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In Vivo Observation of Polypeptide Flux through the Bacterial Chaperonin System

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Summary

The quantitative contribution of chaperonin GroEL to protein folding in E. coli was analyzed. A diverse set of newly synthesized polypeptides, predominantly between 10–55 kDa, interacts with GroEL, accounting for 10%–15% of all cytoplasmic protein under normal growth conditions, and for 30% or more upon exposure to heat stress. Most proteins leave GroEL rapidly within 10–30 s. We distinguish three classes of substrate proteins: (I) proteins with a chaperonin-independent folding pathway; (II) proteins, more than 50% of total, with an intermediate chaperonin dependence for which normally only a small fraction transits GroEL; and (III) a set of highly chaperonin-dependent proteins, many of which dissociate slowly from GroEL and probably require sequestration of aggregation-sensitive intermediates within the GroEL cavity for successful folding.

Introduction

The mechanism of protein folding in the cell remains one of the central concerns of biology. Although the information required for folding is contained in the linear amino acid sequence of the polypeptide chain, efficient realization of that information in the cellular environment in many cases requires a machinery of preexisting proteins that assists newly synthesized polypeptides in folding. The molecular chaperones (Ellis, 1987) have been identified as key molecules in this process, based on their ability to bind to unfolded conformers of other proteins, prevent misfolding and aggregation, and thereby promote correct folding (reviewed in Ellis and van der Vies, 1991; Georgopoulos and Welch, 1993; Hartl, 1996). Much of this concept resulted from the discovery and analysis of the chaperonins (Hemmingsen et al., 1988), represented by GroEL and its cofactor GroES in the cytoplasm of Escherichia coli.

GroEL is a large cylindrical protein complex comprising two heptameric rings of 57 kDa subunits (Braig et al., 1994). Its function in protein folding has been analyzed extensively in vitro (reviewed in Hartl, 1996). Nonnative polypeptide binds in the central cavity of the GroEL cylinder near the outer rim. Folding usually occurs with the aid of GroES, a dome-shaped ring containing seven subunits of ~10 kDa (Hunt et al., 1996; Mande et al., 1996). Binding of GroES to the polypeptide-containing ring of GroEL results in the displacement of polypeptide into an enclosed cage, defined by the GroEL cavity and the dome of GroES, in which aggregation is prevented and folding to the native state is possible (Martin et al., 1993; Chen et al., 1994; Weissman et al., 1995; Hayer-Hartl et al., 1996; Mayhew et al., 1996; Roseman et al., 1996; Weissman et al., 1996). GroES binding to the polypeptide-containing ring of GroEL is accompanied by ATP hydrolysis in that ring (Martin et al., 1993; Todd et al., 1994). GroES is then released upon subsequent ATP hydrolysis on the opposite GroEL ring, permitting native or partially folded proteins to leave the chaperonin. About 20 s pass between GroES binding to GroEL and release (at 25°C) (Todd et al., 1994; Hayer-Hartl et al., 1995). As shown in vitro, usually only a fraction of GroEL-bound molecules complete folding within a single cycle. The remainder is rebound by GroEL and reset to an unfolded state in preparation for another folding trial (Corrales and Fersht, 1996; Mayhew et al., 1996). Some proteins, such as mitochondrial rhodanese, require multiple chaperonin cycles for folding.

While this basic mechanism is relatively well defined, little is known about the overall contribution of the chaperonins to protein folding in vivo. GroEL and GroES were originally identified as host factors for the assembly of phage particles (Georgopoulos et al., 1973), but both proteins provide essential cellular functions under all growth conditions (Fayet et al., 1989). In principle, this could result if GroEL facilitated the folding of just one or a few essential cellular proteins. Indeed, recent estimates suggest that, based on in vitro rates for GroEL-facilitated folding, the cellular complement of GroEL might only be adequate to fold 2%–7% of all newly synthesized proteins (Ellis and Hartl, 1996; Lorimer, 1996). In contrast, the analysis of the lethal phenotype of a temperature-sensitive GroEL mutant strain had previously suggested that about 30% of newly translated polypeptides depend on GroEL (Horwich et al., 1993). Curiously, the interaction between GroEL and its substrates in vivo has never been analyzed directly. Here we have investigated the flux of newly synthesized proteins through GroEL in living E. coli cells and upon cell-free translation.

Results

A Diverse Set of Newly Synthesized Proteins Interacts with GroEL

To determine the extent to which GroEL interacts with newly synthesized proteins, GroEL and its bound

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substrates were isolated by immunoprecipitation from pulse-labeled E. coli. Cells were labeled at temperatures between 30°C and 42°C for 15 s with [^{35}S]methionine followed by the addition of excess nonradioactive methionine. At specific times, cells were removed, rapidly cooled to 4°C, converted to spheroplasts, and lysed in EDTA-containing buffer (low temperature and chelating Mg^{2+} stop ATP-dependent polypeptide release from GroEL [Martin et al., 1991; Mendoza et al., 1991]). Alternatively, spheroplasts were generated first and then pulse labeled. GroEL and its associated polypeptides were isolated from the cytoplasmic extracts with immobilized anti-GroEL immunoglobulins. A labeling time of 15 s is sufficient to synthesize an E. coli polypeptide of average length (~300 amino acid residues; see Figure 2). While shorter polypeptides generally contain fewer methionines (on average there is one methionine every 35 residues) (Neidhardt and Umbarger, 1996), they tend to be fully labeled. Polypeptides larger than 300 residues, though they contain more methionines, would become only partially radiolabeled within the 15 s pulse. These two effects are likely to compensate each other such that the size pattern of labeled GroEL-associated polypeptides approximates the actual size distribution of GroEL substrates. In support of this, the size distribution of cytoplasmic proteins established by this approach is rather similar to that of total E. coli proteins based on genomic analysis (see Figure 2A).

Addition of nonradioactive methionine to intact cells stopped further labeling of newly synthesized polypeptide within seconds. A wide range of radiolabeled proteins immunoprecipitated specifically together with labeled and unlabeled GroEL and disappeared rapidly during the chase period (Figure 1A). Full-length GroEL alone was recovered during immunoprecipitation in the presence of 0.1% SDS, which caused the dissociation of GroEL–substrate complexes. Degradation products of GroEL were not detected (see Experimental Procedures). Addition of an excess of a noncycling GroEL mutant ($GroEL_{TRAP}$) (Weissman et al., 1994) during cell lysis did not change the amount or pattern of GroEL-bound polypeptides (data not shown), indicating that binding to GroEL occurred in the intact cells. In agreement with previous studies, GroEL acts posttranslationally (Gaitanaris et al., 1994; Frydman and Hartl, 1996). Distinct polypeptide species were bound to GroEL rather than a size continuum of nascent chains. During overexpression of specific proteins, only the full-length polypeptides were detected in a complex with GroEL (see below).

The flux of proteins through GroEL was found to be surprisingly rapid under all conditions tested, suggesting that a significant fraction of GroEL substrates escaped detection. We therefore performed similar labeling experiments with viable spheroplasts in which GroEL cycling could be inhibited almost instantaneously by lysing the plasma membrane with a mild detergent, digitonin, in the presence of EDTA. These experiments revealed an increased proportion of shorter polypeptides in association with GroEL (Figure 1B). Thus, the majority of GroEL substrates are between 10 and 55 kDa (Figure 2A). Interestingly, the pattern of polypeptides bound to GroEL differs significantly from that of total cytoplasmic protein labeled under the same conditions.

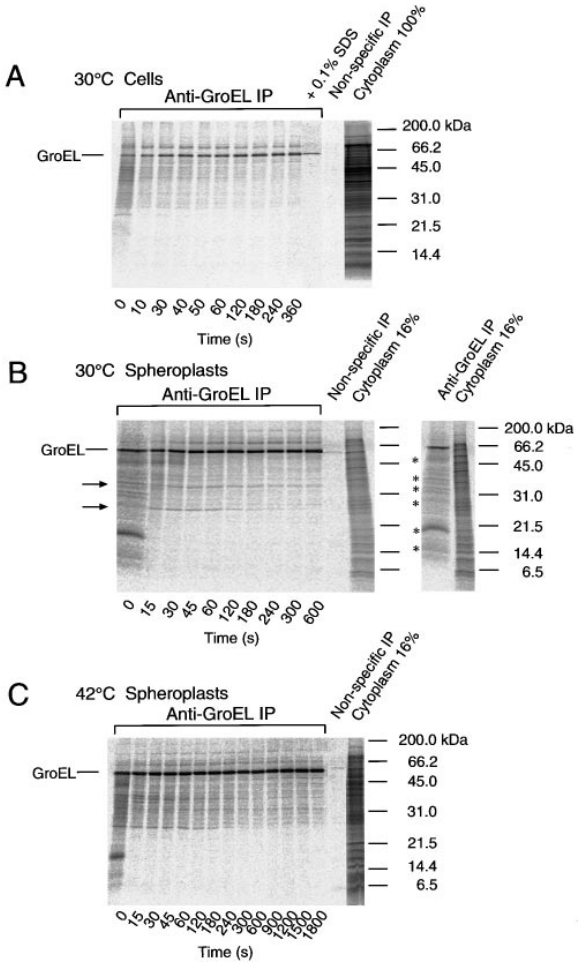

Figure 1. Transit of Endogenous Substrate Proteins through GroEL

E. coli (LMG194) cells and spheroplasts were pulse-chase labeled followed by isolation of GroEL-polypeptide complexes by immunoprecipitation.

(A) 16% SDS-PAGE of GroEL and coimmunoprecipitated polypeptides isolated from E. coli labeled at 30°C. Excess nonradioactive methionine was added at time 0 after a 15 s pulse with [^{35}S]methionine. For comparison, an equivalent portion (100%) of total radiolabeled cytoplasmic proteins was also analyzed.

(B) SDS-PAGE of GroEL and coimmunoprecipitated polypeptides isolated from spheroplasts pulse labeled at 30°C. For clarity, the panel on the right again displays the pattern of proteins immunoprecipitated (shown at time 0) compared to the total pattern of radiolabeled proteins (16% of total). Asterisks indicate polypeptides that appear to be enriched as substrates on GroEL. Arrows indicate proteins that dissociate slowly from GroEL.

(C) Spheroplasts preincubated for 5 min at 42°C were analyzed at 42°C as in (B).

Specific polypeptide bands are enriched in the GroEL-bound fraction (see asterisks in Figure 1B), suggesting that GroEL substrates consist in large part of a specific subset of proteins. Among these polypeptides is a prominent ~18 kDa band. This protein may be identical with the methionine-rich protein Spy that is strongly induced by spheroplasting of E. coli and secreted from the cytoplasm (Hagenmaier et al., 1997). Generally, only a few large polypeptides, up to 150 kDa in size, associated with GroEL (Figure 2A). These proteins, which would be too large to fit into the central GroEL cavity (Braig et

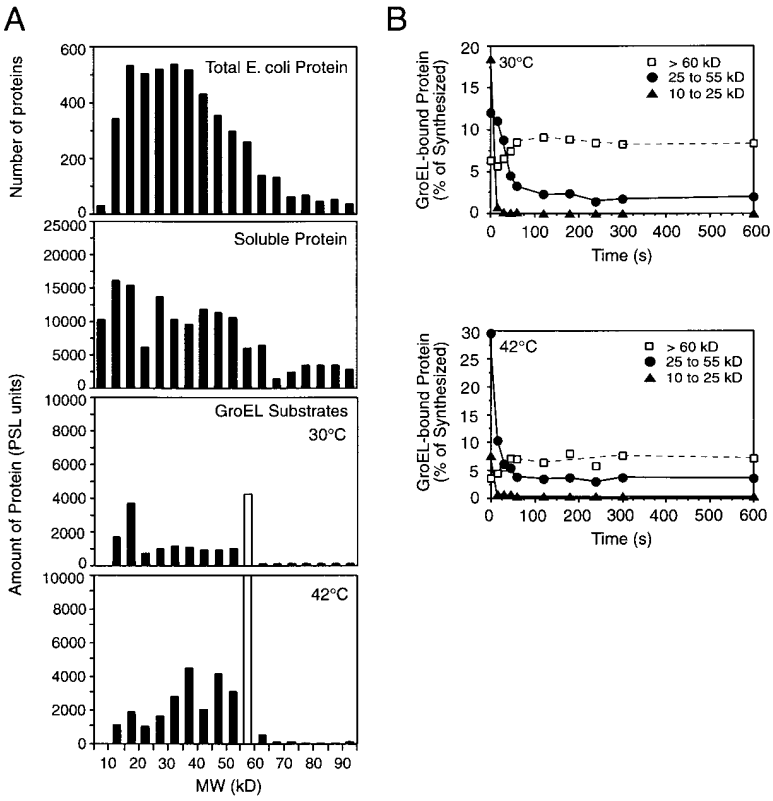

Figure 2. Size Distribution of GroEL-Bound Proteins and Kinetics of Interaction with GroEL

Cytoplasmic and immunoprecipitated fractions of spheroplast pulse-labeling experiments (Figures 1B and 1C) were used to quantitate the [^{35}S]methionine incorporated into proteins of various size ranges. A correction was made for the yield of GroEL immunoprecipitation.

(A) Distribution of E. coli proteins by apparent molecular weight. Top panel represents the number and distribution of proteins encoded for in the E. coli genome (TIGR Microbial Database of the Institute for Genomic Research; http://www.tigr.org:80/tdb/mdb/mdb.html). The average length of all 4285 proteins is 317 amino acids. The second panel displays the pattern of [^{35}S]methionine-labeled cytoplasmic proteins, the third panel displays the distribution of substrate proteins bound to GroEL upon labeling at 30°C for 15 s, and the bottom panel displays the distribution of GroEL substrates upon labeling at 42°C. Labeled GroEL itself is shown as an empty bar.

(B) Kinetics of protein release from GroEL at 30°C and 42°C based on data in Figures 1B and 1C. Proteins were analyzed in three size increments of 10–25 kDa, 25–55 kDa, and >60 kDa, and amounts are given in percent of total synthesized in the respective size region. GroEL itself (57 kDa) was excluded.

al., 1994), did not undergo the time-dependent release observed for most other proteins. They may not require GroEL for folding but may associate with GroEL to fulfill other cellular functions, as exemplified by the 114 kDa protein RNase E (Sohlberg et al., 1993).

When cells were labeled under heat-shock conditions at 42°C (after a 5 min preexposure to 42°C), where GroEL/GroES is rapidly induced, a significantly higher proportion of polypeptides between 25–55 kDa interacted with GroEL (Figures 1C and 2A). Some of these polypeptides were inefficiently released from GroEL, and others seemed to reassociate with GroEL after long chase times, perhaps representing folded proteins that were destabilized at the high temperature.

GroEL complexes with polypeptide substrates were stable during immunoprecipitation (see Experimental Procedures), allowing a quantitative analysis of substrate flux. After correcting for the efficiency of GroEL precipitation, about 12% of all newly synthesized (and ribosome-released) cytoplasmic proteins between 10–55 kDa were in a complex with GroEL at either 30°C or 37°C. Under heat shock, the fraction of GroEL-bound protein increased 2- to 3-fold, as did GroEL itself (Figure 2A). Here we take into account that GroEL does not interact with ribosome-bound chains, and that about 30% of the total radiolabeled polypeptides are still ribosome-bound at the earliest time of analysis, as evidenced by sedimenting ribosome-bound chains (data not shown). Notably, the amount of protein that interacts with GroEL may be somewhat underestimated, due to the fast kinetics of initial protein release from GroEL. Taking this into consideration, we estimate that under nonstress conditions the chaperonin may interact with up to 15% of all newly synthesized polypeptides in the cytoplasm. This fraction increases to 30% or more upon brief exposure of E. coli to 42°C.

Newly Synthesized Polypeptides Transit GroEL Very Rapidly

Two major phases of dissociation of newly synthesized polypeptides from GroEL could be distinguished (Figures 1 and 2B). Most proteins between 10 and 25 kDa (the majority of substrates at 30°C) dissociate from GroEL within 10 s, i.e., they probably occupy GroEL for only a single ATPase cycle of GroES binding and release. In contrast, most proteins between 25–55 kDa (the majority of substrates at 42°C) dissociate within 20–30 s, corresponding to 2–3 cycles. An additional number of proteins, all larger than 25 kDa (Figure 1B, arrows), dissociates from GroEL significantly more slowly with observed half-times of 100–250 s. These differences in transit time probably reflect differences in folding rates, which are generally faster for smaller polypeptides. Thus, the bulk of endogenous E. coli substrates bind GroEL immediately upon synthesis and transit GroEL approximately at the rate of synthesis. This contrasts with observations in vitro that most proteins interact with GroEL through multiple ATPase cycles with half-times of folding between 40 s and 600 s at 37°C (Lorimer, 1996).

The Majority of Newly Synthesized Proteins Can Interact with GroEL

Do endogenous GroEL substrates exclusively consist of a core group of highly GroEL-dependent proteins, or does a small, more slowly folding fraction of most

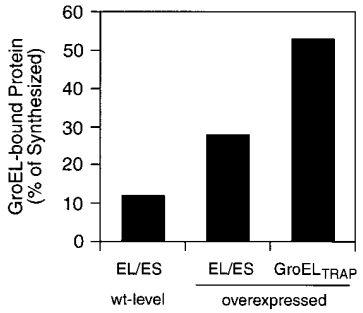

Figure 3. Determining the Pool of Potential GroEL Substrates by Overexpression of GroEL and GroEL$_{TRAP}$

E. coli cells (carrying plasmids pBADESL or pBADGroEL$_{TRAP}$) were grown at 30°C in either glucose (to repress GroES/GroEL expression) or arabinose (to induce GroEL$_{TRAP}$ or GroES/GroEL expression, respectively), and GroEL-bound polypeptides were analyzed as in Figure 1A (after 15 s of labeling), followed by quantification as in Figure 2B. Amounts of GroEL-bound protein are given in percent of total labeled cytoplasmic protein. GroEL/GroES and GroEL$_{TRAP}$ were overexpressed 5- to 6-fold.

proteins utilize GroEL in addition? In the latter case, the proportion of GroEL-interacting proteins should increase significantly with the cytosolic concentration of GroEL. To address this question, we overexpressed either wild-type GroEL/GroES or a dominant negative GroEL mutant (GroEL$_{TRAP}$) under the control of a tightly regulated, arabinose-inducible promoter. GroEL$_{TRAP}$ monomers contain the amino acid substitutions G337S and I349E (Weissman et al., 1994). They assemble into GroEL complexes that bind substrates but do not release them.

Overexpression of GroEL/GroES or GroEL$_{TRAP}$ by ~5-fold (cytosolic concentration of ~15 μM) (see Figure 4A) resulted in 20%–40% slower growth of E. coli. Presumably, a significant fraction of GroEL$_{TRAP}$ is rapidly blocked by polypeptide substrate, explaining the modest growth inhibition. Pulse labeling at 30°C showed that the fraction of total protein interacting with GroEL increased to up to 30% upon overexpression of GroEL/GroES and to more than 50% in the GroEL$_{TRAP}$-expressing cells (equivalent to ~70% of ribosome-released polypeptide) (Figure 3). The pattern and size distribution of GroEL-associated polypeptides remained largely unchanged (data not shown; also see Figure 4), except for an increased proportion of GroEL-bound polypeptides of 25–55 kDa, similar to that observed upon heat shock.

We conclude that the concentration of GroEL in the cytosol is normally limited to permit only a fraction of the possible substrate polypeptides (the majority of total protein) to use GroEL. It is thus likely that for most proteins at least a small fraction of the population, presumably those molecules on a slower folding track, utilize the chaperonin. This fraction may increase under stress conditions.

Different Polypeptides Reveal Distinct GroEL-Binding Properties

In order to understand how individual polypeptides elect to use GroEL, we analyzed the GroEL-binding behavior of specific proteins expressed in vivo. Rhodanese (33 kDa), chloramphenicol acetyl transferase (CAT) (25 kDa), mouse dihydrofolate reductase (DHFR) (20 kDa), and pre-β-lactamase (31 kDa) were selected as examples of polypeptides that might have different requirements for in vivo folding. Denatured rhodanese tends to aggregate during refolding in vitro, but refolds efficiently in the presence of GroEL, GroES, and nucleotide (Martin et al., 1991; Mendoza et al., 1991). Rhodanese folding in mitochondria requires the GroEL homolog, Hsp60 (Rospert et al., 1996). There is evidence that CAT is not dependent on GroEL for folding in vivo (Kim and Kang, 1991). Mouse DHFR refolds spontaneously in vitro, and GroEL actually slows its folding (Martin et al., 1991). The secreted protein pre-β-lactamase can use GroEL for folding in vitro (Laminet et al., 1990) and is affected in its export to the periplasm in chaperonin-deficient E. coli (Kusukawa et al., 1989).

Under normal cellular conditions, 85% of newly synthesized rhodanese accumulated as insoluble aggregates. To test whether overexpression of GroEL and GroES (under non-heat-shock conditions) could alleviate this aggregation, the E. coli strain containing *groES* and *groEL* under control of an arabinose promoter was cotransformed to express rhodanese. Strikingly, when GroES and GroEL levels were raised by 4- to 6-fold (Figure 4A), overexpression of rhodanese produced exclusively soluble, active enzyme in the cytoplasm. Slower growth in minimal media containing arabinose also increased the folding efficiency of rhodanese to 50% even at normal GroEL/GroES levels, presumably by improving the ratio of available GroEL relative to newly synthesized polypeptides.

The interaction of newly synthesized rhodanese with the chaperonin was analyzed at 37°C. In arabinose-grown cells, five major proteins dominated the pattern of [^{35}S]methionine incorporation upon pulse-chase labeling (Figure 4B): GroEL, GroES, rhodanese, CAT, and β-lactamase. (CAT and β-lactamase were used to select for the GroEL- and rhodanese-containing plasmids, respectively.) Both rhodanese and CAT were found associated with GroEL; however, the two proteins showed strikingly different kinetics of GroEL release (Figure 4C). Of the total labeled rhodanese synthesized, the majority accumulated rapidly on GroEL before dissociation with an apparent half-time of ~160 s was observed, corresponding to ~16 cycles of GroEL action. In contrast, only ~15% of newly synthesized CAT was initially associated with GroEL, and this protein dissociated much more rapidly, apparently within a single GroEL cycle. Notably, when GroEL was present at normal levels, only 3%–5% of overexpressed CAT was bound to GroEL, but its folding was nevertheless fully efficient (data not shown), as reported previously (Kim and Kang, 1991). Mouse DHFR interacted with GroEL in the same manner as described for CAT (data not shown). Surprisingly, no pre-β-lactamase was found associated with GroEL during the time course of the experiment. Instead, the protein was exported from the cytosplasm and recovered in the periplasmic fraction from the earliest times analyzed (Figure 4B). Either the interaction of pre-β-lactamase with GroEL is extremely transient, or the involvement of GroEL in β-lactamase export detected in a GroEL mutant strain is indirect (Kusukawa et al., 1989).

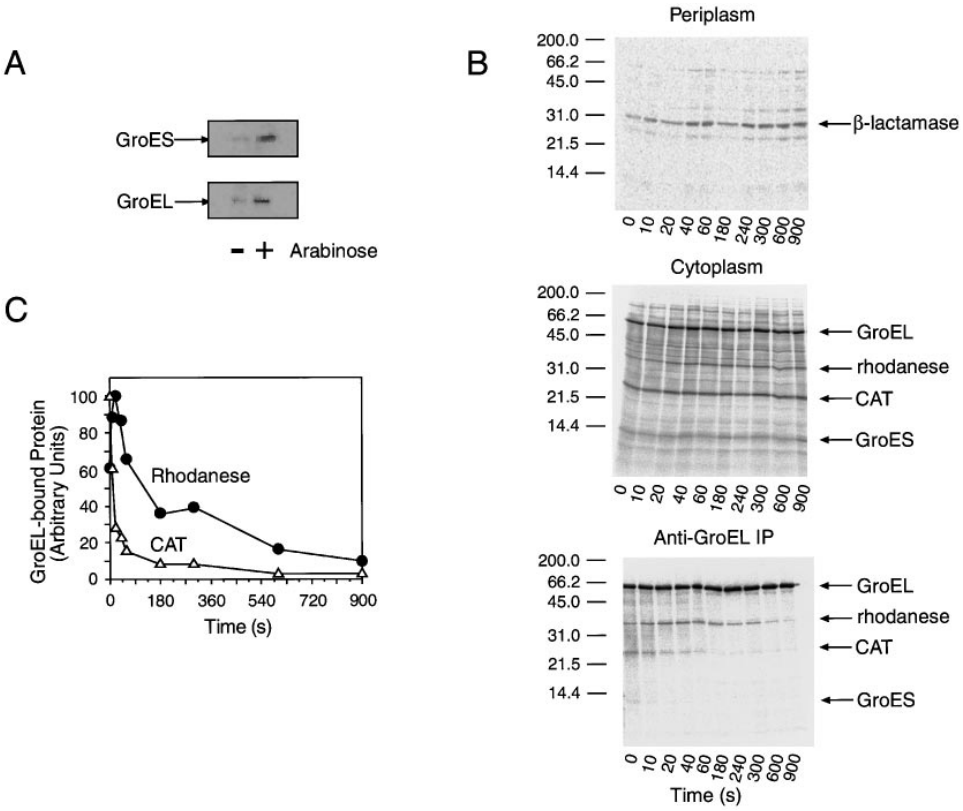

Figure 4. Transit of Rhodanese, CAT, and β-Lactamase through GroEL In Vivo

E. coli containing the plasmids pBADESL and pT7-rhodanese were grown in the presence of 0.2% arabinose to induce overexpression of GroEL and GroES. Expression of rhodanese was induced by IPTG. After 3 hr at 37°C, cells were pulse-chase labeled as in Figure 1 and analyzed by GroEL immunoprecipitation.

(A) Western blot of GroES and GroEL, detected by ^{125}I-protein A, from cells grown in the absence or presence of arabinose.

(B) 15% SDS-PAGE of periplasmic and cytopasmic fractions as well as anti-GroEL immunoprecipitates isolated as a function of time during pulse-chase labeling.

(C) Kinetics of binding and release of [^{35}S]methionine-labeled rhodanese and CAT from GroEL. Maximal amounts of GroEL-bound rhodanese and CAT are set to 100 (arbitrary units).

In addition to rhodanese and CAT, a collection of endogenous E. coli proteins interacted with GroEL in the chaperonin-overproducing strain (Figure 4B). Most of these proteins were released from GroEL rapidly, within 10–30 s, at rates equivalent to those measured in wild-type cells. The apparent rate of CAT and rhodanese dissociation also remained unchanged at different GroEL/GroES levels. Thus the rate of protein release from GroEL seems to be independent of the GroEL concentration in the cytosol, although the fraction of total protein that interacts with GroEL is normally limited by the GroEL concentration. As discussed below, this has implications for the in vivo mechanism of the chaperonin.

In summary, the extent and kinetics of the interaction with GroEL in vivo vary dramatically among different proteins. While a small fraction of CAT (and mouse DHFR) transits GroEL rapidly, reflecting the behavior of the bulk of endogenous substrates, the majority of newly synthesized rhodanese interacts with GroEL through multiple reaction cycles. A similar behavior is seen for a set of endogenous polypeptides, and probably signals a strong chaperonin dependence of these proteins for folding.

Rhodanese Folds as It Is Released from GroEL

Based on in vitro experiments, only a small fraction of the total GroEL-bound rhodanese (5%–10%) completes folding in the GroEL cavity in a single cycle of GroES binding and release; the remainder rebinds to GroEL and is reset to the unfolded state in preparation for a subsequent folding trial. The overall reaction follows first-order kinetics ($t_{1/2}$ of ~5 min at 25°C) (Mayhew et al., 1996), consistent with the slow clearance of rhodanese from GroEL observed in vivo (Figures 4 and 5). Two models have been proposed to explain this behavior. In the first model, all or most of the GroEL-bound polypeptide is thought to be released into the bulk solution in every reaction cycle, the majority (~90% in the case of rhodanese) being in an unfolded conformation that is not yet committed to fold to the native state (Todd et al., 1994; Weissman et al., 1994). These molecules would then partition to another GroEL molecule for a new folding trial. However, unfolded rhodanese is highly aggregation-prone, and the average distance between GroEL complexes in the cytosplasm (3 μM GroEL) is about 100 nm (Ellis and Hartl, 1996). For comparison, the diameter of unfolded rhodanese is less than 5 nm.

An alternative view suggests that in vivo the majority of

rhodanese may be retained by the same GroEL complex throughout successive folding cycles and leave GroEL once it has either completed folding or reached a conformation committed to complete folding (Mayhew et al., 1996; Martin and Hartl, 1997).

To assess these models, we measured the relative time scales of rhodanese synthesis, transit through GroEL, folding, and aggregation under GroEL-limiting conditions where both folding and aggregation of rhodanese occur continuously. Rhodanese was expressed in an E. coli strain containing wild-type levels of GroEL and GroES, grown in minimal media. Spheroplasts were pulse-chase labeled, lysed in the presence or absence of proteinase K, and then fractionated into pellets and supernatants (native folded rhodanese, but not GroEL-bound rhodanese, is soluble and highly proteinase K resistant) (Martin et al., 1991).

Overall, 65% of newly synthesized rhodanese folded into soluble, native protein; the rest aggregated (Figure 5A). In contrast, at 2 min, when incorporation of [^{35}S]methionine into protein was blocked effectively, about 84% of the newly synthesized rhodanese was soluble: 49% was in a complex with GroEL, 16% had folded into the proteinase K-resistant protein, and the remaining 19% was in a nonnative state, but not bound to GroEL. Rhodanese bound to GroEL rapidly, on the same time scale as synthesis, but was released slowly ($t_{1/2}$ of ~2.7 min). Appearance of folded rhodanese occurred with essentially the same rate as that of release from GroEL (Figure 5B). Judged by the amplitudes of the two curves, GroEL-bound rhodanese gave rise to folded protein with almost 100% efficiency (Figure 5A). (Due to the difference between rates of rhodanese synthesis and release from GroEL, nearly complete accumulation of labeled rhodanese on GroEL occurs before release is observed.) In contrast, aggregated rhodanese appeared in the pellet fraction at about twice the rate of that of rhodanese release from GroEL and without an apparent lag phase (Figure 5B). Aggregation of labeled rhodanese was complete at 5 min when only half of the GroEL-bound protein had been released and folded (Figure 5A). Thus, aggregation does not result from rhodanese molecules that are released from GroEL, but rather from unfolded chains that, upon synthesis, fail to bind GroEL.

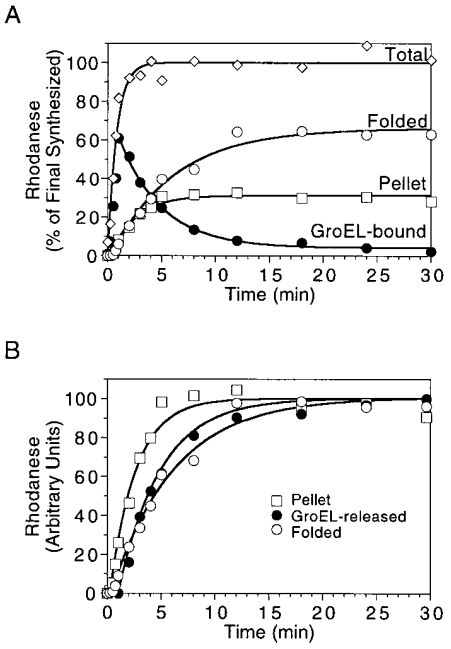

Figure 5. Synthesis, Folding, and Aggregation of Rhodanese In Vivo

Bacterial cells that contain the plasmids pBAD33 (lacking the *groESL* coding sequence) and pT7-rhodanese were grown in the presence of arabinose. Slow growth resulted in increased efficiency of rhodanese folding at wild-type levels of GroEL/GroES. Spheroplasts were labeled at 37°C as in Figure 1B and lysed by dilution into chilled buffer containing digitonin and EDTA in the presence or absence of proteinase K (see Experimental Procedures).

(A) Appearance of rhodanese in the total, pelleted, GroEL-bound, and folded (soluble proteinase K-resistant) fractions. GroEL-bound rhodanese was isolated by immunoprecipitation as in Figure 1 and analzyed as in Figure 2B. Data were fitted to single exponential functions, assuming pseudo-first-order processes, which is probably an oversimplification of the actual kinetics.

(B) Comparison of the rates of rhodanese release from GroEL (the inverse of the GroEL-bound rhodanese), folding, and rhodanese appearance in the pellet fraction. Amplitudes of the three curves in (A) were normalized.

A model in which GroEL discharges essentially unfolded rhodanese into the cytosol in every ATPase cycle is not supported by our observations. Such a model would predict that under GroEL-limiting conditions, where rhodanese aggregation is ongoing, a fraction of the rhodanese molecules released from GroEL would aggregate rather than be recaptured by GroEL. Consequently, aggregation would be expected to proceed throughout the time period of rhodanese dissociation from GroEL. In the experiment shown in Figure 5, unlabeled rhodanese and other polypeptides are continuously synthesized during the chase. These substrates rapidly fill the limited chaperonin pool and would thus effectively compete with the labeled rhodanese for binding to GroEL, if it had been released into solution. In this model, raising the GroEL concentration in the cytosol would be expected to slow the clearance of labeled rhodanese from GroEL, in contrast to the experimental observation (Figure 4C). Thus, proteins with folding properties similar to those of rhodanese are not intermittently discharged from GroEL into the bulk cytosol in an aggregation-sensitive, unfolded state, but rather leave GroEL after having reached a significantly folded, though not necessarily fully native, conformation.

Some Proteins Can Fold Independently of GroEL but Use GroEL In Vivo

The variation in GroEL dependence of different proteins was analyzed further by expressing rhodanese, CAT, and DHFR in an E. coli translation extract. Immunodepletion of GroEL from the extract to more than 95% served to determine the chaperonin requirement for folding in this system. Staphylococcal nuclease (16 kDa) was used as an additional model protein, because it was not associated with GroEL under the conditions of in vitro translation (data not shown), in contrast to all other proteins tested.

Newly synthesized rhodanese failed to fold in the

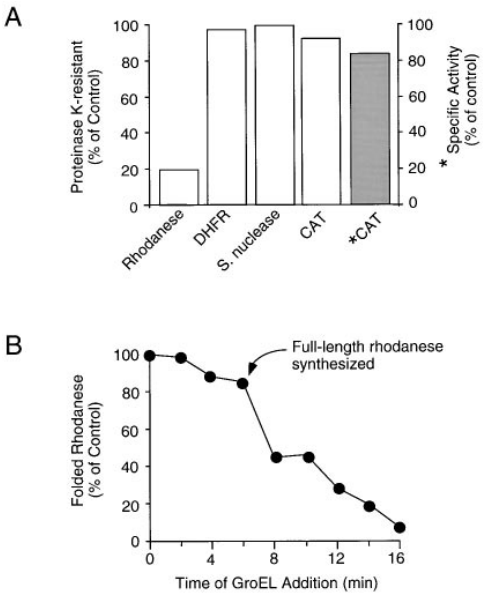

Figure 6. GroEL Dependency for Folding during Translation in S30 Extract

Normal E. coli S30 extract and an extract depleted to >95% of GroEL were programmed to synthesize either [^{35}S]methionine-labeled rhodanese, CAT, DHFR, or S. nuclease.

(A) After completion of translation (20 min at 30°C), the extent of folding of the newly synthesized proteins was determined by the acquisition of protease resistance (rhodanese, DHFR, CAT, S. nuclease) or by the acquisition of enzymatic activity (CAT). Amounts of proteinase K-resistant and enzymatically active protein are expressed as percent of undepleted control lysate (normalized for amounts of protein synthesized).

(B) Delayed addition of GroEL to rhodanese translation reactions. Purified GroEL (3 μM final) was readded to aliquots of this reaction either immediately or at different times after initiating synthesis. After 20 min, the yield of folded soluble rhodanese in each sample was determined by limited proteolysis. Full-length labeled rhodanese was detectable after 6 min of translation.

GroEL-depleted extract (Figure 6A). When purified GroEL was readded at various times after initiation of translation, rhodanese folding was recovered (Figure 6B). However, efficient folding was only achieved when GroEL was supplemented prior to or shortly after completion of synthesis of full-length polypeptide (Figure 6B). Thus, rhodanese has to bind GroEL immediately upon its release from the ribsosome and, as shown in vivo, if this interaction is prevented or delayed under GroEL-limiting conditions, newly synthesized rhodanese aggregates. These observations question the physiologic significance of recent findings that in vitro isolated ribosomes can mediate the refolding of chemically-denatured rhodanese in the absence of chaperonin (Kudlicki et al., 1997).

In contrast to rhodanese, DHFR, S. nuclease, and CAT attained their folded structures with full efficiency in the GroEL-depleted extract (Figure 6A). Thus, although a fraction of the population of each of these proteins interacts with GroEL, this interaction is apparently not required for folding (at least upon in vitro translation), consistent with the efficient folding of overexpressed CAT in cells containing wild-type levels of chaperonin.

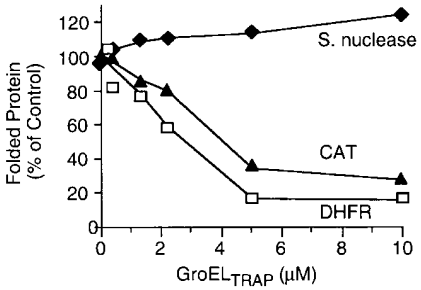

Figure 7. Prevention of Folding by GroEL$_{TRAP}$ in S30 Translation Extract

GroEL$_{TRAP}$ was added to E. coli S30 extracts to the concentrations indicated before initiating translation as in Figure 6. Native protein was quantified after 20 min by proteolytic digestion (DHFR and S. nuclease) or by enzymatic assay (CAT).

The size of the GroEL-interacting population of such a protein is presumably determined by its rate of folding and by the concentration of GroEL.

Indeed, binding to GroEL predominated over chaperonin-independent folding when the translation extract was supplemented with GroEL$_{TRAP}$. The amount of folded DHFR and CAT progressively diminished at increasing GroEL$_{TRAP}$ concentrations until a plateau of only ~20% to 30% remained (Figure 7). In contrast, the folding of S. nuclease could not be inhibited by the addition of GroEL$_{TRAP}$ to the translation (Figure 7). Apparently, this protein folds very rapidly and escapes binding to GroEL$_{TRAP}$ at all concentrations. In conjunction with the results obtained upon overexpression of GroEL$_{TRAP}$ in vivo, it thus appears that a very large fraction of newly translated proteins, exemplified by CAT, can adopt a GroEL-mediated folding pathway in addition to a chaperonin-independent pathway. However, the vast majority of the population of these proteins avoids binding to GroEL at normal GroEL levels.

Discussion

Our results provide a global assessment of protein flux through chaperonin-assisted and -unassisted folding pathways in vivo. Only 10%–15% of all newly synthesized polypeptide chains interact with GroEL under nonstress conditions and transit the chaperonin very rapidly. At least three classes of proteins can be distinguished (Figure 8). (I) A minor fraction of the total polypeptide species neither require GroEL nor interact with it, even at high GroEL concentrations. Most of these proteins, including S. nuclease, are probably small and fold very rapidly. (II) The majority of polypeptides, including CAT, may utilize the chaperonin, but normally have no stringent chaperonin requirement. Typically, only ~5% of the population of a protein in this class binds GroEL, but this fraction appears to increase 2- to 3-fold upon heat stress. (III) A set of proteins (predominantly 25–55 kDa in size) interacts with GroEL quantitatively and probably has a stringent chaperonin requirement for folding. Many of these, represented by rhodanese as a model, fold relatively slowly during multiple GroEL cycles.

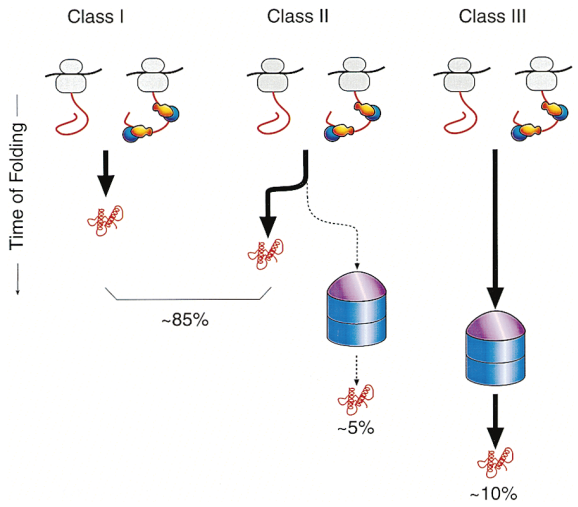

Figure 8. Chaperonin Usage by Newly Synthesized Proteins

Three groups of E. coli proteins are distinguished in this model. Class I proteins are completely independent of the chaperonin in vivo. Class II proteins can fold in a largely GroEL independent manner, although a fraction of each of these proteins utilizes GroEL (~5% of all newly made proteins). Class III proteins display a strict dependence on GroEL for folding (~10% of proteins). Increasing GroEL-dependence correlates with an increase in the time required by these proteins for folding.

About two-thirds of all ~4300 polypeptide species of E. coli are localized in the cytoplasm (Goodsell, 1991). Assuming that all proteins that transit GroEL require the chaperonin for folding, then the maximum number of individual GroEL-dependent polypeptides would be 300–400. However, the actual number of class III proteins must be lower, between 200–300, because at normal levels of GroEL about one-third of the GroEL capacity is likely to be occupied by class II polypeptides. Interestingly, in comparison to GroEL, the chaperonin in the eukaryotic cytosol appears to facilitate the folding of a much smaller number of specific polypeptides (Kubota et al., 1995; Lewis et al., 1996). This would suggest that eukaryotic cells have acquired folding mechanisms that eliminated the need for a general chaperonin.

The approximate size cutoff for GroEL substrates at 55 kDa (~500 residues) is consistent with the size limitations imposed by the central GroEL cavity (upon binding of GroES) (Chen et al., 1994; Hartl, 1996). Although some larger polypeptides bind GroEL, the functional significance of their interaction is unclear, as they decay from GroEL only very slowly. How the efficient folding of large cytoplasmic polypeptides is achieved is not yet known, but it may well involve other chaperone systems. It is remarkable, however, that 87% of all E. coli proteins are smaller than 50 kDa (Figure 2A) and are thus potential GroEL substrates. Indeed, more than half of all newly synthesized polypeptides of 10–55 kDa are capable of binding GroEL in vivo. Given that GroEL does not associate with ribosome-bound chains, this suggests that the majority of E. coli cytoplasmic proteins are selected for rapid posttranslational folding. Furthermore, it appears that the cellular level of GroEL (~3 μM, relative to 30 μM of ribosomes) must be carefully balanced in order to provide assistance of folding rather than hindrance. As shown for proteins like DHFR, a GroEL-mediated folding pathway, while being effective, may actually slow down folding (Martin et al., 1991).

What properties of a newly synthesized polypeptide determine the interaction with GroEL? Our results argue that the extent to which the population of a given protein interacts with GroEL in vivo is generally determined by its rate of folding, the affinity of its folding intermediates for GroEL, and the cellular GroEL concentration. Binding to GroEL is mediated by hydrophobic surfaces exposed by unfolded proteins (reviewed in Hartl, 1996). The bulk of substrates transit GroEL at a very fast rate, equivalent to the speed of synthesis, suggesting that they bury exposed hydrophobic surfaces rapidly. This behavior is most pronounced for proteins smaller than 25 kDa. Many of these probably represent class II proteins that have been slightly delayed in their folding. Binding to GroEL may destabilize these kinetically trapped intermediates, permitting rapid completion of folding. In contrast, GroEL-dependent polypeptides (class III) probably bury their hydrophobic surfaces more slowly during folding and are expected to occupy GroEL predominantly. These are the same proteins that tend to aggregate in vivo and in vitro, as exemplified by rhodanese. A significant number of E. coli polypeptides mimic the behavior of rhodanese in that they transit GroEL with kinetics much slower than synthesis. As shown for rhodanese, these proteins seem to rely predominantly on the capacity of GroEL to sequester aggregation-sensitive folding intermediates within its central cavity (Hayer-Hartl et al., 1996).

Although GroEL-dependent folding intermediates may encounter a free GroEL simply based on their high affinity for GroEL, additional mechanisms may be implemented to ensure their timely interaction, given the vast excess of potential GroEL substrates. Perhaps these proteins have high affinity for an initial set of molecular chaperones that may act to prevent aggregation during translation (Figure 8) and may favor subsequent interaction with GroEL. Candidate chaperones include trigger factor and the Hsp70 system (reviewed in Hartl, 1996). Additionally, specific mechanisms such as the interaction of trigger factor with a polypeptide–GroEL complex, may be necessary to disrupt unproductive GroEL cycling of aberrant substrates (Kandror et al., 1995), causing their transfer to the degradative machinery.

Experimental Procedures

Bacterial Strains and Plasmid Construction

Strains: KS272 (F⁻ Δ*lacX74 galE galK thi rpsL* Δ*phoA* [PvuII]), LMG194 (KS*272* Δ*ara714 leu*::Tn*10*) (Guzman et al., 1995), BL21(DE3), D10 (*relA1*, *spoT1*, *metB1*, *rna-10*) (E. coli Genetic Stock Center).

Plasmids: pT7-rhodanese (Miller et al., 1991), pT7-DHFR, pCAT2.2 (gift from M. Wiedmann), Snuc (Flanagan et al., 1992), pBAD33 (Guzman et al., 1995).

Construction of pBADGroEL$_{TRAP}$: the GroEL$_{TRAP}$ coding region (XbaI-NsiI) was isolated from plasmid T7-TRAP (Mayhew et al., 1996). A 91 bp fragment containing the 3′ end of the GroEL gene with an SphI site inserted downstream was prepared by amplifying plasmid pOF39 (Fayet et al., 1986) with appropriate oligonucleotide primers and cleaving the product with NsiI and SphI. These fragments were inserted into the multicloning site of pBAD33 (Guzman

et al., 1995). $GroEL_{TRAP}$ has a characteristic mobility on native polyacrylamide gels. PCR products were confirmed by sequencing.

Construction of pBADESL: the GroES and GroEL coding region (23 bases upstream of the initiating ATG of GroES to 5 bases downstream of GroEL) of pOF39 was amplified by PCR and ligated into the multiple cloning site of pBAD33.

Overexpression and Purification of Proteins

Cells that carried either pBADESL or $pBADGroEL_{TRAP}$ were grown to midlog phase in M63 medium, 0.2% glycerol, and 0.2% arabinose. For cooverexpression of either DHFR or rhodanese, IPTG was added to 1 mM, and growth was continued for at least 1 hr at 30°C or 37°C prior to pulse labeling. The level of specific protein overexpression was assessed by quantitative Western blotting with ^{125}I-protein A.

$GroEL_{TRAP}$, GroEL, and GroES were purified as described (Clark et al., 1996; Mayhew et al., 1996).

Cell Labeling

Cells were grown to midlog phase at either 30°C or 37°C in M63 medium (0.0005% thiamin, 40 μg/ml amino acids without methionine) supplemented with 0.2% glycerol and either 0.2% glucose or 0.02% arabinose (Ausubel et al., 1992). [^{35}S]Methionine was added to 60 mCi/ml, followed after 15 s by addition of nonradioactive methionine to 1 mM, and 1 ml of solution was transferred to a 20 ml glass beaker in an ice-water bath. Additional aliquots were removed at the times indicated in the figures. Radiolabeled cells (0.8 ml of each aliquot) were converted to spheroplasts (Ausubel et al., 1992). Spheroplasts were isolated by centrifugation at 14000 × g for 40 s, washed with 0.5 ml of 50 mM Tris (pH 8.0), 0.25 M sucrose, and 10 mM $MgSO_4$, and lysed in a hypoosmotic buffer of 50 mM Tris-acetate (pH 8), and 5 mM EDTA. After lysis, NaCl (140 mM) with protease inhibitors (1 mM PMSF, 1 mM pefabloc, 0.5 μg/ml leupeptin, and 2 μg/ml aprotinin) was added. Soluble proteins were separated from pelletable material (14000 × g, 5 min). Portions (5 μl) of periplasmic and cytoplasmic fractions were analyzed for [^{35}S]methionine incorporation by measuring TCA-precipitable radioactivity (Pratt, 1984). Another portion of the cytoplasmic extract was subjected to GroEL immunoprecipitation and then applied to SDS-PAGE. Radiolabeled proteins were detected and quantified with a Fuji Bioanalyzer.

Alternatively, spheroplasts were resuspended in M63 media supplemented with 0.2% glycerol, 0.2% glucose or arabinose, and 0.25 M sucrose and then radiolabeled as above. When necessary, the media contained 1 mM IPTG, chloramphenicol (30 μg/ml), and ampicillin (150 μg/ml). Aliquots (0.5 ml) collected following pulse-chase labeling were diluted into an equal volume of chilled 40 mM Tris (pH 7.4), 10 mM EDTA, and 1% digitonin (with or without 20 μg/ml proteinase K). Proteolysis, used to detect folded soluble rhodanese, was inhibited after 10 min on ice by adding 1 mM PMSF.

Enzyme Assays

Rhodanese (Hayer-Hartl et al., 1996) and CAT (Ausubel et al., 1992) were assayed as described. ATP hydrolysis by GroEL was monitored by the conversion of [α-^{32}P]ATP into [α-^{32}P]ADP (Höhfeld et al., 1995).

Immunoprecipitation

Polyclonal rabbit anti-GroEL immunoglobulins were affinity purified on immobilized GroEL and cross-linked to protein A-Sepharose. Cell extracts (300 μl) were first incubated with protein A-Sepharose (10 μl beads) for 1 hr at 4°C. A portion of the extract was then incubated for 90 min either with anti-GroEL beads (100 μl extract added to 5 μl beads in 200 μl PBS, 0.02% Tween 20) or with a nonspecific immunoadsorbent (the monoclonal antibody w6/32, specific for MHC class II). Beads were washed once with 500 μl of 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% deoxycholate, and 1% Triton X-100, once with 500 μl of 50 mM Tris (pH 8.0), 5 mM EDTA, 500 mM NaCl, and 1% Triton X-100, and twice with 500 μl of either 20 mM Tris, 80 mM KCl, 2 mM EDTA (pH 8), or PBS/5 mM EDTA. Proteins were released from the beads with SDS-buffer and separated by SDS-PAGE. The efficiency of GroEL immunoprecipitation was quantified by Western blotting using ^{125}I-protein A. In different experiments the extent of GroEL immunoprecipition varied between 20% and 90% of the total GroEL in the cell. GroEL degradation products were not detected.

Stability of GroEL-Substrate Complexes

Radioactive cell lysates (see above; ~20 μM protein based on average protein mass of 35 kDa) were denatured in 5 M guanidinium chloride, PBS (pH 5.0), 5 mM EDTA, and 0.02% Tween-20 at 4°C overnight. A complex between GroEL and lysate proteins was formed by diluting 10 μl of unfolded lysate with 500 μl of 0.5 μM GroEL in PBS (pH 7.0), 5 mM EDTA, and 0.02% Tween-20. After 5 min, 200 μl of the mixture was fractionated on a Sephacryl S-300 (Pharmacia Biotech) column in the same buffer. The fraction (300 μl) containing the GroEL peak (well separated from the rest of the protein) was analyzed by anti-GroEL immunoprecipitation, by immunoprecipitation with nonspecific antibody, or by incubation at room temperature for the duration of immunoprecipitation (typically 2 hr) followed by SDS-PAGE. (At least 50% of total labeled protein bound to GroEL with a size distribution very similar to that observed in vivo.) The efficiency of immunoprecipitation was determined by quantitating the amount of GroEL observed on Coomassie-stained gels and the radiolabeled proteins with a Fuji Bioanalyzer. No loss of radiolabeled polypeptides was detected upon immunoprecipitation. In a separate analysis, an aliquot of the isolated GroEL-polypeptide complex was refractionated immediately (as above) or after 2 hr. The elution profiles of radiolabeled protein from both fractionations were identical, further indicating that GroEL–substrate complexes were stable under the conditions of immunoprecipitation.

In Vitro Translation in E. coli S30

Translation reactions (30°C) (Pratt, 1984) contained amino acids (1 mM each minus methionine) and 0.6 mCi/ml [^{35}S]methionine. For T7 plasmids, 1 μg/ml rifampicin (Sigma) and 20 U/ml T7 RNA polymerase (Promega) were added. For GroEL depletion, S30 extract was incubated for 90 min at 4°C with the anti-GroEL immunoadsorbent.

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