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A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates

Alexander Szabo, Richard Korszun[1], F.Ulrich Hartl[2] and John Flanagan[1]

Howard Hughes Medical Institute and Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021 and [1]Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

[2]Corresponding author

The *Escherichia coli* heat-shock protein DnaJ cooperates with the Hsp70 homolog DnaK in protein folding *in vitro* and *in vivo*. Little is known about the structural features of DnaJ that mediate its interaction with DnaK and unfolded polypeptide. DnaJ contains at least four blocks of sequence representing potential functional domains which have been conserved throughout evolution. In order to understand the role of each of these regions, we have analyzed DnaJ fragments in reactions corresponding to known functions of the intact protein. Both the N-terminal 70 amino acid 'J-domain' and a 35 amino acid glycine–phenylalanine region following it are required for interactions with DnaK. However, only complete DnaJ can cooperate with DnaK and a third protein, GrpE, in refolding denatured firefly luciferase. As demonstrated by atomic absorption and extended X-ray absorption fine structure spectroscopy (EXAFS), the 90 amino acid cysteine-rich region of DnaJ contains two Zn atoms tetrahedrally coordinated to four cysteine residues, resembling their arrangement in the C4 Zn binding domains of certain DNA binding proteins. Interestingly, binding experiments and cross-linking studies indicate that this Zn finger-like domain is required for the DnaJ molecular chaperone to specifically recognize and bind to proteins in their denatured state.

Keywords: chaperone/DnaJ/heat-shock protein/zinc finger-like domain

Introduction

The 41 kDa heat-shock protein, DnaJ, is the founding member of a large family of homologous proteins implicated in the regulation of protein conformation (Silver and Way, 1993; Cyr *et al.*, 1994). DnaJ itself was originally identified as an *Escherichia coli* host factor required for the replication of λ phage (Sunshine, 1977; Saito and Uchida, 1978). In this role, DnaJ stimulates the capacity of another host protein, the Hsp70 homolog DnaK, to foster the formation of a replication-competent complex on the λ phage origin of replication (Zylicz *et al.*, 1989; Georgopolous, 1992). The critical step in this process is the ATP-mediated dissociation of the λP protein from the replication initiation complex. Subsequent work has implicated DnaJ and DnaK, as well as their eukaryotic homologs, in a variety of important reactions in the cell in which the unifying mechanism of action involves the capacity to specifically recognize proteins in non-native states and release them in an ATP-dependent manner. These functions include the maturation of newly synthesized polypeptides (Gragerov *et al.*, 1992; Hendrick *et al.*, 1993; Kudlicki *et al.*, 1994), the refolding of proteins after heat denaturation (Skowyra *et al.*, 1990; Schröder *et al.*, 1993) and the regulation of protein degradation (Sherman and Goldberg, 1992). A mammalian homolog of DnaJ, the Hsp40 protein, along with Hsp70, has been detected in a complex with short ribosome-bound nascent polypeptide chains (Frydman *et al.*, 1994). These data suggest that DnaJ homologs may cooperate with Hsp70s in preventing misfolding of proteins that might occur prior to completion of translation.

Hsp70s bind to a variety of protein and synthetic peptide substrates, and it has been proposed that the role of different DnaJ homologs may be to cooperate with individual Hsp70s to modulate or determine their specificity of binding to unfolded polypeptides (Silver and Way, 1993; Cyr and Douglas, 1994; Cyr *et al.*, 1994). The various DnaJ homologs identified to date each contain one or more blocks of sequence homology to DnaJ that may correspond to individual functional domains of the protein. In particular, the N-terminal 70 amino acids or 'J-region' has been implicated in determining the specificity of interactions between DnaJ homologs and Hsp70s. This was originally concluded from the observation that the membrane protein Sec63, which shares only the J-region with DnaJ, cooperates with the Hsp70, Kar2 (BiP), in import reactions into the endoplasmic reticulum (Rothblatt *et al.*, 1989; Sadler *et al.*, 1989; Scidmore *et al.*, 1993). A 108 amino acid fragment of DnaJ containing the J-region can substitute for the intact protein in λ phage replication (Wall *et al.*, 1994). However, DnaJ also possesses a molecular chaperone function of its own, as revealed by its capacity to prevent the aggregation of denatured polypeptides *in vitro* (Langer *et al.*, 1992; Schröder *et al.*, 1993), indicating that DnaJ does not function merely as a regulator of DnaK.

We have demonstrated recently that DnaJ can participate with DnaK and a third protein, GrpE, in an ATP-dependent reaction that results in the *in vitro* refolding of denatured firefly luciferase (Szabo *et al.*, 1994). In this reaction, which may mimic the role of these chaperones in stabilizing nascent polypeptides during translation, DnaJ initially acts alone to bind to and prevent the aggregation of unfolded luciferase. Subsequently, in the presence of ATP, a complex is formed involving luciferase, DnaJ and DnaK. DnaJ appears to act in the formation of this complex by triggering ATP hydrolysis by DnaK, shifting it into the ADP state (Liberek *et al.*, 1991), which binds tightly to

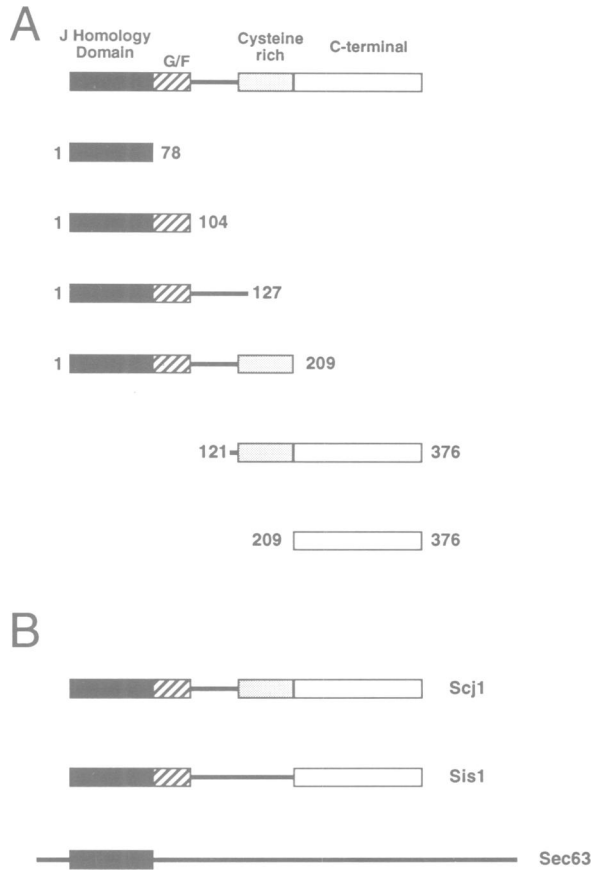

Fig. 1. (**A**) Conserved structural features of *E.coli* DnaJ protein, and (**B**) of the yeast DnaJ homologs Scj1, Sis1 and Sec63. The integral membrane protein Sec63 contains only the DnaJ homology domain (black box) which is exposed to the lumen of the ER.

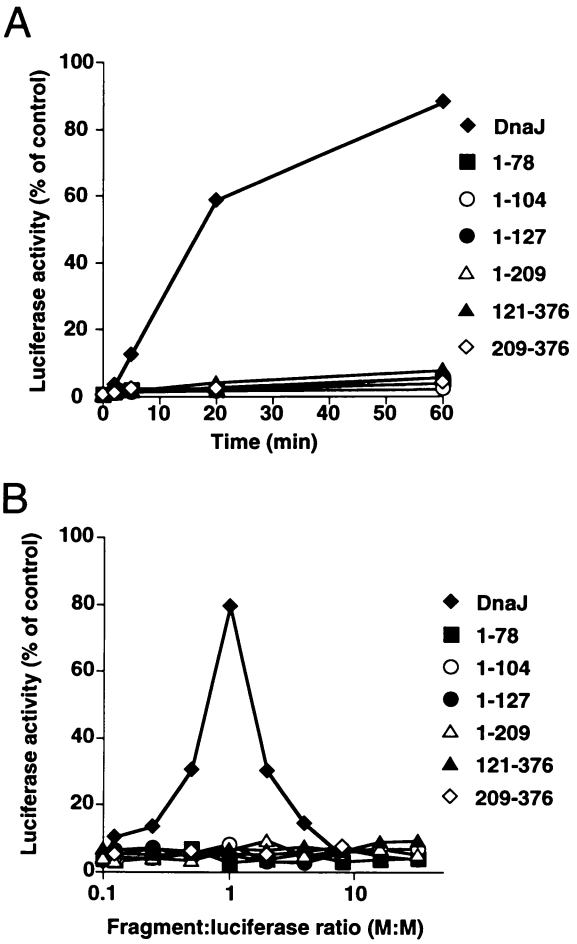

Fig. 2. DnaJ fragments are unable to replace the intact protein in the chaperone-mediated refolding of firefly luciferase. (**A**) Time course of luciferase reactivation in the presence of an equimolar concentration of DnaJ or of the indicated fragments. (**B**) Luciferase refolding at increasing concentrations of DnaJ and DnaJ fragments relative to denatured luciferase. Luciferase activities were measured after incubation for 1 h at 25°C and are expressed as percentage of a native enzyme control (see Materials and methods).

polypeptide substrates (Palleros *et al.*, 1991). GrpE acts to release the bound ADP, labilizing the complex and permitting release of luciferase for refolding upon binding of a second molecule of ATP to DnaK. In order to characterize the role of DnaJ in this process, we engineered protein fragments of DnaJ corresponding to potential functional domains of the protein and analyzed their capacity to substitute for authentic DnaJ in assays testing individual steps in the luciferase refolding reaction. The results indicate that each of four main regions of the protein which have been conserved through evolution (Silver and Way, 1993; Cyr and Douglas, 1994; Cyr *et al.*, 1994) are required for specific functions of the chaperone. The capacity for binding unfolded polypeptide involves the central cysteine-rich region of DnaJ. Surprisingly, we discovered this domain to contain two Zn atoms each coordinated to four cysteine residues.

Results

The complete structure of DnaJ is required for DnaK/DnaJ/GrpE-mediated protein refolding

The primary structure of DnaJ and the fragments used in this study, as well as the structural features of various DnaJ homologs, are shown in Figure 1. In addition to the J-region, consisting of the N-terminal 70 amino acids, three other blocks of sequence present in at least some DnaJ homologs can be identified. These consist of a glycine and phenylalanine (G/F)-rich region located from residues 71 to 108 in DnaJ, a cysteine-rich domain lying between residues 127 and 209, which is present in some, but not all DnaJ homologs, and a C-terminal stretch of ~165 amino acids which again is present in most DnaJ homologs.

DnaJ and the different fragments were purified to >90% homogeneity and in soluble form upon overexpression in *E.coli*. Initially, we examined the capacity of the constructs to replace the intact protein in the luciferase refolding reaction mediated by DnaK, DnaJ and GrpE in the presence of Mg-ATP. The time course of a typical experiment in which DnaJ was added at a 1:1 molar ratio with respect to luciferase diluted from guanidinium chloride (GdmCl) is shown in Figure 2A. None of the DnaJ fragments, when added at equivalent concentrations, was able to support significant levels of refolding. A titration of the full-length protein indicated that addition of DnaJ to concentrations greater than that of luciferase led to a decrease in the yield of refolded protein (Figure 2B). Excess DnaJ may bind to luciferase upon release from its complex with DnaK and DnaJ, preventing renaturation (Szabo *et al.*,

1994). None of the DnaJ fragments was able to support significant refolding, even if present in large excess. This result using the luciferase refolding assay is in contrast to the observation that an N-terminal fragment of DnaJ could replace the full-length protein in an assay measuring λ phage replication (Wall *et al.*, 1994), suggesting a different functional role for DnaJ in this reaction.

DnaJ fragments containing the J-domain and the G/F-rich region stimulate the DnaK ATPase

DnaJ previously has been demonstrated to cause an ~2-fold stimulation of DnaK's weak basal ATPase activity (Liberek *et al.*, 1991). We tested the various fragments for this function. In each case, full-length DnaJ or DnaJ fragment was present in the assay at a molar ratio of 5:1 with respect to DnaK. As shown in Figure 3A, the DnaJ fragments can be divided into two groups either with or without stimulating activity. In the absence of DnaK, none of the fragments alone contained any significant ATPase activity above background (not shown). In general, all of the stimulatory fragments include the N-terminus of the protein, although the fragment corresponding to the J-domain alone is insufficient for stimulation. The minimal fragment identified, therefore, contains both the J-domain and the G/F-rich region up to residue 104. This construct was also able to synergize with GrpE in stimulating the DnaK ATPase in a manner similar to that reported for full-length DnaJ (Liberek *et al.*, 1991) (not shown). These results are in agreement with a recent report that a polypeptide corresponding to the N-terminal 108 amino acids of DnaJ can stimulate the DnaK ATPase activity (Wall *et al.*, 1994).

To determine whether this activation of the DnaK ATPase activity corresponds to an essential interaction between DnaJ and DnaK that is necessary for these molecular chaperones to mediate protein folding, we tested whether the N-terminal fragments could act as competitive inhibitors of DnaJ in the firefly luciferase refolding reaction. Denatured luciferase was first diluted into buffer containing DnaJ. Prior to addition of DnaK, ATP and GrpE, DnaJ fragment was added at a 5-fold molar excess over DnaK. In agreement with the results described above, the DnaJ fragments 1–104, 1–127 and 1–209 all significantly reduced the efficiency by which luciferase was refolded, whereas the 1–78 fragment had no effect (Figure 3B). For the 1–127 fragment, a half-maximal inhibitory effect was observed at a concentration ~5-fold that of DnaK in the reaction (Figure 3C), consistent with the fragment acting by competitively inhibiting an interaction between intact DnaJ and DnaK.

The cysteine-rich domain of DnaJ is involved in binding unfolded polypeptide

Although significant work has gone into characterizing the means by which Hsp70s such as DnaK bind to denatured polypeptide substrates, recent evidence (Hendrick *et al.*, 1993; Schröder *et al.*, 1993; Szabo *et al.*, 1994) indicates that DnaJ may actually be the first molecular chaperone to interact directly with unfolded proteins, both *in vitro* and in the *de novo* folding of nascent polypeptides. In order to determine which region of DnaJ is responsible for the binding specificity of DnaJ for denatured proteins, each of the fragments described in

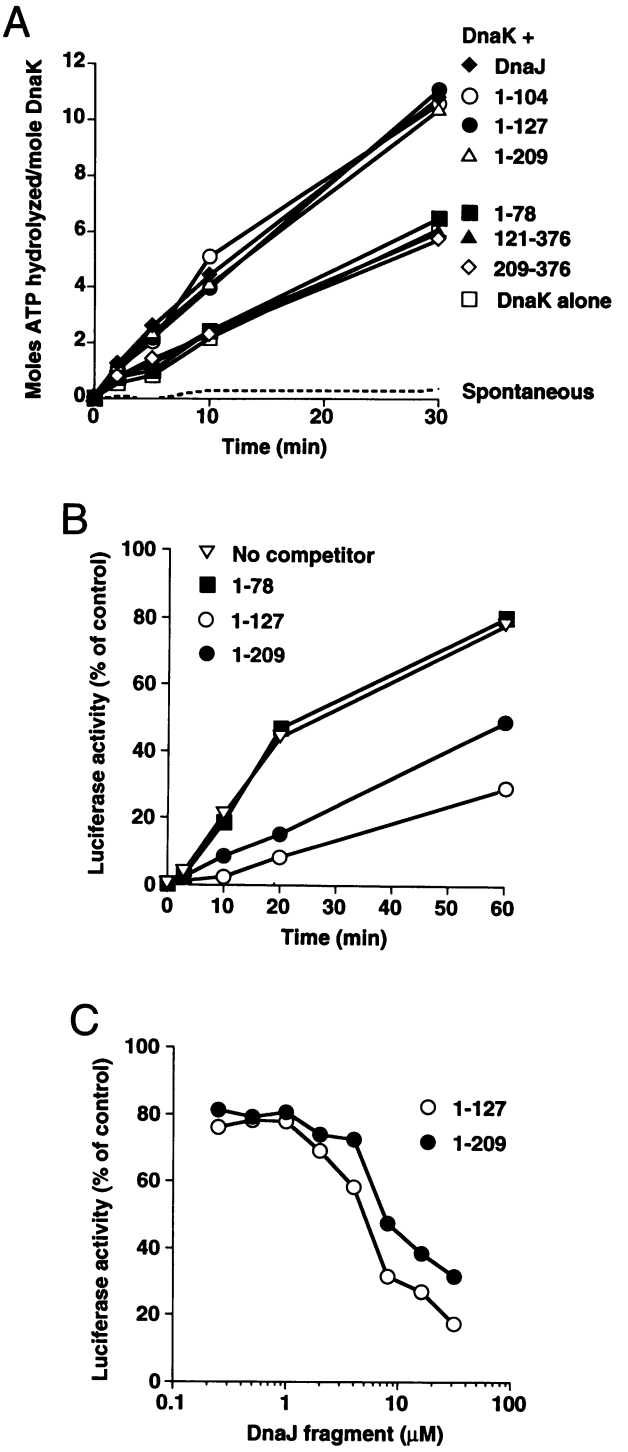

Fig. 3. Interaction of DnaJ fragments with DnaK. (**A**) Time course of ATP hydrolysis by DnaK (1 μM) in the presence of the indicated DnaJ fragments (5 μM). (**B**) Inhibition of chaperone-mediated luciferase reactivation by N-terminal fragments of DnaJ. Luciferase refolding was measured as in Figure 1 at the times indicated, except that GdmCl-denatured luciferase was initially diluted into buffer containing 0.25 μM DnaJ alone. The reactions were incubated for 5 min, then the indicated DnaJ fragments were added at a final concentration of 6.25 μM, followed by the addition of DnaK to 1.25 μM. (**C**) Competition of DnaJ in the luciferase refolding assay by DnaJ fragments 1–127 and 1–209. Luciferase refolding was measured as in (B) after 1 h of incubation in the presence of the DnaJ fragments at the concentrations indicated. Luciferase activities are expressed as in Figure 2B.

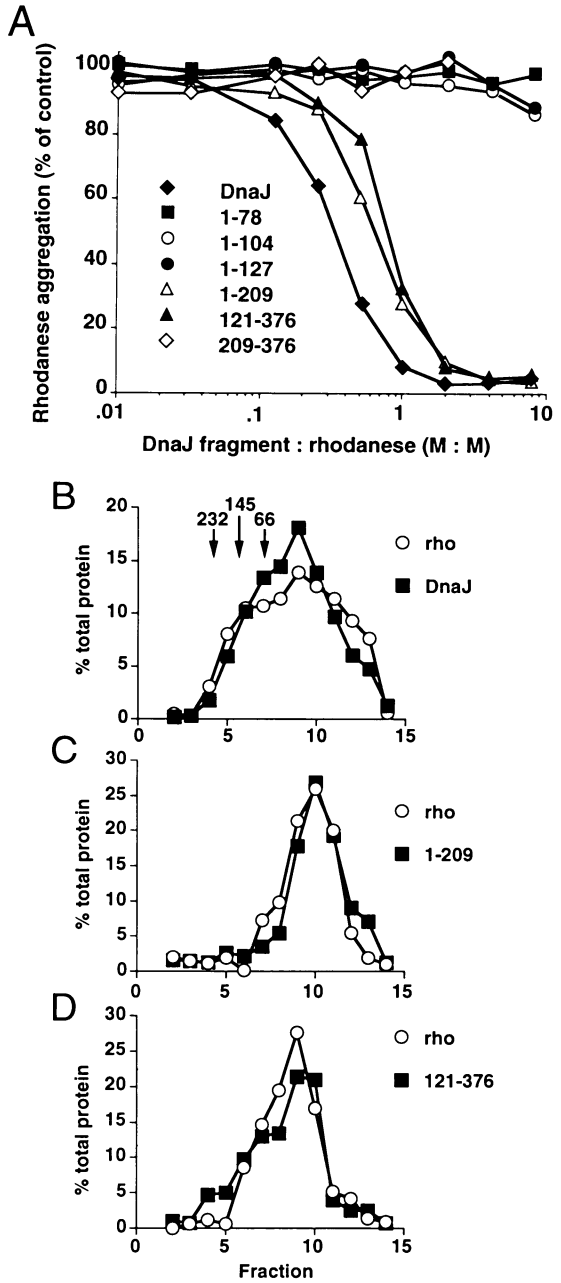

Fig. 4. Interaction of DnaJ and DnaJ fragments with non-native polypeptide. (**A**) Inhibition of aggregation of [^{3}H]rhodanese (final concentration 0.5 μM) upon dilution from denaturant into buffer solution containing the various proteins at the molar ratio over rhodanese indicated (see Materials and methods). Aggregation measured in the absence of chaperone proteins is set to 100%. (**B–D**) Sephacryl S-300 gel filtration chromatography of rhodanese complexed with DnaJ (B), fragments 1–209 (C) and 121–376 (D), respectively. The final concentrations of rhodanese and DnaJ proteins were 0.5 and 1 μM, respectively. Protein amounts were determined by laser densitometry of Coomassie Blue-stained SDS–polyacrylamide gels and are given as percentage of total protein recovered from the column. Recoveries of DnaJ proteins and rhodanese were ~80% of that applied onto the column.

Figure 1 was tested for its capacity to prevent the aggregation of ^{3}H-labeled bovine rhodanese upon its dilution from 6 M GdmCl. Intact DnaJ effectively prevented the formation of pelletable rhodanese aggregates when present at a concentration equivalent to that of rhodanese (Langer *et al.*, 1992) (Figure 4A). Only two other fragments shared this property, the overlapping fragments 1–209 and 121–376, although they were ~2-fold less effective on a molar basis than intact DnaJ. The N-terminal fragments shorter than 1–209 had no significant effect. Gel chromatography indicated that the prevention of aggregation occurs through the formation of a complex between the denatured protein and either DnaJ or the 1–209 or 121–376 fragments. When samples of the reactions were applied to Sephacryl S-300 size exclusion columns, the denatured rhodanese co-eluted with the DnaJ fragment (Figure 4B–D). In the absence of DnaJ, the aggregated rhodanese was not recovered from the column, but could be eluted by washing with denaturant (not shown) (Langer *et al.*, 1992).

The only region in common between the two fragments capable of effectively binding to and preventing rhodanese aggegation is the cysteine-rich domain between residues 121 and 209. Interestingly, the spacing of the eight cysteines in this domain closely matches the spacing of the Zn-coordinating cysteines in C4 Zn finger proteins (Cyr *et al.*, 1994; Schwabe and Klug, 1994) (Figure 5). As shown below, two Zn atoms are indeed coordinated to these cysteines. To test the possibility that the cysteine residues play a critical role in the interaction of DnaJ with unfolded polypeptide, we employed site-directed mutagenesis to create the variants DnaJ(C161/164S) and DnaJ(C183/186S) in which either the last two cysteines in the first Zn finger-like domain or the first pair of cysteines in the second Zn finger-like domain were substituted with serines (Figure 5). These changes decreased the capacity of the variants to prevent rhodanese aggregation ~10-fold (Figure 6A). Furthermore, when substituted for DnaJ in the luciferase refolding assay, the variants were again at least 10-fold less effective than the wild-type protein (Figure 6B). Interestingly, the two mutant proteins behaved somewhat differently in this assay. While DnaJ(C183/186S) could refold luciferase when used at 10-fold higher concentrations than the wild-type, DnaJ(C161/164S) supported only a fraction of the luciferase refolding, even when employed at the highest concentrations. The functional defect of mutants DnaJ(C161/164S) and DnaJ(C183/186S) is unlikely to be due merely to global effects on DnaJ structure, because both mutant proteins behave identically to authentic DnaJ during purification and in their capacity to stimulate the DnaK ATPase (not shown).

To analyze further the function of the central cysteine-rich region (residues 127–209), we took advantage of the fact that apart from the eight cysteines concentrated in this domain, DnaJ contains only two other cysteine residues, both of which are located C-terminal to residue 209. The sulfhydryl-specific heterobifunctional cross-linking reagent *N*-[4-*p*-(azidosalicylamido)butyl]-3′(2′-pyridyldithio) proprionamide was radioiodinated and bound to DnaJ or to the fragment 1–209. At least in the case of the latter protein, the cross-linker must have been bound by a disulfide bond to one or more of the cysteine residues located between residues 121 and 209. The chemical modification of cysteine residues within a C4 Zn finger protein has previously been described (O'Connor *et al.*, 1993). Subsequently, denatured luciferase was diluted into buffer containing an equal concentration of modified DnaJ or fragment 1–209. The reactions were then photolyzed to induce formation of a cross-link with

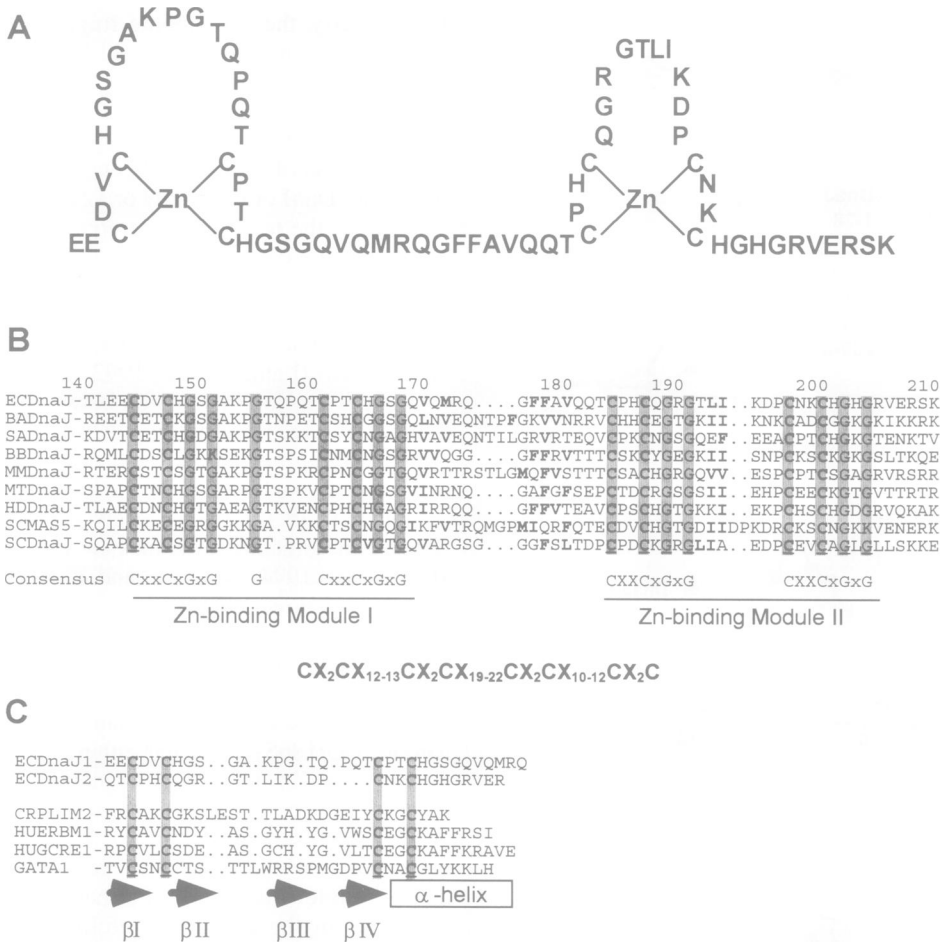

Fig. 5. Alignment of the Zn finger domain present in most DnaJ family members. (**A**) The Zn finger representation of the cysteine-rich region in DnaJ. (**B**) Sequence homologs of *E.coli* DnaJ. Sequences were obtained using the TFastA search program (Pearson and Lipman, 1988) of the GENBANK data base and were aligned using the MULALN program (Copret, 1988). Consensus residues are indicated by the shaded boxes. (**C**) Comparison of the sequence for the two Zn binding domains in DnaJ with C4 type Zn binding domains of proteins where the three-dimensional structures are known and similar spacings between cysteine residues to that in DnaJ are found. The proteins of known structure are: the second Zn binding domain in the LIM motif (Perez-Alvarado *et al.*, 1994), the first Zn binding domains in the estrogen and glucocorticoid steroid hormone receptors (Luisi *et al.*, 1991), and the GATA Zn binding domain (Omichinski *et al.*, 1993). Below the sequence of these domains are shown the location and type of secondary structural elements found in these proteins.

luciferase. After treatment with dithiothreitol (DTT) to induce cleavage of the disulfide bond linking the cross-linker to DnaJ and permit transfer of the radiolabeled cross-linker to the substrate, samples of the reactions were analyzed by reducing SDS–PAGE. For both the complete DnaJ protein and the 1–209 fragment, radiolabel was transferred efficiently from DnaJ to luciferase (Figure 7A, lanes 2 and 4). Furthermore, no label was transferred either to DnaK or GrpE when similar experiments were performed under conditions appropriate for luciferase refolding (Figure 7B, lanes 4, 6 and 8). Notably, no label was transferred to DnaK when DnaJ, luciferase and DnaK were incubated in the presence of Mg-ATP (Figure 7B, lane 6). Under these conditions, the three proteins are involved in a ternary complex (Szabo *et al.*, 1994) in which the DnaJ–DnaK interaction is presumably mediated by the J- and G/F-rich domains (see above). These data indicate that a cross-link was formed specifically between the cysteine-rich domain of DnaJ and the unfolded substrate protein. APDP-modified DnaJ could not support the refolding of luciferase (Figure 7C). However, after addition of DTT to cleave the cross-linker, the chaperone regained its activity in the assay, demonstrating that the effect of the cross-linker was fully reversible. We conclude from these results that the region between residues 121 and 209 is involved in the binding of DnaJ to the unfolded protein substrate.

DnaJ contains two zinc atoms coordinated in two zinc finger-like structures

Atomic absorption spectroscopy demonstrated that DnaJ indeed contains two atoms of Zn per protein monomer (Table I). Furthermore, both the 1–209 and 121–376 fragments contain similar amounts of Zn, indicating that the Zn binding domains are structurally intact in both fragments and represent relatively independent structural modules. Only one atom of Zn per protein monomer is found in mutants (C144/147S), (C161/164S), (C183/186S) and (C197/201S), confirming that these cysteine residues are critical for Zn binding. No Zn was detectable in fragments 1–127 and 210–376.

The region containing the eight cysteine residues within the central domain of DnaJ also contains a number of histidine residues that could, in principle, be involved in

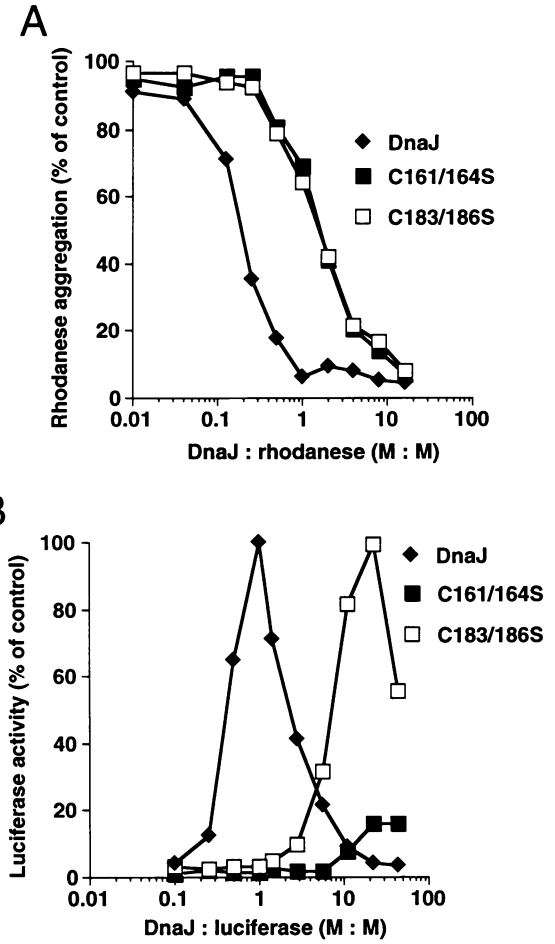

Fig. 6. Functional analysis of cysteine–serine variants of DnaJ. (**A**) Inhibition of [^{3}H]rhodanese aggregation by DnaJ and cysteine–serine variants of DnaJ. Aggregation was measured as in Figure 4. (**B**) Luciferase reactivation in the presence of increasing concentrations of DnaJ and cysteine–serine variants of DnaJ (see legend to Figure 2B and Materials and methods). Luciferase activities are expressed as percentage of the reactivation obtained with 1 μM DnaJ.

the chelation of the Zn atoms. X-ray absorption fine structure spectroscopy (EXAFS) was therefore employed to characterize further the metal binding of DnaJ. Like atomic absorption, this form of spectroscopy is diagnostic for specific heavy atoms and, in addition, provides detailed information concerning the number and types of ligands chelating the metal. We compared the EXAFS spectrum of purified DnaJ with two model compounds, Zn(II) tetrathiophenolate [$Zn(SPh)_4^{2-}$] and Zn(II) EDTA. The Zn atom in the former of these two compounds has been shown by X-ray crystallography to be tetrahedrally coordinated by four sulfur atoms, while in the latter compound the Zn atom is coordinated by four oxygen and two nitrogen atoms. Figure 8A shows the background-corrected EXAFS spectrum of DnaJ plotted in reciprocal Å, and Figure 8B shows the Fourier transform of this spectrum. The amplitude of peaks in the EXAFS Fourier transform corresponds to scattering between the Zn atom and its nearest neighbors. Consequently, the single dominant peak in this representation suggests symmetric chelation of Zn by one type of ligand.

The environment of the Zn atoms within DnaJ was compared with that within the model compounds by filtering the nearest-neighbor peaks from each Fourier spectrum and back-transforming them into momentum space. The DnaJ profile (Figure 8C, top, solid line) is typical of the backscatter from sulfur, as can be seen by comparing the DnaJ spectrum with that of [$Zn(SPh)_4^{2-}$] (Figure 8C, bottom, solid line). By contrast, the spectrum of Zn(EDTA) (Figure 8C, bottom, dashed line) is clearly different. In order to determine nearest-neighbor coordination parameters of the Zn in DnaJ, a least-squares method was used to fit a mathematical model to the DnaJ spectrum. The coordination number, ligand bond distance and disorder parameter (σ^2) were varied independently during the fitting procedure. This process yielded an optimal fit (Figure 8C, top, dashed line) corresponding to a coordination number of 4.0 ± 0.3 sulfur atoms for each of the two Zn atoms with an average Zn–ligand distance of 2.32 ± 0.02 Å. This value is nearly identical to the previously reported Zn–sulfur distances for two C4 DNA binding proteins, $TFIII_A$ (2.30 ± 0.02 Å) and the glucocorticoid receptor (2.32 ± 0.02 Å), and for *E.coli* aspartate transcarbamylase (2.34 ± 0.03 Å) (Phillips *et al.*, 1982; Diakun *et al.*, 1986; Freedman *et al.*, 1988). It is also in close agreement with the Zn–sulfur value of 2.353 ± 0.016 Å obtained for $Zn(SPh)_4^{2-}$ (Swenson *et al.*, 1978). The Debey–Waller-like factor $\Delta\sigma^2$ gives an indication of the thermal and static disorder in the system. For DnaJ, this factor refined to 0.001 ± 0.002 $Å^2$, indicating that the Zn–ligand distances for the two Zn atoms in DnaJ are very similar. In addition, the X-ray absorption near-edge structure (XANES) portion of the EXAFS spectra for DnaJ is qualitatively similar to that reported for the glucocorticoid receptor (data not shown) (see Freedman *et al.*, 1988). The XANES includes multiple scatter effects and reflects the symmetry of chelation of the heavy atom. Based on these data, we conclude that the substrate binding domain of DnaJ contains two Zn atoms and has a structure closely resembling that of other previously identified Zn finger proteins.

Discussion

Using purified fragments of DnaJ, we have demonstrated that DnaJ contains at least four functionally distinct sequence elements. While specific functions in the protein folding reaction could be ascribed to these individual domains (Figure 9), only the complete DnaJ sequence was able to cooperate effectively with DnaK and GrpE in the ATP-dependent reactivation of denatured firefly luciferase. Full function in the refolding assay apparently requires the interaction of DnaJ and DnaK, both with each other and with substrate. Remarkably, the capacity of binding unfolded polypeptide involves a Zn finger-like structural motif in the cysteine-rich domain of DnaJ.

According to our recent model for the reaction cycle of the DnaK/DnaJ/GrpE chaperone system (Szabo *et al.*, 1994), refolding of denatured protein requires the formation of a ternary complex between DnaJ, DnaK and the substrate protein. As a key step, formation of this complex involves the interaction of DnaJ with DnaK to stimulate the hydrolysis of ATP by DnaK (Liberek *et al.*, 1991). In addition, DnaJ stabilizes the ADP state of DnaK over the ATP state. The interaction between the two chaperones is mediated by the N-terminal segment of DnaJ. Residues

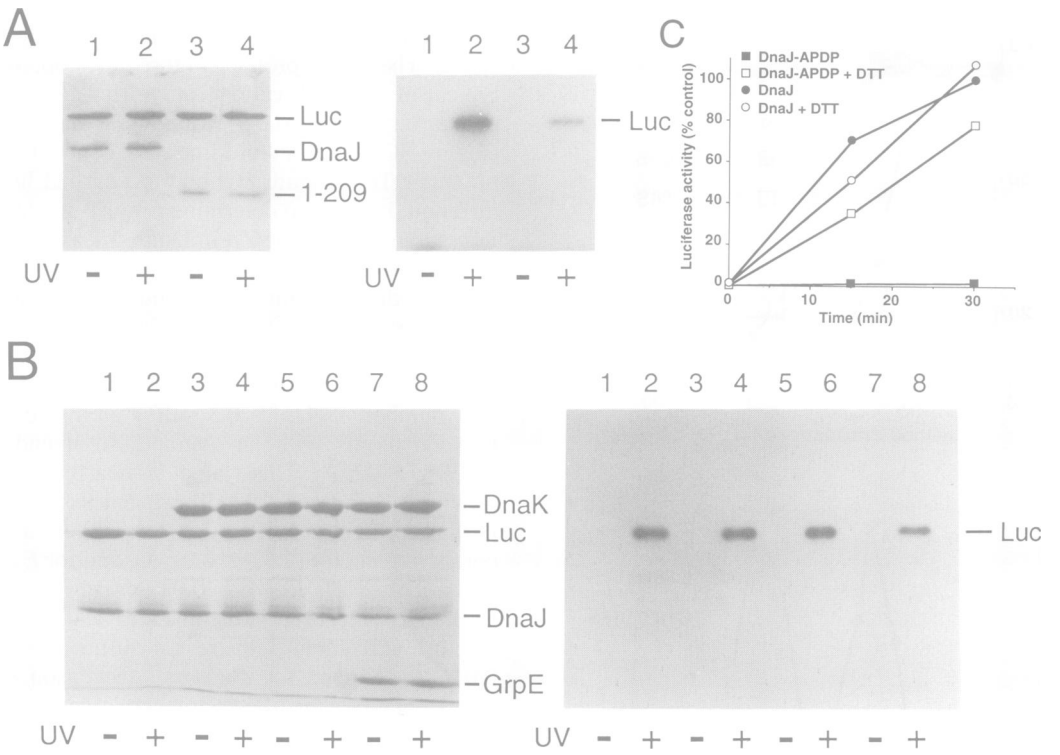

Fig. 7. Interaction of the cysteine-rich domain of DnaJ with unfolded substrate protein. (**A**) DnaJ (lanes 1 and 2) or DnaJ fragment 1–209 (lanes 3 and 4) (0.5 μM each) was labeled with iodinated APDP and incubated with denatured luciferase with or without exposure to UV light as indicated (see Materials and methods for details), resulting in transfer labeling of luciferase. Reactions were divided, separated by reducing SDS–PAGE and analyzed by Coomassie staining (left panel) or autoradiography (right panel). Note that DnaJ stains weakly with Coomassie Blue and that iodinated APDP–DnaJ 1–209 has a lower specific activity than APDP–DnaJ. (**B**) Transfer labeling of luciferase from 0.5 μM APDP-labeled DnaJ (lanes 1–8) in the presence of 2.5 μM DnaK (lanes 3–8), 2.5 μM GrpE (lanes 7 and 8) and 5 mM Mg-ATP (lanes 5–8). Reactions were analyzed as in (A). (**C**) Time course of luciferase refolding by DnaK, GrpE and APDP-modified DnaJ after 30 min pre-incubation of DnaJ in the absence or presence of 25 mM DTT at 25°C.

1–104, including the J-domain and the G/F-rich region following it, comprise the minimum fragment necessary and sufficient to stimulate the DnaK ATPase activity and to act as a competitive inhibitor of intact DnaJ in the luciferase refolding reaction (Figure 9). In contrast to the results obtained with the assay testing luciferase refolding, this fragment has been reported to substitute for authentic DnaJ in an assay masuring λ phage replication (Wall *et al.*, 1994). In the latter reaction, DnaK acts primarily to modulate the conformation of a stable protein, the λP protein, to induce the initiation of DNA replication. The capacity of DnaJ to stimulate the DnaK ATPase may be sufficient to support this function in the absence of DnaJ's ability to interact with unfolded polypeptide. Interestingly, neither DnaJ nor any of the fragments tested was able to stimulate the isolated DnaK ATPase domain, suggesting that both the ATPase and substrate binding domains of DnaK are involved in the interaction with DnaJ (not shown). Consistent with this, a conserved EEVD motif of the human Hsp70 homolog, located C-terminal to its substrate binding domain, is required for interaction with the DnaJ homolog HDJ-1 (Freeman *et al.*, 1995).

Mutational studies on DnaJ and its homologs have indicated that substitutions within the tripeptide segment HPD in the J-domain (residues 33–35 in DnaJ) abrogate the interaction with the corresponding Hsp70 (Wall *et al.*, 1994). According to the recent NMR structure of DnaJ1–108 (Szyperski *et al.*, 1994; Hill *et al.*, 1995), this tripeptide lies at the solvent-accessible tip of a scaffolding of α-helices. The hydrophobic G/F-rich region is relatively disordered and is attached at the opposite end of the scaffold, although some of its residues make contacts with residues near the HPD loop (K.Huang, J.Prestegard and J.M.Flanagan, unpublished data). The G/F-rich region may play merely an auxillary role in stimulating the ATPase activity of DnaK, since variants of DnaJ in which only the G/F-rich region has been deleted are indistinguishable from wild-type DnaJ in this activity (Wall *et al.*, 1994). It seems likely that the active conformation of the J-

Table I. Summary of atomic absorption data for various forms of DnaJ

Protein	Putative zinc binding motifs	Moles Zn per mole protein ($n \geqslant 3$)
DnaJ	+/+	2.1 ± 0.3
DnaJ (1–127)	−/−	<0.2
DnaJ (1–209)	+/+	1.9 ± 0.3
DnaJ (121–376)	+/+	2.2 ± 0.3
DnaJ (210–376)	−/−	<0.2
DnaJ C144S+C147S	−/+	0.9 ± 0.3
DnaJ C161S+C164S	−/+	0.7 ± 0.4
DnaJ C183S+C186S	+/−	1.1 ± 0.2
DnaJ C196S+C199S	+/−	0.8 ± 0.3

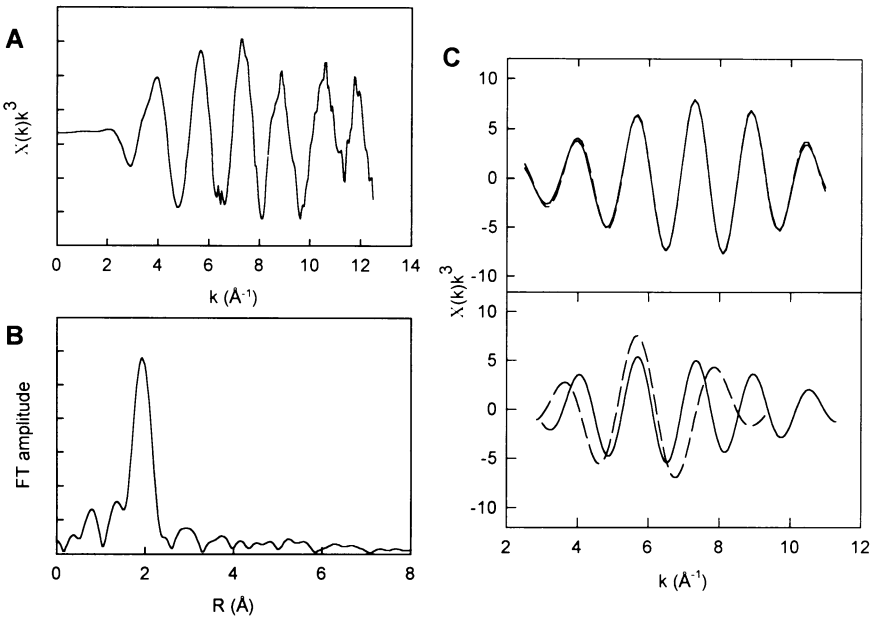

Fig. 8. Zn EXAFS analysis of 0.5 mM DnaJ. (**A**) The EXAFS portion of the k^3 weighted k-space spectrum. (**B**) The Fourier transform of the function of $k^3 \times (k)$ spectrum in (A) to give the R-space spectrum. (**C**) The Fourier-filtered data from the major peak in (B) for DnaJ (top, solid line) generated by inverse Fourier transform from R-space to k-space, and the calculated model for eight sulfur atoms tetrahedrally coordinated to two Zn atoms (top, dashed line). For comparison, the bottom panel shows the spectra of $Zn(SPh)_4^{2-}$ (bottom, solid line) and Zn(EDTA) (bottom, dashed line).

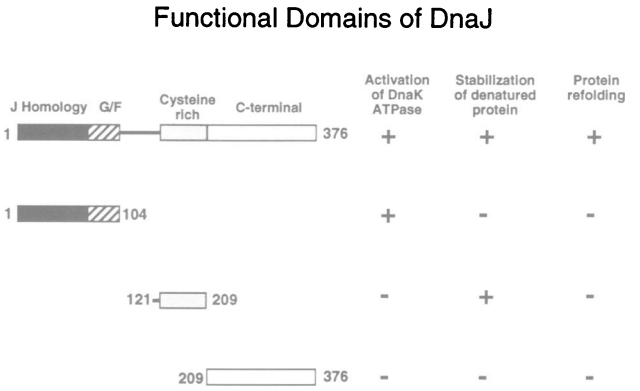

Fig. 9. Functional domains of *E.coli* DnaJ protein.

domain is stabilized through interactions with residues outside of this domain. A subset of these interactions, present in the mutant proteins lacking only the G/F-rich domain, may be sufficient to stabilize the active conformation. In this context, it is worth noting that the sequence of the G/F-rich region conforms closely to the alternating hydrophobic/aromatic motifs identified in peptide substrates for DnaK and other Hsp70s (Blond-Elguindi *et al.*, 1993; Fourie *et al.*, 1994). It seems possible that the G/F-rich region of DnaJ interacts with DnaK by mimicking a DnaK peptide substrate.

In the *in vitro* refolding reaction, DnaJ must first bind to the unfolded protein upon its dilution from denaturant, thereby stabilizing it against aggregation (Schröder *et al.*, 1993; Szabo *et al.*, 1994). Our results implicate the central, cysteine-rich domain of DnaJ, which contains two Zn finger structures, in this function. Three separate types of experiments support this conclusion. (i) Only DnaJ fragments containing this region inhibit the aggregation of denatured rhodanese, and fragments co-migrate in gel chromatography with denatured substrate. (ii) Disruption by mutagenesis of either of the two Zn finger motifs strongly reduces the capacity of DnaJ to bind unfolded polypeptide. (iii) Experiments in which the heterobifunctional cross-linker APDP was pre-bound to a cysteine(s) in the Zn finger-like domain before photocross-linking indicate that this region is in close proximity to the polypeptide substrate. Atomic absorption and EXAFS spectroscopy was used to demonstrate that the eight cysteine residues within this domain are involved in chelating two Zn atoms. The role of the bound Zn is probably to provide a structural cross-strut, similar to a pair of disulfide bonds tethering the protein at four different points (Schwabe and Klug, 1994). In DnaJ, the spacings between the cysteine residues chelating the Zn are similar to those found in a family of C4 Zn fingers (Figure 5B and C), suggesting an overall similar fold of this domain.

How the Zn finger-like domain of DnaJ participates in the interaction with the denatured polypeptide substrate is still unclear. Most molecular chaperones recognize many different substrate proteins in their non-native states, independently of a consensus binding motif. Members of the Hsp70 and Hsp60 (chaperonin) classes of molecular chaperones are thought to interact with extended peptides enriched in hydrophobic residues (Flynn *et al.*, 1991; Landry and Gierasch, 1991; Blond-Elguindi *et al.*, 1993; Fourie *et al.*, 1994) and with hydrophobic surfaces exposed by partially folded polypeptides, respectively (Distel *et al.*, 1992; Hayer-Hartl *et al.*, 1994; Hlodan *et al.*, 1995). It seems likely that DnaJ also recognizes hydrophobic surface properties of an unfolded polypeptide (Langer *et al.*, 1992; Szabo *et al.*, 1994). Recognition of DNA by the Zn finger domains of the steroid hormone receptors occurs primarily through interactions between DNA and residues in the region between the two Zn fingers, including a short α-helix and a relatively flexible linker (Luisi *et al.*, 1991). An inspection of the region between the two Zn finger structures in DnaJ (Figure 5A) identifies a conserved

cluster of hydrophobic residues, including a methionine, two phenylalanines, an alanine and a valine, which may be involved in binding complementary regions of a non-native polypeptide. We assume that the Zn finger regions in DnaJ may play predominantly a structural role in stabilizing the presumably flexible polypeptide binding region of the protein. While the C-terminal domain of DnaJ (residues 209–376) is unable to stabilize unfolded proteins in the absence of the Zn finger-containing domain, parts of the C-terminal domain may contribute to the interaction with polypeptide substrates. This may explain why the 1–209 fragment of DnaJ cannot replace intact DnaJ in the refolding reaction. Certain DnaJ homologs, such as yeast Sis1p and human Hsp40, lack the cysteine-rich domain altogether (see Figure 1B). Nevertheless, these proteins seem to be capable of interacting with polypeptide substrates (Frydman *et al.*, 1994).

Materials and methods

Expression and purification of proteins

DnaK, DnaJ and GrpE proteins were purified from overexpressing *E.coli* strains as previously described (Szabo *et al.*, 1994). All DnaJ fragments were expressed from a pET11 vector (Studier *et al.*, 1990). Authentic DnaJ, DnaJ(C161/162S), (C183/186S), 1–209 and 121–376 were purified as described previously for DnaJ (Zylicz *et al.*, 1985). DnaJ 1–79, 1–104, 1–127 and 209–376 were all found in the soluble phase after cell lysis in low salt buffer. These proteins were purified as previously described for DnaJ 1–79 (Hill *et al.*, 1995). Protein amounts were determined by quantitative amino acid analysis.

Luciferase refolding reactions

Luciferase refolding reactions were prepared as previously described (Szabo *et al.*, 1994). Briefly, refolding reactions were performed at 25°C in buffer B (10 mM MOPS pH 7.2/50 mM KCl) with Mg-ATP (5 mM Mg acetate/1 mM ATP) and typically contained DnaK at 1.25 μM and DnaJ at 0.25 μM (unless otherwise indicated). Luciferase, chemically denatured in buffer A (6 M GdmCl in 30 mM Tris–HCl, pH 7.4/5 mM DTT; 30 min at 25°C) was diluted 100-fold to 0.25 μM final concentration into a reaction containing the above chaperone components. After a 5 min incubation, refolding was initiated by addition of GrpE to 1.25 μM. Luciferase activities were measured as described (Frydman *et al.*, 1994).

Measurement of DnaK ATPase activity

Reactions (50 μl) containing 1 mM [γ-^{32}P]ATP (20 mCi/mmol, final specific activity) and 5 μM of the indicated DnaJ fragments were prepared in buffer B. At time zero, DnaK was added to a final concentration of 1 μM. At the indicated times, 5 ml samples were removed and added to a 500 μl slurry of acidified activated charcoal (Norite, United States Biochemical Corp.) (Sadis and Hightower, 1992). The slurry was vortexed briefly, centrifuged and the supernatant was removed for liquid scintillation counting to determine the amount of free γ-phosphate produced by hydrolysis.

Rhodanese aggregation assays and gel chromatography

Reductively methylated [^{3}H]rhodanese (92.6×10^3 c.p.m./μg) (Hlodan *et al.*, 1995) was denatured at 50 μM in 6 M GdmCl. The rhodanese was diluted 100-fold into 50 μl reactions containing a range of concentrations of DnaJ or DnaJ fragments in buffer B with 50 mM NaCl substituted for KCl. After a 10 min incubation at 25°C, the samples were centrifuged for 10 min at 20 000 *g*. The supernatants were aspirated and the pellets were resolubilized in 100 μl of 1% SDS which was then assayed for radioactivity by liquid scintillation counting. For gel chromatography, rhodanese was diluted to 0.5 μM into 500 μl of buffer B containing DnaJ or DnaJ fragment as indicated in the figure legend. After a 10 min incubation, the sample was applied to a 30×1 cm Sephacryl S-300 column equilibrated in the same buffer. The first 15 ml were discarded and 1 ml fractions were collected. Proteins in each fraction were precipitated by addition of trichloroacetic acid to 10% and then quantified by SDS–PAGE and laser densitometry.

Cross-linking

The heterobifunctional cross-linker APDP was radioiodinated and bound to DnaJ or DnaJ fragment 1–209 according to the protocol of the supplier (Pierce). Briefly, DnaJ was fully reduced by a 30 min incubation in the presence of 25 mM DTT at 30°C, then separated from reducing agent by centrifugation through a Bio-Rad Bio-spin 6 gel filtration column. APDP was radiolabeled by a 30 s incubation in the presence of 500 μCi ^{125}I and an Iodobead (Pierce), after which the sample was removed from the vessel and the iodination reaction quenched by the addition of 25 mM *p*-hydroxyphenylacetic acid. Fifty μg of DnaJ or 1–209 fragment was added and the reaction was incubated for 30 min at room temperature to allow the cross-linker to bind to free sulfhydryls in the substrate protein. The modified proteins were then separated from free APDP and iodine by rapid gel filtration using a Nick column (Pharmacia) and concentrated to ~5 mg/ml using a Microcon filtration apparatus (Millipore). Specific activity of the modified proteins was 1.3×10^5 c.p.m./μg for DnaJ–APDP and 0.8×10^5 c.p.m./μg for 1–209–APDP. Cross-linking reactions were performed after dilution of GdmCl-denatured luciferase to a final concentration of 0.5 μM into buffer containing an equal concentration of modified DnaJ or 1–209 fragment and after removal of aggregates by centrifugation for 10 min at 20 000 *g*. After photolysis with a 320 nm lamp for 5 min, the reactions were incubated for 30 min with 25 mM DTT to allow transfer labeling and then electrophoresed on a 15% SDS–polyacrylamide gel under reducing conditions.

X-ray absorption fine structure spectroscopy (EXAFS)

The EXAFS spectra were collected at Brookhaven National Laboratory NSLS beamline X9A. DnaJ, prepared as described above, was dialyzed exhaustively against 50 mM Tris–HCl pH 8.0, 500 mM KCl, 0.1 mM TCEP–HCl and 0.05% Brij-58. The spectrum was recorded in the fluorescence mode using a Canberra 13 element Ge solid-state detector array windowed on the Zn signal. Data collection was terminated at 10^6 effective counts per data point. The raw EXAFS data consists of the sum of seven scans. This spectrum was corrected by removal of a linear background, extrapolated from the pre-edge region. In addition, it has been normalized to its K-edge jump and converted into momentum (k-space) space. The photoelectron wave-vector k is

$$k = 2\pi/h\ [2m_e(E-E_0)]^{1/2}$$

where E and E_0 are expressed in electron volt (eV) and k has the units of inverse angstroms (Å^{-1}). The value of E_0 was estimated from DnaJ's Zn K-edge inflection point. A background correction was made using a cubic polynomial with a single knot in the center of the spectrum. Analysis of the EXAFS spectrum was performed by multiplication by k^3 and Fourier transformed into coordinate space [R] using data in the range of $3.0\leq k\leq12$ Å^{-1}. The first shell contribution to the EXAFS spectra was isolated with a 1.5 window centered on the largest peak. All data analysis and model fitting was performed using standard procedures (Sayres *et al.*, 1971), and the theoretical calculations developed by Rehr *et al.* (1989).

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