GroEL Binds to and Unfolds Rhodanese Posttranslationally*
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The Escherichia coli chaperone GroEL is a member of a class of molecular chaperones that possesses a stacked double ring structure containing seven subunits per ring, with approximately 60-kDa subunits. It has been suggested that newly synthesized proteins may interact with a eukaryotic homolog of GroEL co-translationally, thereby sequestering the unfolded protein from other proteins in the cell. To test whether it is essential for GroEL to form a stable interaction with a nascent polypeptide co-translationally, we translated the well studied GroEL substrate rhodanese in bacterial and wheat germ translation extracts. We found that rhodanese formed stable complexes with GroEL solely post-translationally. Upon binding to GroEL, the protease resistant N-terminal domain of rhodanese unfolds. This interaction with GroEL leads to productive folding of the full-length rhodanese. We conclude that GroEL is able to assist in the folding of newly synthesized proteins following release from the ribosome and that GroEL can unfold a trapped protein folding intermediate of rhodanese.

Two different sets of molecular chaperones are likely to facilitate the folding of newly synthesized proteins in the cytosol. One set includes the 70-kDa family of heat shock proteins (hsp70) and a family of 40-kDa heat shock proteins (hsp40) related to the DnaJ protein from Escherichia coli (1). The other set of molecular chaperones is comprised of a family of proteins that are functionally related to the 60-kDa family of heat shock proteins (2). Whereas the members of the hsp60 class have little primary sequence similarity, all are polypeptides of approximately 60 kDa, which form stacked oligomeric ring structures (3-5). In eukaryotic translation extracts there is evidence that both sets of chaperones engage the nascent polypeptide chain co-translationally. (6, 7). Presumably, hsp40 and hsp70 are interacting with exposed hydrophobic segments on the unfolded partially synthesized proteins (8, 9).

The hsp70, hsp40, and hsp60 chaperones are also found in prokaryotes. In fact, the molecular chaperones from E. coli have been studied more extensively than from any other organism, and much is known about their requirements in vivo and their mechanism of action in vitro (for a review, see Ref. 10). The E. coli hsp60 homologue, GroEL, cooperates with the hsp70/hsp40 chaperones (DnaK/DnaJ) to facilitate protein folding in the cell (1, 11). It is not clear if the hsp70/hsp40 chaperone system acts prior to the hsp60 along the same folding pathway, or if the two systems work along parallel pathways. Both molecular chaperone systems can function independently to assist the refolding of proteins in vitro (12, 13). However, at least one model protein can be released from DnaK/DnaJ in a state competent to bind to GroEL, but not vice versa, suggesting that the chaperones may work along the same folding pathway (1).

Two models have been proposed for the mechanism of GroEL action. In the first model, sometimes called the infinite dilution model, the role of GroEL is to sequester the protein folding intermediates, thereby preventing aggregation of the folding protein (14). The protein is released from the GroEL oligomer only after it achieves its native state. In a contrasting model, the role of GroEL is to assist unproductive, or trapped, folding intermediates back onto the productive protein folding pathway (15-17). We will call this second model the “kinetic detrapping” model. GroEL binds to partially folded proteins in solution and then uses the energy of ATP hydrolysis to drive the protein to a folding-competent state. The cycle of binding and release may occur on average several times before a high percentage of a model protein has obtained its native structure.

To distinguish between the infinite dilution and kinetic trapping models we investigated whether the hsp60 chaperone encounters a polypeptide attached to a ribosome. The infinite dilution model would be most compatible with a co-translation interaction between a folding protein and GroEL. This model predicts that as a protein is being synthesized it is immediately sequestered from solution to prevent aggregation. Co- translational interactions would seem unnecessary in the kinetic detrapping model, since the fully translated protein may release from and rebind to GroEL multiple times during the chaperone assisted folding.

In this study we followed the interactions between GroEL and newly synthesized proteins in bacterial translation extracts, to test when this bacterial hsp60 first encounters a folding protein in the cell.

EXPERIMENTAL PROCEDURES

DNA and RNA—Polymerase chain reaction was performed using pRhOΔ (18) as a template to generate a rhodanese gene flanked with BamHI and HindIII restriction sites. The primers used for rhodanese polymerase chain reaction were: start, 5'-TAG GAT CCA TGC ATC and end, 5'-AT AAG CCT CCC ACT CTG CCC CTC'. The BamHI site is immediately 5' of the translation start codon and the HindIII site replaced both the stop codon and the C-terminal alanine codon of rhodanese. This fragment was ligated into the plasmid vector pGEM-3Z (Promega), transformed into bacteria, and purified (19) for subsequent wheat germ translations or into pAED2 (20) for bacterial S30 translations. pAED2 transcripts code for a 14-amino acid leader peptide (21) and were transcribed in vitro (Promega Riboprobe) to generate truncated mRNA.

For T4 lysozyme transcription and translation, pT4L in pAED2 was used as template (20). pT4L codes for the TA, or cysteinless “pseudo wild type” lysozyme. This lysozyme construct codes for a flexible linker extension on the C terminus (AGTTGTGGCGTGGCGCC), which also contains a NarI restriction site used to generate the stop-codonless truncation in this study. NarI-linearized T4 lysozyme DNA was tran-
Posttranslational Interaction of Rhodanese with GroEL

**RESULTS**

Posttranslational Binding of Rhodanese to GroEL—Rhodanese, a 33-kDa monomer, has been used as a model protein in a number of chaperone assisted folding studies (14, 24). When rapidly diluted into a buffer from denaturant, rhodanese tends to aggregate in the absence of detergent or chaperones (25). Rhodanese renatured from urea binds tightly to GroEL and can be reactivated with the addition of the hsp60 co-factor GroES and MgATP (24). To address whether GroEL is able to bind rhodanese co-translationally, we generated stalled translation complexes with bound nascent rhodanese. Stalled ribosome-nascent chain complexes were created by translating mRNA in vitro lacking stop codons. In the absence of a termination codon, termination factors cannot bind and release the nascent chain from the ribosome (26).

For the following experiments, 35S-labeled proteins were synthesized in vitro using either a wheat germ or a bacterial S30 translation extract with mRNA lacking stop codons. Of two model proteins studied in detail, both yielded relatively stable stalled mRNA-ribosome-nascent chain complexes, as determined by sedimentation over a sucrose gradient. Controls were performed to determine the sedimentation positions of small soluble proteins, GroEL, ribosomes, and polysomes on identical gradients (not shown). About 70% of full-length proteins remained bound to the ribosomes. The efficiency of release varied with the protein tested. For example, approximately 90% of T4 lysozyme could be released upon addition of the aminoacyl-tRNA analog puromycin, yet only about 70% of rhodanese was released under similar conditions. Release of the nascent chain was monitored by the sedimentation rate of the labeled protein through a sucrose gradient. As shown in Fig. 1, the bound 35S-labeled bacteriophage T4 lysozyme sedimented at the position of monosomes and polysomes (Fig. 1a). Following a brief puromycin treatment, the lysozyme sedimented more slowly and barely entered the sucrose gradient (Fig. 1b, lanes 1–3). This position on the gradient is consistent with the released protein behaving as a monomer. In contrast, truncated rhodanese released with puromycin sedimented over a very broad range of values, indicating that the puromycyl-rhodanese ei-
ther aggregates or binds to other proteins in the wheat germ translation extract (Fig. 1c). Rhodanese found at the bottom of the gradient (fraction 14) likely represents aggregated rhodanese and not unreleased polysomes, as the characteristic high molecular weight peptidyl-tRNA band was not evident.

As with T4 lysozyme, truncated rhodanese remains bound to ribosomes following translation (Fig. 2a), and sediments at the position of monosomes and polysomes (fractions 9–11 and fraction 14). If GroEL is added prior to puromycin addition, rhodanese released from the ribosome no longer was distributed over the entire sucrose gradient as seen in Fig. 1c, but was instead found mainly in the same fractions as GroEL (Fig. 2b, fractions 3 and 4). This peak of radioactivity could be well resolved from both monomeric rhodanese (see Fig. 3b, lane 1) and the monosome and polysomes (see Fig. 2a, lanes 10 and 14, respectively). Other proteins tested (α-luciferase and β-luciferase) also bound to GroEL following release from the ribosome (data not shown). In contrast, puromycin-released T4 lysozyme did not bind stably to GroEL (Fig. 1), as expected for this protein, which can spontaneously refold (27). It should be noted that the TA, or pseudo wild type lysozyme, which lacks disulfide-forming cysteines and is nearly as stable as wild type T4 lysozyme (65.8°C versus 67.2°C), was used in this study (28–30).

Although these sucrose gradients suggest that stable binding of rhodanese to GroEL can occur posttranslationally, they do not address when GroEL is first able to bind to the nascent polypeptide chain. In order to determine whether GroEL can bind nascent rhodanese on the ribosome, immunoprecipitations were performed on sucrose gradient fractions shown in Fig. 2, a and b, using anti-GroEL antisera. Radiolabeled rhodanese in the GroEL containing peak (Fig. 2b, lanes 3 and 4) could be co-immunoprecipitated with GroEL following release with puromycin. In contrast, no 35S-labeled rhodanese could be detected in immunoprecipitations on the monosome or polysome fraction prior to (Fig. 2c, fractions 9–14) or following puromycin treatments (Fig. 2d, fractions 9–14). Apparently, the anti-GroEL antisera was unable to co-immunoprecipitate the radiolabeled rhodanese in these fractions. It is difficult to imagine that the polyonal antisera was simply unable to access and immunoprecipitate GroEL bound to a ribosome, since GroEL is such a large complex, consisting of two seven-membered rings stacked back to back (31). Moreover, GroEL was found only in fractions 2–5 (with a peak in fractions 3 and 4), and not in the monosome or polysome fractions, on all the gradients probed by immunoblotting, both before and following release of the nascent chain (not shown). These results indicate that stable interactions between GroEL and rhodanese occur solely posttranslationally on the full-length protein. Identical results were obtained in both wheat germ and bacterial translation extracts (data not shown). Similar results were recently reported for bacterial proteins in the cell. In a pulse-chase experiment a large number of unidentified bacterial proteins were found to interact with GroEL solely posttranslationally (32).

Similar immunoprecipitation experiments were also performed with monoclonal antibodies against DnaK. No radiolabeled rhodanese could be detected in these precipitates from GroEL-rhodanese complexes or in the monosome or polysome fractions from bacterial extract translations (data not shown). It remains possible, however, that the epitope recognized by the monoclonal DnaK antibody was blocked in a DnaK containing complex.

As described above, addition of GroEL prevents the puromycin-released rhodanese from aggregating or binding nonspecifically to other proteins. Like the full-length protein binding to GroEL upon dilution from urea, the rhodanese released from the ribosome binds tightly enough to GroEL to be isolated on a sucrose gradient (22). Even after a prolonged attachment to
Proteinase K digestions of 35S-labeled rhodanese. a and b, digestion of 35S-labeled rhodanese released from GroEL (a) and bound to GroEL (b). Puromycin-released rhodanese-GroEL complexes were purified by sucrose gradient sedimentation. GroEL-rhodanese complexes were incubated for 2 h with (a) or without (b) GroES and ATP at 30°C. Proteinase K was then added to a final concentration of 10 μg/ml and incubated for the times shown. c, digestion of rhodanese-GroES ribosome complex. Stalled rhodanese-ribosome complexes were generated by translation in bacterial S30 extracts. Lane 1 shows the material prior to incubation with protease. Lane 2 is the product of a 10-min incubation with 20 μg/ml proteinase K at 30°C. d, proteinase K digestion of full-length and truncated (amino acids 1–206 rhodanese) stalled translation complexes. Full-length rhodanese and amino acids 1–206 truncated rhodanese stalled ribosomal complexes were made in bacterial S30. Lane 1 shows the undigested full-length complex. Lanes 2 and 3 show 10-min protease digestions at 20 and 100 μg/ml proteinase K, respectively. Lane 4 shows undigested amino acids 1–206 rhodanese translation complexes, and lanes 5 and 6 show digestions of amino acids 1–206 rhodanese complex at 20 and 100 μg/ml proteinase K, respectively. Proteolysis was quenched by the addition of 1 mM phenylmethanesulfonyl fluoride, and samples were analyzed by SDS-PAGE and fluorography.

GroEL, urea-denatured rhodanese can be renatured with the addition of ATP and the GroES co-factor. To test whether the GroEL bound form of the translated puromycin-released rhodanese was capable of folding, GroEL-rhodanese complex isolated from a sucrose gradient (Fig. 2a, fractions 3 and 4) was incubated with ATP and GroES for 2 h at 30°C. Following this treatment, most of the rhodanese was released and sedimented near the top (Fig. 3b, fraction 1) of a second sucrose gradient. The labeled rhodanese remained bound to GroEL on the second sucrose gradient if GroES and ATP were omitted from the incubation (Fig. 3a, fractions 3 and 4).

Although the radiolabeled rhodanese could be released from GroEL and sedimented similar to monomeric rhodanese, it was possible that the protein remained in a non-native structure. We therefore tested the folding state of the GroEL released rhodanese by protease sensitivity. Following an incubation of isolated GroEL-rhodanese complexes in which GroES and ATP were omitted, the radiolabeled rhodanese, still bound to GroEL, is protease-sensitive, and is completely digested with proteinase K (10 μg/ml) within 5 min (Fig. 4b). When released from GroEL in the GroES- and ATP-containing incubation, the radiolabeled rhodanese was much more protease-resistant. A substantial portion of the rhodanese released from GroEL remained intact upon a 30-min incubation under identical conditions (Fig. 4a). Taken together, these results suggest that the rhodanese released from GroEL with GroES and ATP is in a more folded state than in the GroEL bound state. However, we were unable to determine the fraction of rhodanese that was able to obtain its native state, since the quantities synthesized in these translation extracts were too low to measure the rhodanese enzymatic activity.

Rhodanese Synthesis and Folding—Since GroEL only binds rhodanese after its release from the ribosome, we were able to test whether GroEL-independent folding of nascent rhodanese occurs co-translationally. Co-translationally folding has been observed for both the β subunit of tryptophan synthetase and firefly luciferase, using antibody binding and proteolysis, respectively (7, 33). Rhodanese is a 33-kDa enzyme composed of two structural domains. These two domains, the N- and C-terminal domains, are structurally homologous despite little amino acid sequence similarity (34). In order to determine whether any domains of rhodanese form co-translationally, the protease sensitivity of the ribosome attached full-length rhodanese was tested. The product of this digestion with proteinase K is shown in Fig. 4c. A digestion product of approximately 17 kDa appeared under a range of protease concentrations, suggesting that a stable domain of rhodanese formed co-translationally (Fig. 4c, lane 2). This domain was stable at proteinase K concentrations of at least 100 μg/ml (data not shown). Furthermore, the domain is protease-resistant for at least 90 min under the conditions used for Fig. 4c.

Because approximately 35 amino acid residues of the C terminus of rhodanese are likely to be buried within the ribosome, and therefore unavailable for folding (35, 36), it is reasonable to propose that the ribosome bound full-length rhodanese consists of a protease stable N-terminal domain and a less well folded C-terminal domain. This was tested directly by generating a stalled translation complex of rhodanese lacking the C-terminal domain. The mRNA from this truncated version of the rhodanese message (amino acids 1–206 rhodanese) would then allow the translation of the N-terminal domain, the short linker region between the two domains, and the first 35 amino acids of the C-terminal domain. Therefore only the N-terminal domain and the linker region would be protease-accessible in the stalled translation complexes. The amino acids 1–206 rhodanese stalled translation complex was formed and digested with proteinase K as described above (Fig. 4d). As seen with the full-length rhodanese, a 17-kDa protease-resistant fragment was generated from the ribosome bound truncated rhodanese (lanes 5 and 6). It appears that the fragment generated by protease treatment of the stalled rhodanese ribosome complex corresponds to the N-terminal region that is similar or identical to the N-terminal structural domain of the native protein.

It should be noted that the 17-kDa protease-resistant domain did not appear to be present on all nascent rhodanese molecules. Quantitation of the 17-kDa domain generated under a range of protease concentrations and time points indicate that about half of the full-length molecules do not have a protease-resistant N-terminal domain. In other words, at the first time point in typical rhodanese digestions, around 50% of the original signal is lost. It is unlikely that the loss of half the nascent N-terminal domains represents a rapid digestion of this domain under these conditions, however. As shown in Fig. 5a, for example, the protease-insensitive N-terminal domain that does form is quite stable for at least 90 min under relatively high concentrations of protease.

Effect of GroEL on Rhodanese Structure—In the previous experiments we observed that the N-terminal domain of rhodanese formed co-translationally, but that GroEL binding only occurred posttranslationally. This order of events raises a new
points and quenched in 1 mM phenylmethylsulfonyl fluoride. This allows us to explore whether the bacterial chaperone GroEL binds to newly synthesized polypeptide chains immediately prior to or following the completion of translation. In the relatively slow process of translation, the growing polypeptide may expose regions that become quickly buried upon release from the ribosome. An incompletely translated polypeptide bound to a molecular chaperone may exist in a conformation different from that found in the full-length protein bound to the chaperone upon dilution from urea. Thus, the structure of a GroEL-bound protein in vitro may not reflect the structure of the polypeptide initially encountered by GroEL in the cell.

The model protein used in our study, rhodanese, has been used in several GroEL-assisted refolding studies (14, 15, 22, 24) and was therefore a good candidate to probe for co-translational interactions with GroEL. Our studies demonstrate, however, that the first stable binding between GroEL and rhodanese occurs posttranslationally. We would not have detected a transient interaction between GroEL and a partially translated rhodanese in this study. Therefore, it remains possible that GroEL needs access to the end of an unfolded protein in order to bind. Once the N-terminal domain rhodanese has folded, GroEL cannot rebind to the unfolded C-terminal domain until after the chain has been released from the ribosome. Since the first stable binding is found only after the entire polypeptide chain has been translated and released from the ribosome, it is reasonable to suppose that the rhodanese structure first recognized by GroEL following protein synthesis in the cell is similar or identical to the rhodanese structure first recognized by GroEL in refolding studies with full-length protein.

A co-translational interaction between hsp60s and a nascent chain has been observed in a study using firefly luciferase as a model protein (7). The cytosolic hsp60 homologue in eukaryotes called TRiC or TCP-1 bound stably to this protein when translated in rabbit reticulocyte lysate. It was not determined whether the translation product interacted directly with TRiC or whether it remained bound to the other proteins in the complex, which included hsp70 and hsp40. These results are consistent with a sequestration, or infinite dilution function for hsp60s. In this model, even as the protein is being synthesized, it is being threaded into a chaperone, which effectively segregates the folding protein from all prospective aggregation partners. Through repeated rounds of ATP hydrolysis, the folding chain matures within or on a single GroEL oligomer. Only after the nascent protein obtains its native state is it released.

A schematic model that summarizes our results is shown in Fig. 6. Only the N-terminal domain of the full-length rhodanese has folded while bound to the ribosome. This partially folded state (I) is maintained following release from the ribosome (I). Upon release, the nascent chain is now able to bind to the molecular chaperone GroEL (2), thereby causing the unfolding of the N-terminal domain (U). Since the binding to GroEL is sufficient to unfold the substrate protein, the energy of ATP hydrolysis is likely be used mainly to release the unfolded substrate from the GroEL surface (3). As suggested in the detrapping model, the unfolded and released rhodanese can (4) either fold to its native state (N), rendering it unable to rebind to rhodanese or can once again form a GroEL binding competent state (I). It is not clear if rhodanese would again pass through the same I state upon release from GroEL.

Weissman et al. (15) have presented evidence that full-length rhodanese exchanges between GroEL oligomers, indicating that the protein does not remain sequestered within a single GroEL throughout the folding pathway. They also observed that the structure of rhodanese was essentially unchanged in successive GroEL-bound states. In their kinetic detrapping model of GroEL-assisted folding, the folding of rhodanese does

**DISCUSSION**

The generation of stalled translation complexes has allowed us to explore whether the bacterial chaperone GroEL binds to
not occur on GroEL but in solution. The function of the chaperone is then to assist rhodanese back onto the productive folding pathway by successive rounds of binding and release.

Our results demonstrating that GroEL actually unfolds rhodanese upon binding are more consistent with the kinetic detrapping model for GroEL function. In the infinite dilution model, the folding develops on the GroEL oligomer; GroEL must therefore be able to bind to a variety of folding intermediates. In contrast, in the kinetic detrapping model, either the binding of the protein to GroEL, or ATP hydrolysis, helps unfold kinetically trapped intermediates. We have found that the binding to GroEL is sufficient to unfold the co-translationally formed N-terminal domain of rhodanese. This domain had not been observed in previously published studies using rhodanese refolded from chemical denaturants. In the GroEL-bound form, as noted in previously published work, rhodanese does not generate any protease-resistant fragments (14). The GroEL bound rhodanese is still competent to fold into a more protease-resistant form in the presence of ATP and the co-chaperone GroES.

Using a coupled transcription/translation bacterial extract, Kudlicki et al. (37) have recently shown that puromycin released rhodanese possesses no enzymatic activity. Two possible explanations for this are that the puromycin group interferes directly with the active site of rhodanese or that proper folding is inhibited by puromycin. Our data support the former suggestion, as we have found that puromycin-released rhodanese can go on to become soluble and protease-resistant.

Are the hsp60 class of molecular chaperones involved in folding and assembly? If assembly occurs through protein subunits free in solution, it is difficult to imagine how GroEL could be assisting in this process through an infinite dilution type mechanism. Individual subunits in some multisubunit proteins do not fold into their proper tertiary structure until they assemble. Therefore the subunits would need to be released from GroEL in a partially folded state. There is little distinction between protein folding and assembly in the kinetic detrapping model (Fig. 6). In both cases the partially folding individual polypeptides can either proceed into their final native structure or can branch off the folding/assembly pathway to a non-native, possibly aggregated state. GroEL binding would help to reverse the off pathway reaction by generating a free folding/assembly-competent state. The folding and assembly would then occur free in solution.

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Fig. 6. Model for chaperone-assisted rhodanese folding prior to release from the ribosome, the N-terminal domain of rhodanese (dark stripes) forms a protease-resistant domain, yet the C-terminal region (light stripes) remains protease-sensitive. Following release (1), this folding state is maintained (I). GroEL can then bind to the released rhodanese (2), causing the unfolding of the N-terminal domain (U). GroES and ATP facilitate the release (3) and folding of rhodanese (N).
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