

Functional Dissection of Trigger Factor and DnaK: Interactions with Nascent Polypeptides and Thermally Denatured Proteins

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In *Escherichia coli*, the ribosome-associated Trigger Factor (TF) cooperates with the DnaK system in the folding of newly synthesized cytosolic polypeptides. Here we investigated the functional relationship of TF and DnaK by comparing various functional properties of both chaperones. First, we analyzed the ability of TF and DnaK to associate with nascent polypeptides and full-length proteins released from the ribosome. Toward this end, we established an *E. coli* based transcription/translation system containing physiological ratios of TF, DnaK and ribosomes. In this system, TF can be crosslinked to nascent polypeptides of σ^{32} . No TF crosslink was found to full-length σ^{32} , which is known to be a DnaK substrate. In contrast, DnaK crosslinked to both nascent and full-length σ^{32} . DnaK crosslinks critically depended on the type of chemical crosslinker. Crosslinks represent specific substrate-chaperone interactions since they relied on the association of the nascent polypeptides with the substrate binding pocket of DnaK. While DnaK is known to be the major chaperone to prevent protein aggregation under heat shock conditions, we found that TF did not prevent aggregation of thermally unfolded proteins *in vitro* and was not able to complement the heat-sensitive phenotype of a $\Delta dnaK52$ mutant *in vivo*. These data indicate that TF and DnaK show strong differences in their ability to prevent aggregation of denatured proteins and to associate with native like substrates, but share the ability to associate with nascent polypeptides.

Key words: Hsp70/Prevention of aggregation/Protein folding/Protein synthesis/ σ^{32} .

Introduction

In *Escherichia coli*, Trigger Factor (TF) as well as the DnaK and the GroEL chaperone systems assist the folding of newly synthesized proteins. TF associates in an apparent 1:1 stoichiometry with ribosomes and displays chaperone and peptidyl-prolyl-*cis/trans* isomerase (PPIase) activities *in vitro* (Stoller *et al.*, 1995; Hesterkamp *et al.*, 1996; Scholz *et al.*, 1997). TF interacts with virtually all nascent chains, even as short as 57 aa, and is located on the large ribosomal subunit near the exit channel for polypeptides. It was therefore proposed that TF is the first chaperone that interacts with nascent chains and assists co-translational folding processes (Valent *et al.*, 1995, 1997; Hesterkamp *et al.*, 1996; Bukau *et al.*, 2000). TF is present in a 2–3 fold molar excess over ribosomes in the cell (Lill *et al.*, 1988). Up to now it is not known whether TF, which is not in complex with ribosomes, has additional functions *in vivo*.

Beyond the interaction with the ribosome associated TF a subset of newly synthesized polypeptides requires further assistance for folding by the DnaK or/and the GroEL system (Hartl, 1996; Bukau *et al.*, 2000). GroEL associates with approximately 10–15% of newly synthesized polypeptides predominantly in a post-translational manner (Fayet *et al.*, 1989; Horwich *et al.*, 1993; Ewalt *et al.*, 1997). DnaK interacts with approximately 9–18% of newly synthesized proteins including polypeptides *in statu nascendi*, indicating that the DnaK system may act co- and post-translationally (Deuerling *et al.*, 1999; Teter *et al.*, 1999).

Deletion of the *dnaK* gene leads to a cold- and heat-sensitive phenotype, but the cells grow between 30 °C and 37 °C and have no severe folding defects at the permissive temperature (Bukau and Walker, 1989a; Hesterkamp and Bukau, 1998). Cells with a deletion of the *tig* gene show no defects and are viable between 15 ° and 42 °C. However, deletion of both genes, *dnaK* and *tig*, causes synthetic lethality at all temperatures tested (Deuerling *et al.*, 1999; Teter *et al.*, 1999) and goes along with extensive aggregation of more than 300 cytosolic proteins (Deuerling *et al.*, 1999; Deuerling and Bukau, unpublished results). *E. coli* thus tolerates the loss of either DnaK or TF in the presence of the other chaperone, suggesting complementary functions of the two chaperones. This is further substantiated by the finding that in cells lacking TF a 2–3 fold higher amount of newly synthesized proteins associate with DnaK (Deuerling *et al.*, 1999; Teter *et al.*, 1999).

In this study we dissected the functional properties of TF and DnaK. In order to study association with nascent

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polypeptides, we developed a cell-free transcription/translation system with physiological molar ratios of ribosomes, TF, DnaK and its co-chaperones. Furthermore, we analyzed to which extent TF can back up the DnaK system with regard to DnaK's function as a modulator of the heat shock response and as the major chaperone in preventing aggregation of heat denatured proteins. We found that DnaK is a highly versatile chaperone, which associated with protein substrates in nascent, unfolded and native states. In contrast, TF is functionally restricted to nascent substrates. This chaperone is inefficient in prevention of aggregation of thermally unfolded cellular proteins and in association with full-length σ^{32} , the transcription activator of the heat shock response and natural substrate of DnaK in its native state.

Results

Elaboration of a Transcription/Translation System with Defined Chaperone Content

To investigate the functional overlap between TF and the DnaK system we analyzed their association with substrates in the nascent state and in the native state after release from the ribosome. For that purpose we developed a cell-free transcription/translation system (t/t-system) derived from the *E. coli* strain MC4100, a wild-type (wt) strain with respect to TF and DnaK. The t/t-system was prepared by the separation of the soluble lysate (S150 extract) from ribosomes and subsequent fractionation of the S150 lysate. The method used (Behrmann *et al.*, 1998) provided a concentrated and efficient t/t-system that is free of P48 (Ffh), SecA and SecB activities important for protein secretion (Beck *et al.*, 2000). However, this system was not further characterized for its

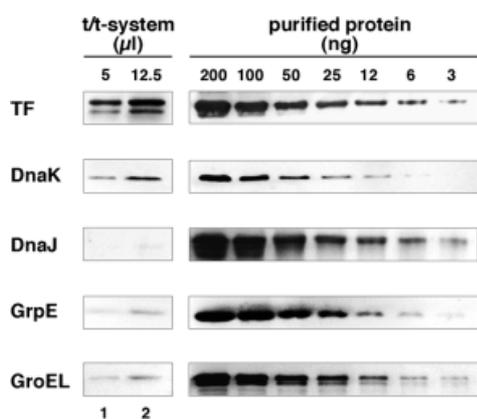


Fig. 1 Levels of Cytosolic *E. coli* Chaperones in the t/t-System. The *in vitro* t/t-system contained 6 μ l translation extract, 100 nm ribosomes and 10 units T7 polymerase per 25 μ l synthesis reaction. Five μ l and 12.5 μ l aliquots of this mixture were subjected to immunoblot analysis using chaperone-specific antisera (lane 1 and 2) except for GroEL where 12.5 and 25 μ l were applied. Serial dilutions of purified proteins served as a standard and allowed quantification of the signals in the linear range.

content of TF, the components of the DnaK system (DnaK, co-chaperones DnaJ and GrpE) and the GroEL system (GroEL and co-chaperone GroES). In *E. coli* the physiological ratios of TF:DnaK:GroEL in their active oligomeric states are approximately 3:2:0.2:1 compared to ribosomes (Lill *et al.*, 1988; Hestekamp and Bukau, 1998; Mogk *et al.*, 1999; Teter *et al.*, 1999). We determined the chaperone content of our t/t-system by quantitative immunoblotting (Figure 1) and found that all chaperones except GrpE were underrepresented compared to the ribosome concentration (100 nm) used in this system. Therefore we added purified chaperones to obtain final concentrations of 300 nm TF, 200 nm DnaK and 20 nm DnaJ, which represents physiologically relevant relative molar ratios of these chaperones. We did not supplement the GroEL system since this chaperone is supposed to act exclusively post-translationally (Ewalt *et al.*, 1997).

Crosslinking of DnaK to Nascent Polypeptides Depends on the Chemical Crosslinker

It has been reported that arrested nascent chains of proOmpA efficiently crosslink to TF in a t/t-system as

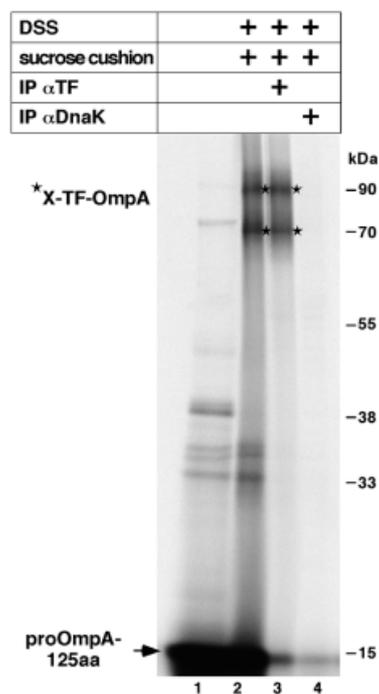


Fig. 2 Trigger Factor But Not DnaK Can Be Crosslinked to Nascent proOmpA by DSS.

Arrested nascent proOmpA polypeptides of 125 aa length were synthesized in the t/t-system in the presence of physiological chaperone ratios (lane 1; please note that additional minor bands visible in the autoradiography correspond to either residual full-length proOmpA or endogenous background products of the t/t-system). After crosslinking with DSS RNCs were purified by centrifugation through a sucrose cushion (lane 2). Aliquots were immunoprecipitated using antisera against TF (lane 3) and DnaK (lane 4). All samples were analyzed by SDS-PAGE; [35 S]-methionine labeled proteins were detected by phosphorimaging. The asterisks indicate crosslinking of TF.

prepared here using the chemical crosslinker DSS (disuccinimidyl suberate) (Beck *et al.*, 2000). We generated [³⁵S]-methionine labeled arrested nascent proOmpA polypeptides of 125 aa length as a model system to establish the optimal crosslinking not only for TF but also for DnaK. To create nascent chains of defined lengths, we used an antisense oligonucleotide approach, in which a DNA oligonucleotide hybridizes with the mRNA and the hybrid region is subsequently hydrolyzed by RNaseH, resulting in truncated mRNA (Behrmann *et al.*, 1998). Ribosome/nascent chain complexes (RNCs) were crosslinked to associated chaperones using DSS. Two crosslinking products of approximately 70 and 90 kDa were obtained (Figure 2), both of which were identified as complexes between nascent proOmpA and TF. These two TF crosslink adducts were already found earlier using this t/t-system (Beck *et al.*, 2000). The 70 kDa complex corresponds arithmetically to the complex of TF and proOmpA, whereas the 90 kDa complex migrates with an aberrant mobility for unknown reasons. In contrast to earlier studies (Behrmann *et al.*, 1998; Beck *et al.*, 2000), our t/t-system was supplemented with the DnaK system to establish physiological ratios of chaperones, ribosomes and nascent polypeptides. We nevertheless failed to detect any crosslinking between DnaK and nascent proOmpA. The used DSS crosslinker is a homo-bifunctional NHS-ester which crosslinks primary amines not more than 11.4 Å apart from each other. Targets are preferentially N-termini of polypeptides and ε-amines of lysyl residues. To test whether DSS is suitable to generate crosslinks between

nascent proOmpA polypeptides and DnaK, we localized the lysyl residues on the surface of the DnaK substrate binding domain structure (Zhu *et al.*, 1996). In Figure 3 lysyl residues surrounding the actual substrate binding pocket are indicated by numbers. The nearest lysyl residue (residues 446 of the DnaK sequence) that may be crosslinked to a substrate bound in the substrate binding pocket is approximately 10 Å away from the binding cavity. This distance is at the borderline for DSS crosslinking. Considering that the partner lysyl residue of the bound substrate is perhaps not in optimal distance, this may lead to inefficient or no crosslinking using DSS, although the chaperone-substrate complex is actually formed.

Therefore, we next tested the hetero-bifunctional crosslinker EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide]. EDC first reacts with carboxyl groups thereby forming an amine reactive intermediate. Subsequently, amino groups are covalently linked to the carboxyl-termini of the first reaction partner without any spacing. In the substrate binding domain of DnaK five amino acids with carboxyl side chains are located adjacent to the substrate binding pocket (Glu402, Glu430, Glu431, Glu402, Asp530, Asp540) and offer additional crosslinking possibilities within a distance ranging from approximately 8 Å for Glu402 and Glu430 up to about 15 Å for Asp530 (Figure 3). The results of crosslinking of [³⁵S]-labeled arrested proOmpA nascent chains (125 aa) with EDC in the t/t-system are shown in Figure 4. Two crosslinking products were identified, a major adduct at approximately 70 kDa and a minor product of approximately 90 kDa. Co-im-

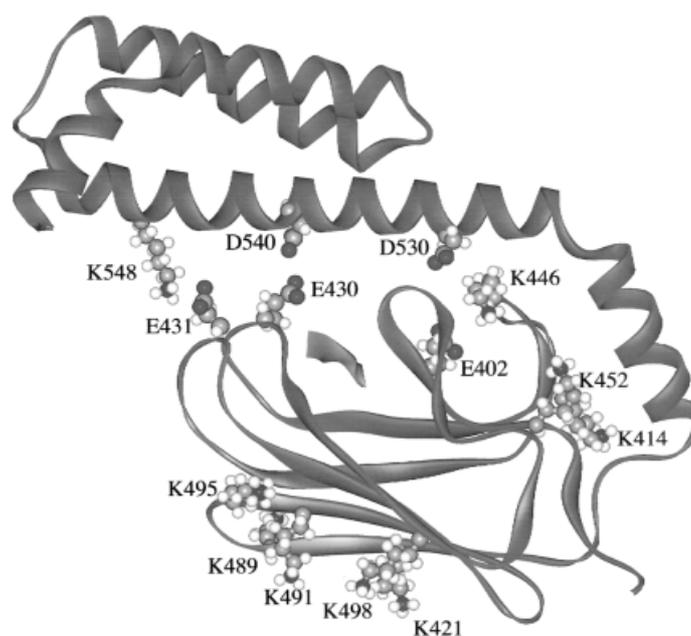


Fig. 3 Potential Amino Acid Side Chains for Crosslinking in the Substrate Binding Domain of DnaK. Secondary structure representation of a DnaK fragment representing most of its substrate binding domain (Zhu *et al.*, 1996). The amino acid side chains of lysyl, aspartyl and glutamyl residues that are potential targets of the chemical crosslinkers DSS (lysyl residues) and EDC (lysyl, aspartyl or glutamyl residues) are shown in space filling representation and indicated by their residue number of the full-length DnaK. The co-crystallized bound peptide substrate is represented by the isolated fragment in the center. The representation of the DnaK substrate binding domain was created using WebLabViewer.

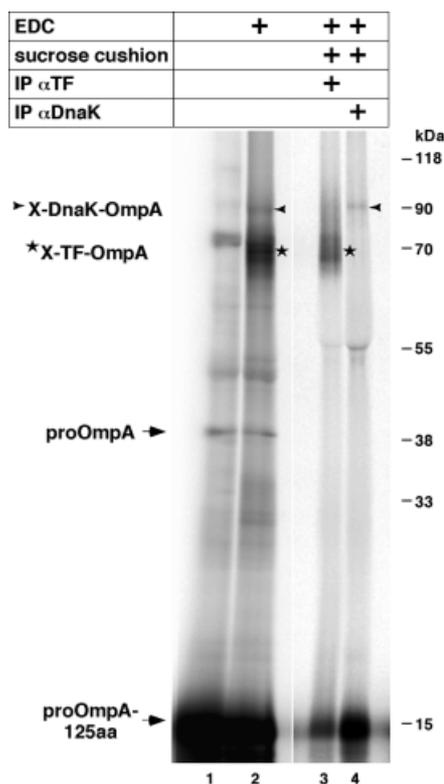


Fig. 4 EDC Crosslinking Reveals Interaction of both Trigger Factor and DnaK with Nascent proOmpA.

ProOmpA nascent chains of 125 aa length were synthesized in the t/t-system in the presence of physiological chaperone ratios (lane 1). Crosslinking with EDC (lane 2) was followed by centrifugation through a sucrose cushion and immunoprecipitation with anti-TF and anti-DnaK antibodies (lanes 3 and 4). The samples were analyzed by SDS-PAGE and phosphorimaging. Crosslinking products with DnaK (arrow) and TF (asterisk) are indicated.

munoprecipitation identified TF as the crosslinking partner of the 70 kDa product and DnaK as partner of the 90 kDa product. The fuzziness of the crosslinking products is likely due to the fact that the chemical crosslinker may link each protein at various positions.

We showed that TF as well as DnaK could be crosslinked to nascent proOmpA. Furthermore, we conclude that a careful selection of the appropriate crosslinking agent is necessary to exclude wrong negative results, since crosslinking of DnaK to substrates critically depended on the specificity of the crosslinker and the accessibility of the residues for crosslinking in the chaperone and the substrate.

Crosslinking of DnaK to Polypeptides Represents a Specific Chaperone-Substrate Interaction

In order to prove that the crosslinking products obtained in our t/t-system represent real substrate-chaperone complexes, we made use of two variants of DnaK. The DnaK-V436F mutant protein is dramatically impaired in substrate interaction due to F436 blocking the hydrophobic substrate binding pocket (Mayer *et al.*, 2000). The

DnaK-Q424C mutant protein can be modified with the UV-inducible crosslinker BPIA (benzophenone-4-iodoacetamide) via the single C424 residue, thereby allowing specific crosslinking between DnaK and substrates bound to the substrate binding cavity (Laufen *et al.*, 1999). To probe the interaction of the two DnaK variants with nascent substrates, we used proOmpA nascent chains generated in our t/t-system. Since this system contains already 60 nM wt DnaK, we added the DnaK mutant proteins or wt DnaK as control in a 20-fold molar excess over ribosomes to efficiently compete with the endogenous DnaK. UV exposure of the t/t-system led to crosslinking of DnaK-Q424C carrying BPIA to nascent proOmpA. Figure 5 (lanes 5, 6) showed two crosslinking products of about 90 and 85 kDa, respectively. Although crosslinking efficiency was much lower compared to EDC crosslinking, the 90 kDa UV-adduct corresponds to the 90 kDa crosslink detected with EDC (Figure 5, compare lanes 2 and 3 with lanes 5 and 6). Furthermore, addition of DnaK-V436F mutant protein and subsequent EDC crosslinking led to a dramatically decreased efficiency of formation of the 90 kDa crosslink product between DnaK and the substrate. The residual crosslink visible in Figure 5, lane 8, may result from the endogenous wild-type DnaK. Taken together, these experiments show that crosslinking with EDC specifically monitors chaperone-substrate interactions.

TF Does Not Associate with Native σ^{32} , a Substrate Protein of DnaK

σ^{32} is the heat shock gene-specific sigma subunit of RNA polymerase and is, in its native state, a substrate of DnaK *in vivo*. The association with DnaK modulates the activity and half-life of σ^{32} (Georgopoulos *et al.*, 1994). We chose σ^{32} as a model substrate to investigate the extent by which the functions of TF and DnaK overlap. We first analyzed the association of both chaperones with nascent σ^{32} by generating arrested nascent chains of 215 aa length in the t/t-system. EDC crosslinking resulted in several products of sizes ranging from 70 kDa to 120 kDa (Figure 6A, lane 2). Co-immunoprecipitation revealed that the crosslinking products correspond either to crosslinks of nascent σ^{32} with TF (70 and 85 kDa adducts) or with DnaK (75, 85, 100 and 120 kDa). No crosslinking product was large enough to accommodate the simultaneous crosslinking of both chaperones to the same nascent chain, although formation of a ternary σ^{32} -DnaK-TF complex cannot be excluded. Several contact sites between nascent σ^{32} and each chaperones appear to exist since multiple crosslinking species with diverse mobilities in the SDS-PAGE were detectable.

We then analyzed the interaction of TF and DnaK with full-length σ^{32} in the t/t-system. After synthesis of full-length σ^{32} and its release from ribosomes, the folding to its native state was allowed to proceed by incubation at 37 °C for at least 10 min. Subsequently EDC was added to crosslink chaperones associated with native σ^{32} (Fig-

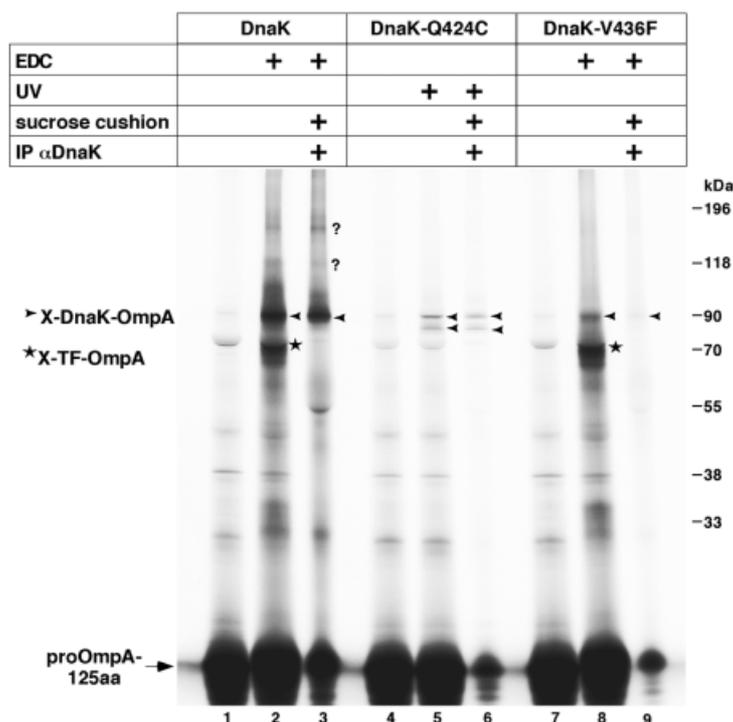


Fig. 5 DnaK Crosslinks Represent Specific Chaperone-Substrate Interactions.

Arrested nascent proOmpA polypeptides of 125 aa length were synthesized in the t/t-system in the presence of 2 μ M DnaK, DnaK-Q424C or DnaK-V436F and 0.2 μ M DnaJ. For crosslinking the samples were either treated with EDC or (in the case of DnaK-Q424C) UV-irradiated as indicated. After crosslinking the RNCs were purified by centrifugation through a sucrose cushion and immunoprecipitated using antisera against DnaK. All samples were analyzed by SDS-PAGE and phosphorimaging. Crosslinking products with DnaK (arrow) and TF (asterisk) are indicated. Question marks indicate DnaK crosslinks of high molecular size only present when DnaK was added in 20-fold molar excess over ribosomes. Lane 9: please note that co-immunoprecipitation efficiency of DnaK crosslink products is low most probably due to less efficient recognition of DnaK-V436F by the DnaK antibodies.

ure 6B, lanes 1 and 2). While no crosslinking to TF occurred (Figure 6B, lanes 2 and 3) crosslinking to DnaK resulted in an adduct of about 100 kDa (Figure 6B, lanes 2 and 4). Three additional crosslinking products were found in the high molecular weight region >120 kDa, which probably represent complexes between RNA polymerase subunits (RNAP) and σ^{32} . The addition of an excess of DnaK abolished the three high molecular weight products (Figure 6B, lane 6). Since DnaK is known to compete with RNAP for association with native σ^{32} (Arsène *et al.*, 1999), this further substantiates that these crosslinks correspond to the association of σ^{32} with the RNAP in the t/t-system.

Taken together, these results show that DnaK associates with both, nascent and native σ^{32} . TF, however, specifically crosslinks to nascent σ^{32} but not to the native protein, indicating that TF has no effect on the regulatory interaction of DnaK with σ^{32} .

TF Cannot Substitute for DnaK in Preventing the Aggregation of Unfolded Proteins

DnaK is known to be the most efficient chaperone in preventing aggregation of thermally unfolded proteins in *E. coli* (Mogk *et al.*, 1999). To further analyze the extent by

which TF and DnaK overlap in their chaperone activities, we compared the capacity of both chaperones to prevent aggregation of thermally denatured proteins. We prepared cell extracts from Δ dnaK52 cells grown at 30 °C. Heat treatment of this extract caused massive aggregation of proteins as evidenced by the electrophoretic separation of the aggregated pellet fraction on SDS-PAGE (Figure 7, lane 1: control with BSA added; Mogk *et al.*, 1999). The exogenous addition of DnaK and its co-chaperones prior to the heat treatment suppressed aggregation in a concentration dependent manner (Figure 7, lanes 2 and 3). In contrast, when TF was added to the same or even higher concentration than DnaK, protein aggregation was not efficiently prevented. The concentration of aggregating proteins in this extract was approximately 5–10 μ M (Mogk *et al.*, 1999). The supplementation with 7.5 up to 15 μ M TF established at least a 1:1 stoichiometry of non-ribosomal TF and potential substrates, irrespective of the endogenous TF partially complexed with ribosomes. To test whether TF and DnaK act cooperatively, we supplemented the extract with DnaK and TF in various combinations (Figure 7, lanes 6–8). However, the degree of aggregation prevention depended only on the presence of the DnaK system and was not altered by further addition of TF. We conclude that, in

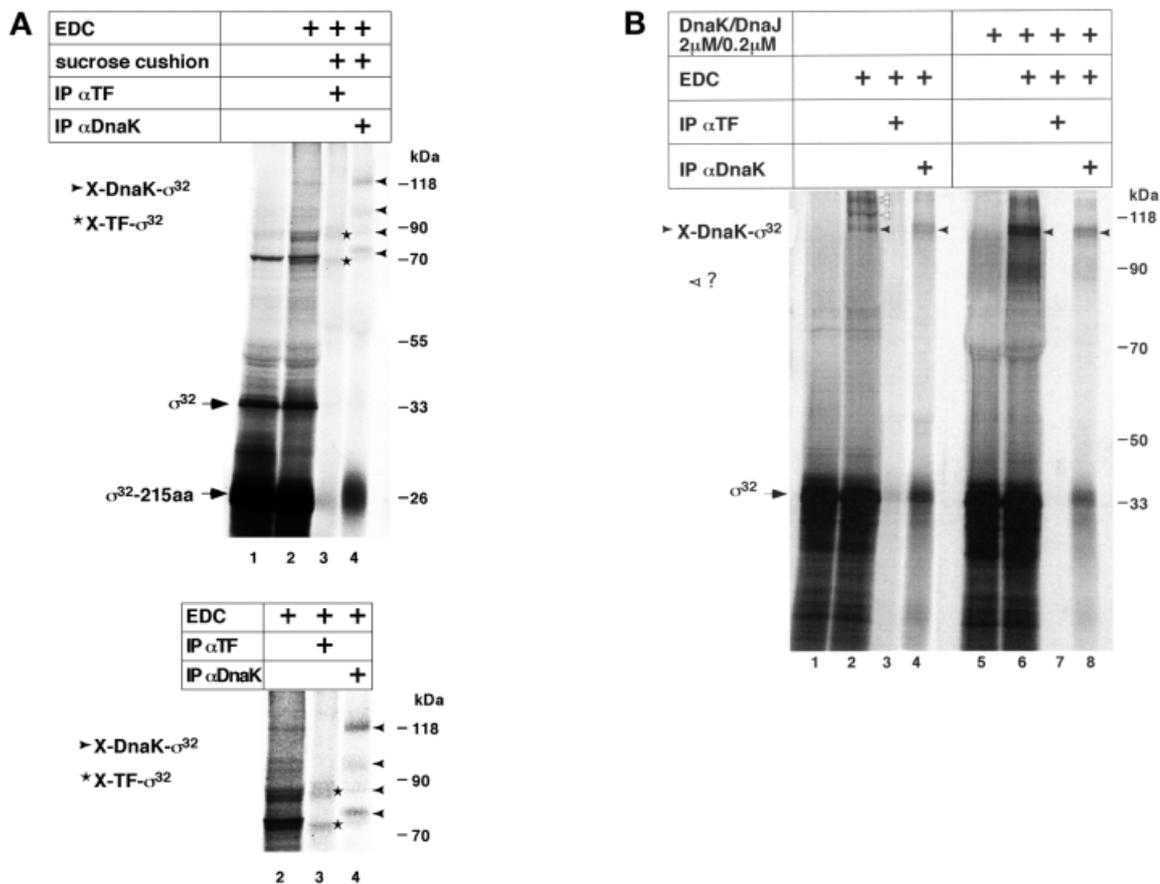


Fig. 6 Trigger Factor and DnaK Both Bind to Nascent σ^{32} Polypeptides Whereas Only DnaK Crosslinks to Native σ^{32} . (A) σ^{32} arrested nascent chains of 215 aa length were synthesized in the presence of physiological ratios of chaperones (lane 1). After crosslinking with EDC (lane 2) the RNCs were purified by centrifugation through a sucrose cushion and subjected to immunoprecipitation using antisera directed against TF and DnaK (lanes 3 and 4). Crosslinking products were analyzed by SDS-PAGE. The panel at the bottom is a magnification of the lanes 2 to 4 for better visualization. (B) Full-length σ^{32} was generated in the t/t-system under physiological chaperone ratios (lane 1) or in the presence of 2 μ M DnaK and 0.2 μ M DnaJ (20-fold molar excess over ribosomes, lane 5). After synthesis EDC was added for crosslinking (lane 2 and 6). Aliquots were immunoprecipitated using antisera against TF (lane 3 and 7) and DnaK (lane 4 and 8). All samples were analyzed by SDS-PAGE and phosphorimaging. The black arrow indicates the position of the crosslinking product specific for DnaK, white arrows mark potential crosslinks with RNA polymerase, and asterisks in (A) indicate TF crosslinks.

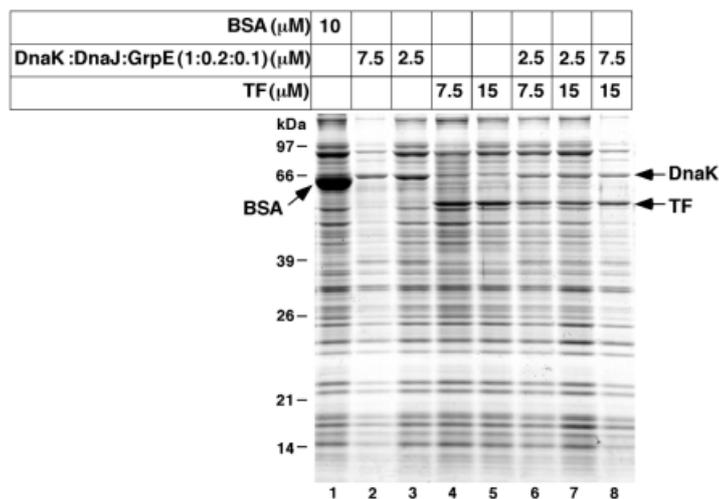


Fig. 7 TF Cannot Efficiently Prevent the Aggregation of Thermally Unfolded Proteins *in Vitro*. Coomassie Blue stained SDS-PAGE of heat-denatured aggregated proteins. Extracts of total soluble cellular proteins isolated from Δ dnaK52 cells grown at 30 °C were incubated for 15 min at 45 °C in the presence of BSA (control) or chaperones. Aggregated material was pelleted as described (Mogk *et al.*, 1999), solubilized in 30 μ l SDS-sample buffer and separated by electrophoresis using a 12% SDS-PAGE.

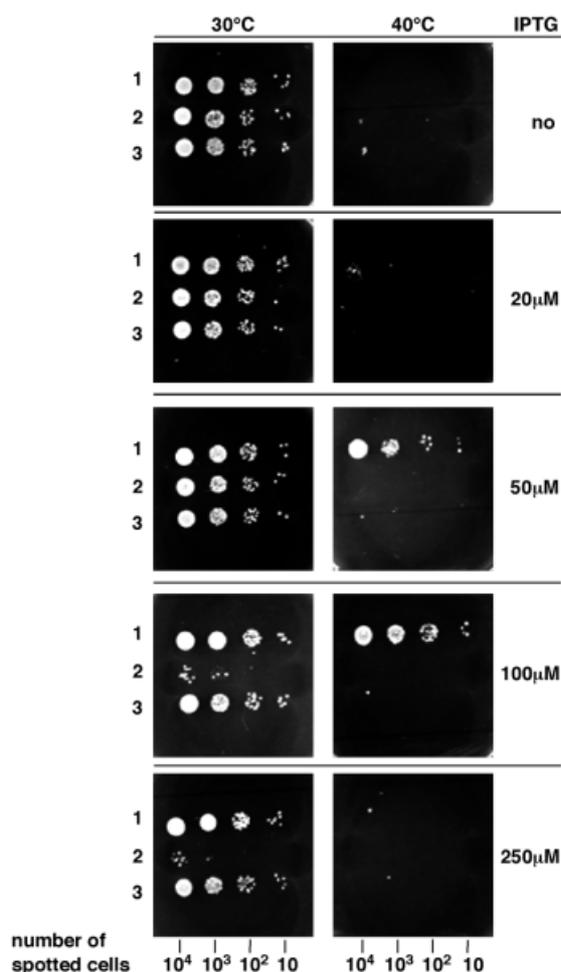


Fig. 8 Trigger Factor Is Unable to Complement the Heat-Sensitive Phenotype of $\Delta dnaK52$ Cells.

$\Delta dnaK52$ cells carrying a plasmid expressing $lacI^Q$ repressor were transformed with plasmids either expressing TF (lanes 2) or DnaK (lanes 1) by IPTG induction. For a control cells were transformed with the vector plasmid (lanes 3). Cells were spotted in different dilutions on agar plates supplemented with 20, 50, 100 and 250 μ M or without IPTG. Plates were incubated at 30 or 40 $^{\circ}$ C for 24 h.

contrast to DnaK, TF is inefficient in suppression of aggregation of thermolabile proteins *in vitro*. TF neither substitutes for DnaK nor cooperates with the DnaK system in preventing the aggregation of bulk *E. coli* proteins during heat treatment.

To investigate whether this conclusion is also valid for the *in vivo* situation, we tested whether overexpression of TF can rescue the temperature-sensitive phenotype of a $\Delta dnaK52$ strain (Figure 8). Cells lacking DnaK do not form colonies at 40 $^{\circ}$ C (Bukau and Walker, 1989b). Overexpression of the *dnaK* gene encoded by a plasmid under the control of an IPTG inducible promoter complements the heat-sensitive growth of $\Delta dnaK52$ cells at 40 $^{\circ}$ C (Figure 8, lanes 1). This phenotype could not be complemented by the overproduction of TF encoded by a plasmid under the control of an IPTG regulatable promoter. Moreover, the massive overproduction of TF after induc-

tion of its synthesis with 100 μ M IPTG (corresponding to approx. 10 to 15-fold of cellular TF levels) was lethal for $\Delta dnaK52$ cells at 30 $^{\circ}$ C (Figure 8, lanes 2). This observation was also made with wt *E. coli* cells overproducing TF (S. Vorderwülbecke and E. Deuring, unpublished results).

In summary, these data indicate that TF does not interact productively with thermally unfolded protein substrates to prevent their aggregation and therefore cannot substitute for DnaK during thermal stress conditions both *in vitro* and *in vivo*.

Discussion

The association of TF and DnaK with nascent polypeptides was investigated by using an *in vitro* *E. coli* based transcription/translation system. To work close to the physiological ratios of chaperones we determined the amount of chaperones present in our t/t-system and supplemented the system with chaperones that were under-represented compared to their estimated cellular concentration (TF, DnaK, and its co-chaperone DnaJ). This approach created a transcription/translation system with defined amounts of ribosomes and chaperones and provided conditions comparable to the *in vivo* situation. To our knowledge this is the first described t/t-system operating under defined ribosome and chaperone conditions. This system will also be a very good tool for further investigations of chaperone functions *e. g.* by preparation of the t/t-system from chaperone mutant strains.

Using the chemical lysyl-specific crosslinker DSS we found exclusively TF covalently linked to nascent pro-OmpA chains whereas the bifunctional EDC crosslinker gave crosslinking adducts with TF as well as with DnaK. These results clearly indicate that crosslinking critically depends on the specificity of the used crosslinking agent and on the formed chaperone-substrate complex. Negative evidence therefore has to be interpreted with caution since it may result simply from the inability of the chemical crosslinker to covalently fix the chaperone-substrate interaction. This might be the reason that earlier studies did not detect DnaK as crosslinking partner of nascent polypeptide chains (Valent *et al.*, 1995, 1997; Beck *et al.*, 2000).

We investigated the functional redundancy of the ribosome-associated chaperone TF and the cytosolic DnaK chaperone system. This study indicates that TF function *in vitro* and *in vivo* is restricted on substrates in the nascent polypeptide state. This was shown by different approaches: (i) TF crosslinked to nascent σ^{32} but an association with released full-length σ^{32} could not be detected. In contrast, DnaK associated with both stages of this protein. (ii) We could not detect an efficient prevention of aggregation by TF of thermally denatured *E. coli* proteins. Obviously soluble TF is not able to associate efficiently with unfolded proteins, a finding that is supported by recent results showing that soluble TF has a K_D for peptide

substrates in the high μM range (approx. 50 μM , D. Brehmer and B. Bukau, unpublished results). (iii) TF cannot complement the heat-sensitive phenotype of ΔdnaK52 mutant cells.

DnaK, like TF, has the capacity to associate with nascent substrates of both, proOmpA and σ^{32} derived nascent polypeptides. Since DnaK has this broad functional spectrum, the loss of TF can be tolerated in the presence of DnaK functioning as a back up system for TF either by acting on nascent polypeptides or by assisting the re-folding of unproductively folded proteins post-translationally. Conclusions in respect to the quantitative nature of the association of DnaK with nascent polypeptides could not be drawn. Recent results indicate that TF and DnaK efficiently compete with each other for binding to nascent proOmpA due to the fact the substrate binding sites of both chaperones overlap in this substrate (E. Deuerling und Bukau, unpublished results). Most likely the localization of TF next to the polypeptide exit of the ribosomes gives TF the advantage to associate much more efficiently with nascent polypeptides compared to DnaK.

Materials and Methods

Culture Conditions

For complementation analysis ΔdnaK52 cells carrying a plasmid (spectinomycin^R) expressing lacI^Q repressor were transformed with plasmids (ampicillin^R) either expressing TF (Hesterkamp *et al.*, 1997) or DnaK (Tomoyasu *et al.*, 1998) by IPTG induction. Cells were grown overnight at 30 °C in LB with 50 $\mu\text{g}/\text{ml}$ spectinomycin and 100 $\mu\text{g}/\text{ml}$ ampicillin and subsequently spotted on LB plates containing 50 $\mu\text{g}/\text{ml}$ spectinomycin and 100 $\mu\text{g}/\text{ml}$ ampicillin.

Isolation of Cell Extracts and Preparation of Aggregates

Cells of the *Escherichia coli* MC4100 ΔdnaK52 mutant strain were grown in 100 ml LB medium at 30 °C up to exponential growth phase, immediately chilled on ice for 10 min and subsequently harvested by centrifugation (10 min, 4000 g, 4 °C). Pellets were resuspended and washed twice in breakage buffer (50 mM HEPES pH 7.6, 150 mM KCl, 20 mM MgCl₂, 10 mM DTT) and lysed in a precooled french press cell at 5.6 MPa. After centrifugation (30 min, 30 000 g, 4 °C), the concentration of soluble proteins in the supernatant was determined in a Bradford assay with BSA as standard. For thermal aggregation 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 for 15 min to 45 °C 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 using 12% SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue staining.

In Vitro Transcription/Translation and Chemical Crosslinking

Preparation of translation extracts from MC4100 *E. coli* cells and generation of arrested nascent chains were performed as described (Beck *et al.*, 2000). Extracts were analyzed for chaper-

one contents by quantitative immunoblotting with specific antisera as described (Mogk *et al.*, 1999). To obtain concentrations close to physiological molar ratios, purified TF (300 nM, final concentration), DnaK (200 nM, final conc.) and DnaJ (20 nM, final conc.) were added to 100 nM of ribosomes in the translation extracts. For the generation of σ^{32} the plasmid pFN476 (Sankar *et al.*, 1993) and for proOmpA the p717OmpA plasmid (Beck *et al.*, 2000) was used. For transcription 10 ng/ μl of plasmid and 0.4 U/ μl T7 polymerase were added to the t/t-system (final volume of 25 μl) and reactions were started by supplementation with 0.3 $\mu\text{Ci}/\mu\text{l}$ [³⁵S]-methionine. Arrested nascent chains were produced by the addition of 0.16 $\mu\text{g}/\mu\text{l}$ antisense-oligonucleotide (5'-CG-GTTTGCCGCCTGCTCTTCCCAGC-3' for σ^{32} and 5'-TAAACGT-TGGATTAGTGTC-3' for proOmpA) together with 0.12 $\mu\text{g}/\mu\text{l}$ anti-ssrA oligonucleotide (5'-TTAAGCTGCTAAAGCGTAGTTTT-CGTCGTTTGCGACTA-3'). After 30 min, the crosslinker DSS (in DMSO, 2.5 mM final concentration) or EDC (in H₂O, 80 mM final concentration) were added for 30 min at 30 °C, the reaction was quenched with 50 mM Tris/HCl pH7.5 or 100 mM glycine, 10 mM NaHCO₃, respectively, for 15 min on ice and ribosomal complexes were purified as described (Hesterkamp *et al.*, 1997). The pellet was resolubilized in PBS buffer and co-immunoprecipitation performed with DnaK- or TF-specific antisera as described (Deuerling *et al.*, 1999). Co-immunoprecipitated material was solubilized in 25 μl SDS-sample buffer for 6 min at 100 °C. For control of crosslinking products one sample of the t/t-extract was precipitated with 5% TCA immediately after crosslinking and the pellet was resolubilized in 20 μl sample buffer and incubated at 100 °C for 5 min. Samples were loaded on an SDS-PAGE and crosslinking products subsequently visualized by autoradiography.

UV-Crosslinking Experiments

For crosslinking of DnaK-Q424C to nascent σ^{32} the cysteine reactive crosslinker BPIA (benzophenon-4-iodoacetamid) was attached to C424 of DnaK according the protocol of Laufen *et al.* (1999).

After generation of arrested σ^{32} nascent polypeptides the t/t system was exposed to UV light (365 nm, 100 W, 5 cm distance between the light source and the sample) for 5 min. After sucrose cushion centrifugation the DnaK-Q424C crosslinking products were co-immunoprecipitated as described (Deuerling *et al.*, 1999) and analyzed as described above.

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References

- Arsène, F., Tomoyasu, T., Mogk, A., Schirra, C., Schulze-Speckling, A., and Bukau, B. (1999). Role of region C in regulation of the heat shock gene-specific sigma factor of *Escherichia coli*, σ^{32} . *J. Bacteriol.* 181, 3552–3561.
- Beck, K., Wu, L.-F., Brunner, J., and Müller, M. (2000). Discrimination between SRP- and SecA/SecB-dependent substrates involves selective recognition of nascent chains by SRP and trigger factor. *EMBO J.* 19, 134–143.

- Behrmann, M., Koch, H.-G., Hengelage, T., Wieseler, B., Hoffschulte, H.K., and Müller, M. (1998). Requirements for the translocation of elongation-arrested, ribosome-associated OmpA across the plasma membrane of *Escherichia coli*. *J. Biol. Chem.* **273**, 13898–13904.
- Bukau, B., and Walker, G.C. (1989a). Cellular defects caused by deletion of the *Escherichia coli dnaK* gene indicates roles for heat shock protein in normal metabolism. *J. Bacteriol.* **171**, 2337–2346.
- Bukau, B., and Walker, G.C. (1989b). $\Delta dnaK$ mutants of *Escherichia coli* have defects in chromosomal segregation and plasmid maintenance at normal growth temperatures. *J. Bacteriol.* **171**, 6030–6038.
- Bukau, B., Deuerling, E., Pfund, C., and Craig, E.A. (2000). Getting newly synthesized proteins into shape. *Cell* **101**, 119–122.
- Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. (1999). Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature* **400**, 693–696.
- Ewalt, K.L., Hendrick, J.P., Houry, W.A., and Hartl, F.U. (1997). *In vivo* observation of polypeptide flux through the bacterial chaperonin system. *Cell* **90**, 491–500.
- Fayet, O., Ziegelhoffer, T., and Georgopoulos, C. (1989). The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.* **171**, 1379–1385.
- Georgopoulos, C., Liberek, K., Zylicz, M. and Ang, D. (1994). Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response. In: *The biology of heat shock proteins and molecular chaperones*, R.I. Morimoto, A. Tissières and C. Georgopoulos, eds. (Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press), pp. 209–250.
- Hartl, F.U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571–580.
- Hesterkamp, T., and Bukau, B. (1998). Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in *E. coli*. *EMBO J.* **17**, 4818–4828.
- Hesterkamp, T., Hauser, S., Lütcke, M., and Bukau, B. (1996). *Escherichia coli* trigger factor is a prolyl-isomerase that associates with nascent polypeptide chains. *Proc. Natl. Acad. Sci. USA* **93**, 4437–4441.
- Hesterkamp, T., Deuerling, E., and Bukau, B. (1997). The amino-terminal 118 amino acids of *Escherichia coli* trigger factor constitute a domain that is necessary and sufficient for binding to ribosomes. *J. Biol. Chem.* **272**, 21865–21871.
- Horwich, A.L., Brooks Low, K., Fenton, W.A., Hirshfield, I.N., and Furtak, K. (1993). Folding *in vivo* of bacterial cytoplasmic proteins: role of GroEL. *Cell* **74**, 909–917.
- Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Reinstein, J., and Bukau, B. (1999). Mechanism of regulation of Hsp70 chaperones by DnaJ co-chaperones. *Proc. Natl. Acad. Sci. USA* **96**, 5452–5457.
- Lill, R., Croke, E., Guthrie, B., and Wickner, W. (1988). The 'trigger factor cycle' includes ribosomes, presecretory proteins and the plasma membrane. *Cell* **54**, 1013–1018.
- Mayer, M.P., Schröder, H., Rüdiger, S., Paal, K., Laufen, T., and Bukau, B. (2000). Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nature Struct. Biol.* **7**, 586–593.
- Mogk, A., Tomoyasu, T., Goloubinoff, P., Rüdiger, S., Röder, D., Langen, H., and Bukau, B. (1999). Identification of thermolabile *E. coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* **18**, 6934–6949.
- Sankar, P., Hutton, M.E., VanBogelen, R.A., Clark, R.L., and Neidhardt, F.C. (1993). Expression analysis of cloned chromosomal segments of *Escherichia coli*. *J. Bacteriol.* **175**, 5145–5152.
- Scholz, C., Stoller, G., Zarnt, T., Fischer, G., and Schmid, F.X. (1997). Cooperation of enzymatic and chaperone functions of trigger factor in the catalysis of protein folding. *EMBO J.* **16**, 54–58.
- Stoller, G., Rücknagel, K.P., Nierhaus, K.H., Schmid, F.X., Fischer, G., and Rahfeld, J.-U. (1995). A ribosome-associated peptidyl-prolyl *cis/trans* isomerase identified as the trigger factor. *EMBO J.* **14**, 4939–4948.
- Teter, S.A., Houry, W.A., Ang, D., Tradler, T., Rockabrand, D., Fischer, G., Blum, P., Georgopoulos, C., and Hartl, F.U. (1999). Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell* **97**, 755–765.
- Tomoyasu, T., Ogura, T., Tatsuta, T., and Bukau, B. (1998). Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in *E. coli*. *Mol. Microbiol.* **30**, 567–581.
- Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B., and Lührink, J. (1995). Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. *EMBO J.* **14**, 5494–5505.
- Valent, Q.A., de Gier, J.-W.L., von Heijne, G., Kendall, D.A., ten Hagen-Jongman, C.M., Oudega, B., and Lührink, J. (1997). Nascent membrane and presecretory proteins synthesized in *Escherichia coli* associate with signal recognition particle and trigger factor. *Mol. Microbiol.* **25**, 53–64.
- Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M., and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* **272**, 1606–1614.

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