The Molecular Chaperone DnaJ Is Required for the Degradation of a Soluble Abnormal Protein in *Escherichia coli*§

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In addition to promoting protein folding and translocation, molecular chaperones of Hsp70/DnaJ families are essential for the selective breakdown of many unfolded proteins. It has been proposed that chaperones function in degradation to maintain the substrates in a soluble form. In *Escherichia coli*, a nonsecreted alkaline phosphatase mutant that lacks its signal sequence (PhoAA2–22) fails to fold in the cytosol and is rapidly degraded at 37 °C. We show that PhoAA2–22 is degraded by two ATP-dependent proteases, La (Lon) and ClpAP, and breakdown by both is blocked in a dnaJ259-ts mutant at 37 °C. Both proteases could be immunoprecipitated with PhoA, but to a much lesser extent in the dnaJ mutant. Therefore, DnaJ appears to promote formation of protease-substrate complexes. DnaJ could be coimmunoprecipitated with PhoA, and the extent of this association directly correlated with its rate of degradation. Although PhoA was not degraded when DnaJ was inactivated, 50% or more of the PhoA remained soluble. PhoA breakdown and solubility did not require ClpP. PhoA degradation was reduced in a thioredoxin-reductase mutant (trxB), which allowed PhoAA2–22 to fold into an active form in the cytosol. Introduction of the dnaJ mutation into trxB cells further stabilized PhoA, increased enzyme activity, and left PhoA completely soluble. Thus, DnaJ, although not necessary for folding (or preventing PhoA aggregation), is required for PhoA degradation and must play an active role in this process beyond maintaining the substrate in a soluble form.

Molecular chaperones have been implicated in this degradative process in both bacterial and eukaryotic cells (7, 8). In addition to participating in the folding of nascent polypeptides, their translocation across membranes, and the assembly of oligomeric complexes (9, 10), molecular chaperones of the Hsp70/DnaK family and the cofactors of the DnaJ families have recently been shown to play an essential role in protein degradation (6, 11, 12). A characteristic feature of Hsp70/DnaK is its ability to bind selectively to hydrophobic oligopeptides or unfolded proteins in extended conformations, where such domains may be exposed (13–15). DnaJ stimulates the ATPase activity of DnaK (16, 17) and by itself can also bind to certain unfolded proteins (18–21). Both components can thus possibly facilitate the recognition of substrate conformations or act as cofactors in the degradative process.

Among the first examples of the involvement of DnaJ/DnaK in the degradation of a specific abnormal protein was alkaline phosphatase (PhoA61), which is not secreted from the cytosol due to a missense mutation in its signal sequence (6). Its rapid degradation at 37 °C is mediated in part by the ATP-dependent protease La (Lon) and also requires DnaK, because a dnaJ-null mutant completely prevents its breakdown. Interestingly, dnaK756, a missense mutation that fails to release substrates in response to ATP, was found to enhance PhoA61 degradation, whereas a dnaJ mutant, which reduced PhoA61 association with DnaK, slowed its turnover. Thus, complex formation between PhoA61 and DnaK appears essential for its rapid degradation. Presumably, the successful folding or secretion of a normal polypeptide involves only transient association with DnaK. We therefore proposed that the chaperone, through its prolonged association with a nonfoldable protein, may trigger proteolysis, perhaps by helping to stabilize an abnormal polypeptide in an unfolded, extended conformation that favors recognition or digestion by the protease.

One characteristic feature of unfolded proteins is their tendency to aggregate, and an important function of molecular chaperones of the Hsp70 and DnaJ families is to prevent this process *in vivo* (15, 22). In addition, DnaK, DnaJ, and GrpE, together with ClpP, can promote the solubilization of aggregated proteins (23, 24) in *Escherichia coli*, as can their homologs in yeast. It has been suggested that the primary function of DnaJ/DnaK in proteolysis is to help maintain unfolded proteins in a soluble form, which should render them more susceptible to proteolytic attack (25). In several cases, degraded abnormal proteins have been found to accumulate as intracellular aggregates. For example, in *E. coli*, the degradation of the short-lived transcription factor, RcsA, by protease La was retarded in a dnaJ mutant, where a significant fraction of RcsA was found in particulate form (26). Similarly, in yeast mitochondria, the degradation by the ATP-dependent protease, Pim1 (a homolog of La), of two fusion proteins, bovine lactalbumin and cytochrome b5-DHFR, requires the DnaK homolog.
DnaJ and PhoA Degradation

mt-Hsp70 and the DnaJ homolog Mdj1 (11). In the absence of Mdj1, these fusion proteins formed insoluble aggregates. However, in these chaperone-deficient cells, there was no evidence that the aggregation of these substrates actually caused the block in proteolysis and was not a consequence of the reduced degradation. In other words, if the chaperones are required directly in the degradative process, when degradation is blocked in the chaperone mutants, the unfolded polypeptides that accumulate may then tend to aggregate. Thus, establishing direct involvement of DnaK/DnaJ (Hsp70/40) in the proteolytic pathway is difficult, because the association of these chaperones with the substrate may be important both for facilitating proteolytic attack and for promoting the proper folding of the substrate.

To distinguish these possibilities and to clarify the role of the chaperones in the breakdown of unfolded proteins, we have studied the cytosolic degradation of a nonsecreted alkaline phosphatase lacking the entire signal sequence (PhoAΔ2–22) (27). The present studies have focused on the role(s) of DnaJ, DnaK and some PhoA(

2–22

thioredoxin reductase (trxB), the cytosol has less reducing potential, and some PhoA(Δ2–22) has been shown to fold into an active enzyme (29). We have therefore also used the trxB strain to test whether the rapid degradation of PhoA is in fact due to its failure to achieve the native conformation and to determine what role DnaJ might play when folding and degradation of this protein can both occur.

EXPERIMENTAL PROCEDURES

Materials and Strains—The E. coli strains used in this study are listed in Table I. All strains are isogenic, constructed by P1vir transduction into the WP551 host strain (a kind gift of Dr. W. Prinz) according to standard bacterial genetics procedures (30). Cells were grown and maintained in M9 minimal medium supplemented with essential amino acids, thiamine, 0.5% glucose, and appropriate antibiotics at 30 °C. The polyclonal antibody against PhoA was obtained from 5 Prime (Boulder, CO) and purified by adsorption to extracts of a strain lacking the PhoA gene. The polyclonal anti-DnaJ antibody was a kind gift of Dr. R. McMacken (Johns Hopkins University), and anti-La, anti-ClpA, and anti-ClpP antibodies were a kind gift of Dr. C. H. Chung (Seoul National University, Korea). Protein A-Trisacryl beads were obtained from Pierce, and 125I-protein A was from ICN. All other reagents were purchased from Sigma and were of the highest purity available.

Assays of PhoA Degradation—The rate of PhoA degradation was measured either by Western blot analysis or by a pulse-chase and immunoprecipitation protocol as described before (6, 31). Both approaches gave similar results, and therefore only the Western blot method was routinely used. Cells were grown at 30 °C to mid-logarithmic phase and then shifted to 37 °C in the presence of 2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 20 min to induce PhoA expression. Degradation was assayed at 37 °C after blocking protein synthesis with the addition of a mixture of antibiotics, including chloramphenicol (100 μg/ml) and rifampicin (300 μg/ml) at t = 0. Aliquots of cell culture were taken in duplicates, immediately and at the indicated times there- after. Cell proteins were precipitated with 10% trichloroacetic acid, washed with acetone, resuspended in SDS-PAGE sample buffer, and boiled. Samples were resolved on 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and blotted with an anti-PhoA antibody and 125I-protein A. Quantification of PhoA band intensity was performed with a PhosphorImager, and the duplicates agreed to within 5%.

Assays of PhoA Activity—Assay of alkaline phosphatase activity was performed using a procedure slightly modified from the one described previously (27, 29). After the induction of PhoA expression at 37 °C as described above, cell cultures in duplicates were incubated on ice for 20 min in the presence of 100 mM iodoacetamide, which prevents possible spontaneous renaturation of PhoA(Δ2–22). Cells were then washed and lysed by SDS/CHCl3, and PhoA activity was assayed at 28 °C by the addition of a chromogenic substrate p-nitrophenyl phosphate and detected at Aε405. Assays were performed in duplicates, and the results varied by less than 2%.

Immunoprecipitation—After the induction of PhoA expression at 37 °C (as described above), cells were harvested, resuspended in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 100 mM KCl, and disrupted by sonication. A soluble extract was obtained by taking the supernatant after centrifugation at 15,000 × g for 15 min. Immunoprecipitation of PhoA was carried out by the addition of an anti-PhoA antibody to the soluble extract for 1 h on ice, followed by the addition of bovine serum albumin-coated protein A-Trisacryl beads. The mixture was rotated at 4 °C for an additional 30 min and washed extensively with the same buffer. Precipitates were resolved on 12% SDS-PAGE and transferred onto a nitrocellulose membrane, and blotted with an anti-PhoA antibody and [125I]-protein A. Quantitation of band intensity was performed on a PhosphorImager.

Fractionation of Cell Lysates—As described for immunoprecipitation, cells were disrupted by sonication to yield total cell lysates. Centrifugation of the total lysate at 15,000 × g for 15 min produced the low speed pellet and supernatant fractions. The supernatant was subsequently subjected to ultracentrifugation at 100,000 × g for 1 h to give the high speed pellet and supernatant fractions. The pellets from both centrifugation steps were resuspended in the same buffer and in the same volume as the recovered supernatants. Equal volumes of the fractions were subjected to SDS-PAGE, and the amount of PhoA present in each fraction was detected by Western blot with an anti-PhoA antibody and 125I-protein A. To prevent the possible renaturation of PhoA, which may affect its degree of aggregation, the same buffer containing 100 mM iodoacetamide was used in parallel experiments, but no differences were observed.

RESULTS

Degradation of PhoA(Δ2–22) Involves Multiple ATP-dependent Proteases—Without its signal sequence, E. coli alkaline phosphatase (PhoA) is not secreted into the periplasm, and in the reducing environment of the cytoplasm, it fails to form the disulfide bonds necessary for its native conformation and enzymatic activity (5). To follow the fate of this protein, we used a strain (WP551) that carries on a plasmid a wild-type alkaline phosphatase gene that lacks a signal sequence, under the control of the inducible tac promoter (PhoAΔ2–22) (32). After induction of PhoA for 20 min, protein synthesis was blocked, and the loss of PhoA antigen with time was measured. The newly synthesized PhoA(Δ2–22) was rapidly degraded. Its rate of degradation increased sharply with the growth temperature, and the half-life of PhoA ranged from more than 30 min at 30 °C (not shown) to about 10 min at 37 °C (Fig. 1). These findings resemble closely our earlier observations (6) with another nonsecreted inactive variant of PhoA (PhoAΔ61), which has a point mutation in its signal sequence. It had previously been hypothesized that this altered signal sequence might serve as the recognition element for the protease or a molecular chaperone cofactor. Because a similar temperature-dependent degradation occurred with the two nonsecreted variants, the cytoplasmic degradative system does not recognize the signal sequence, and the half-life of PhoA is apparently determined by the conformation of the PhoA polypeptide itself.

To identify the responsible proteolytic pathway, we constructed by P1vir transduction mutant strains in which genes coding for different ATP-dependent proteases are disrupted (Table I). As shown in Fig. 1, inactivation of the lon gene reproducibly increased the half-life of PhoA(Δ2–22) from 10 to 20 min at 37 °C. This 2-fold stabilization in the lon mutant is similar to that observed with PhoAΔ61 in lon− cells (6), but the

1 The abbreviations used are: IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; TF, Trigger Factor.
protease responsible for the residual degradation had not previously been identified. To test if the remaining degradation was by another ATP-dependent protease, we measured the half-life of PhoA(Δ2–22) in a clpP mutant. Its rate of degradation was also about 2-fold slower in this mutant than in the wild-type (Fig. 1). Because ClpP can function in complex with either the ClpA or ClpX ATPase (33, 34), we also tested if inactivation of one of these ATPases affected PhoA degradation. In a clpA mutant, the half-life of PhoA(Δ2–22) increased to a very similar extent as in the clpP mutant (t1/2 ~ 20 min, Fig. 1). Thus, protease La (Lon) and the ClpAP protease (also called Ti) are both involved in the degradation of this substrate. Accordingly, in a lon-clpP double mutant, PhoA(Δ2–22) was dramatically stabilized (t1/2 > 2 h) (Fig. 1). Therefore, both La and ClpAP proteases contribute approximately equally to the very rapid breakdown of PhoA in the cytosol, but each can function by itself in this process.

*DnaJ Is Required for PhoA Degradation by Proteases La and ClpAP—Previous studies demonstrated that DnaK and its co-factors are required for the degradation of PhoA61, and that the rate of degradation correlates with the extent of association between the substrate and DnaK (6). Because DnaJ has been shown also to bind directly to certain unfolded proteins (18–21), we investigated the possible role of DnaJ in the breakdown of PhoA(Δ2–22). A dnaJ259-ts mutation was transduced into WP551. The resulting temperature-sensitive strain, HHJ1 (Table I), grew well at 30 °C or 37 °C, but did not grow at all at 43 °C (data not shown). These cells were grown at 30 °C to mid-logarithmic phase, shifted to 37 °C in the presence of IPTG for 20 min to induce PhoA expression, and then the degradation of PhoA(Δ2–22) was measured. In these cells, this degradative process was completely blocked at 37 °C (Fig. 2), even though cell growth still occurred at this temperature. Because the requirement for DnaJ for PhoA degradation is more stringent than that for cell growth, DnaJ must be serving some highly specific, essential role in this degradative process. Moreover, because PhoA(Δ2–22) was completely stabilized in the dnaJ mutant (Fig. 2), this chaperone is required for the degradation of PhoA by both proteases La and ClpAP, each of which accounts for about half the degradation seen (Fig. 1).

*DnaJ Influences the Association of PhoA with the Proteases—One possible function of the chaperone could be to facilitate the binding of the substrate to protease La and ClpAP. We therefore measured the amount of these proteases associated with PhoA by immunoprecipitating PhoA from soluble cell extracts with an anti-PhoA antibody, followed by quantitative Western blot analysis of the precipitates with antibodies against La or ClpP. (The ClpA subunit was assayed in this experiment, because the anti-ClpA was more specific than the anti-ClpP antibody we used.) Cell lysates were prepared from wild-type and dnaJ ts-mutant cells, which had been shifted to 37 °C for 20 min. In extracts of wild-type cells, some La and ClpP could be coimmunoprecipitated with PhoA, as shown in Fig. 3, but none was detected in control immunoprecipitations from cells lacking PhoA(Δ2–22) or when a mock immunoprecipitation was carried out with only protein A-beads or preimmune serum (not shown). By contrast, after the inactivation of DnaJ at 37 °C, the amount of ClpA or La that coprecipitated with soluble PhoA was much lower in the mutant than in the wild-type. This inactivation of DnaJ did not affect the association between ClpA and ClpP, because the amount of ClpP that coprecipitated with an anti-ClpA antibody was similar in the wild-type and in the dnaJ mutant (data not shown).

Further evidence that the dnaJ mutant reduces the association of PhoA and the ClpAP protease was obtained when the converse experiment was performed, in which ClpA was immunoprecipitated from extracts of wild-type and mutant cells at 37 °C with an anti-ClpA antibody. When the amount of PhoA present in the precipitates was analyzed by Western blot with an anti-PhoA antibody (not shown), much less PhoA was present in the dnaJ mutant than in controls. Furthermore, in the dnaJ756 mutant, where PhoA degradation was accelerated (Ref. 6, and see below), much more ClpA was coprecipitated with PhoA than in wild-type cells (not shown). Additional ex-

![Fig. 1. Rapid degradation of PhoA(Δ2–22) involves both ATP-dependent proteases La and ClpAP.](http://www.jbc.org/)

**Table I**

*List of strains used in this study*

| Strain | Source |
|--------|--------|
| WP551  | W. Prinz/J. Beckwith |
| HHL3   | This study |
| HHP1   | This study |
| HHA4   | This study |
| HHL2   | This study |
| HHH1   | This study |
| WHP52  | W. Prinz/J. Beckwith |
| HHX6   | This study |

*Plasmid pAID135 expresses PhoA(Δ2–22) under the control of tac promoter as described previously (27).
DnaJ and PhoA Degradation

% PhoA remaining

minute

wt

dnaK756

dnaJ

100

30°C

37°C

wt (30°C)

wt (37°C)

dnaJ259 (37°C)

dnaK756 (37°C)

30°C

37°C

Relative intensity (arbitrary units)

PhoA

DnaJ

FIG. 2. DnaJ function is required for the degradation of PhoA(Δ2-22). Degradation at 37 °C was assayed in isogenic wild-type (wt) and mutant strains as in Fig. 1. The effect of the dnaJ259-ts mutation was compared with that of a dnaK756 mutation, which had been shown to prevent PhoA release from DnaK in response to ATP (6). Similar results were obtained in at least two additional experiments.

FIG. 3. DnaJ promotes the association of PhoA(Δ2-22) with proteases La and ClpAP. The wt and dnaJ259 cells were grown at 30 °C, and PhoA expression was induced at 37 °C. PhoA was immunoprecipitated from the soluble cell lysates with an anti-PhoA antibody. The precipitates were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane for Western blot analysis. The amounts of protease La and ClpA that coprecipitated with PhoA were assayed with an anti-La or anti-ClpA antibody followed by 125I-protein A. Similar results were obtained in several additional experiments. No protease La or ClpAP was detected in similar immunoprecipitations from cells lacking PhoA(Δ2-22) in mock immunoprecipitations using preimmune serum.

Experiments indicated that the chaperone, although affecting protease-substrate interactions, is not necessary for the formation or maintenance of active proteases, because the specific peptide substrates of protease La, Suc-Ala-Ala-Phe-4-methoxy-2-naphthylamide, and ClpAP, Suc-Leu-Tyr-MNA, were cleaved at similar rates in the wild-type and dnaJ mutants growing at 37 °C. Thus, DnaJ's ability to support PhoA breakdown correlates with its ability to enhance the association of this substrate with these ATP-dependent proteases.

Amount of PhoA Associated with DnaJ Also Correlates with Its Degradation Rate—Because the association of a protein with DnaK or DnaJ is likely to be quite brief during successful protein folding but much longer with a mutant protein that is unable to fold correctly, we proposed that the prolonged association of PhoA61 with DnaK might be the critical factor leading to its degradation (7). Accordingly, the extent of DnaK binding to PhoA was found to correlate with its rate of degradation (6). Because DnaK has also been shown to form complexes with many unfolded proteins and with DnaK (20), a substrate that dissociates poorly from DnaK might also be found in a prolonged association with DnaJ. To test if DnaJ also associates with PhoA during its degradation, we first immunoprecipitated PhoA from soluble cell extracts using an anti-PhoA antibody and then performed quantitative Western blot on the immunoprecipitates with an antibody against DnaJ. The amount of precipitated PhoA was confirmed by Western blot, and the presence of DnaJ that coprecipitated with PhoA was detected with an anti-DnaJ antibody. The amounts of PhoA from the different strains were normalized, and the amount of DnaJ that coprecipitated was expressed relative to that in the wt at 37 °C. The intensities of PhoA and DnaJ bands in the wt were set as 100. Quantitation of band intensity was performed with a PhosphorImager. Similar results were obtained in at least two additional experiments.

To further test whether the extent of the association with DnaJ correlated with PhoA degradation rates, we constructed an isogenic dnaK756 mutant (HHK2), and measured the rate of PhoA(Δ2-22) breakdown as well as the amount of DnaJ in complex with this substrate. This mutation was previously shown to cause a failure of PhoA61 (and other polypeptides) to be released from DnaK, even in the presence of ATP (6). The rate of degradation of PhoA(Δ2-22) clearly increased in this mutant, from a t1/2 of about 10 min to one of less than 5 min at 37 °C (Fig. 2), and the relative amount of DnaJ in complex with
soluble PhoA (Δ2–22) was almost 5-fold higher than in wild-type (Fig. 4). In control (mock) immunoprecipitations from all three strains, using only protein A-beads, no DnaJ was detected (data not shown). Thus, when PhoA was degraded faster, more of it was present in complexes with DnaJ (as well as with DnaK (6)). These findings with the dnaK756 and the dnaJ mutants suggest a link between enhanced or reduced chaperone binding and changes in degradative rates.

It should be noted that, in our previous studies with PhoA61, which had clearly indicated that rapid degradation also correlated with the extent of Dnak binding, no DnaJ was found to coprecipitate under the conditions used. However, in those experiments, we had included Triton X-100 in the immunoprecipitation buffer (6). In the present studies, DnaJ was detected in complexes with PhoA (Δ2–22), because Triton X-100 was removed from the buffer. Also, the amount of DnaJ that coprecipitated with PhoA was not affected by the addition of Mg-ATP or EDTA, in contrast to Dnak, which dissociated from PhoA upon ATP addition (data not shown).

If DnaJ Is Inactivated, Some PhoA Aggregates—Unfolded proteins tend to aggregate in the absence of molecular chaperones (22–24), and it has been proposed that the role of DnaJ and Dnak in protein breakdown is primarily to help maintain the substrate in a soluble form that allows proteolysis to occur (25). To determine whether the DnaJ requirement for PhoA (Δ2–22) degradation was to prevent its becoming insoluble, we examined whether the nondegraded protein was soluble or in particulate form. After shifting the cells grown at 30 to 37 °C, PhoA expression was induced for 20 min. Cells were then disrupted by sonication (22–24) and centrifugation at 100,000 × g for 15 min to remove large insoluble protein aggregates, and subsequently the supernatant was ultracentrifuged at 100,000 × g for 1 h. These pellets were resuspended in the original buffer volumes, and the amount of PhoA in each fraction was measured by quantitative Western blot. In wild-type (WP551) cells, all of the PhoA protein was found in the soluble fractions (i.e. 100,000 × g supernatant) (Fig. 5). In the lon-clpP double mutant, where degradation of PhoA was completely abolished (Fig. 1), it remained almost entirely soluble even after the ultracentrifugation step (Fig. 5). However, in the dnaJ mutant, where PhoA was also not degraded, ~50% of the protein was recovered in the 15,000 × g pellet, and the remainder was soluble. The degree of PhoA aggregation was also determined after a 1-h incubation at 37 °C, and the amount of PhoA in the 10,000 × g or 100,000 × g pellets did not increase further during this period (data not shown). Thus, DnaJ clearly helps to maintain some of the nondegraded PhoA in a soluble form. However, even without functional DnaJ, half of these molecules remained soluble after centrifugation at 100,000 × g (Fig. 5). Because degradation of both the soluble and insoluble PhoA was completely blocked under these conditions (Fig. 2), DnaJ must play a direct role in the degradation of soluble PhoA, beyond any additional role in preventing aggregation of some of the nondegraded molecules.

PhoA Degradation and Solubility Do Not Require ClpB—Recently, the Dnak/DnaJ/GrpE system was shown to function together with another molecular chaperone, ClpB, in disaggregation and refolding of protein aggregates (23). Similarly in yeast, the homologous chaperones, Hsp70 and Ydj-1, have been shown to function with the ClpB homolog, Hsp104, in solubilizing protein inclusions (35). These observations raised the possibility that DnaJ and Dnak promote PhoA (Δ2–22) degradation by functioning together with ClpB to solubilize degraded PhoA molecules. To test whether ClpB is also essential in PhoA degradation, a clpB deletion (strain kindly provided by Dr. T. Baker, MIT) was introduced into WP551 background by P1 transduction. Using this strain, we tested whether ClpB was a cofactor in PhoA degradation. Although its degradation was consistently reduced to a slight extent in the clpB mutant (Fig. 6A), this reduction was much smaller than that seen upon DnaJ inactivation or upon loss of Dnak, which caused a complete stabilization of PhoA. Thus, at 37 °C, ClpB is not an essential cofactor in Dnak/K-dependent degradation of PhoA. Moreover, in the clpB mutant at 37 °C, nearly all the PhoA remained soluble (Fig. 6B), in contrast to the dnaJ mutant, where about 50% of the protein was insoluble. Thus, ClpB also is not important to maintain PhoA soluble at this temperature.
FIG. 7. Proper folding of PhoA in a trxB mutant retards its degradation, and DnaJ is required for this degradation but not for PhoA folding. The isogenic wt, trxB, and trxB-dnaJ259 strains were grown and lysed, and PhoA was induced at 37 °C as in Fig. 5. A, PhoA degradation was assayed at 37 °C as in Fig. 2. B, alkaline phosphatase activity in these strains was measured as described under “Experimental Procedures.” Similar results were obtained in three separate experiments.

Proper Folding of PhoA in a trxB Mutant Retards Its Degradation—The rapid hydrolysis of PhoA(Δ2–22) at 37 °C results presumably from its failure to form sulphydryl bridges in the cytosol and to achieve a stable conformation. The active form of PhoA (alkaline phosphatase) contains two intra-chain disulfide bonds. Beckwith and coworkers (29) have shown that cytosolic proteins are maintained in a reduced state in part due to the function of thioredoxin reductase (TrxB). In a trxB mutant, all the nonsecreted PhoA(Δ2–22) was able to fold into disulfide bridges, and a substantial portion could fold into a native form in the cytosol of a nonsecreted alkaline phosphatase molecule, PhoA(Δ2–22). Normally, when a signal peptide is present, DnaJ, together with DnaK and GrpE, promotes translocation of this enzyme into the periplasm (36, 37). When export is impossible, DnaJ, apparently with DnaK and GrpE (6), carries out some function essential for rapid degradation of this unfolded protein by the ATP-dependent proteases La (Lon) and ClpAP. Although DnaJ was found to help maintain this protein in a soluble form (for example, in the trxB mutant cell, where a large fraction was particulate (Fig. 8)), therefore, in an oxidizing environment, where PhoA can form disulfide bridges, both native and non-native, DnaJ is apparently not necessary for the folding process or for maintaining it in a soluble form, even though this chaperone remains absolutely essential for PhoA degradation.

DISCUSSION

The present studies indicate that the molecular chaperone, DnaJ, plays multiple roles in determining the fate in the cytosol of a nonsecreted alkaline phosphatase molecule, PhoA(Δ2–22). Normally, when a signal peptide is present, DnaJ, together with DnaK and GrpE, promotes translocation of this enzyme into the periplasm (36, 37). When export is impossible, DnaJ, apparently with DnaK and GrpE (6), carries out some function essential for rapid degradation of this unfolded protein by the ATP-dependent proteases La (Lon) and ClpAP. Although DnaJ was found to help maintain this protein in a soluble form (for example, in the lon-ClpP mutant cell, where degradation is not possible), DnaJ clearly must play additional role(s) in PhoA degradation beyond preventing the substrate from forming insoluble aggregates. When DnaJ was inactivated, at least half of the PhoA molecules remained soluble at 100,000 × g, yet they were not degraded. Furthermore, when a favorable reducing environment was provided (i.e. in the trxB mutant cells), in which some PhoA can fold into its native form, DnaJ was still required for its degradation, but was not necessary for the prevention of aggregation or for the proper folding of the active enzyme.

Previous findings in E. coli (6, 38) and yeast mitochondria (11) have implicated the DnaK/DnaJ/GrpE chaperones in the rapid breakdown of certain abnormal proteins by ATP-depend-
dent proteases. Both the DnaK and DnaJ families of chaperones are known to bind selectively to unfolded proteins (9, 15–17), and in this way, they may promote the degradative process by facilitating the recognition of unfolded proteins as substrates by the proteases. Alternatively, by maintaining the substrates in an unfolded conformation, these chaperones may function as cofactors that facilitate digestion by the proteases. Studies with another nonsecreted variant, PhoA61, which contains a point mutation in the signal sequence, had suggested a kinetic partitioning model for chaperone function, in which a prolonged association of the substrate with DnaK would favor proteolytic attack and help distinguish appropriate substrates for degradation from normal cell constituents (7). Successful folding of a protein, as occurs normally for most proteins, would involve only a transient association with the chaperones, whereas an extended association, as may occur with a highly abnormal mutant protein or an irreversibly damaged polypeptide, would favor its proteolytic digestion.

The present finding that the association of PhoA with DnaJ correlates with its rapid degradation is consistent with this proposed mechanism. At 30 °C, where PhoA was relatively stable, or at 37 °C in the dnaJ259 mutant, where PhoA degradation was blocked, much less soluble PhoA was associated with DnaJ than in the wild-type at 37 °C. By contrast, in a strain carrying a nondissociating DnaK mutation (dnaK756), where the amount of DnaJ complexed with PhoA was at least 5-fold higher than in the wild-type, PhoA degradation was enhanced at least 2-fold. In our prior studies, we had observed that the extent of DnaK association with PhoA61 (in dnaK756 and dnaK deletion strains) also correlated with the rate of its hydrolysis (6). (Those earlier studies, in contrast with the present ones, failed to demonstrate an association of PhoA with DnaJ because of the use of the detergent Triton X-100 in the buffers.) Thus, these findings together strongly suggest that prolonged association of this unfolded protein with DnaJ and DnaK facilitates its hydrolysis. However, these coimmunoprecipitation data only demonstrate correlations with the amount of PhoA present in complexes with the chaperones; the data do not distinguish whether rapid degradation may in fact be due to the presence of more molecules of the chaperone in complexes with the substrate or to a more prolonged or a tighter association of the substrate with the same number of chaperone molecules.

Although not directly demonstrated in these experiments, it seems very likely that DnaJ and DnaK are present together in the same complexes with PhoA, as was found for certain other denatured proteins (16, 17, 20, 21). For example, in the dnaK756 mutant, which is defective in ATP-dependent dissociation from PhoA, more PhoA was associated with DnaJ than in the wild-type. Also, the dnaJ259 mutation, which is located in the highly conserved “J” domain that interacts with DnaK and thus causes a defective association with DnaK (39) showed a dramatically reduced association with PhoA. This in vivo failure of dnaJ259 to bind to PhoA is noteworthy, because no deficiency had been observed previously in its binding to model substrates in vitro. It is not clear if DnaJ and DnaK influence degradation rates independently or if one chaperone acts by influencing binding of the other as suggested by the results with the dnaK756 mutant. Such mechanistic questions will be best analyzed with pure components in vitro.

The present results, although indicating a requirement of DnaJ for proteolysis, also support the view that one function of this chaperone in cells is to prevent PhoA aggregation into insoluble inclusions, because 30–50% of the newly synthesized PhoA accumulated in rapidly sedimenting fractions in the dnaJ mutant. A role for DnaJ in maintaining denatured proteins in a soluble form has been established in several studies (28, 40), especially in concert with DnaK (23, 24) and also ClpB (23). In mitochondria, the rapid breakdown of two fusion proteins by the Pim1 protease (a homolog of protease Lp) required the mitochondrial DnaJ homolog Mrp-1, and in its absence, some of these proteins accumulated as insoluble aggregates (11). Similarly, in E. coli, the rapidly degraded transcriptional activator RcsA was stabilized in a dnaJ mutant, and a large fraction of the RcsA was found in insoluble aggregates (26). It was therefore proposed that the primary role of DnaJ, and by extension DnaK and GrpE, in protein breakdown is to maintain the substrate soluble and therefore susceptible to the protease (25). However, there is no evidence that maintenance of these molecules in a soluble form is actually essential for proteolysis. Although it is likely that proteolytic attack on aggregated substrates may be slower and less efficient than degradation of soluble proteins (at comparable concentrations), the automatic assumption (25) that polypeptides in particulate fractions cannot be digested by soluble proteases seems unwarranted. Indeed, it has long been known that denatured proteins, once in large inclusion bodies, can still be rapidly hydrolyzed to amino acids (41, 42) and that protease La can digest insoluble, membrane-associated proteins (43).

Rapid digestion of such aggregated species may also require the involvement of molecular chaperones to promote substrate solubilization, and for this reason, we investigated the possible involvement of PhoA breakdown of ClpB, which can function with DnaK/DnaJ/GrpE to solubilize aggregated proteins (23). ClpB, unlike DnaK and DnaJ, was not necessary for proteolysis. However, the clpB strain did consistently show a small reduction in PhoA breakdown, perhaps because a minor portion of the substrate was particulate and may require this chaperone for solubilization prior to DnaJ-dependent degradation.

Although earlier studies (26), like the present ones, have demonstrated a tendency of the stabilized abnormal proteins to aggregate, they did not show (but assumed) that aggregation is the cause of the stabilization. On the contrary, the present findings clearly indicate an important additional function of chaperones in proteolysis, because in the dnaJ mutant, degradation was abolished completely, although at least half of the nondegraded PhoA remained soluble at 100,000 × g. Perhaps the best evidence for a role of DnaJ in facilitating the degradative process came from the trxB strains, where the dnaJ mutant completely blocked PhoA degradation, even though none of the substrate was aggregated.

In these trxB mutants, where a large fraction of the PhoA was able to fold into the active conformation, the inactivation of DnaJ led to a 3-fold increase in PhoA enzymatic activity. This increase in activity was most likely due to the increase in the amount of soluble PhoA that resulted from blocking its degradation. In other words, by promoting PhoA degradation, DnaJ appears to suppress the folding process. Such a role for DnaJ is supported by the finding that overexpression of DnaK and DnaJ reduced the cytosolic accumulation of a cloned human polypeptide (SPARC), presumably by increasing its degradation, and it also reduced the yield of SPARC with correct disulfide bond formation in a trxB mutant (44). Interestingly, DnaJ itself has been reported to possess disulfide oxidoreductase activity in vitro (45), and it is conceivable that this activity may help maintain certain proteins, such as PhoA, in an unfolded conformation in vivo (e.g. in a trxB cell).

These findings suggest that an essential function of DnaJ and DnaK in proteolysis is to facilitate the binding of substrates to the ATP-dependent proteases, ClpAP or La, or to stabilize these enzyme-substrate complexes once they are formed. The chaperones may promote the recognition of the
partially unfolded protein by maintaining certain domains in a conformation that is particularly susceptible to association with the proteases. The critical finding here is that, when DnaJ was defective, very little Lj or ClpAP appeared to be associated with PhoA. These results indicate for the first time a requirement for DnaJ for the formation of degradative complexes between a substrate and the proteases that digest it. An analogous role of a DnaJ homolog in targeting a substrate to proteolysis has been proposed in yeast cytosol, based on the finding that binding of the DnaJ homolog Ydj1 was necessary for ubiquitin conjugation to a rapidly degraded protein (12). An additional example of Ydj1 maintaining a substrate in a conformation that allows enzymatic attack is in the ubiquitination of the Cln3 cyclin. In this case, Ydj1 binding to Cln3 was required for Cln3 phosphorylation by p34<sup>Cdc28</sup>, which in turn triggers ubiquitin-dependent proteolysis (46). Other chaperone systems have also been implicated in “presenting” substrates for proteolysis or helping maintain them in conformations that allow proteolytic attack; for example, the rapid degradation of the fusion protein, CRAG, in <i>E. coli</i> requires a specific association between the substrate GroEL and Trigger Factor (TF) (31, 47, 48).

It is very unlikely that the requirement for DnaJ in proteolysis is for the folding, assembly, or activation of these two ATP-dependent proteases. Inactivation of these enzymes seems unlikely in these experiments, because the cells were grown at 30 °C and switched to 37 °C for only 20 min, which was sufficient to prevent complex formation between the protease and the substrate. In addition, hydrolysis of specific fluorogenic peptide substrates by these proteases occurred at similar rates in extracts of the wild-type and <i>dnaJ</i> mutant strains, and complex formation between ClpAP and ClpP occurred whether or not DnaJ was functional. Finally, Jubete et al. (26) have noted that inactivation of DnaJ stabilized some protein substrates of protease Lj, but not others. Therefore, the chaperone must instead be necessary for substrate recognition or rapid digestion.

An important feature of the degradation of PhoA shown here is that this process is catalyzed by two very different proteases, Lj and ClpAP. The two ATP-dependent proteases contribute about equally in vivo, and each can degrade the substrate apparently without the involvement of the other. Degradation by both proteases is likely to occur in a highly processive fashion, because no intermediate fragments of PhoA were observed; also with each, relatively stable substrate-protease complexes could be captured by communoprecipitation. Previous findings with PhoA61 (6) and CRAG (31) had raised the possibility of a specific collaboration between the DnaK/DnaJ/GrpE chaperone system and protease Lj, and between the GroEL/GroES/TF chaperones and the ClpP protease. The present findings, however, indicate otherwise, that DnaJ somehow can facilitate the interaction of PhoA with either protease Lj or ClpAP. Also, in related studies, we have found that GroEL/ GroES/TF can promote degradation of other substrates by Lj (48). Thus, the specific chaperones required for proteolysis seem to be dictated by the nature of the substrate, and not by the protease that is involved.

A fundamental unresolved issue is what feature(s) of the nonsecreted PhoA are recognized by DnaJ and/or DnaK; it is also unclear which chaperone binds first. Because PhoA(Δ2–22), which lacks the entire signal sequence, was degraded rapidly in a similar process as PhoA61, substrate recognition cannot be through chaperone binding to the unfolded signal sequence. That mechanism was initially an attractive one, because an uncleaved signal sequence would be a simple method for recognition of a protein that failed to be successfully translocated into the periplasm (49). Presumably, exposed hydrophobic domains in the PhoA polypeptide itself are recognized by DnaJ or DnaK (14). Other important questions include the precise roles of DnaJ and DnaK in allowing PhoA to interact with the proteases. It remains to be determined, for example, if binding of these chaperones leads to the exposure of the C-terminal region of PhoA, to which ClpAP may bind through the recently identified PDZ-like domain common to the Clp family (50). Possibly, prolonged association of a protein with both chaperones may be specifically recognized by the proteases, or may maintain the polypeptide in a conformation long enough for the proteases to bind or to stay bound to the substrate. In either case, the molecular chaperones would appear to be functioning as enzymatic cofactors of the ATP-dependent proteases, rather than indirectly promoting proteolysis.

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The Molecular Chaperone DnaJ Is Required for the Degradation of a Soluble Abnormal Protein in *Escherichia coli*

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