentrations used for immunoprecipitation were such that the targeted subunit was completely precipitated under denaturing conditions, as determined by immunoblot analysis of the residual supernatants (28). We have recently reported (28) that immunoprecipitation of CCT subunits from cell extracts is dramatically dependent on the nucleotide content of the extract. Briefly, most CCT subunit-specific antibodies co-precipitated the other seven subunits of CCT from cell extracts prepared in the absence of added nucleotide, i.e. the “core” hetero-oligomeric chaperonin particle was immunoprecipitated. However, addition of 2 mM ATP to cell extracts tended to yield immune precipitates of the targeted subunit only, with the exception of the CCTγ-directed antibody, which still co-precipitated other CCT subunits, albeit to a lesser extent than in the absence of nucleotides (see Fig. 8 in Ref. 28). At this time we routinely used 90 mM KCl in our cell extraction buffer. We now report that this effect of nucleotide is highly dependent on the $K^+$ ion content of the extract. There was co-precipitation of CCT subunits in the presence of 2 mM ATP in the absence of $K^+$ ions and this gradually diminished as $K^+$ ion concentration was increased (e.g. Fig. 1A, upper panel), and at physiological levels of $K^+$ ion (140 mM), immunoprecipitation of the single, targeted subunit prevailed. $K^+$ ions could not be replaced by Na+ ions in this respect (e.g. Fig. 1A, lower panel). At a KCl concentration of 140 mM and ATP concentrations in excess of 1 mM, i.e. physiological concentrations, all CCT subunit-specific antibodies immunoprecipitated the targeted subunit only, while in the absence of added ATP, immunoprecipitation of the core CCT particle prevailed (e.g. Fig. 1B). Exceptions to this were the anti-CCTδ (Fig. 2) and anti-CCTθ (Fig. 1B) antibodies, which, in the absence of ATP, only precipitated small amounts of the targeted subunit (presumably representing the small amounts of free CCT subunits present in cell extracts prepared under these conditions (28). It would therefore appear that within the chaperonin particle the CCTδ and CCTθ COOH termini were not accessible to antibody binding, but did become accessible once dissociated, either by ATP addition (e.g. Fig. 1B) or by immunoprecipitation under denaturing conditions (Fig. 2). It can also be noted from the data in Fig. 1B that particular subunits dissociated more readily from the chaperonin particle as ATP levels increased, with CCTβ and CCTθ dissociating at much lower ATP concentrations than CCTγ, with other subunits showing intermediate sensitivities to ATP concentration.

The extent of $K^+$-ATP-induced, free subunit immunoprecipitation was assessed in two ways. Immunoblot analysis of subunit content of original extracts and supernatants remaining after immunoprecipitation by anti-CCTα and by anti-CCTε antibodies (data not shown), indicated that, in the absence of ATP, 50% and 80%, respectively, of the targeted subunit were immunoprecipitated along with 40–80% of the remaining CCT subunits. At 2 mM ATP, 50% and 70%, respectively, of the targeted subunit were immunoprecipitated with 0% of the other CCT subunits. These data were confirmed and extended by a comparison of CCT subunits in immune complexes prepared under native conditions in the presence and absence of ATP with those precipitated under denaturing conditions and detected by autoradiography (Fig. 2). Taking the subunit content of immune precipitates prepared under denaturing conditions as 100% of the cell extract subunit content (28), quantitative analysis of the data in Fig. 2 revealed that immune precipitates prepared in the presence of $K^+$-ATP contained 100, 60, 50, 80, 60, and 100% of the extract content of CCTβ, CCTγ, CCTδ, CCTε, CCTζ, and CCTθ, respectively. From these data it would appear that a proportion of certain CCT subunits becomes inaccessible to immunoprecipitation by COOH-termi-
precipitated, with the possible exception of CCTγ-targeted immunoprecipitation (data not shown). Concentrations of GTP that yielded single subunit immunoprecipitates were approximately double the effective concentrations of ATP (compare upper (ATP) and middle (GTP) panels in Fig. 1D). The non-hydrolyzable ATP analog, AMP-PNP, was completely ineffec-
tual in yielding single subunit immune precipitates (Fig. 1D, lower panel), although a significant decrease in core particle precipitation was noted with increasing AMP-PNP concentrations. This last observation suggested that ATP hydrolysis might be required to generate the immunoprecipitable free CCT subunits.

**CCT ATPase Activity Is K⁺-Ion-dependent**—CCT ATPase activity has been determined in a number of reports with quite a range of specific activities from 2.5 nmol/min/mg protein (15) to 25 nmol/min/mg protein (43) quoted, the adrenal medullary isoform of CCT, chromabindin A, being the most extensively characterized CCT ATPase (44, 45). In this present investigation, the ATPase activity of CCT purified from ND7/23 cells, by sucrose gradient fractionation followed by anion exchange chromatography, was measured. This preparation was free, as judged by immunoblot analysis (data not shown), of hsc70, a common contaminant of CCT preparations (46). The CCT ATPase was highly dependent on K⁺ ion concentration (Fig. 4) and over the range of K⁺ ion concentration examined, the specific activities varied over the range reported in the literature for CCT ATPase activity (probably a reflection of the variation in K⁺ ion concentrations used in these reports). As with the K⁺ ion dependence of single subunit immunoprecipitation, Na⁺ ions could not effectively replace the K⁺ requirement of the CCT ATPase (Fig. 4). The Kᵣ for KCl at 2 mM ATP was determined as 175 mM with a Vᵣ of 37.1 nmol/min/mg protein (Fig. 4). The Kᵣ for ATP at 140 mM KCl was 129 μM with a Vᵣ of 23.3 nmol/min/mg protein (data not shown). These latter figures are in good agreement with those quoted for chromabindin A (43). Like chromabindin A (43), the ND cell ATPase was inhibited by high (>1 mM) concentrations of Ca²⁺ ions, as was ATP-induced single-subunit immunoprecipitation (data not shown). In general, an excellent correlation existed between the level of CCT ATPase activity and the generation of immunoprecipitable free CCT subunits.

**Size-exclusion Chromatography Shows K⁺-ATP-dependent Dissociation of CCT to Smaller Oligomer(s) and Free Subunits**—The generation of immunoprecipitable free CCT subunits at physiological levels of K⁺ and ATP could have reflected *bona fide* disassembly under these conditions or could have been consequent to ATP binding/hydrolysis weakening subunit binding within the chaperonin double torus, followed by complete dissociation of targeted subunit on antibody binding. Against this latter possibility was the observation that, while AMP-PNP, like ATP, is known to cause extensive loss of contacts between subunits in just one of the CCT rings (31), this non-hydrolyzable analog did not support the generation of immunoprecipitable free subunits (Fig. 1D, lower panel). However, it was considered necessary to assess the extent of CCT disassembly in cell extracts by a second method. Gel filtration over a Superose 6 column readily resolved the ~950-kDa chaperonin particle from the majority of the cell extract proteins.
K\(^{+}\)-ATP-dependent Disassembly of the CCT Chaperonin

Fig. 5. Size-exclusion chromatography of ND cell extracts and of urea-treated rat testis CCT. 100 µl (0.5 mg of protein) (A and B) or 400 µl (2 mg of protein) (C) of ND cell extract prepared in basic buffer plus the indicated additions were resolved over a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated, and eluted with the same buffer as used for extract preparation at 0.5 ml/min, collecting 1-ml fractions. Eluted proteins were resolved by SDS-PAGE and detected by Coomassie stain (A, upper panel) or by immunoblot (A, middle panel; B, upper panel; C) probed for CCT\(\gamma\) (upper band), CCT\(\alpha\) (middle band), and CCT\(\beta\) (lower band). For A and B (lower panels only), proteins in 100-µl aliquots of fractions were immunoprecipitated with rabbit anti-CCT\(\gamma\) and the immunoblots of SDS-PAGE resolutions of the precipitated proteins probed with rat anti-CCT\(\alpha\). Buffer additions were as follows: A, 140 mM KCl; B and C, 140 mM KCl + 2 mM ATP. In D, purified rat testis CCT was exposed to 2.5 mM urea for 1 h at 4 °C, then dialyzed against 20 mM HEPES-NaOH, pH 7.2, containing 25 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. Dialysate was clarified by brief centrifugation and 100 µl (40 µg protein) loaded onto a Superose 6 column equilibrated and eluted with dialysis buffer at 0.5 ml/min, collecting 1-ml fractions. Immunoblots of SDS-PAGE resolutions of fractions were probed for: CCT\(\gamma\) (upper band), CCT\(\alpha\) (middle band), and CCT\(\beta\) (lower band). Size exclusion chromatography molecular mass markers in (A, upper panel) were from the left, thyroglobulin (669 kDa, apoferritin (443 kDa), and bovine serum albumin (66 kDa) and SDS-PAGE mass markers (A, upper panel) were as in Fig. 2.

(Fig. 5A, upper panel) including free CCT subunits and microcomplexes. The CCT polypeptides in ND cell extract prepared in extraction buffer containing 140 mM KCl co-eluted (Fig. 5A, middle panel) just prior (fraction 14) to the thyroglobulin size marker (669 kDa) with very small amounts of some subunits, notably CCT\(\beta\), in the microcomplex size range (fractions 18 and 19). Prior exposure of this cell extract to 2 mM ATP (Fig. 5B), followed by gel filtration in the same ATP-containing buffer, in addition to increasing the amounts of free subunits (fractions 19 and 20) rather than microcomplexes, also caused a consistent shift of the CCT oligomer from fraction 14 to fractions 15 plus 16. Since the apoferritin marker (443 kDa) eluted in fraction 16, this ATP-induced shift in oligomer elution position could well have been due to disassembly of the CCT double torus to single rings. The quantitation of subunit immunoprecipitation data had already indicated that, in the presence of ATP, a portion of CCT polypeptides becomes inaccessible to immunoprecipitation. Immunoprecipitation of gel filtration fractions (Fig. 5, A and B, lower panels) confirmed that CCT COOH-terminal epitopes in the oligomeric species eluting in fractions 15 and 16 in the presence of ATP, were indeed much less accessible to immunoprecipitation than those in the nucleotide-free CCT double torus. As with the CCT ATPase activity, and with the generation of immunoprecipitable free subunits, Na\(^{+}\) ions could not replace K\(^{+}\) ions in increasing the amounts of free subunits, nor the shift to the smaller oligomeric species, detectable on gel filtration in the presence of ATP (data not shown).

This gel filtration analysis of cell extracts did not confirm the extent of disassembly of the CCT particle to free subunits which had been indicated by the immunoprecipitation experiments. Most notably, CCT\(\gamma\) remained entirely, and CCT\(\alpha\), \(\delta\), and \(\zeta\), largely, in oligomeric species. More extensive disassembly was observed when the gel filtration load, and therefore the spread of eluted peaks, was increased 4–5-fold (Fig. 5C). These observations pointed to the possibility that gel filtration was removing factor(s) required for the more extensive disassembly of CCT indicated by the data from immunoprecipitation in cell extracts.

The CCT particle could also be partially dissociated by exposure to moderate concentrations of urea (47). Gel filtration analysis of purified rat testis CCT, previously exposed to 2.5 mM urea, revealed that several subunits, specifically CCT\(\beta\), \(\theta\), and \(\epsilon\), fractionated entirely as free subunits and, once again, CCT\(\gamma\) remained in the oligomeric species that eluted in fractions 15 and 16 (Fig. 5D), i.e. in the same position as the oligomeric species generated by exposure to K\(^{+}\)-ATP. There was therefore reasonably good agreement between these three assessments of CCT disassembly in terms of the ease with which particular subunits could dissociate from the core particle, i.e. CCT\(\gamma\) least readily, CCT\(\beta\) and \(\theta\), most readily, with CCT\(\alpha\), \(\delta\), \(\zeta\), and \(\epsilon\), ranked in order of increasing ease of disassembly, lying between these two extremes.

Homology modeling of the CCT subunit sequences (14) onto the crystal structure of the \(\alpha\)-subunit of the related type II chaperonin from the archaeabacterium Thermoplasma acidophilum (21) identified a loop of variable length extending from the outer edge of the lower hinge region between the equatorial domain and the intermediate domain of CCT subunits (Fig. 6, upper panel). A similarly positioned loop was also noted in the \(\alpha\)-subunit of the Sulfolobus shibatae chaperonin, although not in the \(\beta\)-subunit, when these two subunits were homology modeled on the \(\alpha\)- and \(\beta\)-thermosome subunit structures, respectively. The length of this loop in CCT subunits (Fig. 6, lower panel) correlated well with the order of subunit disassembly determined experimentally, with CCT\(\gamma\) extending the longest loop and CCT\(\beta\), the shortest. Since this loop is in a good position to contribute to intra-ring subunit interactions, and is near the hinge region about which a large conformation change occurs on ATP binding (31), its length could well be a determining factor in the ease with which subunits can exit the core chaperonin particle. It is also interesting to note that the two subunits with the longest loop sequences (\(\gamma\) and \(\alpha\)) would be disposed opposite to each other in the ring orientation proposed by Liu and Willison (26).

Additional Factor(s) Are Required for K\(^{+}\)-ATP-induced Disassembly of CCT—The requirement for additional factor(s) in
the K⁺-ATP-induced disassembly of the CCT particle indicated by the gel filtration analysis, was confirmed. Immunoprecipitation of the 20 S CCT particle purified by sucrose gradient fractionation (Fig. 7, middle panels), and of CCT additionally purified by anion exchange chromatography (Fig. 7, right panels), did not yield the single subunit immune precipitates observed with cell extracts (Fig. 7, left panels), but rather, reduced amounts of the core chaperonin complex. It is important to note that, due to dilution of sample by sucrose gradient fractionation followed by anion exchange chromatography, the concentration of CCT in the immunoprecipitation reactions for sucrose gradient samples was less than in the starting extract (gradient load). Nevertheless, even at this low concentration, core particle precipitation prevailed over single subunit precipitation for this partly purified CCT preparation. This, together with our observation that K⁺-ATP single subunit precipitation was unaffected by extract protein concentration over the 40-fold concentration range examined (Fig. 3), argues against the generation of single CCT subunits being a dilution artifact. K⁺-ATP-dependent immunoprecipitation of single subunits could be restored by the addition of a pool of the other fractions from the sucrose gradient fractionation, but not by the addition of resuspended pellet from the sucrose gradient (Fig. 8A, panels i-iv). Fraction-wise analysis revealed the additional component(s), which were non-dialyzable, to center on fractions 23 and 24 of a sucrose gradient resolved into a total of 24 fractions (Fig. 8B). i.e. smaller than the bulk of proteins fractionating toward the top of the gradient (Fig. 8C).

**DISCUSSION**

We are not aware of any previous reports specifically defining the K⁺ ion requirement of the CCT ATPase, although a range of K⁺ ion concentrations (0–100 mM) have been used in investigations of CCT structure and function. This is, in retrospect, surprising in view of the K⁺ ion dependence of other molecular chaperone ATPases including GroEL (48), mammalian hsp60 (49), and mammalian hsc70 (50), this latter showing a dependence on physiological levels of K⁺ ions (but not Na⁺ ions) similar to those effective in activating the CCT ATPase. In this report we have demonstrated an excellent correlation between the level of CCT ATPase activity and the liberation of free subunits from the core chaperonin complex. Specifically, an increase in both with increasing K⁺ and ATP concentrations, a decrease in both with increasing Ca²⁺ ion concentration and the higher K_m (43) and lesser effectiveness of GTP in liberating free CCT subunits. Collectively, then, our data show that, at intracellular levels of K⁺ ions and ATP, there is likely to be considerable disassembly of the CCT complex to a smaller oligomeric species and free subunits (not microcomplexes).
These data would therefore explain the degree of non-colocalization of CCT subunits, which we have consistently observed by immunofluorescence microscopy (28, 32). Also, standard procedures for purification of the CCT particle would dilute K\(^+\) ions and ATP down to levels where reassembly of the core particle could be expected, based on our observation of the reversal of disassembly by appyrase addition, and would therefore lead to the bulk of CCT subunits purifying as the heterooligomeric core particle, as has been observed.

There seems to be a propensity toward varying degrees of ATP-dependent dissociation of the double toroid structure even within group I chaperonins. GroEL itself can undergo a K\(^+\)-ATP-dependent heptamer exchange reaction with a second group I chaperonin (51), presumably via single ring intermediates (52), though this process is not reversible. The eukaryotic organellar group I chaperonins (mitochondrial hsp60 and plastid cpn60) dissociate not only to single heptameric rings, which in the case of hsp60 is folding-competent (13) and is probably the normal oligomeric state of this chaperonin (12), but also to monomers in the presence of MgATP, and particularly at low temperatures (53–55). Most of these changes in oligomeric status do not occur in the presence of non-hydrolyzable ATP analogs (51, 54). However, although there are parallels between these dissociation phenomena and the disassembly of CCT reported here, there are significant differences. The dissociation of plastid cpn60 is strongly dependent on protein concentration (56, 57) and is therefore considered unlikely to be significant at the high concentrations of cpn60 inside the chloroplast. Dissociation of plastid cpn60 is highly specific for ATP; in contrast to the disassembly of CCT, GTP will not replace ATP in plastid chaperonin disassembly (53). We reported here that Ca\(^{2+}\) ions inhibit both CCT ATPase and the generation of free CCT subunits, whereas Ca\(^{2+}\) can replace Mg\(^{2+}\) in ATP-induced disassembly of plastid cpn60. Finally, disassembly of type I chaperonins does not appear to be dependent on additional factors, e.g., mitochondrial hsp60 dissociation is observed with highly purified protein (55). In contrast, we found K\(^+\)-ATP-induced disassembly is not an intrinsic property of CCT, but rather, is dependent on the presence of additional factor(s). So, it is possible that the capacity for dissociation present in group I chaperonins has become more extensive, more readily reversed and regulated by additional factors in group II chaperonins, to the extent that it may have become an integral part of the functional cycle of this type of chaperonin.

What is the functional significance of the K\(^+\)-ATP-induced disassembly of CCT? It could be involved in the biosynthesis of CCT via the semi-conserved replication model put forward by Willison’s group (29), or, of course, it could be an integral part of the protein folding cycle of the CCT complex, as has been suggested for the Sulfolobus chaperonin (30). This will be determined, once the additional components (Fig. 8) have been isolated, by folding assays with pure CCT in the presence and absence of disassembly factor(s). Availability of pure components will also be required for an electron microscopic study of K\(^+\)-ATP-induced CCT disassembly. From the report of Llorens et al. (31), we know at present that ATP, like AMP-PNP, binding to CCT causes extensive opening out of subunits within one of the two chaperonin rings, but since these specimens were prepared in the absence of K\(^+\) ions, structural changes consequent to ATP hydrolysis and the presence of the additional disassembly factor(s) could not have been observed. An electron microscopic examination will be particularly informative also because some idea of the time scale of the disassembly process will be obtained. The observations described in this present report are all based on quite prolonged (minimally 3 h) experimental procedures.

If CCT disassembly is to a single ring plus monomers, then our finding that some CCT subunits, e.g. CCT\(\gamma\) and \(\delta\), dissociate more readily than others, e.g. CCT\(\gamma\), would argue against the CCT particle being two identical rings, each one containing all eight CCT polypeptides. However, in the absence of definitive electron microscopic data, it is not possible at present to say whether the residual oligomeric species is a single ring enriched in particular subunits such as CCT\(\gamma\), or is two identical partial rings from which certain subunits, e.g. CCT\(\delta\), have dissociated. The answers to these questions, and an assessment of the functional significance of the K\(^+\)-ATP-induced disassembly of CCT, must await further experimentation. However, this previously overlooked property of CCT is likely to be
of fundamental significance in understanding the mode of action of the eukaryotic cytosolic chaperon in the environment of the cell.

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