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Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB

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We systematically analyzed the capability of the major cytosolic chaperones of *Escherichia coli* to cope with protein misfolding and aggregation during heat stress *in vivo* and in cell extracts. Under physiological heat stress conditions, only the DnaK system efficiently prevented the aggregation of thermolabile proteins, a surprisingly high number of 150–200 species, corresponding to 15–25% of detected proteins. Identification of thermolabile DnaK substrates by mass spectrometry revealed that they comprise 80% of the large (≥90 kDa) but only 18% of the small (≤30 kDa) cytosolic proteins and include essential proteins. The DnaK system in addition acts with ClpB to form a bi-chaperone system that quantitatively solubilizes aggregates of most of these proteins. Efficient solubilization also occurred in an *in vivo* order-of-addition experiment in which aggregates were formed prior to induction of synthesis of the bi-chaperone system. Our data indicate that large-sized proteins are most vulnerable to thermal unfolding and aggregation, and that the DnaK system has central, dual protective roles for these proteins by preventing their aggregation and, cooperatively with ClpB, mediating their disaggregation.

Keywords: chaperones/heat-shock response/Hsp70/protein denaturation/thermotolerance

Introduction

Misfolding and aggregation of proteins are major damaging consequences of stress situations such as heat shock and pathophysiological states (Morimoto *et al.*, 1994; Horwich and Weissman, 1997; Lindquist and Schirmer, 1999). The central cellular defense against such damage is molecular chaperones, which prevent aggregation, assist refolding and mediate degradation of misfolded proteins (Morimoto *et al.*, 1994; Hartl, 1996; Bukau, 1999). Chaperones can cooperate *in vitro* as part of a functional network in which 'holder' chaperones prevent aggregation of misfolded proteins, whereas 'folder' chaperones actively assist refolding (Langer *et al.*, 1992; Buchberger *et al.*, 1996; Freeman and Morimoto, 1996; Ehrnsperger *et al.*, 1997; Johnson and Craig, 1997; Veinger *et al.*, 1998). The contributions of individual chaperones to this folding network *in vivo* and the identity of the stress-sensitive cellular proteins remain unknown. Moreover, since cells have only a limited chaperone capacity to prevent protein aggregation under stress conditions (Craig and Gross, 1991; Tatsuta *et al.*, 1998; Tomoyasu *et al.*, 1998), it becomes important to determine the extent to which chaperones can resolubilize aggregates of proteins that escaped the protective function of holder chaperones.

For *Escherichia coli* it has been shown that the GroEL chaperone with its GroES co-chaperone assists folding of a subset of newly synthesized proteins and refolding of misfolded proteins (Hartl, 1996; Bukau and Horwich, 1998). Furthermore, overproduction of this chaperone system suppresses heat-induced aggregation of proteins in regulatory *rpoH* mutant cells that are defective in the synthesis of major chaperones (Gragerov *et al.*, 1992). The DnaK (Hsp70) chaperone and its DnaJ and GrpE co-chaperones participate in the folding of a subset of newly synthesized proteins (Deuerling *et al.*, 1999; Teter *et al.*, 1999), prevent protein aggregation at heat-shock temperatures (Gragerov *et al.*, 1992; Hesterkamp and Bukau, 1998) and refold misfolded proteins (Schröder *et al.*, 1993). The DnaK system has also been reported to dissolve aggregates of RNA polymerase and DnaA (Hwang *et al.*, 1990; Skowyra *et al.*, 1990; Ziemienowicz *et al.*, 1993), but it is inefficient in disaggregating other substrates including heat-aggregated firefly luciferase and malate dehydrogenase (Schröder *et al.*, 1993; Veinger *et al.*, 1998). Null mutations in the *clpB* gene encoding the *E.coli* Hsp104 homolog, ClpB, are retarded in the removal of a cell fraction assumed to be enriched in protein aggregates (Laskowska *et al.*, 1996a), but another study reported that Δ*clpB* mutants show no increase in protein aggregation at 46°C (Thomas and Baneyx, 1998).

The present work provides a systematic analysis of the roles of major chaperones of the *E.coli* cytosol in preventing and reverting heat-induced aggregation of proteins. This analysis was performed both *in vivo* and in cell extracts to allow independent validation of the *in vivo* results and to evaluate the usefulness of an *in vitro* approach. We identified for the first time a large set of thermolabile *E.coli* proteins, found to be enriched in large-sized proteins, and showed that the DnaK system is the single most effective chaperone in preventing their aggregation. Our findings thus provide the first identification of thermolabile *in vivo* substrates for an Hsp70 chaperone. Furthermore, we describe a powerful bi-chaperone system, consisting of ClpB and the DnaK system, which resolubilizes a wide variety of protein aggregates.

Results

The DnaK system is the most effective chaperone in preventing protein aggregation in extracts

The capacity of cytosolic chaperones to prevent heat-induced aggregation of *E.coli* proteins was determined in total soluble extracts of [^{35}S]methionine-labeled wild-type cells (MC4100). Heat treatment of extracts for 15 min at 45°C, a temperature within the growth temperature range of *E.coli*, caused denaturation and aggregation of 10–15% of the proteins despite the presence of endogenous chaperones (data not shown). We considered the possibility that many of the aggregating proteins are not thermolabile *per se*, but were co-precipitated through unspecific association with aggregating thermolabile proteins. We performed a seeding experiment in which [^{35}S]methionine-labeled cell extracts kept at 30°C were mixed with equimolar amounts of aggregating proteins of non-labeled cell extracts isolated at various time points after heat shock to 45°C. However, the mixing with aggregating proteins did not cause significant aggregation of the native, labeled proteins (Figure 1B), indicating that for most proteins, aggregation at 45°C is caused by thermal unfolding.

The aggregation of thermolabile proteins in cell extracts allowed us to test the potential of exogenously added chaperones in the presence of Mg^{2+}/ATP to prevent protein aggregation upon a subsequent heat shock. Chaperones were added to concentrations between 2.5 and 10 μM of monomers, a range corresponding to the concentration of aggregating proteins (~5–10 μM). Of the five chaperone systems tested (DnaK/DnaJ/GrpE, GroEL/GroES, ClpB, HtpG, IbpB), only the DnaK system suppressed protein aggregation in a concentration-dependent manner, with 90% efficiency reached at 7.5 μM DnaK (Figure 1A). For reasons explained below we tested a combination of the DnaK system and ClpB and found that it does not further increase the potential of the DnaK system to prevent protein aggregation (data not shown). We also investigated whether a crowded environment, as it exists in the *E.coli* cytosol (~170 mg proteins/ml *in vivo* versus 4 mg/ml in the extract), is sufficient to protect proteins from heat-induced aggregation. The presence of up to 20% (w/v) Ficoll 70 in cell extracts, which mimics the molecular crowding of the cellular environment (Martin and Hartl, 1997), caused no significant differences in protein aggregation (Figure 1A) and, in addition, did not alter the protection capacity of the DnaK or GroEL system (data not shown).

To judge whether the chosen concentration range of chaperones is physiological, we determined the chaperone levels in wild-type cells grown at 30°C or subjected to heat shock. The concentrations of all chaperones were in the low micromolar range and thus within the range used in the *in vitro* experiments described above. With respect to molar concentrations and the number of monomers, DnaK and GroEL were the most abundant chaperones at both temperatures (Table I), consistent with earlier determinations (Neidhardt and VanBogelen, 1987). However, since most chaperones are active as oligomers (hexamer for ClpB; dimer for HtpG; monomer for DnaK; tetradecamer for GroEL; unclear for IbpA/B), DnaK is at least 8-fold more abundant as active species compared

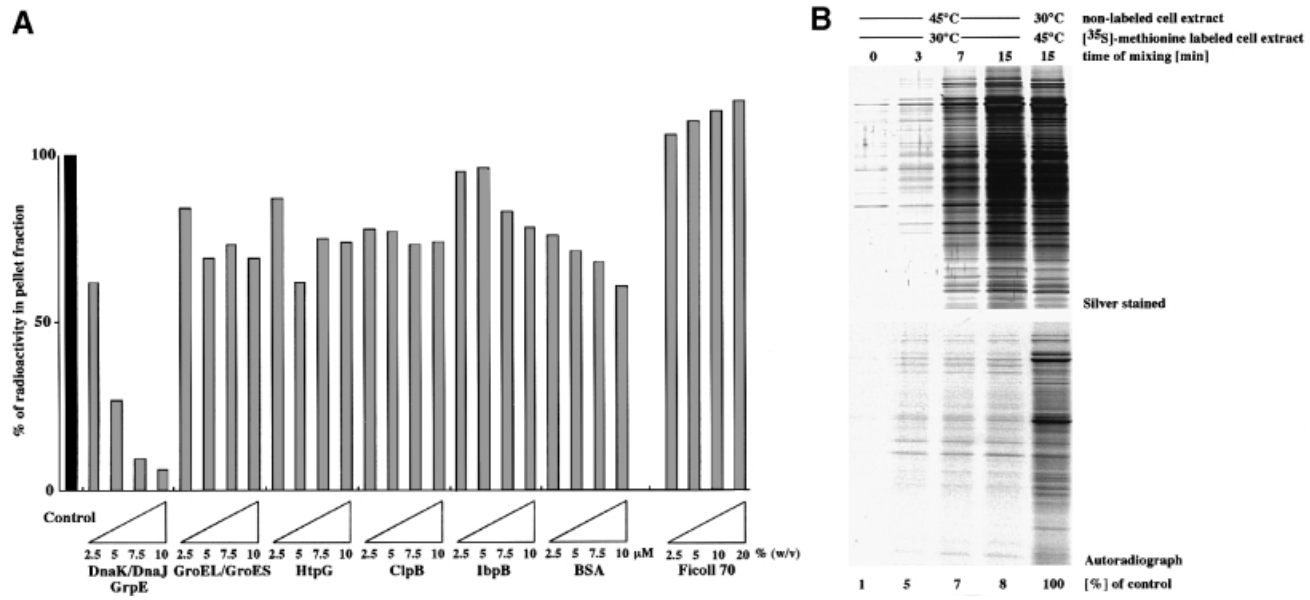

Fig. 1. (**A**) Prevention of heat-induced protein aggregation in *E.coli* cell extracts by chaperones. [^{35}S]methionine-labeled cell extracts were incubated for 5 min at 30°C and then 15 min at 45°C in the absence or presence of added chaperones, bovine serum albumin (BSA) or Ficoll 70 at the indicated concentrations [2.5–10 μM monomers or 2.5–20% (w/v)]. Aggregated proteins were isolated by centrifugation, boiled in 100 μl of SDS sample buffer and finally quantified by scintillation counting. Aggregation of heat-labile proteins in the absence of added chaperones was set at 100%. Protein ratios in the DnaK chaperone system were 1:0.2:0.1 (DnaK:DnaJ:GrpE) and 1:1 (GroEL:GroES) in the GroE chaperonin system. All experiments were carried out three times with <10% deviations. (**B**) Seeding of cell extracts kept at 30°C with aggregating proteins does not induce further protein aggregation. [^{35}S]methionine-labeled or non-labeled cell extracts were incubated at 30 or 45°C. At the time points indicated, extracts were mixed and incubated for a further 10 min at 30°C. Cell extracts kept at 45°C were cooled for 15 s on ice before addition. Insoluble cell fractions were isolated by centrifugation and analyzed by SDS–PAGE followed by silver staining. Dried gels were scanned using a phosphoimager (FLA-2000) and quantified by MacBAS software (Fuji film). The amount of labeled proteins in the pellet fraction of cell extracts kept at 45°C for 15 min was set at 100%.

with all other chaperones tested (Table I). Its high cellular concentration and high capacity to prevent protein aggregation in cell extracts qualifies DnaK as the central 'holder' chaperone of the *E.coli* cytosol.

The thermolabile proteins that showed increased aggregation in heat-treated cell extracts and were prevented from aggregation by the DnaK system were identified by two-dimensional (2D) gel electrophoresis of the aggregated protein fraction. This fraction consisted of ~250 protein species (Figure 2) corresponding to ~30% of all detected protein species. Addition of 5 μM DnaK and its co-chaperones (DnaJ, 1 μM; GrpE, 0.5 μM) to the extract before heat treatment prevented, at least partially, the aggregation of >90% of these protein species.

The DnaK system is the most effective chaperone system in preventing protein aggregation in vivo

The roles of chaperones in preventing thermal aggregation of proteins were determined *in vivo*. This analysis extends former studies that allowed only limited conclusions due to differences in the experimental and genetic conditions and partially controversial results (Gragerov *et al.*, 1992; Hesterkamp and Bukau, 1998; Thomas and Baneyx, 1998; Kedzierska *et al.*, 1999). We investigated the extent of heat-induced protein aggregation in wild-type and isogenic mutant cells carrying knockout mutations (Δ*clpB::kan*, Δ*htpG::lacZ*, Δ*ibpAB::kan*, Δ*dnaK52::cat*) or conditional mutations conferring temperature-sensitive growth at 42–43°C (*groEL44*, *groEL140*). The cells were grown at 30°C and then subjected to mild (60 min at 42°C) or severe (60 min at 45°C) heat-shock treatment. Within the time course of the experiment, none of the mutant strains showed growth defects as judged by increases in optical density of the cultures. Δ*dnaK52::cat* and *groEL* mutant cells stopped growing only 2–3 and 1–2 h after temperature upshift to 42 and 45°C, respectively. Δ*htpG::lacZ* mutants had a reduced growth rate after heat shock to 45°C, as reported previously (Bardwell and Craig, 1988).

Heat-shock treatment at 42°C caused strong protein aggregation in Δ*dnaK52::cat* mutants (Figure 3), but no detectable aggregation in the wild-type and other mutant cells tested (data not shown). Treatment at 45°C caused even stronger protein aggregation in Δ*dnaK52::cat* mutants, minor accumulation of aggregated proteins in *groEL44*, *groEL140* and Δ*clpB::kan* mutant cells, and no detectable protein aggregation in Δ*htpG::lacZ* and Δ*ibpAB::kan* cells (see Figure 8; data not shown). In the case of the conditional *groEL* mutants we can not exclude the possibility that some remaining activity of GroEL makes a potential contribution of GroEL to aggregation prevention. We consider this contribution as minor, given that the onset of growth arrest of these mutant cells at 42°C was earlier than that of Δ*dnaK52::cat* mutant cells and the GroEL system was inefficient in aggregation prevention in cell extracts.

Our *in vivo* data are consistent with and confirm the results of the *in vitro* experiments described above. DnaK is thus the most effective chaperone to prevent aggregation

Table I. Cellular levels of cytosolic chaperones of *E.coli*

Protein	Concentration [μM] (monomers)		Number per cell (monomers/active species)	
	30°C	42°C	30°C	42°C
ClpB	8.7	19.4	3000/500	6700/1115
HtpG	5.8	21.2	2100/1050	7700/3850
DnaK	27	54	9900/9900	20 000/20 000
GroEL	47	99	17 200/1230	36 200/2590
IbpA/B	1.5	11.2	600/<600	5200/<5200

Wild-type cells (MC4100) were grown logarithmically in LB medium at 30°C and aliquots were further incubated at 42°C for 30 min. Aliquots of total cell extracts were subjected to SDS–PAGE followed by immunoblot analysis using chaperone-specific antisera. Serial dilutions of purified proteins served as a standard and allowed quantification of the signals in the linear range. The number of active species per cell was calculated on the basis of the known oligomeric state of the chaperones. IbpB purifies as a heterogeneous mixture of monomers and oligomers.

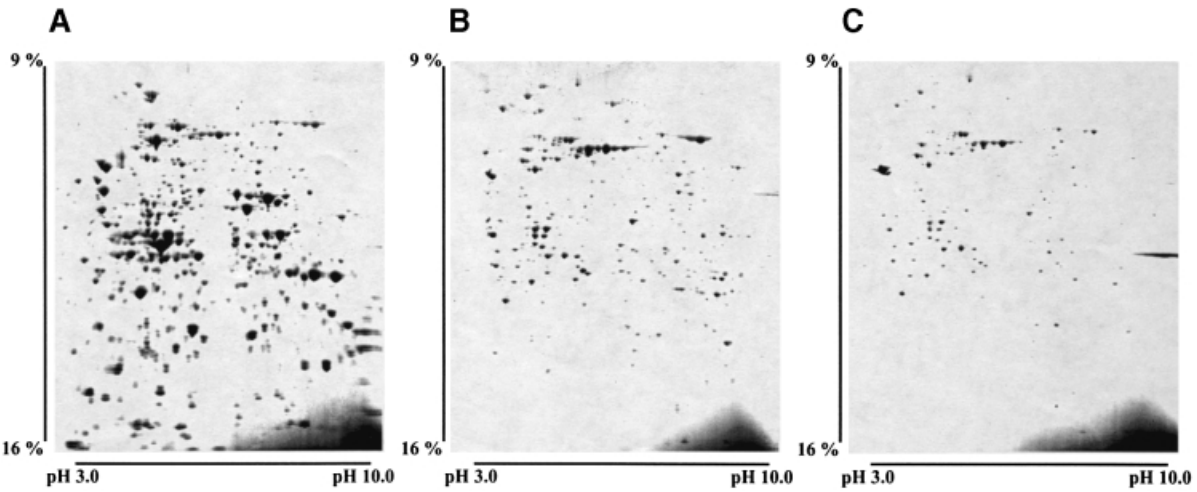

Fig. 2. Heat-induced protein aggregation in cell extracts: dependence on protein size and general protection by DnaK. Cell extracts were heated to 45°C for 15 min in the absence or presence of exogenously added DnaK/DnaJ/GrpE. Soluble and insoluble fractions were separated by centrifugation and analyzed by 2D gel electrophoresis. (**A**) Total soluble proteins of wild type (MC4100). (**B**) Aggregated proteins after heat treatment for 15 min at 45°C. (**C**) Protection of heat-labile *E.coli* proteins from aggregation by the DnaK chaperone system *in vitro*. DnaK (5 μM), DnaJ (1 μM), GrpE (0.5 μM) and ATP (10 mM) were added prior to heat shock. Aggregated heat-labile proteins were isolated and analyzed by 2D gel electrophoresis.

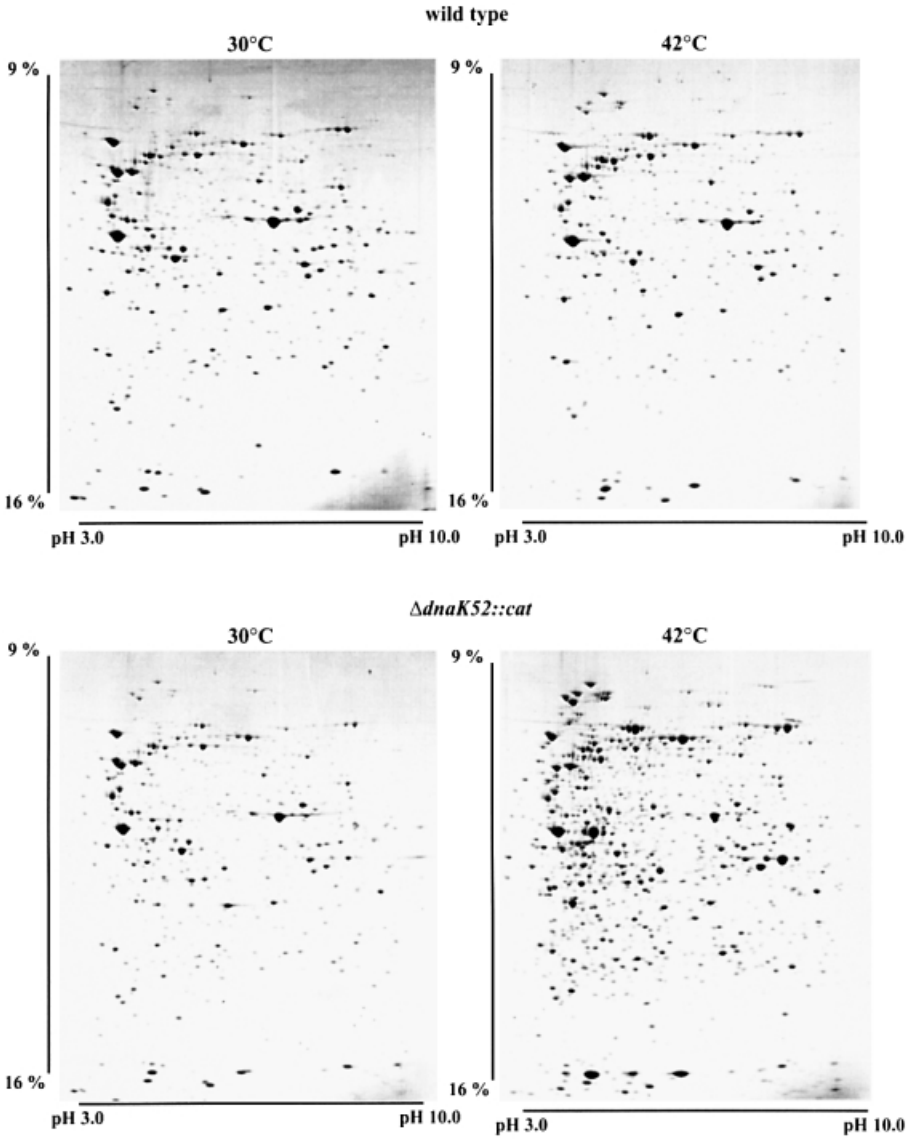

Fig. 3. Two-dimensional gel electrophoresis of insoluble cell fractions of *E.coli* MC4100 wild-type and Δ*dnaK52::cat* mutant cells, isolated at 30°C or 60 min after heat shock to 42°C. Cultures of wild-type and mutant strains were grown in M9 minimal medium supplemented with 19 L-amino acids except L-methionine at 30°C to logarithmic phase and shifted to 42°C for 60 min. Insoluble proteins were analyzed by 2D gel electrophoresis. Pellet fractions contain membrane proteins and aggregated protein material.

of thermolabile proteins in the cell, and this activity explains the temperature-sensitive growth phenotype of Δ*dnaK52::cat* mutant cells. The inability of the other chaperones to prevent protein aggregation efficiently *in vitro*, and the lack of strong protein aggregation phenotypes of the mutants lacking functions of these chaperones, indicate that they play only minor roles in aggregation prevention. This finding was further supported by tests for complementation of the temperature-sensitive growth phenotype of Δ*dnaK52::cat* mutant cells by overproduction of other chaperones. Isopropyl-β-D-thiogalactopyranoside (IPTG)-controlled overproduction of GroEL/GroES, ClpB, HtpG and IbpA/B did not restore the ability of Δ*dnaK52::cat* mutant cells to grow at 42°C (Table II). Partial complementation was observed at 40°C by overproduction of GroEL/GroES. This finding was consistent with the observation that a massive overproduction of GroEL/GroES (~30% of total protein) partially suppressed protein aggregation at 42°C in Δ*dnaK52::cat* cells, while massive overproduction of all other chaperones tested had only minor (IbpA/B) or no (HtpG, ClpB) effects in preventing heat-induced protein aggregation (data not shown). It is important to emphasize that these effects were only observed upon overproduction of the chaperones to very high levels and are therefore considered unphysiological.

Identification of natural DnaK substrates

We first analyzed the proteins that aggregated in Δ*dnaK52::cat* mutant cells after shift to 42°C by 2D gel electrophoresis of the insoluble fractions (Figure 3). These fractions contained membrane proteins and protein aggregates if present. The membrane proteins served as an internal loading control since their amount stayed constant for all *E.coli* strains and temperatures tested. Removal of most membrane proteins from the insoluble

Table II. Complementation of the temperature-sensitive growth phenotype of Δ*dnaK52::cat* mutants by plasmid-encoded chaperone alleles

Allele	Colony formation											
	30°C		40°C					42°C				
	0	500	0	50	100	250	500	0	50	100	250	500
dnaK, *dnaJ*	+	+	−	(+)	+	+	+	−	(+)	+	+	+
groEL, *groES*	+	+	−	−	(+)	(+)	−	−	−	−	−	−
clpB	+	+	−	−	−	−	−	−	−	−	−	−
htpG	+	+	−	−	−	−	−	−	−	−	−	−
ibpA, *ibpB*	+	+	−	−	−	−	−	−	−	−	−	−

Δ*dnaK52::cat* mutants that carry pDMI,1 (*lacI*^{q}) as well as IPTG-inducible expression vectors (pUHE derivatives) (Lutz and Bujard, 1997) carrying the given chaperone alleles were tested for growth at 30, 40 and 42°C at IPTG concentrations as indicated in the top row (μM). Growth was assayed by determining the ability of the cells to form colonies on LB/Ap/Km agar plates. +, normal number and size of colonies; (+) reduced number and size of colonies; –, no colonies.

fractions in the presence of 2% (v/v) NP-40 during the extraction did not change the amount or pattern of the aggregated proteins (data not shown).

For wild-type cells kept at 30°C or incubated for 60 min at 42°C, the insoluble fractions showed no differences in the overall pattern of the proteins, of which the most prominent are membrane proteins (Figure 3). Only a slight increase in the amount of insoluble proteins was detected in Δ*dnaK52::cat* mutant cells kept at 30°C, consistent with earlier findings that DnaK is dispensable for folding of the majority of newly synthesized proteins at 30°C (Hesterkamp and Bukau, 1998). Increases in spot intensities compared with wild type were reproducibly detected for a few cytosolic proteins, including AceE (E1 component of pyruvate dehydrogenase), β and β′ subunits of RNA polymerase, PurL (formylglycinamidine synthase) and CarB (carbamoylphosphate synthetase). In heat-treated Δ*dnaK52::cat* mutants, ~10% of the amount of pre-existing cytosolic proteins of Δ*dnaK52::cat* mutant cells aggregated within 1 h of incubation of the cells at 42°C. Of the ~800 proteins resolved in total soluble cell lysate, the aggregating proteins comprised 150–200 species, defined as spots whose intensity is at least 4-fold increased compared with the 30°C Δ*dnaK52::cat* and wild-type controls. Thus, ~15–25% of the detected soluble protein species showed increased aggregation in Δ*dnaK52::cat* mutant cells at 42°C. The degree to which individual proteins aggregated varied between 30 and 70% of the particular spot.

The aggregated proteins were identified by subjecting 2D gel spots to Lys-C digestion followed by mass spectrometry of the peptides. In total lysates of Δ*dnaK52::cat* mutant and wild-type cells grown at 30°C, we identified 268 proteins of the soluble fraction and 14 proteins of the insoluble fraction that were membrane proteins. Fifty-seven of the identified soluble proteins specifically aggregated in Δ*dnaK52::cat* cells upon incubation at 42°C (Table III). These proteins were exclusively cytosolic and did not include any of the 28 periplasmic proteins or 14 membrane proteins identified.

We then determined the relationship between the aggregated proteins of heat-treated Δ*dnaK52::cat* mutant cells (Figure 3) and those of heat-treated cell extracts (Figure 2B) using comparative spot matching analysis and mass spectrometry. There was a good overall match between the two protein populations. More than 80% of the aggregating proteins identified *in vivo* and in cell extracts were identical (see Table III for summary); moreover, many of these proteins were identical to those co-immunoprecipitated with DnaK at 45°C (see below; Figure 4; Table III). These findings indicate that most of the proteins that aggregate in Δ*dnaK52::cat* mutant cells at 42°C are the major thermolabile proteins of *E.coli* as identified in cell extracts and are natural substrates for the DnaK chaperone system.

We defined the features of the aggregation-prone, thermolabile proteins that are substrates for DnaK. We compared the proteins that aggregated in Δ*dnaK52::cat* mutant cells at 42°C with total soluble proteins of cells kept at 30°C, and included the 2D gel spots identified by mass spectrometry and additional detectable spots evaluated by ImageMaster software (Pharmacia). No correlation was observed with respect to cellular function, pI value or oligomeric state. In contrast, a strong correlation existed with respect to molecular weight. The total soluble proteins detected in the 2D gels showed a broad size distribution with an average molecular weight of 42 kDa (Figure 5A). These characteristics were similar to those calculated on the basis of the entire *E.coli* genome (Netzer and Hartl, 1998), taking into account that very small proteins <10 kDa can not be resolved in the 2D gel system used. The aggregated proteins isolated from heat-treated Δ*dnaK52::cat* mutant cells, or from heat-treated cell extracts, showed a 2- to 4-fold increase in the relative number of large proteins (>70 kDa) and a 2- to 3-fold decrease in the relative number of small proteins (<30 kDa). Similar results were obtained when the intensities of spots were taken as a basis for this evaluation (data not shown). These characteristics have dramatic consequences with respect to the aggregation behaviour of large- and small-sized proteins. Approximately 80% of the soluble proteins >90 kDa that were detected in the soluble fraction of wild-type cells showed increased aggregation in heat-treated Δ*dnaK52::cat* mutants and extracts of wild-type cells (Figure 5A). In contrast, only 9 and 18% of the identified soluble proteins <20 and <30 kDa, respectively, showed a tendency to aggregate. Thus, the most striking finding of this analysis is that most large-sized cytosolic proteins are thermolabile even under mild heat-shock conditions, but are prevented from aggregating by the DnaK system.

Table III. Aggregation-prone *E.coli* proteins and their interaction with the DnaK system and ClpB

	Name	Size (kDa)	Function	Aggregation		CoIP		Disaggregation	
				in vivo	extract	*in vivo*	extract	*in vivo*	extract
1	RpoB	151	RNA polymerase	+	+	(+)	+	+	+
2	PutA	145	proline dehydrogenase	+	+			+	+
3	PurL	142	formylglycinamidine synthase	+	+		(+)	+	+
4	NarG	141	nitrate reductase (respiratory chain)	+	+			+	+
5	MetH	137	methionine biosynthesis	+	+	+	+	+	+
6	NifJ	130	oxidoreductase (respiratory chain)	+	+			+	+
7	TrcF	130	transcription repair coupling factor	+	+			+	+
8	CarB	118	carbamoylphosphate synthetase	+	+		(+)	+	+
9	SucA	106	E1 component of oxoglutarate dehydrogenase	+	+	+	+	+	+
10	AceE	100	E1 component of pyruvate dehydrogenase	+	+	+*	+*	+	+
11	AdhE	96	alcohol dehydrogenase	+	+	+	+	+	+
12	AcnB	94	aconitase	+	+	+	+	+	+
13	Lon	88	ATP-dependent protease	+	+	+		+	+
14	MetE	85	methionine biosynthesis	+	+	+	(+)	+	+
15	YghF	82	unknown	+	+	+		+	+
16	EF-G	78	elongation factor of translation	+	+	+	(+)	+	+
17	AckA	77	acetyltransferase	+	+	+*	+	+	+
18	ThrS	75	threonine tRNA synthetase	+				+	
19	Dxs	68	transketolase	+		+		+	
20	SfcA	66	probable malate oxidoreductase	+	+	+	+	+	+
21	TypA	66	unknown, EF-G homolog	+	+	+	+	+	+
22	AceF	66	E2 component of pyruvate dehydrogenase	+				+	
23	SdhA	65	succinate dehydrogenase	+	(+)			+	
24	FumA	61	fumarase	+	+	+		+	+
25	GuaA	59	glutamine amidotransferase	+	(+)			+	+
26	LysS	57	Lys-tRNA synthetase	+	+			+	+
27	AsnS	53	asparagine-tRNA synthetase	+	(+)			+	
28	GlnA	52	glutamine synthetase	+				+	
29	ImdH	52	IMP dehydrogenase	+	(+)			+	
30	HslU	50	subunit of ATP-dependent protease	+	+	+*	+	+	+
31	ClpX	47	subunit of ATP-dependent protease	+	+		+	+	+
32	ThrC	47	threonine biosynthesis	+	+	+*	+	+	+
33	DeoA	47	thymidine phosphorylase	+	+		+	+	+
34	Rho	47	termination of transcription	+	+			+	+
35	MurA	45	cell wall synthesis	+			(+)	+	
36	SerA	44	3-phosphoglycerate dehydrogenase	+	+	(+)	(+)	+	+
37	Tgt	43	quenine-tRNA ribosyl transferase	+	(+)	(+)	(+)	+	
38	Glf	43	LPS synthesis	+	+	+	+	+	
39	CarA	42	carbamoylphosphate synthetase	+	(+)			+	
40	RfbB	41	DTDP-glucose-4,6-dehydratase	+	+		(+)	+	+
41	Pgk	41	phosphoglycerate kinase	+				+	
42	FtsZ	40	cell division	+	+			+	+
43	YchF	40	probable GTP-binding protein	+	+		+	+	+
44	RpoA	37	RNA polymerase	+	+		(+)	+	+
45	AsnA	37	asparagine synthetase	+	+	(+)	(+)	+	+
46	GAP-DH	36	3-glyceraldehyde dehydrogenase	+	+		+	+	
47	MhpE	34	3-hydroxyphenylpropionate degradation	+	(+)	+	+	+	+
48	EntB	33	isochorismatase	+	+	+	+	+	+
49	MetF	33	methionine biosynthesis	+	(+)		(+)	+	
50	GatY	31	fructose bisphospate aldolase	+	+		+	+	
51	MinD	30	cell division	+	+			+	+
52	GpmA	29	phosphoglycerate mutase	+	+	(+)		+	+
53	ArcA	27	response regulator (oxygen control)	+	+	+		+	+
54	UbiB	26	flavoprotein oxidoreductase	+	+			+	
55	PyrH	26	UMP kinase	+	+	+		+	
56	NusG	21	antiterminator of transcription	+	+			+	
57	IbpB	16	small HSP (chaperone)	+				+	

Fifty-seven proteins were identified by mass spectrometry based on their specific aggregation in Δ*dnaK52::cat* mutant cells (BB1553, grown at 30°C to log phase) that were incubated at 42°C for 60 min (Aggregation, *in vivo*). This list comprises proteins identified in cells grown in LB or M9 minimal media, on the basis of at least four independent experiments each. Protein names, sizes and functions are indicated. These proteins were compared with the aggregated protein fraction of extracts of wild-type cells (MC4100) subjected to a 30–45°C heat shock for 15 min (Figure 2), and proteins co-immunoprecipitated with DnaK-specific antiserum from wild-type spheroplasts (MC4100) (CoIP, *in vivo*) and cell extracts (CoIP, extract) subjected to heat treatment (Figure 4). In addition, the disaggregation of heat-aggregated DnaK substrates by ClpB and the DnaK chaperone system in cell extracts (Disaggregation, extract) (Figure 7) and in Δ*dnaK52::cat* mutant cells (Disaggregation, *in vivo*) are indicated (Figure 9). (+), only minor amounts were detected. +*, proteins could also be co-immunoprecipitated at 30°C.

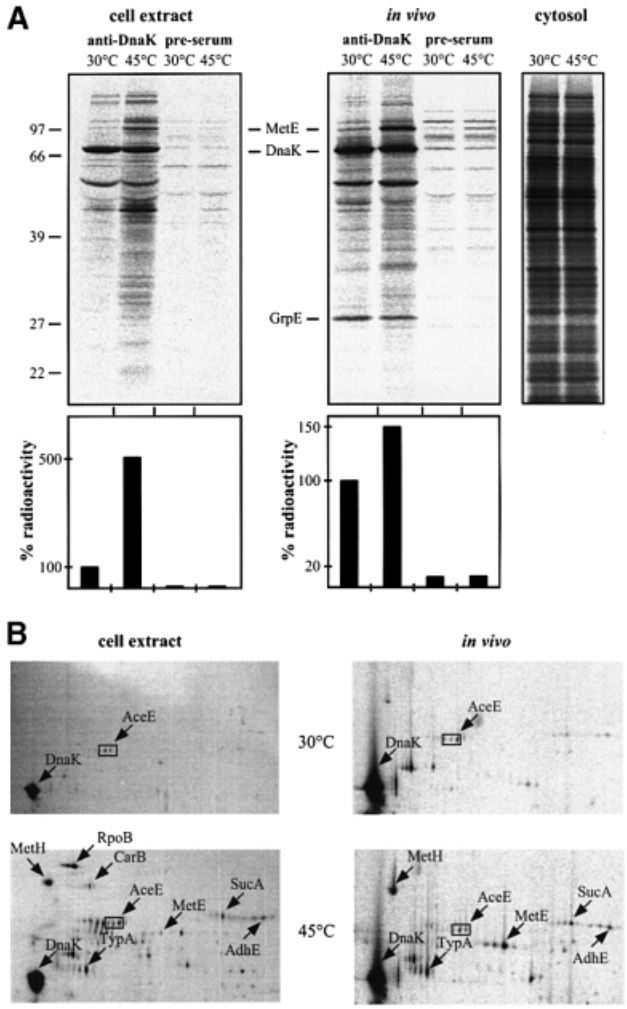

Fig. 4. Temperature-dependent association of protein substrates with DnaK. [^{35}S]methionine-labeled cell extracts (4 mg/ml, 10 mM ATP) were incubated at 30°C or shifted to 45°C for 15 min in the presence of exogenously added 7.5 μM DnaK, 1.5 μM DnaJ and 0.75 μM GrpE. [^{35}S]methionine-labeled spheroplasts (*in vivo*) were incubated at 30°C or shifted to 45°C for 5 min and lysed by addition of EDTA (100 mM) and sonification. All reactions were subjected to co-immunoprecipitation using polyclonal antibodies raised against the ATPase domain of DnaK or pre-serum as a control. Immunoprecipitated material was analyzed by one- and two-dimensional gel electrophoresis. (**A**) Autoradiograph of immunoprecipitated material, isolated by incubation with DnaK-specific antiserum or pre-serum. The lower panel shows quantification of the immunoprecipitated material by scintillation counting and MacBAS software. The amount of radiolabeled immunoprecipitated material at 30°C was set at 100%. [^{35}S]methionine-labeled cytosolic extracts from *E.coli* MC4100 cells are given. (**B**) Identical parts of 2D gels of immunoprecipitated material. Upper part, co-immunoprecipitation at 30°C; lower part, co-immunoprecipitation at 45°C. Identified proteins are indicated.

DnaK interacts with a broad variety of thermolabile proteins in extracts and in vivo

We investigated by co-immunoprecipitation whether the protective role of the DnaK system in cell extracts and *in vivo* relies on direct association with the aggregation-prone proteins. This approach is expected to detect only a subfraction of the DnaK substrates, given the highly transient nature of DnaK–substrate complexes and unavoidable losses during co-immunoprecipitation that limit the detectability of less abundant substrates. First, [^{35}S]methionine-labeled extracts supplemented with

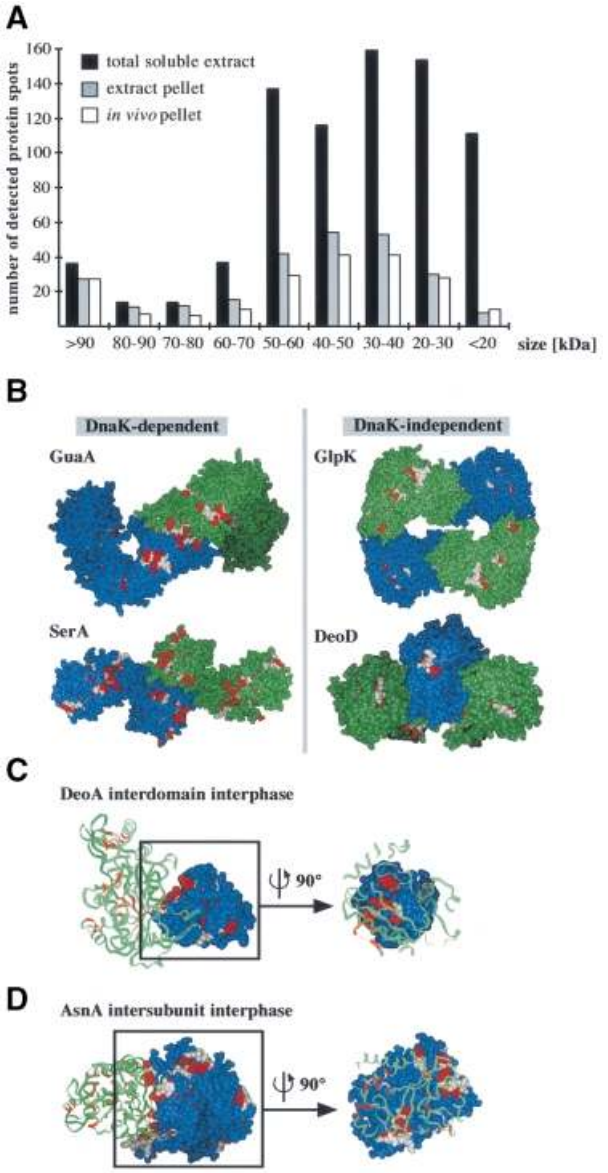

Fig. 5. (**A**) Protein aggregation in wild-type extracts and Δ*dnaK52::cat* mutant cells depends on protein size. Two-dimensional gels representing total soluble proteins of wild type (MC4100), aggregated proteins of wild-type cell extracts after incubation at 45°C and aggregated proteins in a Δ*dnaK52::cat* mutant after heat shock to 42°C were analyzed by ImageMaster software (Pharmacia Biotech). Molecular masses of protein spots were calculated following manufacturers' protocols using known masses of identified proteins. Total numbers of detected protein spots are compared within defined molecular weight groups. (B–D) Examples of thermolabile DnaK substrates with increased surface hydrophobicity. Potential DnaK-binding sites had been identified using the algorithm described previously (Rüdiger *et al.*, 1997b) and localized in the three-dimensional structures of proteins that are aggregation prone (GuaA, SerA, DeoA, AsnA) or stable (GlpK, DeoD) in the absence of DnaK at 42°C. The residues of the hydrophobic core of DnaK-binding sites with a prediction value of less than –4 are shown in gray, the hydrophobic side chains are shown in red. (**B**) GuaA and SerA expose several patches of hydrophobic side chains of potential DnaK-binding sites. GlpK and DeoD expose only a few sites. (**C**) The N-terminal domain of DeoA (blue space filling) exposes DnaK sites on the contact site to the C-terminal domain (green ribbon). (**D**) The homodimeric AsnA exposes DnaK-binding sites on the dimerization interphase (one subunit is shown as blue space fillings, the other as green ribbon).

Mg^{2+}/ATP were incubated at 30 or 45°C in the presence of added DnaK, DnaJ and GrpE. When incubated at 30°C, DnaK was co-immunoprecipitated with only few labeled proteins (Figure 4A). Seventy-five to eighty-five percent of the labeled protein corresponded to endogenous DnaK itself. After heat treatment, the amount of co-immunoprecipitated proteins increased by 5- to 6-fold compared with the 30°C control. Secondly, [^{35}S]methionine-labeled spheroplasts of *E.coli* wild-type cells, which are readily lysed and immediately accessible for co-immunoprecipitation, were subjected to heat treatment at 45°C. Before the temperature upshift an excess of unlabeled L-methionine was added to avoid detection of newly synthesized, nascent polypeptide chains, which may associate with DnaK. Seventy percent of the labeled protein corresponded to endogenous DnaK itself. After heat treatment, a 1.5-fold increase in the amount of co-immunoprecipitated proteins was observed compared with the 30°C control (Figure 4A). The lower number of co-immunoprecipitated proteins in heat-treated spheroplasts compared with heat-treated cell extracts may be due to differences in the DnaK concentrations. In the extracts, we used DnaK concentrations that were sufficient to allow complete prevention of protein aggregation. In contrast, the *in vivo* concentration of DnaK is limiting for aggregation prevention at 45°C, as shown by substantial transient aggregation of proteins in wild-type cells subjected to a 45°C heat treatment (see Figure 8).

Two-dimensional gel electrophoresis of the co-immunoprecipitated proteins revealed that they represent a broad spectrum of species (Figure 4B shows segments of the gels). Spot matching with 2D gels of heat-treated cell extracts and spheroplasts showed that many of the proteins protected by DnaK from thermal aggregation in extracts and *in vivo* were found associated with the chaperone after a 45°C treatment (Table III). We noted that AceE, which showed slightly enhanced aggregation in Δ*dnaK52::cat* mutant cells even at 30°C, could be co-immunoprecipitated at that temperature, pointing to a possible role of DnaK in *de novo* folding or assembly of the pyruvate–dehydrogenase complex. Together, these co-immunoprecipitation experiments detected >60 proteins associated with DnaK predominantly at high temperature both in spheroplasts and extracts, of which >25 were identified by comparative spot matching with reference gels. These proteins represent a subpopulation of those detected as aggregated proteins in Δ*dnaK52::cat* mutant cells and cell extracts (~200), of which 57 proteins were identified by mass spectrometry and no additional protein species were observed. The similarity of the patterns of identified DnaK substrates in spheroplasts and in extracts further strengthens the physiological relevance of the results obtained with extracts. In consideration of the mentioned difficulty in catching many of the unstable DnaK–substrate complexes, these findings indicate that the prevention of aggregation of thermolabile proteins by the DnaK system involves at least in many cases a direct interaction of DnaK with the substrate.

ClpB and the DnaK system cooperate to dissolve protein aggregates in extracts

We tested the capability of cytosolic chaperones to dissolve protein aggregates by using [^{35}S]methionine-labeled soluble extracts of wild-type cells incubated for 15 min at 45°C to induce protein aggregation (see Figure 1). The protein aggregates were isolated by centrifugation, resuspended in buffer with or without combinations of chaperones and ATP, and incubated at permissive temperature (30°C) for 4 h, followed by separation of the soluble and insoluble fractions (Figure 6). No disaggregation occurred in the absence of added chaperones or upon separate addition of the GroEL system, IbpB, HtpG and ClpB. Limited solubilization of only 3–5% of the aggregated protein was observed upon incubation with the DnaK system. In sharp contrast, the simultaneous addition of ClpB and the DnaK system resulted in highly efficient solubilization of ~50% of the aggregated proteins. This disaggregation activity strictly depended on the presence of ATP and was highly efficient since the chaperones were added at substoichiometric amounts (2 μM of ClpB and DnaK monomers) with respect to the proteins (5–10 μM) that aggregated. Furthermore, it exhibited broad substrate specificity as revealed by 2D gel electrophoretic analysis of the soluble and insoluble fractions (Figure 7). Of the aggregated protein species, 20% (50 proteins) were nearly completely disaggregated, 56% (140 proteins) were solubilized by ~50%, and only 24% (60 proteins) were poorly or not disaggregated. We conclude that the DnaK chaperone system cooperates with ClpB to solubilize at least 75% of the thermally aggregated *E.coli* proteins in cell extracts.

Disaggregation of heat-denatured proteins is impaired in dnaK and clpB null mutants

We investigated the potential of ClpB and the DnaK system to reverse protein aggregation *in vivo* by comparing the wild type with isogenic Δ*clpB::kan* and Δ*dnaK52::cat* mutants. Cells of these strains were grown at permissive temperature (30°C) followed by incubation at 45°C for 30 min to induce protein misfolding, and a 3 h recovery period at 30°C to allow protein disaggregation. We noted that the temperature upshift of wild-type cells to 45°C caused a minor and transient accumulation of aggregated proteins, which was not observed after heat shock to 42°C (data not shown). This transient aggregation is consistent with earlier observations (Kucharczyk *et al.*, 1991) and probably results from limitations in chaperone capacity at severe stress conditions (Tomoyasu *et al.*, 1998). Among the transiently aggregated proteins, we identified by mass spectrometry especially large-sized proteins including the β and β′ subunits of RNA polymerase.

In heat-treated Δ*dnaK52::cat* mutant cells, the extensive aggregation of proteins was irreversible (Figure 8), indicating that protein disaggregation depends on DnaK *in vivo*. In heat-treated Δ*clpB::kan* mutant cells, a diversity of proteins aggregated to low amounts that were comparable to those of the transient aggregates observed in wild-type cells (Figure 8). In contrast to wild type, however, these aggregates were not solubilized during the recovery phase. A comparison by 2D gel electrophoresis revealed that the same proteins aggregated transiently in wild-type cells and stably in Δ*clpB::kan*. These proteins form a subset of those proteins that aggregate in *ΔdnaK52::cat* mutant cells (data not shown).

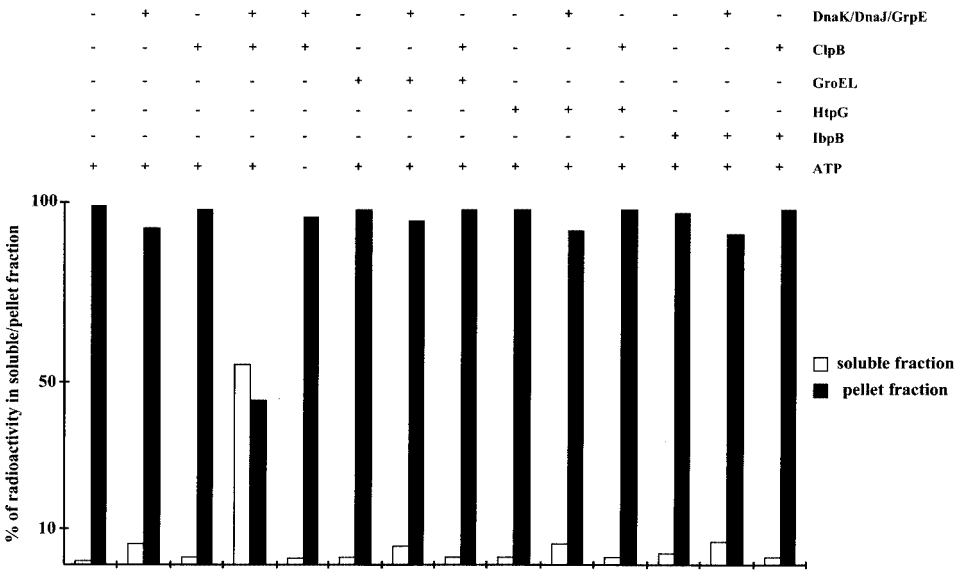

Fig. 6. Disaggregation of pre-existing aggregates of heat-labile *E.coli* proteins in cell extracts. Protein aggregates of [^{35}S]methionine-labeled cell extracts were isolated after heat treatment to 45°C by centrifugation. Pellets were incubated with 2 μM of the indicated chaperone combinations in the presence of 5 mM ATP and an ATP-regenerating system for 4 h at 30°C. Resolubilized protein aggregates and still insoluble protein material were separated by centrifugation. The amount of radioactivity of both fractions (soluble fraction, white bars; pellet fraction, black bars) was determined by scintillation counting. The amount of radioactivity in the control reaction (no chaperone added) before the disaggregation reaction was set at 100%. All reactions were carried out three times with <5% deviations.

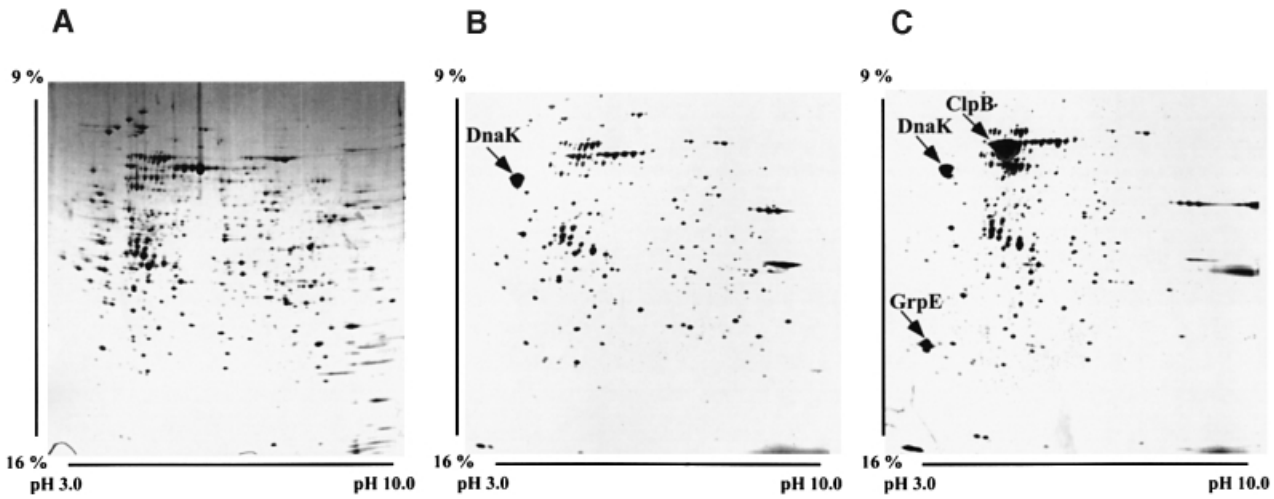

Fig. 7. ClpB and the DnaK system resolubilize pre-existing aggregates of heat-denatured proteins with a broad specificity. Cell extracts were heated to 45°C. Heat-aggregated proteins were isolated by centrifugation and incubated for 4 h at 30°C in the presence of ClpB, the DnaK system and an ATP-regenerating system. Resolubilized and insoluble protein fractions were separated by centrifugation and analyzed by 2D gel electrophoresis. (**A**) Heat-aggregated proteins of *E.coli* cell extract. (**B**) Insoluble and (**C**) soluble (representing resolubilized protein aggregates) protein fractions after the incubation of heat-aggregated proteins with ClpB and the DnaK system.

Reversal of protein aggregation in ΔdnaK52::cat mutants by induced synthesis of ClpB and the DnaK system

To test rigorously whether the extensive aggregation of proteins in heat-treated Δ*dnaK52::cat* mutant cells can be reversed by ClpB and the DnaK system, we performed an *in vivo* order-of-addition experiment. Expression plasmids for *clpB*, *dnaK* and *dnaJ* in various combinations were constructed to allow tight repression of these genes in the absence of inducer and overexpression in the presence of IPTG. Overexpression of *grpE* was not required for these experiments, since Δ*dnaK52::cat* mutant cells have high endogenous GrpE levels due to defects in negative autoregulation of heat-shock genes (Bukau and Walker, 1990). With the continuous presence of IPTG in the growth medium, cells carrying appropriate expression plasmids had levels of ClpB, DnaK and DnaJ that were 5- to 10-fold higher than in wild type. The induced expression of *dnaK* and *dnaJ* without *clpB* (pBB525), or *dnaK* and *dnaJ* with *clpB* (pBB526), rescued the temperature-sensitive growth phenotype of Δ*dnaK52::cat* mutants at 42°C (Table II) and prevented protein aggregation in Δ*dnaK52::cat* mutant cells after the shift from 30 to 42°C (data not shown).

To test for protein disaggregation, Δ*dnaK52::cat* mutant cells carrying appropriate expression plasmids were grown at 30°C in the absence of IPTG, followed by incubation for 30 min at 45°C to allow extensive formation of protein

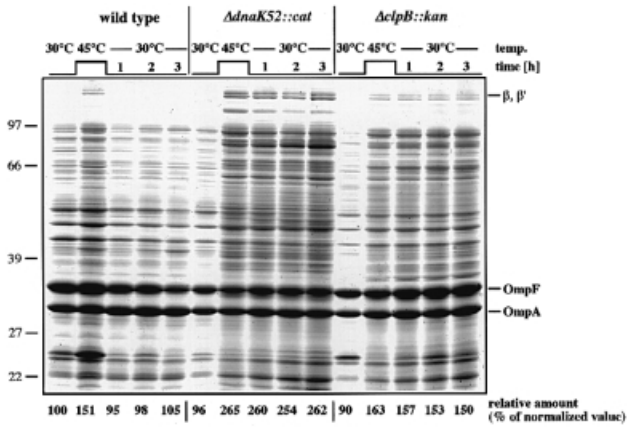

Fig. 8. Disaggregation of heat-induced protein aggregates is impaired in Δ*dnaK52::cat* and Δ*clpB::kan* mutants. Cultures of wild-type and mutant strains were grown in LB medium at 30°C to logarithmic phase, then shifted to 45°C for 30 min, followed by incubation at 30°C for 3 h. Insoluble fractions, containing membrane proteins and aggregated proteins, were analyzed at the indicated time points by SDS–PAGE followed by staining with Coomassie Brilliant Blue. Selected proteins were identified by immunoblot analysis using specific antisera. The insoluble cell fractions were quantified by MacBAS software (Fuji film) and normalized to the band intensities of OmpA and OmpF, which serve as internal loading controls. The amount of insoluble cell fraction of wild-type cells at 30°C before heat shock was set at 100%.

aggregates (Figure 9). After temperature downshift to 30°C, the cells were incubated with or without IPTG and assayed for protein disaggregation. Cells carrying either the vector control (pBB527) or pBB521 (*clpB*) showed no alteration of the high aggregation level in the presence or absence of IPTG (data not shown). Furthermore, even massive IPTG-induced overproduction of other chaperones (GroEL/GroES, HtpG, IbpA/B) did not disaggregate heat-aggregated proteins in Δ*dnaK52::cat* cells (data not shown). In contrast, cells carrying pBB525 (*dnaK dnaJ*) could solubilize ~50% of the pre-existing aggregates when IPTG was present (data not shown). Thus, some solubilization occurs by the DnaK system, probably together with ClpB produced from the chromosomal *clpB* gene. It should be noted that in the Δ*dnaK52::cat* cells, ClpB levels were increased compared with the wild type due to the defects in regulation of heat-shock genes including *clpB* (Bukau and Walker, 1990). The strongest effect was observed for Δ*dnaK52::cat* cells overexpressing *clpB*, *dnaK*, *dnaJ* from pBB526. At least 95% of the heat-aggregated protein species were disaggregated within 2 h at 30°C, provided that IPTG was present after temperature downshift (Figure 9). Unpublished experiments showed that overproduction of the DnaK system in Δ*rpoH* cells, which lack σ^{32} and therefore have strongly reduced levels of all major cytosolic chaperones including ClpB, did not allow solubilization of protein aggregates formed at 42°C. In these cells, co-overproduction of ClpB and the DnaK system was necessary and sufficient to allow complete disaggregation of heat-aggregated proteins (T.Tomoyasu, A.Mogk and B.Bukau, unpublished results). Together, these results demonstrate the generality and high efficiency of the protein disaggregating activity of ClpB and the DnaK system in *E.coli* cells.

MetE: destiny of a thermolabile model protein of E.coli

To strengthen the results obtained in this study and to dissect further the fate of thermolabile proteins, we investigated the most prominent heat-aggregated protein of Δ*dnaK52::cat* cells grown in M9 minimal medium, MetE. Immunoblot analysis revealed that MetE transiently aggregated in wild-type cells subjected to a heat shock to 45°C, but stably aggregated in heat-treated Δ*dnaK52::cat* and Δ*clpB::kan* mutant cells (Figure 10A). Temperature-dependent aggregation and protection by the DnaK system were confirmed in cell extracts and in an *in vitro* system with purified MetE (Figure 10B and C), demonstrating the direct association of the DnaK system with MetE. Efficient resolubilization of pre-formed MetE aggregates by ClpB and the DnaK system was reached in cell extracts and *in vitro* with purified components (Figure 10B and C). These findings confirm for a major natural thermolabile substrate identified in this study, that the DnaK system and ClpB are directly involved in aggregation prevention and disaggregation after temperature-induced unfolding.

Interestingly, MetE was degraded in heat-treated wild-type cells if they were kept at 45°C. This degradation was Lon dependent since MetE was largely stable at 45°C in a *lon* null mutant (Figure 10A), but not in *clpA* or *clpB* null mutants (data not shown). This finding indicates that aggregation-prone substrates that are kept or rendered soluble by the DnaK system may become susceptible to proteolysis.

Discussion

This study shows that the DnaK system is the central 'holder' chaperone of the cytosol that prevents aggregation of a wide variety of heat-denatured proteins. This central function of DnaK is also reflected by its substantially higher effective cellular concentration compared with the other major cytosolic chaperones. We identified for the first time thermolabile proteins of *E.coli* that misfold and aggregate under physiological heat stress. Almost all of them become protected from aggregation by the DnaK system, in at least many cases through direct association with DnaK, which defines them as natural DnaK substrates. Finally, a bi-chaperone system is described consisting of the DnaK system and ClpB, which efficiently disaggregates proteins that escaped the holder function of DnaK.

The thermolabile proteins were identified in heat-treated extracts and *in vivo*. Both populations were qualitatively highly similar, thus validating the *in vitro* experiments, but the extent of aggregation differed strongly between extracts and cells. An identical heat treatment resulted in stable aggregation of 10–15% of the proteins in wild-type extracts, but only transient aggregation of ⩽3% of the soluble proteins in wild-type cells. This difference is unlikely to result from the buffer conditions or the chaperone composition in the extracts since they were chosen to be near physiological. We considered that the crowded environment of the *E.coli* cytosol (~170 mg/ml *in vivo* versus 4 mg/ml in the extract) protects proteins from aggregation. A commonly used unspecific crowding agent, Ficoll 70, did not provide protection to proteins against aggregation in cell extracts, even when added to a high concentration of 20% (w/v) (Figure 1). We

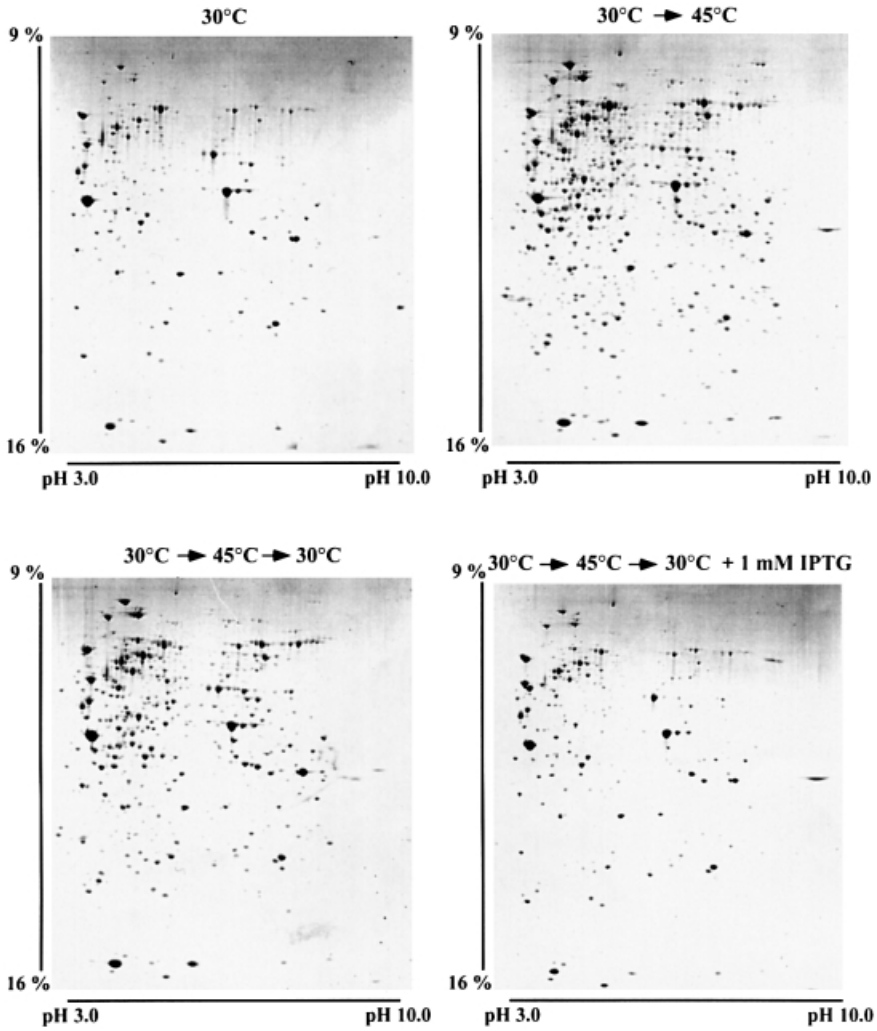

Fig. 9. Disaggregation of heat-induced protein aggregates in Δ*dnaK52::cat* mutant cells by ClpB and the DnaK chaperone machinery. Δ*dnaK52::cat* mutant cells carrying pBB526 (allowing overexpression of *clpB*, *dnaK*, *dnaJ*) were grown in LB medium at 30°C to logarithmic phase and shifted for 30 min to 45°C. Cultures were split after heat treatment and further incubated for 2 h at 30°C with or without IPTG (1 mM). Insoluble cell fractions containing membrane proteins and aggregated proteins were prepared and analyzed by 2D gel electrophoresis.

therefore assume that more specific interactions of the aggregation-prone proteins with other cytosolic chaperones and protectants are responsible for the reduced protein aggregation in wild-type cells compared with cell extracts.

The unique ability of the DnaK system to prevent aggregation of a large variety of thermolabile proteins efficiently was detected *in vivo* and in cell extracts. Incubation of Δ*dnaK52::cat* mutant cells at 42°C caused aggregation of ~10% of the soluble proteins after 60 min. Incubation at 45°C increased the aggregated fraction of each thermolabile protein, but did not change the spectrum of aggregated proteins. The missing function of the DnaK system can thus not be replaced *in vivo* by other chaperones, even though their levels are 2- to 3-fold increased in Δ*dnaK52::cat* mutant cells compared with wild type (Bukau and Walker, 1990). This interpretation is further supported by the findings that IPTG-controlled overproduction of GroEL/GroES, ClpB or IbpA/B did not rescue the temperature-sensitive growth phenotype of Δ*dnaK52::cat* mutant cells at 42°C, and that mutations in the genes encoding these chaperones and HtpG did not cause strong protein aggregation at 42°C. Finally, the experiments using cell extracts showed that only the DnaK system efficiently prevented heat-induced protein aggregation.

This low capacity as holder chaperone was expected for ClpB, since, like its yeast homolog Hsp104, it fails to prevent aggregation of heat-denatured test substrates (Glover and Lindquist, 1998; Goloubinoff *et al.*, 1999). For HtpG our findings agree with earlier reports that HtpG is poorly active in preventing aggregation of unfolded citrate synthase (Jakob *et al.*, 1995). The low capacity of IbpB in cell extracts was unexpected given that stoichiometric amounts prevented aggregation of heat-denatured malate dehydrogenase (Veinger *et al.*, 1998; Shearstone and Baneyx, 1999). However, our findings are consistent with the observed lack of protein aggregation phenotypes in heat-treated Δ*ibpAB* null mutants (this study; Thomas and Baneyx, 1998). The low capacity of the GroEL system may be explained by the ability of GroES and ATP to disfavor the GroEL-bound state of thermolabile proteins. Also, the tetradecameric state of the active form of GroEL would demand a 14-fold molar excess of GroEL monomers over the aggregation-prone proteins to reach equimolarity. Such a high GroEL concentration is unphysiological (~3 μM at 30°C), but strong overproduction of GroEL

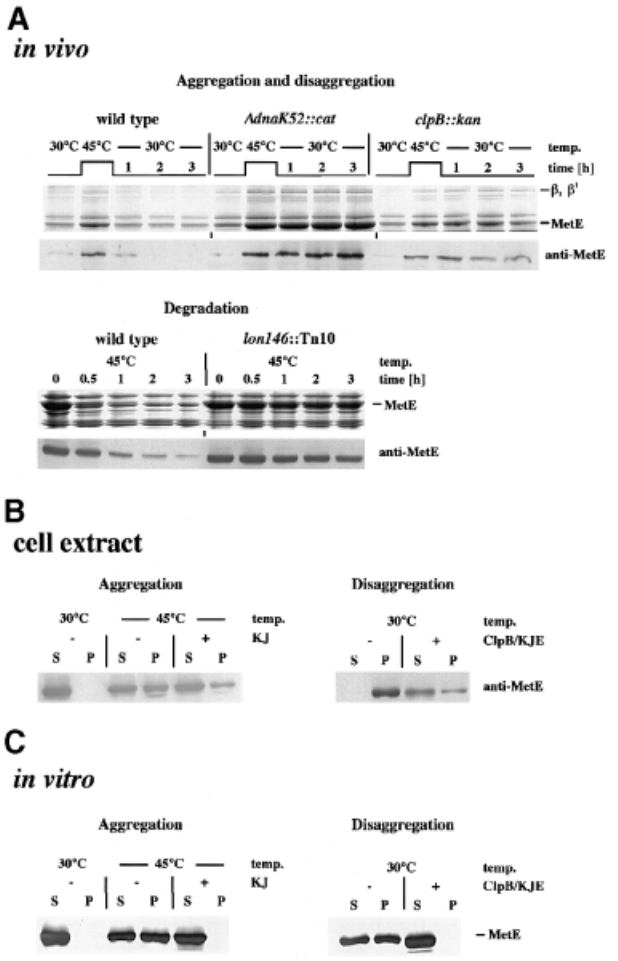

Fig. 10. Aggregation, disaggregation and degradation of MetE. (**A**) Disaggregation of heat-aggregated MetE is impaired in Δ*dnaK52::cat* and Δ*clpB::kan* mutants. Cultures of wild-type and mutant strains were grown in M9 minimal medium supplemented with 19 L-amino acids except L-methionine at 30°C to logarithmic phase, then shifted to 45°C for 30 min, followed by incubation at 30°C for 3 h. Insoluble fractions, containing membrane proteins and aggregated proteins, were analyzed at the indicated time points by SDS–PAGE followed by staining with Coomassie Brilliant Blue. Transient or stable aggregation of MetE was verified by immunoblot analysis using MetE-specific antisera. MetE is degraded by Lon after heat shock to 45°C in wild-type cells. Cultures of wild-type and *lon146*::Tn*10* mutant strains were grown in supplemented M9 minimal medium at 30°C to logarithmic phase, then shifted to 45°C for 3 h. Soluble fractions were analyzed at the indicated time points by SDS–PAGE followed by staining with Coomassie Brilliant Blue or by immunoblot analysis using MetE-specific antisera. (**B**) Aggregation and disaggregation of MetE in cell extracts. Cell extracts (4 mg/ml) were heated to 45°C for 15 min in the absence or presence of added 5 μM DnaK and 1 μM DnaJ. Soluble (S) and insoluble (P) fractions were separated by centrifugation and analyzed by immunoblotting using MetE-specific antisera. In addition, heat-aggregated proteins were isolated by centrifugation and incubated with 2 μM ClpB and DnaK, 0.4 μM DnaJ and 0.2 μM GrpE in the presence of 5 mM ATP and an ATP-regenerating system for 4 h at 30°C. Resolubilized and insoluble MetE were separated by centrifugation and analyzed by immunoblotting using MetE-specific antisera. (**C**) Aggregation and disaggregation of purified MetE *in vitro*. MetE (100 nM) was incubated at 30 or 45°C for 15 min in the absence or presence of DnaK (1 μM) and DnaJ (0.2 μM). Soluble (S) and insoluble (P) fractions were separated by centrifugation and analyzed by silver staining. In addition, ClpB (100 nM) and DnaK/DnaJ/GrpE (100/20/10 nM) were added after heat treatment of MetE and incubated for 4 h at 30°C. Soluble (S) and insoluble (P) fractions were separated by centrifugation and analyzed by silver staining.

and GroES can indeed suppress protein aggregation in chaperone-deficient Δ*rpoH* or *dnaK756* mutant cells (Gragerov *et al.*, 1992; Kedzierska *et al.*, 1999). On the other hand, overproduction of GroEL and GroES could not complement the temperature-sensitive growth phenotype of Δ*dnaK52::cat* mutant cells at 42°C, and only partially suppressed protein aggregation in Δ*dnaK52::cat* at 42°C (this study). These findings confirm the unique role of DnaK as the central protection system of *E.coli* at high temperatures and support results from previous studies (Kusukawa and Yura, 1988). Previously published data (Horwich *et al.*, 1993; Ewalt *et al.*, 1997; Bukau and Horwich, 1998), together with ours, strongly suggest that the primary role of the GroEL system is to promote folding and refolding, rather than holding, of newly synthesized and misfolded proteins.

The number of aggregation-prone *E.coli* proteins is surprisingly high (150–200 *in vivo* and 250 in extracts, out of 800 detectable proteins) given that the chosen heat-shock temperatures are within the growth temperature range. Most aggregation-prone proteins are substrates for DnaK since: (i) addition of DnaK protects their aggregation in cell extracts and *in vivo*; (ii) a highly similar set of proteins aggregates in heat-treated Δ*dnaK52::cat* mutant cells; and (iii) many of these proteins can be co-immunoprecipitated with DnaK-specific antisera in heat-treated cell extracts and *in vivo*. Furthermore, for a major aggregation-prone protein identified *in vivo* and in cell extract, MetE, we showed a direct role for DnaK in aggregation prevention. Purified MetE was found to be thermolabile and prone to aggregation at 45°C *in vitro* and to become protected from aggregation by addition of DnaK and DnaJ. Interestingly, at heat-shock temperatures, MetE aggregated in Δ*dnaK52::cat* mutant cells but was degraded in wild-type cells in a Lon-dependent manner. This example indicates that the fate of thermolabile proteins that were prevented from aggregation by the DnaK system or resolubilized by ClpB and the DnaK system (see below) can also be to be targeted for degradation. Degradation is likely to explain why the patterns of DnaK substrates identified *in vivo* and *in vitro* show some differences.

All identified proteins are cytoplasmic and participate in various cellular processes including metabolism, transcription, translation, cell wall synthesis and cell division, and in particular include key proteins of the transcription and translation apparatus. Many of these proteins have essential functions, and aggregation of any one of them may cause the temperature sensitivity of Δ*dnaK52::cat* mutants. The thermosensitivity of key proteins of transcription (RNA polymerase) and translation (elongation factors) may have protective roles in heat-treated cells, since it may slow down protein synthesis when the stress state limits the availability of chaperones. The insoluble fraction of heat-treated Δ*dnaK52::cat* mutant cells and cell extracts also contained the proteases Lon, ClpX and HslU, as well as IbpB (not in cell extracts). These components of the cellular folding system may not be thermolabile but rather associate with denatured and aggregated protein substrates as already reported for IbpB (Allen *et al.*, 1992; Laskowska *et al.*, 1996b). In isolated cases proteins may become trapped in aggregates by specific association with a thermolabile protein as part of

an oligomeric complex (e.g. Rho). We consider this possibility as an exception, since most aggregation-prone proteins that we identified participate in different cellular processes. Furthermore, we have excluded experimentally the possibility that the aggregation of thermolabile proteins causes unspecific co-precipitation of stable proteins.

Earlier attempts to identify the parameters determining the thermolability of model proteins gave no generally applicable answer (Jaenicke and Bohm, 1998). Our study provides a different access to this problem by identifying a large number of aggregation-prone proteins of *E.coli* and showing that they are substrates for DnaK upon thermal unfolding. The most striking feature of this class is that large-sized proteins are strongly enriched. It is tempting to speculate that the appearance of large, multi-domain proteins in evolution was accompanied by the appearance of a powerful DnaK chaperone system. Three features of large proteins may contribute to their aggregation propensity. First, misfolded conformers of larger proteins may statistically expose more hydrophobic surface patches than smaller proteins. Since such patches are considered to be involved in, or even trigger, intermolecular aggregation (Mitraki and King, 1989; Seckler and Jaenicke, 1992), there is a higher probability that larger proteins will aggregate. Secondly, large proteins are composed of more domains than small proteins. In many proteins the interactions between domains are flexible and subject to regulation. Interdomain surface contacts may be particularly vulnerable to heat and, if they expose hydrophobic surfaces, initiate intermolecular aggregation. Thirdly, the rates of refolding of unfolded conformers of large proteins may be slower than that of small proteins, which consequently favors competing aggregation reactions.

All features involve the surface exposure of hydrophobic patches, which may be the connection between intermolecular aggregation and DnaK association. We previously established that DnaK recognizes hydrophobic patches consisting of 4–5 consecutive residues enriched in large hydrophobic and aromatic residues and flanked by regions enriched in basic residues (Rüdiger *et al.*, 1997a,b). An algorithm predicting DnaK-binding sites in protein sequences was established (Rüdiger *et al.*, 1997b) and used here to compare 32 thermolabile proteins shown to be DnaK substrates by co-immunoprecipitation with DnaK-specific antisera and/or shown to become aggregated or disaggregated in a DnaK-dependent fashion with 27 thermostable control proteins. The majority of the thermolabile and stable proteins have statistically one DnaK-binding site every 26 and 35 residues, respectively. Nineteen percent of the thermolabile subset have a higher frequency (compared with 4% of the thermostable subset), while 41% of the stable subset have a lower frequency (compared with 16% of the thermolabile subset). There is thus some correlation between the frequency of predicted DnaK-binding sites and the propensity to aggregate or associate with DnaK. It is interesting that of all *E.coli* proteins >700 residues investigated, only FumC (1010 residues) was stable in Δ*dnaK52::cat* mutants at 42°C, the protein that has the lowest frequency of DnaK-binding sites of all proteins investigated (one binding site per 92 residues).

One can not predict the particular hydrophobic patch(es) of a thermolabile protein involved in aggregation and DnaK binding, given that aggregation is primarily driven by unfolding kinetics and properties of the unfolded state. We nevertheless investigated whether thermolabile DnaK substrates share features of their native structures, especially regarding the localization of potential DnaK-binding sites. We compared the atomic structures of DnaK substrates (GlnA, GatY, EF-G, AsnA, DeoA, CarA, CarB, SerA and GuaA) with those of stable proteins (DeoD, FabF, PckA, FbaB, EF-Ts, PykF, GlpK and FumC). In eight out of nine thermolabile proteins analyzed (all except DeoA), at least one patch of three hydrophobic side chains, constituting the core of a DnaK-binding site, is surface exposed, and in three of these nine proteins several such patches are exposed [SerA and GuaA (Figure 5B); complex of CarA and CarB (not shown)]. In contrast, only one of the eight stable proteins exposes such a hydrophobic patch (FbaB). It should be emphasized that the mere surface exposure of these sites in the native structures is insufficient for DnaK association. The architecture of the substrate-binding cavity of DnaK indeed requires significant separation of the binding segment from the remainder of the polypeptide chain (Zhu *et al.*, 1996). One possible scenario may therefore be that these surface-exposed sites become even more exposed upon thermal unfolding of the proteins and trigger protein aggregation. Furthermore, we studied the contact sites between protein domains and subunits and found potential DnaK-binding sites on interdomain contact surfaces, particularly of smaller domains (Figure 5C) and some inter-subunit surfaces (Figure 5D). Although these features are not unique for the proteins of the subset of thermolabile proteins, the accessibility of such contact sites may be increased during thermal unfolding in the case of thermolabile proteins.

Our study provides evidence for a highly efficient bi-chaperone system, composed of the DnaK system and ClpB, for dissolving a wide spectrum of aggregated proteins in extracts and *in vivo*. These results extend the pioneering findings of Lindquist and coworkers (Sanchez *et al.*, 1992; Parsell *et al.*, 1994; Glover and Lindquist, 1998), which for the first time established a protein disaggregating activity for the yeast homologs Hsp104 and Ssa1/Ydj1. ClpB and the DnaK system are both required for this activity and can not be replaced by any of the other chaperones tested. Substoichiometric amounts of both chaperone systems (2 μM for ClpB and DnaK) were sufficient to solubilize ~50% of the amount of aggregated proteins (5–10 μM) present in heat-treated cell extracts, with at least partial solubilization of 75% of the aggregated protein species. These findings show the high efficiency and broad specificity of this bi-chaperone system. The efficiency was even higher *in vivo* than *in vitro*, as it allowed complete solubilization of pre-formed, large-sized aggregates. A central role of this bi-chaperone system in stress protection of *E.coli* is indicated by the failure of Δ*dnaK52::cat* and Δ*clpB::kan* mutant cells to disaggregate protein aggregates formed during severe heat treatment. In agreement with our data, *clpB* null mutants and *dnaK* point mutants are retarded in removal of a cell fraction assumed to be enriched in protein aggregates (Laskowska *et al.*, 1996a; Kedzierska *et al.*, 1999).

Our findings functionally explain genetic evidence that suggests a stress-related role of the cooperative activity

of the DnaK system and ClpB. First, in several bacteria the heat-shock-inducible *clpB*, *dnaK* and *dnaJ* genes are organized in an operon (Falah and Gupta, 1997; Osipiuk and Joachimiak, 1997), or are at least regulated by a common, specific mechanism (Grandvalet *et al.*, 1999). Secondly, knockout mutations in *clpB* of *E.coli* and *Helicobacter pylori*, and in *hsp104* of *Saccharomyces cerevisiae* prevent the cells from acquiring thermotolerance and surviving severe stress conditions (Lindquist, 1990; Squires *et al.*, 1991; Sanchez *et al.*, 1992; Lindquist and Kim, 1996; Allan *et al.*, 1998; Thomas and Baneyx, 1998). Thermotolerance and survival of bacteria and yeast under heat stress are thus linked to the ability of these cells to reverse protein aggregation, an activity that is performed by the bi-chaperone system.

Materials and methods

Strains and culture conditions

Escherichia coli strains used were derivatives of MC4100 [araD139 Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*]. Mutant alleles of chaperone genes were introduced by P1 transduction into MC4100 background to generate strains BB1553 (Δ*dnaK52::cat sidB1*) (Bukau and Walker, 1990), BB2395 (*lon*146::Tn*10*), BB2396 (*clpA*82::Tn*10*) (Gottesman and Maurizi, 1992), BB4561 (Δ*clpB::kan*) (Squires *et al.*, 1991), BB4562 (Δ*htpG1::lacZ zba315::kan*) (Bardwell and Craig, 1988), BB4563 (Δ*ibpAB::kan*) (S.Vorderwülbecke, E.Deuerling and B.Bukau, unpublished), BB4564 (*groEL140 zjd::*Tn*10 zje*::ΩSpc^r/Str^r) and BB4565 (*groEL44 zjd*::Tn*10 zje::kan*) (Zeilstra-Ryalls *et al.*, 1993). Bacterial strains were cultured at 30°C in Luria broth (LB) or M9 minimal medium supplemented with 0.2% (w/v) glucose and 19 L-amino acids (Sigma LAA-21; 50 μg/ml/amino acid). Chloramphenicol, kanamycin, spectinomycin, tetracycline and ampicillin were used at final concentrations of 20, 20, 50, 10 and 100 μg/ml, respectively. Temperature-shift experiments were performed in orbital shaking water baths. For pulse-labeling with [^{35}S]methionine, cells were grown in M9/glucose with all L-amino acids except L-methionine. Labeling was done by adding [^{35}S]methionine (Amersham SJ1515; 15 mCi/ml, 1000 Ci/mmol) to 30 μCi/ml cell culture for 2 min, followed by addition of unlabeled L-methionine to 200 mg/ml. In co-immunoprecipitation experiments, cells were pulse-labeled three times to increase the labeling efficiency of the proteins.

Protein purifications

Chaperones were purified according to standard protocols (Jakob *et al.*, 1995; Buchberger *et al.*, 1996; Kim *et al.*, 1998; Veinger *et al.*, 1998). The DnaK and GroEL systems, IbpB and HtpG, were active in preventing aggregation and assisting refolding of denatured model substrates (malate dehydrogenase, firefly luciferase, mitochondrial citrate synthase) similar to published efficiencies (Jakob *et al.*, 1995; Buchberger *et al.*, 1996; Veinger *et al.*, 1998). MetE was purified following published protocols (Gonzalez *et al.*, 1992).

Preparation of soluble cell extracts and spheroplasts

Cell cultures were grown to mid-exponential phase and cooled rapidly in an ice-water bath to 0°C. Cells were harvested by centrifugation for 10 min at 5000 *g* and 4°C. Cell pellets were washed twice and resuspended in ice-cold breakage buffer (50 mM HEPES pH 7.6, 150 mM KCl, 20 mM $MgCl_2$, 10 mM DTT) and lysed in a precooled French pressure cell at 20 000 lb/in^2. Total cell protein was quantified by Bradford assay using BSA as standard. Spheroplasts were prepared following published protocols (Ewalt *et al.*, 1997). For co-immunoprecipitations spheroplasts were resuspended in M9/glucose medium containing all L-amino acids except L-methionine and 15% (w/v) sucrose.

Aggregation and disaggregation of proteins in cell extracts

Soluble cell extracts (4 mg/ml) were pre-incubated for 5 min at 30°C in the absence or presence of chaperones. ATP (10 mM) was added 2 min prior to heat shock. Samples were shifted to 45°C for 15 min and aggregated proteins were pelleted by centrifugation for 15 min at 15 000 *g* and 4°C. Pellets were washed twice with ice-cold breakage buffer and analyzed by electrophoresis. For disaggregation of aggregated proteins the washed protein pellet was resuspended in breakage buffer by pipetting. Chaperones (2 μM DnaK, ClpB; 0.4 μM DnaJ; 0.2 μM GrpE), ATP (5 mM) and an ATP-regenerating system (3 mM phospho-enolpyruvate, 20 ng/ml pyruvate kinase) were added and incubated for 4 h at 30°C. Resolubilized and still aggregated proteins were separated by centrifugation (15 min, 15 000 *g* at 4°C). Pellets were washed twice with ice-cold breakage buffer and the soluble and insoluble fractions were further analyzed by gel electrophoresis.

Preparation of insoluble cell fractions

Aliquots (10–40 ml) of bacterial cultures were cooled rapidly to 0°C in an ice-water bath and within a few minutes centrifuged for 10 min at 5000 *g* and 4°C to harvest the cells. Pellets were resuspended in 10× lysis buffer [100 mM Tris pH 7.5, 100 mM KCl, 2 mM EDTA, 15% (w/v) sucrose, 1 mg/ml lysozyme] according to their optical density (50 ml of lysis buffer for 10 ml of culture of OD_{600} = 1) and frozen at –80°C. Samples were then thawed slowly at 0°C followed by addition of a 10-fold volume of ice-cold water and mixing. The viscous, turbid solution was sonicated with a Branson Cell Disruptor B15 (microtip, level 5, 50% duty, four cycles) while cooling. Intact cells were removed by centrifugation at 2000 *g* for 15 min at 4°C. The insoluble cell fraction was isolated by subsequent centrifugation at 15 000 *g* for 15 min at 4°C. Pellets were washed twice with ice-cold water and analyzed by gel electrophoresis.

Co-immunoprecipitation

[^{35}S]methionine-labeled soluble cell extracts (4 mg/ml) were incubated at 30 or 45°C in the presence of added DnaK, DnaJ and GrpE (7.5, 1.5 and 0.75 μM, respectively). After incubation for 15 min at the temperature indicated, ice-cold EDTA (100 mM) was added and the samples were kept on ice to disfavor dissociation of DnaK from the substrates. [^{35}S]methionine-labeled spheroplasts of MC4100 cells were incubated at 30 or 45°C for 5 min, lysed by dilution into 10 vols of ice-cold EDTA (100 mM), followed by sonication. Intact cells and membrane fractions were removed by subsequent centrifugation at 15 000 *g* for 15 min at 4°C. Aliquots of 80 μl were incubated with 50 μl of protein A–Sepharose beads in phosphate-buffered saline (PBS), 4 μl of polyclonal antisera raised against the ATPase domain of DnaK, and 1 ml of co-immunoprecipitation (CoIP) buffer [50 mM Tris pH 7.5, 10 mM EDTA, 150 mM KCl, 0.5% (v/v) NP-40] for 1 h at 4°C. Beads were washed twice with 1 ml of CoIP buffer and once with 1 ml of PBS containing 5 mM EDTA. Proteins bound to protein A–Sepharose were analyzed by gel electrophoresis. Dried gels were scanned using a phosphoimager (FLA-2000) and quantified by MacBAS software (Fuji film).

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed essentially as described previously (Bjellqvist *et al.*, 1993) with minor modifications. Nonlinear immobilized pH 3–10 gradient strips (18 cm; Pharmacia Biotech) were rehydrated in 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.4% (w/v) DTT, 2% (v/v) resolyte pH 3–10 (Pharmacia Biotech). Total soluble cell extracts were dialyzed twice against 2 l of 5 mM HEPES pH 8.0, containing 10% (v/v) glycerol and 1 mM EDTA. After dialysis, urea (8 M), CHAPS [2% (w/v)], DTT [0.4% (w/v)] and 0.5% (v/v) resolyte pH 3–10 were added and ~1 mg of total soluble protein was applied to both the cathodic and anodic ends of the rehydrated strips. In the case of insoluble cell fractions the pellets were dissolved in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.4% (w/v) DTT and 0.5% (v/v) resolyte pH 3–10. The proteins were focused first for 2 h at 150 V, then for 5 h at 300 V. The voltage was finally increased to 1500 V for 10 h and to 3500 V for 48–60 h. The proteins were separated on 9–16% linear gradient polyacrylamide gels at 5 mA/gel for 2 h, followed by 40 mA/gel for 7 h. The gels were stained with colloidal Coomassie Blue (Novex) and destained with water for protein detection.

Identification of proteins by mass spectrometry

For protein identification the Coomassie-stained spots were cut out of the gel and destained by a solution of 100 mM ammonium bicarbonate in 50% acetonitrile/water. The gel pieces were then prepared and digested with endoproteinase Lys-C essentially as described previously (Langen *et al.*, 1997). Elution of the resulting peptides and determination of their masses followed published protocols (Fountoulakis and Langen, 1997). Proteins were also identified by protein spot matching with reference gels using the ImageMaster software (Pharmacia).

SDS–PAGE, immunoblotting and quantifications

Gel electrophoresis was carried out according to published protocols (Laemmli, 1970) using 12% SDS–PAGE and stained with Coomassie

Brilliant Blue or silver nitrate. Immunoblotting was performed according to standard procedures, using rabbit antisera specific for the relevant protein as primary antibody, and developed with a Vistra ECF fluorescence immunoblotting kit (Amersham) using alkaline phosphatase/conjugated anti-rabbit IgG as secondary antibodies (Vector Laboratories). Developed immunoblots were scanned using a fluoroimager (FLA-2000) and quantified using MacBAS software (Fuji Film). To determine the cellular levels of relevant proteins, different amounts of total and soluble cell extracts were subjected to SDS–PAGE. For immunoblot quantification and determination of the linear range of detection, serial dilutions of purified proteins (ClpB, HtpG, DnaK, GroEL, IbpB) served as an internal standard. Protein molecules/cell were calculated on the basis of the known weight of proteins per cell (0.154 pg) (Neidhardt and VanBogelen, 1987).

Construction of plasmids

Control plasmid pBB527 (pZE4 *lacI*) was derived from pZE GrpE (laboratory collection, derivative of pUHS4 IntI) (Lutz and Bujard, 1997). pZE GrpE and pBB509 (Tomoyasu *et al.*, 1998) were digested by *Avr*II or *Eco*RI and the sticky ends created were filled in with T4 DNA polymerase (Boehringer Mannheim). Both plasmids were then digested with *Aat*II and the isolated *lacI* fragment was subsequently cloned into engineered pZE pzl GrpE. pBB525 (pZE4 *lacI* pA1/lacO1 *dnaK*, *dnaJ*) was derived from pBB518 (pZE1 *lacI* pA1/lacO1 *dnaK*, *dnaJ*; laboratory collection). pBB518 and pZE GrpE were digested with *Aat*II and *Avr*II. The isolated *lacI*, *dnaK*, *dnaJ* containing fragment was ligated into engineered pZE GrpE. The *clpB* gene from *E.coli* was amplified by PCR using native Pfu DNA polymerase (Stratagene), pClpB (Squires *et al.*, 1991) as template, forward primer 5′-AGGAAGATCTATGGGAGGAGTTATGCGTCT-3′ and reverse primer 5′-GGCCTCTAGATTACTGGACGGCGACAATCC-3′. The engineered *Bgl*II and *Xba*I sites were used for subsequent cloning of the *clpB* gene fragment into the single *Bam*HI and *Xba*I restriction sites of pUHE21-2fdΔ12 (Buchberger *et al.*, 1994) followed by sequencing of the entire gene. The resulting plasmid pBB519 (pUHE21-2fdΔ12 *clpB*) was digested with *Xho*I and *Xba*I, and ligated into *Xho*I–*Avr*II-digested pZE GrpE. The isolated *clpB* fragment was subsequently cloned into engineered pZE GrpE, yielding pBB521 (pZE4 pA1/lacO1 *clpB*). pBB526 (pZE4 lacI pA1/lacO1 *dnaK*, *dnaJ*, *clpB*) was constructed by digesting pBB525 with *Avr*II and pBB521 with *Xho*I, and the sticky ends created were filled in with T4 DNA polymerase. Both resulting plasmids were cut with *Aat*II afterwards and the *lacI*, *dnaK*, *dnaJ* containing fragment was cloned into engineered pBB521. The *ibpAB* operon from *E.coli* was amplified by PCR using native Pfu DNA polymerase, chromosomal DNA from *E.coli* C600 as template, forward primer 5′-AGGAGGATCCATGCGTAACTTTGATTTATCC-3′ and reverse primer 5′-AGGAAAGCTTTTAGCTATTTAACGCGGGAC-3′. The engineered *Bam*HI and *Hin*dIII sites were used for subsequent cloning of the *ibpAB* operon into single *Bam*HI and *Hin*dIII restriction sites of pUHE21-2fdΔ12 followed by sequencing of the entire operon. The *groESL* operon from *E.coli* was amplified by PCR using native Pfu DNA polymerase, chromosomal DNA from *E.coli* C600 as template, forward primer 5′-AGGAGTCGACATGAATATTCGTCCATTGCAT-3′ and reverse primer 5′-AGGAGTCGATTTACATCATGCCGCCCATGC-3′. The engineered *Sal*I sites were used for subsequent cloning of the *groESL* operon into the single *Sal*I restriction site of pDS56 followed by sequencing of the entire operon. The *htpG* gene from *E.coli* was amplified by PCR using native Pfu DNA polymerase, chromosomal DNA from *E.coli* C600 as template, forward primer 5′-GGCCATGGATCCATGAAAGGACAAGAAACTCGTGGT-3′ and reverse primer 5′-GGCCATAAGCTTTCAGGAAACCAGCAGCTGGTTCAT-3′. The engineered *Bam*HI and *Hin*dIII sites were used for subsequent cloning of the *htpG* gene into single *Bam*HI and *Hin*dIII restriction sites of pUHE21-2fdΔ12 followed by sequencing of the entire gene.

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