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Monitoring Protein Conformation along the Pathway of Chaperonin-Assisted Folding

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SUMMARY

The GroEL/GroES chaperonin system mediates protein folding in the bacterial cytosol. Newly synthesized proteins reach GroEL via transfer from upstream chaperones such as DnaK/DnaJ (Hsp70). Here we employed single molecule and ensemble FRET to monitor the conformational transitions of a model substrate as it proceeds along this chaperone pathway. We find that DnaK/DnaJ stabilizes the protein in collapsed states that fold exceedingly slowly. Transfer to GroEL results in unfolding, with a fraction of molecules reaching locally highly expanded conformations. ATP-induced domain movements in GroEL cause transient further unfolding and rapid mobilization of protein segments with moderate hydrophobicity, allowing partial compaction on the GroEL surface. The more hydrophobic regions are released upon subsequent protein encapsulation in the central GroEL cavity by GroES, completing compaction and allowing rapid folding. Segmental chain release and compaction may be important in avoiding misfolding by proteins that fail to fold efficiently through spontaneous hydrophobic collapse.

INTRODUCTION

Many newly synthesized polypeptides require assistance by molecular chaperones in order to reach their folded states efficiently and at a biologically relevant timescale (Frydman, 2001; Hartl and Hayer-Hartl, 2002). A subset of cytosolic proteins in bacteria strictly depend on the GroEL-GroES chaperonin system for folding (Kerner et al., 2005). GroEL acts posttranslationally and receives its substrates through transfer from upstream chaperones, such as DnaK/DnaJ (Hsp70) (Kerner et al., 2005; Langer et al., 1992), which function in shielding hydrophobic regions exposed by newly synthesized polypeptides. The basic mechanism of GroEL and its cofactor GroES involves encapsulation of a single molecule of nonnative protein in a cage-like structure, thereby allowing folding to occur unimpaired by aggregation (Mayhew et al., 1996; Weissman et al., 1996). Additionally, the environment of this nanocage appears to modulate the energy landscape and the trajectories along which folding proceeds, resulting in accelerated folding for some proteins (Brinker et al., 2001; Tang et al., 2006). Understanding the mechanisms underlying this process of in vivo folding requires information about the conformations populated by protein substrates along the chaperone pathway.

The bacterial chaperonin system has been the subject of extensive structural and functional analysis (summarized in Saibil and Ranson, 2002; Fenton and Horwich, 2003). GroEL is an ~800 kDa cylindrical complex with ATPase activity consisting of two heptameric rings of 57 kDa subunits. Each ring encloses a central cavity exposing hydrophobic surfaces for the binding of nonnative protein. GroES, a heptameric ring of ~10 kDa subunits, caps the ends of the GroEL cylinder. This step is dependent on ATP binding to the interacting GroEL ring (the *cis* ring) and results in the displacement of bound protein into an enclosed cage, large enough for proteins up to ~60 kDa. Upon binding of ATP and GroES, GroEL undergoes allosterically regulated rigid-body movements that result in the transition of the cavity environment from hydrophobic to hydrophilic, allowing folding of the enclosed protein. Substrate proteins remain encapsulated for ~10 s, the time needed for hydrolysis of the 7 ATP in the *cis* GroEL ring. Following hydrolysis, ATP binding to the *trans* GroEL ring causes the dissociation of GroES and release of the protein substrate. Incompletely folded protein is rapidly recaptured by an open GroEL ring for another folding attempt. How the conformation of bound substrate is affected by rigid-body movements of GroEL and whether such effects

contribute to productive folding is not yet understood. Based on ensemble measurements, GroEL-bound proteins are largely disordered and devoid of stable tertiary interactions (Chen et al., 2001; Hayer-Hartl et al., 1994; Horst et al., 2005; Robinson et al., 1994; Zahn et al., 1996). GroES binding may cause further unfolding of substrate protein, and iterative steps of unfolding during GroEL-GroES cycling have been proposed to remove kinetic folding traps (Shtilerman et al., 1999).

We performed single-molecule fluorescence resonance energy transfer (FRET) with pulsed interleaved excitation (PIE) (Kapanidis et al., 2004; Muller et al., 2005) to probe the conformational properties of a substrate protein along the chaperone pathway. As shown recently, single-molecule FRET has the potential to provide unanticipated structural information regarding the composition of conformational ensembles (Coban et al., 2006; Kapanidis et al., 2004; Schuler et al., 2002; Tezuka-Kawakami et al., 2006). A slow-folding double mutant form of maltose binding protein (DM-MBP) expressed in the cytosol was chosen for these experiments, because this protein was previously shown to experience an ~10-fold rate acceleration of folding by GroEL/GroES (Tang et al., 2006). Moreover, like authentic GroEL substrates, folding of DM-MBP is largely inhibited by the Hsp70-system (DnaK, DnaJ, GrpE), and rapid folding occurs only upon protein transfer to GroEL/GroES. Our single-molecule analysis reveals that GroEL unfolds DM-MBP from collapsed Hsp70-bound states and stabilizes the protein in a dynamic ensemble of locally expanded conformations. ATP-induced domain movements in GroEL cause transient further unfolding and rapid mobilization of protein segments with moderate hydrophobicity. The more hydrophobic regions are released only upon subsequent protein encapsulation by GroES, completing compaction and folding to the native state. Segmental chain release and compaction may be important for some proteins in avoiding the formation of kinetically trapped intermediates during folding in vivo.

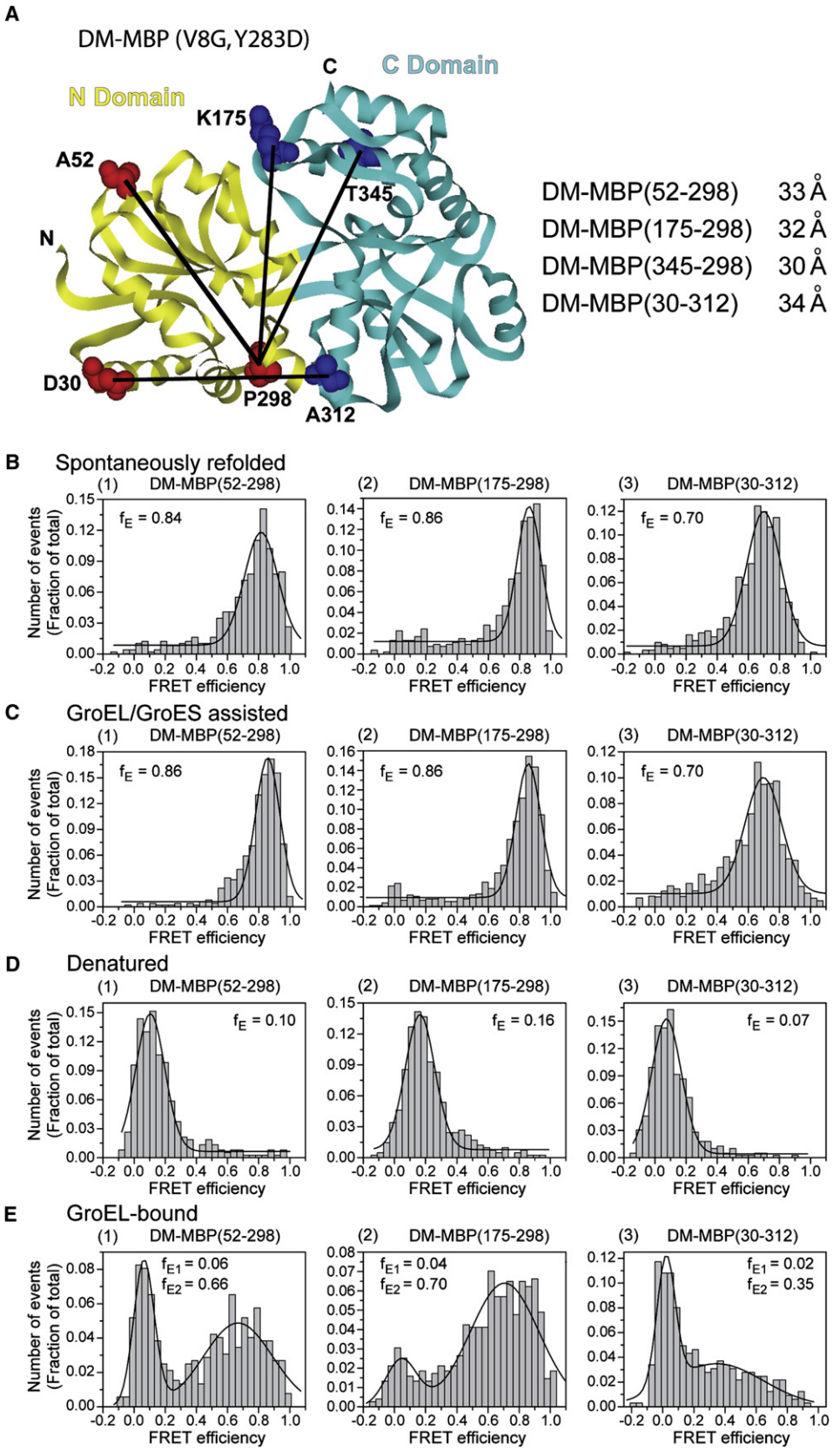
RESULTS

Conformational Distribution of GroEL-Bound Protein

DM-MBP (41 kDa) is a double mutant of the maltose-binding protein containing mutations V8G and Y283D in the N-terminal domain. Spontaneous folding of this protein is slow ($t_{1/2}$ of ~20 min) but is accelerated by chaperonin (Tang et al., 2006). To obtain insight into the conformational states populated during chaperonin-assisted folding, single-pair cysteine mutants of DM-MBP (52-298, 175-298, and 30-312) were labeled with Atto532 as the donor and Atto647N as the acceptor dye for single-molecule and ensemble FRET experiments (Figure 1A). This dye pair is appropriate for distance estimates in the range of 30–80 Å (R_0 ~46 Å). Position 298 was labeled specifically with acceptor, taking advantage of its differential solvent accessibility in the presence and absence of maltose (see Supplemental Experimental Procedures). Dye modification did not affect the folding rates of the cysteine mutants or the ability of the refolded proteins to bind maltose and folding was typically accelerated ~6-fold by GroEL/GroES (Table S1). Single-molecule distributions of FRET efficiencies (f_E) were determined by confocal fluorescence spectroscopy in solution. Single-pair (sp)FRET measurements combined with pulsed interleaved excitation (PIE) (Kapanidis et al., 2004; Muller et al., 2005) allow for the selective analysis of donor-acceptor double-labeled molecules by exciting the donor and acceptor dye alternately on a timescale much faster than the diffusion of the particles through the probe volume. Only molecules that have both an active donor and acceptor are included in the FRET analysis. Hence, there is no peak in the f_E histogram due to a donor-only species, and quantitative measurements can be performed even at low FRET efficiencies. The f_E distributions obtained after spontaneous and GroEL/GroES-assisted refolding were very similar in peak values and widths (Figures 1B and 1C; Table S2) and were generally in good agreement with the intramolecular distances calculated from the MBP crystal structure (Spurlino et al., 1991) (Figure 1A; Table S3). Thus, the native states produced in both reactions are indistinguishable for the constructs measured here.

Chemically denatured DM-MBP exhibited low f_E values for all cysteine pairs measured with calculated donor-acceptor distances 1.5- to 2-fold expanded relative to the native state (Figure 1D; Table S3). In contrast, bimodal f_E distributions were observed for the protein bound to GroEL upon dilution from denaturant (Figure 1E). For the distance between residues 52 and 298 (Figure 1E [1]), a broad distribution of molecules (~60% of the total population) centered around a high f_E of ~0.65, considerably more compact than the denatured state (Figures 1B and 1C), whereas another population of molecules (~40%) exhibited a narrow distribution with an f_E of 0.06, roughly similar to the dimensions of the denatured protein in solution (Figure 1D; Table S3). The f_E values cannot be directly compared as the conformation of the denatured polypeptide chain in solution changes dynamically, yielding a different average f_E as when the conformation of the unfolded protein is static (Schuler et al., 2005). Qualitatively similar results were obtained for the distance vector from residue 30–312 (Figure 1E [3]), except that in this case a greater fraction of GroEL-bound molecules (~65% of the total population) exists in the expanded conformation. DM-MBP (175-298) (Figure 1E [2]), on the other hand, exhibited a very broad f_E distribution when bound to GroEL, with only a small fraction of molecules being in the expanded state. Based on these results, the GroEL-bound protein populates a broad distribution of nonnative states including locally highly expanded and more compact conformations. The observation of two distinct peaks in the f_E histograms indicates that an exchange between these two distributions has to be slower than the diffusion of the molecules through the probe volume (~10 ms). A peak width analysis of the spFRET histograms (Coban et al., 2006; Merchant et al., 2007) indicates that both f_E peaks are generally wider than expected due to shot-noise broadening (Table S2). The large heterogeneous width of the high f_E state suggests that this population consists of a broad distribution of conformations. Interestingly, the proportion of molecules in the highly expanded conformation was reduced when DM-MBP was bound to the single-ring mutant of GroEL, SR-EL (Figure S1), which has a lower affinity for unfolded DM-MBP than wild-type GroEL (Figure S2A). Thus, the expanded conformation may result from higher-affinity multivalent binding to GroEL apical domains.

Several control experiments confirmed that the bimodal f_E distribution of the GroEL-bound protein represented conformational heterogeneity: Based on the diffusion rates of DM-MBP:


A
DM-MBP (V8G,Y283D)
C
C Domain
N Domain
K175
A52
T345
N
D30
P298
A312
DM-MBP(52-298) 33 Å
DM-MBP(175-298) 32 Å
DM-MBP(345-298) 30 Å
DM-MBP(30-312) 34 Å
B
Spontaneously refolded
C
GroEL/GroES assisted
D
Denatured
E
GroEL-bound
(1) DM-MBP(52-298)
(2) DM-MBP(175-298)
(3) DM-MBP(30-312)
Number of events (Fraction of total)
FRET efficiency
f_E = 0.84
f_E = 0.86
f_E = 0.70
f_E = 0.86
f_E = 0.86
f_E = 0.70
f_E = 0.10
f_E = 0.16
f_E = 0.07
f_{E1} = 0.06
f_{E2} = 0.66
f_{E1} = 0.04
f_{E2} = 0.70
f_{E1} = 0.02
f_{E2} = 0.35

chaperonin complexes and their equilibrium dissociation constants, only chaperonin-bound protein was analyzed in single-molecule measurements (Figures S2A and S2B; Supplemental Experimental Procedures). PIE in conjunction with fluorescence crosscorrelation spectroscopy (FCCS) (Muller et al., 2005) excluded the presence of GroEL complexes with two substrate molecules bound (Figure S2C). Time-resolved anisotropy decay measurements revealed considerable flexibility of the dye molecules in GroEL-bound DM-MBP (Table S4), suggesting that the observed distributions are not due to biased fluorophore orientation. To further exclude photophysical artifacts due to the environment of GroEL, a 10-residue polyproline ruler was C-terminally attached to DM-MBP, containing donor and acceptor fluorophores at the beginning and at the end of the polyproline sequence. The f_E values measured with this construct were in agreement with the expected distance (Schuler et al., 2005) and were largely unaffected by GroEL binding or enclosure of the protein inside chaperonin (Figure S3).

Unfolding upon GroEL Binding

Stopped-flow ensemble FRET measurements with three distance vectors demonstrated that upon dilution from denaturant the protein collapses from an expanded state (f_E of 0.1–0.15) to a highly compact state (f_E of ~0.71) within milliseconds, i.e., within the dead time of the instrument (Figure 2A). In the presence of GroEL, a rapid expansion of this collapsed state occurred with kinetics similar to GroEL binding ($t_{1/2}$ of ~100 ms), as shown with DM-MBP(52-298) (Figure 2A). Single-molecule FRET analysis confirmed that the protein assumes a uniformly compact conformation within the first 200 s of spontaneous refolding with a peak f_E of 0.84 and an averaged f_E value of 0.73 (Figure 2B). GroEL addition caused a reversion to a bimodal conformational distribution (Figure 2B [2]) similar to that observed upon initial binding (Figure 1E [1]). Thus, upon GroEL binding the slow-folding compact intermediates of DM-MBP unfold and adopt a heterogeneous conformational distribution with a fraction of molecules populating locally expanded states. Note that for technical reasons the precise f_E values from ensemble measurements may differ from the averaged values of single molecule f_E distributions (see Experimental Procedures). However, the changes in average f_E values calculated from the histograms follow those observed in ensemble measurements.

To evaluate the possible biological relevance of substrate unfolding upon GroEL binding, we analyzed the conformational properties of DM-MBP when bound to the Hsp70 chaperone system (DnaK/DnaJ) that acts upstream of GroEL. Denatured DM-MBP bound efficiently to DnaK/DnaJ in the presence of ATP, resulting in inhibition of folding (Figure S4) (Tang et al., 2006). Interestingly, ensemble FRET measurements with DM-MBP (52-298) and DM-MBP (175-298) showed that the DnaK/DnaJ-bound protein was similar in compactness to the spontaneously collapsed intermediate (f_E of ~0.7) (Figure 2C). Single-molecule analysis revealed a unimodal distribution broader than that of the spontaneously collapsed state (data not shown). Addition of the nucleotide exchange factor GrpE, which catalyzes substrate cycling on DnaK, failed to accelerate folding (Figure S4), suggesting that the nonnative states stabilized by DnaK/DnaJ are kinetically trapped. Notably, GrpE-mediated transfer to GroEL(D87K) (ATP-binding-deficient mutant; Farr et al., 1997) resulted in a conformational expansion of DM-MBP as observed upon initial binding to GroEL from denaturant (Figure 2C). This unfolding step may be important in preparing the protein for subsequent accelerated folding (Figure S4).

Segmental Protein Release from GroEL

ATP binding precedes the binding of GroES in the GroEL reaction cycle and causes an anticlockwise twist of the apical GroEL domains, lowering the affinity for unfolded substrate (Saibil and Ranson, 2002). Interestingly, steady-state single-molecule analysis showed that addition of ATP caused the disappearance of molecules that were highly expanded along the distance between residues 52-298 and 175-298 (Figure 3A [1]; data not shown), reflecting a conformational compaction of DM-MBP. ATP addition resulted in a decrease in the affinity of GroEL for DM-MBP from ~50 nM to ~150 nM. Note, however, that DM-MBP remains GroEL bound in the presence of the large excess of GroEL used in single-molecule experiments (Figure S2A). Mutant forms of GroEL defective in ATP binding (GroEL (D87K) or hydrolysis (GroEL (D398A)) (Rye et al., 1997) demonstrated that the shift in conformational distribution of DM-MBP was caused by nucleotide binding, not hydrolysis (Figure S5). Remarkably, the conformational change was completely reversible upon removal of bound nucleotide by addition of the Mg-chelator EDTA (Figure 3A [2]), demonstrating that expanded and more compact nonnative states are interconvertible dependent on the affinity state of GroEL.

To obtain insight into how GroEL binding affects local protein mobility, we performed steady-state anisotropy measurements with the dye Atto532 attached to single-cysteine residues positioned throughout the DM-MBP sequence. Since the lifetime of Atto532 is 4 ns or less, the steady-state anisotropy of Atto532-labeled DM-MBP correlates with the restriction of segmental mobility resulting from binding to GroEL, which has a rotational correlation time orders of magnitude greater than the lifetime of the dye (Lin and Rye, 2004). Interestingly, segmental mobility differed substantially for different regions of the GroEL-bound protein (Figure 3B). A flexible N-terminal segment (approximately residues 1–40) is followed by a highly immobile region (approximately residues 75–200), comprising parts of the discontiguous N and C domains. Notably, this segment contains the most hydrophobic regions of DM-MBP, presumably mediating strong GroEL binding. In contrast,

Figure 1. Single-Molecule FRET Analysis of DM-MBP in Spontaneous and Chaperonin-Assisted Folding

(A) Ribbon diagram of the structure of MBP (Spurlino et al., 1991) (pdb 1OMP) with the N-terminal domain shown in yellow and the C-terminal domain in blue. The positions of engineered cysteines are indicated in red (N domain) and blue (C domain).

(B–E) Single-molecule FRET measurements of double-labeled DM-MBP(52-298) (1), DM-MBP (175-298) (2) and DM-MBP (30-312) (3). GuHCl-denatured double-labeled DM-MBP (3 nM) was diluted 50-fold (60 pM final concentration) either into buffer A alone (B) or into buffer A containing 3 μM GroEL/6 μM GroES/2 mM ATP (C) or 3 M GuHCl (D) or 3 μM GroEL alone (E). Peak values of a Gaussian fit to the FRET efficiency distributions (f_E) are indicated. Representative histograms of at least two independent measurements are shown.

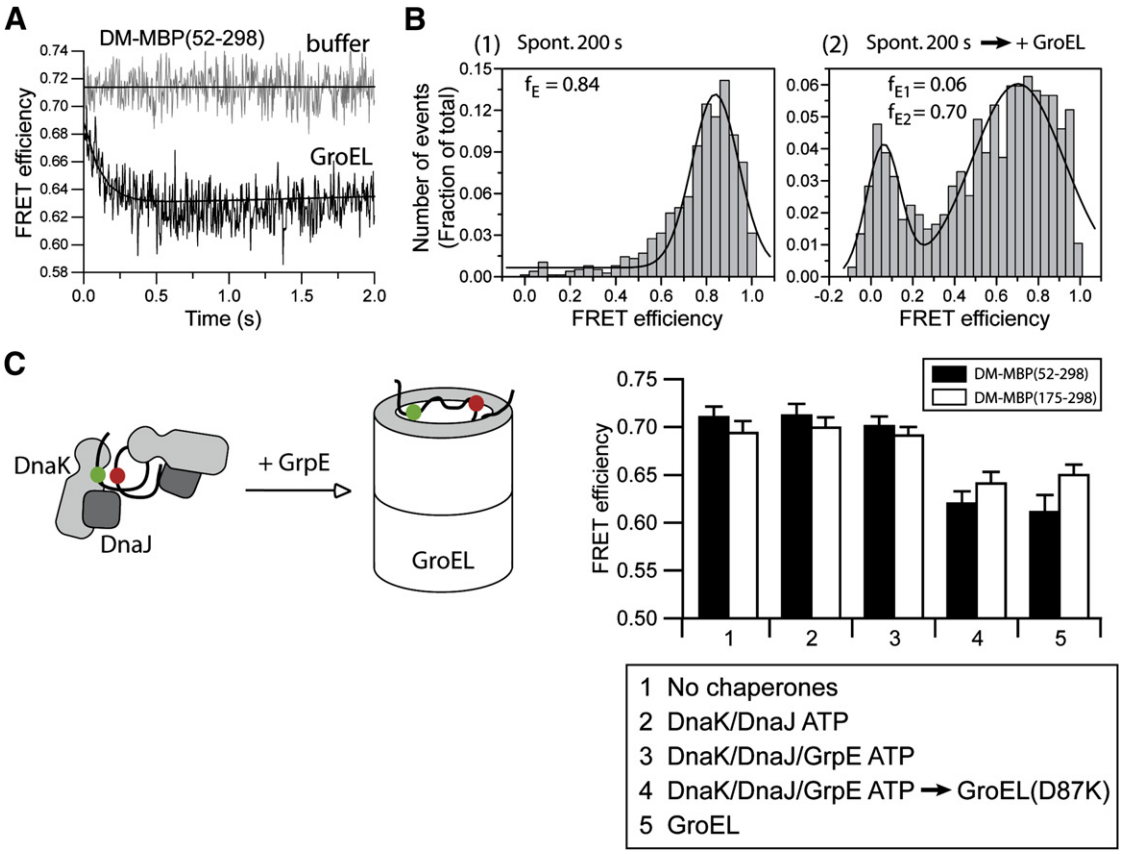


Figure 2. Unfolding upon Protein Binding to GroEL

(A) Denatured, double-labeled DM-MBP (52-298) was 25-fold diluted from 1.5 M GuHCl into buffer A containing GroEL (1 μM) or buffer A alone and the time-dependent change in ensemble FRET efficiency upon stopped-flow mixing was monitored. FRET efficiencies were obtained from identical experiments performed with donor-only-labeled and donor-acceptor-labeled proteins as described in Experimental Procedures. Kinetic traces are averages of 10–12 independent measurements.

(B) Denatured, double-labeled DM-MBP (52-298) was diluted into buffer A as in Figure 1, and single-molecule FRET efficiency distributions were obtained for the first 50 to 250 s of refolding (1). GroEL (3 μM) was added to the refolding reaction after 200 s to obtain the conformational distribution of the GroEL-bound protein (2). Peak values of a Gaussian fit to the FRET efficiency distributions (f_E) are indicated.

(C) Steady-state ensemble FRET measurements with double-labeled DM-MBP (52-298) and DM-MBP (175-298) to compare the conformations of free and chaperone-bound folding intermediates. The cartoon shows a schematic representation of unfolding upon protein transfer from DnaK/DnaJ to GroEL. DM-MBP (5 μM) was diluted 50-fold from 3 M GuHCl into buffer A alone (1), or into buffer containing DnaK (1.25 μM)/DnaJ (0.63 μM)/ATP (5 mM) (2), or DnaK (1.25 μM)/DnaJ (0.63 μM)/ATP (5 mM)/GrpE (1.25 μM) (3), or DnaK (1.25 μM)/DnaJ (0.63 μM)/ATP (5 mM)/GrpE (1.25 μM), after 3 min addition of 3 μM GroEL (D87K) (4), or 3 μM GroEL alone (5). Standard deviations of at least three independent experiments are shown.

residues 260–310, again belonging to the N domain, are more mobile, followed by a mobility-restricted C-domain segment. Addition of ATP had only little effect on the mobility of the stably bound hydrophobic regions (residues 75–200), but markedly increased the mobility of the region between residues 250 and 370, containing the less hydrophobic segments of the bound protein (Figure 3B).

Stopped-flow mixing experiments with DM-MBP labeled at positions 345 or 362 showed that this segmental release occurred rapidly within 50 ms upon ATP addition and was not accelerated by GroES (Figure 3C; data not shown). In contrast, positions 141 or 202 of the highly immobile hydrophobic segment were only mobilized upon addition of ATP and GroES (Figure 3D; data not shown). Significantly, this release was slower than the mobilization of the C-domain segment (Figures 3C and 3D) and occurred with similar kinetics as GroES binding, which was monitored by FRET between GroES and GroEL (Rye et al., 1999) (Figure 3E). Thus, protein collapse upon ATP binding and encapsulation by GroES follows a sequential mechanism, in which less hydrophobic regions are mobilized prior to more hydrophobic ones. In contrast, the observed compaction of DM-MBP at a millisecond timescale upon dilution from denaturant is consistent with an indiscriminate global collapse (Figure 2A; data not shown). Sequential compaction may therefore contribute to avoiding the formation of kinetically trapped intermediates during chaperonin-assisted folding.

Transient Substrate Expansion Accompanies Segmental Mobilization

Following the ATP-induced anticlockwise twist, the apical GroEL domains swing upwards by 60° and undergo a 120° clockwise

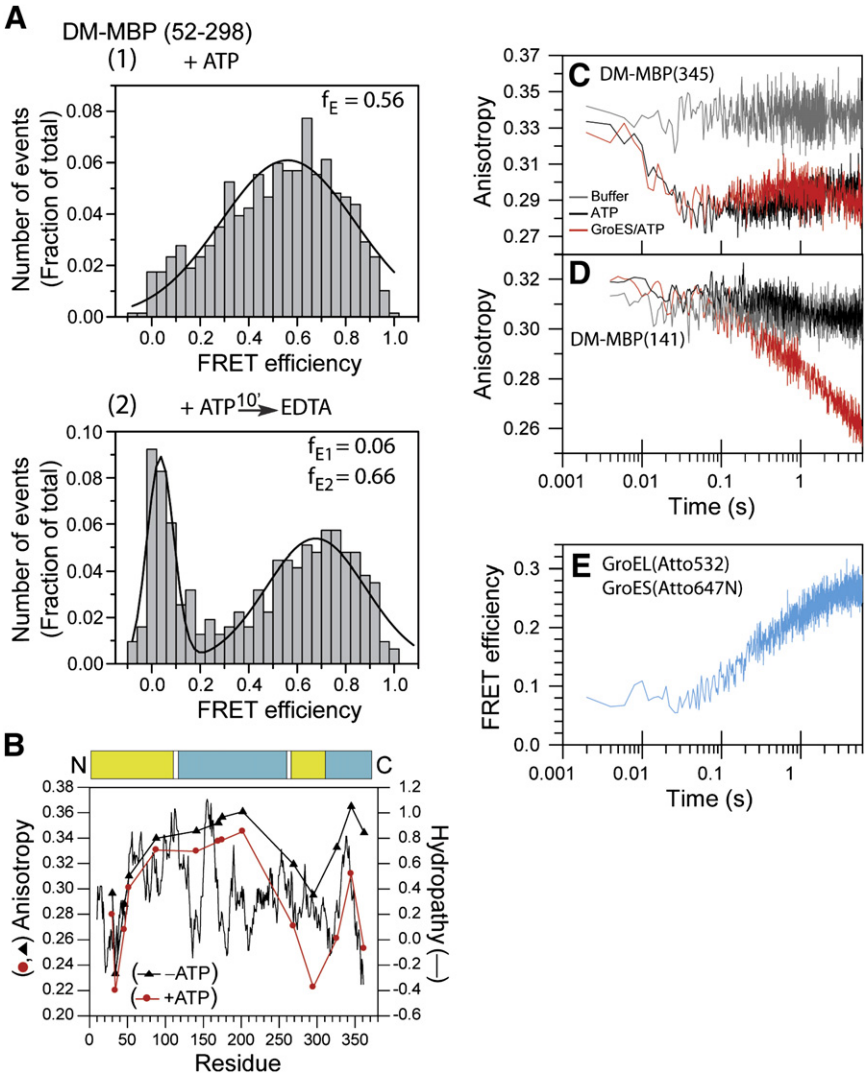


Figure 3. Sequential Release of Protein Segments from GroEL

(A) Single-molecule FRET efficiencies were measured as in Figure 1 for GroEL-bound, double-labeled DM-MBP (52-298) upon addition of 2 mM ATP (1). In reaction (2), 10 mM EDTA was added 10 min after ATP. Peak values of a Gaussian fit to the FRET efficiency distributions (f_E) are indicated. Representative histograms of at least two independent measurements are shown.

(B) Steady-state anisotropy of GroEL-bound single cysteine mutants of DM-MBP labeled with Atto532 at the positions indicated was measured either in the absence of ATP or addition of ATP to the GroEL:DM-MBP complexes. The hydropathy index of DM-MBP (Abraham and Leo, 1987), calculated with a sequence window of 19 residues is shown as a solid black line. The domain demarcation of the protein is displayed as a horizontal bar with yellow representing segments of the N domain and blue of the C domain.

(C and D) Kinetics of release of DM-MBP segments from SR-EL were measured by anisotropy upon stopped-flow mixing of SR-EL:DM-MBP complexes (1 μM SR-EL/0.1 μM DM-MBP) with an equal volume of buffer, 1 mM ATP, or ATP/GroES (1 mM/2 μM). DM-MBP labeled at position 345 (C) and 141 (D) was analyzed.

(E) To obtain the binding kinetics of GroES to GroEL under these conditions, FRET was monitored between Atto532-labeled GroEL E315C and Atto647N labeled GroES 98C by mixing equal volumes of 1 μM GroEL bound to unfolded DM-MBP and 1 mM ATP/2 μM GroES.

rotation upon GroES binding (Saibil and Ranson, 2002). These dramatic rigid-body movements have been suggested to exert a stretching force on GroEL-bound substrate, possibly resulting in unfolding of kinetically trapped states (Shtilerman et al., 1999). To achieve the time resolution necessary to address this issue, we performed ensemble FRET measurements upon stopped-flow mixing of SR-EL:DM-MBP complexes with ATP or ATP/GroES. SR-EL was chosen for these experiments to avoid the complication that GroES may bind to the GroEL ring opposite to bound substrate. Interestingly, addition of ATP alone, but not ADP, resulted in a very rapid expansion of the SR-EL-bound protein ($t_{1/2}$ of ~15 ms) as observed with DM-MBP (52-298) and DM-MBP (30-312) (Figure 4A; data not shown). This effect was transient and was therefore not detected in the steady-state, single-molecule measurements. The rapid expansion was followed by a slow compaction ($t_{1/2}$ of ~10 s), returning the protein to the original f_E value of ~0.71 (data not shown; Figure 4B). This end state corresponds to the broadened conformational distribution of GroEL-bound DM-MBP upon ATP addition observed in the steady-state, single-molecule measurements (Figure 3A). ATP-dependent stretching was also observed with GroEL, albeit with a lower amplitude (data not shown). This suggests that the more compact substrate conformation is mainly affected by the transient stretching as this population is predominant in the complex with SR-EL. Furthermore, expansion of the extended conformation would contribute little to a change in the averaged FRET efficiency. Notably, when SR-EL:DM-MBP complexes were preincubated with ATP, addition of GroES caused no expansion but instead a compaction of the bound protein, concomitantly with its displacement into the GroEL cavity (Figure 4B). On the other hand, when ATP was added together with GroES, transient expansion of the protein ($t_{1/2}$ of ~15 ms) was followed by rapid compaction ($t_{1/2}$ of ~110 ms) (Figure 4B). Interestingly, no ATP-dependent expansion of DM-MBP was observed along the distance vector between residues 345 and 298 (Figure 4C). Instead, ATP addition caused a partial compaction ($t_{1/2}$ of ~15 ms), which was enhanced in the presence of GroES (Figure 4C), consistent with the finding that the C-terminal region around residue 298 and 345 is rapidly released upon ATP addition (see Figure 3C). Thus, ATP-mediated apical domain

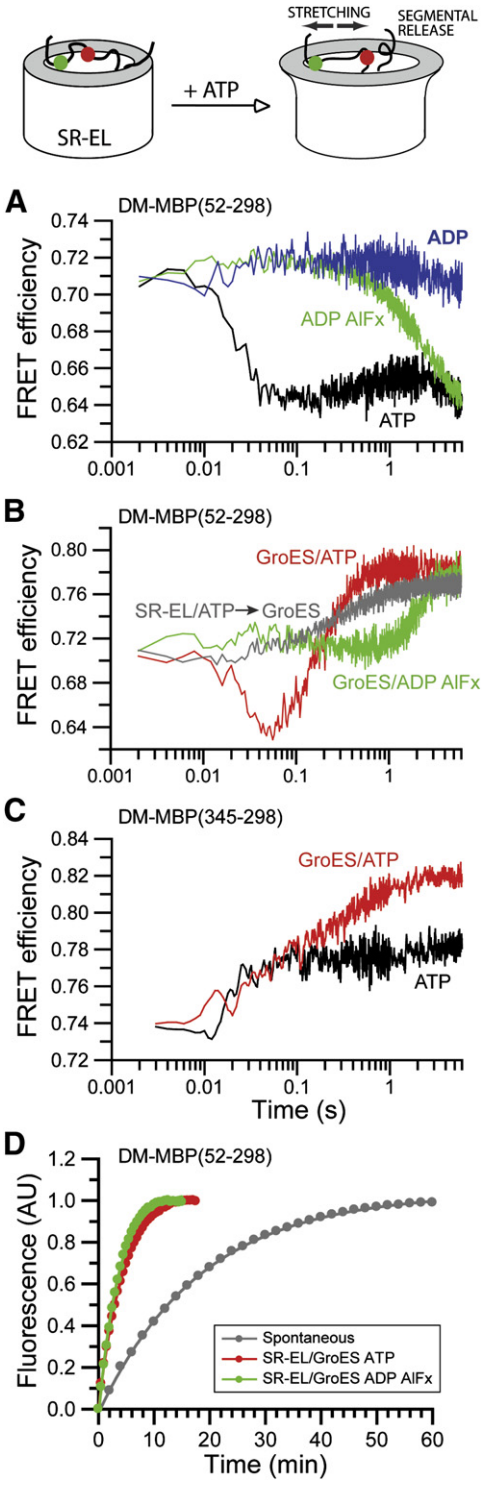


Figure 4. Local, ATP-Dependent Unfolding by GroEL

(A–C) Stopped-flow ensemble FRET measurements were performed to monitor the conformation changes of SR-EL-bound DM-MBP upon binding of nucleotide and GroES. FRET efficiencies were obtained as described in Figure 2A. The cartoon shows polypeptide expansion and segmental release resulting from the ATP-induced movement of the apical domains of SR-EL. In (A), 100 nM DM-MBP (52-298) bound to SR-EL was prepared by 50-fold dilution of GuHCl-denatured protein (5 μM) into buffer A containing SR-EL (1 μM). This was mixed with an equal volume of 1 mM ADP, ATP, or ADP-AlFx. In (B), SR-EL-bound DM-MBP (52-298) was mixed with an equal volume of ATP/GroES or ADP-AlFx/GroES. SR-EL:DM-MBP complexes that had been preincubated with ATP were mixed with GroES (2 μM). In (C), SR-EL-bound DM-MBP (345-298) upon mixing with ATP or GroES/ATP was analyzed as above. (D) Refolding rates of double-labeled DM-MBP (52-298) were measured by monitoring the change in fluorescence of Atto532 in presence or absence of maltose (see Experimental Procedures). Refolding was analyzed upon 50-fold dilution of GuHCl-denatured DM-MBP (52-298) (5 μM) into buffer A alone or into buffer containing SR-EL (1 μM) and GroES (2 μM), and refolding was initiated by addition of ATP or ADP-AlFx.

movements can cause a local expansion in strongly bound protein regions while resulting in the release and partial compaction of more weakly bound segments. Compaction is then completed without further unfolding upon displacement of the protein into the chaperonin cage by GroES.

To investigate whether the ATP-mediated local expansion of DM-MBP is a mechanistic requirement for subsequent accelerated folding, we observed that ADP-AlF$_x$, which mimics the transition state of ATP-hydrolysis, produced a similar degree of expansion of the GroEL-bound protein as ATP but with ~50-fold slower kinetics (Figure 4A). As a consequence, simultaneous addition of ADP-AlF$_x$ and GroES circumvented the transient unfolding step and resulted in delayed GroES-mediated compaction (Figure 4B). Importantly, folding within the GroES-enclosed cage of SR-EL occurred at essentially the same rate in the presence of ATP or ADP-AlF$_x$ (Figure 4D). Thus, ATP-mediated local expansion is not a requirement for subsequent rapid folding inside the chaperonin cage, at least in the case of DM-MBP.

Reversion to a More Expanded Conformation during GroEL Cycling

Since GroES binds and unbinds from GroEL every 10–15 s, folding generally occurs through multiple GroEL/GroES reaction cycles with only a fraction of molecules (~5% in case of DM-MBP), reaching the native state per cycle. Molecules that have not yet reached the native state will rapidly rebind to an open GroEL ring upon GroES release. Since DM-MBP undergoes compaction upon encapsulation (Figure 4B), rebinding possibly results in unfolding. To see how GroEL cycling affects the conformational distribution of the bound state, folding was initiated with ATP/GroES and then stopped at various time points by addition of Mg chelator with apyrase (or without apyrase when folding was to be reinitiated later in the experiment) (Brinker et al., 2001) (Figure 5A). Under these conditions, GroEL is reset to the nucleotide-free state and noncorrectly folded proteins rebind. GroEL:DM-MBP complexes were then isolated from free, native DM-MBP on an amylase column. The rebound protein adopted essentially the same f_E distribution after different times of cycling (Figure 5A, [1]–[3]). Reinitiation of cycling by Mg addition resulted in folding at the same single-exponential rate, independent of the number of chaperonin cycles the protein had previously experienced (Figure 5B).

Next, we tested whether there is sufficient time between cycles for unfolding to occur prior to renewed substrate encapsulation by GroES. Collapsed folding intermediate of DM-MBP (52-298) was mixed with the asymmetric GroEL:GroES:ADP complex (Figure 5C), the chaperonin configuration that rebinds

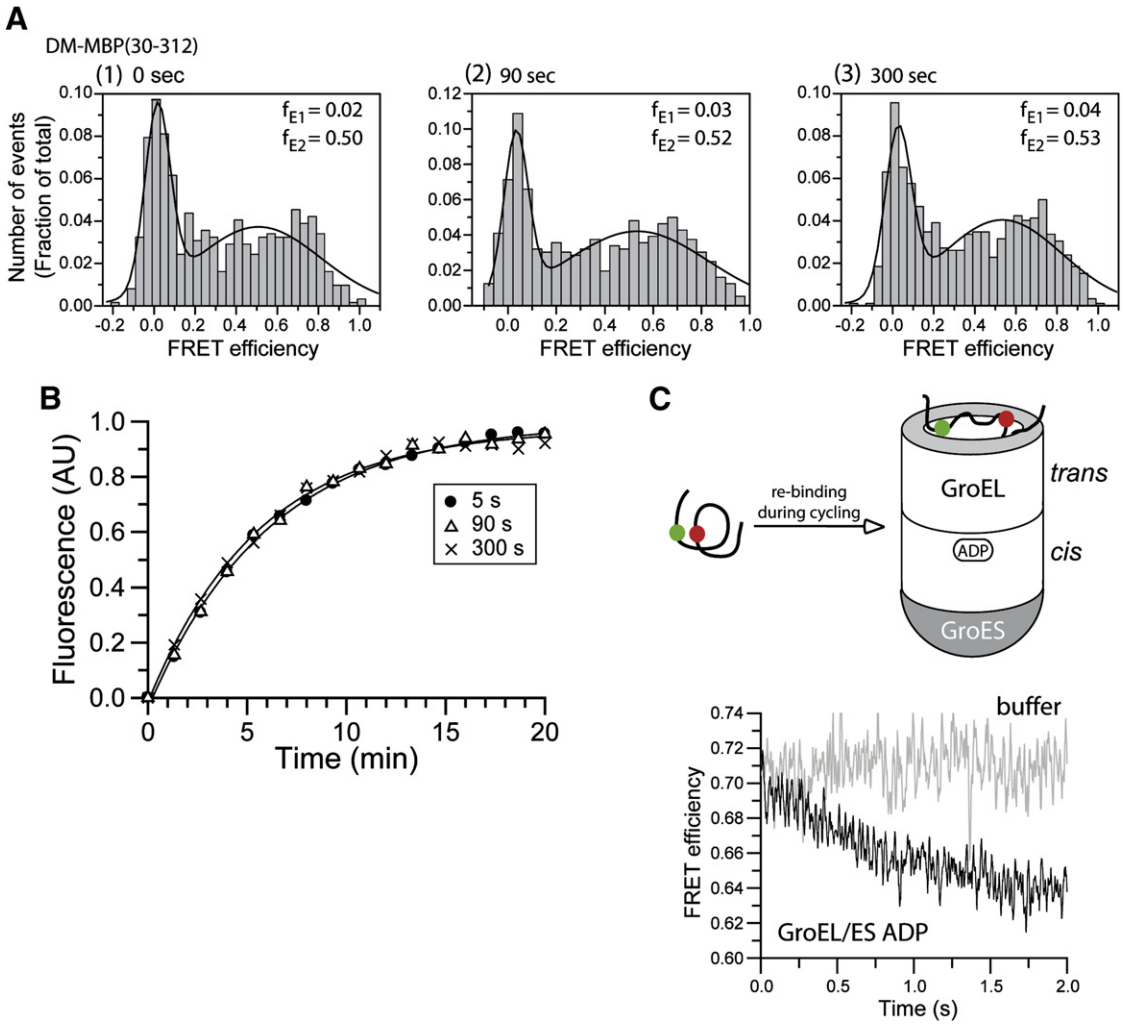


Figure 5. Unfolding between Consecutive Chaperonin Cycles

(A) Single-molecule FRET efficiencies of GroEL-bound, double-labeled DM-MBP (30-312) were measured as in Figure 1E. After initiating folding by addition of ATP/GroES, refolding reactions were terminated after 0 s (1), 90 s (2), or 300 s (3) by addition of EDTA (10 mM) and apyrase (25 units/mL) to revert GroEL to the apo-state with bound DM-MBP. Native protein produced during the course of the reaction was removed by binding to amylose resin and the GroEL-bound substrate measured by spFRET. Peak values of the Gaussian fit to the FRET efficiency distributions (f_E) are indicated. Representative histograms of at least two independent measurements are shown.

(B) Refolding rates of GroEL-bound protein (0.2 μM DM-MBP, 1 μM GroEL, 2 μM GroES, 2 mM ATP) were measured after folding was stopped with 10 mM EDTA after 5 s, 90 s, or 300 s and free protein was removed as above. Folding was reinitiated by addition of 12.5 mM $MgCl_2$ and measured by following the change in Trp fluorescence of unlabeled DM-MBP. Fluorescence intensity reached after 20 min was set to 1.

(C) To obtain the kinetics of the conformational rearrangement of DM-MBP (52-298) as it occurs upon GroEL rebinding during chaperonin cycling, preformed asymmetrical GroEL:GroES:ADP complexes were mixed with denatured DM-MBP as in Figure 2A. As a control reaction, denatured DM-MBP was mixed with buffer. Kinetic traces are averages of 10–12 independent measurements. The cartoon shows a schematic representation of the experiment.

the substrate during cycling (Hayer-Hartl et al., 1999; Rye et al., 1999). A conformational expansion was indeed observed by FRET (Figure 5C), albeit at a somewhat slower rate than unfolding upon binding to free GroEL (Figure 2A). The GroEL:GroES:ADP complex binds unfolded substrate in the *trans* GroEL-ring at the same rate as unliganded GroEL (Rye et al., 1999), but substrate binding destabilizes the GroEL-GroES interaction on the opposite ring (Martin et al., 1993). Thus, the measured rate of unfolding may appear slower due to a fraction of substrate being encapsulated by the released GroES. These data indicate that transient unfolding does occur upon substrate rebinding during successive chaperonin cycles, reverting folding intermediates to a locally more expanded conformational ensemble.

To investigate whether or not repeated unfolding events are required for accelerating folding of DM-MBP, a comparative analysis of refolding with SR-EL/GroES and GroEL/GroES was performed. The folding rates upon single-round enclosure and during cycling were indistinguishable within experimental error (Table S1) (Tang et al., 2006). This indicates that any unfolding during cycling

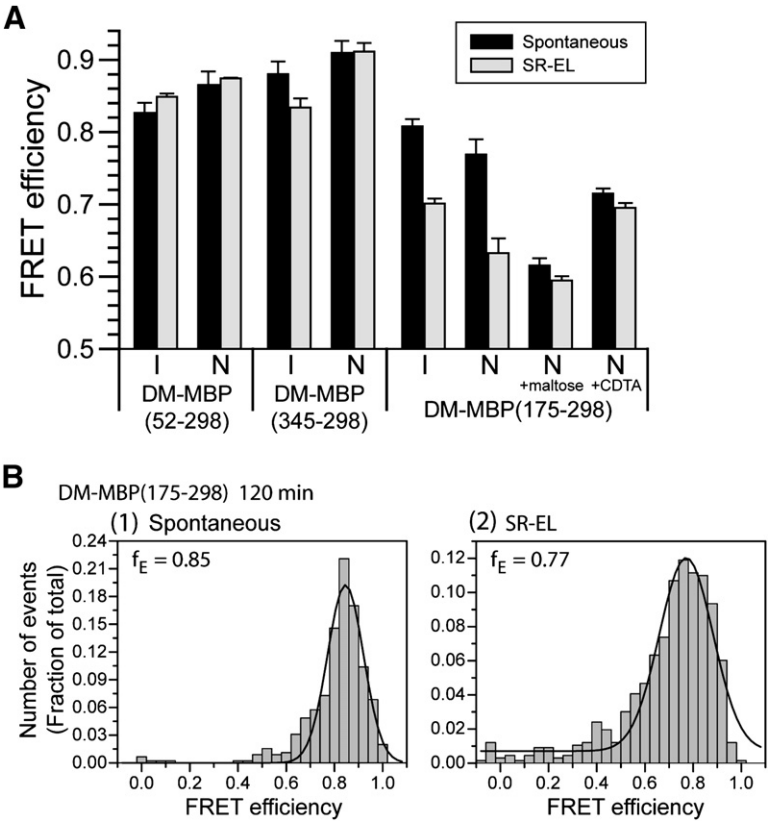


Figure 6. Folding Intermediates inside the Chaperonin Cage and in Solution

(A) Ensemble FRET measurements with double-labeled DM-MBP (52-298), DM-MBP (345-298), and DM-MBP (175-298) were performed to compare the conformational properties of folding intermediates (I) and native states (N) produced upon spontaneous folding and upon encapsulation in the cavity of SR-EL by GroES. In the case of DM-MBP (175-298), FRET efficiencies were also measured upon addition of maltose to the free and chaperonin-enclosed native states. Addition of CDTA (10 mM) results in the release of native DM-MBP from the SR-EL:GroES cage. Measurements were performed as in Figure 2C. Standard deviations of at least three independent experiments are shown.

(B) Distributions of single-molecule FRET efficiencies of DM-MBP (175-298) after 120 min of spontaneous folding (1) or encapsulation inside SR-EL:GroES (2). Chaperonin-assisted folding was initiated by addition of GroES (6 μM) and ATP (2 mM) to 60 pM DM-MBP bound to 3 μM SR-EL, and the measurements were performed as in Figure 1. Peak values of a Gaussian fit to the FRET efficiency distributions (f_E) are indicated. Representative histograms of at least two independent measurements are shown.

does not enhance the overall folding rate and yield of DM-MBP but could potentially be important for other substrates.

Substrate Conformation inside the Chaperonin Cage

Previous studies suggested that the environment of the GroEL-GroES cage critically contributes to substrate compaction and accelerated folding (Brinker et al., 2001; Lin and Rye, 2004; Tang et al., 2006). The conformational distribution of DM-MBP was analyzed upon single-round encapsulation with SR-EL/GroES to investigate the effect of enclosure within the chaperonin cage upon substrate conformation. Ensemble FRET measurements showed that overall the protein adopted a highly compact conformation within 10 s of GroES/ATP addition (Figure 6A). The f_E values of the compact folding intermediates and the native states for the distances between residues 52-298 and 345-298 were very similar upon assisted and spontaneous refolding (Figure 6A). In contrast, the distance between residues 175 and 298 in both the intermediate and the native state was more expanded when inside the chaperonin (Figure 6A). Release of the protein into free solution upon CDTA-induced GroES dissociation resulted in a compaction close to the f_E value of spontaneously folded DM-MBP. On the other hand, maltose binding caused the free native state to adopt the f_E value measured for the encapsulated protein (Figure 6A). Single-molecule measurements confirmed that the end state of folding reached in the chaperonin cavity has a slightly broader distance distribution than the free native protein (Figure 6B; Table S2). Thus, within the cage, DM-MBP adopts a state that appears to be more similar to the maltose-bound conformation, consistent with the ability of the enclosed protein to bind maltose (Table S1). Based on these findings, the space constraints of the chaperonin cavity affect the conformation of both folding intermediates and the native protein, and thus likely also the kinetic partitioning between intermediates along the folding pathway.

DISCUSSION

Chaperone-Bound Nonnative States

Initiation of folding in vitro by removal from denaturant results in the rapid compaction of DM-MBP to conformations with intramolecular distances similar to the native state. In the cell, however, proteins are synthesized on ribosomes and interact with chaperones such as Trigger factor and the Hsp70 system (DnaK/DnaJ) early during translation (Frydman, 2001; Hartl and Hayer-Hartl, 2002). These factors prevent aggregation of nascent polypeptides by shielding hydrophobic amino acid residues. Interestingly, we found that DM-MBP is stabilized by DnaK/DnaJ in collapsed, nonnative conformations (Figure 7). As observed with other GroEL-dependent substrates (Kerner et al., 2005), the DnaK/DnaJ-bound protein fails to fold upon GrpE-mediated Hsp70 cycling and thus represents a kinetically trapped state in the chaperone pathway. Notably, transfer to GroEL results in local unfolding, possibly resolving nonnative interactions and repositioning the protein to a higher point in the energy landscape (Figure 7, step 1).

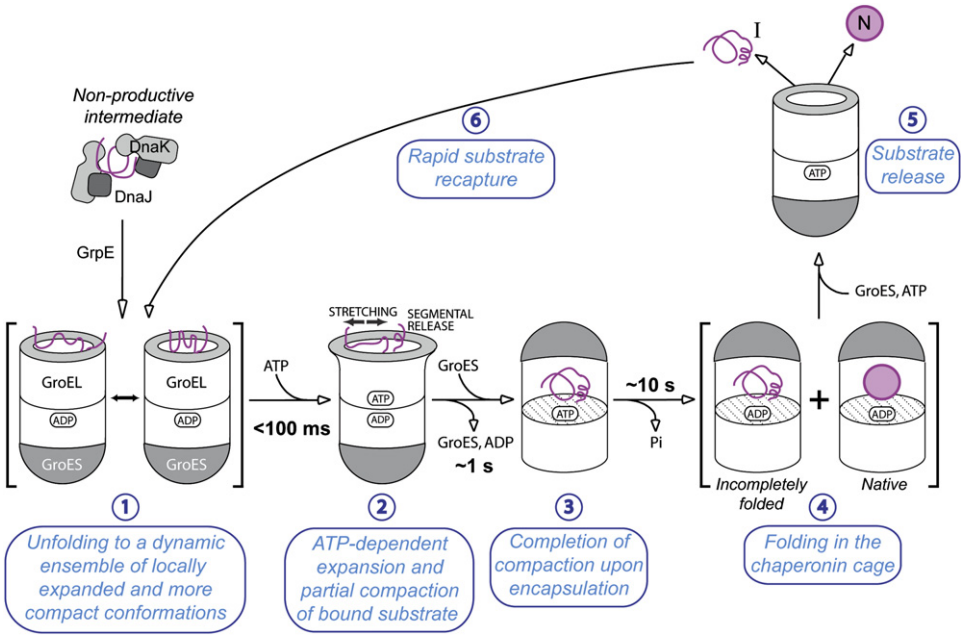


Figure 7. Folding of DM-MBP in the Chaperone Pathway

Working model summarizing the conformational changes in DM-MBP upon transfer from DnaK/DnaJ to GroEL and during GroEL/GroES-mediated folding (see Discussion for details). Note that binding of a second substrate molecule to the open ring of GroEL in steps 4 and 5 is omitted for simplicity. N, native state; I, folding intermediate.

Single-molecule FRET analysis of the GroEL-bound protein revealed bimodal or broad conformational distributions with intramolecular distances ranging from highly expanded to compact, indicating that chaperonin-assisted folding does not initiate from a homogeneous nonnative state. This heterogeneity of the GroEL-bound state could not have been predicted from previous spectroscopic ensemble measurements (Horst et al., 2005; Lin and Rye, 2004; Robinson et al., 1994; Zahn et al., 1996) but is consistent with the observation of different topologies of bound protein in GroEL complexes by cryo-electron microscopy (Elad et al., 2007). The presence of distinct bimodal distance distributions, as detected with the FRET pairs 52-298 and 30-312, suggests that any interconversion between these populations occurs on a timescale longer than 10 ms. The ratio of the expanded and more compact conformations of bound protein may reflect the allosteric equilibrium of GroEL between high-affinity and low-affinity states for substrate, consistent with their convertibility by nucleotide-dependent shifting between these states. The locally highly expanded conformations are likely due to high-affinity interactions of hydrophobic regions with multiple apical GroEL domains. Since the more compact nonnative states predominate in the complex with SR-EL, which has lower substrate affinity but assists DM-MBP folding to the same degree as GroEL, it seems plausible that the local expansion observed upon binding to GroEL is not a prerequisite for the chaperonin-assisted folding of this protein. It remains to be seen whether the remarkable capacity of GroEL to stabilize locally highly expanded states is important in the folding of authentic chaperonin substrates.

Unfolding by GroEL

Unfolding of collapsed DM-MBP intermediate by GroEL occurred with rapid kinetics at the timescale of GroEL binding ($t_{1/2}$ of ~100 ms) (Figure 7, step 1). This effect may be explained by GroEL capturing more expanded states that are present in fast equilibrium with compact intermediates (Walter et al., 1996). However, our observation of coexisting GroEL-bound states, varying in conformational expansion, suggests that local unfolding may occur as the protein interacts successively with an increasing number of apical domain-binding sites (Farr et al., 2000). It has also been suggested that the GroES-induced movement of the apical GroEL domains may exert a stretching force on the bound protein, causing forced unfolding (Shtilerman et al., 1999). We have shown here that GroEL-bound DM-MBP undergoes a conformational expansion upon addition of ATP, independent of GroES (Figure 7, step 2). This expansion occurs very rapidly on the timescale of ATP binding to GroEL and is likely caused by the ATP-dependent turning motion of the apical GroEL domains. However, the resulting unfolding is restricted locally to regions of the polypeptide that are strongly bound. More weakly interacting regions are released upon ATP-binding, initiating step-wise compaction (Figure 7, step 2). Subsequent GroES binding to the complex does not result in any further expansion, but instead in rapid completion of compaction and folding (Figure 7, steps 3 and 4). The functional significance of ATP-mediated unfolding by GroEL remains unclear. We found that under conditions where the nucleotide-induced expansion of DM-MBP is slower than GroES binding (in the presence of ADP-AlF_x), GroES-dependent folding occurs at the same accelerated rate as in the presence of ATP. This indicates that the

ATP-induced expansion is not a prerequisite for accelerated folding, at least in the case of DM-MBP.

The possible significance of unfolding upon initial GroEL binding or rebinding in consecutive chaperonin cycles must be considered separately (Figure 7, steps 1 and 6). Assuming that obligate substrates are delivered to GroEL in intermediate states that fold intrinsically slowly (Kerner et al., 2005), local unfolding upon transfer to GroEL may indeed facilitate productive folding. However, while the unfolding step upon initial binding may be important, we determined that repeated unfolding in consecutive reaction cycles does not affect the folding rate. Fully efficient folding is already achieved upon a single round of GroES-mediated encapsulation in SR-EL. Similar observations were previously made with the obligate GroEL substrate Rubisco (Brinker et al., 2001). In contrast, the iterative annealing model of chaperonin function predicts a substantial rate advantage of the cycling system, assuming that unfolding between cycles allows a fraction of molecules to partition along a faster folding track (Figure S6) (Shtilerman et al., 1999).

Folding upon Controlled Chain Collapse into a Confined Space

How then does the chaperonin system accelerate the folding of proteins such as DM-MBP? We have previously shown that this effect critically depends on the physical environment of the chaperonin cage, which appears to disfavor kinetically trapped states due to a combination of steric confinement and charge-repulsion effects from the cavity wall (Brinker et al., 2001; Tang et al., 2006). The present study revealed an additional mechanistic element that distinguishes chaperonin-assisted from spontaneous folding: chain collapse occurs in a controlled, step-wise manner during polypeptide displacement into the chaperonin cage rather than in an indiscriminate, possibly unimodal process as in spontaneous refolding (Dobson et al., 1998; Radford, 2000). By measuring segmental chain mobility, we demonstrated that regions of lower hydrophobicity are being released from GroEL in a first phase, when GroEL binds ATP, followed by the mobilization of more hydrophobic regions upon subsequent GroES binding, presumably reversing the order of spontaneous collapse (Figure 7, steps 2 and 3). This sequential release occurs within the kinetic framework of the GroEL/GroES reaction cycle. ATP-induced movements are known to reduce the available hydrophobic binding surface of the apical GroEL domains (Saibil and Ranson, 2002), explaining the release of weakly hydrophobic regions, whereas strongly hydrophobic chain segments would experience a stretching force and be released only after further conformation changes of the apical domains induced by GroES. In this mechanism, chain collapse (and folding) can already begin while a substrate protein is still in contact with GroEL, possibly opening up folding pathways that would not be available upon spontaneous hydrophobic collapse. The assisted collapse reaction may thus favor the formation of folding intermediates with a reduced propensity to engage in nonnative hydrophobic interactions.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

DM-MBP (V8G;Y283D) (Tang et al., 2006) was cloned between the NdeI and NheI sites in the pCH vector (Chang et al., 2005) and was expressed in the cytosol under the control of the T7 promoter, inducible with IPTG. Single-cysteine and double-cysteine mutants of DM-MBP were generated by site-directed mutagenesis of the DM-MBP gene. A complete list of single and double cysteine constructs of DM-MBP is provided in Supplemental Experimental Procedures.

Proteins

Chaperone proteins DnaK, DnaJ, GrpE, GroEL, GroEL(D398A), GroEL(D87K), GroEL(E315C), SR-EL, GroES, and GroES(98C), were purified as described previously (Hayer-Hartl et al., 1996; Kerner et al., 2005). Wild-type and cysteine mutants of DM-MBP were purified using an amylose affinity column (New England Biolabs). DM-MBP single-cysteine mutants were labeled with Atto532 maleimide (ATTO-TEC) and the double-cysteine mutants were labeled with Atto532 (donor) and Atto647N maleimide (acceptor; ATTO-TEC) as described in Supplemental Experimental Procedures.

Refolding Measured by Fluorescence Spectroscopy

DM-MBP and its cysteine mutants (5 μM) were denatured in buffer A (5 mM Tris, pH 7.5, 20 mM KCl, 5 mM $Mg[OAc]_2$) containing 3 M GuHCl and refolded upon 50-fold dilution into buffer A in the absence or presence of chaperones at the concentrations indicated in the figure legends. Folding reactions were performed at 25°C and monitored by intrinsic Trp fluorescence on a Fluorolog 3 fluorometer (Spex) taking advantage of the fact that GroEL and GroES lack Trp (Tang et al., 2006). As Trp fluorescence is highly quenched in the double-labeled proteins, the fraction of folded protein in the constructs (DM-MBP (52-298), DM-MBP (175-298), and DM-MBP (345-298)) was quantified by monitoring the fluorescence change (~2.5-fold) of Atto532 in the presence or absence of maltose.

Steady-State and Kinetic Ensemble FRET Measurements

Steady-state ensemble FRET measurements were performed on a Fluorolog 3 fluorometer (Spex) with Atto532 as donor and Atto647N as fluorescent acceptor at 25°C. Stopped-flow experiments were done using an Applied Photo Physics SX.18MV with a 1:1 or a 1:24 mixing ratio at 25°C. Kinetic traces shown are averages of 10–12 independent measurements (see Supplemental Experimental Procedures for details).

Single-Molecule FRET Experiments

Single-molecule spectroscopy (fluorescence correlation spectroscopy [FCS] as well as single-pair FRET [spFRET] measurements) was performed on a confocal system based on an inverted microscope (Zeiss Axiovert 200) using pulsed interleaved excitation (PIE) (see Muller et al. [2005] and Supplemental Experimental Procedures for details). The concentration of double-labeled protein in the sample was diluted to ~60 pM to ensure that the probability of having more than one particle in the probe volume at the same time is negligible (<1 %). For each experiment, at least 500 particles were measured and the experiments were repeated with different protein preparations to verify the reproducibility of the results. Details for spFRET measurements are provided in Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental data include six figures, four tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/133/1/142/DC1/.

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