Rapid Degradation of an Abnormal Protein in Escherichia coli Involves the Chaperones GroEL and GroES*

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In Escherichia coli, the molecular chaperones (DnaK, DnaJ, and GroE) are essential for the rapid degradation of certain proteins. To see if chaperones are involved more generally in proteolysis, we studied the degradation of a short-lived fusion protein, CRAG, which associates with DnaK and GroEL in vivo. Its rapid degradation requires ATP and ClpP, the proteolytic subunit of protease Ti (Clp). However, this process is not reduced in strains lacking the complementary ATPase subunit, ClpA, or its homologs, ClpB and ClpX. At 37 °C, but not at 42 °C, protease La also contributes partially to CRAG degradation. Nevertheless, CRAG is not degraded in cell-free extracts or upon incubation with ClpP or protease La.

We tested whether the chaperones associated with CRAG might be involved in its degradation. CRAG breakdown was accelerated 2-3-fold in strains with high levels of heat-shock proteins (hsp)s, i.e. in those that overproduce the hsp transcription factor (σ^B) or carry a dnaK deletion. A similar stimulation of proteolysis was observed in cells overproducing GroEL or both GroEL and GroES; in these cells, more CRAG was associated with GroEL than in the wild type. In a temperature-sensitive groEL44 mutant at the nonpermissive temperature, CRAG breakdown was accelerated, and more CRAG was found complexed with GroEL. However, in a temperature-sensitive groES mutant, CRAG was completely stable at the nonpermissive temperature and accumulated bound to GroEL. These findings indicate that the association of CRAG with GroEL is a rate-limiting step in CRAG degradation, which also requires a subsequent action of GroES. We propose that if the hsp60/hsp10 chaperonins fail to catalyze the proper folding of a protein, they can facilitate its rapid degradation.

In Escherichia coli, as in eukaryotic cells, proteins with highly abnormal conformations are rapidly hydrolyzed via ATP-requiring pathways (Goldberg, 1992). This degradative system is induced as part of the cell’s heat-shock response (Goff et al., 1988; Gottesman, 1989). The signal for the induction of heat-shock genes is the intracellular accumulation of unfolded polypeptides (Goff and Goldberg, 1985), and the function of a number of heat-shock proteins is to eliminate either by degradation or refolding such damaged polypeptides, whose accumulation could be quite deleterious to the organism. Certain heat-shock proteins (hsp)s in E. coli are ATP-dependent proteases (Goff and Goldberg, 1985; Goff et al., 1984, 1988; Kroh and Simon, 1990), such as protease La (Lon), which is critical in the breakdown of most abnormal proteins. los mutations reduce by severalfold the degradation of polypeptides that are incompletely, contain amino acids analogs, or result from missense mutations (Chung and Goldberg, 1981). Furthermore, when the cellular content of protease La increases (Goff and Goldberg, 1987), as occurs during heat shock (Goff et al., 1984), the cell’s capacity to degrade such abnormal proteins is increased. Protease Ti (Clp) also catalyzes the rapid breakdown of some abnormal proteins, although its effects appear to be generally smaller than those of protease La (Maurizi et al., 1985). However, protease Ti appears to be important in the rapid breakdown of polypeptides with abnormal amino termini (by the N-end rule pathway) (Tobias et al., 1991). Unlike protease La, which is a homotetramer, protease Ti contains two types of subunits: ClpA, which has a protein-activated ATPase activity, and ClpP, which has a proteolytic activity (Hwang et al., 1987, 1988; Katayama-Fujimura et al., 1987).

Other hsp functions as “molecular chaperones” (e.g., DnaK, DnaJ, GroEL, GroES, and GrpE in E. coli), which can promote the refolding or assembly of unfolded polypeptides (Pelham, 1986; Beckmann et al., 1990) or their translocation across membranes (Gething and Sambrook, 1992; Welch, 1991). For example, DnaK, the member of the hsp70 family in E. coli, binds selectively to unfolded proteins and helps prevent their intracellular aggregation (Skowyra et al., 1990). GroEL, the bacterial homolog of the mitochondrial chaperone hsp60, and GroES, the homolog of hsp10 (Pfanner et al., 1990), together promote the ATP-dependent folding of certain polypeptides (Goloubinoff et al., 1989), their assembly into active multimers (Pfanner et al., 1990), or their transport into the periplasm (Wickner, 1989).

Several observations suggested that these chaperones may be important in the degradation of abnormal polypeptides. For example, E. coli mutants with reduced levels of hsp s (rhoH) (Goff et al., 1984; Grossman et al., 1984), or those with mutations in dnaK, dnaJ, grpE, and groE (Simon et al., 1988; Strass et al., 1987) have a reduced capacity to degrade polypeptides containing amino acid analogs or paromycin. However, the mechanism by which hsps promote intracellular proteolysis is unknown. Recently, we showed that DnaK and its cofactors, DnaJ and GrpE, are necessary for the rapid breakdown of a specific mutant protein, a nonsecreted form of alkaline phosphatase (PhoA61) (Sherman and Goldberg, 1992a). The degradation of this polypeptide in the cytosol is rapid at 37 and 41 °C (Sherman and Goldberg, 1992a), and at both temperatures, some of the PhoA61 was found associated with DnaK, protease La, and GrpE. These studies suggested that the for-

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Degradation of Abnormal Protein Involves GroEL/ES

Table I

List of strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>C-600</td>
<td>sup E rad r thi1 thr1 leu lac Y tonA21</td>
</tr>
<tr>
<td>ATC12017</td>
<td>C-600 lac510</td>
</tr>
<tr>
<td>SG12050</td>
<td>C-600 clpP1::Cm'</td>
</tr>
<tr>
<td>SG12051</td>
<td>C-600 lac510 clpP1::Cm'</td>
</tr>
<tr>
<td>OK21</td>
<td>C-600 clpX::Km'</td>
</tr>
<tr>
<td>MG1400</td>
<td>araD (arg F-lac) U169 rpsL18 relA deoC1 pta285 rbsR fblB 5301</td>
</tr>
<tr>
<td>SG21118</td>
<td>MG1400 clpA::Tet'</td>
</tr>
<tr>
<td>SG222080</td>
<td>MG1400 clpX::Km'</td>
</tr>
<tr>
<td>MC1000</td>
<td>Δlac(HOZ) galU galK Δara-lev rpsL spoT relA</td>
</tr>
<tr>
<td>LC23</td>
<td>MC1000 clpB::Km'</td>
</tr>
<tr>
<td>LB24</td>
<td>MC1000 clpB::Km' clpA::Tet' clpP::Cm'</td>
</tr>
<tr>
<td>MP1986</td>
<td>F' supCts (lac (am) trp (am) mal (am) aro phoA61)</td>
</tr>
<tr>
<td>MS41</td>
<td>MPH86 dnaK22::Cm'</td>
</tr>
<tr>
<td>MS60</td>
<td>MPH86 groEL44 Tn10</td>
</tr>
<tr>
<td>OK20</td>
<td>MPH86 groEL44 Tn10 clpP::Cm'</td>
</tr>
<tr>
<td>MS61</td>
<td>MPH86 groES619 Tn10</td>
</tr>
<tr>
<td>CG2245</td>
<td>W3110 galE Tn10</td>
</tr>
<tr>
<td>CG2243</td>
<td>CG2245 groEL140</td>
</tr>
<tr>
<td>CG1921</td>
<td>CG2245 groES30</td>
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MATERIALS AND METHODS

The strains used in these experiments and their sources are listed in Table I. All new strains were constructed by transduction as described by Miller (1972). The cells were routinely grown in Luria broth with aeration at the temperatures indicated in each figure legend. Except where specified, CRAG was expressed constitutively from the RIT2 promoter.
which was isolated by immunoprecipitation with IgG-Sepharose (e.g. Fig. 4). In other experiments, protein synthesis was blocked with antibiotics, and at different times, CRAG levels were measured by quantitative Western blot analysis using IgG and 125I-protein A (e.g. Figs. 1-3, and 6).

Measurements of Protein Half-lives—In all experiments, the rates of CRAG degradation appeared to obey first-order decay kinetics, as was carefully documented in initial control studies with wild-type strains. Therefore, to compare degradative rates, we have routinely calculated the apparent half-lives of the substrate from measurements of three time points. In some strains, the half-lives measured varied by 10-15% in different experiments. The data presented are typical of results obtained at least three times for the control and experimental conditions studied in parallel.

Since the rapid breakdown of many abnormal polypeptides requires ATP (Gottesman, 1989), we tested if CRAG degradation is also ATP-dependent. The bacteria were depleted of ATP by removal of glucose and by addition of α-methylglucoside to block glycolysis and of cyanide and arsenate to block oxidative phosphorylation (Olden et al., 1978). The intracellular level of ATP decreases by >95% within 10 min (Olden et al., 1978). Under these conditions, the degradation of CRAG ceased (Figs. 1B and 2). Thus, ATP appears to be required for this process, either to support the function of an ATP-dependent protease or for some other step(s) in the proteolytic pathway.

Proteases Involved in CRAG Hydrolysis—We therefore used lon and clpP mutants to test if either of the two ATP-dependent proteases, La or Ti, is involved in this process. In a clpP mutant at 37 °C, CRAG was at least 10 times more stable than in wild-type strains (Figs. 1E and 3). Surprisingly, in the lon mutant at this temperature, rates of CRAG degradation also decreased below wild-type rates, but only by ~2.5-fold. Such effects were clearly seen in three independent experiments involving isogenic lon− strains. Thus, the proteolytic subunit of protease Ti (ClpP) is critical for degradation, but at 37 °C, protease La also appears to play some role, but not the major one, in degrading CRAG. Although these findings may suggest, for the first time, that both major ATP-requiring proteases are involved in the degradation of a single protein, it is also possible that the lack of protease La reduces CRAG degradation only by an indirect mechanism. For example, in lon− cells, there is likely to be an accumulation of abnormal proteins that may compete with CRAG for ClpP or for chaperones (see below). In contrast to 37 °C, at 42 °C, the lon mutation had no effect on CRAG breakdown (Fig. 3), while the clpP mutation still greatly stabilized this polypeptide, but not completely. Thus, at 42 °C, the degradative pathway seems to differ from that at 37 °C and appears to involve ClpP, but not protease La. These experiments also indicate that a distinct proteolytic system that does not involve ClpP also functions at 42 °C and is responsible for ~40% of CRAG degradation.

Degradation of Abnormal Protein involves GroEL/ES

Degradation was measured by Western blot analysis at 44 °C (A, C, and D) or at 42 °C (B and E).

ATP hydrolysis by protease ClpP was previously shown to be catalyzed by the ClpA subunit (Hwang et al., 1987; Katayama-Fujimura et al., 1987); however, this ATPase subunit is not necessary for CRAG degradation. At 37 °C, CRAG was degraded at the same rate in both the wild type and the clpA mutant, while at 42 °C, this process was actually much faster in the clpA mutant than in the wild type. One attractive possibility is that in the clpA mutant, the ClpA component is replaced in protease Ti by its homolog, ClpB (Squires et al., 1991). Like ClpA, the ClpB protein exhibits protein-activated ATPase activity (Woo et al., 1992), and ClpB is induced at 42 °C (Squires et al., 1991). We therefore measured the degradation of CRAG in a clpB mutant and in a clpA/clpB double mutant. As shown in Table II, CRAG was degraded faster in these mutants than in the wild type, especially at 42 °C. Thus, neither the ClpA nor the ClpB ATPase is necessary for CRAG degradation by the ClpP peptidase.

Recently, a new homolog of ClpA was discovered, ClpX, which is encoded by a gene adjacent to clpP (Gottesman et al., 1993; Wojtkowiak et al., 1993). This protein also has ATP-binding domains and was recently shown to be involved in ClpP-mediated degradation of 10 protein (Wojtkowiak et al., 1993). Since ClpX may function as an alternative ATPase subunit in the ClpP-dependent degradation of certain substrates, we tested whether the ClpX protein is involved in CRAG breakdown. When a clpX mutation was introduced into E. coli C-600 cells, instead of reducing CRAG breakdown, it actually accelerated this process at 37 °C (Table II): Thus, the critical ATPase involved in CRAG degradation remains unclear, and possibly, the ClpP peptidase may be regulated by a yet undiscovered regulatory subunit(s).

The reasons for the more rapid breakdown of CRAG in the clpA, clpB, clpX mutants and in the clpA/clpB double mutant are unclear. The acceleration of proteolysis in part involves the ClpP peptidase because when we transduced the clpP mutation into the clpA/clpB double mutant, CRAG degradation in this triple mutant was at least 2-fold slower than in clpA/clpB at both 37 and 42 °C (Table II). However, CRAG was still degraded at ~60-80% of the rate in the wild-type strain. Therefore, in cells lacking ClpA and ClpB, a distinct system not involving ClpP must also be functioning to degrade CRAG.
tempted to reconstitute this process in vitro. Radiolabeled in vivo involves additional component(s) lacking in vitro.

In the MC1000 and C-600 wild-type strains, the rates of degradation of CRAG were measured as reciprocal minutes. All the rates of CRAG degradation were calculated as reciprocal minutes. The rate of degradation of each wild-type strain was considered as 100%, and rates in the isogenic strains were expressed relative to the wild type. In the MC1000 and C-600 wild-type strains, the rates of CRAG degradation ranged between 20 and 35 min. However, in MC4100, CRAG breakdown was 45 min, and it reproducibly increased to $t_\text{m}$. The rate of degradation of CRAG, in contrast to our findings on PhoA61 and 42 °C. No degradation of CRAG was observed under any experimental condition tested, although under these same conditions, casein was hydrolyzed in an ATP-dependent manner by protease La, and the specific peptide substrate succinyl-Leu-Tyr-methylcoumarin was hydrolyzed by ClpP. Thus, both proteases were active under these conditions. The inability of the purified proteases to hydrolyze CRAG suggests that its degradation in vivo involves additional component(s) lacking in vitro.

To clarify the mechanism of CRAG degradation, we attempted to reconstitute this process in vitro. Radiolabeled CRAG was incubated with protease La or ClpP in the presence or absence of ATP, and proteolysis was analyzed by the production of fragments soluble in trichloroacetic acid and by SDS-PAGE. The experiments were carried out at 37 and 42 °C. No degradation of CRAG was observed under any experimental condition tested, although under these same conditions, casein was hydrolyzed in an ATP-dependent manner by protease La, and the specific peptide substrate succinyl-Leu-Tyr-methylcoumarin was hydrolyzed by ClpP. Thus, both proteases were active under these conditions. The inability of the purified proteases to hydrolyze CRAG suggests that its degradation in vivo involves additional component(s) lacking in vitro.

Involvement of Chaperones in CRAG Degradation—CRAG is found in vivo in association with DnaK, GrpE, and GroEL (Sherman and Goldberg, 1991), all of which are important for the ATP-dependent breakdown of certain proteins (Keller and Simon, 1988; Strauss et al., 1988; Sherman and Goldberg, 1992a). To test whether these chaperones are required for CRAG degradation, we measured CRAG's half-life in E. coli carrying a dnaK deletion. CRAG was not stabilized in this strain (data not shown). Thus, DnaK is clearly not essential for the degradation of CRAG, in contrast to our findings on PhoA61 (Sherman and Goldberg, 1991). In fact, CRAG degradation was severalfold faster in the dnaK deletion mutant than in the isogenic wild-type cells. It is noteworthy that in this mutant, there was a marked overproduction of all hsps, as reported previously (Craig and Gross, 1990), and this increase in hsps (e.g. protease La, ClpP, or GroEL and GroES) could be responsible for the accelerated hydrolysis of CRAG. Therefore, we tested whether the overproduction of hsps per se enhances CRAG degradation. In a strain with extra copies of the rpoH (htpR) gene on a plasmid under the control of the lac promoter, the addition of isopropyl-1-thio-β-D-galactopyranoside stimulated CRAG degradation by ~2-fold in three different experiments (data not shown). Thus, some hsp(s) under the control of the rpoH product, aside from DnaK, appear to be rate-limiting in CRAG degradation (see below).

To study if the chaperone GroEL or its cofactor, GroES, is important for CRAG degradation, we transformed cells containing CRAG with multicopy plasmids carrying either the groEL gene alone or both the groEL and groES genes under the control of the lac promoter. These proteins are encoded by the neighboring genes and can function together in protein assembly (Hartl, 1991). Even though GroEL is one of the most abundant proteins in normal cells, the overproduction of either GroEL or GroEL plus GroES resulted in a 2-fold enhancement of the rate of CRAG breakdown (Fig. 4), as had been seen upon overproduction of all hsps. This 2-fold stimulation of proteolysis was observed consistently in several different experiments. To learn whether more GroEL is associated with CRAG when

![Fig. 3. Influence of clpP (SG12050), lon (ATC12051), and lon-clpP (SG12051) mutations on degradation of CRAG at 37 and 42 °C. Bacteria were grown at 37 °C until mid-log phase. Half of the culture was shifted to 42 °C for 30 min, and CRAG degradation was measured at 42 and 37 °C by Western blot analysis.](image-url)
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**Table III**

Effect of temperature-sensitive groEL and groES mutations and overexpression on CRAG degradation and amount of GroEL associated with CRAG

<table>
<thead>
<tr>
<th>Strain</th>
<th>Overexpressed genes</th>
<th>Half-life of CRAG</th>
<th>CRAG associated with GroEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPH86 (wild type)</td>
<td>44</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>groEL44</td>
<td>44</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>groEL140</td>
<td>44</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>groES619</td>
<td>44</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>C-600 + pDR82</td>
<td>37</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>C-600 + pDR84</td>
<td>37</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Fig. 4.** Effect of GroEL overproduction on CRAG degradation. E. coli C-600 cells containing plasmid pDR62 or pDR84 were grown until mid-log phase in M9 minimal medium at 37°C. The culture was then divided into two parts. To one of them was added 1 μM isopropyl-1-thio-β-D-galactopyranoside. After incubation for 40 min, CRAG degradation was measured by immunoprecipitation.

degradation increases, we loaded the [35S]methionine-labeled extracts from the CRAG-containing strains onto an IgG-Sepharose column, washed the column extensively, eluted all the bound components with acid, and then measured the amount of [35S]labeled GroEL (Sherman and Goldberg, 1991).

Control studies indicated that the recovery of CRAG from the IgG-Sepharose column by this approach was quantitative and that an insignificant amount of CRAG was lost in the insoluble fraction during preparation of the extract. When we completely solubilized cells in SDS buffer and precipitated CRAG with an antibody against β-galactosidase, the amounts of CRAG in the precipitate were very similar to those obtained with the IgG affinity column from the same amounts of cells.

It is noteworthy that the amount of CRAG recovered in complex with GroEL was ~5-fold higher in the overproducing strains than in the parental strain, where degradation was slower (Table III). When we overexpressed ClpP on a multicopy plasmid, no acceleration of proteolysis was observed, in contrast to our findings when GroEL is overproduced. These findings clearly indicate that it is the cell's content of GroEL, and not ClpP, that is rate-limiting for CRAG degradation.

To study further the involvement of chaperones in CRAG degradation, its half-life was measured in temperature-sensitive groES and groEL mutants. Transferring the temperature-sensitive groES619 mutant strain to the nonpermissive temperature (44°C) slowed the disappearance of CRAG by ~8-fold (Figs. 1D and 5), but no such change was seen in the wild-type cells. In other words, at 37°C, the rate of CRAG degradation was similar in the mutant and isogenic wild-type strains, but it was much slower in the mutant at 44°C. Very similar results were obtained using a different temperature-sensitive groES mutant, groES30 (data not shown). Thus, GroES function is clearly necessary for the degradative process.

When similar experiments were performed with temperature-sensitive groEL mutations, the results obtained depended on the specific temperature-sensitive mutant used. groEL44 is a leaky mutation; at temperatures below 44°C, this strain grows slightly slower than the wild type, but has a major growth defect at 44°C. In this mutant strain, CRAG degradation did not decrease (as it did in the groES mutant), but was actually two times faster than in the isogenic wild type at 37°C and four times faster at 44°C (Figs. 1C and 5). Similar results were obtained for three independent experiments. Interestingly, the amount of CRAG associated with GroEL in the groEL44 mutant at 44°C was at least 20-fold higher than in the wild type (Table III). Thus, in this mutant, the greater tendency for formation of the complex between CRAG and GroEL correlates with faster CRAG degradation, as was also found upon overexpression of GroEL.

In a different temperature-sensitive mutant (groEL140), the rate of CRAG breakdown did not differ from that in the wild type at either the permissive (37°C) or nonpermissive (44°C) temperature. Accordingly, the amount of CRAG found in complex with GroEL was the same as in the wild type at both temperatures (Table III). It was previously reported that this mutation did not affect binding of unfolded proteins to GroEL (Baneyx and Gatenby, 1992). Thus, only certain temperature-sensitive defects in GroEL (i.e. those present in GroEL44) lead to CRAG accumulation on GroEL and to more rapid proteolysis. More information about the functional defects in the groEL44 and groEL140 mutants may help clarify their different effects on CRAG proteolysis. To check that these mutations were not influencing CRAG hydrolysis by some indirect way (e.g. by causing induction of hsps or by causing a general accumulation of abnormal proteins), we also measured the content of GroEL in these various groEL and groES mutants by Western blot analysis with a specific antibody. In all cases, the levels of the chaperone were similar to those measured in the isogenic wild-type strain (data not shown). Thus, at 37 or 44°C, rates of CRAG degradation appear to depend on the amount of CRAG present in complex and on the functional capacity of GroEL and GroES.

Additional experiments (Fig. 6) tested whether the groEL44 mutation stimulates CRAG degradation by the same protease that functions in the wild type (Fig. 3). When the clpP mutation was transduced into the groEL44 strain, CRAG was completely stabilized. Thus, the stimulation of CRAG digestion in groEL44 is dependent on the ClpP subunit of protease Ti, which appears to function together with GroEL in the rapid degradation of CRAG. These data suggest that complex formation between CRAG and GroEL may be a critical step for the subsequent proteolytic attack by ClpP.
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The reasons for these unsuccessful efforts are unclear since other polypeptides (e.g., casein) are degraded in an ATP-dependent manner under these conditions. Possibly, these findings indicate that additional components are involved in CRAG degradation in vivo, and in recent experiments, we have identified one such factor as the polypeptide trigger factor.3

DISCUSSION

Our studies have shown that E. coli contains several surprisingly complex pathways for protein degradation and that hydrolysis of different abnormal polypeptides can involve distinct ATP-dependent proteases as well as different families of chaperones. For example, PhoA61 degradation requires protease La, DnaK, GrpE, and DnaJ (Sherman and Goldberg, 1992a), while CRAG breakdown involves GroEL, GroES, ClpP, and probably other polypeptides. These different requirements are most clear in cells lacking dnaK, in which PhoA breakdown ceased, while CRAG breakdown increased severalfold, apparently due to the induction of other hsp genes. CRAG appears to be hydrolyzed primarily by the ClpP peptidase, although at 42°C, another yet unidentified proteolytic enzyme also appears to catalyze a part of this degradative process. Apparently, protease La (Lon) has no role in CRAG breakdown at 42°C, even though it is induced during heat shock (Goff et al., 1984). It is surprising that at 37°C, lon mutations reduced CRAG degradation partially (although to a much lesser extent than the clpP mutation). These findings may indicate that at 37°C, protease La functions together with ClpP in this process; however, it is also possible that the lon mutation decreases CRAG breakdown only indirectly (e.g., by allowing the accumulation of abnormal proteins that compete for proteolysis). In any case, at both temperatures, the major degradative pathway requires Clp as well as the hsp60/hsp10 family of chaperones.

ClpP has been implicated in the "N-end rule pathway" for the degradation of polypeptides with unusual amino termini. However, this pathway cannot be responsible for CRAG's breakdown since, in related studies, we have determined by sequencing that the NH₂-terminal residue of CRAG purified from E. coli is methionine, which should confer stability (Tobias et al., 1991). Moreover, the selective degradation of such N-end substrates requires both the clpA and clpP gene products, unlike the breakdown of CRAG. These experiments also demonstrate that ClpP, the proteolytic subunit of protease Ti, can function independently of its ATPase subunit, the clpA product. Neither

Fig. 5. Influence of temperature-sensitive groES619 and groEL44 mutations on degradation of CRAG. The groES and groEL mutants and wild-type cells (MPH86) were grown until mid-log phase at 37°C. Half of the cultures were then shifted to the nonpermissive temperature (44°C) for 30 min, and the rate of CRAG degradation was measured by Western blot analysis.

Fig. 6. Effect of clpP mutation on CRAG degradation in temperature-sensitive groEL44 mutant. Isogenic strains (MPH86) carrying the groEL44 mutation, a clpP mutation, or both mutations were grown at 37°C until mid-log phase. They were shifted for 30 min to the nonpermissive temperature (44°C), and the rate of CRAG degradation was measured by Western blot analysis.

Attempts to Reconstitute CRAG Degradation in Vitro—SinceGroEL and GroES appear to be required for CRAG degradation in vivo, we attempted to reconstitute the chaperone-dependent breakdown of CRAG in vitro. 35S-CRAG was purified from labeled cells and then incubated with different amounts of purified protease ClpP or La and the chaperones GroEL and GroES (kindly provided by Dr. U. Hartl) in the presence of ATP and Mg²⁺ at 30, 37, or 42°C for 2 h. No degradation was detected by the release of trichloroacetic acid-soluble counts in the incubation mixture. We also tried unsuccessfully to detect CRAG degradation in E. coli cell lysates (prepared from the wild-type cells or from the strain overproducing haps) or in 30% ammonium sulfate pellets of these extracts. No increase in trichloroacetic acid-soluble counts was found after 2 h of incubation at different temperatures. In addition, we isolated the CRAG-GroEL complex by rapid gel filtration using a Sephadex G-150 column and then studied the degradation of this GroEL-bound CRAG by purified proteases La and ClpP and the complete E. coli lysate. After 2 h of incubation in the presence of ATP, proteins from these mixtures were separated by SDS-PAGE. Neither a decrease in the radioactivity in the CRAG band nor the generation of CRAG fragments was observed under these conditions.
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of these components alone shows ATP-dependent proteolysis (Hwang et al., 1988; Katayama-Fujimura et al., 1987). By itself, ClpP can degrade only small peptides, while ClpA is a protein-activated ATPase, which allows the peptidase to attack large polypeptides (Woo et al., 1989). The association between these two subunits is relatively weak; for example, they are readily separable by phosphocellulose or ion-exchange chromatography (Hwang et al., 1988; Katayama-Fujimura et al., 1987). Most likely, the ClpA subunit can be replaced by another unidentified ATPase.

The obvious candidate for such a role was the homologous protein-activated ATPase, ClpB (Woo et al., 1992), but this hsp was also found to be unnecessary for CRAG degradation. It was reported recently that the breakdown of the mutated repressor for bacteriophage Mu in E. coli also requires ClpB, but not ClpA or ClpB (Geuskens et al., 1992). Similarly, E. coli proteins made during carbon starvation are hydrolyzed selectively upon restoration of glucose by a process requiring ClpP, but not ClpA (Damerau and St. John, 1993). Finally, the newly discovered ClpA homolog, ClpX, which is essential for the rapid breakdown of A0 protein by ClpP (Gottesman et al., 1993; Wojtkowiak et al., 1993), is also not essential for the degradation of CRAG. Therefore, it seems likely that the ClpP peptidase can function in association with a still unidentified ATPase, related to either the clpA or clpX gene product. Alternatively, the GroEL/ES chaperones might function in lieu of the ATPase subunit in CRAG hydrolysis (see below). Interestingly, CRAG degradation was faster in the clpA, clpB, and clpA/clpB mutants at 42 °C and in the clpX mutant at 37 °C than in the wild-type cells. Possibly, the loss of these cofactors of ClpP leaves more of this peptidase available for CRAG degradation. However, approximately half of the accelerated breakdown of CRAG in the clpA/clpB mutant at 42 °C was seen in cells lacking protease ClpP; thus, another proteolytic system independent of proteases ClpP and La seems to be activated or induced in these mutants during heat shock. In the wild type at 42 °C, a fraction of CRAG degradation was also independent of proteases ClpP and La.

To clarify which enzymes function in CRAG degradation, we tried repeatedly to reconstitute this process in crude extracts or using purified proteases La and Ti, but we were unsuccessful, despite our trying a variety of different conditions (e.g. 37 or 42 °C, with or without ATP, etc.). This inability to demonstrate degradation indicates that these proteases by themselves cannot digest CRAG and that the degradative process requires additional components. GroES clearly is one of these additional factors, and it seems very likely that GroEL is also essential. Accordingly, overproduction of normal GroEL with or without GroES enhanced CRAG degradation. The accelerated CRAG degradation seen upon overproducing the heat-shock transcription factor σ8 in or the dnaK deletion strain appears to be due to the increased levels of GroEL under these conditions. By contrast, the overproduction of ClpP did not affect the rate of proteolysis. These findings argue that GroEL is the rate-limiting factor in the degradative pathway, presumably because complex formation with CRAG is the critical initial step. Accordingly, when proteolysis rises or when it is prevented by the inactivation of GroES, CRAG accumulates in association with GroEL. Under these conditions, —5–20% of the CRAG in the cell is found in complex with GroEL, compared with 1% in the wild type (Table III). The dissociation of GroEL from CRAG (Sherman and Goldberg, 1992b), like that of many polypeptides (Hartl, 1991), is dependent on ATP, and so possibly, ATP hydrolysis by GroEL and GroES contributes to the energy requirement for CRAG degradation.

Our many attempts to reconstitute the CRAG degradation system in cell-free extracts in the presence of the chaperones GroEL and GroES did not succeed; in fact, no CRAG degradation was ever measured under any of a large variety of conditions tested. The most likely explanation is that some additional components, aside from ClpP, GroEL, and GroES, are also involved in this process. In fact, the identification of one such component will be presented elsewhere.

At this time, a definitive model to explain our findings with groEL mutants is impossible because the effects of these mutations on substrate binding to GroEL, dissociation, and other processes are still unknown. Although these findings clearly implicate GroEL in proteolysis, the ability of the groEL44 mutation to stimulate CRAG degradation, while groES mutations strongly inhibited this process, may initially appear surprising. In the groEL44 strain, where proteolysis was rapid, much more GroEL was found in complex with CRAG than in the wild type. This correlation supports the conclusion that GroEL binding of CRAG is important for its rapid degradation. It is noteworthy that a different temperature-sensitive groEL mutation (groEL140) did not affect CRAG breakdown at 44 °C and did not affect the amount of GroEL in complex with CRAG. Unfortunately, few mutant forms of GroEL are available, and in no cases related to our studies are the biochemical defects sufficiently defined to clarify the precise role of GroEL in CRAG proteolysis.

It is known that the association of GroEL with unfolded polypeptides prevents their entering unproductive aggregation pathways and thus favors proper folding or perhaps secretion of the polypeptide (Hartl, 1991). However, if a stable conformation fails to develop, as may happen with a recombinant fusion protein like CRAG or with proteins damaged during heat shock, it would seem advantageous for the cell to target such polypeptides for degradation. GroEL can stabilize unfolded polypeptides in molten globule conformations (Martin et al., 1991) that lack a fully defined tertiary structure. This intermediate in the folding pathway constitutes a relatively open conformation, in which the polypeptide chain associated with GroEL is very sensitive to proteolytic attack (Martin et al., 1991). Thus, the function of GroEL/ES in CRAG degradation in vivo is likely to involve facilitating attack by proteases. Since the binding of CRAG to GroEL appears to be the critical rate-limiting step in the degradative pathway, GroES probably functions in some subsequent step linked to proteolytic attack. The large amounts of GroEL associated with CRAG in the groES19 strain suggest that the GroES-dependent dissociation of CRAG from GroEL is critical for its subsequent hydrolysis. Possibly, GroES releases CRAG from GroEL in a conformation that is particularly susceptible to attack by ClpP. It is also possible that GroES may alter the CRAG-GroEL complex and thus facilitates the digestion of CRAG bound to GroEL. In either case, these chaperones appear to serve as recognition elements for the cell's proteolytic machinery and probably also as quality-control elements that monitor the success of protein folding, i.e. if a proper conformation is not achieved, GroEL/ES can facilitate the rapid degradation of the polypeptide.

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References
Degradation of Abnormal Protein Involves GroEL/ES

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