The Roles of the Two Zinc Binding Sites in DnaJ

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Running Title: Functional Analysis of DnaJ’s Conserved Zinc Centers
Abstract

All type I DnaJ (Hsp40) homologues share the presence of two highly conserved zinc centers. To elucidate their function, we constructed DnaJ mutants that had separately replaced the cysteines of either zinc center I or zinc center II with serine residues. We found that in the absence of zinc center I, the autonomous, DnaK-independent chaperone activity of DnaJ is dramatically reduced. Surprisingly, this only slightly impairs the in vivo function of DnaJ and its ability to function as a co-chaperone in the DnaK/DnaJ/GrpE foldase machine. DnaJ’s zinc center II, on the other hand, was found to be absolutely essential for the in vivo and in vitro function of DnaJ. This did not seem to be due to a lack of substrate binding affinity or due to an inability to work as an ATPase stimulating factor. Rather it appears that zinc center II mutant proteins lack a necessary additional interaction site with DnaK, which seems to be crucial for locking-in of substrate proteins onto DnaK. These findings led us to a model, in which ATP hydrolysis in DnaK is only the first step in turning DnaK into its high affinity binding state. Additional interactions between DnaK and DnaJ are required to make the DnaK/DnaJ/GrpE foldase machinery catalytically active.
Introduction

The heat shock proteins DnaJ (Hsp40), DnaK (Hsp70) and GrpE form a highly efficient and highly conserved molecular chaperone machinery. Under non-stress conditions, this chaperone system appears to be involved in the folding of newly synthesized proteins, in protein assembly and disassembly and in the translocation of proteins into organelles. Under stress conditions, it plays a role in the protection of proteins against stress induced protein unfolding and in the refolding of unfolded proteins (1).

The functions of the “foldase” DnaK and the nucleotide exchange factor GrpE are quite well understood. In contrast, however, the roles of DnaJ and its homologues are still somewhat enigmatic (2). DnaJ consists of four domains that are conserved to various degrees among the homologues. The N-terminal 76 amino acid J-domain defines proteins as members of the DnaJ/Hsp40 family and is, thus, present in all DnaJ/Hsp40 family members (3). It contains the conserved tripeptide His-Pro-Asp, which is essential for DnaJ-DnaK interactions and for the stimulation of DnaK’s ATPase activity (4-6). Adjacent to the J-domain resides the glycine and phenylalanine rich G/F domain (residues 77-107). Together with the J-domain, the G/F domain is essential for the maximal stimulation of DnaK’s ATPase activity (7). The central domain of DnaJ is the zinc-binding cysteine rich CR-domain (residues 144-200), which is followed by a poorly conserved C-terminal CTD-domain (8).

Hsp40 homologues that contain all four domains are called class I DnaJ homologues. They exert autonomous, DnaK-independent chaperone activity as well as a DnaK-dependent co-chaperone activity (9). The autonomous chaperone activity is characterized by the ability of excess amounts of DnaJ to bind to unfolded substrate proteins and to prevent their irreversible aggregation (10,11). The co-chaperone activity of DnaJ, on the other hand, is based on the
synergistic interaction of DnaJ and DnaK with substrate proteins and results in the efficient suppression of aggregation at much lower DnaJ concentrations than DnaJ alone is capable of doing. DnaJ works as the “holdase” by binding to and delivering unfolded substrate proteins to the “foldase” DnaK, which folds the protein. Interaction of the DnaJ-substrate protein complex with the DnaK-ATP-substrate protein complex maximally stimulates ATP hydrolysis. This is thought to cause conformational changes in DnaK that increase DnaK’s affinity for unfolded substrate proteins and that cause the lock-in of the substrate proteins to the substrate binding site of DnaK (7,12-15).

Class II DnaJ/Hsp40 homologues lack the central cysteine rich zinc binding domain but have two C-terminal domains, CTD1 and CTD2, instead (16). They have not been found to exert any autonomous, Hsp70-independent ability to interact with unfolded substrate proteins. However, in concert with Hsp70 homologues, they will support the refolding of unfolded proteins such as luciferase in vitro, albeit with about 4 fold less efficiency than class I DnaJ homologues (9). Class III DnaJ representatives only have the J-domain in common. They appear unable to work as molecular chaperones in vitro. In vivo, they have been found to be involved in protein secretion (17).

The cysteine rich zinc binding region of class I DnaJ homologues is thought to be involved in the DnaK-dependent chaperone activity based on domain truncation studies and cysteine mutagenesis (9,18,19). However, conflicting results have been reported concerning the role of the entire zinc binding region in the autonomous DnaK-independent chaperone activity (8, 9,19). Furthermore, the role of the individual zinc centers in the DnaK-dependent and independent chaperone function of DnaJ has not been investigated. The cysteine rich CR-domain contains four repeated C-X-X-C-X-G-X-G motifs, which are invariable to all class I
DnaJ proteins (20) (21). It has long been known that the two sulfur atoms of each consensus motif are involved in zinc coordination, resulting in the tetrahedral coordination of two zinc ions per DnaJ monomer (18,19). However, the identity of the specific residues involved in each zinc coordination remained unclear until the NMR structure of the isolated cysteine rich domain was solved in 2000 (Fig. 1)(21). Prior to the solution of the structure, it was assumed that the four immediately adjacent cysteine residues were involved in each zinc site, with the four N-terminal cysteine residues forming zinc center I and the four C-terminal cysteines forming zinc center II (19). The solution structure clarified the arrangement of the individual zinc centers and revealed that zinc center I is actually formed by the two non-adjacent cysteine motifs 1 (Cys144, Cys147) and 4 (Cys197, Cys200), which are furthest apart in the primary sequence. Zinc center II is coordinated by the conserved cysteines of the two adjacent cysteine motifs 2 (Cys161, Cys164) and 3 (Cys183, Cys186) (21) (Fig. 1). This somewhat surprising arrangement meant that a number of experiments that had been conducted to investigate the function of the zinc centers in type I DnaJ homologues needed to be re-interpreted. For instance, experiments that were designed to disrupt the zinc centers individually, probably only succeeded in altering one zinc center (19). This also meant that no experiments had been conducted so far, that separately investigated the specific role of these highly conserved zinc centers.

To analyze the exact functional role of the two individual zinc centers in type I DnaJ homologues in vitro and in vivo, we constructed two DnaJ mutants, based on the NMR structure, that had either zinc center I or zinc center II disrupted. Our investigations revealed that zinc center I plays an important role in the autonomous, DnaK-independent chaperone activity of DnaJ and that absence of this activity only slightly impairs the in vivo function of DnaJ. Zinc center II, on the other hand, is crucial for the in vivo function of DnaJ. Zinc center II mutants
appear to lack a necessary additional interaction site between DnaJ-substrate complexes and DnaK-ADP substrate protein complexes, which seems to be essential for the refolding activity of the DnaK/DnaJ/GrpE foldase machinery.
Experimental Procedures

Construction of zinc center mutants of DnaJ- The amino acid substitutions Cys144Ser, Cys147Ser, Cys197Ser, Cys200Ser to generate DnaJΔZnI and Cys161Ser, Cys164Ser, Cys183Ser, Cys186Ser to generate DnaJΔZnII were constructed with the Quick Change site-directed mutagenesis kit (Stratagene) using pUH21-12fdΔ12 (gift from Dr. Bernd Bukau) as the parental plasmid. The resulting plasmids were sequenced and transformed into the dnaJ deletion strain PK11 (MG1655 ΔdnaJ Thr::Tn10) (gift from Dr. Elizabeth Craig) for phenotypical studies or into wild type E. coli WM3110 containing the plasmid pDMI (22) for protein purification.

Protein purification- Wild type DnaJ as well as the DnaJΔZnI and DnaJΔZnII mutant proteins were purified as previously described (22) with modifications to remove all residual detergents. After elution of the proteins from S-Sepharose Hitrap-column (Pharmacia), the protein solutions were applied to a hydroxyapatite column, equilibrated in 50 mM Tris-HCl, pH 7.5, 2 M urea, 2 mM DTT, 50 mM KCl, 0.1% Brij58. The column was then washed in 50 mM Tris-HCl, pH 7.5, 2 M urea, 2 mM DTT, 1 M KCl followed by a wash with 50 mM Tris-HCl, pH 7.5, 2 M urea, 2 mM DTT, 50 mM KCl. DnaJ and the mutant proteins were eluted using a gradient from 0 to 400 mM KH₂PO₄. The pooled fractions were diluted into 50 mM Tris-HCl, pH 9.0, 2 M urea, 2 mM DTT, 100 mM KCl and applied onto a Q-Sepharose Hitrap column (Pharmacia). The flow through contained > 99% pure DnaJ or the mutant proteins, respectively. The DnaJ variants were dialyzed against 30 mM HEPES-KOH, pH 8.2, 120 mM KCl, 15 mM NaCl, 4% glycerol and the protein concentration was determined using the extinction coefficient ε₂₈₀ =13.400 M⁻¹ cm⁻¹. Wild type DnaK, overexpressed from the plasmid pMOB45, and GrpE overexpressed from the plasmid pWKG20, were purified as described (22).


**Zinc measurements**- The zinc content of DnaJ was determined using a combined PAR/PMPS assay (23). In this assay, free zinc readily interacts with 4-(2-pyridylazo) resorcinol (PAR) (Sigma) to form a Zn(PAR)\(_2\) complex, which strongly absorbs at 500 nm. To determine the amount of free zinc in the protein solution, 1 µM DnaJ in 30 mM HEPES-KOH, pH 8.2, 120 mM KCl, 15 mM NaCl, 4% glycerol was mixed with 100 µM PAR and the \(A_{500\text{ nm}}\) was monitored. To determine the amount of zinc that is cysteine coordinated in wild type DnaJ and the mutant proteins, 2 µl aliquots of a 1 mM \(p\)-hydroxymercuri-phenylsulfonic acid (PMPS) (Sigma) titration solution (in 100 µM PAR, 40 mM HEPES-KOH, pH 7.5) were added and the changes in \(A_{500\text{ nm}}\) were monitored after each addition, until no further changes were detected (24). PMPS forms stoichiometric mercaptide bonds with thiols. This leads to the release of zinc into the solution, where it is immediately complexed by PAR, thereby turning the solution red. A zinc standard was used to determine the extinction coefficient of the Zn(PAR)\(_2\) complex at 500 nm in 30 mM HEPES-KOH, pH 8.2, 120 mM KCl, 15 mM NaCl, 4% glycerol.

**CD measurements**- All DnaJ proteins were dialyzed against 30 mM phosphate buffer pH 8.2, 120 mM KCl, 15 mM NaCl, 4% glycerol. Then, the protein concentrations were adjusted to 10 µM. Cuvettes with path lengths of 1 or 10 mm were used for far-UV-CD or near-UV-CD, respectively. CD spectra were recorded using a Jasco J810 spectropolarimeter.

**Luciferase aggregation and reactivation assay**- 8 µM firefly luciferase (Sigma) was denatured in 5 M guanidinium-HCl in 30 mM Tris-acetate pH 7.5 for at least 2 hours at RT. Aggregation and reactivation was initiated by a 1:100 dilution into 40 mM MOPS, pH 7.5, 50 mM KCl, 2 mM MgATP at 30°C. The buffers were either not supplemented with any chaperones or supplemented with the indicated concentrations of DnaK, DnaJ and GrpE. The extent of
luciferase aggregation was determined 600 sec after the dilution using light scattering measurements (Hitachi Fluorimeter F4500, $\lambda_{\text{ex/em}} = 320 \text{ nm}$, slit widths 5.0 nm).

To determine luciferase reactivation, aliquots were withdrawn and diluted into assay buffer (100 mM potassium phosphate pH 7.8, 25 mM glycylglycine, 70 µM luciferin, 0.2 mM EDTA, 0.5 mg/ml BSA). Luciferase activity was analyzed by monitoring chemiluminescence one minute after the start of the reaction using a Hitachi Fluorimeter F4500 (900 V, $\lambda_{\text{em}} = 557 \text{ nm}$, 20 nm slit). The chemiluminescence was followed for 45 sec at room temperature, and the initial activity was obtained by linear extrapolation (25).

**Determination of DnaK’s ATPase activity** - The influence of DnaJ on the ATP hydrolysis of DnaK was determined using single turn over experiments (26). Briefly, 10 µM DnaK was incubated with 40 µM ATP, 0.1 – 0.2 µCi/ml [$\alpha^{32}\text{P}$$]\text{ATP}$ in 40 mM MOPS pH 7.5, 50 mM KCl, 10 mM MgCl$_2$ for 2 minutes on ice to allow the DnaK-[${\alpha}^{32}\text{P}$$]\text{ATP}$-complex to form. Free ATP was separated from the complex using a micro Bio-Spin chromatography column (BioRad). DnaK-[$\alpha^{32}\text{P}$$]\text{ATP}$ aliquots were kept frozen until further use. DnaK concentration was determined using a Bradford assay. To determine the rate of ATP hydrolysis, 30 µl reactions containing 0.16 µM wild type DnaJ or the mutants proteins in 40 mM MOPS pH 7.5, 50 mM KCl were prepared and incubated at 25$^\circ$C. Immediately after addition of chemically denatured luciferase (final conc. 0.08 µM), the reaction was started with the addition of 0.8 µM DnaK-[${\alpha}^{32}\text{P}$$]\text{ATP}$ complex. At the indicated time points, 2 µl of the reaction were withdrawn and spotted directly onto a PEI-cellulose TLC-plate (Merck). The plates were developed in 2 M formic acid / 0.5 M LiCl. The amount of ADP formed was determined using a phosphoimager.
Results

Zinc center II - Essential for the in vivo function of DnaJ

DnaJ’s cysteine rich domain forms two zinc centers, each of which is composed of four conserved cysteines (18,19,21) (Fig.1). To investigate the role of the individual zinc centers in the function of class I DnaJ homologues, we decided to construct DnaJ mutants that had either all 4 cysteines of zinc center I (DnaJΔZnI) or of zinc center II (DnaJΔZnII) replaced by serine residues. To test the in vivo function of the two mutant proteins, plasmids expressing either the mutated dnaJ genes or wild type dnaJ were transformed into the dnaJ deletion strain PK11.

DnaJ deletion strains such as PK11 are unable to propagate λ-growth (27), do not grow at heat shock temperatures (≥ 43°C) (28) and are unable to swarm (29). These three phenotypes appear to depend on slightly different functions of the DnaJ protein. We, therefore, decided that analyzing the extent to which the two mutant proteins are able to rescue those diverse phenotypes should provide a valuable first tool to functionally evaluate our mutant proteins. To exclude the possibility that any observed phenotype is due to the under- or overexpression of the mutant proteins, quantitative western blot analysis was performed of PK11 strains expressing the two mutant proteins. We found that both mutant proteins migrated slightly different on SDS-PAGE than wild type DnaJ, but were expressed to levels that were very similar to DnaJ expressed from its own promoter off the chromosome at 37°C (data not shown).

In vivo analysis of the two mutant proteins revealed dramatic functional differences. PK11 strains expressing the zinc center II mutant protein failed to grow at 43°C and formed smaller colonies at 37°C than PK11 strains expressing wild type DnaJ (Fig. 2A, B). Mutants lacking zinc center I, on the other hand, showed no significant growth defect at 37°C and were able to form small colonies at 43°C (Fig. 2A, B). Moreover, zinc center II mutants were non-
motile at 37°C, whereas zinc center I mutants were fully motile (Fig. 2C). These functional differences between zinc center I and II mutants were not due to solubility and/or stability problems of the mutant DnaJ proteins. This became evident when \( \lambda \)-growth was tested in these strains. \( \lambda \)-propagation only requires the presence of a functional N-terminal domain of DnaJ (30) and was found to be nearly indistinguishable in both mutant and wild type strains (Table 1).

These \textit{in vivo} results provided the first evidence that the two highly conserved zinc centers might not form a single functional entity in DnaJ but exert rather distinct functional properties. Absence of zinc center I showed only a slight influence on the DnaJ function at heat shock temperatures and did not appear to affect growth or flagellum synthesis at 37°C. Lack of zinc center II, on the other hand, dramatically influenced the function of DnaJ under both heat shock and non-stress temperature conditions.

**Zinc center I and II of DnaJ form independently**

In order to analyze the \textit{in vitro} function of the two zinc center mutant proteins, we investigated the following properties: i) zinc content, ii) protein conformation, and iii) \textit{in vitro} chaperone activity of the mutant proteins alone and in concert with the DnaK/GrpE machinery. The DnaJ\( \Delta \)ZnI and DnaJ\( \Delta \)ZnII mutant proteins were overexpressed in \textit{E. coli} and purified to homogeneity. The purification of DnaJ\( \Delta \)ZnI and DnaJ\( \Delta \)ZnII mutant protein was performed according to the purification protocol for wild type DnaJ (see material and methods). The behavior of the two mutant proteins and DnaJ wild type was indistinguishable on the chromatography columns, suggesting that the deletion of zinc center I or zinc center II did not dramatically alter the physical-chemical properties of DnaJ.
To determine whether the zinc centers can form independently of each other, the zinc content of the two mutant proteins and wild type DnaJ was analyzed using the PAR/PMPS titration assay (23, 24). In this assay, the total amount of zinc, which is specifically coordinated via thiol groups in a protein, can be determined. The assay is based on the presence of the chelator 4-(2-pyridylazo) resorcinol (PAR), which quickly associates with free zinc and forms a bright red Zn(PAR)$_2$ complex, whose absorption can be determined at 500 nm. When the zinc binding constant of the thiol-containing zinc center exceeds that of the Zn(PAR)$_2$ complex ($K_a = 2 \times 10^{12}$ M in 40 mM HEPES-KOH, pH 7.0), however, addition of PAR is not sufficient to extract zinc from proteins (23). In this case, thiol-modifying agents such as $p$-hydroxymercuriphenylsulfonic acid (PMPS) have to be used, which form stoichiometric thiol-mercaptide bonds and cause the release of zinc. We found that both mutant proteins coordinated close to one molecule of zinc per molecule of protein (0.8 ± 0.1) via the four remaining cysteines of each zinc center (Table 1). The strict requirement for PMPS to release the zinc from the remaining zinc center of the mutant proteins (1 µM) into a solution containing 100 µM PAR, indicated that the zinc binding constant $K_a$ for the individual zinc centers must be still higher than that of PAR under these conditions. These results revealed that each of the two mutant proteins harbor one intact zinc center and suggested that DnaJ mutants, which lack zinc coordination in one zinc center, still retain sufficient protein structure to bind and coordinate zinc in the second zinc center. We, therefore, concluded that the formation of the two individual zinc centers in DnaJ can occur independently.

To determine to what extent the lack of zinc center I or II causes conformational changes in the mutant proteins that might explain the inability of DnaJΔZnII to rescue the observed phenotypes, far and near UV-CD spectra were recorded. These spectra showed that neither the
secondary structure (Fig. 3) nor the tertiary structure (Fig. 3, insert) of DnaJ was significantly disturbed by the lack of the individual zinc centers, suggesting that the individual zinc centers do not play a major role in the overall secondary or tertiary structure of DnaJ. This agreed well with the NMR structure of the isolated zinc binding domain, which showed that this domain forms an autonomous folding domain (21) (Fig. 1).

Zinc center II – Required for optimal refolding activity of the DnaJ/DnaK/GrpE system

The DnaK/DnaJ/GrpE chaperone machinery supports the refolding of denatured proteins both in vivo and in vitro (for recent review see (31)). In vitro analysis of the reactivation of chemically or thermally denatured luciferase in the presence of DnaK/DnaJ/GrpE is a widely used way to measure the activity of the individual protein components (11). This assay has been used, for instance, to show that the presence of full length DnaJ is required to result in high yields of reactivated luciferase, while DnaJ variants that were missing both the cysteine rich zinc binding domain and the C-terminal domain showed no refolding activity (19). DnaJ mutants that were lacking zinc center II because of mutations in Cys 183 and 186 showed 10-fold reduced activity, while mutants that were lacking zinc center II because of mutations in Cys161 and Cys164 had no activity (19). This suggested that zinc center II is important and may even be essential for the DnaK-dependent chaperone activity of class I DnaJ homologues. There was also evidence suggesting that zinc center I might be involved in this activity, because mutants in the yeast homologue Ydj1p that were lacking one of the highly conserved zinc center I cysteines, also revealed a significantly reduced ability to cooperate with yeast Hsp70 in the refolding of luciferase (8).
To clearly define the role of DnaJ’s individual zinc centers in the cooperation with DnaK and GrpE, we utilized this well-established in vitro system to test our mutant proteins. Refolding of luciferase in the absence of any chaperones resulted in less than 10% reactivated luciferase molecules (Fig. 4A, squares). In the presence of a 2:10:10 molar ratio of wild type DnaJ:DnaK:GrpE over luciferase, however, more than 80% of chemically denatured firefly luciferase reactivated within the time frame of the experiment (Fig. 4A, closed circle). These results agreed well with the published data (15).

The DnaJΔZnI mutant protein, which showed slightly reduced activity under heat shock conditions in vivo, displayed near wild type activity in vitro. Presence of a 2:10:10 ratio of DnaJΔZnI mutant protein to DnaK and GrpE supported luciferase refolding to nearly the same levels as wild type DnaJ did (Fig. 4A, open circle), indicating that the absence of the highly conserved zinc center I does not influence the co-chaperone activity of DnaJ. Presence of the DnaJΔZnII mutant protein, on the other hand, resulted in reactivation yields that were only slightly higher than the reactivation yields obtained in the absence of any chaperones (Fig. 4A, triangle). This finding agreed with previous reports (19) and suggested that the observed lack of in vivo function of the DnaJΔZnII mutant protein might be due to a loss of its chaperone activity.

To further analyze the precise extent by which zinc center I and II mutations influence the co-chaperone activity of DnaJ, luciferase refolding assays were performed in the presence of varying concentrations of DnaJ and constant concentrations of DnaK and GrpE. Wild type DnaJ supported luciferase reactivation to the highest extent when present in a 2:10:10:1 molar ratio of DnaJ:DnaK:GrpE:luciferase (Fig. 4B, closed circles)(12,19). DnaJ concentrations higher than this optimum decreased the refolding yield of luciferase, presumably because the autonomous, DnaK-independent chaperone activity of DnaJ competes with DnaK for substrate binding (Fig.
Refolding reactions that contained lower ratios of DnaJ to DnaK:GrpE: luciferase also showed decreased refolding yields. This might be due to denatured luciferase that fails to interact with DnaJ and forms irreversible aggregates.

The zinc center I mutant protein DnaJΔZnI was only slightly less active than wild type DnaJ at lower concentrations (Fig. 4B, open circles). Interestingly, however, the luciferase reactivation yields did not decrease with increasing DnaJΔZnI concentrations. The yields of reactivated luciferase remained at the same high level even when a 100:10:10:1 molar ratio of DnaJΔZnI:DnaK:GrpE:luciferase was used. This was unexpected and actually suggested that the autonomous, DnaK-independent substrate binding affinity of DnaJ might be impaired when zinc center I is absent from the protein, because at high wild type DnaJ concentrations, this autonomous chaperone activity has been shown to interfere with the refolding of luciferase. The co-chaperone activity of DnaJ, which is responsible for binding and delivering substrate proteins to DnaK, on the other hand, appeared not to be significantly influenced by the lack of zinc center I.

Substitution of wild type DnaJ with the DnaJΔZnII mutant protein dramatically reduced the activity of the DnaK/DnaJ/GrpE folding machinery. With a 10:10:10:1 molar ratio of DnaJΔZnII to DnaK:GrpE:luciferase, only 60% of luciferase were reactivated (Fig. 4B, triangles), while lower concentrations of DnaJΔZnII resulted in refolding yields that were only slightly higher than the yields obtained in the absence of any chaperones. Interestingly, in the presence of excess DnaJΔZnII mutant protein, on the other hand, the same reduction in luciferase refolding yields was observed as with wild type DnaJ. A 50:1 molar ratio of either DnaJΔZnII mutant or wild type protein to luciferase completely blocked the refolding of luciferase, indicating that the ability to interact with unfolded substrate proteins might actually not be affected by the deletion of zinc center II. This was in contrast to previous studies, which
concluded that zinc center II mutant proteins are less capable of interacting with unfolded substrate proteins (19). Our results suggested, however, that zinc center II might not play a major role in the autonomous, DnaK-independent chaperone activity of DnaJ but might be specifically involved in the interaction with DnaK. In contrast, zinc center I appeared to be involved in the autonomous, DnaK-independent chaperone activity of DnaJ and dispensable for the DnaK-dependent chaperone activity of DnaJ. These results provided the first evidence that the chaperone activity that DnaJ displays when acting in concert with the DnaK/GrpE machinery might be distinct and can be disconnected from the chaperone activity that DnaJ displays on its own.

**Zinc center II – Involved in a rate limiting step of the DnaK/DnaJ/GrpE mediated luciferase refolding**

In the absence of any chaperones, the refolding rate of luciferase is rather slow. In contrast, however, presence of the optimal 2:10:10:1 molar ratio of DnaJ:DnaK:GrpE:luciferase accelerates the rate of luciferase refolding by about 4-fold (Fig. 4C, closed circles). Under these conditions, no luciferase aggregation is observed and the ATPase activity of DnaK is thought to be optimally stimulated by the DnaJ-substrate protein complex. Lower concentrations of DnaJ cause a slowing down of the refolding reaction. This could be either due to the presence of luciferase micro-aggregates, whose dissociation by the DnaK/DnaJ/GrpE chaperone machinery might become a rate limiting step in the refolding of luciferase, or due to a sub-optimal stimulation of DnaK’s ATPase activity. Higher concentrations of DnaJ also slow down the reactivation rate presumably because of the competitive binding of DnaJ to the refolding substrate proteins.
A quite similar but right-shifted dependence of the reactivation rate on the DnaJ concentration was found when the DnaJΔZnI mutant protein was used instead of wild type DnaJ (Fig. 4C, open circles). Here, the same high reactivation rates were reached, albeit in the presence of higher molar ratios of DnaJΔZnI mutant protein to DnaK:GrpE and luciferase. This was consistent with the slightly lower activity of the DnaJΔZnI mutant protein under heat shock conditions in vivo. The results also showed clearly that optimal luciferase refolding yields do not necessarily require optimal refolding rates and indicated that absence of zinc center I might be involved in some rate limiting steps in the refolding reaction of luciferase that can be compensated by higher concentrations of the DnaJΔZnI mutant protein. In contrast, however, the DnaJΔZnII mutant protein did not accelerate the refolding reaction of luciferase at any concentration used (Fig. 4C, triangles). Even at a 10:10:10:1 molar ratio of DnaJΔZnII to DnaK:GrpE: luciferase, where 60% of luciferase molecules refolded to the native state (Fig. 4B), the refolding rate was still similar to the slow spontaneous refolding rate of luciferase. This suggested that the DnaJΔZnII/DnaK/GrpE complex was no longer catalytically active. Zinc center II in DnaJ appears to mediate a rate-limiting step in the DnaK/DnaJ/GrpE foldase machinery, which allows DnaJ to accelerate the folding of luciferase and, which, in its absence, can not be compensated by higher concentrations of the DnaJΔZnII mutant protein.

Zinc center II - Essential for locking in the substrate onto DnaK?

The inability of the DnaJΔZnII mutant protein to function as a co-chaperone in the DnaK/DnaJ/GrpE machinery might be due to a specific inability of this mutant protein to interact with and transfer substrate to DnaK, and not, as suggested by Szabo and co-workers, due to a general lack in substrate binding affinity (19). To specifically assess the interaction of our two
mutant proteins with substrate proteins and DnaK, the influence of our DnaJ variants on the aggregation of chemically denatured luciferase was investigated in the absence and presence of DnaK. The buffer and reaction conditions that were used for these experiments were identical to the conditions employed for analyzing luciferase reactivation.

As shown in Fig. 5A, in the absence of any molecular chaperones, chemically denatured luciferase quickly aggregated after dilution into refolding buffer. This is well known and explains the very low yield of refolded luciferase that is obtained under these conditions (Fig. 4A). In the presence of a 0.5:1 molar ratio of wild type DnaJ to luciferase, the aggregation of luciferase was suppressed by about 50% (Fig. 5A). Increasing concentrations of DnaJ over luciferase prevented the aggregation even more and a 2:1 molar ratio of DnaJ to luciferase was sufficient to almost completely suppress luciferase aggregation (Fig. 5C). This reflects the autonomous, DnaK-independent chaperone activity of DnaJ. DnaK by itself, on the other hand, does not influence the aggregation of luciferase even when present in a 10:1 molar ratio to luciferase (Fig. 5B). This is due to the negligible affinity of DnaK-ATP complexes to substrate proteins (32,33). Presence of both DnaJ and DnaK in a 0.5:10:1 molar ratio of DnaJ:DnaK:luciferase, however, completely suppressed luciferase aggregation (compare Fig. 5A with Fig. 5B). This synergistic effect is caused by the cooperative chaperone action of DnaJ and DnaK (8). Here, the simultaneous interaction of DnaK with the substrate protein and DnaJ allows DnaJ to stimulate the ATP hydrolysis of DnaK. This is thought to cause the “locking in” of substrate proteins to the substrate binding site of DnaK, which in turn leads to the effective prevention of luciferase aggregation (8,12). Additional presence of GrpE does not influence the aggregation behavior (data not shown) but leads to the efficient refolding of nearly 60% of luciferase under these concentration conditions (Fig. 4B).
Analysis of the DnaJΔZnII mutant protein in this assay showed that absence of zinc center II does not significantly influence the ability of DnaJ to interact with unfolded substrate proteins (Fig. 5A,C) but renders the mutant protein unable to productively cooperate with DnaK (Fig. 5B). In the absence of DnaK, the DnaJΔZnII mutant protein was nearly as efficient as wild type DnaJ in interacting with refolding luciferase intermediates and preventing their irreversible aggregation (Fig. 5A, C). In contrast to the experiments with wild type DnaJ, however, simultaneous presence of DnaJΔZnII and DnaK did not cause a further decrease of the light scattering signal (Fig. 5B). Zinc center II appears, therefore, not to be directly involved in the DnaJ-substrate interaction. This excluded the possibility that the failure of the DnaJΔZnII mutant protein to promote and accelerate the refolding reaction of luciferase (Fig. 4) is due to the inability to interact with substrate proteins.

The observed lack of synergistic interaction between the DnaJΔZnII mutant protein and DnaK is also not substrate specific because very similar results were obtained when chemically unfolded rhodanese was used (data not shown). As in the case with luciferase, the DnaJΔZnII mutant protein was fully able to bind to folding intermediates of rhodanese and to prevent their non-specific aggregation, but additional presence of DnaK did not increase the protective action of the DnaJΔZnII mutant protein.

**Zinc center II – Providing a second necessary interaction site with DnaK**

The observed lack of synergistic DnaJ-DnaK interaction in the absence of zinc center II could be due to several reasons: i) DnaK might be unable to interact with substrate proteins in the presence of the DnaJΔZnII mutant protein, ii) DnaJΔZnII mutant protein might be incapable of effectively stimulating ATP hydrolysis of the DnaK-ATP-substrate protein complexes or iii)
DnaJΔZnII mutant protein might fail to provide a necessary second DnaK interaction site, whose existence has been proposed previously and, which might be necessary for a catalytically active DnaK/DnaJ/GrpE complex (34,35).

One assay that allows us to distinguish between those possibilities is the stimulation of DnaK’s ATPase activity in the simultaneous presence of DnaJ and substrate proteins. Maximal stimulation of DnaK’s ATPase activity has been shown to be achieved when DnaJ’s J-domain interacts with DnaK’s N-terminal ATPase domain as DnaK and DnaJ are binding to the same unfolded polypeptide chain (12,13). This situation is thought to cause the substrate to become locked-in to the high affinity DnaK-ADP complex (12). We, therefore, decided to utilize this assay to investigate the extent to which absence of zinc center II influences this DnaK-unfolded substrate protein-DnaJ interaction. Single turnover experiments were performed under conditions that were identical to the refolding and aggregation assays described above.

In agreement with the literature, we found that in the absence of unfolded substrate proteins, a 2:10 molar ratio of wild type DnaJ to DnaK stimulated DnaK’s ATPase activity approximately 50-fold (Fig. 6, insert). In the simultaneous presence of unfolded luciferase in a 2:10:1 molar ratio of DnaJ:DnaK:luciferase, a more than 600-fold stimulation of DnaK’s ATPase activity was observed (Fig. 6, closed circles, Table 1). When we tested our DnaJΔZnII mutant protein it became evident that this high stimulation of DnaK’s ATPase activity is not sufficient for optimal foldase activity of the DnaK/DnaJ/GrpE chaperone machine. The DnaJΔZnII mutant protein behaved very similarly to wild type DnaJ and stimulated the ATPase activity approximately 70-fold in the absence of substrate (Fig. 6, insert) and about 600-fold in the presence of substrate proteins (Fig. 6, triangles, Table 1). These results indicated that absence of zinc center II in DnaJ neither influences the interaction between DnaK and the substrate protein...
nor the interaction between DnaJ’s N-terminal J-domain and DnaK’s ATPase domain. These findings led us to propose, however, that to be catalytically active, the DnaK/DnaJ/GrpE foldase machinery requires additional interactions between DnaJ and DnaK, which appear to be mediated by zinc center II. These additional interactions between DnaJ and DnaK might cause conformational changes in DnaK that are essential for converting the DnaK-ADP into its high affinity binding state.

**Zinc center I - Important for DnaK-independent chaperone activity of DnaJ**

DnaJΔZnI mutant protein showed an at least 10-fold reduced ability to prevent the aggregation of chemically denatured luciferase (Fig. 5C, open circles) and rhodanese (data not shown) as compared to wild type DnaJ, indicating that the absence of zinc center I causes a strong reduction in DnaJ’s ability to interact with unfolded substrate proteins. Importantly, the significantly reduced ability of the DnaJΔZnI mutant protein to prevent the aggregation of folding intermediates such as luciferase or rhodanese did not appear to influence the DnaK-dependent co-chaperone activity of DnaJ (Fig. 5A, B). In the presence of a 0.5:1 molar ratio of DnaJΔZnI mutant protein to luciferase, no substantial change in the aggregation behavior of luciferase was observed (Fig. 5A). In contrast, simultaneous presence of DnaJΔZnI and DnaK led to a very similar suppression of luciferase aggregation that was observed when wild type DnaJ and DnaK was used (Fig. 5B). These findings indicated that absence of zinc center I did not significantly impair the interaction between DnaJ and DnaK and explained how the DnaJΔZnI mutant protein can be as effective as wild type protein in the DnaK/DnaJ/GrpE mediated refolding of chemically unfolded luciferase (Fig. 4A,B). These results, furthermore,
suggested that a rather transient interaction between DnaJ and substrate proteins must be sufficient for a successful cooperative interaction with DnaK-substrate protein complexes.

Maximal stimulation of DnaK’s ATPase activity requires the simultaneous presence of DnaJ and DnaK bound to the same polypeptide (13). Therefore, more transient interactions between DnaJ and substrate protein should lower the effective concentrations of substrate bound DnaJ, which in turn should decrease the extent of ATPase stimulation. That this was indeed the case is shown in Fig. 6 and summarized in Table I. While the DnaJΔZnI mutant protein was nearly as active as wild type DnaJ in stimulating the ATPase activity of DnaK in the absence of substrate proteins, a lower additional stimulation of DnaK’s ATPase activity was observed when unfolded luciferase was present (Fig. 6, open circles). This might explain why higher concentrations of the DnaJΔZnI mutant protein are required during the luciferase refolding reactions to achieve maximal luciferase refolding rates (Fig. 4C).
Discussion

The two independent zinc centers in DnaJ

What is the specific role of the two highly conserved zinc centers in the widely distributed class I DnaJ homologues? A number of studies have been conducted to study the involvement of this region in the autonomous, DnaK-independent substrate binding activity as well as in the DnaK-dependent co-chaperone activity of class I DnaJ homologues (8,18,19). However, most of these studies were based on the presumption that the four N-terminal cysteines are involved in forming zinc center I and that the four C-terminal cysteines are involved in forming zinc center II. Only when the NMR structure of the cysteine rich domain was solved, the correct zinc coordination became evident (Fig. 1) (21). Zinc center I was found to be formed by the two cysteine motifs that are furthest apart in the primary sequence, while zinc center II was formed by the two central, adjacent cysteine motifs. This knowledge allowed us to specifically disrupt the two zinc centers and to evaluate their precise effects on the in vivo and in vitro function of class I DnaJ homologues.

We constructed the two zinc center mutants by individually replacing the four cysteines of each zinc center with serine residues. Both DnaJΔZnI and DnaJΔZnII mutant proteins were found to be stable proteins in vivo and circular dichroism measurements of the purified proteins did not reveal any major structural changes when compared to wild type DnaJ. This agreed well with the modular character of DnaJ proteins and suggested that the 79 aa cysteine rich domain is also an autonomous folding unit in the full length protein. The results were also in good agreement with studies using metal-free full length DnaJ, which demonstrated that even in the absence of any zinc coordination, DnaJ undergoes probably only local structural changes (36). These local changes most likely include the partial unfolding of the zinc binding region, because
in the isolated cysteine rich domain, absence of zinc has been shown to cause the unfolding of the β-hairpin structure (21). When zinc was titrated back into the metal free cysteine rich domain, zinc center II formed first and independently of zinc center I, while zinc center I only formed when the Zn$^{2+}$ to protein ratio was increased above 1. This led the authors to conclude that folding of the two symmetric zinc sites is zinc dependent and that one site forms before the other (21). Our studies now showed that both mutants of DnaJ are able to correctly assemble the one remaining zinc center. This suggests that the unfolding of one zinc center might not affect the second zinc site. The differences between our results and those obtained previously could be based on the DnaJ constructs used. While we performed our analysis with full length protein, Martinez-Yamout and co-workers used the isolated zinc binding domain. The two zinc centers I and II, which fold into symmetric zinc binding modules, form the “wings” of a V-shaped structure (Fig. 1) (21). Two antiparallel β-strands form the outside of each zinc center. In zinc center II, the β-strands are connected by a β-hairpin loop, while in zinc center I the N-and C-terminal strands need to approach each other to form the β-strands (Fig. 1B). It is, therefore, conceivable that in the isolated cysteine rich domain, absence of zinc coordination in zinc center I causes the separation of the N-and C-termini and, therefore, the spatial separation of the two cysteine motifs of zinc center I. This would make re-coordination of the metal in zinc center I significantly more difficult than in zinc center II, where the four cysteines are relatively close in the primary sequence.

**Zinc center I: High affinity binding site for unfolded substrate proteins**

Functional analysis of our mutant proteins revealed that zinc center I is involved in the high affinity binding of unfolded substrate proteins. This activity, which allows DnaJ to bind to
non native proteins and prevent their non-specific aggregation processes *in vitro*, has been characterized as the autonomous DnaK-independent chaperone activity of DnaJ (10,11). Zinc center I, which is built by two inter-linked turns is connected with zinc center II via a long loop that forms the central base of the “V-apex” (Fig. 1B)(21). This central part of the cysteine-rich domain harbors a groove of conserved and hydrophobic residues, which has been suggested to be a potential substrate binding site of DnaJ (Fig. 1C) (21). It is conceivable that unfolding of zinc center I extends into this adjacent loop region. This could change the conformation of this putative substrate binding site and therefore, reduce its ability to interact with unfolded substrate proteins.

Until now, the autonomous DnaK-independent chaperone function of DnaJ has always been linked to the DnaK-dependent chaperone activity and was thought to be a necessary functional requirement for DnaJ to present unfolded substrate proteins to DnaK. We have now discovered that this high affinity substrate binding activity of DnaJ appears to be largely dispensable for the synergistic interaction of DnaJ with DnaK. Zinc center I mutant proteins, which show at least a 10-fold reduction in their ability to bind unfolded substrate proteins, are not significantly impaired in their cooperative interaction with DnaK to suppress protein aggregation or in their ability to refold unfolded proteins when acting as part of the DnaK/DnaJ/GrpE chaperone machine. This suggested that a much more transient interaction between DnaJ and unfolded substrate proteins must be sufficient for the cooperative interaction with DnaK-substrate complexes. These findings agree with functional studies of class II DnaJ homologues such as Sis1p, which appear to lack the autonomous chaperone activity, but are able to interact with Hsp70 to suppress protein aggregation and to refold non-native proteins (9).
Where is the low affinity substrate binding site located in DnaJ, which appears to be still present in mutant proteins lacking zinc center I and, which seems to be sufficient to bind and present unfolded substrate proteins to DnaK? Earlier studies showed that DnaJ deletion mutants that lack the complete zinc binding region are still capable of interacting with substrate proteins such as $^{32}\sigma$ (18). This suggested that the low affinity binding site might be located in the C-terminus of DnaJ. This agreed with *in vivo* and *in vitro* studies that have shown that residues in the first 68 aa of the C-terminus of class I DnaJ homologues are involved in the DnaK-dependent chaperone activity of DnaJ (37,38). Absence of the highly conserved C-terminal $G_{242}^-D-L-Y-V_{246}$ motif has been found to dramatically influence the synergistic interaction with DnaK, but shows little effect on the ability to suppress the aggregation of unfolded proteins such as luciferase (38). This is presumably because the autonomous, DnaK-independent high affinity binding site of zinc center I is still functional in these mutants.

**Zinc center II—A new interaction site with DnaK**

Previous studies that were conducted to identify the role of zinc coordination in the function of DnaJ led to the conclusion that DnaJ’s zinc center II plays a significant role in the DnaJ/DnaK/GrpE mediated refolding of unfolded proteins (19). The authors explained their observed effects with the significantly reduced ability of these mutant proteins to bind to unfolded substrate proteins and to prevent their aggregation (19). These findings did not agree with our mutant protein studies that showed that lack of the complete zinc center II did not significantly change DnaJ’s ability to interact with unfolded proteins such as luciferase or rhodanese. We do not know the reason for the observed discrepancy but by performing all of our aggregation, refolding and ATPase studies in the same buffer supplemented with identical
amounts of ATP and co-chaperones, we were able to exclude the possibility that even small
differences in the conformational stabilities of our mutant proteins might influence the outcome
of our experiments.

We found that absence of zinc center II specifically affects the ability of DnaJ to convert
DnaK into its high affinity binding state. This is not due to the inability of DnaJ to interact with
unfolded proteins because the DnaJΔZnII mutant protein exerts a similar high affinity for
unfolded proteins compared to wild type DnaJ. We could also exclude that this is not due to
unusually stable complexes between the DnaJΔZnII mutant protein and unfolded substrate
proteins that would prevent substrate transfer between DnaJ and DnaK. The stability of protein-
protein complexes is known to increase with increasing protein concentrations. Therefore,
luciferase refolding would have been expected to decrease with increasing DnaJΔZnII mutant
protein concentrations. Quite in contrast, however, we found that luciferase refolding peaked in
the presence of a 10-fold molar excess of the DnaJΔZnII mutant protein.

We showed that the DnaJΔZnII mutant protein behaved identically to wild type DnaJ and
stimulated DnaK’s ATPase activity maximally when present together with unfolded substrate
proteins. Since it had been demonstrated that only the simultaneous binding of DnaJ and DnaK
to the same polypeptide chain facilitates the high stimulatory effect of DnaJ on DnaK’s ATPase
(13), we must, therefore, assume that our DnaJΔZnII mutant protein is able to interact with
substrate proteins and DnaK like wild type DnaJ. These results provided excellent evidence that
the maximal stimulation of DnaK’s ATPase activity is not, as previously suggested, sufficient for
the “locking-in” of substrate proteins into the substrate binding site of DnaK. It appears,
however, that additional interactions between DnaK and DnaJ are required to promote the
conversion of DnaK into its high affinity binding state, and that these additional interactions are
mediated by a functional zinc center II. In the presence of these additional interactions, the DnaK/DnaJ/GrpE foldase machine becomes catalytically active and accelerates the refolding of luciferase. These findings are in good agreement with studies conducted by Banecki and co-workers, who showed that a DnaJ truncation mutant, which lacks the complete zinc binding region of DnaJ, is able to bind native substrate proteins, and stimulates DnaK’s ATPase activity. This mutant protein was, however, like our zinc center II mutant unable to efficiently convert DnaK’s conformation into its high affinity binding state (18).

Based on earlier models and our studies, we propose a model in which the conversion of DnaK-substrate protein complex into the high affinity binding state requires at least two distinct steps: ATP hydrolysis in DnaK and additional interactions between DnaK and the zinc center II of DnaJ. These interactions might cause DnaJ to release its substrate proteins to DnaK, and allow DnaK to lock in the substrate protein (Fig. 7). The requirement for such additional interactions between DnaJ and DnaK-substrate protein complexes have been suggested before in DnaK suppressor studies that showed that DnaJ binds to at least two sites in DnaK (35). The first interaction, which has been recognized for many years, seems to occur between the N-terminal J-domain of DnaJ and the N-terminal ATPase domain of DnaK. The second interaction site was proposed to be between an unidentified site in DnaJ and the C-terminal substrate binding site in DnaK (35). It appears that we have succeeded in identifying that this required additional interaction site in class I DnaJ homologues is mediated by the highly conserved zinc center II.
References


Footnotes

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Figure legends

Fig. 1 Cysteine rich zinc binding domain of DnaJ

A. Model of the cysteine rich domain of wild type DnaJ according to the solution structure of the isolated CR domain (21). Zinc center I is formed by cysteine motif 1 (Cys144, Cys147) and cysteine motif 4 (Cys197, Cys200). Zinc center II is formed by cysteine motif 2 (Cys161, Cys164) and cysteine motif 3 (Cys183, Cys186). In our study, either all 4 cysteines of zinc center I were replaced by Ser residues to construct zinc center I mutant protein DnaJΔZnI, or all 4 cysteines of zinc center II were replaced by Ser residues to construct the zinc center II mutant protein DnaJΔZnII. B. Secondary structure of the cysteine rich domain of DnaJ (PDB: 1EXK) using povscript+ (39). C. Solvent accessible surface model of the cysteine rich domain of DnaJ using GRASP (40). Only the regions of charged side chains (blue) and of hydrophobic side chains (yellow) are depicted in color.

Fig. 2 Zinc center II is crucial for the in vivo activity of DnaJ

Wild type strain MG1655, dnaJ deletion strain PK11 as well as PK11 expressing either wild type DnaJ or the individual zinc center mutants DnaJΔZnI or DnaJΔZnII were grown on LB plates overnight at (A) 37°C or (B) 43°C. To analyze the (C) motility of these strains at 37°C, dnaJ deletion strain PK11 as well as PK11 expressing either wild type DnaJ or the individual zinc center mutants DnaJΔZnI or DnaJΔZnII were grown on 0.3% LB agar plates for 15 h.

Fig. 3 Near and Far-UV CD spectra of wild type DnaJ and the zinc center mutants

Far-UV CD spectra of 10 μM (——) wild type DnaJ, (••••) DnaJΔZnI and (−−−−) DnaJΔZnII in 30 mM phosphate buffer pH 8.2, 120 mM KCl, 15 mM NaCl, 4% glycerol. Twenty spectra were
accumulated and buffer corrected. **Insert:** Near-UV CD spectra of 10 µM (----) wild type DnaJ, (••••) DnaJΔZnI and (-----) DnaJΔZnII in 30 mM phosphate buffer pH 8.2, 120 mM KCl, 15 mM NaCl, 4% glycerol.

**Fig. 4.** DnaJ’s zinc center II is important for the function of the DnaK/DnaJ/GrpE folding machinery **in vitro**

A Guanidinium-HCl denatured firefly luciferase was diluted 1:100 (final conc.: 0.08 µM) into 40 mM MOPS, pH 7.5, 50 mM KCl, 2 mM MgATP and 0.1 mg/ml BSA supplemented with 0.8 µM DnaK and 0.8 µM GrpE either in the (■) absence of DnaJ or in the presence of 0.16 µM (●) wild type DnaJ, (○) DnaJΔZnI mutant protein or (▼)DnaJΔZnII mutant protein. The activity of luciferase was determined as described. The activity of native luciferase was set to 100%.

B, C Chemically denatured luciferase (0.08 µM) was reactivated in the presence of 0.8 µM DnaK and 0.8 µM GrpE and various concentrations of (●) wild type DnaJ, (○) DnaJΔZnI or (▼) DnaJΔZnII. Either (B) the reactivation yields after 60 minutes of reactivation or (C) the reactivation rates were determined and plotted against the ratio of DnaJ to luciferase.

**Fig. 5.** Chaperone activity of DnaJ and the mutant proteins

A, B. Guanidinium-HCl denatured firefly luciferase was diluted 1:100 (final conc.: 0.08 µM) into 40 mM MOPS, pH 7.5, 50 mM KCl, 2 mM MgATP and 0.1 mg/ml BSA with constant stirring. The incubation reaction was either not supplemented with DnaJ (—••--) or contained 0.04 µM (••••) wild type DnaJ, (-----) DnaJΔZnI or (---) DnaJΔZnII in the (A) absence or (B) presence of 0.8 µM DnaK. Aggregation was determined using light scattering measurements. C. Influence of various concentrations of (●) wild type DnaJ, (○) DnaJΔZnI or
(▼) DnaJΔZnII on the aggregation of chemically denatured luciferase. The conditions used were identical to the ones described above. The light scattering signal after 600 sec in the absence of chaperones is set to 100%.

**Fig. 6. Influence of DnaJ mutants on the ATPase activity of DnaK**

ATP hydrolysis of 0.8 µM DnaK-[α<sup>32</sup>P]ATP in 40 mM MOPS, pH 7.5, 50 mM KCl was determined in the (insert) absence of unfolded substrate proteins or in the presence of 0.08 µM chemically denatured luciferase. The reactions were either (■) not supplemented with DnaJ or supplemented with 0.16 µM (●) wild type DnaJ, (○) DnaJΔZnI mutant protein or (▼) DnaJΔZnII mutant protein. Single turn over experiments were performed and the results of one representative experiment is shown.

**Fig. 7. Model of DnaK/DnaJ/GrpE Action**

DnaK and DnaJ bind simultaneously to distinct sites of an unfolded substrate polypeptide chains (13,33). The interaction of DnaK-ATP to substrate proteins is transient and characterized by high on/off rates (33). Adjacent binding of DnaJ and DnaK promotes their interaction. Interaction between DnaJ’s J-domain and DnaK’s N-terminal ATPase domain, stimulates ATP hydrolysis. This presumably causes conformational changes and might be a requirement for further DnaK-DnaJ interactions. A second interaction between DnaJ and DnaK, which is mediated by zinc center II, seems to be essential for closing DnaK’s substrate binding site and for “locking-in” the substrate. This additional step appears to be crucial for the catalytic activity of the DnaK/DnaJ/GrpE foldase machine. The nucleotide exchange factor GrpE then exchanges ADP with ATP and unlocks DnaK.
Table 1. Summary of the *in vivo* and *in vitro* properties of DnaJ class I zinc center mutants

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Linke et al., Fig. 2

A

MG1655
PK11
pdnaJΔZnI
PK11
dnaJ
PK11
pdnaJΔZnII
PK11

37°C

B

MG1655
PK11
pdnaJΔZnI
PK11
dnaJ
PK11
pdnaJΔZnII
PK11

43°C

C

MG1655
PK11
pdnaJΔZnI
PK11
dnaJ
PK11
pdnaJΔZnII
PK11

motility
Linke et al., Fig. 4A, 4B

**Figure 4A**

Ratio DnaJ : luciferase vs. luciferase activity [%]

- **A**
  - Graph showing luciferase activity [%] over time [min].
  - Axes: time [min] on the x-axis and luciferase activity [%] on the y-axis.
  - Data points and lines indicating different conditions.

- **B**
  - Graph showing luciferase reactivation [%] vs. ratio DnaJ : luciferase.
  - Axes: ratio DnaJ : luciferase on the x-axis and luciferase reactivation [%] on the y-axis.
  - Data points and lines indicating different conditions.
Linke et al., Fig. 4C

ratio DnaJ : luciferase

rate of luciferase reactivation [min\(^{-1}\)]

0.0 0.1 0.2 0.3 0.4

0.0 0.1

0.1 1 10

ratio DnaJ : luciferase

10 100

100
Linke et al., Fig. 5A, 5B

**A**

- DnaK

DnaJΔZnI

DnaJΔZnII

DnaJ WT

**B**

+ DnaK

luciferase

DnaJΔZnII

DnaJ WT

DnaJΔZnI
Linke et al., Fig. 5C

The graph shows the relative light scattering of luciferase [%] as a function of the ratio DnaJ : luciferase. The x-axis represents the ratio DnaJ : luciferase, ranging from 0.125 to 10, while the y-axis represents the relative light scattering, ranging from 0 to 100. The data points are plotted for different conditions, indicated by different symbols. The graph illustrates the effect of varying the ratio of DnaJ to luciferase on the relative light scattering.
Linke et al., Fig. 7

DnaJ  DnaK

GrpE

ATP

ADP

Unfolded protein

ATP hydrolysis

“lock in”

GrpE

ATP

ADP
The roles of the two zinc binding sites in DnaJ
Katrin Linke, Tobias Wolfram, Johanna Bussemer and Ursula Jakob

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