Structure
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Information Encoded in Non-Native States Drives Substrate-Chaperone Pairing

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http://dx.doi.org/10.1016/j.str.2012.06.014

SUMMARY

Many proteins refold in vitro through kinetic folding intermediates that are believed to be by-products of native-state centric evolution. These intermediates are postulated to play only minor roles, if any, in vivo because they lack any information related to translation-associated vectorial folding. We demonstrate that refolding intermediate of a test protein, generated in vitro, is able to find its cognate chaperone, from the whole complement of *Escherichia coli* soluble chaperones. Cognate chaperone-binding uniquely alters the conformation of non-native substrate. Importantly, precise chaperone targeting of substrates are maintained as long as physiological molar ratios of chaperones remain unaltered. Using a library of different chaperone substrates, we demonstrate that kinetically trapped refolding intermediates contain sufficient structural features for precise targeting to cognate chaperones. We posit that evolution favors sequences that, in addition to coding for a functional native state, encode folding intermediates with higher affinity for cognate chaperones than noncognate ones.

INTRODUCTION

Intracellular protein folding is compromised by aggregation problems. To prevent loss-of-function phenotypes or aggregation-associated toxicity, cells have devised a set of proteins, termed molecular chaperones, to assist folding and alleviate aggregation-related problems. In *Escherichia coli*, the simplest model organism, a complex network of chaperones exists to maintain a healthy proteome. Nearly all nascent polypeptide chains during their passage through ribosome exit tunnel interact with Trigger Factor (TF)-a ribosome-associated chaperone. Subsequently depending on the degree of chaperone dependence nascent chains might interact with DnaK/J system-homolog of the Hsp70/40 machinery or further downstream chaperones like GroEL/ES system, to reach the final native structure. Additionally, for specialized functions dedicated chaperones exist; for periplasmic export of proteins, SecB/SecA system is required and ClpP, ClpX proteins are known for refolding and degradation of misfolded proteins. For growth of *E. coli*, none of the chaperones are essential except for GroEL/ES system suggesting sufficient functional redundancies among different chaperone systems. For example, overproduction of GroEL/ES and SecB partially rescue the temperature-sensitive phenotype and folding defects observed in case of DnaK and TF deletions (Ullers et al., 2004; Vorderwülbecke et al., 2004). Conversely, there are indications that DnaK/J and GroEL may assist in export of proteins that can rescue growth defects resulting from SecB deletion (Kusukawa et al., 1989; Wild et al., 1992, 1996).

So far, the features of substrate proteins that target them to particular chaperone machinery are not well understood. As a primitive signature of targeting, primary sequence motifs that bind to certain chaperones have been described. Like, TF and DnaK/J system have been shown to bind to linear hydrophobic stretches of polypeptides. In case of DnaK/J the hydrophobic sequences are usually flanked by positively charged residues (Rüdiger et al., 1997, 2001). Although sequences of short linear peptides that bind DnaK are well-defined, it is unclear if the binding specificity remains unaltered when present in the context of a partially structured folding intermediate. This becomes important in the light of recent evidences that conclusively demonstrate that DnaK can bind native, denatured or aggregated proteins as substrates containing exposed hydrophobic residues that are not necessarily presented to chaperones as linear stretches as generally believed (Schlecht et al., 2011). Similarly, binding specificity of SecB shows that it favors peptides with aromatic and basic residues and disfavors acidic residues (Knoblauch et al., 1999). There is apparently no difference in the occurrence of SecB-binding sequence in the cytosolic and secretory proteins negating the exclusivity of sequence specificity driven substrate binding of SecB. Furthermore, typical SecB substrates seem to contain signal sequences at the N-terminus but it has been shown to be dispensable for precise targeting (Collier et al., 1988; Gannon et al., 1989; Liu et al., 1989; Weiss and Bassford, 1990). Taken together, SecB dependent secretory proteins must contain some unique structural features that target them specifically to the chaperone. The most downstream chaperone, GroEL, is believed to bind to structured intermediates that populate molten-globule like conformation (Martin et al., 1991; Robinson et al., 1994). In spite of all the knowledge that has been gathered on chaperone substrates over the past two decades, it is still unclear how a substrate is able to pair with its target chaperone in the complex intracellular milieu.

In this context, it is reasonable to speculate that folding intermediates of proteins may contain information that can target them to particular chaperones. The folding intermediates that are populated in vivo are difficult to detect and characterize experimentally. On the other hand, in vitro refolding intermediates are well studied and have been characterized for a large number of refolding proteins. However, a major drawback of in vitro refolding reactions is that it lacks any structural information that may result from vectorial folding process, associated with translation. Even with this limitation, it is interesting to note that many of the kinetic intermediates populated during a typical refolding reaction, starting from chemically or thermally denatured state, are prone to aggregation thereby exhibiting hallmarks of chaperone-dependence. Thus, it will be interesting to test if these intermediates that populate in vitro refolding reactions have any physiological significance or are by-products of native state-centered evolution.

Using a model substrate, a slow folding mutant of Maltose binding protein, we investigated if the refolding intermediates formed in vitro are recognized by cellular components and if they contain any pertinent physiological information. Using single molecule fluorescence resonance energy transfer (sm-FRET) measurements and biochemistry, we show that the refolding intermediate is able to bind to its cognate chaperones, SecB and DnaK in a mixed pool that comprised of all the soluble chaperones of *E. coli*. Precise targeting of this substrate is lost when the chaperone concentrations of the cell are altered. We postulate that the folding intermediate contained sufficient information for precise targeting of substrate to its cognate chaperone. Because substrates of GroEL/ES and SecB have been well-characterized, we chose other substrates of these two chaperones to validate the generality of our hypothesis (Baars et al., 2006; Kerner et al., 2005; Knoblauch et al., 1999). Using a library of these substrates, we categorically demonstrate that folding intermediates indeed contain information that determines chaperone-targeting. We posit that ruggedness in folding landscape, encoded by amino acid sequences, are physiologically important for protein targeting and may have evolved under selection pressure to assure faithful association with cognate chaperones.

RESULTS

In Vitro Refolding Intermediate of DM-MBP Is Recognized by Cellular Components

To understand the targeting of kinetic intermediates of substrates within the chaperone network of *E. coli*, we chose a slow folding mutant of Maltose binding protein (DM-MBP) as a model substrate. DM-MBP harbors two substitutions, V8G and Y283D, situated in close spatial proximity in its N-terminal domain that possibly hinder formation of native contacts within this domain, a rate limiting step of wild-type MBP refolding (Figure 1A) (Spurlino et al., 1992; Wang et al., 1998). DM-MBP was chosen based on three major considerations. First, the test protein should populate a long-lived folding intermediate. DM-MBP is known to undergo a rapid compaction (due to hydrophobic collapse) leading to a loosely compact folding intermediate that is long-lived ($t_{1/2}$ ~1,700 s at 25°C and 60 mM GuHCl) and undergoes a slow conversion to native state (Sharma et al., 2008). Second, the test protein should be detectable in low nM concentrations as in vivo concentration of nascent chain of any substrate protein at a given time point is in the low nM range. Thus an ideal model-system for substrate-chaperones pairing requires experimentation at very low nM concentrations of the substrate protein that necessitates the use of highly advanced techniques like single molecule fluorescence spectroscopy. DM-MBP is an ideal candidate for that purpose as it has been extensively used for single molecule spectroscopy (Chakraborty et al., 2010; Sharma et al., 2008). Last, the protein needs to be targeted to specific chaperones. MBP is an excellent choice as it has been shown to interact with SecB machinery in vivo. Additionally, the mutant form, DM-MBP, is known to be a well established substrate of the GroEL/ES system (Chakraborty et al., 2010; Sharma et al., 2008; Sparrer et al., 1997; Tang et al., 2008; Wang et al., 1998). This allows the investigation of conditions that lead to noncognate substrate-chaperone partitioning between two distinct chaperones SecB and GroEL.

To investigate if cellular components are able to recognize and alter the conformation of the kinetic refolding intermediate of DM-MBP (hereafter referred as iDM-MBP) at low nM concentrations, we investigated the effect of cellular chaperones on the structure of iDM-MBP. To set the reference point and delineate the structural heterogeneity of the folding intermediate, conformational distribution of spontaneously refolding molecules of DM-MBP were investigated at single molecule resolution. Double cysteine variant of DM-MBP (134C, 298C) was labeled with maleimide-reactive fluorophores (Alexa 488-C5 maleimide and Alexa 647-C2 maleimide, Molecular Probes) to perform sm-FRET experiments. All sm-FRET measurements were taken at very low concentrations (~50 pM) of labeled molecules to minimize the possibility of contamination with multiple molecules (Figure S1A available online).The distance vector (134–298) probes for distance changes between N-domain and the C-domain of DM-MBP molecule during the refolding process (Figure 1A). Anisotropy of fluorophores conjugated to the cysteine residues (Table S1) showed that free rotation of fluorophores was not hindered at any of these positions. Sm-FRET methodology (modified version of pulsed interleaved excitation [PIE] ALEX and PAX, described in Supplemental Experimental Procedures) (Doose et al., 2007; Kapanidis et al., 2004; Müller et al., 2005), ensured that only molecules with active donor and acceptor fluorophores are considered for FRET ratio (FR) histogram. We first denatured the protein and then diluted it out of the denaturant that allows the protein to refold to native state and we probed alterations in the intramolecular distance during refolding by single molecule FRET. When donor-acceptor labeled DM-MBP was denatured with Gu-HCl (6 M) most of the molecules had low FR (Figure 1C), indicating a large intramolecular distance due to unfolding compared to the native protein that is largely populated by molecules with high FR (Figure 1B). Just after dilution from the denaturant (final concentration of 60 mM Gu-HCl), refolding molecules populated a broad FR distribution with majority of molecules in the low FR region (FR = 0.18) with much broader width than the unfolded state (Figure 1D). The refolding intermediate of DM-MBP thus formed is presumably due to a fast (within first 4 ms of the refolding reaction) hydrophobic collapse of the protein upon diluting out the denaturant (Sharma et al., 2008). To probe structural alterations

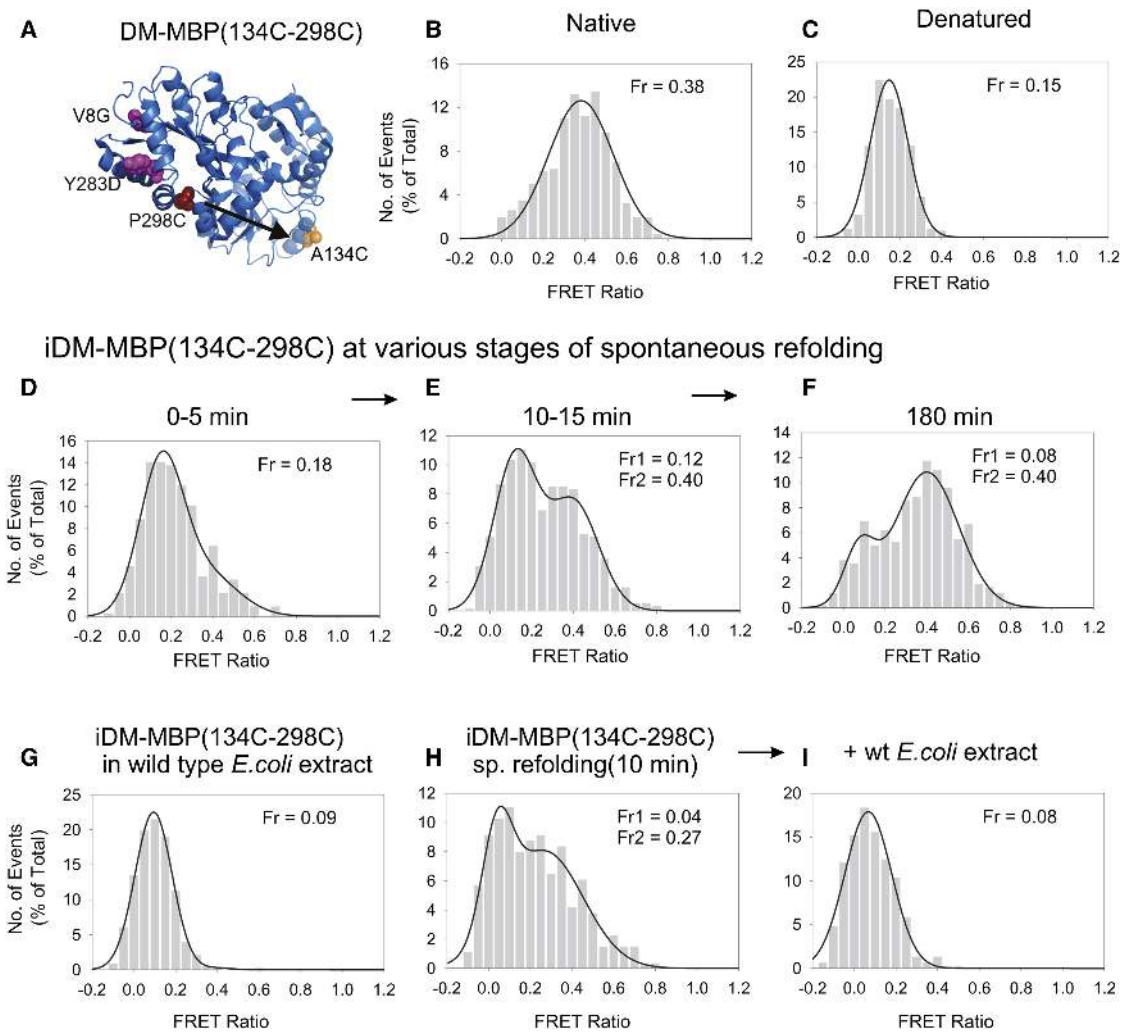

Figure 1. Refolding Intermediate of DM-MBP Undergo Radical Conformational Alterations within Intracellular Milieu

(A) Ribbon diagram of MBP crystal structure (pdb 1OMP) (Spurlino et al., 1992) showing substituted cysteine residues used for labeling (acceptor position shown in red, donor in yellow). Two mutations, V8G and Y283D, are indicated in purple.

(B and C) Sm-FRET ratio histogram of double labeled DM-MBP (134,298) (Alexa 488-maleimide/Alexa 647-maleimide). All sm-FRET experiments were done at ~50 pM of DM-MBP concentrations. Native DM-MBP molecule in buffer A (B). Denatured DM-MBP in buffer B (C).

(D–F) FRET ratio histogram demonstrating structural states populated during different stages of spontaneous refolding of DM-MBP (in final 60 mM GuHCl) during 0–5 min (D), during 10–15 min (E), and spontaneously refolded state after 180 min (F).

(G) Sm-FRET ratio histogram of iDM-MBP (134,298) in cell and membrane free extract of wild-type *E. coli* cells supplemented with 10 mM ATP. 0.5 μM DM-MBP denatured with buffer B was diluted (1:100) in the cell-extract for 10 min. To obtain single molecule resolution, iDM-MBP bound to cell extract was further diluted in buffer C.

(H and I) After initial hydrophobic collapse in buffer, (I) collapsed state (from [H]) was subsequently mixed with wild-type *E. coli* cell extract (1:1) and incubated for 10 min. FRET ratio histograms were obtained thereafter as described in (G). See also Figure S1.

during the rest of the refolding process (slow phase), we obtained FRET-histograms as a function of time that demonstrated accumulation of high FR molecules in a time dependent manner without populating any other intermediate structures (Figures 1E and 1F). This data vividly captures stages of refolding events and demonstrates that the slow phase of iDM-MBP refolding is essentially constituted by a non-native folding intermediate and the native state.

Subsequently, we probed the structural alteration of iDM-MBP in presence of cell and membrane free extracts of *E. coli*, supplemented with exogenous ATP. This reaction contained the full complement of soluble chaperones. First we diluted denatured DM-MBP in wild-type *E. coli* extract for 10 min and then probed the structure of iDM-MBP formed in cell extract by smFRET. Interestingly, we found that almost the entire population of molecules was distributed in a narrow low FR (FR = 0.09) population that was strikingly different from the broad FR distribution of collapsed state in buffer (Figure 1G). This led us to ask whether the expanded state populated in cell extract is formed through prevention of fast hydrophobic collapse or cellular components actively alter the conformation of collapsed intermediate. Toward this, we initiated the refolding reaction in buffer, allowing

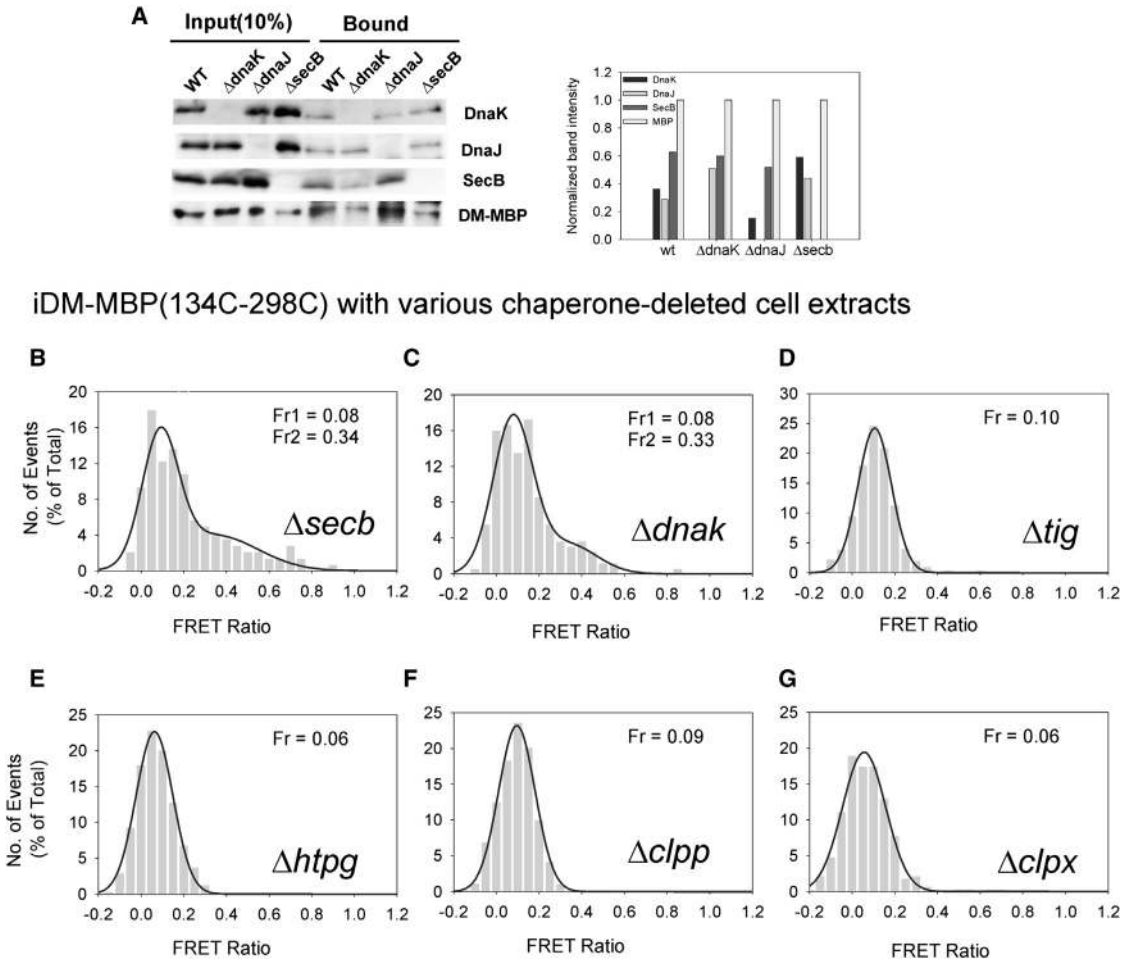

Figure 2. Refolding Intermediate of DM-MBP Is Recognized and Structurally Modulated by Its Cognate Chaperones from a Complex Pool of Chaperones

(A) Left: coimmunoprecipitation of bound chaperones with iDM-MBP after interaction with cell extracts of wild-type, Δ*dnak*, Δ*dnaj*, Δ*secb E. coli* cells. iDM-MBP was bound to cell extract as described in Figure 1G and subsequently immunoprecipitated. Bound fractions were probed with chaperone specific antibodies and anti-MBP antibodies. Right: Band intensities of eluted proteins were quantified (using Image J software) and were normalized to the inputs. Signal of MBP has been normalized to 1 for easy comparisons.

(B–G) Sm-FRET ratio histogram of iDM-MBP molecules in extracts of various single chaperone deleted strains were obtained as described in Figure 1G. Cell extracts used were from Δ*secb* (B), Δ*dnak* (C), Δ*tig* (D), Δ*htpg* (E), Δ*clpp* (F), and Δ*clpx* (G) cells. See also Figure S2.

it to form the collapsed state and subsequently mixed it with the cell extract. Interestingly, even after initial hydrophobic collapse, represented by very broad FR distribution of iDM-MBP (Figure 1H), the refolding intermediate underwent expansion in cell extract (Figure 1I). In order to deconvolute the contribution of cellular components in structural modulation of iDM-MBP, we fractionated the cell extract. Using ultrafiltration, small-molecule components ($\leq$3 kDa) of cell extract were separated. Contribution of small molecules was checked as many of them are osmolytes that can potentially perturb the structural states of non-native proteins. Interestingly, small-molecule complement of the cytosol was unable to affect an expansion of the folding intermediate pointing toward the role of macromolecular components mediating the structural alterations (Figure S1B). Similarly, removal of small molecules by extensive dialysis gave similar conformational state as populated in presence of total cell extract (Figure S1C). This indicated that macromolecular components of the cytosol are necessary and sufficient to sustain the extended state of non-native DM-MBP in cell-extract. Thus, macromolecular components of the cellular extract are able to recognize and alter the conformational distribution of an in vitro refolding intermediate.

Refolding Intermediate of DM-MBP Selectively Binds SecB and DnaK in a Complex Milieu of Soluble Chaperones

To determine if canonical *E. coli* chaperones bind iDM-MBP, we started refolding of DM-MBP in wild-type *E. coli* extract and subsequently immunoprecipitated it to probe for associated chaperones by chaperone-specific antibodies. We found SecB, DnaK, and DnaJ to be associated with iDM-MBP (Figure 2A). To check for any altered interactions with chaperone pool in the

absence of these chaperones, we prepared extracts of *E. coli* cells deleted of single chaperones (Figure S2) and performed similar immunoprecipitation experiments. Importantly, deletion of any chaperone did not significantly alter the interaction with others (Figure 2A). To investigate chaperone-specific conformational alterations of iDM-MBP in cell extract, we probed for the ex vivo structural states of iDM-MBP in absence of particular chaperones by sm-FRET. We found the structure of the refolding intermediate bound to extracts of Δ*secb* (Figure 2B) and Δ*dnak* (Figure 2C) cells to be significantly altered from the one bound to wild-type *E. coli* cell extract (Figure 1G). This indicated that absence of DnaK and SecB individually led to the formation of slightly more compact structures, represented by more molecules in high FR region. To rule out the role of other chaperones indirectly in modulating the structure of iDM-MBP, we probed the structure with extracts deleted of *tig* (Figure 2D), *htpg* (Figure 2E), *clpp* (Figure 2F), and *clpx* (Figure 2G). Absence of none of these chaperones significantly altered the structure of iDM-MBP. Prominent structural alterations specifically in absence of DnaK and SecB led us to probe the interaction of iDM-MBP with these chaperones in a purified system.

Unique Structural Modulations of iDM-MBP by DnaK and SecB Leads to Differential Holdase Activity

To check the interaction of iDM-MBP with purified chaperones, we monitored the interaction to DnaK/J and SecB with the refolding intermediate of his-tagged version of DM-MBP by Ni-NTA pull-down assay. iDMMBP was found to interact with DnaK/J and SecB independently (Figures 3A and 3B). Specificity of iDM-MBP/DnaK interaction as a true substrate/chaperone interaction was verified by its interaction with isolated peptide binding domain (PBD) of the chaperone known to capture substrates independently (Figure 3C). Furthermore, this interaction could be competed out with a DnaK binding peptide P5 (CALLLSAPRR) (Figure 3C) reconfirming that iDM-MBP indeed interacts with DnaK as a substrate (Feifel et al., 1998).

To find out the sites of interaction of non-native DM-MBP with two different chaperones, we took various single cysteine mutants (at positions 21, 88, 141, 170, 190, 202, 269, and 362) of DM-MBP (Sharma et al., 2008) encompassing almost the entire sequence of the protein. We obtained the anisotropy of fluorophore (Alexa647-C2 maleimide) conjugated to cysteine residues of each mutant during spontaneous as well as chaperone-assisted refolding. Alexa647 has a short lifetime ~1.2 ns and is ideal for reporting fast segmental motion as this short lifetime renders it relatively insensitive to protein tumbling that occurs at a much larger timescale (>10 ns). The difference in anisotropy of chaperone bound state of the protein and the free refolding intermediate (ΔAnisotropy) indicates the regions that are affected upon binding of the specific chaperones, thereby creating a unique fingerprint of binding pattern for the different chaperones. Regions that undergo restriction in segmental mobility are marked by positive values whereas the regions that undergo unfolding (or undergo increase in segmental mobility) upon binding of chaperones are marked by a negative value (Figure 3D). From the anisotropy signature of DnaK on DM-MBP, it is evident that fluorophores conjugated at positions 88, 190, 202, and 362 undergo significant anisotropy change indicating structural perturbations upon DnaK binding in these places. This data is in good correlation with the predicated DnaK binding site on DM-MBP using prediction algorithms (Table S2). In contrast, SecB binding leads to anisotropy change in several positions on iDM-MBP primary sequence (21, 88, 141, 190, 202, and 362) and is in excellent agreement with the previous study on mapping of SecB binding sites on MBP sequence (Figure 3D) (Topping and Randall, 1994). Notably, in this study the extreme N- and C-terminal peptides of MBP were not recovered due to technical limitations. In our assay, we could observe that extreme N- and C-terminal ends of DM-MBP also show anisotropy change upon SecB binding indicating interaction of N and C terminii of MBP with SecB.

Furthermore, to underline the specific role of DnaK/J and SecB machinery in altering the conformation of iDM-MBP ex vivo, we initiated refolding of iDM-MBP in presence of purified DnaK/J/GrpE and SecB individually and both chaperone systems together. In presence of DnaK/J/E, the FR distribution was distinctly different from the one bound to cell extract (Figure 3E). On the other hand, the only SecB-bound structure closely resembled the structure bound to cell extract (Figure 3F). When both chaperone systems were present in the refolding reaction, the structure of the iDM-MBP resembled mostly the SecB-bound structure (Figure 3G). In conclusion, in absence of any other cellular components, the conformation of SecB-bound iDM-MBP, faithfully recapitulated the structure of iDM-MBP as in the complete cell extract. Thus, SecB is the chaperone that is primarily responsible for modulating the in vivo structure of iDM-MBP suggesting existence of precise targeting mechanisms of substrates to specific chaperones.

If indeed chaperones like DnaK and SecB bind to iDM-MBP, in addition to conformational alterations, binding might also influence the refolding rate. To monitor chaperone-assisted refolding of iDM-MBP, we probed the conformational distribution after 3 hr of incubation with DnaK/J/E or SecB by which the spontaneously refolding protein folds in buffer. Refolding in presence of purified DnaK/J/E demonstrated conversion to native state with a slower refolding rate that was in excellent correlation with earlier ensemble experiments (Figure S3A) (Sharma et al., 2008; Tang et al., 2006). On the other hand, purified SecB drastically retarded the refolding of iDM-MBP represented by almost no conversion to native state even after 3 hr of initiation of refolding reaction (Figure S3B).

In agreement with in vitro results, refolding in wild-type *E. coli* extract containing both DnaK and SecB demonstrated presence of majority of DM-MBP molecules in the initial low FR region after 3 hr indicating active role of chaperones in preventing refolding of DM-MBP ex vivo (Figure 4A). More importantly, significantly higher percentage of refolding molecules attained native structure when refolding was initiated in extracts Δ*secb* (Figure 4B) or Δ*dnak* cells (Figure 4C), compared to refolding in wild-type extract. This data pointed toward the importance of both of the chaperones in physiological concentrations for maintenance of the extended conformation of iDM-MBP. SecB-mediated inhibition of refolding could be restored by addition purified SecB to Δ*secb* cell extract (Figure S4A). This underlines the specificity of SecB in preventing refolding of DM-MBP. Inclusion of an ATP regeneration system in the refolding reaction yielded similar amount of refolded proteins ruling out the possibility of rapid depletion of ATP from the system (Figure S4B). Refolding in

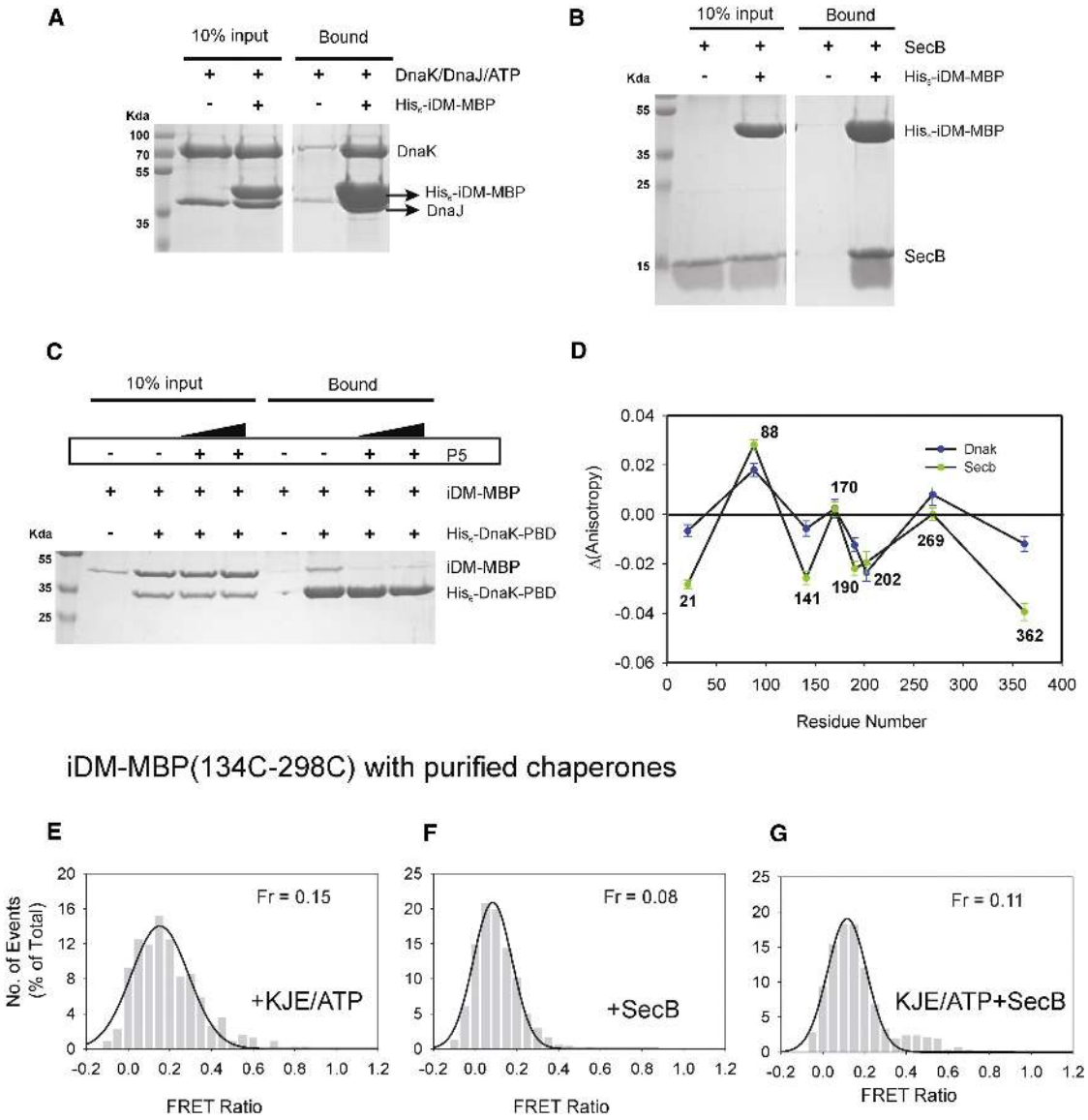

Figure 3. DnaK/J and SecB Independently Bind iDM-MBP and Modulate Its Structure in Unique Manner

(A and B) Interactions of iDM-MBP to DnaK/J and SecB were checked by copurification of bound chaperones with His_6-tagged DM-MBP by Ni-NTA pull down assay. His_6-DM-MBP was denatured by 6 M Gu-HCl and subsequently diluted (1:100) in buffer A containing either SecB or DnaK/J/ATP (final concentrations, iDM-MBP: 1 μM; SecB: 2 μM; K/J: 2/1 μM). Eluted proteins were subjected to SDS-PAGE followed by Coomassie brilliant blue (CBB) staining.

(C) Specificity of iDM-MBP/DnaK interaction as substrate/chaperone interaction was confirmed by its interaction with isolated PBD of DnaK. Refolding intermediate of DM-MBP was similarly generated as in (A) and was allowed to interact with His_6-PBD-DnaK (final concentrations iDM-MBP: 0.8 μM, PBD: 0.4 μM). Eluted proteins were subjected to SDS-PAGE followed by CBB staining. To compete out the iDM-MBP/PBD binding, P5 peptide was added in the similar reactions (~10 μM and 20 μM). Amounts of bound iDM-MBP were compared in absence and presence of the peptide.

(D) Steady-state anisotropy of free refolding and chaperone-bound single cysteine mutants of DM-MBP labeled with Alexa 647 in the indicated positions was measured. The difference of anisotropy of the chaperone bound state and the free refolding state of all positions have been plotted in the y axis as Δ(Anisotropy). Intrinsic errors of anisotropy measurements have been indicated with error bars.

(E–G) DM-MBP (0.02 μM, final) was denatured and subsequently diluted to allow refolding in presence of purified DnaK (0.5 μM)/DnaJ(0.25 μM)/GrpE(0.5 μM)/ATP(5 mM) or SecB(1 μM) (F) or both chaperones together in buffer A (G). After incubation iDM-MBP/chaperone complexes were further diluted in to obtain single molecule resolution (final ~50 pM DM-MBP in 60 mM Gu-HCl) and FRET ratio histograms were plotted. See also Figure S3.

extracts of Δ*tig* (Figure 4D), Δ*htpg* (Figure 4E), Δ*clpp* (Figure 4F), or Δ*clpx* (Figure 4G) cells did not exhibit any significant difference from wild-type extracts. SmFRET experiments were nicely recapitulated by ensemble experiments in wild-type and Δ*secb* extracts: a large fraction of iDM-MBP in Δ*secb* extract reached native state whereas a negligible fraction refolded in the wild-type extract (Figure S4C). Taken together, this data demonstrate that a cooperative holdase activity of DnaK and SecB maintain refolding intermediate of DM-MBP in an expanded state, probably reminiscent of a process that maintains pre-MBP in an

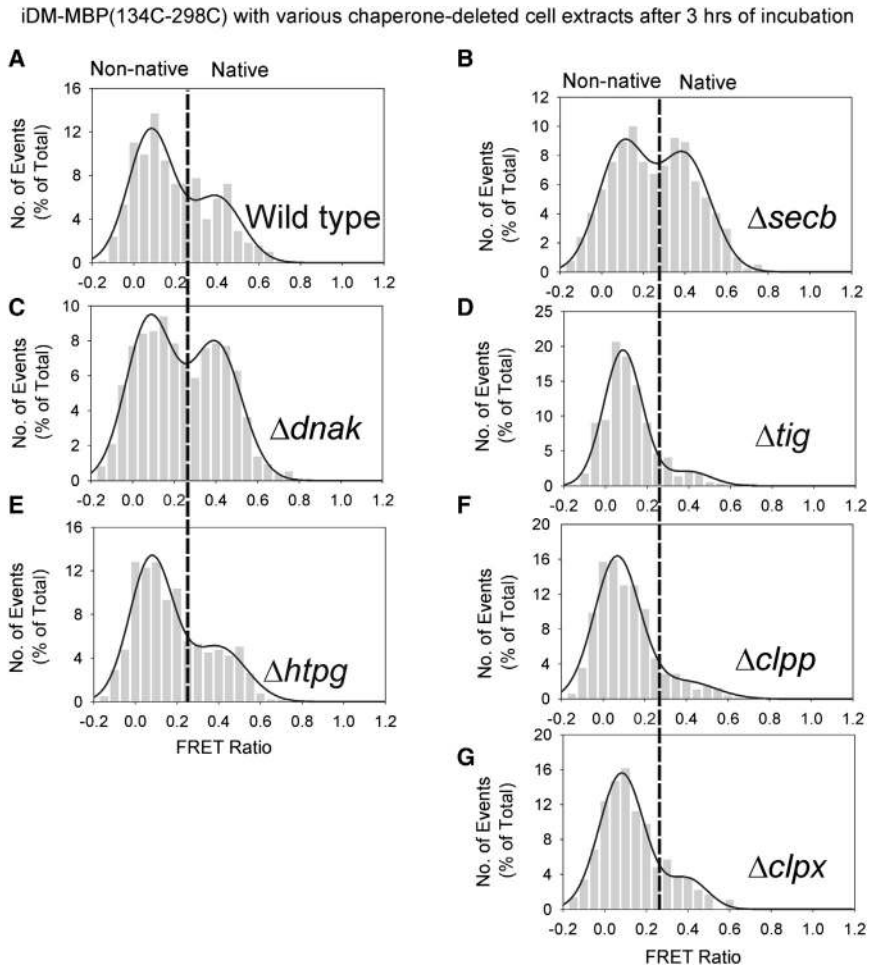

Figure 4. DnaK and SecB Act As Holdases to Keep DM-MBP in an Export-Competent State

FRET ratio histograms showing conformational states of the iDM-MBP molecules after 3 hr of incubation in cell extracts (as described in Figure 2) of wild-type *E. coli* cells (A), Δ*secb* cells (B), Δ*dnak* cells (C), Δ*tig* cells (D), Δ*htpg* cells (E), Δ*clpp* (F), and Δ*clpx* (G). An eye-guide has been used to indicate the fractions of refolding molecules that attained the native state in different cell extracts. See also Figure S4.

extended conformation for subsequent export through SecYEG complex. However, absence of either of them weakens the holdase activity leading to efficient refolding of iDM-MBP. Thus, a kinetic folding intermediate formed during in vitro refolding process is able to precisely recognize its cognate chaperone from a pool of chaperones present in the *E. coli*.

Targeting of iDM-MBP Is Affected upon Chaperonin Overexpression

As GroEL/ES chaperonin system has been shown to accelerate the refolding of iDM-MBP in vitro, we speculated that EL/ES system possibly assists it to refold in absence of either DnaK or SecB. In wild-type *E. coli* cells DM-MBP is present mostly in the inclusion body fraction of the cell. Large fraction of this is rendered soluble in presence of EL/ES overexpression that suggested that the protein is indeed a chaperonin-substrate in vivo (Figure 5A). Solubilization of DM-MBP by EL/ES system exclusively upon overexpression of the chaperonin may be rationalized by two alternate arguments. First, it is possible that EL/ES is saturated by endogenous substrates and hence accommodate DM-MBP only upon overexpression, when a large fraction of EL/ES is free of endogenous folding load. Alternately, it is possible that specific targeting to EL/ES happens only upon GroEL-overexpression through alteration of the holdase to foldase ratio in cells. Our ex vivo folding studies were performed with nanomolar concentrations of DM-MBP, in cell extracts containing micromolar levels (~5 μM) of EL/ES. Even at these low concentrations of substrate protein and in absence of newly-translated nascent chains, when most of the EL/ES system should be free of endogenous substrates, DM-MBP folding was not supported by EL/ES (Figure 4A). On the other hand, extract of EL/ES overexpressing cells accelerates refolding in spite of presence of holdases in its physiological concentration and the refolding is complete by ~20 min (Figure 5B) in ATP-dependent manner (Figure S5A). This rate is in excellent correlation with the EL/ES assisted in vitro refolding rate of DM-MBP (Chakraborty et al., 2010; Tang et al., 2006). Additionally deletions of holdases lead to efficient protein refolding that indicate that holdases indeed affect a block on DM-MBP refolding in the wild-type scenario by preventing the transfer of substrate to EL/ES. This is facilitated by higher affinity of iDM-MBP for SecB (>10^9 M^{-1}) (Figures S5B-S5D) compared to that for GroEL (~2 × 10^7 M^{-1}) (Sharma et al., 2008). Hence relative affinity of the refolding intermediate of a substrate for different chaperones may dictate its folding outcome.

To reinstate the wild-type holdase to foldase ratio under conditions of EL/ES overexpression, we added purified SecB and monitored refolding as a function of SecB concentration. Convincingly, reverting back to higher holdase to foldase ratio by adding SecB, ensured effective repartitioning of DM-MBP to SecB preventing its refolding (Figure 5C). Notably, we obtained that refolding yield and not the refolding rate of EL/ES assisted refolding, to be altered as a function of added SecB (Figure 5D). This suggests that once partitioning of the substrate occurs between EL/ES and SecB based on effective affinities, GroEL-associated iDM-MBP molecules reach native state whereas the ones associated with SecB stay irreversibly bound, mostly due to negligible off-rates. Because substrates cycle in the EL/ES system as a function of ATP-hydrolysis in the rings, we asked if substrate may repartition from EL/ES system to SecB during cycling. We initiated EL/ES assisted refolding

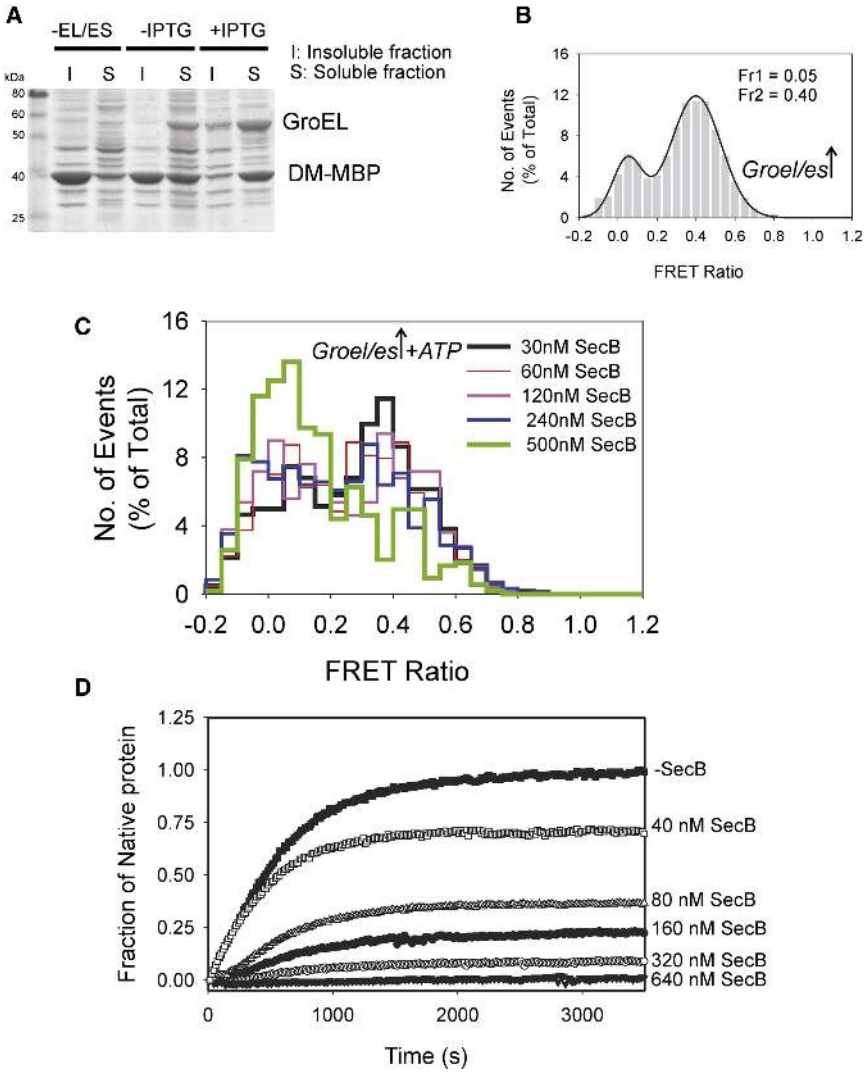

Figure 5. An Altered Holdase/Foldase Ratio Determines the Fate of Client Proteins

(A) Solubility of DM-MBP in absence and presence of EL/ES overexpression in wild-type *E. coli* cells. Overexpression of EL/ES was obtained by IPTG induction. DM-MBP was expressed with 0.1% arabinose from an arabinose-inducible promoter. In all cases cells were disrupted by sonication and equivalent amounts of soluble and insoluble fractions were loaded in SDS-PAGE and stained with CBB.

(B) Sm-FRET ratio histograms of refolding double-labeled DM-MBP (134,298) in *E. coli* cell extract containing overexpressed EL/ES (+10 mM ATP) was obtained as described in Figure 1G.

(C) The same experimental condition as in (B) was used, with increasing concentrations of purified SecB. The FRET ratio histograms obtained with different SecB concentrations are depicted as line plots.

(D) Refolding of double-labeled DM-MBP (52C-298C [Alexa 532-maleimide/Alexa 647-maleimide]) (final 50 nM) was monitored after dilution (1:100) in *E. coli* cell extract containing overexpressed EL/ES with 20 mM maltose and 10 mM ATP, by following the change of donor-dye fluorescence with time. The fraction of protein that reached the native state in presence of GroEL/ES overexpressing cell-extract was set as 1. Similar experiments were performed with increasing concentrations of SecB and the fraction of native protein obtained in each SecB concentration was plotted. See also Figure S5.

reaction in extracts from cells overexpressing EL/ES and supplemented the reaction with purified SecB, 30 s after initiating the refolding reaction (Figure S5E). Refolding was effectively blocked, indicating that SecB is able to associate with substrates during the GroEL/ES cycle. Thus, fates of substrate proteins are critically determined by typical molar ratio of chaperones inside cell. In spite of ample redundancies between different chaperone systems, substrates may have evolved relative affinities to partition to the appropriate system to facilitate chaperone-specific folding or transport across membranes.

Information Encoded in Folding Intermediates Drives Substrate-Chaperone Pairing

Because iDM-MBP targeting was dependent on the ratio of [GroEL]:[SecB] ex vivo, it is possible that relative affinities of the folding intermediates drive targeting. Or more trivially, it is possible that all refolding intermediates generated in vitro stochastically associate preferentially with either of the chaperones, lacking any physiological relevance. To test these possibilities, we purified a number of authentic GroEL and SecB substrates and investigated substrate targeting between these two systems. We took representative members of different substrate groups of GroEL, e.g., Enolase (Eno) as group I substrate, Threonyl tRNA synthetase (Ths) as group II, S-adenosyl methionine synthetase (MetK) and Dihydrodipicolinate synthase (DAPA) as group III substrates (Kerner et al., 2005). We chose OppA and DM-MBP as SecB substrates and TolB, a known SRP substrate as a control (Figure 6A) (Strobel et al., 1993; Zalucki et al., 2011). All substrates were purified by Ni-NTA affinity chromatography by N-terminal hexa-histidine tags. Subsequently, all proteins were first denatured and then diluted out of denaturant to start the refolding reaction. All these proteins populated a bona fide refolding intermediate that bound the hydrophobic dye, 1-anilinonaphthalene-8-sulfonic acid (1-8 ANS) (Figures 6B and S6A). The refolding intermediates of these proteins also contained various degrees of secondary structural elements as probed by circular dichroism (CD) (Figures 6C and S6B). This together with ANS-binding data suggests the formation of kinetically trapped states with molten globule like folding intermediates in majority of the cases. Next, refolding intermediates of substrate proteins were formed in presence of GroEL and SecB in different molar ratios in the physiological range (we found that intracellular SecB to GroEL molar ratio varies from ~1:2 to 1:5) (Data not shown). Non-native substrates are expected to bind to chaperones depending on their relative affinities. Interestingly, type II and type III GroEL substrates were

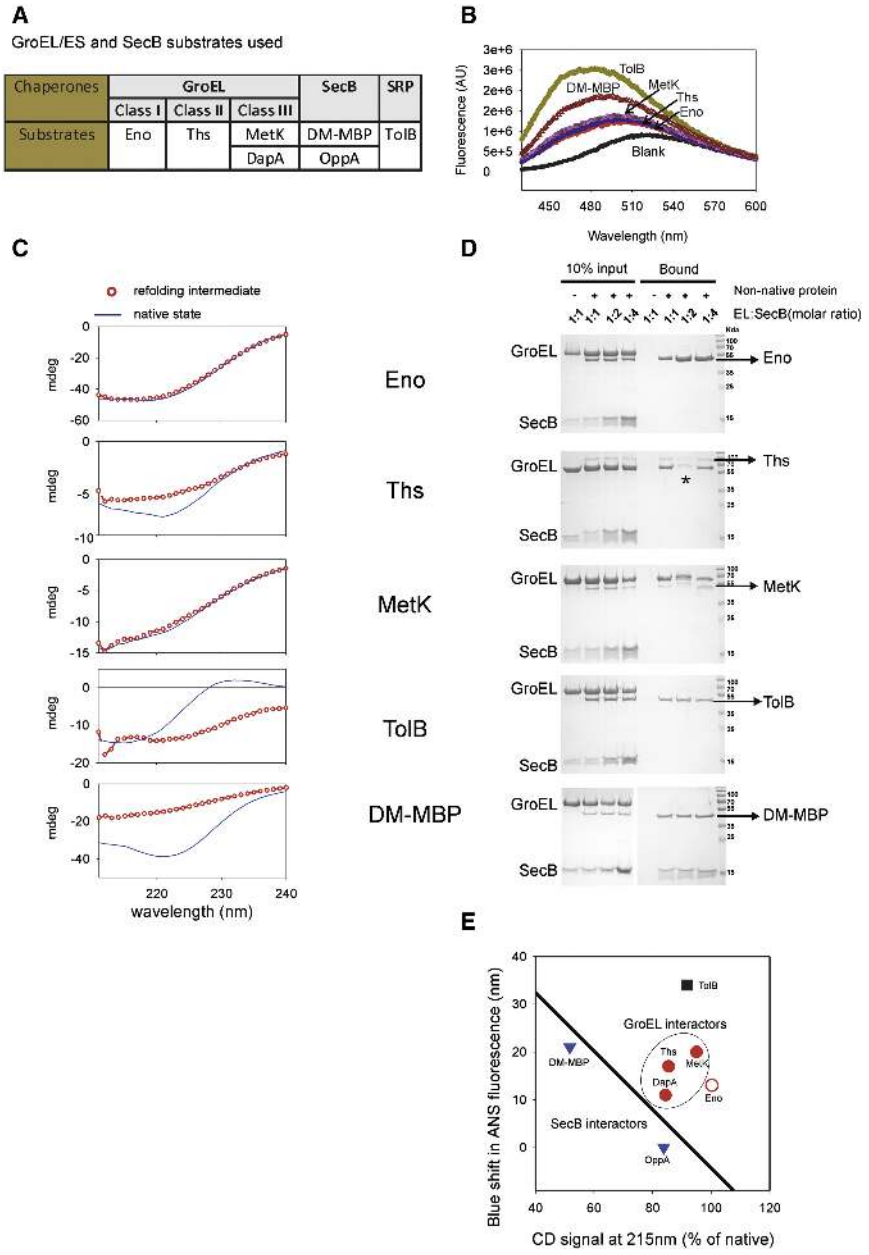

Figure 6. Non-Native States of Protein Contain Sufficient Information for Efficient Chaperone Targeting

(A) A table showing the list of chaperone-substrates used for determining the relative affinity between SecB and GroEL.

(B) Emission spectra of 1-8 ANS (50 μM) in buffer C (blank) or bound to various non-native GroEL and SecB substrates (final 1 μM of non-native protein in 60 mM GuHCl). All substrate proteins were first denatured with 6 M Gu-HCl and was subsequently diluted (1:100) in buffer A containing 50 μM 1-8 ANS. ANS spectra bound to TolB (green circle), DM-MBP (dark red triangle), MetK (pink squares), Ths (blue circles), Eno (red circles), and ANS in buffer (black curve) have been demonstrated. For ANS binding spectra of DapA and OppA see Figure S6A.

(C) Comparative CD spectra of 1 μM of native versus non-native protein have been plotted to compare the secondary structural elements of non-native proteins soon after dilution from denaturant.

(D) Copurification of bound chaperones with His_6-tagged GroEL/ES substrates and SecB substrates by Ni-NTA pull down assay. All substrate proteins were first denatured with 6 M Gu-HCl and then diluted out of denaturant (1:100) in buffer A (final concentrations of non-native proteins were ~1 μM) in presence of SecB and GroEL in varying molar ratios. Bound chaperone was copurified by Ni-NTA pull down assay with His_6-non-native proteins. Eluted proteins from Ni-NTA columns were subjected to TCA precipitation due to large reaction volume and subsequently loaded in SDS-PAGE followed by CBB staining. *Represents inefficient precipitation in the particular lane of SDS-PAGE.

(E) CD signal at 215 nM (% of signal of native protein) of the refolding intermediates of various GroEL, SecB, and SRP substrates are plotted against the blue shift in ANS fluorescence upon binding to the intermediates. See also Figure S6.

efficiently targeted to GroEL with absolutely no targeting to SecB (Figures 6D and S6C). Enolase, a Type I and hence a weak chaperonin substrate, was associated with neither GroEL nor SecB (Figure 6D). DM-MBP, in the physiological molar ratio of SecB to GroEL was specifically targeted to SecB explaining the prevalence of SecB-bound type conformation in wild-type *E. coli* extract (Figure 6D). Notably, OppA, another known SecB substrate, was also unambiguously targeted to SecB with no association with GroEL (Figure S6C). TolB, the SRP substrate was targeted to none of the chaperones ruling out nonspecific interaction of folding intermediates with either of the chaperones (Figure 6D). Interestingly, when the CD signal of the refolding intermediates are plotted against the blue shift in ANS upon binding to the intermediates, the structural features of GroEL-binding and SecB-binding substrates separate out clearly (Figure 6E). This suggests that the structures of refolding intermediates that bind to different chaperones may be different. This result conclusively depicts that non-native structures of substrate proteins contain sufficient information to be efficiently targeted to respective chaperones in spite of redundancies among different chaperone systems.

DISCUSSION

Barring the folding of small, single-domain proteins that have been the model systems for understanding protein folding code, majority of the complex proteins refold in vitro in a non-two state manner: they populate folding intermediates. Many of these intermediates are a manifestation of ruggedness in folding energy landscape. Until now, it is believed that amino acid sequences evolve with the sole aim of achieving and

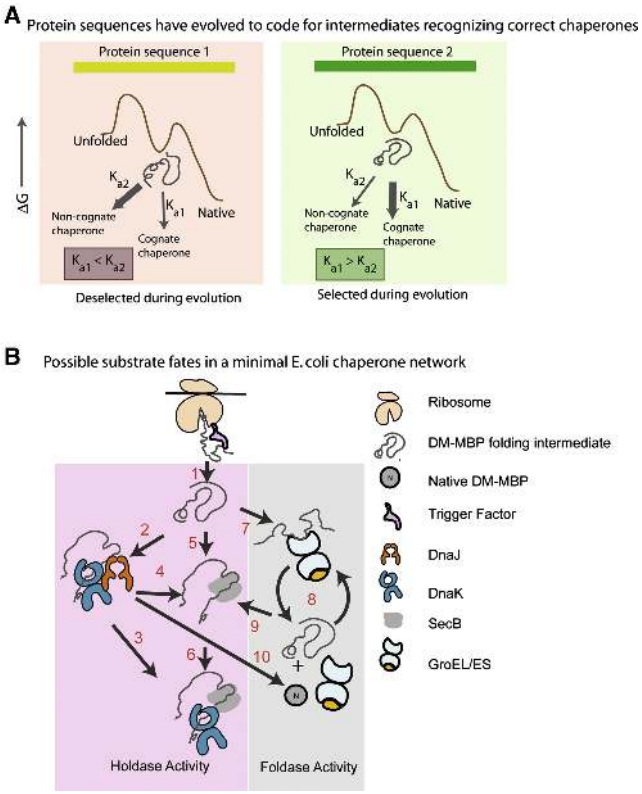

Figure 7. A Comprehensive Model of Substrate Targeting and Partitioning in the *E. coli* Chaperone Network

(A) Model showing that given two amino acid sequences that encodes the same native structure, the one that codes for a folding intermediate that binds to its cognate chaperone preferentially over other chaperones, should be selected in evolution. The sequences that lead to folding intermediates with high affinity for noncognate chaperones should lead to mistargeting and hence will be eliminated in evolution.

(B) After emergence of non-native DM-MBP from the ribosome-Trigger factor complex (step 1) it binds to either SecB (step 5) or DnaK/J (step 2) that by their holdase activity maintains the protein in an extended non-native conformation. Subsequent to DnaK/J binding, the protein may be either handed over to SecB (step 4) or bind additionally to SecB (step 3) to make a multichaperone substrate complex or reach the native state albeit with a slower refolding rate (step 10). SecB may sustain the unfolded conformation of the substrate alone or together with DnaK for subsequent SecYEG-mediated export (step 6). In presence of excess abundance of GroEL/ES system, the protein can be directly targeted to the chaperonin system (step 7) that helps majority of non-native proteins to attain native state. The molecules that require multiple rounds of binding to EL/ES (step 8) may repartition to holdase like SecB (step 9) and remains in an extended non-native conformation. Holdases and the associated reactions for the model protein are shaded in pink whereas the foldase reactions are shaded in gray.

maintaining functional native states (Worth et al., 2009). It is not known whether folding pathways may have any consequence on the evolution of protein sequences (Kim et al., 1998). Our work unravels a potential role of the evolution of rugged landscape in protein folding (summarized in Figure 7A). Non-native intermediates populated during a refolding reaction may have evolved under the constraint of maintaining conserved structural/sequence features that facilitate precise chaperone-targeting. Though the intermediates lack fully folded form, in many proteins they adopt compact molten globule conformations that contain a significant fraction of the secondary structures, and expose hydrophobic patches on the surface. These features are rich in binding information and we show that features present in the folding intermediates are sufficient for cognate-chaperone-substrate binding.

Here, we propose a model of substrate targeting (Figure 7B) within the chaperone network that is based on relative affinity of non-native folding intermediates for different chaperones. We observe that DnaK and SecB act as major holdases for the test protein DM-MBP. Although, DnaK/J binding (Step 2) is weaker than SecB (Step 5), ~25-fold higher intracellular concentration of DnaK over SecB ensures that a significant fraction of the unfolded polypeptide is channeled to the DnaK/J system that may lead to native state albeit at a slower rate (step 10). SecB may sustain the unfolded conformation of the substrate alone or together with DnaK for subsequent SecYEG-mediated export (step 6). More interestingly, partitioning of polypeptides between the holdases and foldases (between steps 2 and 7 or 5 and 7) can be rationalized by the affinity of the substrate for the different chaperone systems. In case of competition between holdases and foldases (pink and gray areas, respectively), holdases like SecB are able to effectively quench EL/ES assisted refolding by binding to substrates during EL/ES cycling event (step 8). This indicates that bona fide GroEL substrates need to minimize repartitioning to holdases (step 9) and thereby require evolution of non-native states with higher affinities for the chaperonin than for other holdases. Furthermore, substrates of SecB that are translocated to periplasmic space are generally more abundant than GroEL substrates. It is therefore crucial for cellular homeostasis that these substrates may evolve higher affinity for SecB than for GroEL, or else they may saturate EL/ES system eventually blocking folding of authentic chaperonin substrates. This is corroborated by the finding that authentic GroEL substrates belonging to type II and type III are efficiently targeted to the chaperonin whereas SecB substrates are directed to SecB in the typical cellular range of molar ratios of chaperones. We found that only large alteration of the cellular molar ratio of GroEL to SecB, by overexpressing the former allow efficient repartitioning (toward step 7 over steps 2 and 5). Thus, substrates may have evolved non-native states with affinities that allow precise targeting at the physiological concentration of different chaperones. Cross-chaperone targeting may be facilitated only at altered molar ratios of the different chaperones. This is consistent with results that show that although overexpression of certain chaperones, like GroEL or SecB is able to rescue phenotypes resulting from double deletion of DnaK and TF, their physiological concentrations are not sufficient to alleviate the phenotype (Ullers et al., 2004; Vorderwülbecke et al., 2004). Conversely, DnaK/J is able to supplement the defect of SecB deletion only upon overexpression (Wild et al., 1992).

Thus, we propose a functional role for the existence of ruggedness in the folding landscape of proteins in facilitating targeting to the right chaperone machinery. Because in vitro refolding intermediates retain the signatures of chaperone targeting, future research will allow the structural dissection of these signatures to unravel the features governing chaperone-recognition.

EXPERIMENTAL PROCEDURES

Proteins

Single and double cysteine mutants of DM-MBP were expressed and purified as described previously (Sharma et al., 2008). DnaK was purified as described previously (Mapa et al., 2010). All concentrations reported for SecB are for the tetrameric protein.

Buffers

All single molecule and ensemble experiments were carried out with buffer A (25 mM Tris-HCl, pH 7.5, 80 mM KCl, 5 mM $MgCl_2$). Proteins were denatured with buffer B (6 M Gu-HCl in buffer A). For single molecule resolution, samples were further diluted to picomolar (~50 pM) concentrations in buffer C (60 mM Gu-HCl in buffer A).

Single-Molecule FRET Experiments

All single molecule FRET experiments were performed in a confocal system based on an inverted microscope (Zeiss Axio Observer Microscope). Detailed methods of spFRET measurements are provided in Supplemental Experimental Procedures.

CD Spectroscopy of Non-Native and Native Substrate Proteins

CD spectra of non-native folding intermediates of all substrate proteins (final 1 μM) were obtained by a JASCO spectro polarimeter using a cuvette of 1 cm path length at 25°C in buffer A just after dilution (1:100) from denaturant (denatured in buffer B). CD spectra of similar concentration of native proteins were obtained in buffer A.

ANS Binding of Non-Native Folding Intermediates of Substrate Proteins

All substrate proteins were first denatured with 6 M Gu-HCl in buffer A and was subsequently diluted out of denaturant (1:100) in buffer A containing 50 μM 1-anilinonaphthalene-8-sulfonic acid (1-8 ANS). Emission spectra of hydrophobic dye 1-8 ANS was obtained in a Fluorolog-3 (Jobin Yvon) instrument after excitation at 365 nm wavelength. Emission spectra of 1-8 ANS (50 μM) in buffer C (blank) was compared to the ones bound to various non-native GroEL and SecB substrates (final 1 μM of non-native protein in 60 mM GuHCl).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.str.2012.06.014.

ACKNOWLEDGMENTS

K.M. is an IYBA Fellow of DBT India. S.M. is a Swarnajayanti Fellow of DST, India. We thank CSIR for providing the infrastructural support and DBT and CSIR for funding. We thank K. Chakraborty for generously providing the mutant DM-MBP constructs, R. Varadarajan for the SecB construct, Arnold Driessen for the anti-SecB antibody, Stefan Rudiger for sharing the DnaK binding site prediction algorithm, and F. Ulrich Hartl for GroEL/ES over expressing plasmid and DM-MBP-pCH construct. We thank NBRP-*E. coli* at NIG for providing the Keio strains. We thank K. Chakraborty, S. SinhaRoy, and S. Sengupta for critically reading the manuscript.

Received: March 20, 2012
Revised: June 20, 2012
Accepted: June 20, 2012
Published online: June 26, 2012

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