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DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage

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Members of the conserved Hsp70 chaperone family are assumed to constitute a main cellular system for the prevention and the amelioration of stress-induced protein damage, though little direct evidence exists for this function. We investigated the roles of the DnaK (Hsp70), DnaJ and GrpE chaperones of *Escherichia coli* in prevention and repair of thermally induced protein damage using firefly luciferase as a test substrate. *In vivo*, luciferase was rapidly inactivated at 42°C, but was efficiently reactivated to 50% of its initial activity during subsequent incubation at 30°C. DnaK, DnaJ and GrpE did not prevent luciferase inactivation, but were essential for its reactivation. *In vitro*, reactivation of heat-inactivated luciferase to 80% of its initial activity required the combined activity of DnaK, DnaJ and GrpE as well as ATP, but not GroEL and GroES. DnaJ associated with denatured luciferase, targeted DnaK to the substrate and co-operated with DnaK to prevent luciferase aggregation at 42°C, an activity that was required for subsequent reactivation. The protein repair function of DnaK, GrpE and, in particular, DnaJ is likely to be part of the role of these proteins in regulation of the heat shock response.

Key words: Escherichia coli/GroEL/heat shock proteins/Hsp70/protein folding

Introduction

Correct folding of proteins in cells often requires the assistance of molecular chaperones such as the Hsp70 (DnaK) and Hsp60 (GroEL) heat shock proteins (Gething and Sambrook, 1992; Hartl *et al.*, 1992). Members of the Hsp70 family maintain proteins in unfolded states, cause the dissociation of protein complexes and have been reported to mediate the reactivation of an oligomeric enzyme (Gething and Sambrook, 1992; Skowyra *et al.*, 1990). Members of the Hsp60 family promote the correct folding of proteins imported into cell organelles as well as the association of oligomeric proteins (Ellis and van der Vies, 1991; Zeilstra *et al.*, 1991; Gething and Sambrook, 1992). Hsp70 and Hsp60 bind to fully or partially unfolded polypeptides and maintain them in loosely folded conformations, thereby preventing non-productive folding reactions. The release of substrates from these chaperones usually requires ATP hydrolysis (Flynn *et al.*, 1989; Goloubinoff *et al.*, 1989). At least for the *Escherichia coli* homologs DnaK and GroEL, ATP-dependent substrate release appears to be controlled by the specific cofactors DnaJ and GrpE (for DnaK) and GroES (for GroEL) (Goloubinoff *et al.*, 1989; Liberek *et al.*, 1991; Martin *et al.*, 1991). In the case of DnaJ and GrpE, these cofactors act co-operatively to stimulate the ATPase activity of DnaK (Liberek *et al.*, 1991). Interestingly, DnaJ has the capability to bind protein substrates independently of DnaK, such as DnaB helicase (Georgopoulos *et al.*, 1990; Liberek *et al.*, 1990), heat shock transcription factor σ^{32} (Gamer *et al.*, 1992), λP (Georgopoulos *et al.*, 1990), plasmid P1-encoded RepA (Wickner, 1990), and unfolded rhodanese (Langer *et al.*, 1992). The functional significance of this substrate binding by DnaJ is unclear.

Given their capacity to assist protein folding, it is not surprising that Hsp70 chaperones have multiple roles in the metabolism of unstressed as well as of stressed cells (Bukau and Walker, 1989a,b; Gething and Sambrook, 1992). One particular role hypothesized for members of the Hsp70 family is that they prevent and/or repair stress-induced damage to proteins. While such a function is consistent with the concept of chaperones, little direct evidence exists for it. The bulk of the information comes from studies of *E.coli* DnaK, which has been shown to bind to various aberrant proteins including protein fragments (Straus *et al.*, 1988; Hellebust *et al.*, 1990), mutant proteins (Clark *et al.*, 1988; Sherman and Goldberg, 1992) and unfolded proteins (Langer *et al.*, 1992). In addition, DnaK appears to be sufficient *in vitro* to protect RNA polymerase from thermal inactivation and to reactivate thermally inactivated RNA polymerase, though these activities require a large excess of DnaK (Skowyra *et al.*, 1990). Finally, it has been demonstrated that strains carrying mutations in *dnaK*, *dnaJ* or *grpE* are defective in the reactivation of a mutant form of λ-repressor (Gaitanaris *et al.*, 1990).

We performed an analysis of the roles of major *E.coli* chaperones in prevention and repair of stress-induced protein damage. We demonstrate that DnaK, DnaJ and GrpE form a chaperone machinery that is essential for the efficient *in vivo* repair of a thermally denatured test protein, luciferase, and is sufficient for *in vitro* repair. DnaJ has a crucial function in this process in that its binding to denatured luciferase suppresses luciferase aggregation and targets DnaK to the substrate. Our data provide further support for a central role of DnaJ in directly transducing stress-induced protein damage to induction of heat shock gene transcription.

Results

Role of heat shock proteins in thermal inactivation of luciferase in vivo

As a suitable thermolabile test substrate for investigation of the role of bacterial chaperones in prevention and repair of thermal denaturation of proteins both *in vivo* and *in vitro*

we considered *Photinus pyralis* luciferase since it fulfils a number of relevant criteria: (i) it is a thermolabile, monomeric protein (Subramani and DeLuca, 1988; Nguyen *et al.*, 1989); (ii) thermally inactivated luciferase produced in cultured mammalian cells can be reactivated *in vivo* (Pinto *et al.*, 1991); (iii) its enzymatic activity, readily determined by a sensitive and highly reliable photometric assay (de Wet *et al.*, 1987), can be utilized to monitor its folding status even at low concentrations; (iv) activity measurements can be performed with whole *E.coli* cells expressing luciferase (Knaus, 1991); and (v) the lack of a physiological role of luciferase in *E.coli* permits study of its thermal inactivation and reactivation *in vivo* without compromising essential metabolic functions.

We first determined the degree of thermolability of luciferase expressed in *E.coli*. For this purpose we used wild type cells (MC4100) which carry pUHE multicopy plasmids expressing the luciferase gene under control of the P_{lac}/lacO3 promoter/operator system (pUHE11-3). Due to deletion of the *lacI* gene in these cells, the plasmid-encoded luciferase gene is constitutively expressed, although at a low level, which leads to accumulation of luciferase to low levels (data not shown). Cells grown at 30°C were treated with high concentrations of kanamycin (100 μg/ml), which is known to prevent completely any further protein biosynthesis in *E.coli* (VanBogelen and Neidhardt, 1990), and were then transferred to heat shock temperatures followed by the determination of luciferase activity at various times after the temperature shift. The complete block of protein synthesis in the cells used in these experiments was verified by protein labeling experiments (data not shown). A heat shock to temperatures as low as 40°C was sufficient to inactivate luciferase. The time course of inactivation was temperature dependent, the half-time of inactivation being 5 min at 40°C, 2 min at 42°C (see Figure 1A) and < 1 min at 44°C. Thus, luciferase is rapidly inactivated at temperatures well within the growth temperature range of *E.coli*.

We then investigated the possibility that heat shock proteins provide a limited protection of luciferase from thermal inactivation in wild type cells. We therefore tested whether the activity of luciferase during steady state growth at 30°C and the kinetics of its inactivation by heat are affected by the Δ*dnaK52*, *dnaJ259*, *grpE280*, *groEL100* and *groES30* null and missense mutations. These alleles either lead to loss of the protein (Δ*dnaK52*) or result in the production of gene products that are functionally defective at high temperatures and lead to temperature sensitive growth of *E.coli*. In strains carrying these alleles only minor differences in luciferase activity (< 3-fold) as compared with wild type were detected during steady state growth at 30°C. Furthermore, neither the rate nor the extent of luciferase inactivation at 42°C was affected by any of these mutations. The example of Δ*dnaK52* mutants is given in Figure 1A.

One interpretation of the failure of these mutants to affect the rate of luciferase inactivation is that major heat shock proteins are already limiting in wild type cells at the heat shock temperature. We tested whether overproduction of DnaK, DnaJ and GrpE alters the kinetics of luciferase inactivation. We used wild type cells that carry the luciferase gene in the chromosome, the $lacI^q$ gene encoding Lac repressor on an F′ episome, and either pUHE multicopy plasmids expressing *dnaK* or *grpE* under control of P_{A1}/lacO3/O4 and P_{N25}/lacO3/O4, respectively, or

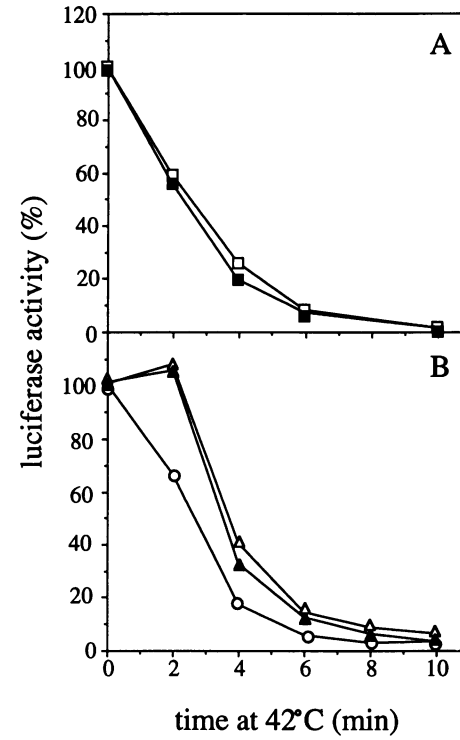

Fig. 1. Thermal inactivation of luciferase in *E.coli*. (**A**) Wild type (MC4100; □) and Δ*dnaK52* mutant (BB1553; ■) cells carrying luciferase-encoding pUHE11-3 were grown at 30°C to logarithmic phase, then treated with kanamycin (100 μg/ml) followed by incubation at 42°C. Luciferase activities were determined before and at the indicated times after shift to 42°C. (**B**) Wild type cells carrying the luciferase gene in the chromosome (BB3030) and pUHE14-1 (*grpE*$^+$) (▲) or Bluescript (*dnaK*$^+$*dnaJ*$^+$) (○) plasmids or no plasmid (△) were grown at 30°C to early logarithmic phase. Then, IPTG (1 mM) was added for 2 h followed by a shift of the cultures to 42°C. Luciferase activities of the cells were determined as for A.

Bluescript plasmids expressing the *dnaKJ* operon under control of P_{lac}. The cellular concentrations of DnaK and DnaJ were raised by adding IPTG to the cultures at 30°C which for DnaK led to an increase of at least 8-fold (for 1 mM IPTG for 2 h) in its cellular level (> 10% of total cellular protein, as estimated from polyacrylamide gels of total cellular extracts) as compared with uninduced cells. Overproduction of DnaK alone (data not shown) or of DnaK and DnaJ did not provide any protection of luciferase from thermal inactivation at 42°C (Figure 1B). We then considered the possibility that GrpE is limiting for DnaK/DnaJ function and that its overproduction might increase their efficiency. However, the inactivation kinetics of luciferase did not change upon overproduction of GrpE (Figure 1B). Together, our results indicate that the DnaK, DnaJ and GrpE chaperones do not act in the prevention of luciferase inactivation by heat.

Thermal inactivation of luciferase is reversible in vivo

We tested whether the inactivation of luciferase by heat is reversible *in vivo*. Luciferase-producing wild type cells were subjected to a 10 min heat shock at 42°C to inactivate luciferase followed by incubation at 30°C to allow recovery of luciferase activity. To ensure that any observable reactivation of luciferase would be independent of *de novo* synthesis of luciferase, a high concentration of kanamycin (100 μg/ml) shown efficiently to inhibit *de novo* protein

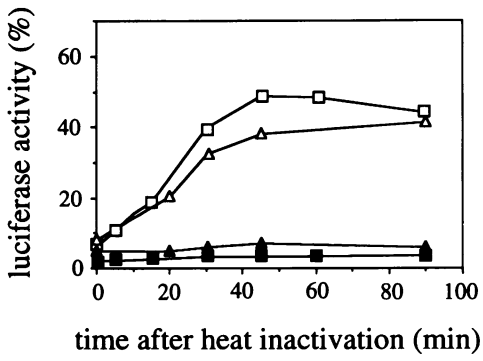

Fig. 2. Reactivation of thermally inactivated luciferase in *E.coli*. Luciferase-producing wild type (MC4100) and Δ*dnaK52* mutant (BB1553) cells carrying pUHE11-3 were grown at 30°C, then incubated for 10 min at 42°C in the presence of kanamycin (100 μg/ml) or tetracycline (25 μg/ml), followed by further incubation at 30°C. Luciferase activities of the cells were determined before and at the indicated times after incubation at 42°C. (□), wild type, Kn; (△), wild type, Tc; (■), Δ*dnaK52* mutant, Kn; (▲) Δ*dnaK52* mutant, Tc.

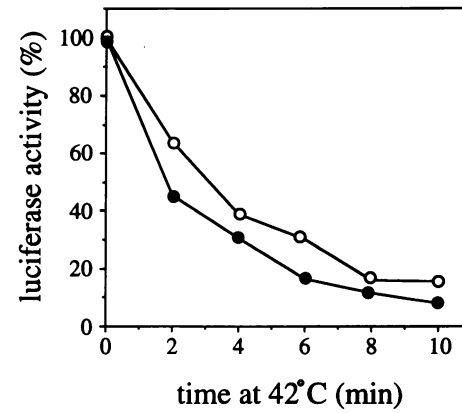

Fig. 3. Thermal inactivation of luciferase *in vitro*. Luciferase (80 nM, in renaturation buffer containing ATP) was incubated at room temperature either in the absence (○) or presence (●) of DnaK (8 μM) followed by incubation at 42°C. Luciferase activities were determined at the indicated times after shift to 42°C.

Table I. Reactivation of thermally inactivated luciferase in strains containing mutations in heat shock genes

Genetic background	Relevant genotype	Reactivation efficiency[a]	Viability after heat shock[b]
MC4100	wild type	49	143
	Δ*dnaK52*	2	84
	dnaJ259	6	104
RB85	wild type	72	n.d.
	dnaK7(am)	3	110
B178	wild type	41	n.d.
	grpE280	14	115
C600	wild type	60	n.d.
	groEL100	9	103
	groES30	10	123

[a]Luciferase activity is expressed as percent of activity present in the cells prior to heat inactivation.
[b]Cellular viability was determined before and after 10 min incubation at 42°C (heat shock) by assaying colony forming units. Viability is expressed as percent relative to viability prior to heat treatment.

synthesis (see above) was added to the culture prior to heat shock. In wild type cells, luciferase was slowly but efficiently reactivated at 30°C and regained ~50% of its initial activity prior to heat inactivation after 45 min (Figure 2). A similar extent of reactivation was also found when kanamycin was replaced with tetracycline (Figure 2) or chloramphenicol (data not shown) at concentrations (25 and 100 μg/ml, respectively) that are much higher than those reported to cause complete block of protein synthesis (VanBogelen and Neidhardt, 1990). We conclude that heat inactivation of luciferase is largely reversible in *E.coli* wild type cells.

Mutations in heat shock genes prevent luciferase reactivation in vivo

The reactivation of thermally inactivated luciferase at 30°C may require the assistance of chaperones. To test this possibility, we investigated whether mutations in genes encoding the major *E.coli* chaperones DnaK, DnaJ and GrpE as well as GroEL and GroES affect the reactivation process. Luciferase-producing Δ*dnaK52* and *dnaK7(am)* mutants were completely unable to reactivate thermally inactivated luciferase (Figure 2 and Table I). Use of tetracycline instead of kanamycin to prevent *de novo* protein synthesis yielded the same results. Furthermore, the ability to reactivate luciferase was abolished in cells carrying the *dnaJ259* missense mutation, and reduced in cells carrying the *grpE280* missense mutation. This inability was not due to a loss of cell viability caused by the heat shock as the applied heat shock regime (10 min at 42°C), while perhaps preventing further growth of the cells at 42°C, did not decrease their viability (Table I). Together, our data indicate that all three components of the DnaK, DnaJ and GrpE chaperone system are required *in vivo* for the efficient repair of thermally inactivated luciferase. It is possible that the requirement for GrpE is even stronger than indicated by our experiments, since the *grpE280* allele encodes a GrpE mutant protein that is partially functional *in vivo* (Ang and Georgopoulos, 1989).

We then investigated whether the GroEL and GroES chaperones are also involved in luciferase reactivation. Strains carrying the *groEL100* and *groES30* missense mutations were largely unable to reactivate luciferase (Table I). This indicates that in addition to DnaK, DnaJ and GrpE, GroEL and GroES may play a role in this process.

Role of heat shock proteins during thermal inactivation of luciferase in vitro

To characterize further the role of heat shock proteins in prevention and repair of thermal denaturation of luciferase, an *in vitro* analysis with purified components was performed. We first examined the ability of heat shock proteins to protect luciferase against thermal inactivation when added prior to heat shock. In the absence of heat shock proteins, the half-time of thermal inactivation of luciferase at 42°C was 2 min, yielding a time course of inactivation similar to that found *in vivo* (Figure 3). The presence of a small molar excess (2- to 5-fold) of DnaK, DnaJ and GrpE in various combinations, or of a large molar excess (up to 100-fold) of DnaK alone, did not alter the time course of inactivation (data not shown). In addition, the presence of high concentrations of DnaK, up to a 100-fold molar excess, did not affect the kinetics of this process (Figure 3). Thus, in agreement with the *in vivo* data presented above, these heat shock proteins do not provide significant protection to luciferase from thermal inactivation *in vitro*.

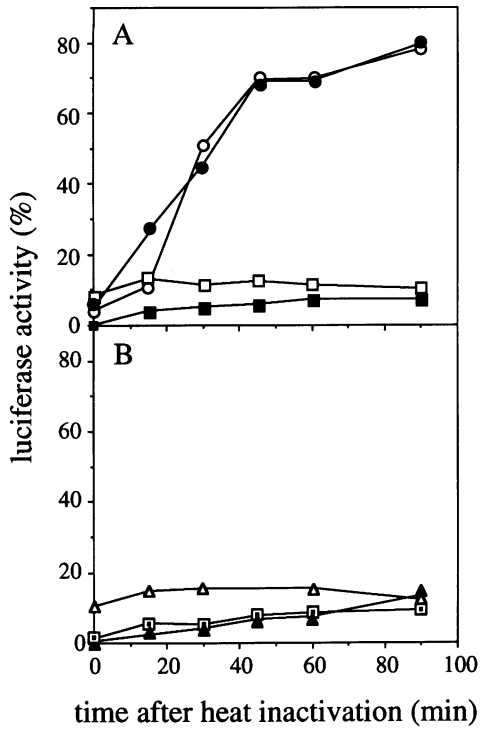

Fig. 4. The role of chaperones in the reactivation of thermally inactivated luciferase *in vitro*. (**A**) Luciferase (80 nM) was preincubated at room temperature in the presence or absence of chaperones, then incubated at 42°C for 10 min followed by incubation at room temperature. ATP was present prior to heat treatment except for reactions containing GroEL and GroES alone where ATP was added after the heat shock. Luciferase activities were determined before and at the indicated times after incubation at 42°C. Chaperones were added as follows: ○, DnaK, DnaJ and GrpE; ●, DnaK, DnaJ, GrpE, GroEL and GroES; ■, GroEL and GroES; □, no added chaperones. The relative stoichiometry of the proteins in the assay was luciferase:DnaK:DnaJ:GrpE:$(GroEL)_{14}$:$(GroES)_7$ = 1:5:2:5:1:1. (**B**) Luciferase (80 nM) in the presence of chaperones and nucleotides as indicated was subjected to heat treatment and luciferase activities were determined as described for A. △, DnaK (luciferase:DnaK = 1:100), ATP; ▲, DnaK, DnaJ, GrpE and AMP-PNP; (⊡), DnaK, DnaJ and GrpE, with no added nucleotides. Unless otherwise indicated, the relative stoichiometries of the proteins were as for A.

DnaK, DnaJ and GrpE are essential for luciferase reactivation in vitro

We then investigated the ability of heat shock proteins to reactivate thermally inactivated luciferase *in vitro*. Luciferase was first incubated at room temperature in either the presence or absence of heat shock proteins, then incubated for 10 min at 42°C (inactivation period), and subsequently incubated at room temperature (recovery period). In the absence of heat shock proteins, luciferase was completely unable to recover from thermal inactivation (Figure 4A). In contrast, when DnaK, DnaJ and GrpE in approximately stoichiometric amounts (luciferase:DnaK:DnaJ:GrpE = 1:5:2:5) and ATP were present prior to heat inactivation, up to 80% of luciferase activity could be recovered (Figure 4A). The reactivation of luciferase proceeded *in vitro* at about the same rate as *in vivo*, with a half-time of reactivation of 20–25 min. Interestingly, the presence of GroEL and GroES did not lead to reactivation of luciferase, whether or not ATP was present during the inactivation period (Figure 4A). Furthermore, the presence of GroEL and GroES did not inhibit the activity of DnaK, DnaJ and GrpE in the reactivation of luciferase. Reactivation of heat-inactivated luciferase required the presence of all three, DnaK, DnaJ and GrpE, since omission of any one of these proteins abolished the reactivation of luciferase. In the particular case of DnaK, a large molar excess, up to 100-fold, of DnaK alone over luciferase was insufficient to promote reactivation (Figure 4B). The DnaK-, DnaJ- and GrpE-dependent reactivation of luciferase was also strictly dependent on hydrolysis of ATP since no reactivation was observed when ATP was omitted or replaced with the nonhydrolyzable AMP-PNP (Figure 4B). We conclude that the combined ATP-dependent activity of DnaK, DnaJ and GrpE is essential as well as sufficient for the efficient reactivation of thermally inactivated luciferase *in vitro*. GroEL and GroES are not productive in this process.

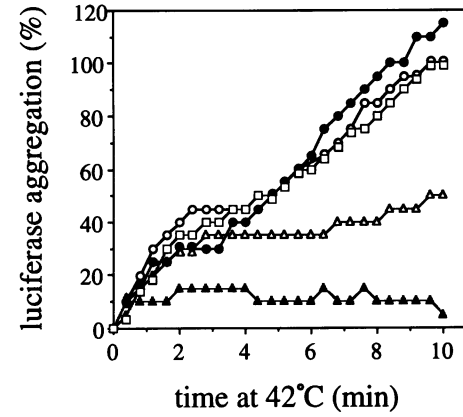

Fig. 5. DnaJ and DnaK co-operatively suppress luciferase aggregation. Luciferase (100 nM), either alone (○) or with DnaK (500 nM; ●), DnaJ (200 nM; △), DnaK and DnaJ (500 and 200 nM, respectively; ▲), or ovalbumin (500 nM; □) was equilibrated to room temperature in renaturation buffer containing 1 mM ATP, followed by incubation at 42°C in a thermostatized cuvette. Light scattering by luciferase aggregation was determined at 320 nm with a Varian DMS200 spectrometer. Aggregation of luciferase in the absence of DnaK and DnaJ after 10 min at 42°C is almost complete and is set as 100%. Aggregation is expressed as a percentage of this value. Omission of ATP in the renaturation buffer did not alter the aggregation behavior of luciferase.

DnaJ and DnaK co-operatively prevent luciferase aggregation during heat shock

Thermal inactivation of luciferase at 42°C was concomitant with its aggregation, as revealed by light scattering experiments (Figure 5). The light scattering of luciferase reached a maximal value after 10 min at 42°C. This time course of luciferase aggregation was similar to that of luciferase inactivation at that temperature. We tested whether the presence of DnaK, DnaJ and GrpE at the molar ratios that were used to obtain efficient reactivation of heat-inactivated luciferase prevents luciferase aggregation. In the presence of DnaK and DnaJ prior to inactivation at 42°C, the aggregation of luciferase was efficiently suppressed (Figure 5). GrpE, interestingly, was not required for this suppression of aggregation. In contrast, the presence of a control protein, ovalbumin, did not suppress luciferase aggregation even at high concentrations, indicating that suppression of luciferase aggregation is an activity specific for DnaK and DnaJ. We then tested whether DnaK or DnaJ alone was sufficient to suppress luciferase aggregation. Surprisingly, the presence of DnaK, even at a 10-fold molar excess, prior to heat treatment did not suppress aggregation,

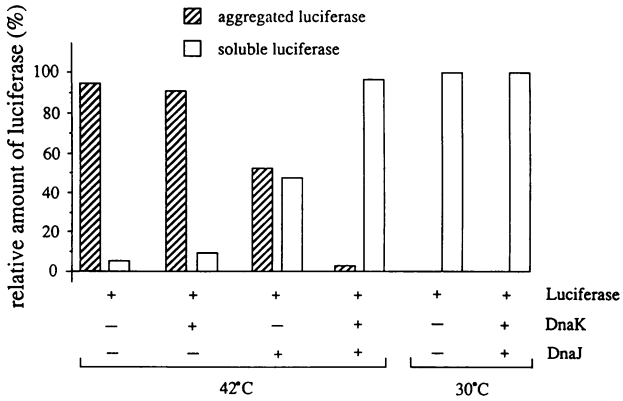

Fig. 6. Quantification of luciferase aggregation. Luciferase in the absence or presence of DnaK and DnaJ was heat-inactivated (10 min at 42°C) or kept at 30°C and analyzed with sucrose gradient centrifugation. Quantification of luciferase was done by densitometry of immunoblots of the fractions using luciferase-specific antisera. Aggregated (hatched bars) and soluble (open bars) luciferase were determined as material at the bottom of the gradient and within the gradient, respectively, and are presented as % of total.

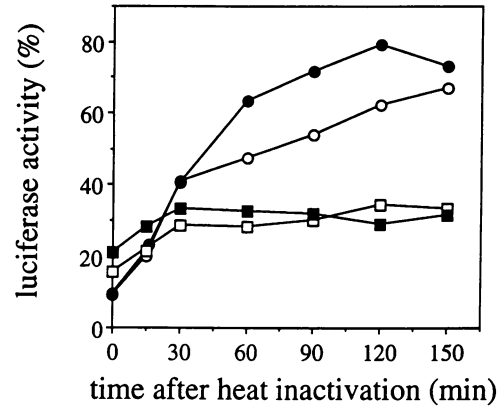

Fig. 7. Prevention of aggregation is crucial for reactivation of thermally denatured luciferase. Luciferase (80 nM, in renaturation buffer containing ATP) was preincubated at room temperature, then incubated at 42°C for 10 min (inactivation period) followed by incubation at room temperature (recovery period). DnaK, DnaJ and GrpE at the concentrations given in the legend of Figure 4A were added as follows: ●, DnaK and DnaJ before and GrpE after the inactivation period; □, DnaK, DnaJ and GrpE after the inactivation period; ■, DnaK before and DnaJ+GrpE after the inactivation period; ○, DnaJ before and DnaK+GrpE after the inactivation period. Luciferase activities were determined at the indicated times during the recovery period.

whereas the presence of DnaJ at a 2-fold molar excess had a clear aggregation-suppressing effect (Figure 5). Increasing the excess of DnaJ (5-fold molar excess over luciferase) did not, however, further improve suppression of aggregation.

To obtain quantitative information on the ability of DnaK and DnaJ to suppress luciferase aggregation, we analyzed the extent of luciferase aggregation in the presence or absence of DnaK and DnaJ by sucrose gradient centrifugation (Figure 6). In the absence of DnaK and DnaJ, >95% of the heat-inactivated luciferase aggregated and was recovered from the bottom of the gradient, compared with <5% of non-heat-treated luciferase. In the presence of DnaK, the relative amount of aggregated luciferase did not decrease, whether or not ATP was present. In contrast, in the presence of DnaJ, the amount of aggregated luciferase was reduced to 43%, while in the presence of DnaJ and DnaK it was further reduced to 3% of the luciferase loaded on the gradient. Taken together, these data show that DnaK and DnaJ act co-operatively to prevent the aggregation of luciferase and, in particular, indicate that DnaJ itself is competent to bind to thermally denatured luciferase and to prevent, at least partially, its aggregation.

Suppression of aggregation by DnaJ and DnaK is crucial for efficient luciferase reactivation

The prevention of aggregation of thermally inactivated luciferase by DnaK and DnaJ might be a crucial prerequisite for its reactivation. To test this hypothesis, we investigated whether luciferase can be reactivated after aggregation had occurred. For this purpose, luciferase was incubated for 10 min at 42°C in the absence of chaperones, leading to its inactivation and aggregation, and then incubated at room temperature in the presence of DnaK, DnaJ and GrpE and tested for the recovery of activity. Within 150 min, luciferase could not be efficiently reactivated (Figure 7). However, addition of DnaJ to luciferase prior to heat inactivation, and addition of DnaK and GrpE after this period, was sufficient to yield a reactivation of luciferase which, although slower, was almost as efficient as when DnaK and DnaJ were added prior to the heat inactivation period (Figure 7). In contrast, addition of DnaK prior to the heat inactivation period, and addition of DnaJ and GrpE after this period, yielded only minor reactivation (Figure 7). This reactivation efficiency is the same as the efficiency obtained when all three chaperones were added after the heat treatment. These results indicate that the presence of DnaJ during the heat inactivation and prior to the aggregation of luciferase is crucial for its efficient reactivation.

Discussion

The central finding of the experiments reported here is that DnaK, DnaJ and GrpE constitute a cellular machinery for the repair of heat-induced protein damage. Their combined activity was essential for the efficient renaturation of a thermally denatured test substrate, firefly luciferase, in *E.coli* cells and was sufficient *in vitro*. Luciferase proved to be a suitable test substrate for this analysis, in particular because of the availability of a rapid and sensitive activity assay that can be performed in living cells expressing luciferase at low levels, the lack of a physiological role in *E.coli* metabolism as well as its extreme thermolability within the growth temperature range of *E.coli*.

Denaturation of luciferase by heat was not prevented by the presence of the major *E.coli* heat shock proteins DnaK, DnaJ, GrpE, GroES and GroEL. *In vivo*, the extent and the kinetics of luciferase inactivation at 42°C were similar for wild type and for mutants lacking functions of any of these heat shock proteins. Furthermore, luciferase denaturation was not prevented even by massive overproduction of DnaK and DnaJ or GrpE, indicating that the lack of protection in wild type cells is not due to limitations in the availability of these heat shock proteins. Consistently, *in vitro*, stoichiometric amounts of all five heat shock proteins, or of even a large molar excess of DnaK alone, failed to provide protection. These results suggest that major *E.coli* chaperones do not act to prevent protein damage by heat, though exceptions may exist. It has been reported that high concentrations of DnaK protect RNA polymerase from

thermal inactivation *in vitro* (Skowyra *et al.*, 1990). However, in this case, DnaK is able to associate even with native RNA polymerase (Skelly *et al.*, 1988). Association with the native protein might be a prerequisite for this special function of DnaK aimed at maximally preventing structural damage to such a key enzyme of cellular metabolism.

Renaturation of heat-denatured luciferase *in vitro* and *in vivo* did not occur spontaneously but was strictly dependent on the activity of DnaK, DnaJ and GrpE. In *E.coli*, renaturation was very efficient in wild type cells, yielding up to 60% of the initial activity, but was abolished in Δ*dnaK52* and *dnaJ259* mutants (which lack DnaK and DnaJ functions, respectively) and was reduced in *grp280* mutants (which produce partially active GrpE). *In vitro*, the renaturation of heat-denatured luciferase in the presence of DnaK, DnaJ and GrpE was even more efficient than *in vivo*, yielding up to 80% of the initial luciferase activity. It is remarkable that all three heat shock proteins were essential for reactivation and, in particular, that DnaK alone, even at a large molar excess, was unable to reactivate luciferase. This is in contrast to the ability of DnaK to reactivate thermally inactivated RNA polymerase in the absence of DnaJ and GrpE (Skowyra *et al.*, 1990). The reason for these apparent substrate-specific differences in the requirement for DnaJ and GrpE is unclear.

A major surprise was that GroEL and GroES were not required for luciferase renaturation *in vitro*, though they appear to bind to heat-denatured luciferase since their presence led to further inactivation of luciferase at 42°C. In contrast to the renaturation of luciferase, most other chaperone-mediated refolding reactions studied so far require the activity of GroEL and GroES. Our results demonstrate that the interaction with DnaJ, DnaK and GrpE alone is sufficient to promote ordered tertiary structure formation, at least in thermally denatured polypeptides. Furthermore, they indicate that DnaK, DnaJ and GrpE and the GroEL and GroES chaperones can act either separately or, as shown for rhodanese (Langer *et al.*, 1992), jointly in the *in vitro* refolding of denatured substrates. The molecular basis for these functional differences is unknown. The role of GroEL and GroES in the renaturation of heat-denatured luciferase *in vivo* is less clear, since mutations in *groEL* and *groES* decreased the yield of luciferase renaturation *in vivo*. We assume that cellular defects in these mutants exist that require the functions of DnaK, DnaJ and GrpE and thereby reduce the availability or activity of these proteins for luciferase reactivation. However, we cannot exclude the possibility that the chaperone requirements for luciferase renaturation are different *in vivo* than *in vitro*.

Several features of the DnaK-, DnaJ- and GrpE-mediated luciferase renaturation have been identified by our experiments. The most interesting of these is that DnaJ alone is able to bind denatured luciferase and is crucial in preventing luciferase aggregation. This activity is essential for luciferase renaturation since aggregated luciferase could not be reactivated efficiently. The key result is that the presence of DnaJ prior to heat denaturation of luciferase is sufficient to suppress partially the aggregation of luciferase and to allow its efficient subsequent renaturation upon addition of DnaK and GrpE. In contrast, DnaK alone does not efficiently bind to heat-denatured luciferase. The presence of DnaK prior to heat denaturation of luciferase is insufficient to prevent aggregation of luciferase and does not allow its subsequent renaturation upon addition of DnaJ and GrpE. Interestingly, though, DnaK and DnaJ when added prior to heat denaturation of luciferase acted co-operatively to prevent its aggregation, indicating that DnaJ is targeting DnaK to the substrate. Finally, we note that luciferase renaturation requires the hydrolysis of ATP as omission of ATP or replacement by nonhydrolyzable analogs of ATP prevent renaturation. An ATP requirement has also been described for other DnaK-mediated activities (Georgopoulos *et al.*, 1990; Skowyra *et al.*, 1990).

These features of the activity of DnaK, DnaJ and GrpE in luciferase refolding are consistent with the following working model. Upon denaturation of luciferase by heat, DnaJ associates with denatured luciferase and stabilizes it in a folding intermediate prior to aggregation. Substrate-bound DnaJ targets DnaK to the DnaJ–luciferase complex thereby causing formation of stable DnaK–DnaJ–luciferase complexes. These complexes are dissociated by a slow, GrpE-controlled process that requires ATP hydrolysis by DnaK. Luciferase might refold either spontaneously after dissociation from the chaperones or by a folding reaction actively promoted by DnaK, DnaJ and GrpE.

The function of DnaK, DnaJ and GrpE in repair of heat-induced protein damage is likely to play a key role in a homeostatic mechanism that controls the expression of heat shock genes in response to environmental stress. DnaK, DnaJ and GrpE are negative modulators of the heat shock regulon (Tilly *et al.*, 1983; Straus *et al.*, 1990). DnaK (Gamer *et al.*, 1992; Liberek *et al.*, 1992) and DnaJ (Gamer *et al.*, 1992) associate with the heat shock transcription factor, σ^{32}, thereby inactivating it (J.Gamer and B.Bukau, unpublished results; C.Georgopoulos, personal communication). In contrast to existing models which predict key regulatory functions for DnaK (Craig and Gross, 1991; Liberek *et al.* 1992), it has been proposed recently that, in addition to DnaK, DnaJ has key functions in regulation (Gamer *et al.*, 1992; Bukau, 1993). This proposal is based on the finding that DnaJ associates with σ^{32}. It was postulated that DnaJ, besides binding to σ^{32}, also binds to stress-damaged proteins; sequestering of DnaJ by binding to damaged proteins may induce a heat shock response. Our demonstration that DnaJ indeed binds to heat-damaged luciferase and, together with DnaK and GrpE, repairs it, strongly supports this model of regulation. However, further substrates of DnaJ will have to be analyzed in order to prove this proposed central role for DnaJ in heat shock gene regulation. It is tempting to speculate that this role as well as other cellular roles of DnaJ are conserved in evolution. Indeed, proteins homologous to DnaJ have recently been identified in several eukaryotes including *Saccharomyces cerevisiae*, *Plasmodium falciparum* and human (Bork *et al.*, 1992) and, for the *S.cerevisiae* homologs YDJ1 and Sec63, functional interactions with Hsp70 proteins have been demonstrated (Cyr *et al.*, 1992; Feldheim *et al.*, 1992).

Materials and methods

Bacterial strains and plasmids

Bacterial strains are listed in Table II. Strain BB3030 was constructed by P1*vir* transduction of a chromosomally integrated luciferase gene controlled by P_{N25}/lacO3 and a linked Kn^r marker of strain RK1 (Knaus, 1991) into the chromosome of XL-1-blue. Plasmids pDMI,1 (*lacI*^{q}, Kn^r) (Lanzer, 1991), pDS12 (Ap^r) (Stüber and Bujard, 1982) and Bluescript (*dnaK*$^+$*dnaJ*$^+$) (McCarty and Walker, 1991) have been described earlier.

Table II. Bacterial strains

Strain	Genotype	Source or reference
MC4100	*Δ(argF-lac) U169 araD139 rpsL150 relA1 deoC1 ptsF25 rpsR flbB301*	Casadaban (1976)
XL-1-blue	*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* F'*(proAB lacI^q lacZ ΔM15* Tn*10)*	Stratagene
C600	F'*supE44 thi-1 thr-1 leuB6 lacY1 tonA21 hsdR*	lab collection
B178	wild type	Tilly *et al.* (1983)
RB85	*thr leu supE thi lacY rpsL*	K.Tilly
BB1553	MC4100 *ΔdnaK52::Cm^R sidB1*	Bukau and Walker (1990)
BB1458	MC4100 *thr::*Tn*10 dnaJ259*	Sunshine *et al.* (1977); Bukau and Walker (1989b)
CG1850	C600 Tn*10 groES30*	C.Georgopoulos
CG1851	C600 Tn*10 groEL100*	C.Georgopoulos
DA16	B178 *pheA::*Tn*10 grpE280*	Saito and Uchida (1978)
RB851	RB85 *thr$^+$ dnaK7(am) tonA*	K.Tilly; Itikawa and Ryu (1979)
BB3030	XL-1-blue, *luci$^+$Kn^R*	this study

The *Photinus pyralis* luciferase gene, derived from pSV2-L-AD5' (de Wet *et al.*, 1987), was cloned into pDS12-derived pUHE plasmids such that its expression is controlled by P_{lac}/lacO3 (pUHE11-3) (Berlin, 1993). The *dnaK* and *grpE* genes were cloned into pUHE plasmids such that their expression is controlled by P_{A1}/lacO3/O4 [pUHE14-2(*dnaK$^+$*)] or P_{N25}/lacO3/O4 [pUHE14-1 (*grpE$^+$*)]. These plasmids were constructed by A.Buchberger and P.Caspers.

Microbiological and genetic techniques

P1*vir* transductions, transformations and DNA preparations were done using standard techniques (Miller, 1972; Sambrook *et al.*, 1989). Bacteria were grown aerobically at 30°C or at the indicated temperatures in Luria broth. Antibiotics were used at the following concentrations: kanamycin (Kn), 20 or 100 μg/ml; ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 25 μg/ml; chloramphenicol (Cm), 100 μg/ml.

Luciferase activity determinations

For the standard luciferase activity assay performed *in vivo*, cultures of *E.coli* cells producing luciferase were grown at 30°C to logarithmic phase (OD_{600} = 0.5). Then, kanamycin (100 μg/ml, final concentration) was added and samples of 1 ml were transferred to prewarmed Eppendorf tubes and incubated at 42°C (inactivation period), followed by incubation at 30°C (recovery period). Aliquots of 100 μl were withdrawn at the indicated times and assayed for luciferase activity in a Berthold Biolumat 8501. The reaction was started by addition of 100 μl of D-luciferin (Sigma) (0.25 mM, final concentration) to the cells. Luciferin can enter unpermeabilized, living *E.coli* cells (Knaus, 1991). Light emission at 560 nm was measured over a period of 10 s. Luciferase activities determined *in vivo* correlate qualitatively with those determined in cell extracts (Knaus, 1991). Specific luciferase activities (light units/min/0.5 OD_{600} units of cells) are presented as percent of luciferase activity present in the cells before the inactivation period. Standard luciferase activity assays *in vitro* were done in 250 μl of renaturation buffer (25 mM HEPES pH 7.6, 5 mM Mg^{2+}-acetate, 50 mM KCl, 5 mM β-mercaptoethanol) containing purified luciferase (Sigma), heat shock proteins and ATP (1 mM, final concentration) as indicated. Heat shock proteins were purified according to standard procedures (Viitanen *et al.*, 1990; Liberek *et al.*, 1991; McCarty and Walker, 1991). The reaction mixture was equilibrated to room temperature, then incubated at 42°C (inactivation period) and subsequently incubated at room temperature (recovery period). Aliquots of 10 μl were withdrawn before and at various times after the temperature shifts, diluted 1:100 in renaturation buffer and analyzed for luciferase activity according to de Wet *et al.* (1987). In general, the mean values of luciferase activities (light units/min/μg luciferase) of three independent experiments were presented as percent of luciferase activity present before the heat shock treatment.

Density gradient centrifugation

200 μl of protein samples containing luciferase (250 nM, in renaturation buffer), DnaK (1.25 μM) and DnaJ (500 nM) as indicated were either subjected to a 10 min heat treatment at 42°C or kept at 30°C and then layered on top of a 4 ml 10–30% sucrose gradient prepared in renaturation buffer. Centrifugation of the gradients was performed in an SW60ti rotor for 12 h at 53 000 r.p.m. and 10°C. ATP was present except in experiments where luciferase was incubated with DnaK alone. Then, fractions of 350 μl were withdrawn from the top of the gradient and were further prepared for electrophoresis.

SDS–PAGE and immunological techniques

Protein samples were precipitated with cold TCA (10%, final concentration), washed with acetone, resuspended in cracking buffer (10 mM EDTA, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 50 mM Tris–HCl pH 6.8), boiled for 5 min and subjected to electrophoresis in 10% polyacrylamide–SDS gels (Laemmli, 1970). Immunoblots on polyvinylidine difluoride membranes (Millipore Inc.) were developed by the Western-Light system (Tropix) according to the manufacturer's instructions, using alkaline phosphatase-conjugated anti-rabbit IgG as secondary antibodies and AMPPD as the chemiluminescent substrate. The membranes were exposed on X-OMAT AR films (Kodak). Luciferase-specific antiserum was diluted 1:7500 for use in immunoblot experiments.

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