Role of DnaJ G/F-rich Domain in Conformational Recognition and Binding of Protein Substrates*

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DnaJ from Escherichia coli is a Type I Hsp40 that functions as a cochaperone of DnaK (Hsp70), stimulating its ATPase activity and delivering protein substrates. How DnaJ binds protein substrates is still poorly understood. Here we have studied the role of DnaJ G/F-rich domain in binding of several substrates with different conformational properties (folded, partially (un)folded and unfolded). Using partial proteolysis we find that RepE, a folded substrate, contacts a wide DnaJ area that involves part of the G/F-rich region and Zn-binding domain. Deletion of G/F-rich region hampers binding of native RepE and reduced the affinity for partially (un)folded substrates. However, binding of completely unfolded substrates is independent on the G/F-rich region. These data indicate that DnaJ distinguishes the substrate conformation and is able to adapt the use of the G/F-rich region to form stable substrate complexes.

The Hsp40 protein family, also called JDP family, exhibits a wide structural and functional diversity (1, 2). All members of this family share a ~70-amino acid J-domain that allows them to cooperate with Hsp70 chaperones in different processes essential for cell life. The Hsp40 family can be divided into three groups depending on domain composition (3). Type I Hsp40s are constituted by a J-domain followed by a G/F-rich flexible linker, a zinc-binding domain and a conserved C-terminal domain involved in substrate binding and dimerization (4, 5). Members of this group are well-known proteins such as Escherichia coli DnaJ (6) and Saccharomyces cerevisiae Ydj1p (7). Type II proteins, e.g. yeast Sis1p (8), are similar to type I but lack the zinc finger domain. Although structurally and functionally related, these two groups contain unique protein modules (8, 9) and have different quaternary structures (10). Type III JDPs contain only the conserved J-domain that can be located anywhere in the protein sequence. To this group belong functionally diverse proteins such as E. coli DjlA, the clathrin-uncoating auxilin (11), mitochondrial Tim14 and Tim16 (12), and endoplasmic reticulum Sec63p (13).

The functionality of Hsp40 proteins is based on their ability to regulate the ATPase activity of Hsp70 chaperones, and to target protein substrates to their Hsp70 partners. By enhancing the ATP hydrolysis rate of Hsp70, Hsp40s facilitate the conformational transition to the high substrate affinity state of the chaperone ensuring tight binding of the polypeptide chain. The activation of E. coli DnaK intrinsic ATPase by DnaJ and the synergistic effect observed in the presence of substrates are strictly dependent on the J-domain and the G/F-rich region (14, 15). Mutations of a conserved HPD motif in the J-domain abolish the functional interaction with Hsp70 (14, 16, 17). The second essential function of Hsp40s is the binding and delivery of substrate proteins to Hsp70 (18, 19), a process not well understood. Type I Hsp40s can act as independent chaperones and bind and suppress aggregation of non-native polypeptides (20, 21), while type II proteins have to cooperate with Hsp70s to prevent aggregation (22). The crystal structure of Ydj1p and Sis1p reveals a hydrophobic pocket in the C-terminal domain that can accommodate short peptides (23, 24). DnaJ has a preference for peptides enriched in aromatic and large hydrophobic residues (25). Binding of substrates by type I Hsp40s also depends on the Zn-binding domain (20, 21). The binding/delivery of substrates to Hsp70s and the stimulation of its ATPase activity are not independent processes and work in a synergic manner.

The aim of this study is to gain knowledge on the interaction of E. coli DnaJ with protein substrates in different conformational states (folded, partially folded, and unfolded). We found that DnaJ distinguishes completely unfolded from partially folded or folded conformations. Of particular importance to recognize these conformations is the G/F-rich domain, which is required for the cochaperone to display a physiologically significant affinity for a folded substrate as RepE, and for folding intermediates of the thermal unfolding pathway of malate dehydrogenase (MDH) 4 and luciferase, but not for chemically unfolded luciferase.

EXPERIMENTAL PROCEDURES

Protein Cloning, Expression, and Purification—DnaJ, DnaJA77p, and DnaJ A107 were amplified by standard PCR techniques and cloned into pET22 vector. Wt DnaJ and mutants were expressed in BL21(DE3) cells and purified as described (26). It should be noted that following the mentioned protocol a small amount of endogenous wt DnaJ could copurify with the mutants. However it was not detected in Coomassie-stained SDS-PAGE (supplemental Fig. S1A). Additionally, the ATPase

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4The abbreviations used are: MDH, malate dehydrogenase; RCMLA, reduced carboxymethylated lactalbumin; GndHCl, guanidinium hydrochloride.
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activity of DnaK was not stimulated by any of the truncation mutants in contrast to wt DnaJ (supplemental Fig. S1B). Thus, the activity (or lack of) of DnaJ_{Δ73} and DnaJ_{Δ100} was not affected by this contamination. DnaK, GrpE, and ClpB were obtained as described (27–29). f-RCMLA was produced from α-lactalbumin (Sigma) (30) and fluorescently labeled using 5′-carboxyfluorescein succinimidyl ester (Invitrogen Molecular Probes) (31). MDH and luciferase were purchased from Sigma and Roche, respectively. Anti-DnaJ antibody was purchased from StressMarq.

RepE expression vector was a kind gift from Prof. D. Bastia, and the protein was purified according to the published protocol (32). For pulldown experiments, an N-terminal His-tagged version of RepE was used. RepE was cloned into pHAT vector and transformed in BL21(DE3) cells. Cell cultures in exponential phase were cooled down to 20 °C and induced with 0.1 mm isopropyl-1-thio-β-d-galactopyranoside. Cells were harvested after 24 h of incubation at 20 °C, resuspended in 40 mm sodium phosphate, pH 8.0, 300 mm NaCl, 10 mm imidazole, 1 mm PMSF, 10% glycerol, and lysed by sonication. After centrifugation at 35,000 rpm for 40 min at 4 °C, 2 ml NiNTA beads (Quia-gen) were added to the supernatant, and the protein was allowed to bind for 2 h at 4 °C. The NiNTA beads were washed with a high salt buffer and the protein was eluted with a 20-mL linear gradient to 500 mm imidazole. RepE-containing fractions were pooled, diluted 5-fold with 40 mm sodium phosphate, pH 6.8, 10% glycerol and loaded in a HiTrap SP 5 ml column (GE Healthcare) equilibrated in 40 mm sodium phosphate, pH 6.8, 100 mm NaCl, 0.1 mm EDTA, 2 mm β-mercaptoethanol, 10% glycerol. The column was eluted with a 100-mL linear gradient, with equilibration buffer containing 1 mm NaCl. HisRePE was concentrated and stored at −70 °C.

Anisotropy Measurements—RepE (1 μM) and f-RCMLA (1 μM) were incubated with increasing concentrations of wt DnaJ or the deletion mutants (0.1–40 μM) in 20 mm Heps/KOH, pH 7.4, 50 mm KCl, 5 mm DTT, 0.1 mm EDTA. Samples were equilibrated overnight at 4 °C following incubation for 1 h at 25 °C before measurements. Fluorescence anisotropy measurements were performed in a Fluorolog spectrofluorimeter (Jobin Ibon) with excitation and emission wavelengths at 280 nm and 345 nm for RepE, and 494 and 518 nm for f-RCMLA. Slit widths were 8 nm. The fraction of RepE bound to DnaJ was calculated and the experimental points were fitted by a non-linear least squares method to a single quadratic equation assuming one binding site.

Cross-linking—RepE (10 μM) and DnaJ (10 μM) were incubated for 1 h at 25 °C in 20 mm Hepes/KOH, pH 7.4, 50 mm KCl, 0.5 mm MgCl₂, Glutaraldehyde (0.005% (v/v)) was added, and samples were incubated for 20 min at 4 °C. Reactions were stopped by addition of 50 mm Tris, pH 7.5, and samples were analyzed by SDS-PAGE.

Trypsin Partial Proteolysis—Proteolysis experiments were performed at 30 °C in 25 mm Hepes/KOH, pH 7.6, 50 mm KCl, 10 mm β-mercaptoethanol, 20 μM DnaJ, and 20 μM RepE were incubated for 2 h at 25 °C to form the complex. Proteolysis was initiated by addition of trypsin at 1:200 or 1:40 (w/w) ratios. Reactions were allowed to occur for different times at 30 °C and stopped by addition of 1 mm PMSF. As a control, the isolated proteins were also treated with trypsin at the same ratios. Proteolysis products were analyzed by SDS-PAGE (12.5% gels) followed by staining with Coomassie Brilliant Blue.

Partial tryptic digestions were analyzed by LC-MS in a Q-ToF Micro (Waters) mass spectrometer interfaced with a CapLC System (Waters). Tryptic digestion was stopped by the addition of formic acid at a final concentration of 0.5% and loaded onto a Symmetry 300 C4 NanoEase Trap precolumn (Waters). The precolumn was connected to a Symmetry 300 C4 analytical column (75 μm × 150 mm, 3.5 μm, Waters) equilibrated in 5% acetonitrile and 0.1% formic acid. Polypeptides were eluted with a 30 min linear gradient of 5–60% acetonitrile directly onto a NanoEase Emitter (Waters) and mass spectra acquisition was performed in MS mode. Mass spectra were manually inspected and combined, and intact mass of the polypeptides was determined by MaxEnt1 software (Waters) using default deconvolution parameters. Mass ranges were selected based on protein sequence information and software was set to iterate to convergence. Experimentally obtained masses were matched to DnaJ and RepE protein sequence fragments using the BioLynx tool embedded in MassLynx 4.1 software (Waters).

Pulldown Experiments—For pulldown of wt DnaJ and mutants, a N-terminal His-tagged version of RepE was used. HisRePE (10 μM) was incubated with NiNTA beads for 90 min at 4 °C with gentle shaking in 20 mm Hepes/KOH, pH 7.4, 50 mm KCl, 0.5 mm MgCl₂, 50 mm imidazole. DnaJ or mutants (10 μM) were added and incubated for 90 min at 25 °C. Unbound proteins were removed by washing three times, and pellets were analyzed by SDS-PAGE.

MDH and Luciferase Aggregation and Refolding—Thermal unfolding of 1 μM MDH and 0.25 μM luciferase was induced by incubation at 47 °C and 42 °C, respectively, in 20 mm Hepes/KOH, pH 7.4, 50 mm KCl, 5 mm MgCl₂, 5 mm DTT in the absence or the presence of DnaJ. Unfolding of luciferase (25 μM) was achieved by incubation in 6 M GndHCl, 30 mm Tris/HCl, pH 7.5, 5 mm DTT for 2 h at 22 °C. The unfolded protein was diluted 100-fold in the above buffer in the absence or presence of DnaJ or DnaJ mutants (0.1–5 μM). Aggregation was monitored by continuous measurement of light scattering at 500 nm (MDH) or 320 nm (luciferase) in a Fluorolog spectrofluorimeter (Jobin Ibon).

Refolding experiments were performed after denaturation of MDH (1 μM) for 20 min at 47 °C or luciferase (80 nm) for 30 min at 42 °C, in the absence or presence of 1 μM DnaJ or DnaJ mutants in the above buffer. For chemical denatured substrates, luciferase (2.5 μM) was incubated in 6 M GndHCl, 30 mm Tris/HCl, pH 7.5, 5 mm DTT for 2 h at 22 °C, and diluted 100-fold in the above buffer in the absence or presence of 1 μM DnaJ or DnaJ mutants. Reactivation was achieved by addition of 2 mm ATP, an ATP-regeneration system (20 ng ml⁻¹ pyruvate kinase, 4 mm PEP), and different combinations of 1 μM DnaK, 1 μM GrpE, and 1.5 μM ClpB. Samples were incubated for 2 h at 30 °C, and MDH and luciferase activity were measured as described (33, 34).

Intrinsic Fluorescence—Luciferase intrinsic fluorescence spectra were recorded in a Fluorolog spectrofluorimeter (Jobin Ibon) using 295 nm as excitation wavelength and 8 nm slit widths. Luciferase concentration was 0.25 μM, and the buffer
was 20 mM Hepes/KOH, pH 7.4, 50 mM KCl, 5 mM MgCl₂, 5 mM DTT. For thermal denaturation, the sample was heated to 42 °C for 20 min in the absence or presence of 5 μM DnaJ. For chemical denaturation, 25 μM luciferase was denatured in 6 M GdnHCl, 30 mM Tris/HCl, pH 7.5, 5 mM DTT for 2 h at 22 °C, and diluted 100-fold in the above buffer containing 6 M GdnHCl or 0.5 μM DnaJ.

**F-APPY Binding Assays**—F-APPY (50 nm) was incubated overnight at 4 °C in the absence of chaperones or with DnaK (1 μM), DnaJ (1 μM), or DnaJ_{Δ107} (1 μM). As a control, recombinant nucleoplasm (1 μM) was used. Buffer was 20 mM Hepes/KOH, pH 7.4, 50 mM KCl, 5 mM MgCl₂. Fluorescence was measured using 492 nm and 516 nm as excitation and emission wavelengths, respectively. The fluorescence intensity of a freshly dissolved peptide solution was set as 100%. For separation in density gradients, F-APPY (0.2 μM) was incubated with 10 μM DnaJ or DnaJ_{Δ107} for 1 h at 25 °C and loaded on top of a 5–30% sucrose gradient. After 22 h centrifugation at 12,600 × g at 4 °C, the gradient was fractionated using a PGFip (Biocomp). F-APPY was detected measuring the fluorescence intensity of the samples, and DnaJ and DnaJ_{Δ107} by SDS-PAGE.

**RESULTS**

**DnaJ Binds RepE with High Affinity**—First, we tested the ability of DnaJ to bind soluble protein substrates such as RepE or reduced carboxymethylated lactalbumin (RCMLA). RepE is the initiation factor of plasmid F and is functionally homologous to RepA of P1 plasmid, which was shown to be a high affinity DnaJ substrate (35). Depending on its oligomeric state, RepE acts as dimer/repressor or monomer/activator of plasmid replication (36). The conversion from dimers to monomers depends on the action of DnaK/DnaJ/GrpE (36). The conversion from dimers to monomers depends on the action of DnaK/DnaJ/GrpE (36). RCMLA adopts a partially folded soluble conformation that makes it a suitable substrate for chaperones (6). Complex formation was first followed by the increase in fluorescence anisotropy of the substrates. RepE–DnaJ interaction was monitored by the intrinsic fluorescence of the substrate, taking advantage of the lack of tryptophan residues in the DnaJ sequence. In the case of RCMLA, the substrate was labeled with fluorescein. As shown in Fig. 1A, DnaJ had a different affinity for the two substrates; while it bound RepE with a \( K_d = 5.2 \pm 0.7 \mu M \), the affinity for RCMLA was much lower, in accordance with previous observations (6, 35). The stability of the DnaJ–RepE complex was also demonstrated by chemical cross-linking with glutaraldehyde (Fig. 1B). Other crosslinkers as BS3 and DSP were also tried, however best results were obtained with glutaraldehyde. Cross-linking of isolated DnaJ or RepE resulted in adducts corresponding to the dimeric forms of both proteins. When preformed DnaJ–RepE complexes were subjected to cross-linking, one adduct, that could be recognized by a DnaJ antibody (Fig. 1B, lower panel), with apparent molecular mass compatible with a cross-link between DnaJ and RepE was also observed. Furthermore, the complex between DnaJ and RepE could be detected by pull-down experiments with NiNTA using an N-terminal His-tagged version of RepE (see below).

**RepE Protects Specific Tryptic Sites in DnaJ**—We used trypsin partial proteolysis to investigate DnaJ sites that were interacting with RepE. This approach assumes that RepE binding would prevent access of trypsin to specific sites within the DnaJ molecule. Isolated DnaJ and RepE, and the complex formed by both proteins were treated with trypsin at low (1:200) and high (1:40) molar ratios for different times. Proteolytic fragments were resolved by SDS-PAGE. To identify the fragments, samples of isolated DnaJ and RepE were digested with trypsin at 1:40 molar ratio and analyzed by mass spectrometry (MS). The presence of DnaJ did not significantly affect RepE proteolytic pattern at any trypsin concentration, however the tryptic pattern of DnaJ was modified upon complex formation with RepE. At low trypsin molar ratios (Fig. 2A), DnaJ was rapidly degraded in the absence of RepE mainly into fragments of apparent molecular mass of ~9 and 28–29 kDa, which can be assigned to the N-terminal J domain, and C-terminal fragments cleaved at positions Arg-108, Arg-110, and Arg-113, just after the G/F-rich region.
In the presence of RepE, full-length DnaJ (marked with an arrow) is protected against proteolysis, indicating that cleavage sites located after the G/F-rich region were not accessible to the protease (Fig. 2A). At 1:40 trypsin molar ratios (Fig. 2B), the 28–29 kDa C-terminal fragments were further degraded in the absence of RepE, giving rise to fragments of 20–22 kDa originated from cleavage within the Zn finger region (Domain II) at positions Lys-153, Arg-173, and Arg-189 (Fig. 2C). These tryptic sites were also protected when DnaJ was complexed to RepE, and degradation of 28–29 kDa fragments was severely impaired (Fig. 2B). In summary, these results suggest that RepE very likely contacts the G/F-rich region and the Zn-binding domain II of DnaJ (Fig. 2C); thus these domains might play an important role in DnaJ-substrate complex formation. Note the absence of tryptic sites within the G/F-rich region that makes impossible the formation of proteolytic fragments starting at this domain.

The G/F-rich Region Is Required for Stable Binding of Substrates—To investigate the role of the N-terminal domains of DnaJ on substrate binding, we produced two deletion mutants, DnaJΔ73 and DnaJΔ107, which lack the J-domain, and J-domain and G/F-rich region, respectively. As previously found (37), none of these deletions compromised the overall stability of the protein as seen by circular dichroism, which showed secondary structure and denaturation temperatures similar to wt DnaJ (supplemental Fig. S2, A and B). The interaction of both mutants with RepE was investigated by fluorescence anisotropy and pull-down experiments. As shown in Fig. 3A, DnaJΔ73 bound RepE with similar affinity to wt DnaJ, while RepE anisotropy did not significantly increase in the presence of DnaJ lacking the J-domain and G/F-rich region within the same concentration range. It should be mentioned that DnaJΔ73 had a tendency to aggregate at high concentrations, possibly because of the exposure of the G/F-rich domain to the solvent. As a consequence, higher anisotropy values were observed with this mutant at concentrations above \(10^{-6}\) M. These data suggest that the G/F-rich domain is required to form a high affinity complex with RepE. Pull-down experiments with His-tagged RepE confirmed these results (Fig. 3B), because DnaJΔ107, in contrast to wt DnaJ and DnaJΔ73, was unable to coprecipitate with RepE above background levels, even when protein concentration was raised to compensate the effect of its reduced affinity.

The role of the G/F-rich region in the stable binding of protein substrates was also investigated using unfolded MDH and luciferase. First, aggregation of thermally denatured MDH and luciferase was followed by light scattering in the absence or presence of DnaJ or of the deletion mutants (Fig. 4, A and B). In both cases DnaJ hindered aggregation in a concentration-dependent manner, higher DnaJ:substrate molar ratios being required to protect luciferase, i.e. equimolar DnaJ significantly reduced aggregation of MDH, and this was completely abolished at 5:1 molar ratio, while a 20:1 DnaJ molar excess was

### TABLE 1

**Identification of tryptic DnaJ fragment by MS**

Proteolysis was performed at 1:40 trypsin:DnaJ molar ratio for 5 min. Reaction was stopped by addition of 1 mM PMSF, and the fragment masses were obtained by LC-MS, as described under “Experimental Procedures.”

<table>
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<th>DnaJ fragment</th>
<th>Molecular mass</th>
<th>Experimental</th>
<th>Theoretical</th>
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<td>29134</td>
<td></td>
</tr>
<tr>
<td>111–376</td>
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<td>28850</td>
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<td>114–376</td>
<td>28549.5</td>
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<td>22143.2</td>
<td>22144</td>
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<td>22067.2</td>
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<td>190–376</td>
<td>20278.1</td>
<td>20279</td>
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**DnaJ-Substrate Interactions**

required to completely protect luciferase. These results might reflect a different affinity of DnaJ for these unfolded substrates as previously observed with RepE and RCMLA (this work; Ref. 35). The ability of DnaJ<sub>Δ73</sub> to protect thermally denatured MDH and luciferase against aggregation was similar to that of wt DnaJ (not shown), in contrast to that of DnaJ<sub>Δ107</sub>, which was severely impaired. DnaJ<sub>Δ107</sub> slightly protected MDH against aggregation at high molar ratios (10:1) and slowed down the kinetics of luciferase aggregation, suggesting that this deletion mutant may interact with these partially (un)folded substrates with a markedly reduced affinity, in agreement with results previously found with Ydj1p (21). Interestingly, a different scenario was found when luciferase was denatured with guanidinium hydrochloride (GndHCl) and substrate aggregation was induced by dilution in the absence or presence of chaperones. Under these conditions wt DnaJ and DnaJ<sub>Δ107</sub> hindered similarly the aggregation of unfolded luciferase (Fig. 4C). These results suggest that the requirement of the G/F-rich region to bind unfolded substrates is complex and depends on the conformational properties of the substrate protein. Whereas GndHCl extensively denatured the protein (λ<sub>em</sub> = 364 nm), thermal denaturation at 42 °C induced a partially (un)folded state (λ<sub>em</sub> = 352 nm). The fluorescence properties of the DnaJ-luciferase complexes were similar (λ<sub>em</sub> = 353 nm), regardless of the denaturation method used, and different from that of native luciferase (λ<sub>em</sub> = 345 nm). Therefore, DnaJ would interact with refolding intermediates of GndHCl-denatured luciferase that would show a larger exposure of tryptophan residues to the solvent (λ<sub>em</sub> varies from 364 to 352 nm), than those that populate its thermal unfolding pathway (λ<sub>em</sub> varies from 345 to 352 nm), and thus that will be more extensively unfolded.

The ability of DnaJ and the deletion mutants to protect aggregation of thermally denatured MDH and luciferase was also tested by assaying substrate refolding. After denaturation of the substrates, as indicated, in the absence or the presence of different DnaJ variants, refolding was started by addition of different combinations of DnaK/GrpE/ClpB and ATP, and incubation of the sample for 2 h at 30 °C (Fig. 4, D and E). Refolding of MDH and luciferase denatured in the absence of chaperones was significant only when the DnaK system and ClpB cooperated, while when 1 μM DnaJ was used to protect aggregation, the DnaK system reactivated MDH and luciferase on its own, albeit to a lower extent. A higher refolding yield was obtained when ClpB was also used in the refolding step in the presence of equimolar DnaJ, reflecting the lower aggregation of the substrate protein, as suggested by light scattering data (Fig. 4, A and B). When the substrates were denatured in the presence of DnaJ<sub>Δ73</sub> the lack of the J-domain hampered reactivation by DnaK/GrpE or DnaK/GrpE/ClpB, despite the protection effect exerted by this mutant as described above. Addition of wt DnaJ together with DnaK/GrpE or DnaK/GrpE/ClpB could reactivate MDH and luciferase denatured in the presence of DnaJ<sub>Δ73</sub> with lower yields, suggesting that the mutant competed with wt DnaJ for substrate binding and, therefore, lowered the final reaction yield. In contrast, when MDH and luciferase were aggregated in the presence of DnaJ<sub>Δ107</sub>, refolding could only be observed when a combination of DnaK/DnaJ/GrpE/ClpB was used, the yield being similar to that of the control experiment. This confirms that under these conditions DnaJ<sub>Δ107</sub> does not interact with or protect aggregation of partially (un)folded MDH and luciferase.

Finally, refolding of chemically denatured luciferase was performed. Aggregates of chemically denatured luciferase were easily reactivated by K/J/E and addition of ClpB did not improve significantly the refolding yield. When DnaJ was present in the dilution buffer, aggregation of luciferase was prevented (Fig. 4C) and higher refolding yields were obtained. DnaJ<sub>Δ73</sub> or DnaJ<sub>Δ107</sub> also avoided aggregation, when present in the dilution step (not shown and Fig. 4C). Because the J-domain is missing in both mutants, refolding could only be achieved when wt DnaJ was added, however the yields were significantly lower (around 20%), meaning that both mutants were competing with wt DnaJ for the substrate as observed above. These results support that DnaJ<sub>Δ107</sub> was able to bind chemically denatured luciferase and prevent its aggregation.

**FIGURE 3. Binding of RepE to DnaJ<sub>Δ73</sub>, deletion mutant is impaired.** A, binding of RepE to DnaJ<sub>Δ73</sub> (closed circles), and DnaJ<sub>Δ107</sub> (triangles). Experimental conditions were as in Fig. 1A. For comparison, the binding isotherm to wt DnaJ is shown (open circles). B, coprecipitation of wt DnaJ and deletion mutants with His-tagged RepE. 10 μM RepE was preincubated with NINTA beads for 90 min at 4 °C, and DnaJ, DnaJ<sub>Δ73</sub>, or DnaJ<sub>Δ107</sub> (10 μM) were added and incubated for 90 min at 25 °C. For DnaJ<sub>Δ107</sub>, pull-down experiments were also performed at 20 μM mutant and RepE (lines marked with an asterisk). Supernatant was separated, and beads were washed three times to remove the unbound protein. Supernatants and pellets were analyzed by SDS-PAGE, and proteins detected by Coomassie Blue staining.
Binding of Peptides Is Not Affected by the N-terminal Deletion—Although DnaJ_{1107} was correctly folded, as mentioned above, it was possible that subtle changes in its tertiary structure affected the stability of the peptide binding site. To investigate this possibility, DnaJ_{1107} was treated with trypsin (1:200 for 30 s), and the fragments obtained analyzed by MS (supplemental Fig. S2C). We found that the deletion did not increase the accessibility to trypsin, and digestion of DnaJ_{3109} gives rise to fragments starting at positions 110 and 113, similar to those found for wt DnaJ. Therefore the stability of the adjacent $\beta$-strand starting at Leu-117 (Ile-116 in Ydj1p), which forms part of the binding pocket, was not affected by the N-terminal deletion. Next, we tested binding of F-APPY peptide (fluorescein-CALLQSRLLLSAPRAAATARY) to DnaJ and DnaJ_{1107}. F-APPY binds Hsp70 proteins with high affinity (34, 38) and contains a sequence similar to peptide p5', known to be a good DnaJ binder (39). Unfortunately neither a significant change in peptide fluorescence nor anisotropy enhancement was observed after binding to DnaJ and, therefore, the $K_d$ of the association reaction could not be estimated. The hydrophobicity of F-APPY promotes its aggregation and only a small percentage of the fluorescence of a freshly dissolved peptide was observed after incubation for 12 h at 4 °C (Fig. 5A). However, in the presence of DnaK, DnaJ, and DnaJ_{1107}, but not of an unrelated protein as nucleoplasmin, aggregation was abolished indicating that F-APPY was bound to the chaperones and thus remained in solution. When isolated F-APPY was loaded in a sucrose density gradient, the peptide was found in two fractions: one, more abundant, at the bottom and a second one at the top (fraction 1) of the gradient corresponding to aggregated and soluble peptide populations, respectively (Fig. 5B). In the presence of chaperones, aggregation was hindered, as mentioned above, and F-APPY co-sedimented around fraction 5 with DnaJ and DnaJ_{1107}. These results demonstrate that the N-terminal deletion does not affect the integrity of DnaJ peptide binding site.

**DISCUSSION**

Chaperones of the Hsp70 and Hsp40 families assist many essential cellular processes due to their ability to interact with (un)folded proteins and remodel their conformation. Hsp40 proteins act as co-chaperones of Hsp70s, modulating their function by two mechanisms: (i) activation of the intrinsically weak ATPase activity of the chaperone that depends on the conserved J domain, present in all Hsp40 proteins; and (ii) delivery of protein substrates, a poorly understood process. In some cases, Hsp40 proteins can act independently and form stable complexes with folded and unfolded polypeptides, avoiding their aggregation. The way they interact with substrates of different conformational properties remains largely unexplored. Type I and Type II Hsp40s, as *E. coli* DnaJ and *S. cerevisiae* Ydj1p and Sis1p, contain a C-terminal domain able to interact with unfolded polypeptides (23, 24). The role of this domain in substrate binding is critical and mutations of residues that form the binding pocket impair the correct function of the protein (4, 22, 40). Hsp40s are modular proteins, and in addition to the C-terminal region, other domains have been involved in the interaction with some substrates (20, 21, 41). Here we have investigated the interaction of *E. coli* DnaJ with folded and (partially) unfolded substrates, focusing on the role of the N-terminal J and G/F-rich domains. We find that DnaJ has a marked substrate specificity as it forms a high-affinity complex with RepE while it interacts with low affinity with RCMLA, in agreement with previous findings (35). DnaJ has a preference to bind peptide stretches of ~8 residues enriched in aromatic and large aliphatic residues (25). Such sequences are found in both RepE and RCMLA, thus it remains unclear.
whether the higher affinity for RepE is due to the greater exposure of a sequence motif or to a specific folding pattern.

Our data indicate that RepE could directly contact the G/F-rich region and Zn-binding domain of DnaJ. The involvement of the Zn-binding domain in the binding of several substrates has been put forward for DnaJ and other Type I Hsp40s as the highly homologous yeast Ydj1p and endoplasmic reticulum ERdj3 (21, 41, 42). Here we give evidence that a substrate as RepE might also interact with this DnaJ domain, as observed by protection against proteolysis of several tryptic sites located within this domain. On the other hand, the role of the G/F-rich region in substrate binding seems controversial. Several reports have shown that this domain is dispensable since deletion or mutation of DIF motifs within this DnaJ domain did not modify the ability to bind either σ22 or chemically unfolded luciferase (15, 43). Similarly, Type I ERdj3 and Type II Sis1p bound several substrates independently of this domain (42, 44). Our data suggest that the requirement of the G/F-rich domain might depend on substrate conformation. Thus DnaJΔ107 interacts with chemically unfolded luciferase with similar affinity than wt DnaJ, resulting in very similar yields of protection against aggregation. However, this deletion mutant cannot efficiently protect aggregation of thermally unfolded luciferase and MDH within the concentration range used, suggesting a decreased affinity for these partially (un)folded conformations. The lower aggregation rate observed at low mutant concentrations might indicate a transient non-stable interaction with the substrate that cannot avoid the aggregation process. These data would suggest that DnaJ differentiates between chemically and thermally denatured luciferase and adapts the use of the G/F-rich domain to stably bind the latter. The main difference between the two denatured conformations of luciferase is the degree of unfolding of the polypeptide chain. Because GndHCl extensively denatures the protein, the cochaperone will probably bind more unordered conformations than during thermal denaturation of the substrate, where luciferase will gradually unfold in the presence of DnaJ, as observed by intrinsic fluorescence. Furthermore, DnaJΔ107 also fails to protect aggregation of thermally denatured MDH. As an indirect method to estimate the apparent affinity of wt DnaJ and DnaJΔ107 for thermally denatured MDH, we have used the percentage of protection against aggregation (not shown). Whereas the estimated $K_d$ for wt DnaJ is 0.45 μM, the affinity drops at least one order of magnitude for DnaJΔ107, making impossible the precise estimation of its value within the protein concentration range used. In agreement with our results, deletion of the G/F-rich domain of Ydj1p also resulted in a lower efficiency to suppress aggregation of rhodanase (21). Finally, the G/F-rich domain is strictly required to bind a folded substrate as RepE, the affinity being drastically reduced, by above two orders of magnitude, for the deletion mutant. Taken together, these results suggest that DnaJ might require a larger interaction surface to bind a folded substrate as RepE, which would be provided by the G/F-rich region and possibly by the Zn-binding domain. Deletion of this domain has been shown to similarly reduce the affinity for both folded (such as the homologous protein RepA and AP1) (41) and unfolded (chemically denatured rhodanase) (20, 21) substrates. Thus, the ability to differentiate the conformation of the substrate protein could mainly depend on the G/F-rich region. A recent study has shown that DnaJ interacts with side chains of a protein substrate in contrast to Dnak, its Hsp70 partner, that establishes contacts with the backbone (45). Binding of unfolded substrates would allow the interaction of DnaJ peptide binding site with exposed hydrophobic side chains, as seen for short apolar peptides, thus making the complex stable in a polar environment. However, as the conformation of the interacting substrate is more stable, or “native,” the hydrophobic side chains would be hidden in the apolar core of the protein, thus the interactions to stabilize the DnaJ-substrate complex would become more polar. As compared with hydrophobic interactions, the number of polar contacts required to stabilize
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the complex would be most likely larger, increasing in parallel with the interacting surface that provides them. This interpretation would explain why the difference in apparent affinities between wt DnaJ and DnaJΔ107 is negligible for chemical unfolded luciferase, around an order of magnitude lower for intermediates of MDH and luciferase thermal unfolding pathways, and two orders of magnitude for a folded substrate as RepE. However, binding of native σ22 to DnaJ seems to contradict this interpretation, since σ22-DnaJ complex does not rely on the presence of the G/F-rich region and Zn-binding domain (15, 41). This apparent disagreement could be rationalized considering that σ22 adopts a loosely folded and highly flexible conformation, as amide hydrogen exchange has demonstrated (46).

Deletion of DnaJ and Sis1p G/F-rich domain in E. coli and yeast cells, respectively, exhibits a poisonous effect (43, 44). This effect was attributed to a possible role of the G/F-rich domain in the “targeting” of substrates to Dnak/Ssa1p and failure to activate the chaperone, which might result in kinetically trapped chaperone-substrate complexes (43, 44). As suggested (43), some of these substrates might be essential proteins for cell survival. In this context, we show that direct binding of several folded and partially folded substrates strictly depends on the G/F-rich region. For substrates that require the G/F-rich domain to bind to DnaJ, this protein domain most likely participates directly in the transfer of substrates to Dnak and activation of the chaperone ATPase activity, possibly by correct positioning of the adjacent J-domain. For other substrates that bind to DnaJ independently of this protein region, the G/F-rich domain might also participate in complex stabilization, through transient interactions with the polypeptide chain.

In summary, the data presented here indicate that DnaJ combines different domains to interact with protein substrates depending on their conformational properties. Whereas binding of small apolar peptides or unfolded proteins does not rely on the presence of the G/F-rich region, complex formation with partially (un)folded or folded substrates requires this protein domain. Therefore the cochaperone can, if necessary, provide an interacting surface to stably bind specific substrates, besides the “binding site” adapted to interact with apolar amino acid stretches at the C-terminal domain. The generalization of this interpretation to rationalize the interaction of protein substrates with DnaJ would require a systematic analysis of more substrates. However the number of native substrates available is rather limited, and only two proteins (RepA, a RepE homolog, and σ22) have been described as high-affinity DnaJ binders. This study points to the importance of the substrate conformational properties in the interaction with DnaJ, which in turn determines the cochaperone domains involved in the formation of stable complexes.

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