Both the *Escherichia coli* Chaperone Systems, GroEL/GroES and DnaK/DnaJ/GrpE, Can Reactivate Heat-treated RNA Polymerase

**DIFFERENT MECHANISMS FOR THE SAME ACTIVITY**

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In this work we show that the GroEL (Hsp60 equivalent) chaperone protein can protect purified *Escherichia coli* RNA polymerase (RNAP) holoenzyme from heat inactivation better than the DnaK (Hsp70 equivalent) chaperone can. In this protection reaction, the GroES protein is not essential, but its presence reduces the amount of GroEL required. GroEL and GroES can also reactivate heat-inactivated RNAP in the presence of ATP. The mutant GroEL673 protein, with or without GroES, is incapable of reactivating heat-inactivated RNAP. GroEL673 can only protect RNAP, and this protecting ability is not stimulated by GroES. The mechanism by which the DnaJ and GrpE heat shock proteins contribute to DnaK's ability to reactivate heat-inactivated RNAP GroEL673 has also been investigated. We found that the DnaJ protein substantially reduces the levels of DnaK protein needed in this reactivation assay. However, the observed lag in reactivation is diminished only in the additional presence of the GrpE protein. Hence, DnaJ and GrpE are involved in both steps of this reactivation reaction (recognition of substrate and release of chaperone from the substrate-chaperone complex) while, in the case of the GroEL-dependent reaction, GroES is involved only during the release of chaperone from the substrate-chaperone complex.

All organisms respond to environmental stresses, such as heat or ethanol, by the rapid and transient acceleration in the rate of synthesis of a specific group of proteins, the so-called heat shock proteins (HSPs) (1). Prototypes of these HSPs are the DnaK and GroEL proteins of *Escherichia coli*, members of the hsp70 and hsp60 families, respectively (1, 2–5). These classes of proteins have been universally conserved throughout evolution, their eukaryotic and prokaryotic members being at least 50% identical at the amino acid sequence level (1).

The *E. coli* dnaK and *groEL* genes were originally discovered because mutations in them block the growth of bacteriophage λ (3, 6). In the case of *dnaK*, it was subsequently shown that bacteriophage growth is blocked at the level of replication, whereas in the case of *groEL*, growth is blocked at the level of bacteriophage morphology (7). In addition, through genetic analyses, the *dnaK* and *groEL* gene products have been shown to perform essential bacterial functions. With respect to *groEL*, this function is essential at all temperatures (7). With respect to *dnaK*, deletion of the gene can be tolerated, but only within a narrow temperature range, and, even then, extragenic suppressors accumulate rapidly (8, 2) These genes are now known to encode products that are members of a family of proteins called "molecular chaperones." The function of these proteins, first proposed by High tower (9) and Pelham (10), is to protect other proteins from thermal inactivation and to reaggregate protein aggregates formed under stress conditions. Given the essentiality of these genes even under nonstress conditions, the cell apparently requires this protecting activity during normal growth as well as under heat shock conditions. Other studies suggest a variety of processes in which "chaperoning" activity is required to maintain proteins in an unfolded state, preventing them from premature, incorrect interactions (e.g. nascent polypeptide folding and protein export).

The various members of the DnaK and GroEL chaperone families share a number of properties. (a) GroEL members possess a weak ATPase activity, and DnaK members an even weaker one; (b) both bind to some forms of unfolded polypeptides, perhaps as these polypeptides emerge from the ribosomes or into specialized cellular compartments (11, 12); and (c) members of both families have been shown to functionally interact with other *E. coli* HSPS, DnaJ and GrpE in the case of DnaK and GroES in the case of GroEL (4). However, differences have been observed in the way these corresponding HSPs interact with their larger partner, DnaK or GroEL. In the case of DnaK, DnaJ and GrpE stimulate DnaK's ATPase activity up to 50-fold (13). In this reaction, DnaJ specifically accelerates the hydrolysis of bound ATP to ADP, while GrpE specifically stimulates the release of both ADP and ATP from DnaK (13). In sharp contrast, GroES has been shown to inhibit the ATPase activity of GroEL (14, 15).

2 A. Ziemienowicz, D. Skowyra, J. Zeilstra-Ryalls, O. Fayet, C. Georgopoulos, and M. Zylicz, unpublished observations.
while DnaJ has been shown to bind to some unfolded proteins (16), no such binding has ever been demonstrated for GroES.

Previously, we were able to show that DnaJ can both protect RNA polymerase (RNAP) from thermal inactivation and, using energy derived from ATP hydrolysis, reactivat RNAP by dissolving the large protein aggregates formed during incubation at high temperature (17). Here we show that GroEL is also able to carry out the same functions. Demonstration of this in vitro activity provides an important correlation with a previously observed phenotype noted in both dnaK and groE temperature-sensitive (Ts) strains, that is, a reduction in global RNA synthesis at nonpermissive temperatures (3, 18). We also demonstrate that the roles of the partner HSPs, DnaJ and GrpE, and GroES, in the functioning of DnaK and GroEL, respectively, are not the same due to differences in their mechanisms of action.

**MATERIALS AND METHODS**

**Bacterial Strains**

The *E. coli* strains B178, B178 (pMOB45 dnaK*), B178 (pMOB45 dnaJ*), C600 dnaE103, B178 (pF39 groES* groEL*), used in the purification of RNAP, DnaK, DnaJ, GrpE, GroEL, and GroES wild type proteins, respectively, have been described previously (7, 19). Mutant GroEL673 protein was overproduced from a bacterial strain that carries the groEL673 mutation on a multicopy plasmid, as well as at its normal chromosomal position (20).

**Proteins**

Highly purified proteins (90% or greater purity) were used. Their specific activities were as follows: DnaK, 3 × 10^6 units/mg (40); DnaJ, 4 × 10^5 units/mg (purified as described by Zylicz et al. (22) with the modifications described by Zylicz et al. (19)); GrpE, 5 × 10^5 units/mg (23). A unit of activity catalyzes the incorporation of 1 pmol of deoxynucleotides/min into trichloroacetic acid-insoluble material under the standard in vitro DNA replication assay conditions (19). Wild-type RNAP enriched with α were purified as described by Burgess and Jendrisak (24).

**Purification of GroEL and GroES Proteins**—*E. coli* strain T1244 (pOF39) bacteria were cultured in 10 liter of LB medium at 37 °C to OD_600 ~ 1.2. Cells were harvested by centrifugation (8,000 rpm for 10 min at 4 °C in a Beckman J21B rotor) and washed with ice-cold T-buffer (50 mM Tris/HCl, pH 8.0, 100 mM KCl, 5 mM β-mercaptoethanol). After centrifugation cells were resuspended in 10 ml of T-buffer containing 10% sucrose and frozen in liquid nitrogen. After thawing the cells in a 4–6 °C water bath for 6 min, 2 ml of lysozyme (10 mg/ml) in cold distilled H_2O, and enough T-buffer to reach a final volume of 200 ml were added. The mixture was incubated on ice for 1 h, shifted to a 37 °C water bath for 5 min, and then returned to ice. Debris was pelleted by centrifugation (10,000 rpm for 80 min at 4 °C in a Beckman J21B rotor). To the cleared supernatant, solid ammonium sulfate was added to 30% saturation, stirred gently on ice for 30 min, then centrifuged (10,000 rpm for 160 min at 4 °C in a Beckman J21B rotor). Ammonium sulfate was added to the supernatant to 50% saturation, stirred gently on ice for 30 min, then centrifuged as before. Precipitated proteins were dissolved in 10 ml of B-buffer (50 mM Tris/HCl, pH 8.0, 10% (v/v) glycerol, 5 mM β-mercaptoethanol, 5 mM EDTA, 0.05% Triton X-100, and 1 mM KCl); half of this was applied to a Sepharose CL-4B column (2.7 × 70 cm; 7 ml/h). The GroEL and GroES peak fractions, as determined by SDS-polyacrylamide gel electrophoresis, were pooled separately, and the purification was continued as described below.

**GroEL**—After dialysis in T-buffer, GroEL fractions were applied to a Q-Sepharose column (1.7 × 10 cm, 20 ml/h) equilibrated with T-buffer. The column was washed with one column volume of T-buffer, then three washed volumes of 200 mM KCl in T-buffer, followed by a 200–600 mM KCl gradient in T-buffer (100 ml). The peak fractions were pooled. Ammonium sulfate was added to a final concentration of 0.3 M, and precipitation allowed to proceed for an additional 30 min at 0 °C. Precipitated proteins were pelleted by centrifugation, resuspended above and below the gradient in a minimal volume of T-buffer prior to dialysis in T-buffer overnight. Aliquots were frozen in liquid nitrogen and stored at -70 °C. The final concentra-
Previously we showed that nearly equivalent levels of protection are afforded by the DnaK756 mutant protein (17). The addition of purified GroEL673 mutant protein also results in protection of RNAP activity at levels close to those obtained with the wild type GroEL protein (Fig. 1). The results with the mutant proteins are not necessarily surprising. Since the DnaK and GroEL functions are essential at all temperatures, under permissive conditions the mutant proteins must retain some chaperone activity to allow E. coli growth. In the case of GroEL673, this partial functionality has been demonstrated in vivo (20).

Both genetic and biochemical studies have established that the GroEL and GroES proteins interact (3). Studies on refolding of a variety of polypeptide substrates have shown that GroES increases recovery of refolded substrates (26). Based on these observations, we asked whether GroES influences the ability of GroEL to protect RNAP from heat inactivation. Fig. 2 shows that the presence of GroES improves protection of RNAP by the wild type GroEL protein, but has no effect on protection by the GroEL673 mutant protein. The optimal molar ratio of GroES to wild type GroEL in this reaction was 1 or 2 GroES 7mers: 1 GroEL 14mers. The inability of GroES to enhance protection of RNAP by the GroEL673 mutant protein suggests that GroEL673 is defective in its ability to interact with GroES. In control experiments using size chromatography and native electrophoresis, we have shown that the presence of either the GroEL/S or DnaK/J/E chaperone systems prevents formation of large RNAP aggregates (results not shown).

Wild Type DnaK and GroEL Chaperone Systems Can Reactivate Heat-treated RNAP—Given the fact that RNA synthesis is affected in both groE and dnaK mutants (3), we decided to test if the GroEL/GroES chaperone system shows the same ability as DnaK to reactivate heat-inactivated RNAP. After incubation for 10 min at 45 °C, less than 10% of the RNAP activity (measured by the transcription assay at 30 °C) remained compared to untreated RNAP. In this case, more than 90% of RNAP appeared in the void volume of the Bio-Gel A-15 gel filtration column, as was previously shown (results not shown; see also Fig. 3 of Ref. 17). However, when GroEL, GroES, and ATP were added to heat-treated RNAP in a ratio of 10 GroEL 14mers: 20 GroES 7mers: 1 RNAP, reactivation of RNAP activity was observed following a 10–20-min lag. After 60 min of incubation with GroEL, GroES, and ATP, more than 60% of the RNAP activity was recovered (Fig. 3).

Surprisingly, when heat-inactivated RNAP was preincubated for 10 min at 30 °C with only GroEL, followed by addition of GroES and ATP at the start of the transcription assay, no lag in the kinetics of RNA reactivation was observed. This result suggests that the presence of GroES may interfere with the binding of GroEL to the heat-inactivated RNAP. Preincubation of RNAP with GroES, or GroES and ATP, or GroEL and ATP did not reduce this lag period (results not shown). In control experiments, we showed that GroEL, GroES, and ATP are all absolutely required for efficient reactivation of heat-inactivated RNAP (Fig. 3; results not shown).

Although both purified DnaK756 and GroEL673 mutant proteins can protect (Ref. 17; Fig. 1), neither of the mutant proteins can function in the reactivation of heat-treated RNAP (Fig. 4). We previously showed that the wild type DnaK protein alone could carry out the reactivation reaction (17), but the DnaK756 mutant protein did not have this ability. Since, for GroEL, this activity absolutely requires GroES (Fig. 3), we asked whether GroEL673 can reactivate heat-treated RNAP in the presence of GroES. Under these conditions, the GroEL673 mutant protein showed no ability to reactivate heat-treated RNAP (Fig. 4). This result correlates with the previous observation that addition of GroES did not improve the ability of the GroEL673 mutant protein to protect RNAP from heat inactivation (see Fig. 1).

Effect of DnaJ and GroE Proteins on DnaK-dependent Reactivation of RNAP—Previously, we showed that it was necessary to add a large excess of DnaK (156 molecules of DnaK/molecule of RNAP, (17) to restore RNAP activity,
The final concentrations of RNAP, GroES, and ATP were 6,400 cpm.

AB buffer, heat-inactivated by incubation for 10 min at 45°C, and the 8-min transcription assay was initiated as described under "Materials and Methods." The final concentrations of RNAP, GroES, and ATP. Under these conditions, 100% was 6,400 cpm.

FIG. 3. GroEL and GroES are both required for ATP-dependent reactivation of heat-inactivated RNAP. RNAP was diluted 20-fold by AB buffer and heat-inactivated by a 10-min incubation at 45°C. The mixture was then transferred to 30°C and preincubated for 15 min with GroEL/GroES/ATP (•—•) or GroES (—) alone. At time = 0, GroES and ATP were added where missing, preincubation was allowed to proceed for the indicated time, and the 8-min transcription assay was initiated as described under "Materials and Methods." A-•—• indicates the control where heat-inactivated RNAP was incubated with GroEL but without subsequent addition of GroES (similar results were obtained with GroES alone, result not shown). -—• indicates the control where heat-inactivated RNAP was incubated with GroEL and GroES in the absence of ATP. The final concentrations of RNAP, GroEL, and GroES in the reactivation assays were 0.12, 0.94, and 0.86 μM, respectively. Relative RNAP activity was calculated taking as 100% the activity of control unheated RNAP assayed in the presence of GroEL, GroES, and ATP. Under these conditions, 100% was 6,400 cpm.

FIG. 4. Mutant GroEL673 cannot reactivate RNAP even in the presence of GroES and ATP. RNAP was diluted 20-fold by AB buffer, heat-inactivated by incubation for 10 min at 45°C, transferred to 30°C, and preincubated for 15 min with GroEL and GroES (○—○), GroEL673 (□—□), and GroEL673 and GroES (●—●). Incubation proceeded for the indicated times, and 8-min transcription assays were initiated as described under "Materials and Methods." The final concentrations of RNAP, GroEL, GroEL673, and GroES in the reactivation assay were 0.12, 0.86, 0.86, and 0.94 μM, respectively. Relative RNAP activity was calculated taking as 100% the activity of control unheated RNAP assayed in the presence of GroEL, GroES, and ATP. Under these conditions, 100% was 5,800 cpm.

FIG. 5. DnaK-dependent reactivation of heat-inactivated RNAP. RNAP was diluted 20-fold and heat-inactivated as described under "Materials and Methods." The reaction mixture was then transferred to 30°C. ATP added to a final concentration of 4 μM, and increasing amounts of DnaK protein added in the presence or absence of DnaJ and GrpE. DnaK alone (□—□); DnaK and DnaJ (○—○); and DnaK, DnaJ, and GrpE (●—●). The final volume of each reaction was adjusted to 16 μl, and incubation proceeded for 30 min at 30°C, followed by initiation of the 8-min transcription assays as described under "Materials and Methods." The final concentration of ATP, DnaJ, and GrpE in the reactivation assay were 0.12, 0.12, and 2 μM, respectively. Relative RNAP activity was calculated taking as 100% the activity of control unheated RNAP assayed in the presence of 18.08 μM DnaK in the presence or absence of DnaJ and GrpE protein (DnaK alone, 10,000 cpm; DnaK and DnaJ, 11,200 cpm; DnaK, DnaJ, and GrpE, 14,800 cpm).

Whereas a limited amount of DnaK (19 molecules per molecule of RNAP) did not reactivate heat-treated RNAP (Fig. 5; Ref. 17). However, when DnaJ was added to the reaction, efficient reactivation was observed with the limited amount of DnaK (Fig. 5). Titration of DnaJ protein in this reaction clearly showed that less than one dimeric molecule of DnaJ per molecule of RNAP is sufficient to saturate the reaction (Fig. 5). Thus, in this assay system it is possible to reduce the concentration of DnaK protein needed to achieve a given level of reactivation by the addition of DnaJ.

The effect of adding GrpE to the reactivation reaction catalyzed by DnaK alone is minimal (data not shown), but there is pronounced enhancement when GrpE is added together with DnaJ to the reaction (Figs. 5 and 6). This stimulatory effect is maximal at a 1:1 ratio of GrpE to DnaK (Fig. 7). As a control, neither DnaJ alone nor DnaJ together with GrpE was able to restore RNAP activity (Fig. 6). This established that the optimal reactivation by DnaK is achieved when both DnaJ and GrpE are present.

During these studies, it became apparent that the time of preincubation of the various proteins with heat-inactivated RNAP was a critical factor in the reactivation reaction, an observation that has already been noted for the GroEL system (see Fig. 3). To more clearly resolve the kinetics of reactivation by the DnaK system, we used a brief 2.5-min transcription assay. When a large amount of DnaK protein alone was used (156 molecules of DnaK per molecule of RNAP), a lag of approximately 10 min was observed, followed by a slow reactivation reaction (Fig. 7). An 8-fold reduction in the amount of DnaK protein (19 molecules per molecule of RNAP) resulted in very little, if any, reactivation of RNAP (Fig. 7). The addition of DnaJ to this limiting amount of DnaK reduced the lag period to 5 min, and thereafter the reactivation reaction proceeded with kinetics very similar to those observed when large amounts of DnaK protein alone were used (Fig. 7).

GrpE with DnaK had very little, if any, effect on the kinetics of the RNAP reactivation reaction. However, when
The concentration, of RNAP, DnaJ, and GrpE used in these reactions was 0.12, 0.12, and 2 \mu M, respectively. Relative RNAP activity was calculated taking as 100% the activity of control unheated RNAP, assayed in the presence of all other proteins (DnaK alone, 7,890 cpm; DnaJ and GrpE, 7,200 cpm; DnaK and DnaJ, 8,400 cpm; DnaK, DnaJ, and GrpE, 9,600 cpm).

To examine whether all three HSPs must be present during all stages of the RNAP reactivation assay, we performed a series of experiments in which different combinations of these proteins were present either during a 5-min preincubation step with heat-inactivated RNAP or were added only during the short 2.5-min transcription assay for RNAP activity. A comparison of the net effect of the various combinations is presented in Table I, while the GrpE dependence of this reactivation reaction under these different conditions is shown in Fig. 8. These results reveal the following. With the relatively short 5-min preincubation, no significant level of DnaK- and DnaJ-dependent reactivation of RNAP was ob-

![Molar ratio (DnaJ/RNAP)](image)

**Fig. 6. Reactivation of heat-inactivated RNAP as a function of DnaJ protein concentration.** RNAP was diluted and heat-inactivated as described under "Materials and Methods." The reactivation reaction (16 \mu l) proceeded for 30 min at 30 °C in the presence of increasing amounts of DnaK protein in the presence or absence of the other two proteins. DnaJ alone (\(\Delta\Delta\)); GrpE and DnaJ (\(\square\)); DnaK and DnaJ (\(\bullet\)); DnaK, DnaJ, and GrpE (\(\bigcirc\)). The final concentrations of RNAP, DnaK, and GrpE used in the reactivation assay were 0.12, 2.26, and 2 \mu M, respectively. Relative RNAP activity was calculated taking as 100% the activity of control unheated RNAP, assayed in the presence of DnaK protein (0.12 \mu M) in the presence or absence of DnaK and GrpE proteins (DnaJ alone, 7,890 cpm; DnaJ and GrpE, 7,200 cpm; DnaK and DnaJ, 8,400 cpm; DnaK, DnaJ, and GrpE, 9,600 cpm).

![Kinetics of DnaK-, DnaJ-, and GrpE-dependent reactivation of RNAP](image)

**Fig. 7. Kinetics of DnaK-, DnaJ-, and GrpE-dependent reactivation of RNAP.** RNAP was diluted and heat-inactivated as described under "Materials and Methods." After transfer to 30 °C, the reactivation reaction (16 \mu l) was allowed to proceed in the presence of 4 mM ATP and: DnaK* (18.08 \mu M) (\(\Delta\Delta\)); DnaK (2.26 \mu M) (\(\bigcirc\)); DnaK (2.26 \mu M) and GrpE (\(\bigcirc\)); DnaK (2.26 \mu M) and DnaJ (\(\bigcirc\)); DnaK (2.26 \mu M), DnaJ, and GrpE (\(\bigcirc\)). The concentration of RNAP, DnaJ, and GrpE used in these reactions was 0.12, 0.12, and 2 \mu M, respectively. Following incubation at 30 °C for the indicated period of time, 2.5-min transcription assays were initiated by addition of 9 \mu l of AB buffer (W). Relative activity was calculated taking as 100% the activity of control unheated RNAP assayed in the presence of DnaK protein (0.12 \mu M) in the presence or absence of DnaK and GrpE proteins (DnaJ alone, 7,890 cpm; DnaJ and GrpE, 7,200 cpm; DnaK and DnaJ, 8,400 cpm; DnaK, DnaJ, and GrpE, 9,600 cpm).

![Molar ratio (GrpE/DnaK)](image)

**Fig. 8. GrpE-dependent reactivation of heat-inactivated RNAP.** The RNAP was diluted and heat-inactivated as described under "Materials and Methods." The reactivation reaction was preincubated for 5 min at 30 °C in the presence of different combinations of DnaK, DnaJ, and GrpE, and ATP. The amounts of RNAP, DnaK, DnaJ, and GrpE proteins in the 16-\mu l reactivation reaction were: 0.12, 2.26, 0.12, and 2 \mu M, respectively. Subsequently, the 2.5-min transcription assay was initiated by addition of 9 \mu l of mix II (see "Materials and Methods"), supplemented with 6 \mu l of AB buffer containing the proteins missing during the preincubation step.

The amounts of RNAP, DnaK, DnaJ, and GrpE present in the 16-\mu l reactivation reaction were 0.12, 2.26, 0.12, and 2 \mu M, respectively. Subsequently, the 2.5-min transcription assay was initiated by addition of 9 \mu l of mix II (see "Materials and Methods"), supplemented with 6 \mu l of AB buffer containing the proteins missing during the preincubation step.

**Table I** Reactivation of heat-inactivated RNAP: DnaK, DnaJ, GrpE-dependent system

| Inactivation of RNAP (10 min, 45 °C) | Pre-incubation (5 min, 30 °C) | Transcription (2.5 min, 30 °C) | Percent reactivation |
|-----------------------------------|-----------------------------|-----------------------------|-------------------|
| ATP, DnaK, DnaJ, GrpE | 1.0 | 1.0 | 0.0 |
| ATP, DnaK, GrpE | 1.0 | 1.0 | 0.0 |
| ATP, DnaJ, GrpE | 1.0 | 1.0 | 0.0 |
| ATP | 1.0 | 1.0 | 0.0 |

**Symbols**: K, J, and E represent DnaK, DnaJ, and GrpE, respectively. Symbols in parentheses represent those proteins which were present during the 5-min preincubation period. 100% of the activity of control unheated RNAP assayed in the presence of DnaK, DnaJ, and GrpE was 1,830 cpm.
served in the absence of GrpE (Table I, line 6); reactivation was absolutely dependent on the presence of GrpE (Fig. 8; Table I, line 7). Apparently the relatively short, 2.5-min transcription assay period was insufficient time to reactivate RNAP since the addition of DnaK, DnaJ, GrpE, and ATP at the start of the transcription assay resulted in no reactivation (Fig. 8; Table I, line 4). When DnaK, DnaJ, and GrpE were preincubated with heat-inactivated RNAP in the presence of ATP, only 37% of the activity was recovered (Fig. 8; Table I, line 2). When DnaJ was preincubated alone with heat-inactivated RNAP, followed by addition of DnaK and GrpE at the start of the transcription assay, a more efficient reactivation was found to occur (Fig. 8; Table I, line 10). Further stimulation was achieved when both DnaJ and DnaK were present (Table I, line 11). The most dramatic stimulation of the reactivation reaction was observed when DnaK, DnaJ, and GrpE were preincubated with the heat-inactivated RNAP for 5 min, in the absence of ATP (ATP was added upon initiation of the transcription assay). Under these conditions 83% of the initial RNAP activity could be recovered (Fig. 8; Table I, line 3). This result cannot be explained by the stimulatory effect of DnaJ and GrpE on DnaK’s ATPase activity (13). Rather, this result suggests that DnaJ and GrpE positively regulate the binding of DnaK to heat-inactivated RNAP.

**DISCUSSION**

Our results show that both DnaK and GroEL can protect RNAP from heat inactivation. Furthermore, both the DnaK/ DnaJ/GrpE and GroEL/GroES chaperone systems can reconstitute activity from heat-treated RNAP. The molecular mechanism of reactivation of heat-inactivated RNAP is still not clear. Recently, our laboratory is addressing the possibility that during heat inactivation, the $\sigma^w$ subunit is released from the RNAP holoenzyme, and the remaining RNAP core molecules aggregate. According to this hypothesis, the chaperone systems, GroEL/S or DnaK/J/E, may not necessarily work directly to disaggregate the aggregated core proteins of RNAP, but rather may promote the reassociation of $\sigma^w$ with the RNAP cores. This would result in the disaggregation of RNAP, leading to reactivation of its enzymatic activity. Such an indirect role of the chaperone proteins on the disaggregation reaction of RNAP may explain why in other systems a direct involvement of chaperone proteins in the disaggregation of protein aggregates is hard to prove (27, 28). Of course another possibility is that the “quality” of the various aggregates may be different in the various systems used, with the RNAP aggregate representing a “mild” version.

The RNAP reconstitution system has allowed us to compare the mechanisms by which the DnaK/DnaJ/GrpE and GroEL/GroES chaperone systems carry out the same function. We conclude that the rate-limiting step for both mechanisms is the binding of chaperone to substrate. This is based on the lag in reactivation seen when there is no preincubation of heat-treated RNAP with either DnaK or GroEL. The activity of DnaK is dramatically enhanced in the presence of DnaJ. However, the lag phase in reactivation is substantially diminished only when the third protein of the DnaK chaperone system, GrpE, is present as well. The stimulation of DnaK activity by DnaJ in this system differs from the behavior of DnaK and DnaJ in the $\lambda$ DNA replication system. For that system, the need for a high concentration of DnaK protein could not be overcome by DnaJ alone, but only by both DnaJ and GrpE (19, 29). The observed lag phase in the case of DnaJ-, DnaK-dependent reactivation of heat-inactivated RNAP, and its disappearance when all three proteins were preincubated with heat-treated RNAP (in the absence of ATP) suggest that the GrpE protein is also involved in the binding of DnaK to RNAP. This finding is new, and sheds further light on the mechanism of DnaJ and GrpE action. It is well established that binding of DnaJ protein to different polypeptides increases the affinity of DnaK for these substrates. Examples are RepA (30), $\lambda$ protein (31), and $\sigma^w$ (32).

Recently, we have shown that, in the presence of GrpE protein, DnaK protein binds more efficiently to the $\lambda$-DnaJ complex than to $\lambda$ protein (29, 31). We call this the “discrimination” function of GrpE. Perhaps in the reactivation of RNAP, GrpE can change the conformation of DnaK in such a way that DnaK recognizes better those RNAP molecules that are already “bound” to DnaJ.

Apparently, the rate-limiting step is the binding of DnaK to aggregated RNAP. Since ATP brings about a dramatic conformational change in DnaK that leads to its release from substrate proteins (32-34), it is not surprising that the best reactivation of RNAP was achieved when all three proteins of the DnaK chaperone system were first preincubated with heat-treated RNA, in the absence of ATP. These conditions would shift the equilibrium of the reaction towards binding of heat shock proteins to RNAP. The subsequent addition of ATP would then induce a change of conformation of DnaK (33) and perhaps RNAP, thus favoring release of RNAP from DnaK. The data presented here suggest that the fundamental difference in the DnaK and GroE chaperone systems lies in the affinity of DnaK and GroEL for substrate polypeptides. As shown here for RNAP and for other substrates, e.g. $\lambda$ protein (29, 31), DnaK has an inherently low affinity for substrates which, when coupled to its promiscuity may prevent it from localizing at specific, critical sites on the RNAP aggregate. This problem may be bypassed by the presence of DnaJ and GrpE which may help DnaK to improve its affinity for certain substrates. In the GroE system, GroEL has an intrinsically high affinity for substrate (RNAP), which needs to be reduced for the release and subsequent recycling of GroEL. This reduction in affinity is brought about by GroES. A similar conclusion for the role of GroES was recently presented by Bochkareva et al. (35).

Consistent with our results, Martin et al. (36) have recently reported both in vitro and in vivo data demonstrating that the Hsp60 family of proteins is capable of preventing protein denaturation and aggregation. Similarly, Mendoza et al. (37) have shown that GroEL can prevent in vitro rhodanese enzyme from heat inactivation. Recently Hartman et al. (38) showed that substoichiometric amount of GroEL and GroES prevents thermal denaturation and aggregation of mammalian mitochondrial malate dehydrogenase in vitro. In this case GroEL alone did not protect dehydrogenase activity against thermal inactivation but kept the denatured protein soluble and thereby prevented its aggregation (38).

Two recent reports have suggested a synergistic role of the DnaK and GroEL chaperone systems in preventing polypeptide misfolding and aggregation. The _in vitro_ system of Langer et al. (16) follows the sequential _in vitro_ binding of unfolded rhodanese by DnaK and DnaJ, and its ATP- and GrpE-dependent release and subsequent binding by the GroEL chaperone. In this system, GrpE appears to assist in the release of bound rhodanese from DnaK/DnaJ. Contrary to these results, GrpE can assist in both the binding of DnaK to $\lambda$ in the absence of ATP, as well as the release of $\lambda$ from DnaK (in the presence of ATP hydrolysis) (31). This apparent contradiction in GrpE action can be due either to the use of

K. Liberek and C. Georgopoulos, manuscript submitted for publication.
The in vivo report of Gragerov et al. (39) demonstrates that newly synthesized polypeptide aggregate in E. coli mutants unable to mount a proper heat shock response (deleted for rpoH, coding for α32). This aggregation can be reversed through the overproduction of either DnaK/DnaJ or GroES/GroEL. Alternatively, physiological levels of DnaK/DnaJ or GroES/GroEL can also prevent protein aggregation in the rpoH mutant host. The fact that the grpE gene can be expressed from either a α32- or α7-dependent promoter ensures the presence of GrpE protein in the rpoH-deleted host. Hence, our in vitro results with purified proteins are in complete agreement with these in vivo findings. Most likely, the demonstrated overlap in function of the DnaK and GroEL chaperone ensures that important molecules, such as RNAP, will stay functional under adverse physiological conditions, such as those encountered under heat stress, and thus assure the probability of survival.

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