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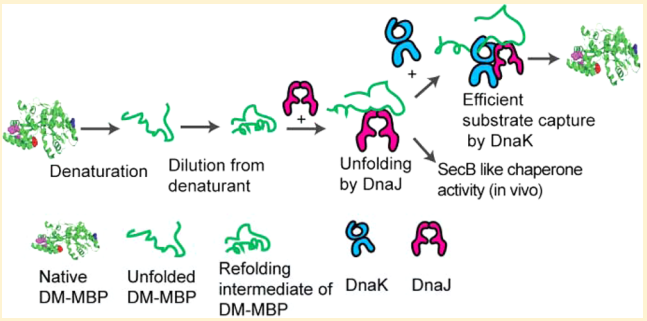
Unique Structural Modulation of a Non-Native Substrate by Cochaperone DnaJ

Satyam Tiwari, Vignesh Kumar, Gopal Gunanathan Jayaraj, Souvik Maiti, and Koyeli Mapa*

Proteomics and Structural Biology Unit, CSIR-Institute of Genomics and Integrative Biology, South Campus, Mathura Road, Delhi 110020, India

ABSTRACT: The role of bacterial DnaJ protein as a cochaperone of DnaK is strongly appreciated. Although DnaJ unaccompanied by DnaK can bind unfolded as well as native substrate proteins, its role as an individual chaperone remains elusive. In this study, we demonstrate that DnaJ binds a model non-native substrate with a low nanomolar dissociation constant and, more importantly, modulates the structure of its non-native state. The structural modulation achieved by DnaJ is different compared to that achieved by the DnaK–DnaJ complex. The nature of structural modulation exerted by DnaJ is suggestive of a unique unfolding activity on the non-native substrate by the chaperone. Furthermore, we demonstrate that the zinc binding motif along with the C-terminal substrate binding domain of DnaJ is necessary and sufficient for binding and the subsequent binding-induced structural alterations of the non-native substrate. We hypothesize that this hitherto unknown structural alteration of non-native states by DnaJ might be important for its chaperoning activity by removing kinetic traps of the folding intermediates.



In almost all subcompartments of eukaryotic cells, Hsp70 chaperones accomplish numerous cellular activities like folding of newly synthesized polypeptide chains, disaggregation and refolding of misfolded proteins, transport across organellar membranes, and degradation of terminally misfolded proteins.[1−3] Two types of cochaperones functionally cooperate with Hsp70s, namely, J-domain proteins (JDPs, also known as Hsp40s) and nucleotide exchange factors (NEFs). Hsp70s are functionally weak ATPases having a slow ATP cycling rate that is accelerated by JDPs, resulting in efficient substrate binding by Hsp70s. Subsequently, to complete a functional chaperone cycle, the NEFs facilitate ADP–ATP exchange. The functional versatility of Hsp70 molecules is feasible because of the involvement of a single Hsp70 with multiple cochaperones, mainly consisting of various JDPs.[4−6] Evolutionarily, JDPs are more diverse than their Hsp70 counterparts. They comprise structurally diverse groups of proteins and are classified as class I, II, and III JDPs. Class I JDPs contain an N-terminal J-domain followed by a glycine and phenylalanine (G/F)-rich region, a cysteine-containing zinc binding motif, and a C-terminal substrate binding domain. Class II JDPs are similar in domain organization except for the absence of the zinc binding motif. Type III JDPs include all those proteins harboring J-domains that are not necessarily in the N-terminus of the protein but are devoid of other structural elements found in typical type I or type II JDPs.[4,7]

Hsp70 of *Escherichia coli*, also known as DnaK, is an archetypal Hsp70 that has been studied extensively. Most of the mechanistic insights into Hsp70 have been elucidated by studying the DnaK system.[5,6,8−11] Substrate binding of DnaK is assisted and regulated by cochaperones DnaJ (JDP) and GrpE (NEF). The classification of JDP has been based on the domain architecture of DnaJ, it being considered as the prototype class I JDP. DnaJ is well understood to be an enhancer of ATPase activity of DnaK, leading to effective substrate binding by DnaK. Other studies suggest that DnaJ can bind to native as well as unfolded substrate proteins in a manner independent of DnaK and probably binds unfolded proteins before DnaK.[12−14] However, it remains to be tested if DnaJ can function as an independent chaperone.

In this study, we demonstrate that DnaJ unaccompanied by DnaK interacts with a model non-native protein, DM-MBP (double-mutant maltose binding protein), with high affinity. By employing single-molecule fluorescence resonance energy transfer (smFRET), we further demonstrate that upon binding the non-native substrate, DnaJ modulates its structure. The nature of structural alterations of the non-native state by DnaJ indicates its potential unfoldase activity on the folding intermediate. We delineate that the cysteine-containing zinc binding motif along with the C-terminal substrate binding domain of DnaJ is sufficient for binding and structural modulation of the non-native substrate. Notably, a type I JDP from *Saccharomyces cerevisiae* with a similar domain organization does not bind the same non-native protein, indicating the specificity of the DnaJ–non-native DM-MBP interaction. Importantly, DnaJ-induced structural alterations are

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similar to the structural alterations exhibited by the SecB chaperone on the same non-native substrate, which is suggestive of its possible role as an individual chaperone in the subsequent periplasmic translocation of the substrate.[15,16] These results uncover an unexpected role of DnaJ in the conformational modulation of non-native states of substrates, underlining its potential as a bona fide chaperone assisting protein folding as well as in substrate protein translocation.

MATERIALS AND METHODS

Proteins. Single- and double-cysteine mutants of DM-MBP were expressed and purified as described previously.[17]

DnaJ, its truncated mutants, and Ydj1 were cloned in the pETDuet-1 vector with N-terminal hexahistidine tags. All proteins were purified following the same protocol as described for Mdj1.[18] In brief, *E. coli* transformants were grown in Luria-Bertani medium at 37 °C until OD_{600} reached 0.6−0.7, and then induction was conducted with IPTG to a final concentration of 0.5 mM. After being induced at 30 °C for 6 h, cells were harvested at 6000 rpm and 4 °C, resuspended in buffer S [20 mM K^+-HEPES (pH 7.5), 20 mM imidazole, 2 mM DTT, 10% glycerol, and 0.5 mM phenylmethanesulfonyl fluoride], and disrupted by sonication . The sonicated cell suspension was centrifuged for 30 min at 15000 rpm and 4 °C. The pellet was resuspended in 40 mL of buffer S containing 1% Triton X-100, 500 mM KCl, and 3 M urea and incubated for 3 h at 4 °C. After insoluble material had been removed by centrifugation for 30 min at 15000 rpm, the supernatant was mixed with 2 mL of a 1:1 aqueous Ni-NTA/agarose suspension (Sigma Aldrich) and incubated for 1 h at 4 °C. The beads were washed once with 25 mL of buffer S containing 1% Triton X-100 and 3 M urea, once with 25 mL of buffer S containing 0.5% Triton X-100 and 2 M urea, and finally once with 25 mL of buffer S . Proteins were eluted with 8 mL of 20 mM K^+-HEPES (pH 7.4), 10% glycerol, 100 mM KCl, 2 mM DTT, and 300 mM imidazole, and buffer was exchanged with 20 mM K^+-HEPES (pH 7.4), 10% glycerol, 300 mM KCl, and 2 mM DTT. The protein was >90% pure as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). All purification steps were conducted at 4 °C. DnaJ Δ107 could also be purified from the soluble fraction.

For purification of DnaK, the cell pellet containing overexpressed DnaK was resuspended in 50 mM Tris-HCl (pH 7.5), 250 mM KCl, 5 mM $MgCl_2$, and 5% glycerol and loaded on a His_6Mge1 bound to Ni-NTA-agarose column. Bound DnaK was eluted with 2 mM ATP in the same buffer. The proteins were stored at −80 °C in 20 mM HEPES-KOH (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, and 5% glycerol.

Fluorophore Labeling of DM-MBP. Cysteine mutants of DM-MBP (100 μM) were labeled with Alexa 488-C5 maleimide in phosphate-buffered saline (PBS, pH 7.8) containing 50 mM maltose for 2 h at 4 °C in the presence of a 1.1-fold molar excess of the fluorophore. This labeling step was conducted in the presence of maltose as binding of maltose to native MBP leads to burial of position 298, resulting in preferential labeling at position 134. After incubation with Alexa 488, unbound fluorophores were removed using a NAP5 column (Amersham Biosciences) equilibrated in PBS (pH 7.8). Maltose was also removed from the donor-labeled protein. Thereafter, the second step of labeling with the acceptor fluorophore was conducted under similar conditions with a 5-fold molar excess of Alexa 647-C2 maleimide (Molecular Probes) in the same buffer in the absence of maltose.

Buffers. For single-molecule and ensemble experiments, buffer A [25 mM Tris-HCl (pH 7.5), 80 mM KCl, and 5 mM $MgCl_2$] was used. Proteins were denatured with buffer B (6 M GuHCl in buffer A). For single-molecule resolution, DM-MBP−chaperone complexes were diluted to picomolar concentrations in buffer C (60 mM GuHCl in buffer A).

Single-Molecule FRET Experiments. The methodology of smFRET measurements has been described in detail previously.[19] In short, all single-molecule FRET measurements were performed on a confocal system on an inverted microscope (Zeiss Axiobserver Microscope) using chambered cover glasses (Lab-Tek) as the sample holders. Our methodology is derived from previously described methods like PIE (pulsed interleaved excitation),[20] ALEX (alternate laser excitation),[21] and PAX[22] to detect doubly labeled molecules with an active donor−acceptor pair. We simultaneously used one continuous laser source at 488 nm and a pulsed laser source of 630 nm set at a repetition frequency of 20 MHz to excite a small confocal detection volume.

Screening of Cellulose-Bound Peptides (peptide scans). A cellulose-bound peptide tiling array from the sequences of wild-type MBP (maltose binding protein) was synthesized from JPT Peptide Technologies GmbH (Berlin, Germany). The array consists of 13mer peptides with a 10-amino acid overlap. Before binding the proteins, the dry membranes were incubated with methanol for 5 min followed by 3 × 10 min washing steps in TBS [10 mM Tris and 0.9% NaCl (pH 8.0)]. The membranes were blocked with 3% BSA in TBS (pH 8.0) at room temperature for 2 h followed by a short washing step with TBS-T (0.05%, v/v). Purified DnaJ or Ydj1 was then allowed to react overnight with the peptide library at a final concentration of 5 μg/mL in blocking buffer at 4 °C with gentle shaking. Unbound proteins were removed via washing with TBS-T, and peptide-bound DnaJ or Ydj1 was electro-transferred onto a polyvinylidene difluoride (PVDF) membrane using a semidry blotting apparatus. The PVDF membrane was sandwiched between blotting papers soaked in anode buffer I (30 mM Tris and 20% methanol), anode buffer II (300 mM Tris and 20% methanol), and cathode buffer (25 mM Tris, 40 mM 6-aminohexanoic acid, and 20% methanol). Electro-transfer was performed three times at a constant power of 1 mA/cm^2 in the cellulose membrane for 30 min. Transferred proteins were detected by immunodecoration with respective antibodies and electro-chemiluminescence (ECL, Sigma). The membrane was regenerated according to the manufacturer's protocol after experiments with a single protein.

RESULTS

DnaJ Binds to the Non-Native Intermediate of DM-MBP with High Stability. DnaK/J chaperones are thought to bind to linear polypeptide stretches enriched with hydrophobic sequences at the core flanked by positively charged residues of non-native substrate proteins and thus exert their chaperoning activity.[23,24] Structural features of model substrate proteins and aggregation prone proteins bound to DnaK or its substrate binding domain have been studied in detail,[25−27] although structural alterations of non-native folding intermediates of substrate proteins due to binding of DnaK/J have remained largely elusive. In a recent study, Sharma et al. showed that DnaK/J/E can unfold misfolded luciferase in substoichiometric amounts.[28]Very recently, we have also described the DnaK/J binding-induced structural alterations on a model non-native substrate, a slow folding mutant of maltose binding protein

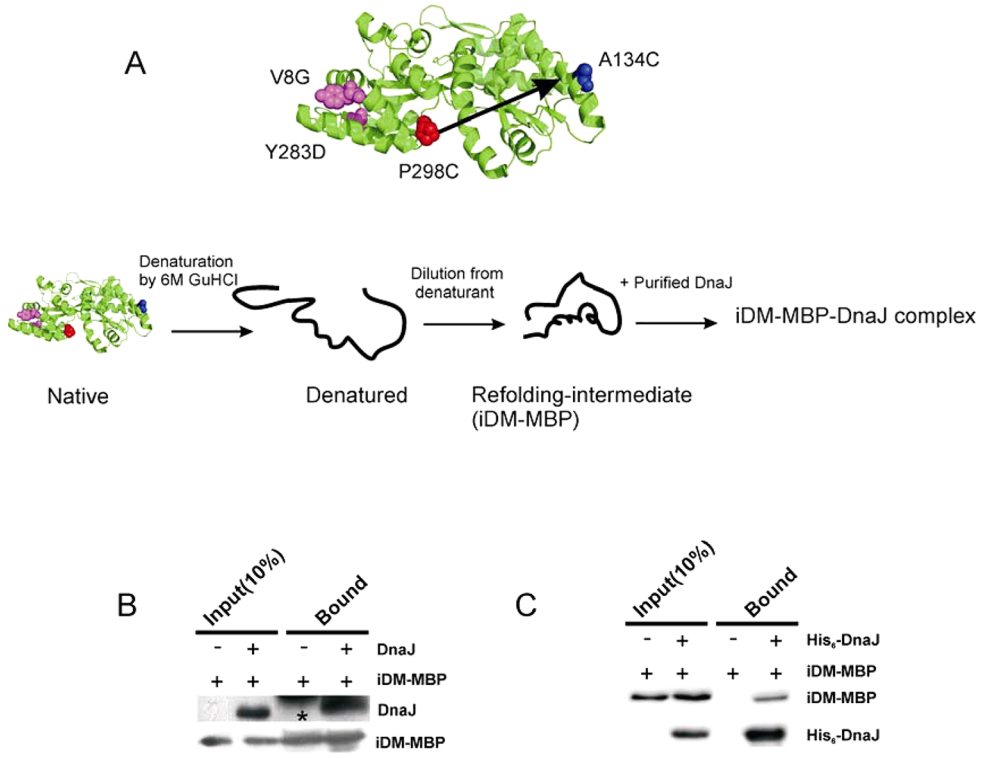

Figure 1. DnaJ interacts with the refolding intermediate of DM-MBP on its own. (A) Ribbon diagram (top) of the crystal structure of MBP (Protein Data Bank entry 1OMP)[37] depicting two mutations, V8G and Y283D, present in DM-MBP (purple). The positions of cysteine substitutions used for fluorophore labeling for conducting ensemble and smFRET studies are also shown. The cysteine residue labeled with an acceptor fluorophore is colored red and the donor fluorophore blue. The distance vector between residues 134 and 298 used for smFRET measurements is indicated with an arrow. Unfoding–refolding reactions (bottom) of DM-MBP with DnaJ, represented schematically. (B) Co-immunoprecipitation of the refolding intermediate of DM-MBP bound to purified DnaJ. Refolding DM-MBP (0.2 μM, final concentration) [denatured with buffer B and diluted (1:100) in buffer A containing the chaperone] was bound to DnaJ (0.5 μM) for 10 min at 25 °C. After binding, iDM-MBP was immunoprecipitated with anti-MBP antibodies, and the bound chaperones were probed with specific antibodies; 10% of the reaction mixture was loaded as input. The asterisk denotes the ECL signal from HRP-conjugated secondary antibodies bound to anti-MBP IgGs. (C) The refolding intermediate of DM-MBP was similarly made in buffer containing His_6-DnaJ, and after being incubated for 10 min at 25 °C, reaction mixtures were bound to Ni-NTA agarose. The bound proteins were eluted with 300 mM imidazole. The refolding intermediate of DM-MBP without DnaJ was used as a negative control; 10% of the reaction mixture was used as input. Input and bound fractions were subjected to SDS–PAGE followed by Western blotting and immunodecoration with specific antibodies. Immunoblotting was performed instead of staining of the SDS–PAGE gel as DM-MBP and His_6-DnaJ run in identical places.

(DM-MBP, V8G and Y283D) (Figure 1A, top panel).[19] We conclusively demonstrated that during DnaK/J/E-mediated refolding, the non-native intermediate adopts a different conformation, finally reaching the same native conformation, albeit with a slower refolding rate. Interestingly, this non-native substrate is specifically targeted to three chaperones of *E. coli*, namely DnaK, SecB, and DnaJ, in an intracellular milieu indicating physiological roles of these chaperone–substrate interactions. More importantly, even in the absence of DnaK, the targeting to DnaJ remains unaltered, pointing to the independent chaperoning effect of DnaJ on the non-native substrate other than its cochaperone functions.[19]

To delineate the individual chaperone function of DnaJ, we have reconstituted the interaction of the refolding intermediate of DM-MBP (termed iDM-MBP) with DnaJ. To this end, iDM-MBP was made by denaturing DM-MBP with 6 M GuHCl followed by dilution from denaturants (Figure 1A, bottom panel). The refolding intermediate or iDM-MBP thus formed was allowed to interact with purified DnaJ in the absence of DnaK. This interaction was confirmed by co-immunoprecipitation of DnaJ along with iDM-MBP with the anti-MBP antibody indicating a stable complex between the refolding intermediate of DM-MBP and DnaJ (Figure 1B). To reconfirm the DnaK-independent interaction of DnaJ with the non-native DM-MBP, we also performed a Ni-NTA pull-down assay with hexahistidine-tagged DnaJ with iDM-MBP. Pull downs reconfirmed that DnaJ alone forms a stable complex with non-native DM-MBP (Figure 1C).

DnaJ Interacts with the Refolding Intermediate of DM-MBP with High Affinity. The stable association of DnaJ with iDM-MBP prompted us determine the affinity of the non-native protein with DnaJ using dual-labeled DM-MBP (at amino acid positions 134 and 298). The protein was specifically labeled with two fluorophores utilizing the differential solvent accessibility of position 298 in the presence and absence of ligand maltose as described previously.[17] The flexibility of fluorophores conjugated to each position was not hindered as was judged by measuring the fluorescence anisotropy of dyes conjugated to each of these positions as described previously.[19] We observed that DnaJ binding was associated with a decrease in the FRET ratio of dual-labeled DM-MBP, which could be due to conformational alterations of the non-native protein upon binding to the chaperone. The apparent rate of change in the FRET ratio was found to vary linearly with DnaJ concentration, suggesting that the conformational change is kinetically inseparable from the binding event (Figure 2A,B).

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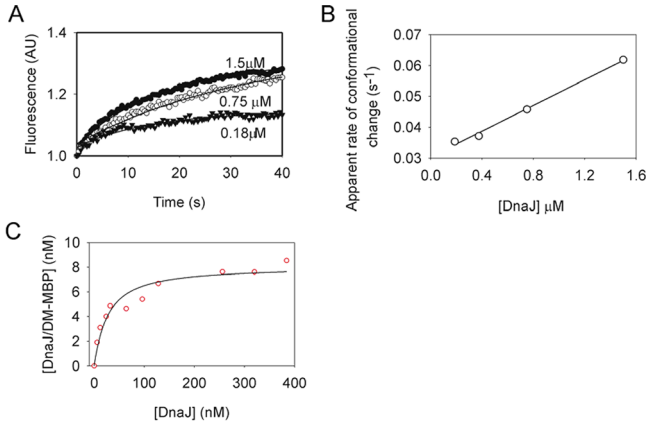

Figure 2. DnaJ interacts with the refolding intermediate of DM-MBP with high affinity. (A) The kinetics of the conformational change of DM-MBP (residues 134−298) due to DnaJ binding was monitored by following the change in donor fluorescence (Alexa 488 maleimide) of DM-MBP labeled with the donor−acceptor pair (Alexa 488 maleimide and Alexa 647 maleimide). Refolding of DM-MBP is nearly arrested at 120 mM GuHCl. We took advantage of this fact and mixed in a 1:1 ratio 16 nM refolding intermediate of DM-MBP stabilized at 120 mM GuHCl with increasing concentrations of purified DnaJ, in a stopped-flow mixing device attached to a fluoromax spectrofluorimeter. Kinetic traces for two of the highest concentrations were fit with Berkeley Madonna simulation-based fitting software to obtain the apparent rate of association. (B) Apparent rate of association of DnaJ with DM-MBP obtained from the fitting parameters from curves in panel A. (C) Determination of the equilibrium dissociation constant (K_D) of the DM-MBP−DnaJ complex by experimentally determined concentrations of the DM-MBP−DnaJ complex from the fluorescence change as described for panel A. The data were fit using the same equation as described elsewhere.[19]

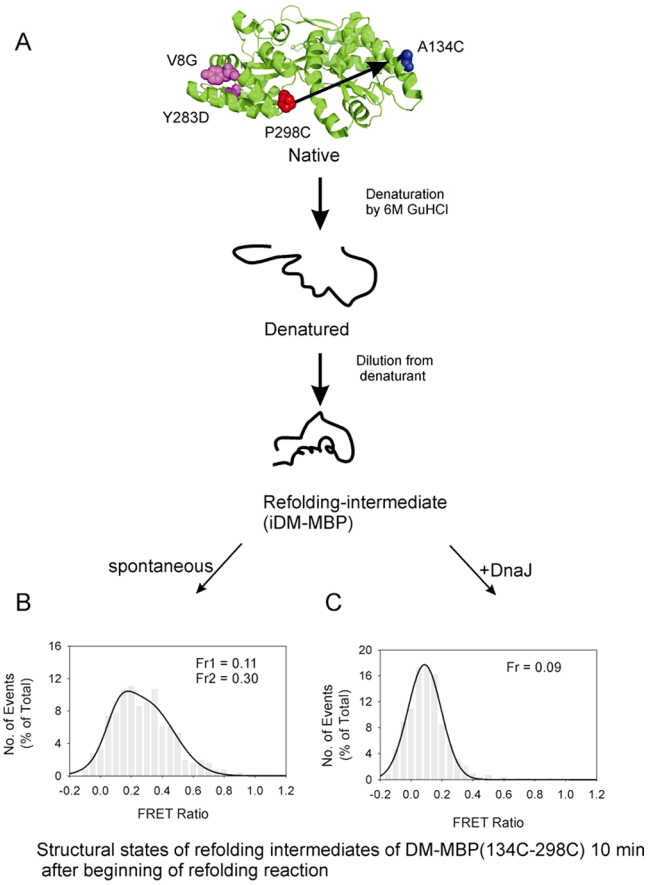

Figure 3. DnaJ confers distinct structural alterations of the refolding intermediate of DM-MBP. (A) Schematic representation of smFRET experiments used to obtain the smFRET ratio histogram of dual-labeled DM-MBP (residues 134−298) (Alexa 488 maleimide and Alexa 647 maleimide). Dual-labeled DM-MBP was first denatured with 6 M GuHCl in buffer A and subsequently diluted (1:100) in buffer A in the absence (B) or presence of DnaJ (0.25 μM) (C). After a 10 min refolding reaction, the structural states of refolding intermediates (spontaneous or chaperone-bound) were probed by smFRET. To obtain single-molecule resolution, we further diluted refolding reactionmixtures in buffer C. All smFRET experiments were conducted at a final DM-MBP concentration of ∼50 pM and a final GuHCl concentration of 60 mM.

Using the change in the FRET ratio to monitor DnaJ association, we obtained an apparent on rate of ∼5 × 10^5 M^{-1} s^{-1} with iDM-MBP with a K_D of ∼30 nM (Figure 2C). These data conclusively demonstrate that DnaJ has a significantly high affinity for non-native proteins, supporting earlier reports,[5] compared to peptide substrates (K_D ∼ 2−10 μM).[6,29]

DnaJ Exhibits Striking Alterations of the Non-Native Conformation of the Refolding Intermediate of DM-MBP. To unravel whether the structures of the non-native states are altered upon binding to DnaJ, we compared the structural states of freely refolding (chaperone-unassisted) iDM-MBP with that of iDM-MBP bound to DnaJ (Figure 3A). In our recent study, we successfully employed smFRET spectroscopy to delineate the structural states of spontaneously refolding DM-MBP as well as various chaperone-assisted refoldings of DM-MBP to its native state.[19] Using the same methodology, we determined the DnaJ-induced structural alterations of iDM-MBP. Single-molecule FRET allows us to probe structural states that are highly flexible or dynamic, a key feature of the non-native proteins that hinders its structural investigations by conventional methods. We performed smFRET using dual-labeled DM-MBP used earlier for the ensemble FRET-based affinity measurements. When denatured DM-MBP is diluted from the denaturant in buffer, it forms a refolding intermediate (iDM-MBP) that adopts a broad and heterogeneous conformation indicated by a broad FRET ratio histogram (Figure 3B). By contrast, when the refolding reaction was started in buffer containing DnaJ, the structure of DnaJ-bound iDM-MBP was starkly different. The majority of DnaJ-bound DM-MBP molecules populated a very low-FR state (FR = 0.10), indicating an opening of the non-native protein plausibly because of unfolding of the non-native protein by the chaperone (Figure 3C) providing experimental evidence of structural alterations of substrate proteins by a J-domain protein and its potential unfoldase activity.

The C-Terminal Zn Binding Motif Along with the Substrate Binding Domain of DnaJ Is Sufficient for Binding and Unfolding of the Non-Native Substrate. DnaJ is the prototypic type I J-protein containing several functional domains. We sought to demarcate the role of different domains of DnaJ in the structural alterations of the non-native substrate. We constructed different truncation mutants of DnaJ to check the minimal region of DnaJ essential for the binding and unfolding activity of the chaperone. One mutant had the deletion of the first 73 amino acids (JΔ73), which is deleted from the J-domain (Figure 4A). Another mutant retains only the zinc binding domain with the CTD but

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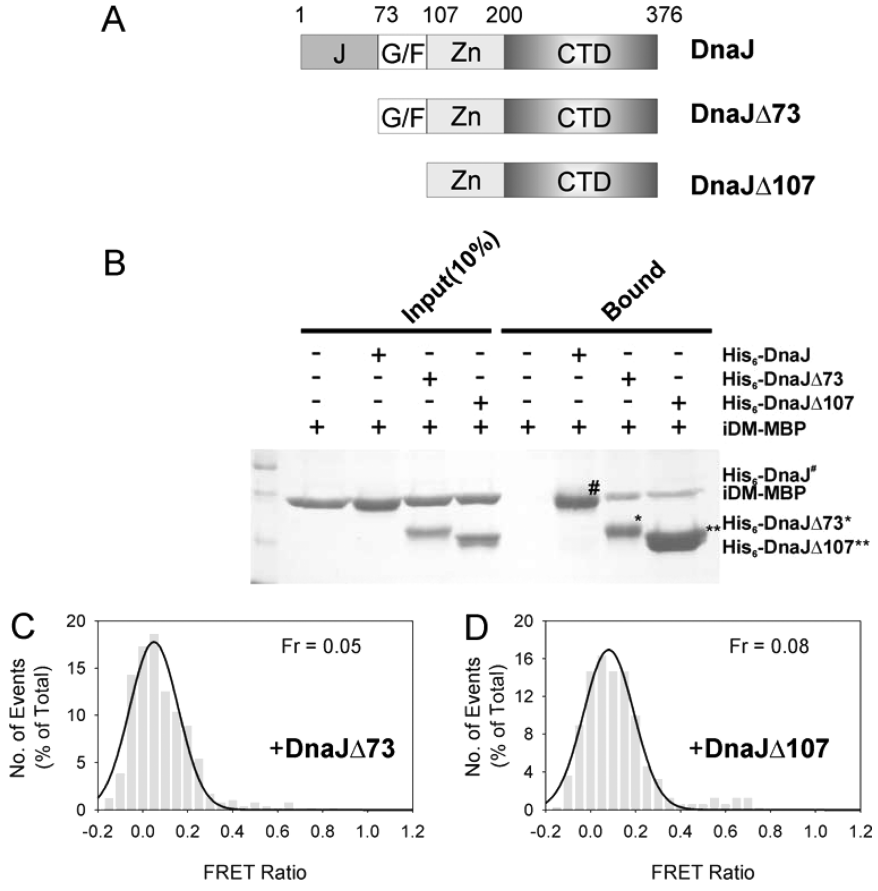

Figure 4. Non-native substrate binding property and its subsequent structural alterations conferred by C-terminal domains of DnaJ. (A) Schematic representations of the domain architecture of DnaJ and its truncation mutants used in the study. (B) The refolding intermediate of DM-MBP was similarly bound to (as described in the legend of Figure 1B) His_6-DnaJ and His-tagged truncation mutants of DnaJ and subsequently bound to Ni-NTA agarose. The bound proteins were eluted with 300 mM imidazole. The refolding intermediate of DM-MBP without DnaJ was used as a negative control; 10% of the reaction mixture was used as input. Input and bound fractions were subjected to SDS−PAGE with Coomassie brilliant blue staining. His_6-DnaJ and iDM-MBP bands overlap in the SDS−PAGE gel. (C and D) smFRET ratio histogram of the refolding intermediate of DM-MBP bound to DnaJ truncation mutants obtained as described for full-length DnaJ in the legend of Figure 3C.

is subjected to deletion of the first 107 residues harboring the J-domain and the G/F-rich region (JΔ107) (Figure 4A). JΔ73 and JΔ107 were expressed in *E. coli* with N-terminal hexahistidine tags and purified. As the Zn binding domain of DnaJ has been described to play a crucial role in non-native protein binding, we retained the Zn binding region in the DnaJ deletion mutants.[14] Both mutants were found to interact independently with the non-native folding intermediate of DM-MBP as indicated by copurification of iDM-MBP in a Ni-NTA pull-down assay with His-tagged versions of the mutant proteins (Figure 4B). Thus, it is evident that the zinc binding domain and the C-terminal substrate binding domain of DnaJ are sufficient for binding of the non-native intermediate of DM-MBP. These data also support the previous observations of nonessentiality of the DnaJ G/F-rich region for binding chemically denatured non-native substrate proteins.[13] Furthermore, to check if N-terminal domains of DnaJ play any role in the conformational alterations of the non-native protein, we probed the structure of iDM-MBP bound to DnaJ deletion mutants. Notably, after binding to iDM-MBP, JΔ73 as well as JΔ107 altered the conformation of iDM-MBP to the typical low-FR conformation as exhibited by full-length DnaJ, demonstrating that the J-domain and the G/F region are dispensable not only for binding iDM-MBP but also for structural alterations of iDM-MBP (Figure 4C).

Isolated DnaJ and the DnaK−DnaJ Complex Have Unique Binding Signatures on iDM-MBP. As isolated DnaJ- induced unique structural alterations of non-native DM-MBP were in stark contrast to DnaK/J-induced structural alterations (as described previously),[19] it seemed interesting to elucidate the binding pattern of the DnaK−DnaJ complex and DnaJ in isolation on the non-native substrate. For that purpose, we measured the anisotropy of the fluorophore (Alexa 647-C2 maleimide, Molecular Probes) conjugated to different single-cysteine mutants with substitutions at positions 21, 88, 141, 170, 190, 202, 269, and 362, spanning the entire protein sequence.[17,19] The difference in the anisotropy of the chaperone-bound non-native substrate and freely refolding substrate [Δ(anisotropy)] at different amino acids to which the fluorophore is conjugated follows a unique pattern depending on the sites of interaction with the chaperone. Alexa 647 was specifically chosen for this purpose as its short lifetime, ∼1.2 ns, makes it a perfect reporter of fast segmental motions. The short lifetime of the fluorophore makes it insensitive to protein tumbling that occurs on a much longer time scale (>10 ns). Positions with positive Δ(anisotropy) values are suggestive of regions of the non-native protein that undergo restricted segmental mobility because of the interactions with the chaperone. On the other hand, positions with negative values belong to regions of the non-native proteins that undergo

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increases in segmental mobility upon binding of chaperones. As is evident from the Δ(anisotropy) signature of the DnaJ-bound iDM-MBP, the N- and C-termini of the non-native protein exhibited strong increases in Δ(anisotropy), indicating binding of DnaJ to these regions (Figure 5). The binding signature of

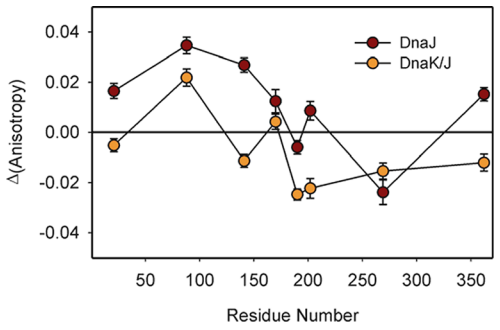
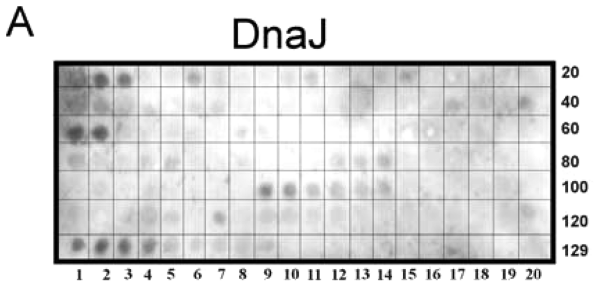
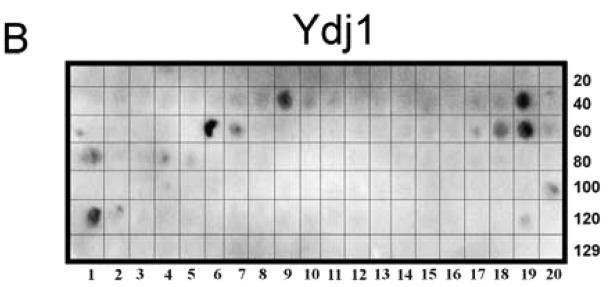


Figure 5. DnaK–DnaJ complex and DnaJ have unique binding signatures on the refolding intermediate of DM-MBP. Steady state anisotropies of Alexa 647 maleimide fluorophores conjugated to single-cysteine mutants of DM-MBP were measured. Δ(anisotropy) (described in Results) at different positions for DnaK–DnaJ complex-bound or DnaJ-bound iDM-MBP is plotted on the *Y*-axis. Intrinsic errors of anisotropy measurements are indicated with error bars.

the DnaK–DnaJ complex resembled the DnaK-bound structure as reported earlier, compared to the DnaJ-bound structure as is evident from our current findings.[19] It is important to note that although DnaK plays a major role in determining the conformation of the DnaK–DnaJ complex-bound state, there are subtle alterations in the structure in the presence of the DnaK–DnaJ system that are absent in the DnaK-bound state. This becomes evident because Δ(anisotropy) at residue 269 in the DnaK–DnaJ complex-bound state resembles more closely that of the DnaJ-bound state than that of the DnaK-bound structure (Figure 5). Thus, the combination of DnaK and DnaJ chaperones modulates the structure of the non-native state of DM-MBP in a unique manner different from structural modulation by individual chaperones.

Primary Sequence Recognition Drives DnaJ Binding on the Refolding Intermediate of DM-MBP. We also asked if the high-affinity interaction between DnaJ and iDM-MBP is governed by the primary sequence specificity of DnaJ. To this end, we probed the binding of DnaJ to a tiling peptide array (consisting of 13mer peptides with a 10-residue overlap) covering the MBP sequence (Figure 6A). DnaJ bound extensive stretches at the N-terminal end and at the extreme C-terminal end of MBP, which correlated well with the anisotropy data already described in Figure 5 (Figure 6A, top and bottom panel). To check for the specificity of DnaJ in recognizing these peptide signatures, we used another type I J-domain protein from *S. cerevisiae* Ydj1. When Ydj1 was bound to iDM-MBP, it did not exhibit any stable interaction with iDM-MBP (data not shown). Correspondingly, Ydj1 binds to only a few peptide spots in the tiling array (Figure 6B, top panel) with different sequence specificities, mainly to the spots with highly enriched





	Spot no. on the array	Peptide sequence
DnaJ binding peptides	1	MKIKTGARILALS
	2	KTGARILALSALT
	3	ARILALSALTTMM
	41	DAVRYNGKLIAYP
	42	RYNGKLIAYPIAV
	72	AKAGLTFLVDLIK
	73	GLTFLVDLIKNKH
	74	FLVDLIKNKHMNA
	89	KVNYGVTVLPTFK
	90	YGVTVLPTFKGQP
	91	TVLPTFKGQPSKP
	92	PTFKGQPSKPFGV
	93	KGQPSKPFVGVLS
	94	PSKPFVGVLSAGI
	121	QMSAFWYAVRTAV
	122	AFWYAVRTAVINA
	123	YAVRTAVINAASG
	124	RTAVINAASGRQT

	Spot no. on the array	Peptide sequence
Ydj1 binding peptide	29	IIFWAHDRFGGYA
	39	LYPFTWDAVRYNG
	58	ALMFNLQEPYFTW
	59	FNLQEPYFTWPLI
	61	YFTWPLIAADGGY
	100	NKELAKEFLENYL
	101	LAKEFLENYLLTD
	119	IMPNIPQMSAFWY

Figure 6. Interaction of DnaJ with non-native DM-MBP is driven by primary sequence recognition. (A) DnaJ binding to a cellulose-bound peptide tiling array (top) derived from the primary sequence of MBP (maltose binding protein). Each spot consists of 13mer peptides with a 10-amino acid overlap. The numbers of the last spots of each row are given at the right. Table (bottom) showing the spot number and sequence of the respective peptides bound to DnaJ. (B) Ydj1 binding to the same peptide array (top) from the MBP sequence. Table (bottom) showing the spot number and sequence of the respective peptides bound to Ydj1.

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aromatic residues (Figure 6B, bottom panel). This suggests that primary sequence recognition by the type I J-proteins is necessary for substrate binding. In conclusion, the binding signature probed in the context of the full-length non-native substrate protein using anisotropy correlates well with the peptide binding signatures indicating that binding of DnaJ to iDM-MBP may be driven by primary sequence recognition that is specific for the substrate-binding domain of DnaJ.

DISCUSSION

The DnaK/J chaperone system, the hub of the *E. coli* proteostasis network, is one of the central machineries in maintaining a healthy *E. coli* proteome.[2,30] Although DnaJ and other J-domain proteins have been shown to bind to native or non-native substrate proteins,[13,31] their binding has been posited to be mainly required to assist binding of the substrate to the Hsp70 group of chaperones.[5,6,32] In the study presented here, we describe the high-affinity binding of DnaJ, a bona fide cochaperone of DnaK, to a model non-native substrate in the absence of DnaK. Interestingly, upon binding the non-native substrate protein, DnaJ modulates the structure of non-native states of the substrate. Rather surprisingly, it exhibits a distinct unfoldase activity on the non-native substrate. Recently, an elegant study using H−D exchange has demonstrated that DnaJ induces a local opening of the native sigma-32 structure, probably facilitating subsequent DnaK binding.[31] In the study presented here, we have uncovered the fact that DnaJ is capable of structural alterations of not only the native substrates but also the non-native states of substrates and can exert unfolding forces on the non-native substrates. In the context of protein folding, it is reasonable to speculate that this function of DnaJ may allow efficient elimination of any non-native contacts formed during protein folding or aggregation, thereby allowing proteins to circumvent kinetic traps during folding. Furthermore, we could demarcate the areas of non-native DM-MBP that are in close contact with the chaperone−cochaperone complex when bound to the DnaK−DnaJ complex and to the individual DnaJ chaperone. We found that the binding signature of DnaJ is clearly distinct from that of the DnaK−DnaJ complex, explaining the different types of conformational states of the non-native states upon binding of these chaperones. We speculate that this happens because of the loading of DnaK on the DnaJ-bound conformation. This may allow binding of DnaK to unique sites: (1) through the exposure of novel sites due to unfolding of the substrate upon DnaJ binding and (2) through the recruitment of DnaK on proximal sites by DnaJ molecules that are bound to the substrate.

Although the DnaJ-bound conformation of non-native DM-MBP was unique and was starkly different from the DnaK/J-bound structure, it closely resembled the SecB-bound structure. SecB is an *E. coli* chaperone mainly involved in periplasmic translocation of secretory proteins, and our previous findings have demonstrated that SecB maintains non-native DM-MBP in a unique conformation.[19] Similar structural alteration of the non-native states of presecretory proteins by DnaJ and SecB may explain their functional redundancies. The functional overlap between these two chaperone systems is underlined by the fact that double deletion of *dnaj* and *secb* is synthetically lethal in *E. coli*.[33] Furthermore, it is also known that DnaJ works as a competent alternate chaperone system for SecB-dependent secretory proteins by efficiently protecting them from lon protease degradation in the Δ*secb* strain.[34] We speculate that DnaJ serves as an effective alternate chaperone to SecB by structurally modulating non-native secretory proteins to lon protease-protected conformations, which is normally achieved by SecB. In addition, DnaJ-induced unique structural alterations of non-native substrates can also very well justify the previous finding that SecB-dependent secretory proteins are more protected from lon protease by DnaJ binding than by binding to the complete DnaK/J/E system. This is probably due to a protease resistant conformation facilitated by DnaJ binding that is not achieved by binding to DnaK/J/E.[34] Simultaneously, it was shown that DnaJ mutant H33Q, which is deficient in accelerating the ATPase activity of DnaK but capable of binding substrates, was more efficient than its wild-type form in rescuing the cold sensitive phenotype of Δ*secb* strains.[34] In light of our current findings, it is tempting to speculate that the H33Q mutant of DnaJ, by losing its cochaperone function, is exclusively involved in binding to unfolded proteins, thereby exhibiting better SecB complementation activity than wild-type DnaJ.

Furthermore, we have conclusively proven that the interaction of non-native DM-MBP with DnaJ is highly specific. Even the close structural homologue of DnaJ from yeast *S. cerevisiae* does not interact with non-native DM-MBP. This may be due to the difference in the primary sequences of non-native substrates that these (co)chaperones recognize, indicating divergence in substrate specificity between different J-proteins.

We propose that J-domain proteins may perform important functions to modulate the structure of non-native proteins and hence may play crucial role in protein folding, disaggregation, and translocation in addition to their role as a cochaperone. The open question is how DnaJ or other J-domain proteins release the bound substrate in the absence of an ATP-dependent substrate binding and release cycle. This may be achieved through association and dissociation rate-dependent substrate partitioning, a mechanism generally proposed for other ATP-independent chaperones like SecB or Trigger Factor.[35,36] Whether DnaJ and other J-domain proteins universally follow this substrate release mechanism to achieve controlled substrate release remains to be explored.

AUTHOR INFORMATION

Corresponding Author

*E-mail: koyeli.mapa@igib.in. Phone: +91-11-29879-107. Fax: +91-11-2766-7471.

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Notes

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REFERENCES

(1) Genevaux, P., Georgopoulos, C., and Kelley, W. L. (2007) The Hsp70 chaperone machines of *Escherichia coli*: A paradigm for the repartition of chaperone functions. *Mol. Microbiol. 66*, 840−857.

(2) Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011) Molecular chaperones in protein folding and proteostasis. *Nature 475*, 324−332.

(3) Hartl, F. U., and Hayer-Hartl, M. (2009) Converging concepts of protein folding in vitro and in vivo. *Nat. Struct. Mol. Biol. 16*, 574−581.

(4) Kampinga, H. H., and Craig, E. A. (2010) The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat. Rev. Mol. Cell Biol. 11*, 579−592.

(5) Karzai, A. W., and McMacken, R. (1996) A bipartite signaling mechanism involved in DnaJ-mediated activation of the *Escherichia coli* DnaK protein. *J. Biol. Chem. 271*, 11236−11246.

(6) Laufen, T., Mayer, M. P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J., and Bukau, B. (1999) Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proc. Natl. Acad. Sci. U.S.A. 96*, 5452−5457.

(7) Sahi, C., and Craig, E. A. (2007) Network of general and specialty J protein chaperones of the yeast cytosol. *Proc. Natl. Acad. Sci. U.S.A. 104*, 7163−7168.

(8) Harrison, C. (2003) GrpE, a nucleotide exchange factor for DnaK. *Cell Stress Chaperones 8*, 218−224.

(9) Kityk, R., Kopp, J., Sinning, I., and Mayer, M. P. (2012) Structure and Dynamics of the ATP-Bound Open Conformation of Hsp70 Chaperones. *Mol. Cell 48*, 863−874.

(10) Mayer, M. P., Rudiger, S., and Bukau, B. (2000) Molecular basis for interactions of the DnaK chaperone with substrates. *Biol. Chem. 381*, 877−885.

(11) Mayer, M. P., Schroder, H., Rudiger, S., Paal, K., Laufen, T., and Bukau, B. (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat. Struct. Biol. 7*, 586−593.

(12) Cajo, G. C., Horne, B. E., Kelley, W. L., Schwager, F., Georgopoulos, C., and Genevaux, P. (2006) The role of the DIF motif of the DnaJ (Hsp40) co-chaperone in the regulation of the DnaK (Hsp70) chaperone cycle. *J. Biol. Chem. 281*, 12436−12444.

(13) Perales-Calvo, J., Muga, A., and Moro, F. (2010) Role of DnaJ G/F-rich domain in conformational recognition and binding of protein substrates. *J. Biol. Chem. 285*, 34231−34239.

(14) Szabo, A., Korszun, R., Hartl, F. U., and Flanagan, J. (1996) A zinc finger-like domain of the molecular chaperone DnaJ is involved in binding to denatured protein substrates. *EMBO J. 15*, 408−417.

(15) Wild, J., Altman, E., Yura, T., and Gross, C. A. (1992) DnaK and DnaJ heat shock proteins participate in protein export in *Escherichia coli*. *Genes Dev. 6*, 1165−1172.

(16) Wild, J., Rossmeissl, P., Walter, W. A., and Gross, C. A. (1996) Involvement of the DnaK-DnaJ-GrpE chaperone team in protein secretion in *Escherichia coli*. *J. Bacteriol. 178*, 3608−3613.

(17) Sharma, S., Chakraborty, K., Muller, B. K., Astola, N., Tang, Y. C., Lamb, D. C., Hayer-Hartl, M., and Hartl, F. U. (2008) Monitoring protein conformation along the pathway of chaperonin-assisted folding. *Cell 133*, 142−153.

(18) Horst, M., Oppliger, W., Rospert, S., Schonfeld, H. J., Schatz, G., and Azem, A. (1997) Sequential action of two hsp70 complexes during protein import into mitochondria. *EMBO J. 16*, 1842−1849.

(19) Mapa, K., Tiwari, S., Kumar, V., Jayaraj, G. G., and Maiti, S. (2012) Information encoded in non-native states drives substrate-chaperone pairing. *Structure 20*, 1562−1573.

(20) Muller, B. K., Zaychikov, E., Brauchle, C., and Lamb, D. C. (2005) Pulsed interleaved excitation. *Biophys. J. 89*, 3508−3522.

(21) Kapanidis, A. N., Lee, N. K., Laurence, T. A., Doose, S., Margeat, E., and Weiss, S. (2004) Fluorescence-aided molecule sorting: Analysis of structure and interactions by alternating-laser excitation of single molecules. *Proc. Natl. Acad. Sci. U.S.A. 101*, 8936−8941.

(22) Doose, S., Heilemann, M., Michalet, X., Weiss, S., and Kapanidis, A. N. (2007) Periodic acceptor excitation spectroscopy of single molecules. *Eur. Biophys. J. 36*, 669−674.

(23) Rudiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997) Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *EMBO J. 16*, 1501−1507.

(24) Rudiger, S., Schneider-Mergener, J., and Bukau, B. (2001) Its substrate specificity characterizes the DnaJ co-chaperone as a scanning factor for the DnaK chaperone. *EMBO J. 20*, 1042−1050.

(25) Chen, Z., Kurt, N., Rajagopalan, S., and Cavagnero, S. (2006) Secondary structure mapping of DnaK-bound protein fragments: Chain helicity and local helix unwinding at the binding site. *Biochemistry 45*, 12325−12333.

(26) Kurt, N., and Cavagnero, S. (2008) Nonnative helical motif in a chaperone-bound protein fragment. *Biophys. J. 94*, L48−L50.

(27) Palleros, D. R., Shi, L., Reid, K. L., and Fink, A. L. (1994) hsp70-protein complexes. Complex stability and conformation of bound substrate protein. *J. Biol. Chem. 269*, 13107−13114.

(28) Sharma, S. K., De los Rios, P., Christen, P., Lustig, A., and Goloubinoff, P. (2010) The kinetic parameters and energy cost of the Hsp70 chaperone as a polypeptide unfoldase. *Nat. Chem. Biol. 6*, 914−920.

(29) Feifel, B., Schonfeld, H. J., and Christen, P. (1998) D-peptide ligands for the co-chaperone DnaJ. *J. Biol. Chem. 273*, 11999−12002.

(30) Calloni, G., Chen, T., Schermann, S. M., Chang, H. C., Genevaux, P., Agostini, F., Tartaglia, G. G., Hayer-Hartl, M., and Hartl, F. U. (2012) DnaK functions as a central hub in the *E. coli* chaperone network. *Cell Rep. 1*, 251−264.

(31) Rodriguez, F., Arsene-Ploetze, F., Rist, W., Rudiger, S., Schneider-Mergener, J., Mayer, M. P., and Bukau, B. (2008) Molecular basis for regulation of the heat shock transcription factor sigma32 by the DnaK and DnaJ chaperones. *Mol. Cell 32*, 347−358.

(32) Han, W., and Christen, P. (2003) Mechanism of the targeting action of DnaJ in the DnaK molecular chaperone system. *J. Biol. Chem. 278*, 19038−19043.

(33) Ullers, R. S., Ang, D., Schwager, F., Georgopoulos, C., and Genevaux, P. (2007) Trigger Factor can antagonize both SecB and DnaK/DnaJ chaperone functions in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3101−3106.

(34) Sakr, S., Cirinesi, A. M., Ullers, R. S., Schwager, F., Georgopoulos, C., and Genevaux, P. (2010) Lon protease quality control of presecretory proteins in *Escherichia coli* and its dependence on the SecB and DnaJ (Hsp40) chaperones. *J. Biol. Chem. 285*, 23506−23514.

(35) Kaiser, C. M., Chang, H. C., Agashe, V. R., Lakshmipathy, S. K., Etchells, S. A., Hayer-Hartl, M., Hartl, F. U., and Barral, J. M. (2006) Real-time observation of trigger factor function on translating ribosomes. *Nature 444*, 455−460.

(36) Krishnan, B., Kulothungan, S. R., Patra, A. K., Udgaonkar, J. B., and Varadarajan, R. (2009) SecB-mediated protein export need not occur via kinetic partitioning. *J. Mol. Biol. 385*, 1243−1256.

(37) Spurlino, J. C., Rodseth, L. E., and Quiocho, F. A. (1992) Atomic interactions in protein-carbohydrate complexes. Tryptophan residues in the periplasmic maltodextrin receptor for active transport and chemotaxis. *J. Mol. Biol. 226*, 15−22.