Molecular chaperones are necessary for the breakdown of many abnormal proteins, but their functions in this process have remained obscure. The rapid degradation of the abnormal fusion protein CRAG in \textit{Escherichia coli} requires the molecular chaperones GroEL, GroES, and trigger factor and proceeds through the formation of a CRAG-GroEL-trigger factor complex. Also associated with GroEL are smaller discrete fragments of CRAG. Pulse-chase experiments showed that these fragments were short-lived intermediates in CRAG degradation formed by C-terminal cleavages. Thus, CRAG degradation is not highly processive. In cells lacking the ClpP protease, the generation of these fragments and their subsequent degradation were much slower than in the wild type. Dissociation of CRAG from GroEL was necessary for its digestion by the ClpP protease, because in a \textit{groES} temperature-sensitive mutant, CRAG was stable and accumulated on GroEL. Furthermore, the expression of a dominant GroEL mutant defective in substrate dissociation slowed degradation of both CRAG and the fragments. Therefore, we suggest that CRAG degradation proceeds through multiple rounds of substrate binding to GroEL, followed by their GroES-dependent dissociation, which allows further digestion by the protease. In this multistep process, GroEL and GroES function repeatedly, apparently to allow further degradation of CRAG and its fragments by the protease.

In addition to their roles in protein folding and translocation (1–4), molecular chaperones are also necessary for the selective degradation of certain proteins with highly abnormal conformations. This role for the molecular chaperones is best documented in \textit{Escherichia coli} but has also been demonstrated in yeast, animal cells, and mitochondria (5, 6). Moreover, the breakdown of different abnormal polypeptides appears to require different chaperones and distinct ATP-dependent proteases (7, 8). For example, if alkaline phosphatase fails to be secreted into the periplasm, it does not fold correctly and is rapidly degraded by protease La (\textit{lon}) in a process requiring DnaK (the Hsp70 homolog in bacteria), and its cofactors, DnaJ and GrpE (7). By contrast, the rapid breakdown of the recombinant fusion protein, CRAG, requires the ClpP protease as well as GroEL, GroES, and trigger factor (TF) (8, 9).

The precise roles of chaperones in these degradative processes are still uncertain. One critical property of the molecular chaperones is that they selectively bind to unfolded proteins, and it has been suggested that association of an unfolded polypeptide with the chaperone may serve to promote substrate recognition by the cell's ATP-dependent proteases (5, 6). Alternatively, the chaperones may function together with proteases during the degradative process, preventing aggregation of the unfolded substrates, promoting their unfolding, or helping to maintain them in a conformation that can be readily digested by cellular proteases.

The major goal of the present study was to clarify the role of the molecular chaperones, GroEL and GroES, in protein breakdown. The unique structure of the fusion protein CRAG provides many experimental advantages for such studies. This rapidly degraded protein contains at its N terminus an unfolded 12-amino acid domain of the Cro repressor, followed by an IgG-binding domain of protein A, and 14 amino acids derived from \(\beta\)-galactosidase at its C terminus (10). Because of the protein A domain, CRAG can be easily isolated from cell extracts together with associated proteins by affinity chromatography on an IgG-Sepharose column. By this approach, GroEL, TF, and CRAG were shown to form \textit{in vivo} ternary complexes, containing one TF molecule, one GroEL dodecamer, and one CRAG molecule (or CRAG fragment) (11). The formation of these complexes appears to be an initial, rate-limiting step in CRAG degradation, and increased expression of GroEL/GroES promotes complex formation and CRAG degradation (8, 9). Even in cells not expressing CRAG, some TF forms complexes with GroEL (11), and these GroEL-TF complexes show a higher affinity for CRAG and for various other unfolded proteins than does GroEL alone (11). This capacity of TF to enhance that association of GroEL with CRAG can account for the finding that increased expression of TF also promotes CRAG degradation (9).

To elucidate the degradative pathway and the precise roles of GroEL and GroES in CRAG degradation, we have studied the fate of CRAG molecules after binding to GroEL. We show here that the digestion of CRAG occurs not by a single highly processive mechanism but through the formation of discrete short-lived polypeptide intermediates. Moreover, this process appears to involve multiple cycles of binding of these polypeptides to GroEL followed by GroES-mediated dissociation from GroEL, which seems to allow proteolytic digestion by ClpP. Degradation of CRAG, like that of most proteins, requires ATP, but the biochemical basis for this energy requirement is unclear (8), because none of the ATPases that are known to associate with ClpP (ClpA, ClpB, or ClpX) are essential for this degradative process (8). Data presented in this paper suggest that the energy required for GroEL/GroES function might account for the ATP utilized in the degradation of CRAG.

**EXPERIMENTAL PROCEDURES**

Cell Extracts—To prepare cell extracts, cells were harvested by centrifugation and resuspended in 50 mM Tris (pH 8.0), 5 mM EDTA, 2 mg/ml lysozyme. This suspension was frozen, thawed, and then sub-

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‡ Present address: Boston Biomedical Research Inst., Boston, MA 02115

1 The abbreviations used are: TF, trigger factor; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-\(\beta\)-\(\gamma\)-galactopyranoside.

**Rapid Degradation of an Abnormal Protein in \textit{Escherichia coli}**

Proceeds through Repeated Cycles of Association with GroEL*  

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Olga Kandror, Michael Sherman‡, and Alfred Goldberg  

From the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115  

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jected to brief sonication. After centrifugation at 14,000 x g for 10 min to remove cell debris, the soluble cell extract was used in further experiments.

Rate of CRAG Degradation—The rate of CRAG degradation in the cells was measured either by the pulse-chase protocol followed by immunoprecipitation or by Western blotting (8). For the pulse-chase, cells were grown in minimal medium M9 containing essential amino acids, thiamine, and 0.5% glucose until mid-log phase. 10 μCi/ml of [35S]methionine (ICN) were added for 1 min (if not specified differently in the figure legends), and then nonradioactive methionine (final concentration, 0.3 mM) and chloramphenicol (0.1 mg/ml) were added to stop 35S incorporation. Aliquots (0.5 ml) were taken at different time points and put on ice. The cells were pelleted by centrifugation, resuspended in 20 μl of 50 mM Tris-HCl (pH 7.5) with 0.3% SDS, and boiled for 3 min. Then 2 μl of α-β-galactosidase antibodies (Sigma) and 20 μl of protein A immobilized on Trisacryl (Pierce) in the immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.5) were added in a total volume of 1 ml. The mixture was incubated for 2 h at 4 °C with rotation and then centrifuged, after which the pellets were washed three times with 1 ml of the immunoprecipitation buffer containing 0.1% SDS and subjected to SDS-PAGE. The gels were dried, and the amount of radioactive CRAG was determined using a PhosphorImager. Electrophoresis and Western blot analysis was performed as described previously (8).

To measure CRAG degradation by Western blot analysis, cell cultures were grown at 37 °C in LB medium mid-log phase. Then protein synthesis was blocked by the addition of chloramphenicol (0.1 mg/ml), and aliquots were taken at different time points after addition of chloramphenicol. Cell proteins were precipitated with 10% trichloroacetic acid, and pellets were washed with acetone and resuspended in Laemmli sample buffer. Samples were analyzed by SDS-PAGE followed by Western blot using anti-β-galactosidase antibody to detect CRAG.

IgG-Sepharose Column (Sigma) was equilibrated with buffer A (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol). Crude extract of [35S]-labeled cells (5 mg at 1×10⁶ cpm/mg) or combined fractions from gel filtration were applied to 2-ml columns, which were then washed with buffer A until the radioactivity in the eluted material had fallen to 5–8 × 10⁵ cpm/mg (about 20 column volumes). To elute all the proteins from the column (including CRAG) 100 mM acetic acid (pH 2.5) was used. Eluted proteins were precipitated with 10% trichloroacetic acid, washed with acetone, and analyzed by SDS-PAGE followed by autoradiography or by Western blot.

CRAG Column—CRAG protein was isolated from a lon-clpP double mutant strain using an IgG-Sepharose column, as described above. The IgG column was washed first with 10 mM Mg-ATP, followed by 1 mM acetic acid (pH 4.5) to strip all the proteins from CRAG. CRAG was then eluted with 100 mM acetic acid, immediately neutralized by 1 M Tris-HCl (pH 8.0), and concentrated to 1 mg/ml. CRAG column was prepared as in “Experimental Procedures”. Associated proteins were eluted with acid and analyzed by SDS-PAGE and autoradiography. The fractions from gel filtration containing free proteins were also combined and analyzed by similar procedures. CRAG fragments containing GroEL were collected, combined, and loaded onto a 2-ml IgG-Sepharose column (see “Experimental Procedures”). Associated proteins were eluted with acid and analyzed by SDS-PAGE and autoradiography. The fractions from gel filtration containing free proteins were also combined and analyzed by similar procedures.

RESULTS

CRAG Fragments Are Found in Association with GroEL—To understand the function of GroEL and TF in CRAG turnover, we purified CRAG-GroEL-TF complexes from cell extracts. These extracts were subjected to sucrose gradient centrifugation (or gel filtration in certain experiments) to separate GroEL together with associated proteins (including CRAG) from free CRAG molecules. After centrifugation, CRAG was soluble (none was found at the bottom of the gradient or as large aggregates). CRAG was present either at the top of the gradient or in association with GroEL. CRAG-GroEL complexes were then isolated from the high molecular weight gradient fractions by affinity chromatography on an IgG-Sepharose column (9). As noted previously, these purified complexes contained both intact CRAG molecules (32,000) and smaller fragments of CRAG (e.g. of 26,000 and 18,000) (Fig. 1A). Because these fragments had bound to the IgG-Sepharose column, they must contain an exposed protein A domain that is accessible for interaction with IgG, whereas the polypeptide is still attached to GroEL. The 10 N-terminal residues of the 26,000 and 18,000 fragments perfectly matched those of the CRAG N terminus (9). Thus, the fragments found in association with GroEL and TF must have been generated by proteolytic trimming of the C-terminal region of CRAG.

To prove that these fragments were generated in the intact cells and were not formed by proteolytic cleavages after cell disruption or during isolation on the affinity column, we performed the following experiment. After labeling the cell proteins with [35S]methionine for 30 min, the culture was divided into two parts. One portion was sonicated and a cell extract was prepared as in Fig. 1A. CRAG and its fragments were then isolated by affinity chromatography on the IgG column. The other portion of the culture was centrifuged, and cell proteins were immediately denatured by boiling in a buffer containing 0.3% SDS (8). This treatment prevented the action of proteases that might be released from the periplasm or outer membrane, when cells were disrupted by sonication (as in Fig. 1A). CRAG and its fragments containing the protein A domains were then isolated by immunoprecipitation, as described previously (8).

Two major labeled fragments, which were of similar size to the ones found in association with GroEL (Fig. 1A), were isolated by both procedures (Fig. 1B). Thus, these CRAG fragments were generated prior to cell disruption.

CRAG Fragments Are Intermediates in Its Degradation—Experiments were carried out to test whether the CRAG fragments that we found associated with GroEL were in fact intermediates in CRAG degradation and not some proteolytic end products unrelated to the major degradative pathway. Wild type cells were labeled by a very short pulse (10 s) with [35S]methionine, and then a large excess of nonradioactive
methionine and a mixture of antibiotic inhibitors of protein synthesis were added to prevent methionine reincorporation. Aliquots were taken at different times during the chase period, the cells were collected by centrifugation, and cell proteins were denatured by boiling in SDS (as described above). CRAG and its fragments were immunoprecipitated and analyzed by SDS-PAGE followed by autoradiography. In this wild type strain, the full-length CRAG was degraded with a $t_{1/2}$ of about 20 min. As this molecule disappeared, CRAG fragments were generated and then disappeared (Fig. 2). Thus, the fragments behave as short-lived intermediates in CRAG degradation. In other words, CRAG degradation is not highly processive but proceeds through C-terminal cleavages, which lead to the formation of relatively stable degradative intermediates.

Because the rates of CRAG degradation vary significantly depending on the genetic background and growth rate of the cell, all experiments and controls presented in each figure were carried out in parallel on the same day. CRAG degradation in MPH86 (Fig. 2) was faster than in the C600 strain, which was used in most of our experiments. Although the absolute rates of degradation differed in these two wild type strains, the quantity of the fragments in both strains increased and then decreased with time. Presented in Fig. 2 are the data with MPH86, where the kinetics of generation and disappearance of the fragments were more pronounced.

CRAG Fragments Are Generated Mainly by the ClpP Protease—We have previously shown that the ClpP protease is mainly responsible for CRAG degradation (8). To test whether ClpP also catalyzes the formation and degradation of CRAG fragments, pulse-chase experiments were carried out in a wild type strain and in a mutant lacking the ClpP protease. The pattern of the CRAG fragments generated in these strains, and the kinetics of their appearance and disappearance were compared. In these experiments, we could not use a 10-s pulse, as in Fig. 2, because the mutant cells grow more slowly than the wild type, and in 10 s, the mutants do not incorporate sufficient $^{[35]S}$methionine into cell proteins to be studied. Therefore, the cells were labeled for 1 min, and as a consequence, in the wild type strain, degradation intermediates were already evident at the zero time point. It is also noteworthy that after the 1-min pulse, more labeled fragments were evident (Fig. 3) than following a 30-min labeling (Fig. 1), because of the rapid turnover of the more labile fragments and the accumulation of the more stable ones.

In the wild type C600 cells at 37 °C, where full-length CRAG was degraded with a half-life of about 40 min, four labeled fragments (28, 24, 18, and 16 kDa) were present at the earliest time point, and the two major ones, 26 and 18 kDa, corresponded to those previously seen in complex with GroEL (Fig. 1). These four fragments disappeared during the chase period with half-lives of about 30, 15, 20, and 7 min, respectively (Fig. 3). In the mutant lacking ClpP, where full-length CRAG is much more stable, all these fragments appeared much later than in the wild type cells and reached their maximal level only after 10 min of the chase period (Fig. 3). In addition, the fragments were much more stable, showing half-lives that ranged from 30 to 90 min (Fig. 3). Thus, ClpP is the protease responsible primarily for both the formation of the intermediates and their subsequent degradation.

Although CRAG degradation was strictly ATP-dependent, it was not reduced in strains lacking either of the ATPases known to form complexes with ClpP, ClpA, or ClpX and in strains lacking the homologous ATPase, ClpB. We therefore proposed that ATP hydrolysis by GroEL/GroES contributes to the energy requirement for CRAG degradation (8). To rule out the possibility that either ATPase alone can support this process, we followed CRAG degradation in a mutant strain lacking all three ATPases, ClpA, ClpB, and ClpX (kindly provided by Susan Gottesman). As shown in Fig. 4, the rate of CRAG degradation was not slower in the triple mutant; thus none of these regulatory ATPases is involved in this ATP-dependent process.

Protease La Catalyzes the ClpP-independent Generation of CRAG Fragments—In the absence of ClpP, some other protease(s) can catalyze CRAG degradation, although at a significantly slower rate. Interestingly, this residual protease(s) generates a rather similar pattern of CRAG fragments that also behave as intermediates in the degradative pathway (Fig. 3A). Our prior data have show that the lon gene product, protease La, contributes in part to CRAG degradation (8). To test whether this protease is responsible for the residual degradation in the clpP mutant (Fig. 3A), analogous pulse-chase experiment was carried out in lon-clp double mutant. In this strain, lacking both proteases, full-length CRAG is completely stable (Fig. 3B), and no fragments are generated. Thus, protease La is responsible for this residual slow rate CRAG degradation and fragment generation. The finding that fragments of roughly similar sizes are produced by two very different proteases suggests that these fragments accumulate because they are inherently resistant to proteolytic attack rather than to any specific feature of the proteases ClpP or La.

Dissociation from GroEL Is Essential for CRAG Degradation—Because the N-terminal fragments of CRAG were found in complexes with GroEL, we attempted to clarify the role of GroEL in fragment formation. One possibility is that the CRAG-GroEL complexes are the sites of proteolysis by ClpP. Although this protease was not found in the complex (not shown), ClpP may attack CRAG while it is bound to the GroEL molecule, and the resulting fragments may then remain associated with GroEL, perhaps in a form that favors further proteolysis. In this case, degradation would not require dissociation of the CRAG molecule from GroEL. Alternatively, after initial binding, the CRAG molecule might dissociate from GroEL in an ATP- and GroES-dependent reaction, in an unfolded form that is particularly susceptible to cleavage by ClpP (and more slowly by protease La).

To distinguish between these two possibilities, we used a temperature-sensitive groES619 mutant in which the dissociation of the substrate from GroEL is blocked at the nonpermissive temperature (8). When the degradation of CRAG was studied in a pulse-chase experiment with this groES mutant, CRAG was completely stable at the nonpermissive temperature, and the shorter fragments were not generated (Fig. 3). Thus, when dissociation could not occur, the degraded full-length CRAG accumulated in cells associated with GroEL. These experiments indicate that in the course of degradation, the CRAG molecule initially binds to GroEL and then dissociates from the chaperone, and this dissociation step is necessary for proteolytic attack by ClpP.

Do GroEL and GroES Function Once or Repeatedly during CRAG Degradation?—To address this question, we used a dom-
inant negative GroEL Trap mutant (337/349), which binds unfolded proteins, but is defective in their dissociation, despite the presence of ATP and GroES (12). This mutant was previously used by Horwich and co-workers (12) to demonstrate that multiple rounds of GroEL/GroES action are necessary for the folding of some proteins. To achieve a high level of its expression, we subcloned this mutant form of GroEL together with wild type GroES in a multicopy plasmid (pBluescriptSK) under the regulation of the \( \text{lac} \) promoter. To confirm that this mutant GroEL can bind CRAG but is defective in its release, equal amounts of the extracts (by protein) from cells overexpressing normal or mutant GroEL were passed over affinity columns with CRAG covalently bound to Sepharose (see “Experimental Procedures”). The columns were extensively washed with buffer A, and then the associated proteins were eluted first with ATP, which causes normal GroEL to dissociate from the complexes with CRAG (7, 8), and then with acid to elute all proteins bound to the column. As shown in Fig. 5, much more GroEL bound to the column from the extract of the Trap mutant than from the one overexpressing normal GroEL, although the rate of expression was similar in both strains. Of the total amount of GroEL bound from the Trap extract, only about 30% could be dissociated by ATP, and this amount is probably due to the chromosomally encoded wild type GroEL present in the cells before induction. In contrast, almost 80% of the bound GroEL were released by ATP in the control extract. These results confirm that most of the CRAG bound by the mutant GroEL was bound irreversibly, and thus, the Trap mutant can be used to define the role of GroEL in this degradative pathway.

To test directly whether dissociation from GroEL is critical for the degradation of CRAG and its fragments, we co-expressed CRAG together with the Trap-GroEL mutant and followed CRAG degradation in this strain to learn whether overexpression of the dominant inhibitor (upon induction with IPTG) would trap some CRAG molecules and thus slow its degradation. As predicted, the expression of this mutant GroEL resulted in a reduced rate of CRAG degradation (Fig. 6A). The rate of cell growth did not change upon induction with IPTG; therefore, the overproduction of the GroEL mutant was not generally harmful to the cells, and it is unlikely that CRAG degradation was decreased by some nonspecific, indirect mechanism. Moreover, we have previously shown that a similar overproduction of the normal GroEL/GroES had the opposite effect, i.e. that the wild type chaperonin stimulated CRAG breakdown in contrast to the inhibition seen with this non-dissociating mutant (8).

To test whether GroEL is also involved in the degradation of

![Fig. 3](image1.png)

**Fig. 3.** A, the pattern and rate of CRAG fragment generation depends mainly on ClpP and GroES. Wild type (WT) C600 and clpP strains were grown in minimal medium at 37 °C until mid-log phase. Cell proteins were labeled with \( \text{[35S]} \)methionine for 1 min. Aliquots were taken at different times of the chase, and CRAG and its fragments were immunoprecipitated. The \( \text{ts-groES} \) mutant was grown at 37 °C until mid-log phase and shifted to 44 °C for 0.5 before labeling. Inactivation of ClpP led to slower generation and slower degradation of intermediates than in the wild type. Inactivation of GroES prevented CRAG breakdown and the appearance of the intermediates. B, no fragments are generated in \( \text{lon-clpP} \) double mutant. The pulse-chase experiment with \( \text{lon-clpP} \) double mutant was carried out at 37 °C as in A.

![Fig. 4](image2.png)

**Fig. 4.** CRAG degradation is not altered in cells lacking ClpA, ClpB, and ClpX ATPases. Wild type (WT) MC4100 strain and the triple mutant, clpABX (SG22091), were transformed with pRIT2 plasmid carrying CRAG under the \( \text{pl} \) promoter. Both cultures were grown at 37 °C in LB medium to mid-log phase. CRAG degradation was measured by Western blot analysis.
the CRAG fragments, we compared the relative amounts of CRAG and the fragments in the wild type cells and the cells overproducing Trap-GroEL. The protein A domain of the CRAG molecule must protrude from the GroEL cavity, because CRAG in complex GroEL was isolated from the extracts by affinity chromatography on an IgG-Sepharose column. After elution with acid, proteins were precipitated in 10% trichloroacetic acid and analyzed by SDS-PAGE followed by autoradiography.

Because we were unable to demonstrate any GroES in the GroEL-CRAG complexes (8, 11), it is most likely that GroES binds to CRAG (9); 2) GroEL is found in vivo in complexes with both full size CRAG and the smaller fragments, which were shown to be intermediates in CRAG degradation; and 3) both the formation and further breakdown of these intermediates requires binding to GroEL and GroES-dependent dissociation of CRAG from GroEL into the cytosol, indicating that the chaperonin must function repeatedly in the course of degradation.

Several mechanisms have been proposed to account for the requirement for molecular chaperones in protein degradation; for example, the chaperones have been proposed to maintain substrates in a soluble nonaggregated form (13, 14) or to facilitate their initial recognition in prokaryotes by cellular proteases (7) or in eukaryotes by ubiquitination enzymes (15, 16). These models can not apply to CRAG and its fragments, which are all soluble, monomeric proteins. The results presented here demonstrate that GroEL and GroES play a quite different role in this nonprocessive pathway, in which the chaperones and the proteases carry out complementary reactions at multiple steps during the degradation of CRAG, as summarized in Fig. 7.

The pathway proposed in Fig. 7 is strongly supported by several observations: 1) GroEL, especially when in complexes with TF, binds strongly to CRAG (9); 2) GroEL is found in vivo in complexes with both full size CRAG and the smaller fragments, which were shown to be intermediates in CRAG degradation; and 3) both the formation and further breakdown of these intermediates requires binding to GroEL and GroES-dependent dissociation of CRAG from GroEL into the cytosol, indicating that the chaperonin must function repeatedly in the course of degradation.
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7) resembles and is consistent with the type of reiterative mechanism proposed earlier for GroEL-assisted protein folding (12, 17, 18). Accordingly, the initial binding of unfolded proteins by GroEL depends on interactions between the hydrophobic inner surface of GroEL and exposed hydrophobic domains on the substrate (19). The subsequent binding of ATP and GroES to the cis GroEL ring creates an enlarged cavity in which the substrate can fold in an isolated environment (20–22). When ATP binds to the trans GroEL ring, the GroES cap dissociates from the GroEL ring (23), and the protein is ejected from the cavity, whether or not it has folded successfully (12).

Apparently, some substrates are able to refold within the central cavity of GroEL; however, other polypeptides, especially mutant or damaged proteins, such as CRAG, are unable to reach a stable, native conformation, by the time of GroES-induced release (12, 24, 25). Such polypeptides appear to dissociate from the chaperonin in an unfolded form that allows refolding or in the case of CRAG, degradation. The kinetic partitioning between these alternative fates must depend on the structural properties of the polypeptide and its fragments, which determine whether they fold, are susceptible to digestion, or rebind to GroEL for another folding attempt. Because CRAG, like many abnormal proteins (and presumably a fraction of normal gene products), never achieves a properly folded structure, it is temporarily unfolded by GroEL and released in a form that can be hydrolyzed by cytosolic proteases, primarily by ClpP, or at a slower rate by protease La.

After release from GroEL, CRAG is cleaved by the ClpP (or La) protease in its C-terminal region, because all the fragments isolated from the cell contain the normal N-terminal sequence. To be degraded by ClpP, a polypeptide must enter within the degradative chamber of ClpP, formed by its two rings. Presumably, the role of GroEL/GroES is to release the CRAG molecule in a conformation capable of entering into the ClpP complex. It seems likely that GroEL unfolds CRAG, including its tight protein A domains, which otherwise probably could not be digested by the ClpP protease. GroES then causes release of CRAG or its fragments in a conformation in which the C-terminal end is susceptible to proteolysis by ClpP (at least temporarily).

Presumably, the degradative intermediates of CRAG, e.g., the 26,000 and 18,000 fragments, are relatively stable and can be isolated because their structure retards further digestion, unless they rebind to GroEL and undergo another round of ATP-GroES-mediated unfolding. This mechanism implies that there are probably two competing processes, in which digestion by ClpP competes with the tendency of CRAG (and its fragments) to quickly reacquire a tight globular conformation that prevents further digestion. Thus, these intermediates probably exist free in the cell until they are recaptured by GroEL and released in a more unfolded, more readily digested conformation. In this way, smaller and smaller N-terminal fragments are generated by ClpP (Fig. 7). The final steps in the degradative pathway are uncertain, because the present approach could not isolate smaller intermediates that have lost the protein A domain.

The finding that CRAG degradation proceeds through the formation of relatively stable polypeptide intermediates that can be isolated from the cell was surprising. The major ATP-dependent proteases in E. coli, Lon, ClpAP, and HslUV (26) degrade model proteins in a highly processive manner without releasing partially digested proteins (27–29). In eukaryotic cells and archaea, proteasomes also function in a highly processive way (30, 31). This processive behavior must be advantageous for the cell because it prevents the appearance in the cytosol of partially digested fragments, which could interfere with normal metabolic regulation and protein-protein interactions. However, with many partially folded proteins, there may be internal features (e.g., tight globular domains in CRAG) that prevent rapid proteolysis and lead to substrate dissociation from the protease complex. Further degradation of the released fragments would thus require this unfolding by chaperones. Accordingly, when GroEL or GroES were inactivated, CRAG degradation was completely blocked (Fig. 3).

Protein degradation by ClpP (8) normally requires the function of an associated ATPase subunit (32, 33). Two ATPases, ClpA (32, 33) and ClpX (34, 35), can function in the ClpP-dependent degradation of different proteins and confer substrate specificity on this process (34, 35). Both ClpA and ClpX appear to possess some “chaperone-like activity,” because by themselves they can promote the disassembly of specific protein complexes in vitro (36, 37). Surprisingly, although CRAG degradation by ClpP is strictly ATP-dependent (8), CRAG degradation is not reduced in the triple clpABX mutant strain (Fig. 4). Thus, ClpP functions in this process without any of these ATPases, and the energy requirement for CRAG breakdown appears, by exclusion, to be due to the involvement of GroEL/ES. Because ClpP by itself has been shown to degrade only oligopeptides, it seems likely that molecular chaperones GroEL/GroES substitute for the ATPases in CRAG degradation by unfolding the CRAG molecule or its fragments and presenting them in a form susceptible to the ClpP protease. Because ClpP was never found in these CRAG-GroEL complexes, the unfolding must be a distinct GroEL-mediated event taking place prior to the substrate’s entry into the degradative chamber.

It seems quite unlikely that CRAG degradation is a special mechanism. On the contrary, GroEL and GroES are likely to play a similar role in degradation of other proteins. Also, whereas GroEL and GroES are absolutely required for this process, some CRAG breakdown occurs, albeit 4–5-fold more slowly, in the clpP strain. This residual proteolysis, although catalyzed by the ATP-dependent protease La, still requires involvement of GroEL/ES and is not processive. Other cytosolic protease complexes (e.g. HsUlV and 20S and 26S proteasomes) also have ring-like structures that require polypeptide unfolding for entry into their central degradative chambers. Therefore, it seems likely that the chaperones may function, as they seem to in CRAG degradation, to facilitate the hydrolysis of polypeptides or fragments that otherwise resist digestion by such proteases.

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