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The role of DnaK/DnaJ and GroEL/GroES systems in the removal of endogenous proteins aggregated by heat-shock from *Escherichia coli* cells

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Abstract The submission of *Escherichia coli* cells to heat-shock (45°C, 15 min) caused the intracellular aggregation of endogenous proteins. In the wt cells the aggregates (the S fraction) disappeared 10 min after transfer to 37°C. In contrast, the S fraction in the *dnaK* and *dnaJ* mutant strains was stable during approximately one generation time (45 min). This demonstrated that neither the renaturation nor the degradation of the denatured proteins was possible in the absence of DnaK and DnaJ. The *groEL44* and *groES619* mutations stabilised the aggregates to a lesser extent. It was shown by the use of cloned genes, *dnaK*|*dnaJ* or *groEL*|*groES*, producing the corresponding proteins in about 4-fold excess, that the appearance of the S fraction in the wt strain resulted from a transiently insufficient supply of the heat-shock proteins. Overproduction of the GroEL/GroES proteins in *dnaK756* or *dnaJ259* background prevented the aggregation, however, overproduction of the DnaK/DnaJ proteins did not prevent the aggregation in the *groEL44* or *groES619* mutant cells although it accelerated the disappearance of the aggregates. The properties of the aggregated proteins are discussed from the point of view of their competence to renaturation/degradation by the heat-shock system.

Key words: Heat-shock; Endogenous protein aggregation; Removal of the aggregate; *Escherichia coli*

1. Introduction

The roles and mechanisms of action of different heat-shock proteins (molecular chaperones and proteases) were established using either reactions in vitro or experimental systems in which proteins unnatural for *Escherichia coli* like gene fusion products, or eukaryotic gene products were introduced into cells. We found a mean for the observation of endogenous proteins aggregation upon heat-shock inside cells, which makes the processes of aggregation and processing or the degradation of the aggregates amenable to research in the entirely natural system.

The purpose of this work was to elucidate to what extent the two main molecular chaperone systems are necessary for the quick disappearance of endogenous proteins aggregated by heat-shock inside *E. coli* cells. We have shown previously that a fraction composed of the aggregated proteins (S fraction) rose transiently after the transfer of a bacterial culture from 30°C to 45°C [1]. It reached its maximum 15 min after the temperature change and disappeared in the next 10 min during growth at 37°C in wt strains. The proteins forming the S fraction have specific properties. It was reported that the proteins aggregating upon heat-shock in *E. coli rpoH* are mostly those newly synthesised [2]. We found that the S fraction cosediments with membranes in a two step sucrose density gradient (SG0) ultracentrifugation but it is separable in refined gradients (SGI). Analyses showed that the fraction contains proteins but no marker components of IM and OM: NADH-oxidase activity, lipids, OmpA protein or lipopolysaccharides. The rapid disappearance of the S fraction from wt cells was attributed to the heat-shock response because in the *rpoH* mutant, which was unable to induce the response [3], the S fraction was much larger than in wt strains and stable over 45 min (approximately one generation time) [1].

Hsps DnaK, DnaJ (most of it) [1] and sHSPs, IbpA and IbpB [4] were incorporated into the S fraction, as was shown by immunoblotting. This result was in general agreement with the results obtained by others in different experimental systems [5–12]. The association with HSPs and sHSPs must be responsible for conferring the specific properties to the S fraction. The most important of them is the competence of the proteins for renaturation or proteolysis (in 10 min in the wt strain). Interesting features were revealed by the use for comparison of (i) the S fraction from wt strain, (ii) soluble proteins released from the same cells grown at 30°C and denatured in vitro (15 min, 45°C: Laskowska, unpublished results) and (iii) casein as possible substrates for the HtrA heat-shock protease in vitro [13]. The S fraction and the casein were digested by HtrA, while the cellular proteins denatured in vitro were not at all recognised as a substrate. However, the two substrates had different properties. For example, the reaction with the natural substrate, the S fraction, was stimulated by Mg^{2+} ions, while that with a fortuitous substrate, the casein, was not. The addition of DnaJ inhibited the digestion of the S fraction, but not that of the casein. The inhibition of the digestion of the S fraction by DnaJ seemed to be an important observation and was interpreted as the reflecting protection in vivo of the aggregated, endogenous proteins by DnaJ from excessive digestion by HtrA (and perhaps by other heat-shock proteases for which the S fraction probably contains substrates [13]). Such protective interaction of DnaJ with the aggregated proteins might underlie the promotion of renaturation versus proteolysis in a cell. These results demonstrated that the use of unnatural substrates may bring incomplete information in some cases.

Moreover, the proteins of the S fraction compared with those denatured in vitro showed a difference in the sedimentation pattern in the before mentioned sucrose density gradients [14]. In summary, the properties of the two kinds of denatured proteins might be explained by a difference in their

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conformation depending on high levels (in vivo) or low levels (in vitro) of HSPs and sHSPs during the denaturation. The difference resulted from the induction of the heat-shock genes during thermal denaturation of the cellular proteins within cells. The denaturation in a cell-free system must have proceeded at the constitutive level of the HSPs and sHSPs present in the extract submitted to denaturation. These findings point to the necessity of maintaining cellular, denatured proteins in specific conformations permissive for their processing or degradation.

In contrast to DnaK and DnaJ, the GroES (unpublished results of E. Laskowska), GroEL and GrpE [1,15] proteins did not associate with the proteins of the S fraction and were detectable exclusively in the soluble, CP (cytoplasmic and periplasmic) fraction. Preliminary experiments [14] with the *dnaK* and *dnaJ* or *groEL* and *groES* mutants revealed that the *dnaK* and *dnaJ* mutations stabilised the S fraction like the *rpoH* mutation. The *groEL* and *groES* mutations considerably retarded the S fraction disappearance. In this work we have estimated (by aggregated protein measurement) the accumulation of the S fraction in wt and in the *dnaJ259*, *dnaK756*, *groES619* and *groEL44* mutant cells and examined the results of the chaperone proteins overproduction from plasmid genes.

2. Materials and methods

2.1. Bacterial strains and growth conditions

HB101 [16] (F, *hsdS20* (r^-, m^-), *rec*A13, *ara*14, *pro*A2, *rps*L20, *xyl-5*, *mlt-5*, *sup*E-44, λ^-), B178 (W3101 *galE*, sup^+) *dnaK756* [17,18], B178 *dnaJ259* [19], B178 *groEL44* [20] and B178 *groES619* [20] were obtained via M. Żylicz (Gdańsk).

Mutations in either *dnaK* or *dnaJ* block the λ DNA replication at the non-permissive temperature and interfere with the *E. coli* DNA synthesis [17–19]. Mutations in either *groES* or *groEL* generally display the same phenotypes, they block the λ head morphogenesis and are temperature sensitive for bacterial growth [20].

The bacteria were grown in 100 ml of LB with aeration to $A_{600} = 0.25$, then transferred to 45°C for 15 min and further grown at 37°C. Expression of plasmid genes was induced by the addition of IPTG (1 mM) or chlorotetracyclin (12.5 μg/ml) 45 min and 2 h before a temperature shift to 45°C, respectively.

2.2. Plasmids

pJZ·589 was obtained from D. Wall (Stanford) via M. Żylicz (Gdańsk). It is a derivative of pTTQ19 (Amersham Corp.) containing the promoter *tac* inducible by IPTG and bearing ampicillin resistance: pGELS2 is a derivative of the pCattTrE18 vector (constructed in the laboratory of W. Szybalski (University of Wisconsin) by M. Koob) carrying the *groEL groES* genes [21]. The vector contains the p_{tetA} promoter controlled by the *tetR* repressor. Transcription from p_{tetA} may be derepressed by chlorotetracyclin.

2.3. Methods

Cells were fractionated according to Kucharczyk et al. [1]. Samples of bacterial cultures (100 ml) were taken at time 0 (before the temperature shift to 45°C), after 15 min at 45°C and at 25, 35, 45 min during growth at 37°C for either cell fractionation or for preparation of whole cell lysates. The samples were quickly chilled by pouring them onto 100 ml of frozen 10 mM Tris-HCl, pH 7.2 and cells were harvested by centrifugation for 10 min at 7000×*g* in a Beckman J2-HS centrifuge. The cells were suspended in 200 mM Tris-HCl buffer pH 8.0 so as to obtain $A_{600} = 28$ U/ml and the spheroplasts were made according to Witholt et al. [22]. The suspension was diluted with an equal volume of 1 M sucrose in 200 mM Tris-HCl pH 8.0 and then supplemented with egg white lysozyme solution (12 mg/ml in 100 mM EDTA pH 7.6) to a final concentration of 60 μg/ml. After 4 min on ice an equal volume of ice-cold distilled water was added. After an additional 10 min on ice the spheroplasts were subjected to sonification. Unbroken cells were removed by centrifugation at 1200×*g* for 15 min. The supernatant was used for the membrane fractionation, as described by Ishidate et al. [23] and modified by Kucharczyk et al. [1]. The supernatant was layered on a two step SG0 composed of 1 ml 55% and 6 ml 17% (w/w) sucrose in 3 mM EDTA solution pH 7.6 and centrifuged for 90 min in a Beckman SW41 Ti rotor at 240 000×*g*. Four 1 ml CP subfractions were collected from the top of the gradient. They contained soluble cytoplasmic and periplasmic proteins. Opalescent fractions of OM and IM also containing the heat-aggregated proteins were collected together (crude membrane fraction) and submitted to fractionation in a six step SGI which consisted of: 55% (1.4 ml), 50%, 45%, 40% (2.3 ml of each), 35% (1.4 ml) and 30% (0.8 ml) (w/w) sucrose in 3 mM EDTA solution pH 7.6. After centrifugation in a Beckman SW41 Ti rotor at 240 000×*g* for 16 h, 30 subfractions were collected from the bottom (total volume 12 ml). Aliquots were taken from SGI subfractions for determination of the protein concentration. The control of the exactness of separation was performed as summarised by Laskowska et al. [4]. The subfractions corresponding to discernible fractions were pooled and denoted S (buoyant density, 1.26 g/ml), OM (1.22 g/ml), IM (1.14 g/ml).

2.4. Analytical methods

Total protein was determined as described [24]. The Laemmli method [25] was used for 0.1% SDS-15% PAGE.

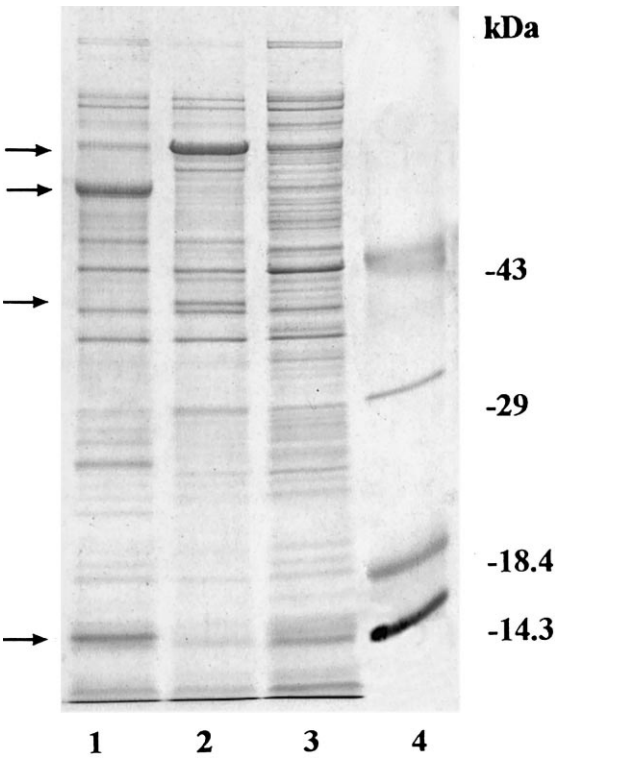

Fig. 1. Comparative estimation of the levels of the HSPs, DnaK and GroEL in cells of *E. coli* HB101(pGELS2) and HB101(pJZ·589) induced for overproduction of GroEL/GroES and DnaK/DnaJ proteins (respectively) and HB101 after heat-shock (after 15 min of the experiment). 30 μl samples were taken from lysates obtained by sonification of the spheroplast preparations (described in Section 2). Equal amounts of Laemmli lysis buffer were added to each sample and after 5 min incubation in a boiling water, bath 0.1% SDS-15% PAGE was performed. The gel was stained with Coomassie brilliant blue R250. Densitometry was carried out by scanning electropherograms by the UVP (Ultra-Violet Products) Easy densitometry system (Cambridge, UK). Lanes: (1) HB101(pGELS2), (2) HB101(pJZ·589), (3) HB101, (4) molecular mass standards. (Arrows from top to bottom show the positions of DnaK, GroEL, DnaJ and GroES).

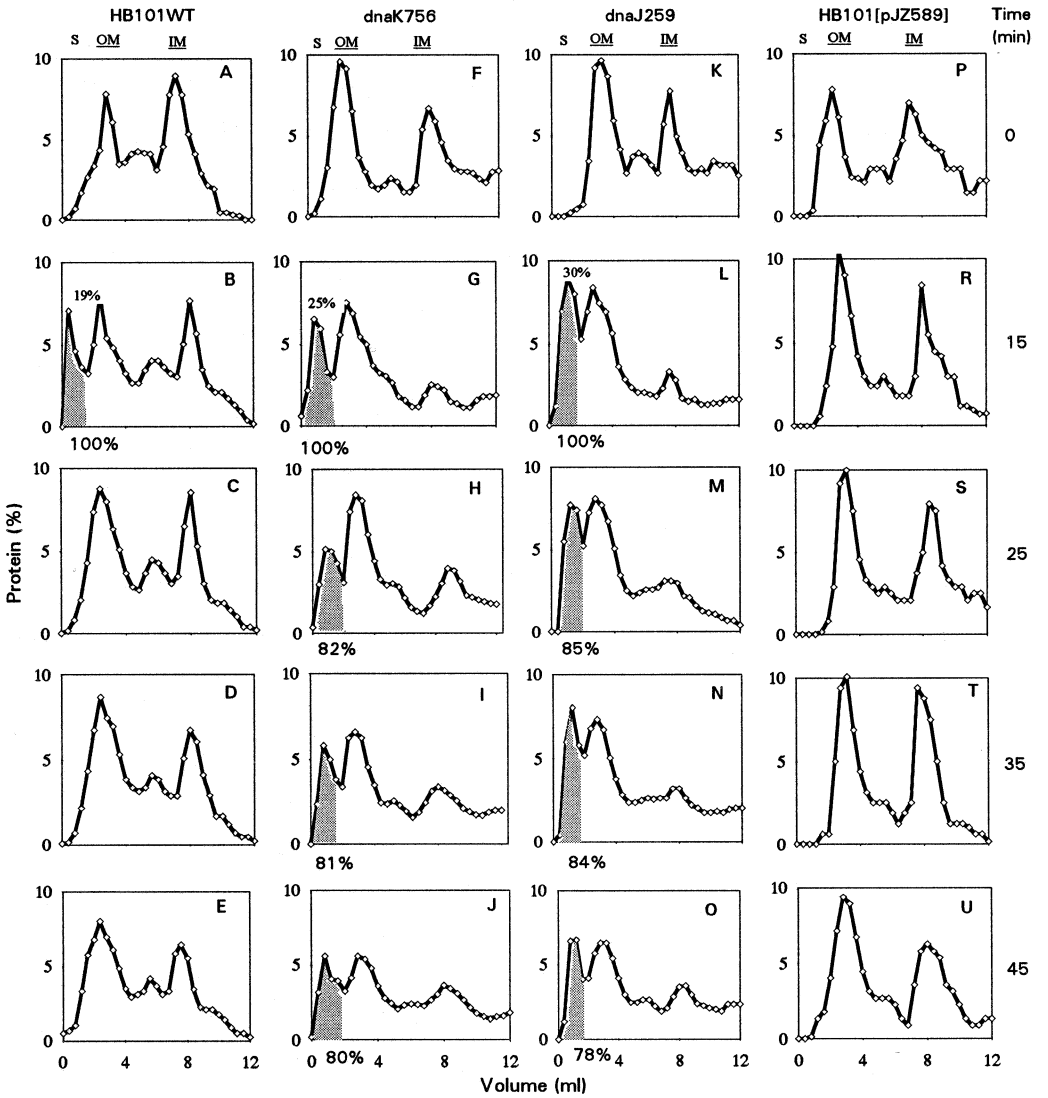

Fig. 2. The rise and decay of the fraction of aggregated proteins (S fraction) in *E. coli*: HB101WT, *dnaK756*, *dnaJ259* and HB101(pJZ·589) after heat-shock. The S fraction is shadowed. The S, OM and IM peaks are marked on the top of the figure. The % values given over the S peaks (B, G, L) are related to the total insoluble protein from the SG0 gradient (introduced onto the SGI gradient). The % values given under the S peaks are related to the total protein of the S fraction (H–J, M–O). 100% of the total protein of the S fraction corresponded to its maximal size after 15 min of the experiment after transfer of the bacterial cultures from 30°C to 45°C. The time of the removal of the samples of the bacterial cultures for analyses is marked on the right side. time 0 was immediately before the transfer to 45°C.

3. Results

The levels of the GroEL and DnaK Hsps in HB101(pGELS2) and HB101(pJZ·589) strains were compared to those in HB101 after induction by heat-shock. The comparison was based on the densitometry of electrophoretically separated cellular proteins stained with Coomassie brilliant blue (Fig. 1). The experiment showed that the the production of GroEL from pGELS2 was 3.8-fold higher than in the induced HB101 and that of DnaK from pJZ·589 was 3.6-fold higher. It was not possible to estimate the increase of the levels of DnaJ and GroES because these proteins were hardly discernible in the parent strain.

The protein content in the S fraction in wt strains corresponded to 4–5% of the total cellular protein and 13–19% of the insoluble proteins [4] as estimated by the Bradford method [24].

The rise and decay of the S fraction in the wt (control) strain, *dnaK756* and *dnaJ259* mutants and in the pJZ·589 transformant are shown in Fig. 2. The HB101 strain was used as the preferable carrier for plasmids. Samples were taken from bacterial cultures at time 0 (immediately before heat-shock), 15 min after the transfer to 45°C and further at 10 min time intervals during growth at 37°C. The bacteria were gently lysed and submitted to fractionation by sucrose density gradient ultracentrifugation. The level of the S fraction after heat-shock (Fig. 2B, the shadowed area) and the time of its disappearance in the HB101 strain was comparable to those in other strains bearing wt heat-shock genes (see discussion in [13]). The striking difference in cells their ability to cope with the aggregated proteins was introduced by *dnaK756* and *dnaJ259* mutations (Fig. 2G,J,L–O correspondingly). The S fraction was so stable in these strains that about 80% of the aggregated proteins were still present after 45 min of the ex-

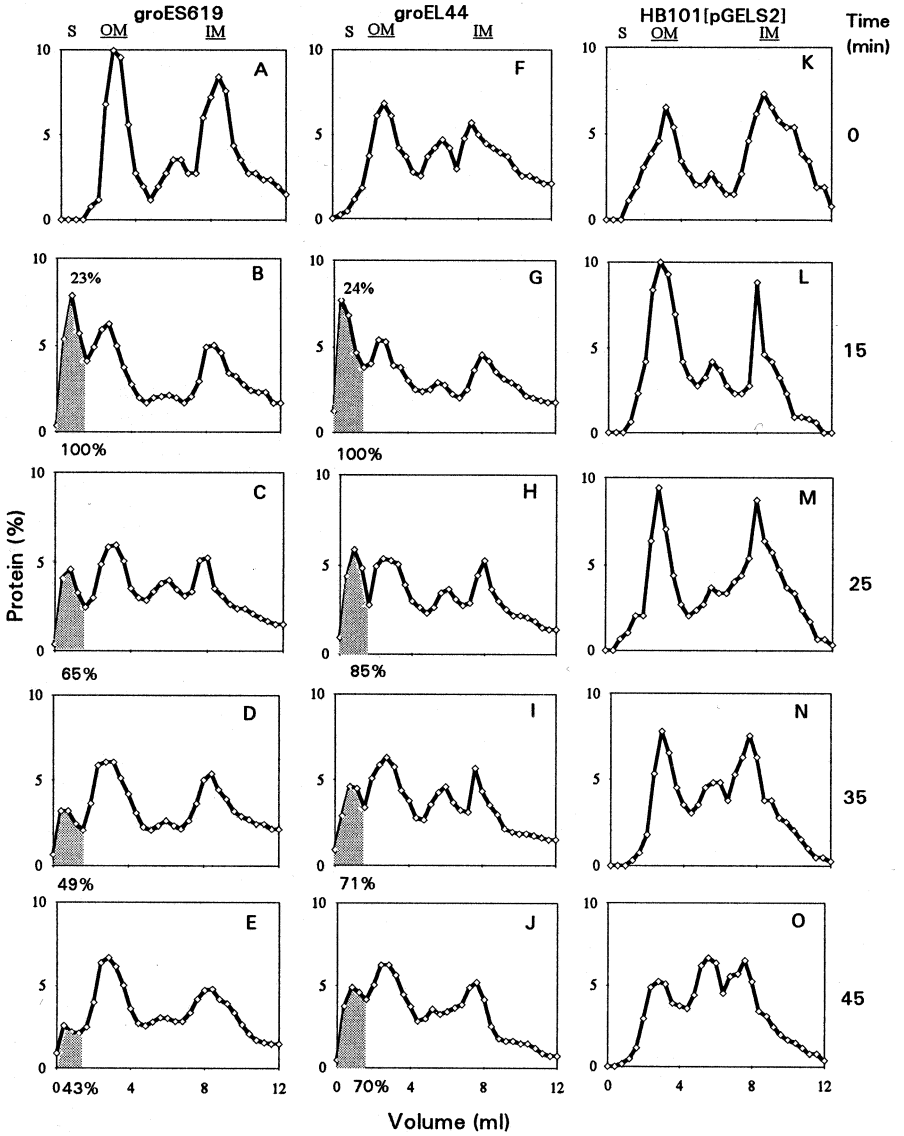

Fig. 3. The rise and decay of the S fraction in *E. coli*: *groES619*, *groEL44* and HB101(pGELS2) are submitted to heat-shock. The designations are explained under Fig. 2.

periment in both cases. Overproduction of DnaK and DnaJ proteins in HB101(pJZ·589) entirely prevented the rise of the S fraction (Fig. 2P–U).

Mutations in *groES* and *groEL* genes, similarly to the previous case, affected the stability of the S fraction. But after 45 min of the experiment 43% of the aggregated proteins remained in the *groES619* mutant, i.e. considerably less than in the *dnaK756* or *dnaJ259* mutant cells, and 70% of the aggregates were stable in the *groEL44* mutant. (Fig. 3A–E and F–J respectively). Transformation of the HB101 with pGELS2 overproducing GroEL and GroES proteins prevented the appearance of the S fraction after heat-shock like the DnaK and DnaJ overproduction in the previous experiment (Fig. 3K–O).

This seemed to mean that the two chaperone systems may mutually replace each other in the process of the removal of the aggregated proteins or in the protection of cellular proteins from aggregation since the excess of either of them prevented the appearance of the S fraction in the wt strain. Therefore, in the next set of experiments the *dnaK756* and *dnaJ256* mutants were transformed with pGELS2 and the *groES619* and *groEL44* mutants were transformed with pJZ·589. The transformants were subjected to heat-shock and fractionation. It appeared that the overproduction of the GroES and GroEL proteins in *dnaK756* or *dnaJ259* mutants (Fig. 4B–E and G–J respectively) again completely prevented the rise of the S fraction.

The situation was different in the case of the overproduction of DnaK and DnaJ proteins in the *groES619* or *groEL44* mutants. The excess of DnaK and DnaJ did not prevent the formation of the S fraction during heat-shock (i.e. after 15 min) in *groES619* mutant cells (Fig. 4L) but accelerated its disappearance so that after 25 min 31% remained and perhaps a trace of the aggregated proteins after 35 min (Fig. 4M,N respectively) which should be compared with 65% and 49% of the remains of the S fraction after 25 and 35 min in the *groES619* mutant (Fig. 3C,D). In *groEL44* mutant cells the S fraction rose to 21% of cellular insoluble proteins after 15 min and persisted to the 35th min of the experiment (15% of the S fraction) (Fig. 4R–T), in spite of the overproduction of

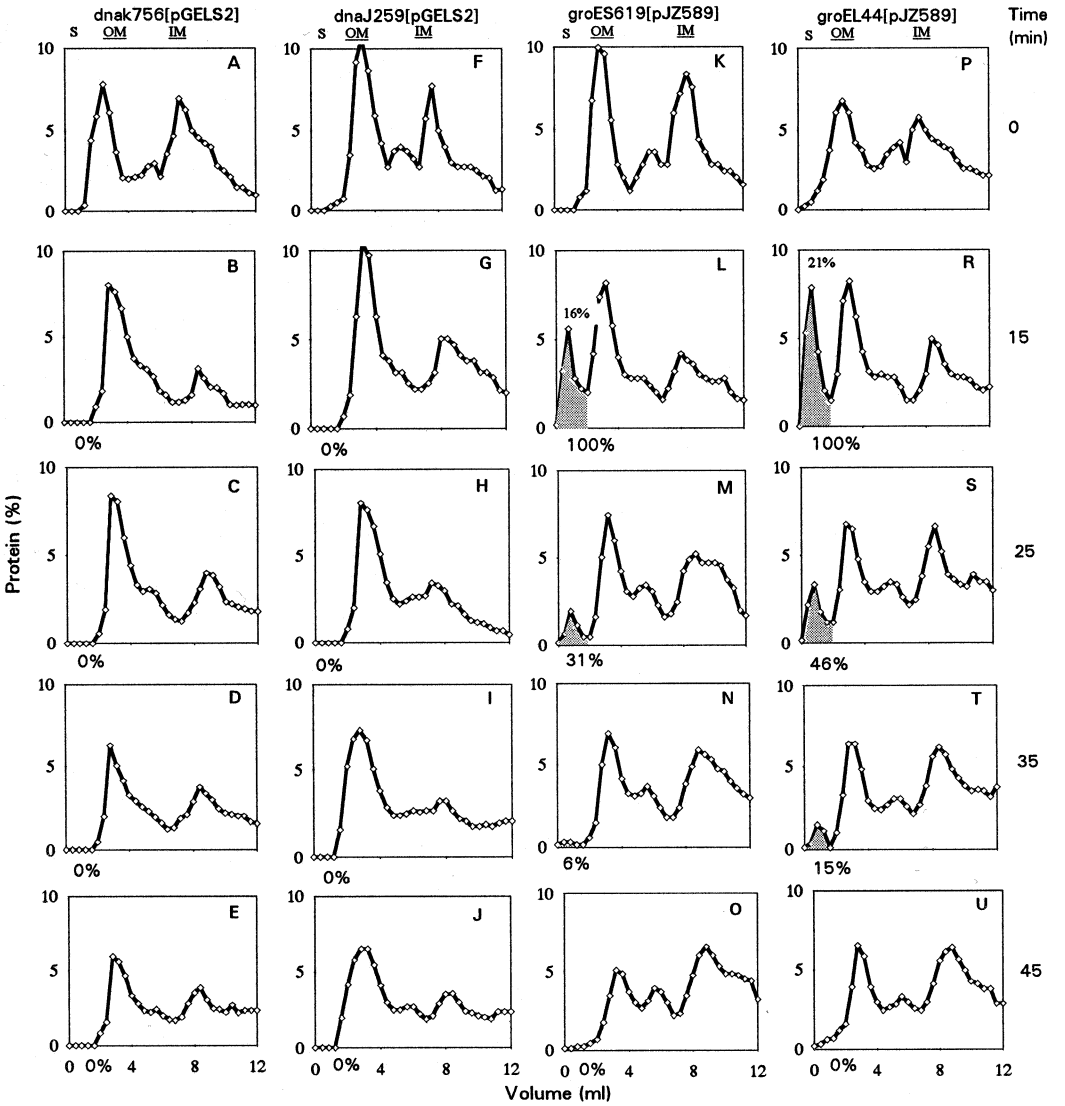

Fig. 4. The rise and decay of the S fraction in *E. coli*: *dnaK756*(pGELS2), *dnaJ259*(pGELS2), *groES619*(pJZ·589) and *groEL44*(pJZ·589) are submitted to heat-shock. The designations are described under Fig. 2.

the DnaK and DnaJ proteins. However, the disappearance of the S fraction was considerably accelerated in comparison with that in the *groEL44* mutant cells not transformed with pJZ·589. After 45 min, these cells were free of the aggregates (Fig. 4O,U). These results point to the specific role of GroEL/GroES, which cannot be substituted by DnaK/DnaJ for some proteins.

In summary, the about 4-fold excess of GroES and GroEL in the *dnaK756* and *dnaJ259* mutant strains compensated for the effects of these mutations and even prevented the rise of the S fraction 15 min after the transfer of the bacteria to 45°C. But the similar excess of DnaK and DnaJ did not fully compensate for the *groES619* and *groEL44* mutations, though acceleration of the S fraction removal was observed in comparison with the not transformed mutant cells.

4. Discussion

Protein aggregation resulting from heat-shock occurs in the cytoplasm and in the periplasm of *E. coli* as one can judge from known localisations of the HSPs playing important roles in the removal of the aggregated proteins, for example: the cytoplasmic GroEL and GroES [1,26] and the HtrA protease [13,27] associated with the inner membrane on the periplasmic side [28].

Our observations concerned the size (measured as protein content) and the time of persistence of the fraction of endogenous proteins aggregated by heat-shock, the S fraction. However, the employed technique did not allow for distinguishing whether the proteins of the S fraction disappear from cells due to their resolubilisation and renaturation or due to proteolysis. The experimental system used here permitted the avoidance of overproduction of model proteins from plasmids, which usually creates abnormally high concentrations of singular HSP substrate proteins. The large amount of unnatural protein would deplete the system of (a) particular kind(s) of chaperone(s) thus, limiting its (their) use for other processes requiring them and thus changing the overall response of the cell. In contrast to that, in the natural system, in vivo, many kinds of cellular proteins undergo denaturation at the same

time, thus require various kinds of chaperones. A similar problem concerning the aggregation of eukaryotic proteins overproduced in *E. coli* was thoroughly reviewed in [29].

The experiments presented here revealed a summary issue of the in vivo activity of the heat-shock system rescuing cells from the consequences of heat-shock, seen as the appearance/disappearance of the aggregated proteins in different genetic backgrounds (i.e. *dnaK756*, *dnaJ259*, *groEL44*, *groES619*). The deficiency of the active HSPs in cells bearing either *rpoH* [1,2], *dnaK756* or *dnaJ259* mutations, resulted in an almost complete (80%) stabilisation of the S fraction, during approximately one generation time (45 min), as we show here. This indicated that neither, renaturation nor proteolysis was possible for most of the aggregated proteins in the absence of DnaK or DnaJ in the mutant cells containing levels of GroEL and GroES normal for them and other HSPs and sHSPs. Mutations in *groES* or *groEL* caused a stabilisation of 43% and 70% of the aggregates (correspondingly) during the same time.

About a 4-fold excess of HSPs, as in the cases of HB101(wt) transformed with pJZ·589 or pGELS2 and overproducing DnaK/DnaJ or GroEL/GroES proteins (respectively), prevented completely the transient appearance of the S fraction in the 15th min of the experiment. Therefore, the appearance of the S fraction in the wt strains resulted from a transient insufficiency of a supply of HSPs after the sudden temperature increase. But the induction of the heat-shock genes in wt cells would provide soon (in 10 min) an adequate amount of HSPs, as indicated by the disappearance of the S fraction during this time [1].

The excess of GroEL/GroES also prevented the rise of the S fraction in *dnaK756* and *dnaJ259* backgrounds, which seemed to be in accord with the results of Gragerov et al. who used a *rpoH* mutant in which the expression of *dnaK* and *dnaJ* is suppressed [30]. However, the 4-fold overproduction of DnaK/DnaJ did not prevent the high rise of the S fraction in the *groES619* and *groEL44* mutant strains, though it accelerated the rate of the disappearance of the S fraction in comparison with that observed in the non-transformed mutant strains. Perhaps, in the absence of GroEL, the final steps of renaturation are impossible for a number of proteins and so they must be destined to degradation. Based on this observation one may suppose that different mechanisms may underlie the disappearance of the S fraction as a whole in the mutant backgrounds, in the sense of the prevalence of either renaturation or proteolysis of the denatured proteins.

Taking together our results and those from other laboratories, one can assume that various events occur in parallel in a cell after the temperature rise, since many proteins, different and denatured to a varied extent, are involved simultaneously.

After heat-shock, the order of events in *E. coli* cells might be as follows: cellular proteins undergoing denaturation (the S fraction) sequester DnaJ and DnaK [1,31]. These proteins negatively modulate the transcription of the heat-shock regulon [32–35] by facilitating the proteolysis of σ^{32} effected by the HflB ATP-dependent protease [36,37]. The sequestration corresponds to an emission of the signal inducing the heat-shock response dependent (among other reactions) on the stabilisation of σ^{32} [5,34,38]. Before the synthesis of HSPs and sHSPs reaches high levels, the accumulation of the S fraction occurs and attains its maximum after 15 min at 45°C. The sHSPs, IbpA and IbpB, easily inducible to high levels [39], bind to the aggregating cellular proteins [4], stabilising them and facilitating interaction with DnaJ and DnaK [12] to prevent their misfolding. IbpA and IbpB have no ATPase activity, therefore they are not supposed to refold denatured proteins by themselves. The binding of HSPs and sHSPs to the denatured proteins proceeds as they are supplied from the induced genes (practically all the cellular DnaJ was found in association with the aggregating proteins [1]). This may serve different tasks, such as protection of the proteins from aggregation, preserving them in a form competent for reactivation and renaturation of some of the proteins. For example, DnaK alone protected in vitro RNA polymerase from thermal inactivation in an ATP independent reaction and reactivated it in a process dependent on ATP [40]. Also DnaJ was able to bind (independently of DnaK) denatured rhodanese [41] and luciferase [42,43], prevent their aggregation and reactivate them. Moreover, DnaJ may protect the aggregated proteins from degradation by the heat-shock proteases as was shown for the HtrA protease [13]. There are also examples of protein reactivation in vitro by the DnaK/DnaJ not requiring cooperation of the GroEL/GroES system [42]. For some of the proteins the two chaperone systems DnaK/DnaJ and GroEL/GroES may compete for repetitive binding in the process of renaturation [44] or replace each other [45]. Nevertheless, a portion of the denatured proteins requires a GrpE-mediated and ATP-dependent transfer from the DnaK/DnaJ to the GroEL/GroES system acting in the cytoplasm [41]. An ATP-dependent iterative mechanism [46] would reactivate some of the proteins or direct for proteolysis [47] those which were irreversibly damaged. When the proteolysis was examined in the *lon, clpB, clpA, clpX, clpP and htrA* mutants it appeared that the proteins of the S fraction were the substrates [13] for the corresponding ATP-dependent, cytoplasmic, heat-shock proteases (Lon, ClpAP, ClpXP) and for the ATP-independent, periplasmic HtrA protease [27,28]. One may suppose that the other proteases [47] ClpY/ClpQ or [36] HflB, so far not tested against the S fraction, may also participate in the degradation of the damaged proteins. The ATPase subunits of the proteases also have independent chaperone activities [48,49]. A role of the two ClpB proteins (ClpB 93 kDa and ClpB 79 kDa), coded by the same gene [50] and possessing ATPase activities [51], should also be recollected here. We have shown previously that the *clpB* mutation stabilised 45% of the protein of the S fraction, measured after 45 min of the experiment, and the *clpBclpP* double mutation −88% [13]. Therefore ClpB seems to play an important role in the renaturation or proteolysis of the aggregated proteins. This finding was strengthened by the observation [52] that the viability of the Δ*clpB* strain fell down for six orders during incubation at 46°C for 4 h in contrast to the wt strain. The mechanism of action of ClpB is not known. One can suppose that it might participate in the resolubilisation of aggregated proteins since such a function was ascribed to its eukaryotic homologue, Hsp104 [53]. This seems to be a plausible explanation for the importance of ClpB in the removal of the S fraction from *E. coli* cells. In *E. coli* wt cells, these processes will take about 10 min, but they will need a much longer time in different mutant cells as indicated by the disappearance of the S fraction.

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