Role of the Heat Shock Protein DnaJ in the Lon-dependent Degradation of Naturally Unstable Proteins*

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We have investigated the role of DnaJ in protein degradation by examining the degradation of intrinsically unstable proteins by Lon protease in vivo. In Escherichia coli, Lon protease is responsible for the rate-limiting step in degradation of highly unstable proteins such as SulA, RcsA, and λN protein, as well as for about 50% of the rapid degradation of abnormal proteins such as canavanine-containing proteins. We found that Lon-dependent degradation of both SulA and λN protein was unaffected in cells lacking functional DnaJ, whereas Lon-dependent turnover of canavanine-containing proteins was slower in dnaJ mutant cells. DnaJ also affected the slow SulA degradation seen in the absence of Lon. The rate of degradation of RcsA was reduced in dnaJ mutants, but both Lon-dependent and Lon-independent degradation was affected; abnormal, canavanine-containing proteins were similarly affected. Both the RcsA that accumulated in dnaJ mutant cells and the SulA that accumulated in lon dnaJ mutant cells was aggregated. The abnormal proteins that partitioned to the insoluble pellet became solubilized over time in dnaJ− cells but not in dnaJ+ cells. Therefore, the co-chaperone DnaJ is not essential for Lon-dependent degradation and may act in protein turnover only as an accessory factor helping to maintain substrates in a soluble form. Such an accessory factor is apparently necessary for abnormal proteins and for RcsA. The relative rates of degradation and aggregation of specific protein targets may determine the importance of the chaperone systems in turnover of a given protein.

Energy-dependent proteases in both prokaryotes and eukaryotes participate in the degradation of abnormal proteins (such as mutant proteins, protein fragments, proteins in the incorrect place or proteins in excess of stoichiometric requirements for multi-protein complexes) as well as in the degradation of specific, short-lived regulatory proteins (for reviews, see Refs. 1–3).

Heat shock proteins have also been implicated in protein turnover in a number of systems. In Escherichia coli, mutations in dnaK, dnaJ, or grpE, which encode components of the DnaK molecular chaperone system, interfere with the turnover of abnormal proteins (4–6). Degradation of these abnormal proteins is also partially dependent on the ATP-dependent protease Lon (4, 5, 7). Since chaperone systems such as DnaK and its co-chaperones DnaJ and GrpE also help in the renaturation of proteins, the involvement of chaperones in degradation as well as renaturation suggests that they act at a decision point in determining the fate of improperly folded proteins. To understand the nature of their action at this decision point, we have investigated in more detail the role of DnaJ, the probable specificity element for the DnaJ/DnaK/GrpE chaperone system (8–10), in Lon-dependent degradation in vivo.

We have tested two possible models for DnaJ action by examining Lon-dependent degradation of both intrinsically unstable proteins and abnormal proteins (11) in the absence of DnaJ. If the DnaJ/DnaK/GrpE molecular chaperone system is an essential component of the degradative machinery, all Lon-dependent protein degradation in cells mutated in dnaJ or dnaK would be blocked. This model predicts that degradation of all intrinsically unstable Lon targets would require the participation of the DnaK molecular chaperone system. However, there has been no in vitro demonstration of a role for the heat shock chaperones in Lon-dependent degradation. In fact, a number of Lon substrates, including the A anti-termination protein N, a His-tagged derivative of the cell division inhibitor SulA, and the F plasmid antidote protein CcdA, can be degraded in vitro by purified Lon protease in an ATP-dependent manner (12–14).

Alternatively, the effect of the chaperone proteins on degradation of abnormal proteins may be more indirect. For instance, they might simply act to maintain abnormal proteins in conformations that are susceptible to degradation by Lon or other cytoplasmic proteases. Many abnormal proteins accumulate as insoluble aggregates that may be resistant to proteolysis. The heat shock chaperones decrease the degree of protein aggregation (9, 15–18), possibly by maintaining substrate proteins in a more unfolded state, by blocking the abnormal protein-protein interactions which lead to aggregate formation, or by actively solubilizing aggregated proteins. If this indirect role is the only necessary role for chaperone proteins in proteolysis, we would predict that specific, intrinsically unstable proteins, which presumably have protease-sensitive conformations under normal physiological conditions, will not necessarily require chaperone proteins to be degraded.

We have examined the role of DnaJ in the Lon degradation in vivo of three naturally unstable proteins known to be dependent on Lon for rapid turnover, SulA, λN protein, and the transcriptional activator RcsA. Our results support the idea that DnaJ is not an essential component of the Lon protease, but helps to maintain some classes of unstable proteins in a form susceptible to Lon proteolysis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**—A set of four isogenic strains, YJ1020 (∆arg lon-510 dnaJ−), YJ1021 (∆arg lon+ dnaJ−), YJ1033 (∆arg lon+ dnaJ+), and YJ1037 (∆arg lon-510 dnaJ+), was used in these studies. This paper is available on line at http://www-jbc.stanford.edu/jbc/
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**RESULTS**

Degradation of ΔN and Sula Protein by Lon Protease in Vivo

Is Independent of DnaJ—ΔN protein, the transcriptional anti-termination protein for λ lytic growth, is degraded in a Lon-dependent fashion (30). ΔN protein was expressed from plasmid pMJD2 (p lac- N) by induction with IPTG. Cells were pulse-labeled with [35S]methionine for 1 min and chased with non-radioactive methionine. The ΔN protein was immunoprecipitated from samples removed after different chase times and quantitated after washing the pellet with acetone, drying, and dissolving it in SDS buffer. All measurements were normalized to a constant volume of the original culture.

**FIG. 1. Stability of ΔN protein.** Cells containing plasmid pMJD2 carrying the gene for ΔN protein were grown in M63 minimal medium (22) with 100 μg/ml ampicillin at 30 °C, induced with IPTG for 10 min, and then labeled for 1 min with [35S]methionine, and chased with non-radioactive methionine. At the times indicated, samples were removed for immunoprecipitation (see "Experimental Procedures"). Time zero corresponds to the start of the chase. Each decay curve was determined at least three times in independent experiments; the results shown are those for a typical experiment. The relative half-lives were always as shown. Host strains used were YJ1020 (Δlon-510), Y1021 (wild-type), YJ1033 (Δlon-510 dnal-1), and YJ1034 (Δlon-1).
Lon-independent Degradation of N is not known, but it is growth without IPTG. Both proteases other than Lon.

DnaJ activity is functional. Therefore, SulA made degradation in a filamentation of cells carrying pGC165 (half-life about 2–3 min) and was similar in a mutant (half-life of 1.5 min) (Fig. 2).

Each decay curve was determined at least three times in independent experiments; the results shown are those of a typical experiment. The host strains were those shown in the legend to Fig. 1.

To test whether DnaJ affected the activity of SulA under the conditions used to study SulA turnover in vivo, we examined the filamentation of cells carrying pGC165sfiA⁺ (25) after induction of SulA. IPTG was added to growing cells for 30 min and cells were suspended in fresh medium for 3 h of further growth without IPTG. Both lon⁺ and lon⁻ cells, whether dnaJ⁺ or dnaJ⁻, filamented after SulA induction (data not shown), indicating that dnaJ mutations did not interfere with either transient (in lon⁺ cells) or irreversible (in lon mutants) filamentation. Therefore, SulA made in vivo in the absence of DnaJ activity is functional.

Lon-independent Degradation of N Protein and SulA—As expected from previous results (30, 31), the degradation of both λN and SulA were dramatically slowed in lon mutant cells (Figs. 1 and 2). dnaJ mutations did not further slow λN protein degradation in a lon mutant (Fig. 1). The protease responsible for Lon-independent degradation of N is not known, but it is not the ClpAP protease (19). In contrast to λN, the half-life of SulA was considerably longer (32 min) in lon dnaJ mutant cells than in lon mutants alone (9 min), suggesting that the heat shock protein DnaJ does affect the slower SulA turnover by proteases other than Lon.

Degradation of Abnormal Proteins—These results, which indicate that SulA and λN can be recognized and degraded by Lon without the participation of DnaJ, contrast with those seen for degradation of abnormal proteins (4, 5, 34). Proteins synthesized with the arginine analog, canavanine, are degraded much more rapidly than normal proteins (35). Although Lon accounts for about 50–60% of the turnover observed (7, 34), cells mutant in the heat shock genes, most notably dnaJ, are defective for degradation of canavanyl proteins and other abnormal proteins (4, 5). We measured the degradation of canavanyl proteins in the same isogenic strains used to measure the Lon and DnaJ dependence of SulA and λN protein turnover. Degradation of canavanyl proteins to trichloroacetic acid-soluble fragments was reduced about 36–46% during the first 2 h in lon mutant cells (Fig. 3A and data not shown), which is slightly less than had been observed previously (7). A dnaJ::kan mutation had little effect in the first 30 min but caused a substantial decrease with time (nearly 50% by 2 h), again somewhat less than what had been found in previous studies (4, 5). These differences in the percentage of degradation dependent on Lon and DnaJ may reflect differences in the strain backgrounds. The combined lon and dnaJ mutations decreased degradation of canavanyl proteins 70–75%.

The release of trichloroacetic acid-soluble radioactivity from canavanyl proteins reflects the fate of the total population of proteins. We had previously found that lon mutants block initial steps in degradation of specific abnormal proteins synthesized in the presence of canavanine (36). We repeated these experiments and asked if dnaJ mutations affected degradation of the same abnormal proteins stabilized in lon mutants. Fig. 3B shows a polyacrylamide gel of abnormal proteins pulse-labeled with [35S]methionine in the presence of canavanine and chased with non-radioactive methionine. Of the subset of cellular proteins visible on the gel, only a few were rapidly degraded in wild-type cells. Among these, most were stabilized in both lon and dnaJ mutants (indicated by arrows in Fig. 3B). Thus, DnaJ is required in vivo for the initial step in degradation of those abnormal proteins degraded by Lon.

Together these results suggest that degradation of SulA and λN protein by Lon is qualitatively different from the initiation of degradation of abnormal proteins. While both SulA and λN degradation in vivo by Lon is unaffected by a null mutation in dnaJ, the degradation of abnormal proteins requires the participation of the heat shock DnaJ protein. Heat shock proteins may be necessary either for the in vivo recognition of abnormal proteins by Lon or to maintain abnormal proteins in a state accessible to Lon, but DnaJ is not an essential component for all Lon-dependent degradation in vivo.

Degradation of RcsA in dnaJ Mutant Hosts—When we examined the involvement of DnaJ in the degradation of a third specific substrate of Lon, RcsA, the results were similar to those seen for abnormal proteins. RcsA is a transcriptional activator of the genes for colanic acid synthesis (cps genes). In lon mutant cells, stabilization of RcsA results in overproduction of the colanic acid capsular polysaccharide (23).

As previously shown (23, 26), RcsA synthesized from the multicopy plasmid pATC400 had a short half-life in wild-type cells (5 min), but a much longer half-life (25 min) in lon mutant cells (Fig. 4). In dnaJ mutants, the half-life of RcsA was increased 3-fold (half-life, 14 min) compared to a dnaJ⁺ host (Fig. 4), suggesting that DnaJ contributes to the in vivo Lon-dependent degradation of RcsA, as it does for abnormal proteins. In cells carrying both lon and dnaJ mutations, RcsA had a half-life of 36 min. Therefore, in contrast to the situation with SulA and λN protein, at least some of the very rapid turnover of RcsA in lon cells, as well as the residual turnover in lon mutant cells, requires active DnaJ.

Aggregation of Proteins in the Absence of DnaJ—We investigated the state of RcsA, SulA, and canavanine-containing proteins in cells carrying the dnaJ mutation. As noted above, many abnormal proteins are known to aggregate in the absence of heat shock proteins (9, 15–18). We were interested in determining if protein is aggregated in the absence of DnaJ, and whether aggregation is seen specifically when degradation is slowed in the dnaJ mutant. We used the distribution of RcsA and SulA between supernatant and pellet fractions as an indication of the degree of aggregation.
In wild-type cells carrying an rcsA+ multicopy plasmid, the rapid turnover of RcsA prevented its detection by Western blot (Fig. 5). In lon mutants, most of the protein was found in the soluble fraction. In dnaJ mutants, whether lon+ or lon−, the majority of the protein was found in the pellet. Therefore, RcsA appears to be aggregated in dnaJ mutants. This aggregation correlates with the longer half-life of RcsA in dnaJ mutant cells.

Relatively little SulA accumulates in lon+ cells, whether they are dnaJ+ or dnaJ−, consistent with the rapid degradation of SulA in these cells (Figs. 2 and 5). We examined the state of the SulA protein in lon− cells, where the presence or absence of DnaJ does affect the rate of SulA degradation (Fig. 2). In lon mutants, the majority of the SulA protein that accumulated was found in the supernatant (Fig. 5), while in lon− dnaJ− hosts most of the SulA was found in the pellet. These results indicate that SulA tends to aggregate and that DnaJ is required to help prevent aggregation. The decreased rate of degradation in lon dnaJ double mutants, like the slower degradation of RcsA in a dnaJ mutant, most likely reflects the inaccessibility of aggregated SulA to degradation by other proteases in the absence of DnaJ.

The distribution of canavanyl proteins into soluble and insoluble fractions was also analyzed. For lon mutants, total protein degradation (Table I) was similar to that found above; degradation was decreased by about 40%. However, degradation was essentially abolished in both the dnaJ and lon dnaJ mutants. At the start of the chase period, a larger proportion of the protein was found in the insoluble fraction in the dnaJ mutant hosts (51 and 63%) than in the dnaJ− hosts (35 and 38%). In cells wild-type for dnaJ, a significant fraction of the protein that was initially insoluble moved out of the insoluble fraction (Table I). In cells wild-type for dnaJ, essentially all of the protein lost from the insoluble fraction could be accounted for by degradation; in the lon− host, where degradation was relatively slow, protein could be detected accumulating in the soluble fraction (Table I). In the dnaJ mutants, there was very little or no decrease in protein in the insoluble pellet during the same period. These results are consistent with DnaJ aiding degradation of this class of abnormal proteins by increasing the availability of the protein in the supernatant and allowing recovery, and therefore degradation, of protein from an insoluble state.
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Fig. 4. Stability of RcsA. Cells containing the rcsA+ plasmid, pATC400, were grown, pulse-labeled, and immunoprecipitated as for Fig. 1. Each decay curve was determined at least three times in independent experiments; the results shown are those of a typical experiment.

Fig. 5. Aggregation state of RcsA and SulA overexpressed from a high copy plasmid. Top panel, cells carrying the rcsA+ plasmid, pATC400, were grown to an OD600 of 0.3, collected, and disrupted in a French pressure cell. Cell lysates were fractionated, proteins were separated by SDS-PAGE, and RcsA was detected by Western blotting, as described under “Experimental Procedures.” Host strains used were YJ1067 (dnaJ::kan), SG20884, (Δlon-510 dnaJ::kan), SG20781 (lon dnaJ+), and SG20780 (Δlon-510). P, pellet fraction of centrifugation of cell extract at 13,000 for 20 min; S, supernatant fraction after centrifugation. Bottom panel, cultures of cells carrying the sulA+ plasmid, pG165 sfaA+, were grown to an A600 of 0.3–0.5 and induced for 60 min with IPTG. Cells were collected and treated as described under “Experimental Procedures.” Host strains were the same as those in panel A. Lanes marked total protein show the SulA detectable in cell lysates before separation of the soluble and insoluble fractions. The distribution of SulA into the soluble and insoluble fractions is shown only for the lon and lon dnaJ strains because no SulA was detectable in either of the lon+ strains.

Table I

Distribution of canavanine-containing protein in lon and dnaJ mutants

Strains were grown and labeled at 30 °C as described under “Experimental Procedures.” The total amount of radioactive methionine incorporated during the labeling period was similar for all four strains. Values in the table are for 3 h after the chase was added; qualitatively similar results were found at 1.5 h. Protein degraded was calculated from the net loss of trichloroacetic acid-precipitable protein 3 h after adding cold methionine and arginine. Soluble and insoluble protein was determined as described under “Experimental Procedures.” For each time point, the sum of the protein in both fractions was normalized to the total trichloroacetic acid-insoluble protein determined for the same volume of the culture. Using these normalized numbers, the fractional change in insoluble and soluble protein was calculated by dividing the change in counts in the appropriate protein fraction from 0 to 3.0 h of chase by the total counts in protein at 0 min. For all strains, the total number of counts detected at later times increased over those calculated at earlier times. Therefore, although no significant counts were lost from the trichloroacetic acid precipitable fraction in the dnaJ and lon dnaJ mutants, there was an increase in counts detected in the trichloroacetic acid-soluble fraction.

| Strain | Fraction of total trichloroacetic acid-precipitable protein lost | Fractional change in protein distribution |
|--------|---------------------------------------------------------------|------------------------------------------|
|        | Insoluble | Soluble | Insoluble | Soluble |
| Wild-type | 0.34 | -0.33 | -0.014 |
| lon | 0.17 | -0.35 | +0.18 |
| dnaJ | <0.01 | -0.35 | +0.005 |
| lon dnaJ | <0.01 | +0.012 |

We demonstrate here that there is no requirement for DnaJ for the rapid, Lon-dependent degradation of AN protein and SulA in the same cells in which DnaJ is necessary for the initial cleavage of a number of abnormal proteins. This result rules out a direct and necessary participation of DnaJ in Lon-dependent protein turnover.

The role of DnaJ in the degradation of a third Lon substrate, the transcriptional activator RcsA, bore more similarity to its role in the turnover of abnormal proteins. While the turnover of RcsA is slowed in dnaJ mutant hosts, the RcsA protein that accumulates is largely aggregated. The simultaneous stabilization and aggregation of RcsA are consistent with a model in which DnaJ (and possibly DnaK and GrpE as well) acts to stimulate proteolysis indirectly by helping to maintain RcsA in a soluble conformation. The effect of DnaJ on RcsA solubility is not restricted to the protein overproduced from the plasmid. We have found that the chromosomally-encoded RcsA is mostly soluble in lon− hosts but insoluble in lon+ dnaJ− hosts; furthermore, insolubility is associated with a failure of RcsA to function in dnaJ mutants.2 Consistent with this role of DnaJ in keeping RcsA soluble and thereby indirectly stimulating proteolysis, we found that SulA also became relatively insoluble in the absence of DnaJ in a lon mutant, conditions under which the degradation of SulA is dependent on DnaJ.

Previous work on the role of chaperones in degradation have focused exclusively on abnormal proteins, but there is no reason to believe that the degradation of naturally unstable proteins by Lon and the degradation of abnormal proteins is qualitatively different. If we generalize the results seen with RcsA and SulA to the similar stabilizing effect of dnaJ, dnaK, and grpE mutations on the degradation of abnormal proteins (4, 5, 38), we would conclude that there is no necessity to invoke a direct role for these chaperones in protein turnover. There is ample evidence that aggregated proteins accumulate in hosts mutant for the heat shock proteins, and that refolding (for instance by overproduction of GroE) is an alternative pathway to degradation (39, 40). We extended these observations to the

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abnormal, canavanine-containing proteins studied here; as predicted, a greater proportion of these proteins are found in a pellet fraction in dnaJ mutants, and movement of proteins out of the pellet requires DnaJ. Therefore, all the observed effects of heat shock mutants on abnormal protein turnover in vitro are consistent with a role in keeping (or returning) potentially unstable proteins in soluble form, accessible to the proteases. Heat shock proteases would provide a pathway toward degradation and away from aggregation, and, where possible, restore a folded structure which would neither aggregate nor be targeted for degradation.

A possible explanation for this behavior is that hydrophobic regions normally buried in stable proteins may be exposed when these proteins are perturbed by mutation, high temperatures, or other stresses, and may serve as the target sites for proteases such as Lon, as the interaction surfaces leading to aggregation and away from aggregation, and, where possible, restore a folded structure which neither aggregate nor be targeted for degradation.

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