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GroEL/GroES-Mediated Folding of a Protein Too Large to Be Encapsulated

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Summary

The chaperonin GroEL binds nonnative proteins too large to fit inside the productive GroEL-GroES *cis* cavity, but whether and how it assists their folding has remained unanswered. We have examined yeast mitochondrial aconitase, an 82 kDa monomeric Fe_4S_4 cluster-containing enzyme, observed to aggregate in chaperonin-deficient mitochondria. We observed that aconitase folding both in vivo and in vitro requires both GroEL and GroES, and proceeds via multiple rounds of binding and release. Unlike the folding of smaller substrates, however, this mechanism does not involve *cis* encapsulation but, rather, requires GroES binding to the *trans* ring to release nonnative substrate, which likely folds in solution. Following the phase of ATP/GroES-dependent refolding, GroEL stably bound apoaconitase, releasing active holoenzyme upon Fe_4S_4 cofactor formation, independent of ATP and GroES.

Introduction

The double-ring chaperonin GroEL has been shown to mediate ATP-dependent folding of a variety of proteins (for reviews, see Horovitz, 1998; Sigler et al., 1998; Feltham and Gierasch, 2000). This is achieved for such proteins as monomeric rhodanese (33 kDa) and the subunits of malate dehydrogenase (33 kDa) and Rubisco (50 kDa) by binding of nonnative protein in an open GroEL ring through multiple hydrophobic contacts with the apical domains (Farr et al., 2000), serving to forestall misfolding and aggregation (Goloubinoff et al., 1989; Martin et al., 1991; Ranson et al., 1995), followed by folding in an encapsulated, now hydrophilic, cavity formed upon ATP/GroES binding to the same (*cis*) ring (Sigler et al., 1998). For these substrate proteins, multiple rounds of binding and attempted folding in the *cis* cavity are required, with only ~2%–5% of substrate protein molecules reaching native form in any single cycle of a stoichiometric reaction (Weissman et al., 1994; Ranson et al., 1997; Rye et al., 1997). Other, typically smaller, proteins may be assisted by transient binding and release into solution from an open GroEL ring (Coyle et al., 1999), without requiring GroES, although a physiologic requirement for chaperonin assistance by such species seems unlikely.

The lifetime of a folding-active *cis* ternary GroEL-GroES-polypeptide complex is governed by the nucleotide cycle, which controls the binding and discharge of ligands from GroEL. Regarding complex formation, GroES binding to a GroEL ring requires ATP binding to the same ring. Because the two rings of GroEL behave asymmetrically with respect to ATP binding, acting cooperatively within a ring but anticooperatively between the rings (Yifrach and Horovitz, 1995), GroES binding occurs with corresponding asymmetry. The dissociation of a *cis* ternary ATP complex is then governed by *cis* ATP hydrolysis ($t_{1/2}$ ~10 s, the slowest step of the reaction cycle), because conversion of ATP to ADP permits rapid binding of ATP to the opposite (*trans*) ring, triggering allosteric discharge of the *cis* ligands (GroES, polypeptide, and ADP) (Rye et al., 1997, 1999). This step of *trans* ATP-mediated discharge is accelerated by binding of nonnative polypeptide on the *trans* ring, but is unaffected by added GroES. Subsequent ordered binding of GroES to the ATP-polypeptide-bound ring sets up a new folding-active *cis* complex. In this way, GroEL alternates its rings back and forth, forming and dissociating chambers that support the productive folding of substrates small enough to be encapsulated.

In the case of larger proteins—those >60 kDa, which are too big to fit within the *cis* cavity of GroEL-GroES—neither a requirement for the chaperonins nor a possible mechanism for assisted folding has been established. However, GroEL has been shown to be able to bind to such larger species. For example, when total ^{35}S-labeled *E. coli* proteins were unfolded in denaturant, then incubated with GroEL, many species larger than 60 kDa became associated with the chaperonin (Viitanen et al., 1992). Similarly, when pulse-radiolabeled *E. coli* cells or spheroplasts were lysed and immunoprecipitated with anti-GroEL antiserum, larger species were again observed to be associated (Ewalt et al., 1997; Houry et al., 1999); however, if pulse-chase was carried out in vivo, many of these species remained stably associated over a long period.

The action of GroEL on several specific larger proteins has been examined. GroEL binds nonnative forms of the 70 kDa tailspike protein of phage P22 both in vivo (Gordon et al., 1994) and in vitro (Brunschier et al., 1993), but releases it without refolding and with no effect of GroES. Chuang and coworkers observed that GroEL stably binds an 86 kDa $\alpha\beta$ heterodimer composed of 48 kDa and 38 kDa subunits, proposed to be an assembly intermediate of the $\alpha_2\beta_2$ E1 enzyme of mammalian branched chain ketoacid dehydrogenase (Chuang et al., 1999; Song et al., 2000). Upon addition of ADP and GroES, the heterodimer was reported to become encapsulated in *cis* underneath GroES, as it exhibited resistance to protease digestion. When ATP and GroES were added, active E1 tetramer was recovered, albeit over a period of hours. Contrasting results were obtained for

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an 86 kDa MBP-E1α fusion protein, which could not be encapsulated by GroES but required it for similar, very slow recovery of E1 activity (Huang and Chuang, 1999).

Given the potential difficulties in interpretation inherent in studying either a multimer or a chimeric protein, it seemed desirable to study a natural monomeric protein of large size to resolve whether and how GroEL/GroES assist folding of larger species (e.g., by a *cis* versus *trans* mechanism). Here, we have carried out a study of such a protein, yeast mitochondrial aconitase, a monomeric, 82 kDa, Fe_4S_4 cluster-containing enzyme of the Krebs cycle that catalyzes the isomerization of citrate to isocitrate. Rospert and coworkers reported earlier that, when the precursor form of this protein is imported into mitochondria deficient in either Hsp60 (GroEL) or Hsp10 (GroES), the imported protein lodged in insoluble aggregates, as compared with being fully soluble after import into wild-type mitochondria (Dubaquié et al., 1998), implying that the complete chaperonin system is required for proper folding of this enzyme. Studies here of the mature form of this enzyme, both expressed in intact *E. coli* and in vitro, confirm a requirement for both GroEL and GroES to enable production of active enzyme and elucidate a mechanism for GroEL-GroES-mediated folding of larger proteins.

Results

Both GroEL and GroES Are Required for Production of Native Yeast Aconitase in *E. coli*

When mature yeast mitochondrial aconitase was expressed in *E. coli* from a *lac*-regulated promoter on a high-copy plasmid (pAco), a large amount of enzymatic activity was detected in the cell extract (Figure 1a, lane 2), amounting to at least 30-fold more than in an equivalent amount of cell extract from untransformed *E. coli* (lane 1). When constitutively overexpressed GroEL and GroES were present, the activity recovered was increased an additional 1.5-fold (lane 3). Examination of cell extracts by SDS-PAGE revealed that, in the absence of additional GroEL/GroES, ~30% of the expressed enzyme was soluble, while the rest had apparently misfolded and aggregated (Figure 1b, lanes 3 and 4). In the presence of overexpressed GroEL/GroES, the percentage in the soluble fraction increased to ~40% (lanes 5 and 6), corresponding to the increase in activity. Strikingly, the recovery of activity required the coexpression of both GroES and GroEL. When aconitase was induced in the presence of overexpressed GroEL alone, no appreciable activity was recovered ([a], lane 4), and the expressed aconitase was found almost entirely in the insoluble fraction ([b], lanes 7 and 8). Overexpression of GroEL alone apparently prevented the expressed aconitase from reaching native form, either through sequestration and/or through unproductive release that resulted in aggregation. Notably, pulse-chase experiments using anti-GroEL antiserum to recover complexes showed that, in the setting of expression of GroEL alone, newly synthesized aconitase became bound to GroEL and remained bound during a chase period (Figure 1c). In contrast, in a strain overexpressing both GroEL and GroES, GroEL-bound aconitase was released during the

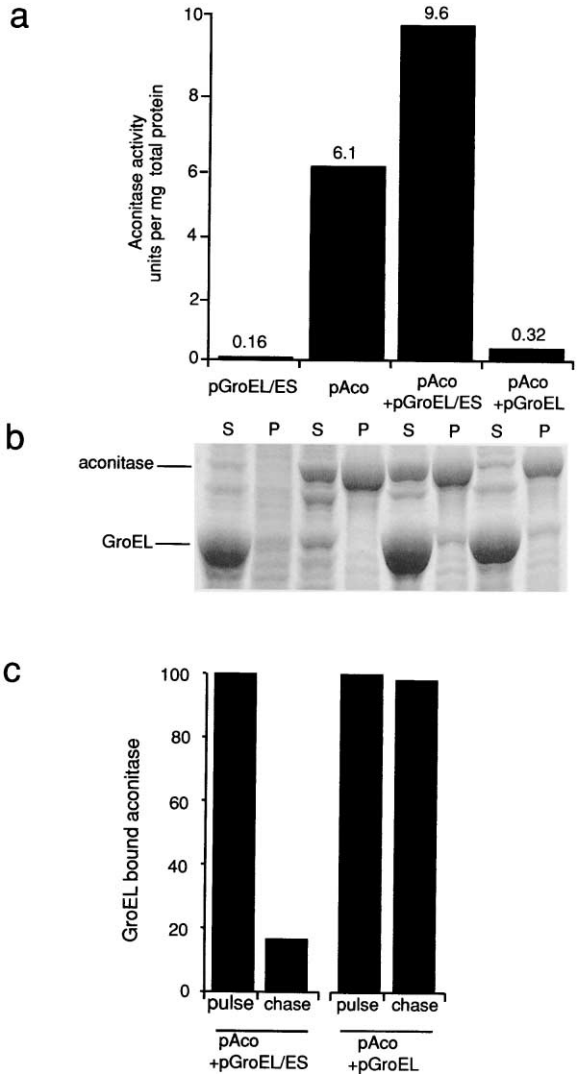

Figure 1. GroEL and GroES Are Both Required for Recovery of Yeast Aconitase Activity in *E. coli*

(a and b) Cooverexpression of GroEL/GroES increases the recovery of soluble active aconitase, but expression of GroEL alone blocks recovery of activity and, correspondingly, aconitase partitions entirely to the insoluble fraction. Extracts were prepared by sonication from equal amounts of cells expressing GroEL and GroES (pGroEL/ES) or aconitase (pAco) alone, aconitase with GroEL/GroES (pAco + pGroEL/ES), or aconitase with GroEL only (pAco + pGroEL). The extracts were centrifuged 10 min at 14,000 × g, and the soluble fraction assayed for enzymatic activity (a). In parallel, equivalent amounts of the soluble, S, and insoluble, P, fractions of the extracts were solubilized in SDS sample buffer and analyzed in SDS-PAGE (b).

(c) Newly translated aconitase in *E. coli* is bound to GroEL and released from it in the presence but not absence of GroES. Association of newly translated aconitase with GroEL and release from it were measured by immunoprecipitation with anti-GroEL antiserum of extracts of pulse-radiolabeled and chased cells expressing aconitase with both GroEL and GroES (pAco + pGroEL/ES) or aconitase with GroEL alone (pAco + pGroEL). Cultures were pulsed with [^{35}S]methionine, aliquots were chased with unlabeled methionine, and extracts prepared as in Experimental Procedures. GroEL in the soluble fraction was immunoprecipitated with anti-GroEL antiserum, and the amount of aconitase associated with it quantitated by Phosphorimager analysis after SDS-PAGE of the immunoprecipitates. In each case, the pulse value was set to 100.

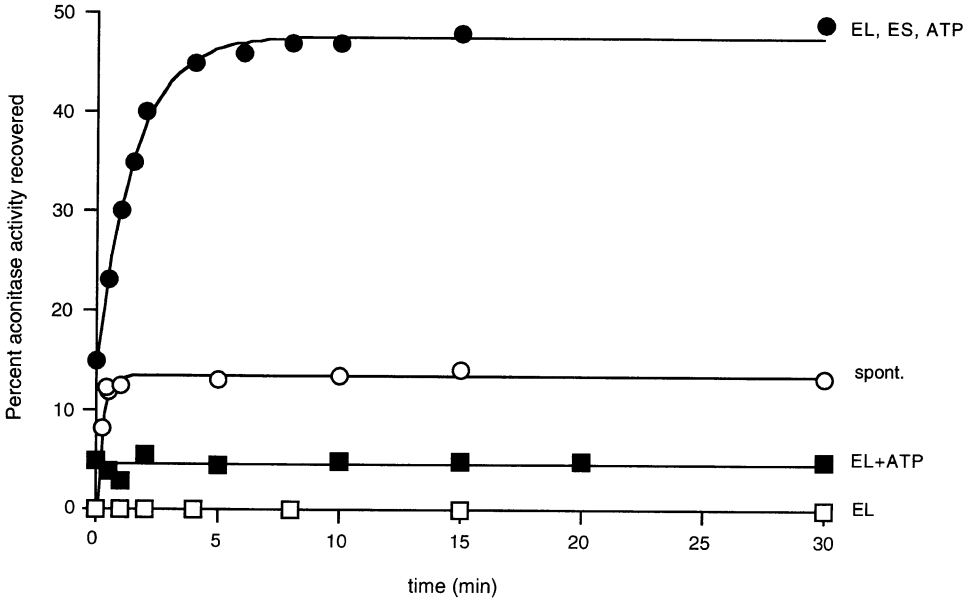

Figure 2. GroEL, GroES, and ATP Mediate Refolding of Aconitase In Vitro

Aconitase unfolded in acid was diluted directly into refolding buffer containing the indicated additions: GroEL (EL), GroES (ES), ATP, or none (spont.), and refolding was allowed to proceed without the isolation of binary complexes. At the times indicated, the reaction was halted by addition of hexokinase/glucose. Iron-sulfur cluster formation was then carried out to convert refolded apoaconitase to the holo form, and aconitase activity was assayed and expressed as a percentage of that obtained with an equivalent aliquot of aconitase that had not been acid unfolded.

chase (Figure 1c). This suggested that GroES might be required for timely release of GroEL-bound aconitase.

Both GroEL and GroES Are also Required for Reconstitution of Aconitase Activity In Vitro

To further evaluate the role of GroES in assisting productive folding of aconitase by GroEL, a chaperonin-mediated refolding reaction was reconstituted in vitro to produce apoenzyme, followed by addition of the Fe_4S_4 cluster under anaerobic conditions to produce active holoenzyme. When aconitase was unfolded in glycine phosphate (pH 2) then diluted into buffer with the complete chaperonin system, i.e., GroEL, GroES, and ATP, ~50% of activity was recovered by 4 min (Figure 2). Denaturation in 8 M urea or 6 M guanidine HCl gave similar results (not shown). By contrast, only ~10%–15% activity was recovered in the absence of chaperonin (spontaneous). The zero time point of this latter trace corresponds to direct addition of the Fe_4S_4 cluster-generating reagents to the denatured protein, showing that they are insufficient alone to produce proper refolding and recovery of activity. Moreover, apoaconitase was not recovered if GroEL/GroES/ATP were added 5 or 10 min after dilution of aconitase from denaturant (not shown). These observations are consistent with the earlier observation of wholesale aggregation of aconitase imported into Hsp60-deficient mitochondria (Dubaquié et al., 1998). Thus, aconitase appears to require assistance from the chaperonin system both in vivo and in vitro. This likely extends to the case of the yeast enzyme expressed in *E. coli* where, even without GroEL/GroES overexpression, productive folding was likely reliant on the endogenous GroEL/GroES proteins.

In parallel with the results from intact *E. coli*, the addition of GroEL alone to an in vitro refolding mixture abrogated the refolding of aconitase diluted from denaturant (Figure 2). This was associated with formation of a stable binary complex of aconitase with GroEL (see Figure 7), as well as with aggregation of a fraction of the input protein (not shown). The addition of ATP did not improve the recovery of activity (Figure 2). These results in vitro, in agreement with those in vivo, indicated a role for GroES in chaperonin-mediated folding of aconitase, potentially at the level of release of substrate protein.

GroES Fails to Encapsulate GroEL-Bound Aconitase

To further examine the requirement for GroES, we asked whether the cochaperonin could bind to the same ring as aconitase and encapsulate the protein in a *cis* complex, the normal site of productive folding of smaller substrate proteins such as rhodanese (33 kDa), MDH (33 kDa), and Rubisco (50 kDa) (Weissman et al., 1994; Ranson et al., 1997; Rye et al., 1997). Radiolabeled aconitase was diluted from acid into buffer with GroEL, forming a binary complex that was purified by gel filtration. The complex was mixed with GroES in the presence of various nucleotides, and protection of aconitase from digestion by proteinase K was assessed as a measure of GroES encapsulation. In all cases, the protein was degraded (Figure 3), as compared with substantial protection of Rubisco as a control (not shown). Included was a test of the ability of the transition state analog of ATP, ADP·AlF_x, to produce GroES encapsulation. Such incubation has recently been shown to trigger productive folding of such smaller proteins as rhodanese and

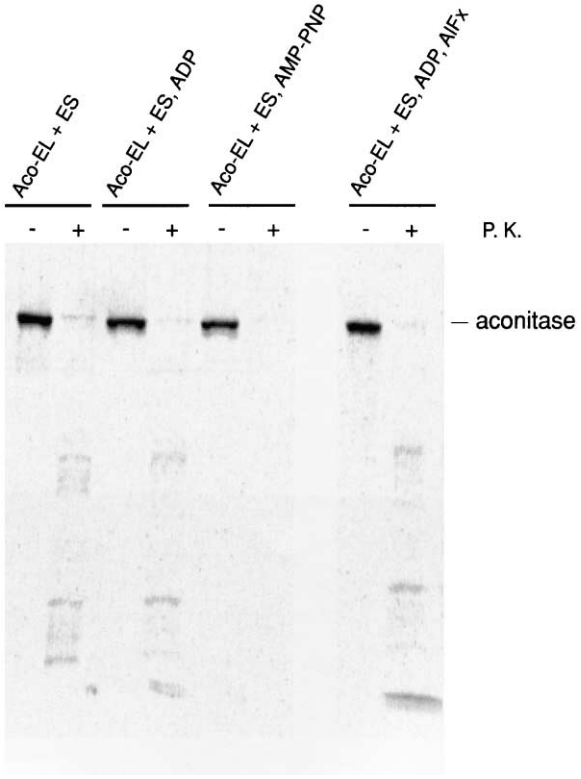

Figure 3. GroES Does Not Encapsulate GroEL-Bound Aconitase

Addition of GroES and nucleotide to aconitase-GroEL binary complexes is unable to protect aconitase from digestion by added proteinase K. Aconitase-GroEL binary complexes were formed by dilution of [^{35}S]aconitase from acid into buffer containing GroEL, and the complexes were purified by gel filtration. GroES was added in 2-fold molar excess in the presence of the indicated nucleotides. For the ADP and AMP-PNP reactions, proteinase K was directly added to half of the sample; after 10 min, PMSF was added, and the reaction mixture was applied to SDS-PAGE. For ADP·AlF$_x$, the mixture was gel filtered after adding ADP and AlF$_x$ to remove unbound nucleotide and AlF$_x$, then treated with proteinase K in the same manner.

Rubisco inside *cis* complexes that are stable for many hours (C. Chaudhry, A.H., and H. Rye, unpublished). Yet with ADP·AlF$_x$ as well, aconitase failed to be protected from proteolysis (Figure 3). Thus, GroES could not form a *cis* ternary complex with aconitase-GroEL, and this suggested that the function of GroES in supporting aconitase folding might be supplied in *trans*, i.e., by binding the ring opposite that containing the nonnative protein. Previous studies have indeed indicated that a ring opposite one bound by ATP/GroES lacks significant affinity for polypeptide (Yifrach and Horovitz, 1996; Rye et al., 1999), so, in the case of aconitase, its release might be accomplished by such binding of ATP/GroES in *trans*.

GroES Functions by Binding in *trans*

The topological requirement for GroES in *trans* was investigated further by asking whether SR1, a single ring version of GroEL, could produce native aconitase upon addition of ATP/GroES. Because of the absence of a second ring, GroES acts obligately only in *cis*, on the polypeptide-bound ring. Both SR1-aconitase and double-ring (wild-type) GroEL-aconitase binary complexes were formed and purified by gel filtration. Upon addition of ATP/GroES, whereas wild-type GroEL complex produced ~75% recovery of activity of the aconitase bound in the starting binary complex, SR1 complex produced only ~15% recovery of the aconitase activity (Figure 4). To more directly assess the requirement for interactions of GroES with a ring in *trans*, a similar experiment was carried out with a mixed double-ring complex, MR1, able to bind ligands normally on one wild-type ring, but unable to bind GroES or polypeptide on the opposite ring as the result of mutations in its apical domains. Aconitase diluted from acid was as efficiently captured by MR1 as by wild-type GroEL (not shown), but when the gel filtration-purified complex was incubated with ATP/GroES, only ~10% of activity was recovered (Figure 4). We thus conclude that ATP/GroES binding to the ring in *trans* to bound aconitase is required for productive folding.

GroES Binding in *trans* Drives Release of Nonnative Aconitase

To directly address whether the specific role of GroES binding in *trans* is to trigger release of aconitase, another form of GroEL was required to capture any released, nonnative aconitase (note that "nonnative" refers to an ensemble of unfolded and partially folded conformations, and specifically excludes apoenzyme). Unfortunately, the "trap" variants of GroEL used previously for this purpose did not bind aconitase sufficiently strongly to be used (not shown). Therefore, a biotinylated version of GroEL was produced, GroEL-bio, in which the three native cysteines of GroEL were substituted with alanine and in which Asp473 at the outside aspect of the equatorial domain was changed to cysteine and modified with biotin maleimide. The GroEL-bio molecule was observed to bind aconitase diluted from denaturant as efficiently as wild-type GroEL, and the biotinylated molecule was quantitatively captured by incubation with streptavidin magnetic beads, whereas wild-type GroEL did not associate with the beads. To measure aconitase release during folding, binary complexes of ^{35}S-methionine-labeled aconitase and wild-type, SR1, or MR1 complexes were formed and purified by gel filtration. GroEL-bio was added (in 2-fold molar excess), followed by addition of ATP/GroES (Figure 5a). After various times, the reaction was quenched with hexokinase/glucose, streptavidin beads were added, and after brief incubation, the beads were magnetically separated from the mixture. Scintillation counting of bead and supernatant fractions was carried out to address whether the [^{35}S]aconitase had been released and partitioned to GroEL-bio.

In control reactions in the absence of added ATP or GroES, a small amount (<10%) of the input aconitase was recovered with the avidin beads, but this occurred regardless of whether GroEL-bio had been added to the mixture and thus likely represents a background of nonspecific trapping of soluble GroEL-aconitase complex with the avidin beads. When ATP/GroES was added to the aconitase-GroEL binary complexes and incubation carried out for 10 min in the presence of GroEL-bio, ~50% of the input aconitase became associated

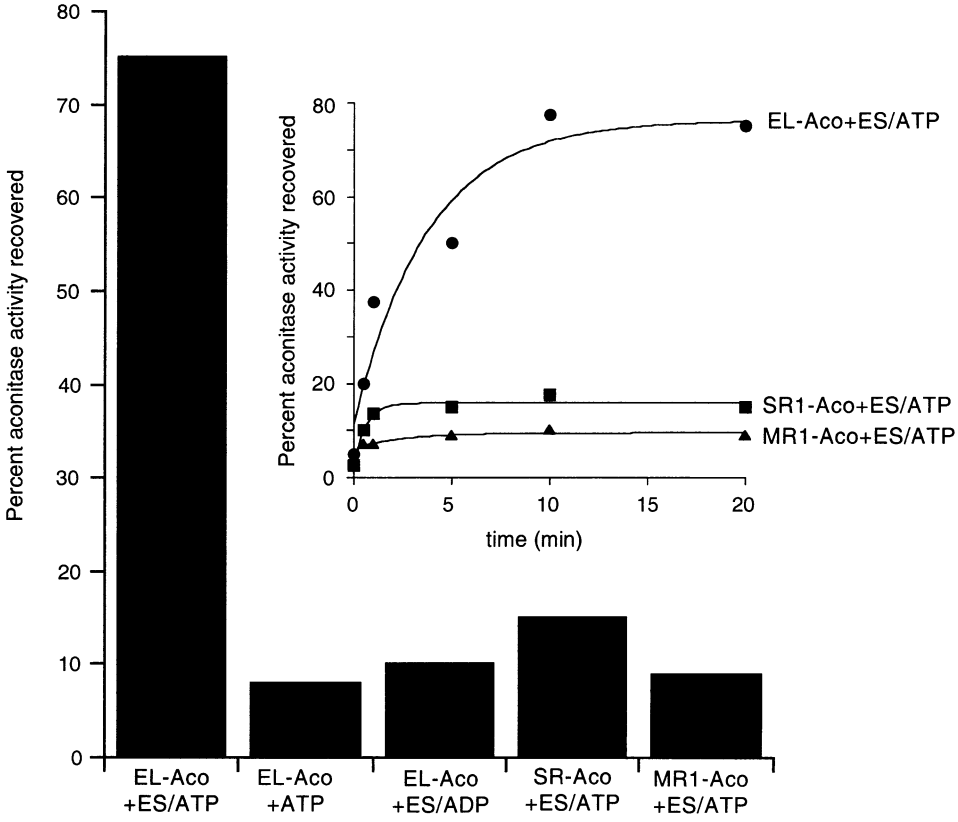

Figure 4. Productive Folding Requires GroES Binding in *trans*

Productive folding occurs upon addition of ATP/GroES to aconitase-GroEL binary complex, but not upon addition to binary complexes with SR1, a single-ring version of GroEL, or with MR1, a mixed double-ring version, able to bind polypeptide and GroES on only one ring. Binary complexes between aconitase and the respective GroEL molecules were formed by dilution of aconitase from acid into buffer containing the chaperonin; the binary complexes were purified by gel filtration, and ATP alone, GroES/ADP, or GroES/ATP were added. The reactions were quenched by addition of hexokinase/glucose, iron-sulfur reconstitution was carried out, and aconitase activity was assayed. Bar graph shows extent of refolding after 20 min reaction, as a percentage of the potential aconitase activity in the respective binary complexes. The inset shows the time course for the GroES/ATP-mediated reactions.

with the avidin beads (Figure 5b, upper panel). This reflects release of the bound substrate protein and capture by GroEL-bio, because it was dependent on the presence of GroEL-bio and on addition of both ATP and GroES. In particular, in the absence of GroEL-bio, only ~10% of the aconitase was found with the beads, corresponding to the level of background trapping (not shown). Likewise, addition of ATP alone produced only slightly greater radioactivity associated with the avidin beads than background (Figure 5b, upper panel). These results thus provide direct evidence that ATP/GroES is required for release of nonnative aconitase. In addition, such release was mediated in *trans*, because no transfer above the background was observed upon addition of ATP/GroES to binary complexes of [^{35}S]aconitase with either SR1 or MR1 (Figure 5b, lower panel). Thus, the folding deficiency observed for SR1 and MR1 complexes is directly attributable to their inability to release bound aconitase.

Based on the behavior of the chaperonin system with other substrate polypeptides, it seems likely that all of the bound nonnative aconitase molecules are discharged with each round of the chaperonin cycle. The recovery of ~50% of the initially GroEL-bound aconitase with GroEL-bio following addition of ATP/GroES probably reflects both the inability of GroEL-bio to irreversibly capture nonnative aconitase molecules and the resulting competition for binding between GroEL-bio and the wild-type GroEL molecules present in the solution.

The observation that ATP/GroES is required in *trans* to trigger release of aconitase differs strikingly from earlier studies of the discharge of encapsulated substates from *cis* ternary GroEL-GroES-polypeptide complexes, where ATP binding alone in *trans* was sufficient to trigger dissociation of the *cis* ligands (see Introduction and Rye et al., 1997). In particular, in these cases, GroES in *trans* was neither required for release nor had any effect on the kinetics of release (Rye et al., 1999).

Multiple Rounds of Release/Rebinding of Aconitase during Productive Folding

The foregoing experiments indicate that productive folding of aconitase is associated with at least one round of release from GroEL. But does a substantial fraction of aconitase molecules require multiple rounds of release and rebinding before reaching the apo form, as is the case for GroES-encapsulated substrates such as rhodanese, MDH, and Rubisco? To address this ques-

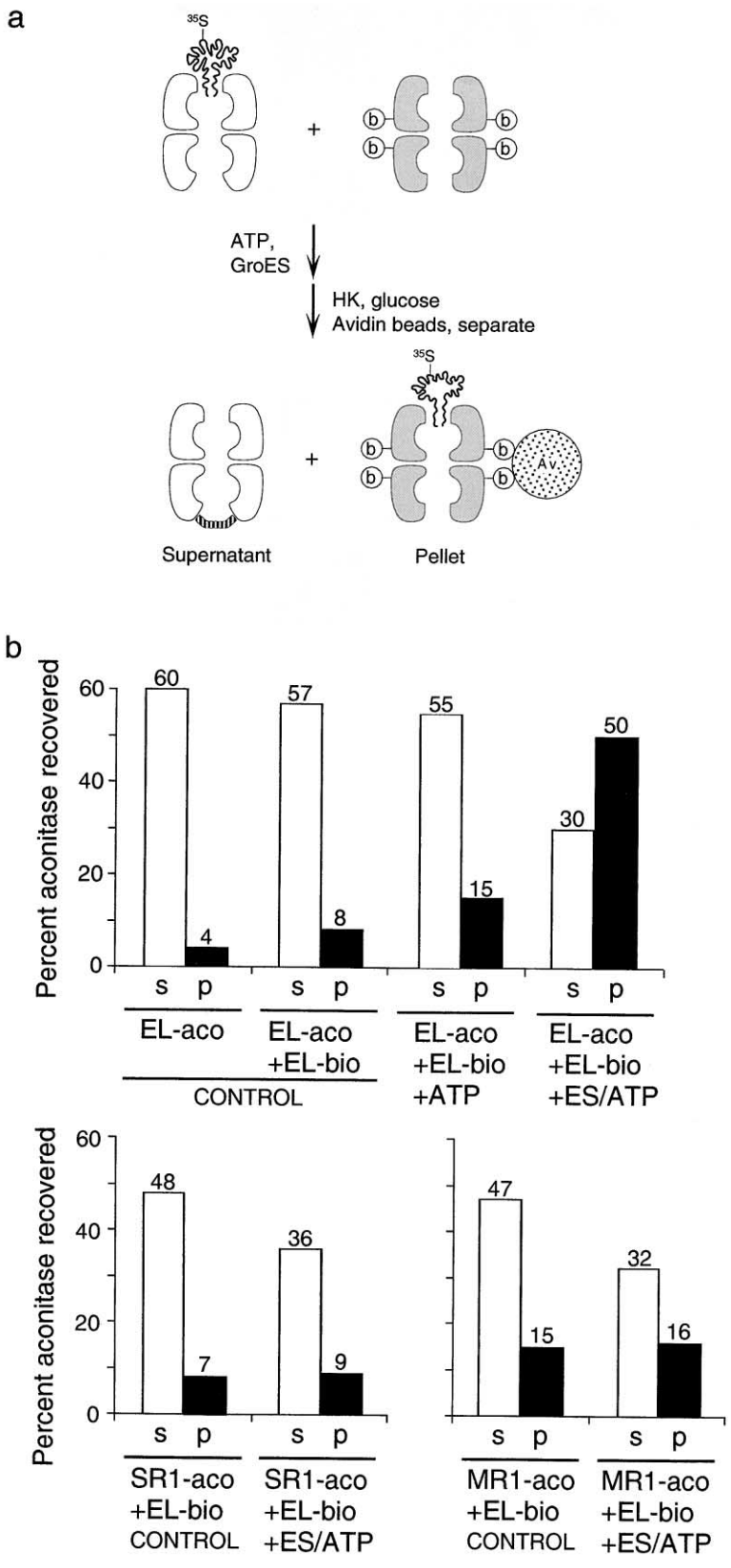

Figure 5. The Action of GroES in *trans* Triggers Release of Aconitase

Release of aconitase, detected via capture by GroEL-bio, is observed from wild-type GroEL, but not SR1 or MR1, upon addition of GroES/ATP.

(a) Experimental scheme. A binary complex of [^{35}S]aconitase and the respective chaperonin was formed and purified by gel filtration and mixed with GroEL-bio. ATP and GroES were added, and, after 10 min, the reaction was quenched with hexokinase/glucose, streptavidin magnetic beads were added, and the beads collected magnetically. Release from GroEL was detected by transfer of radioactivity to GroEL-bio associated with the beads. Note that GroEL-bio is not a "trap" molecule—transfer in the reverse direction, back to wild-type, can occur.

(b) Transfer of aconitase to GroEL-bio from wild-type GroEL, but not from SR1 or MR1. The upper panel shows experiments with wild-type GroEL-aco complex (EL-aco), the lower panel with the corresponding SR1 and MR1 complexes. Control experiments were without GroEL-bio addition or with GroEL-bio alone added to each binary complex. Experimental incubations were with addition of GroEL-bio and either ATP (+EL-bio+ATP) or GroES and ATP (+EL-bio+ES/ATP). Data are presented as the percentage of the input [^{35}S]aconitase recovered in the supernatant (s) or the magnetic beads (p) after separation. There is a small percentage of transfer to GroEL-bio in the control incubations, ranging from 4%–15%, but note that neither ATP alone in the case of wild-type GroEL, nor GroES/ATP for SR1 or MR1, produced significant increases in transfer above this background. Note also that recoveries of radioactivity were typically ~50%–70% of the input, with significant losses on tube walls even in the control samples.

tion and determine what fraction of aconitase reaches native form in one round of release, we utilized the GroEL mutant, D398A (Rye et al., 1997). This mutant binds ATP with an affinity similar to wild-type, but hydrolyzes it at a rate only ~2% that of wild-type. Accordingly, addition of ATP/GroES to an Aco-D398A binary complex should

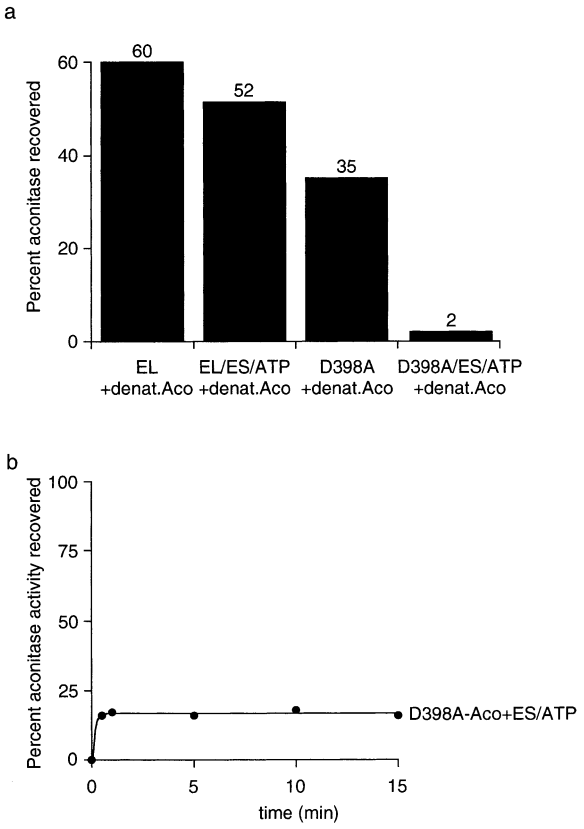

Figure 6. Only a Fraction of Aconitase Molecules Bound to GroEL D398A Reach apo Form in One Round of Release and Folding

(a) Denatured aconitase binds to unliganded D398A, but not to a D398A-GroES-ATP complex. [^{35}S]Aconitase was acid-denatured and diluted into refolding buffer containing wild-type GroEL (EL) or D398A or containing the respective chaperonins incubated briefly with GroES (ES) and ATP to permit formation of GroEL-GroES-ATP complexes. The mixtures were subjected to gel filtration chromatography as described. The numbers over the bars are the percentage of the input radioactivity recovered with the chaperonin peak.

(b) Recovery of aconitase activity from D398A-aconitase after addition of ATP/GroES and reconstitution. Binary complexes of aconitase with D398A, prepared and purified as above, were incubated with GroES and ATP for the indicated times and quenched with hexokinase/glucose. Holoaconitase was reconstituted and its activity measured. The ordinate reflects the percentage of the potential aconitase activity in the binary complex recovered after reconstitution.

produce *trans*-driven release of aconitase and one round of folding. However, released nonnative molecules should be unable to rebind to the chaperonin and to undergo further rounds of release and folding, because the open ring of the asymmetric complex will lie opposite a ring still occupied by GroES and unhydrolyzed ATP, a state unable to bind polypeptide (Rye et al., 1999). Indeed, when [^{35}S]aconitase is diluted from denaturant into a mixture of D398A, GroES, and ATP, it fails to bind to the chaperonin (Figure 6a).

A folding reaction was carried out with Aco-D398A binary complex and ATP/GroES, quenched at various times with hexokinase/glucose. At the earliest time point (30 s), approximately 20% of aconitase activity was recovered (Figure 6b). As predicted, at later times up to 15 min, no additional activity was recovered (Figure 6b). By comparison, in a similar reaction with a wild-type GroEL-aconitase complex (Figure 4), the recovery of activity at 30 s was similar ($\sim$20%), but it increased during the subsequent minutes to $>$70%. Thus, we conclude that aconitase refolding involves multiple cycles of binding by GroEL and GroES-driven release in *trans*, with $\sim$20% of the molecules reaching the folded apo form in any given round.

Refolded Apoaconitase Remains Bound to GroEL and Is Released by Fe_4S_4 Cluster Formation

To our surprise, following completion of GroEL-GroES-ATP-directed refolding, aconitase was recovered in gel filtration exclusively in association with GroEL (800 kDa fraction) (Figure 7, panel 4), as opposed to migrating as a released monomer (82 kDa). Remarkably, addition of Fe(II) and Na_2S, under anaerobic and reducing conditions to allow Fe_4S_4 cluster formation (Kennedy and Beinert, 1988), prompted release of the holoenzyme from GroEL (Figure 7, panel 5). This step did not require ATP/GroES, nor was it affected by their presence (Figure 7, cf. panels 4 and 5 and Supplemental Figure S1, available online at http://www.cell.com/cgi/content/full/107/2/235/DC1). If the apo form produced by chaperonin-mediated refolding is the same as that produced by removal of the Fe_4S_4 cluster from the holoenzyme, we would predict that the latter species would also associate with GroEL. Indeed, when the holoenzyme was incubated with EDTA/$K_3Fe(CN)_6$ and then with GroEL, the apo form produced was now observed to associate with GroEL (Figure 7, panel 6). As with the refolded apo form, this complex could again form the holoenzyme by readdition of Fe(II) and Na_2S.

Discussion

Folding of a Larger Protein by GroEL through Release into Solution and Rebinding

The experiments reported here indicate that the GroEL chaperonin can assist the folding of a larger protein, too big to fit within the central (*cis*) cavity of a GroES-bound GroEL ring, by a mechanism that nevertheless relies on GroES, in *trans*, to trigger polypeptide release and enable productive rounds of folding (Figure 8). While the nucleotide cycle of this *trans*-operating mechanism appears to be the same as that employed in *cis* folding, and while there are also cycles of polypeptide binding and release as with the *cis* folding reaction, there are fundamental differences between the two mechanisms, concerning both the fate of polypeptide and the action of GroES (see following section). Regarding the polypeptide, the larger nonnative protein is released into the bulk solution, instead of into the sequestered and hydrophilic chamber produced when GroES binds to a GroEL ring containing a smaller protein that can be encapsulated within the *cis* space. Correspondingly, the steps of productive aconitase folding seem likely to be taking place in the bulk solution, although the dwell time in that location may be far shorter ($t_{1/2} < 1$ s) than that experienced by a polypeptide in the *cis* cavity ($t_{1/2} \sim 10$ s) (Weissman et al., 1994; Rye et al., 1999). Therefore, the question arises as to what the advantage is to the cell of em-

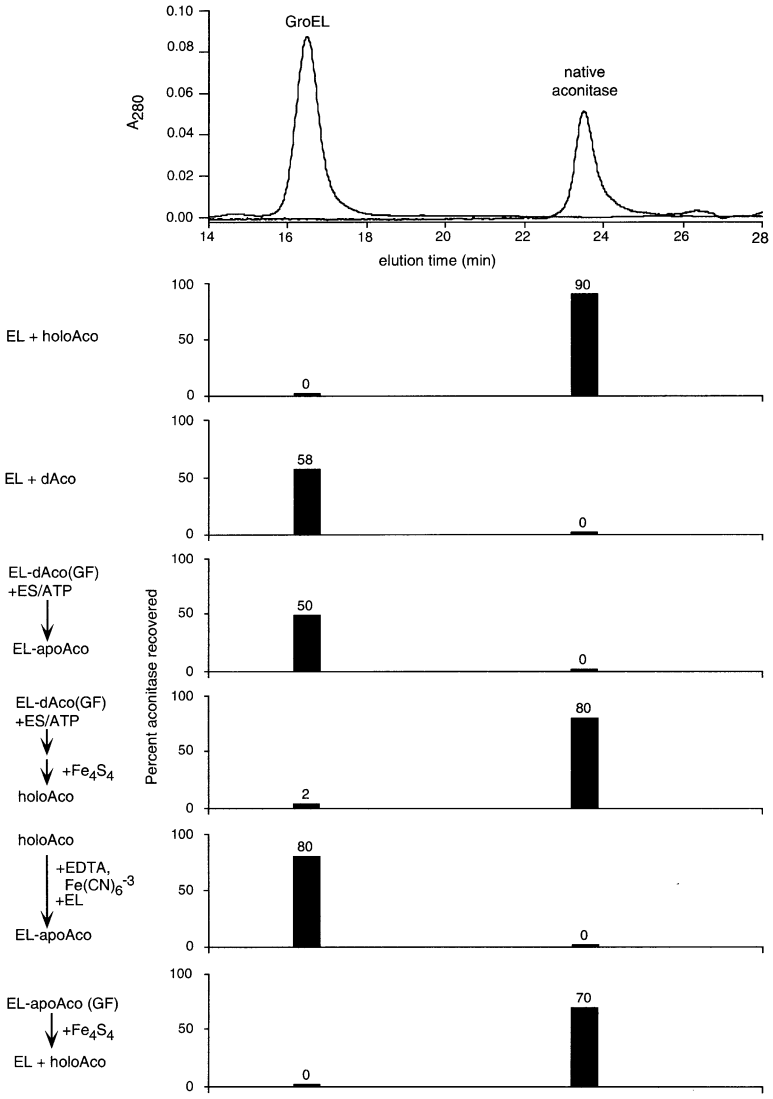

Figure 7. Refolded Apoaconitase Is Stably Bound by GroEL, and Release Is Triggered by Iron-Sulfur Cluster Formation

[^{35}S]Aconitase and GroEL were mixed under a variety of conditions, and the reaction mixtures were applied to a gel filtration column. The top panel shows typical elution profiles for GroEL and native holoaconitase overlaid on the same graph. The bar graphs in the other panels indicate the fraction of the input radioactivity recovered at the elution positions of GroEL and aconitase. Second panel, GroEL plus native holoaconitase; third panel, aconitase denatured in acid (dAco) and diluted into buffer with GroEL; fourth panel, gel filtration purified binary complex (EL-dAco [GF]) incubated with GroES and ATP for 20 min; fifth panel, the mixture used for panel four further incubated to produce the Fe_4S_4 cluster and holoaconitase; sixth panel, holoaconitase treated to remove the Fe_4S_4 cluster, then incubated with GroEL; seventh panel, gel filtration purified GroEL-aconitase complex (EL-apoAco[GF]) from panel six incubated with reagents to form the Fe_4S_4 cluster and holoenzyme.

ploying the GroEL system for folding a larger protein if it cannot be encapsulated in a *cis* complex. This almost certainly must lie at the step of polypeptide binding, where, as with smaller substrates that ultimately fold in the *cis* cavity, the binding to an open GroEL ring serves to prevent irreversible misfolding and aggregation and has even been suggested to be able to reverse incipient misfolding (Ranson et al., 1995).

Presumably only a portion of a large protein like 82 kDa aconitase could be bound within the central cavity, while the remainder must lie outside in the bulk solution. As suggested earlier (Dubaquié et al., 1998), it may be that only one domain of the four in aconitase has kinetic difficulty reaching native form, and it is this domain that becomes recruited to the central cavity, while the other three domains lying in the bulk solution are essentially native and would not themselves recruit chaperones to their surfaces. Insofar as the most COOH-terminal domain of native aconitase lies somewhat apart in the crystal structure from the bodies of the other three, which house the Fe_4S_4 cluster (Lauble et al., 1992), this domain could potentially be responsible for misfolding. Various topology and crosslinking studies should be able to resolve whether aconitase is bound to GroEL with a characteristic topology.

The potential unfolding action exerted by an open GroEL ring on nonnative polypeptides, or on nonnative portions of larger proteins, has been described in terms of two potential mechanisms, which are currently under experimental study for smaller proteins. One mechanism involves thermodynamic partitioning, in which GroEL exhibits greater affinity for less-folded states among an ensemble of conformers that are in equilibrium with each other, effectively shifting that equilibrium by mass action toward less-folded states (Zahn and Pluckthun, 1994; Walter et al., 1996). Thus, GroEL would preferentially bind less-folded states without affecting the energy barriers that lie between the states. Studies with a mutant RNase T1 and with several other small proteins provide experimental support for such action (Walter et al., 1996; Clark and Frieden, 1999; Bhutani and Udgaonkar, 2000). The other mechanism involves catalysis of unfolding, in which binding by GroEL lowers the energy barriers between nonnative states, allowing for global unfolding

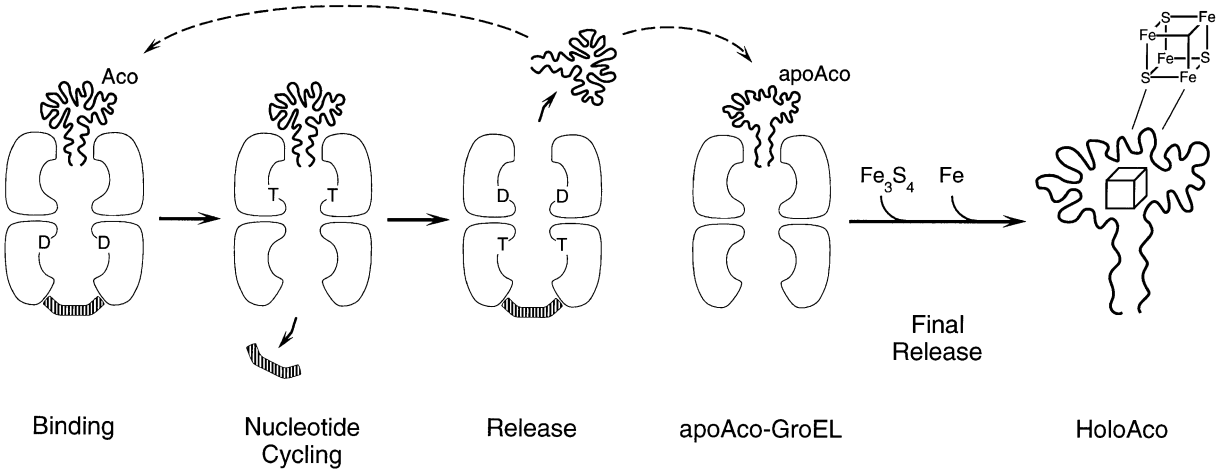

Figure 8. Model of GroEL-Mediated Folding of Aconitase, an 82 kDa Protein Too Large to Be Encapsulated by GroES, Showing Cycles of Binding and Release Driven by GroES in *trans*

In vivo, nonnative aconitase likely binds to the open ring of a GroEL-GroES-ADP complex (first panel), which then undergoes a round of ATP binding in the polypeptide-bound ring and associated GroES release from the opposite ring (second panel). Subsequent binding of ATP and GroES to the ring in *trans* to polypeptide releases the nonnative aconitase into solution, where a fraction of the molecules fold to apoaconitase and bind stably to GroEL (fourth panel). Those molecules not refolded during their lifetime in solution rebind to GroEL (first panel) to undergo another round of release and refolding. Aconitase is finally released from GroEL during the formation of the Fe_4S_4 cluster to produce holoenzyme (fifth panel). Two steps are shown in the formation of the cluster, reflecting the lability of the fourth iron in the native enzyme (Kent et al., 1985).

of a bound polypeptide. A hydrogen-deuterium exchange experiment with the 6 kDa protein, barnase, supports this, observing global unfolding with catalytic amounts of GroEL (Zahn et al., 1996). The multivalent nature of polypeptide binding of such substrate proteins as MDH (33 kDa) and Rubisco (50 kDa) by multiple apical domains simultaneously (Farr et al., 2000) could support either mechanism of unfolding action.

Release during Folding Driven by GroES Binding in *trans*

Whereas smaller substrates folding in *cis* GroEL-GroES complexes have been observed to be efficiently released by binding of ATP alone to the *trans* ring, without any requirement or kinetic effect of added GroES (Rye et al., 1997, 1999), here, ATP alone was insufficient to trigger significant discharge of bound nonnative aconitase, and only the presence of both ATP and GroES could accomplish release (Figure 5). This is likely a function of the state of the aconitase-bound GroEL ring, whose apical domains almost certainly occupy a very different topology from that occupied in a GroES-bound *cis* ring. In the latter case, as shown by both cryoEM and X-ray studies (Roseman et al., 1996; Xu et al., 1997), the apical domains are elevated $\sim$60° and twisted clockwise $\sim$90° (relative to an unliganded ring), with their hydrophobic polypeptide binding surfaces removed from the central cavity. Here, the apical domains of the ring occupied with bound aconitase are likely to remain in a state that is similar to the unliganded (binding-active) topology and far from that of a GroES-bound *cis* ring. As such, binding of ATP in the opposite *trans* ring may not be sufficient to produce allosteric apical movement that could eject aconitase. Only with the subsequent binding of GroES would a larger extent of apical movement that could release the substrate be allosterically transduced. Consistent with the last possibility, a cryoEM reconstruction of a GroEL-GroES-ATP complex showed that the apical domains of its *trans* ring underwent significant twisting movements that disrupted the polypeptide binding surface; moreover, this ring had no significant affinity for nonnative polypeptide (Rye et al., 1999).

In sum, then, a novel action for GroES is observed here in mediating the folding of a substrate protein too large for encapsulation, involving binding of GroES in *trans* to produce an allosteric change in the polypeptide-bound ring that ejects polypeptide. In some respects, this action may result from the failure of GroES to encapsulate the larger substrate protein in *cis*, leaving the *cis* ring in a state similar to an open ring. Does GroES have other particular actions in assistance of a larger substrate protein? Could it, for example, facilitate aconitase binding to the open ring of a GroEL-GroES-ADP acceptor complex? Notably, no such preference for binding aconitase was observed with this complex as compared with unliganded GroEL alone (see Supplemental Figure S2, available online at http://www.cell.com/cgi/content/full/107/2/235/DC1).

GroEL as a Holding Chamber for the apo Form of an Fe_4S_4 Cluster Protein

The observation that the refolded apo form of aconitase remains bound to GroEL is surprising, because in the absence of chaperonin, apoaconitase produced by removing the Fe_4S_4 cluster from the holoenzyme is stable in solution and remains competent to reform the holoenzyme for several hours (T.C., unpublished observations). While apoaconitase can be recruited to GroEL, it is not subject to misfolding and aggregation in its absence.

GroEL thus appears to act as a "holding tank" for the apo protein, which is released by subsequent cluster formation (Figure 8). Yet the surfaces on both GroEL and aconitase that retain the apo form at GroEL may be significantly different from those involved in retaining the nonnative state(s) of the protein, because apoaconitase is not released by ATP/GroES (Figure 7 and T.C., unpublished data). Could this imply that the apo form is held by contacts other than the hydrophobic ones involved in binding nonnative proteins? Further topologic studies and examination of the ability of various GroEL cavity mutants to bind nonnative and apo forms should resolve this.

Interestingly, involvement of chaperonins in the biogenesis of two other metal cluster-containing proteins has been reported recently. Formation of the Fe_2S_2 cluster in bovine apoadrenodoxin (14 kDa) was accelerated in vitro by substoichiometric amounts of GroEL alone, with no added effect of ATP and/or GroES (Iametti et al., 2001). In contrast with the present study, however, no stable complex between apoadrenodoxin and GroEL was observed. Rather, a transient interaction, with GroEL serving as a scaffold for the metal cluster insertion process, was proposed. Less well-resolved is a second report of a requirement for both GroEL and MgATP in the insertion of iron-molybdenum cofactor into the molybdenum-iron (MoFe) protein in the large nitrogen-fixing nitrogenase enzyme of *A. vinelandii* (Ribbe and Burgess, 2001). Based on the studies here, it seems possible that this action could lie at the level of folding, preceding or associated with cluster formation, and that this could potentially involve binding of a species larger than one able to fit inside a *cis* complex. Notably, the MoFe protein that receives the cofactor is a 230 kDa $\alpha_2\beta_2$ tetramer. Although both aconitase and nitrogenase are larger proteins bearing metal clusters, our conjecture is that other large proteins lacking metal clusters/cofactors will be found that also employ GroEL and GroES and a *trans*-operating mechanism for productive folding.

Experimental Procedures

Proteins

Chaperonins were expressed and purified as previously described (Weissman et al., 1995; Rye et al., 1997). MR1, containing one wild-type ring and a ring with the substitutions Y203E, G337S, and I349E, was prepared as previously described (Burston et al., 1996). GroEL D473C (in a cysteine-to-alanine substituted GroEL background) was generated by site-directed mutagenesis. Rubisco was expressed and purified as described (Rye et al., 1997). Isocitrate dehydrogenase was obtained from Sigma and hexokinase was obtained from Roche.

Aconitase Preparation

Aconitase holoenzyme was produced in *E. coli* from plasmid pAco, which contains the predicted mature coding sequence of the yeast mitochondrial enzyme, preceded by codons for Met and Leu, cloned into pQE60 (Qiagen). This plasmid was carried in strain M15 (Qiagen), which had been transformed with a second plasmid, pACYCGroESL, bearing the GroE operon and constitutively overproducing both chaperonins, or pACYCGroEL, identical except for deletion of GroES codons 38–94. Cultures grown at 25°C to an OD_{650} of 0.6 were induced with 50 μM IPTG, grown overnight at 25°C, and harvested. Cells were resuspended in 50 mM HEPES (pH 7.4), 0.5 mM $MgCl_2$, 1 mM DTT, and disrupted in a microfluidizer (Microfluidics Corp., Newton, MA), followed by centrifugation at 143,000 × g for 45 min. The lysate was applied to a 60 ml Q Sepharose Fast Flow column (Pharmacia) attached in tandem to a 25 ml CM Sepharose Fast Flow column (Pharmacia). The columns were washed with 20 mM HEPES (pH 7.4) until no absorbance at 280 nm was detected. The Q Sepharose column was removed, and the CM Sepharose column was further washed with 50–75 ml 20 mM HEPES (pH 7.4). Aconitase was eluted with 20 mM HEPES (pH 7.4), containing 0.5 mM *cis*-aconitate. Typically, 3 mg of pure aconitase was obtained from 6 liters of culture. Apoaconitase was prepared from holoenzyme by removing the Fe_4S_4 cluster using the procedure of Kennedy and Beinert (1988).

Aconitase Refolding

Denatured aconitase was prepared by diluting 100 μM holoenzyme 10-fold into 25 mM glycine phosphate (pH 2), and incubating at 25°C for 30 min. GroEL-aconitase binary complexes were formed by 10-fold dilution of denatured aconitase into refolding buffer (50 mM HEPES [pH 7.4], 10 mM KCl, and 10 mM $MgCl_2$) containing 1 μM wild-type GroEL, SR1, MR1, or D398A. Binary complexes were purified by gel filtration using a G4000SW_{xl} column (Tosoh Biosep) eluted with 50 mM HEPES (pH 7.4), 10 mM KCl, and 0.2 mM DTT, concentrated using a Centricon 30 at 25°C, and quantitated. Recovery of aconitase in the binary complexes was typically 60%–70% with wild-type GroEL and MR1, 20%–30% with SR1, and 30%–40% with D398A. Refolding reactions contained ~1 μM binary complex (based on chaperonin content) in refolding buffer and 2-fold molar excess of GroES relative to GroEL. Refolding was initiated by addition of ATP to 5 mM. At the indicated time points, refolding reactions were quenched with hexokinase and glucose (final concentrations 0.1 U/μl and 10 mM, respectively). In order to avoid interference by GroEL, the quenched mixtures were diluted 10-fold with refolding buffer before reconstitution.

Reconstitution of Apoaconitase to Holo Form

Addition of an Fe_4S_4 cluster to preformed apoaconitase (either after refolding or after removing the cluster from holoenzyme) was accomplished under anaerobic conditions following a procedure modified from Kennedy and Beinert (1988). Briefly, separate solutions containing either ~0.1 μM apoaconitase in refolding buffer, 20 μM ferrous ethylenediamine, 100 mM dithiothreitol (DTT), or 20 μM Na_2S were deoxygenated by incubation under flowing argon for 60 min at 25°C. Then, 0.1 ml ferrous ethylenediamine, 0.1 ml DTT, and 0.1 ml Na_2S were added sequentially in this order via gas-tight syringe to 1 ml of apoenzyme in a septum-sealed vial under argon. The mixture was incubated one hr at 25°C, then one hr at 4°C, before enzyme assay.

Aconitase Assay

Aconitase activity was quantitated using a coupled enzyme assay in which conversion of citrate to isocitrate by aconitase was detected by the subsequent conversion of isocitrate to α-ketoglutarate by isocitrate dehydrogenase with concomitant reduction of NADP to NADPH (Morrison, 1954). The aconitase assay was performed by mixing 500 μl of aconitase (3–5 μg, 35–60 pmol) with 500 μl of assay solution (0.1 M Tris-HCl [pH 8.0], 1.3 mM sodium citrate, 1.3 mM $MnSO_4$, 1 mg/ml β-NADP, and 0.34 mg/ml isocitrate dehydrogenase), and the reaction was followed at 340 nm.

Pulse-Chase Labeling

Pulse-chase experiments were carried out using spheroplasts according to Ewalt et al., 1997, with modifications. *E. coli* carrying pAco and either pACYCGroESL or pACYCGroEL were grown at 25°C in M63 minimal medium supplemented with 0.0005% thiamin, 0.2% glycerol, 0.2% glucose, and 40 μg/ml amino acids except methionine. Cells were induced at mid-log phase with 50 μg/ml IPTG, grown for 4 hr, harvested, and converted to spheroplasts as described. Spheroplasts, resuspended in the supplemented M63 medium, were labeled for 1 min with 860 μCi/ml [^{35}S]methionine, and either immediately recovered by centrifugation (14,000 × g, 1 min) and suspended in lysis buffer (50 mM Tris [pH 8], 5 mM EDTA, 2 mM unlabeled methionine, 2 mM cycloheximide, 0.5% Triton X-100, and 25 μg/ml RNase) or chased with 2 mM unlabeled methionine for 2 hr before recovery and lysis. The lysates were centrifuged 15 min at 15,000 ×

g, and GroEL and associated proteins were recovered from the soluble fraction by immunoprecipitation with anti-GroEL antiserum and protein A-Sepharose beads (Pharmacia). Proteins were released from the beads with SDS sample buffer and separated by SDS/PAGE. The gels were dried and subjected to Phosphorimager analysis.

Proteinase K Assay for GroES Encapsulation

Binary complexes were formed between 0.4 μM GroEL and 0.4 μM [^{35}S]aconitase as above and were incubated with 0.8 μM GroES in the presence of ADP or AMP-PNP for 10 min at 25°C. Proteolytic digestion was carried out with proteinase K at 400 ng/ml for 10 min at 25°C, followed by addition of PMSF to a final concentration of 1 mM. For an encapsulation test in the presence of ADP•AlF_x, binary complexes were first purified by gel filtration, then incubated for 10 min with 0.8 μM GroES, 5 mM ADP, 30 mM KF, and 3 mM $KAl(SO_4)_2$. The resulting complexes were purified by gel filtration and treated with proteinase K as above. After electrophoresis of the samples on SDS/polyacrylamide gels, the recovery of labeled aconitase was quantitated by Phosphorimager analysis.

Biotinylation of GroEL D473C

D473C GroEL (4 mg) was incubated overnight at 25°C with 1 mM 3-(*N*-maleimidylpropionyl)biocytin (MPB; Molecular Probes) in 0.4 ml 25 mM potassium phosphate (pH 7.4). The reaction was quenched by addition of 0.2 ml 0.1 M reduced glutathione. After 15 min at 25°C, glutathionyl-MPB and excess glutathione were removed by gel filtration on a PD-10 column (Pharmacia) in 25 mM potassium phosphate (pH 7.4), and the biotinylated D473C (GroEL-bio) was concentrated on a Centricon 30. The extent of biotinylation of GroEL-bio was estimated from the loss of free sulfhydryl groups in GroEL, as measured with 5,5′-dithio*bis*(2-nitrobenzoic acid).

Transfer of Aconitase to GroEL-bio

Binary complexes of [^{35}S]aconitase and chaperonins were formed, purified as above, and used in refolding reactions as described, except that a 2-fold excess of GroEL-bio relative to GroEL was added, and the GroES concentration was twice that of the total GroEL. The reaction was initiated by adding ATP and terminated after 10 min with hexokinase/glucose, as above. Washed Magna-Bind streptavidin beads (100 μl in 100 mM potassium phosphate [pH 7.4] and 150 mM NaCl [Pierce]) were added to the reaction mixture, incubated 5 min at room temperature, then separated magnetically and the radioactivity in the supernatant solution and beads determined.

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