DsbA is a switchable mechanical chaperone

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Abstract:

Force is a crucial protein denaturant in cells, occurring during processes including translation, degradation, and translocation. In bacteria, many toxins and virulence factors pass through a translocon pore as unfolded polypeptides en route to periplasmic and extracellular compartments. DsbA is a ubiquitous bacterial oxidoreductase that associates with substrates during and after translocation, yet its involvement in protein folding and translocation remains an open question. Here, using magnetic tweezers-based single molecule force spectroscopy, we characterize a mechanical chaperone activity of DsbA on a cysteine-free domain from the protein L super-antigen. Interaction of DsbA with unfolded protein L substrates prominently increases the fraction of time that protein L spends in its native folded state. This chaperone activity is tuned by the oxidation state of DsbA; oxidized DsbA is a strong promoter of folding, but the effect is weakened by reduction of the catalytic *CXXC* motif. We further localize the chaperone binding site of DsbA using a seven residue peptide which effectively blocks the chaperone activity. We calculated that DsbA assisted folding of proteins in the periplasm generates enough mechanical work to decrease the ATP consumption needed for periplasmic translocation by up to 33%. In turn, pharmacologic inhibition of this chaperone activity may open up a new class of anti-virulence agents.

Introduction

Force is a key physiological denaturant. For example, proteins unfold or refold under a pulling force during, protein degradation, stretching of elastic tissues, chaperone induced unfolding, or protein translocation (*1–5*). Translocation is of utmost importance in bacteria, where a large number of proteins, including virulence factors, must pass through the translocon pore in the unfolded state and properly fold into the native configuration in the periplasm (*6*, *7*). Geometric confinement in the translocon channel forces these polypeptides into an extended conformation and as such they emerge into the periplasm under an effective stretching force (*8*).

Interestingly most of these translocated extracellular proteins in bacteria contain disulphide bonds which are introduced by oxidoreductase enzymes of the Dsb-family that share a conserved thioredoxin-type fold (*9*). The prototypical family member DsbA is necessary for the maturation of a range of virulence factors including flagellar motors and pilus adhesins, Type III secretion systems, and heat-labile and heat-stable enterotoxins and has emerged as a target for novel antibiotic development (*10–13*). Notably, DsbA is an ongoing target for drug development due to its direct relevance in urinary tract infections, bacteremia, and biological weapons development (*13–16*). To date, a set of small molecules, peptide compounds have been developed in one direction that block the oxidoreductase activity of this enzyme (*17*).

There is evidence that members of the thioredoxin fold family, such as human protein disulfide isomerase (PDI) and *E. coli* DsbC, are able to act as chaperones themselves(*18*, *19*). These oxidoreductases are able to reactivate denatured enzymes that contain cysteines but lack disulfide bonds. DsbA is also known to be required for efficient periplasmic secretion of the enzyme pullulanase that has been mutated to prevent internal disulfide formation (*20*). By what mechanism is DsbA facilitating secretion of this substrate, if not through catalysis of disulfide bond formation?

The link between the chaperone binding and protein secretion has been extensively discussed, even implicated in translocation of eukaryotic peptides by chaperones BiP and HSP70 into the endoplasmic reticulum and mitochondria, respectively (*21–23*). It is believed that chaperone binding to an unfolded substrate protein on one side of the membrane can induce biased transport

of a polypeptide through a Brownian ratchet mechanism (*24–27*). This relies not on the energy of hydrolysis of ATP but on the differential concentrations of the chaperones on either side of the membrane. Simple binding of chaperones on one side of the membrane prevents backsliding of the protein into the pore, providing directional transport of the polypeptide.

There is strong evidence that DsbA is required for the secretion of disulfide containing proteins, but the understanding of DsbA interaction with peptides under force is poorly understood(*6*, *20*). Here using single molecule magnetic tweezers, we aim to mimic the stretching forces experienced by polypeptides in the translocon pore. The substrate used in these experiments is the cysteine-free globular fold of protein L which undergoes an equilibrium between the folded and unfolded states under small pulling forces (4-9 pN). We observe a signature of DsbA binding and chaperone activity on the folding dynamics of protein L despite the fact that this substrate contains no cysteine residues: The presence of soluble DsbA in the experimental buffer greatly increases the residence time of protein L in the folded state and allows the protein L domain to refold at higher forces. Moreover, we find that this chaperone activity is dependent on the redox state of the catalytic thiols of DsbA, with >15-fold higher concentrations of reduced DsbA required to achieve the same chaperone activity as the oxidized form. Addition of a short peptide, known to block DsbA oxidoreductase activity, to the reaction buffer is found to knock out DsbA chaperone activity as well, likely through competition for the hydrophobic groove surrounding the catalytic site.

These results suggest that DsbA is a mechanical foldase that assists with folding of peptides emerging from the translocon pore. DsbA drives protein folding at higher forces. Thus, we suggest translocation is not only increased by chaperone binding as suggested by the Brownian ratchet mechanism, but is further directed by the mechanical foldase activity of DsbA chaperone, which prevent backsliding of proteins into the translocon pore. We calculate that the work done by chaperone assisted protein folding on the periplasmic side of the translocon pore could be an energy source that greatly decreases the ATP consumption of the SecA translocation motor. Furthermore, our data provides the first report for redox control of chaperone affinity in the DsbA enzyme, which will inform ongoing efforts to develop novel anti-DsbA therapeutics.

Results

Single molecule measurement of protein folding by magnetic tweezers – We recently developed a single molecule assay to study chaperone activity using magnetic tweezers-based force spectroscopy that overcomes limitations of bulk studies of chaperones (*28*). Bulk studies require high salt concentrations, denaturants, or mutations to the substrate protein to study chaperone-client interactions in an environment far from *in vivo*. In these studies, denaturants such as guanidine, low pH, or high temperature necessarily perturb the structure of the chaperone as well. The single molecule magnetic tweezers has the unique advantage of applying force to unfold only the substrate protein without disturbing whatever protein species, such as chaperones, that are in solution (*28*).

Mechanical unfolding of individual proteins is achieved through attachment to microscopic probes (Fig 1B). In magnetic tweezers-based force spectroscopy, proteins are tethered at their N-terminus to a glass surface via HaloTag-Halo ligand covalent chemistry (*29*, *30*), and at their C-terminus to a paramagnetic bead via biotin-streptavidin. Positioning of a permanent magnet at fixed distances from the paramagnetic bead applies a passive force clamp that can explore a broad range of forces up to 100 pN. Further, the long-term stability of magnetic tweezers enables measurement of slow rates, with successive measurements from a single protein achieving a record of two weeks (*29*).

The B1 antibody-binding domain of protein L from *Finegoldia magna* is a model substrate, well described by bulk biophysical and single-molecule force spectroscopy techniques (*29*, *31–33*). Protein L is 62 residues in length with a simple α/β fold (*34*). Significantly, protein L lacks metal cofactors, or cysteine or proline residues, and therefore folds independent of oxidoreductase or prolyl isomerase activities. In all studies herein, we employ an octameric tandem modular protein L, flanked with N-terminal HaloTag and C-terminal biotin for tethering (Fig 1B). With application of a high mechanical force (45 pN in Fig 1A, Pulse I), the eight protein L repeats unfold as eight discrete stepwise extensions of 15 nm, providing the distinct single-molecule fingerprint of the polyprotein (Fig 1A, inset). Upon a decrease in force, the total length of the fully unfolded polypeptide collapses due to polymer entropy, followed by discrete stepwise contractions from individual protein L domain refolding (9.0 nm; Fig 1A, Pulse II). Eventually, an equilibrium is reached between the stepwise extensions of unfolding and the stepwise contractions of refolding, with an identical length for each transition (Fig 1A, Pulse II). The

equilibrium behavior is reported by the folding probability P_f, which describes the likelihood of a protein L domain to be folded at a particular force (equation, Fig 1A, see also Methods) (*28*). The equilibrium position between folding and unfolding is sharply force-dependent (Fig 2B), with the folding probability shifting from >99% at 4 pN to <1% at 12 pN. The 50% folding probability is found at 8.0 pN (*28*).

DsbA shifts the equilibrium towards folding – Having extensively characterized the force-dependent folding profile of protein L alone, we asked how DsbA might alter the energy landscape and folding dynamics of this substrate. Upon addition of 3 µM of oxidized DsbA to the magnetic tweezers experiment, a shift in the folding probability is observed (Fig 2A). At an equilibrium force of 8.9 pN, the protein L polyprotein hops between its 5th, 6th, and 7th folded state in the presence of oxidized DsbA (Fig 2A, red trace), whereas in the absence of oxidized DsbA the polyprotein hops between the fully unfolded and the 1st folded state (Fig 2A, black trace).

At the extremes of 4 pN and 12 pN, folding is unaffected, with either 100% of the substrate folded (at 4 pN) or unfolded (at 12 pN), independent of the enzyme. However, at intermediate forces, the effect of DsbA becomes more pronounced (Fig 2B). The largest shift is observed at 8.9 pN (dotted line, Fig 2B), where the protein L substrate has a 0.66 ± 0.05 probability of being folded when in the presence of oxidized DsbA, versus a 0.20 ± 0.04 probability of being folded in its absence.

The DsbA foldase activity is redox-state dependent – Within the Gram-negative periplasm, oxidized DsbA represents the active fraction that is capable of forming mixed disulfides between enzyme and substrate peptide. After transferring the disulfide bond into its substrate, DsbA is released with both cysteines of its active site *CXXC* motif reduced. We therefore asked whether the chaperone-like activities of DsbA might depend on its redox state, which is expected only if the chaperone activity originates from the hydrophobic groove encapsulating the catalytic site. We reduced DsbA with an overnight incubation in 100 µM TCEP, and repeated the magnetic tweezers-based mechanical foldase assay. Whereas 3 µM of oxidized DsbA shifts the probability of substrate folding to 0.66 at 8.9 pN, 3 µM of reduced DsbA induces no such shift in substrate folding (Fig 3A blue curve). Instead, experiments with reduced DsbA track the folding probability of the protein L substrate alone over the range of 5 – 11 pN (Fig 3A). A comparable

foldase effect with reduced DsbA requires 50 µM, a ~17-fold excess over oxidized DsbA (Fig 3B, green curve). Importantly, these experiments with reduced DsbA were performed with 100 µM of reducing agent TCEP in solution to prevent against spontaneous re-oxidation of the catalytic disulfide bond.

A peptide antagonist blocks the chaperone activities of DsbA – DsbA has emerged as an attractive therapeutic target due to its vital role in the maturation of bacterial virulence factors, including toxins, adhesins, and secretion machinery (*17*, *35*). In principle, inhibition of DsbA could lessen the severity of bacterial infections without a selective bactericidal activity that would foster resistance. DsbA proteins have multiple binding interfaces: a hydrophobic patch immediately adjacent to the catalytic *CXXC* motif, and a non-catalytic groove on the opposite face involved in protein-protein interactions (*12*, *36*). Both sites have successfully been targeted with peptide and small molecules.

For DsbA, several peptide inhibitors have been developed based off the oxidoreductase interacting partner DsbB, which binds at and oxidizes the catalytic *CXXC* via a short loop with the sequence PFATCDF(*10*). A single point mutation of the N-terminal phenylalanine to tryptophan (and substitution of the N-terminal residue to serine to improve solubility) produced the peptide PWATCDS with high affinity for the hydrophobic groove of DsbA from *Proteus mirabilis* in a co-crystal structure (Fig 4C)(*11*) . Binding of PWATCDS effectively blocks the oxidoreductace activity of DsbA. We therefore asked whether this peptide binding at the hydrophobic groove neighboring the *CXXC* motif would also inhibit the mechanical foldase activity of DsbA.

We repeated the mechanical foldase assay with protein L and either reduced or oxidized DsbA using magnetic tweezers, now in the presence of the PWATCDS peptide. With 3 µM oxidized DsbA at 8.9 pN, the folding probability of protein L is found to be 0.66, however, with the addition of the peptide at 100 µM, the folding probability shifts to 0.21, suggesting a loss of the enzyme's foldase activity (Fig 4A, pink curve). Furthermore, the inhibitory effect is evident at all forces tested (4 – 12 pN), as the folding probability of protein L in the presence of oxidized DsbA and 100 µM peptide closely tracks the folding probability of the control experiment. The residual foldase activity with 50 µM reduced DsbA is also lost upon addition of 100 µM PWATCDS peptide (Fig 4B, gold curve). A comparison at the highly sensitive equilibrium force

of 8.9 pN demonstrates the large shifts in the folding probability of protein L with or without the catalytic thiols oxidized, and in the presence or absence of the PWATCDS peptide (Fig 4D).

Discussion

Oftentimes, chaperones are initially identified and described by passive prevention of protein aggregation (*37*, *38*) or active ATP-driven acceleration of refolding (*39–41*). However, the mechanisms by which ATP independent chaperones can accelerate refolding remain unclear. A common shortcoming of bulk assays is that they have trouble distinguishing competing pathways. The single molecule assays used here, and previously used by the authors to study *E. coli* Trigger Factor(*28*), operate under approximately infinite dilution of the substrate so that aggregation pathways are avoided. This allows the influence of the chaperone on the folding pathways from the unfolded to the native state to be monitored independently from any competing reaction pathways.

Here, applying single-molecule force spectroscopy by magnetic tweezers, we have provided an empiric description and measurement of a chaperone-like behavior of *E. coli* DsbA. This periplasmic enzyme acts as a mechanical chaperone to accelerate refolding to the native state and to shift the folding probability under force. Protein L notably lacks any cysteine residues and thus allows us to probe the mechanism of DsbA chaperone activity independent from its oxidoreductase activity. We previously studied DsbA-catalyzed oxidative folding on a disulfide-containing protein using AFM-based force spectroscopy (*42*). In those studies, it was discovered that DsbA-catalyzed oxidative folding occurs ~3-fold faster than folding of the reduced protein. This acceleration in the folding rate with DsbA versus reduced substrate is likely an indirect indication of the chaperone activity of the enzyme. These studies by atomic force microscope (AFM) lacked sensitivity required to resolve folding events at low force, which we can now directly observe in the magnetic tweezers. New data using the magnetic tweezers supports the idea that for oxidoreductase enzymes involved in formation and isomerization of disulfide bonds, chaperone activity is likely necessary to drive the substrate into a near-native conformation where the cysteines are close enough to finally form a disulfide bond (Eckels et al., S*ubmitted*, 2018).

By using a substrate that lacks cysteines, it is now clear that the chaperone activity of DsbA is separable from its oxidoreductase activity. In other words, acceleration of folding can be

attributed to some interactions along the interface between DsbA and its substrate that does not involve thiol/disulfide exchange with the substrate. We have been able to localize this interface to the hydrophobic groove surrounding the *CXXC* catalytic motif by taking advantage of the reactivity of the catalytic thiols, as well as the groove affinity for its DsbB electron shuttling partner. Reducing the catalytic thiols or binding of the PWATCDS peptide both alter the interface between DsbA and the substrate protein L such that chaperone activity is weakened or abrogated. Determining if these two modifications of the DsbA enzyme alter electrostatics, hydrophobicity, or local geometry would help to elucidate the mechanism(*1*) by which DsbA recognizes and folds its protein L substrate.

Although this is the first report for DsbA, there are several other instances of chaperones with redox activated switches. Such a mechanism can be rationalized as a protective response to increased oxidative stress, which is known to alter side chain chemistry and cause an accumulation of misfolded proteins. Peroxiredoxins act as oxidoreductases in monomeric form but oligomerize into molecular chaperones under conditions of oxidative stress (*43*, *44*). S-nitrosylation, a by-product of reactive nitrogen stress, at a non-catalytic thiol of human Thioredoxin regulates its redox and anti-apoptotic activity(*45*). Of high relevance to our current findings is regulation of retrograde transport of cholera toxin from the endoplasmic reticulum to the cytosol. Tsai and colleagues demonstrated that reduced PDI binds to and unfolds the cholera toxin subunit A (*46*). When the disulfide bridge is transferred to PDI, it releases the toxin in the unfolded form, which can then escape through a molecular pore to the cytosol where it acts as a ribosyltransferase. This instance of PDI acting as an unfoldase demonstrates evidence of redox regulated chaperone activity coupled to peptide transport.

DsbA has been shown to bind to translocating polypeptides in the periplasm, both co-translationally and post-translationally (*6*). Here we propose an alternative version of chaperone biased polypeptide translocation based on our experimentally observed results that could be important for post-translationally secreted polypeptides (Fig 5). We suggest that DsbA enhanced folding of protein domains on the periplasmic mouth of the Sec pore can generate a large pulling force that transfers its strain to the polypeptide in the translocon tunnel, and to any portion still in the cytosol. Chaperone assisted folding on the periplasmic side of the membrane would then in turn decrease the energetic costs of protein translocation.

During translocation, a protein is first maintained in the unfolded state by the secB chaperone which carries the unfolded polypeptide to the SecA motor for transport of the polypeptide through the SecYEG pore using ATP hydrolysis (*47*). It has been shown for the SecA motor that one round of ATP binding and hydrolysis is responsible for the translocation of 20 amino acid residues through the translocon pore (*48*), so a single protein L domain with 60 residues participating in the fold would require 3 ATP molecules for translocation. Assuming a mechanochemical coupling efficiency of ~50% for the SecA motor (*49*), hydrolysis of 3 ATP molecules would generate 150 zJ of mechanical work (100 zJ per ATP * 50% efficiency * 3 molecules ATP)(*50*). Although there are no single molecule force spectroscopy measurements of the forces generated by the SecA motor, it is thought to operate optimally over the force range of 5 – 11 pN (*50*), stalling at the higher forces, which holds true for most protein translocating motors (*5*, *51*). This range coincides with the forces over which DsbA can assist protein folding, reaching a maximal effect at 9 pN (Fig 2B). Refolding of a single protein L domain on the periplasmic side at 9 pN with a step size of 9.5 nm potentially generates 85 zJ (9 pN * 9.5 nm) of work, although the average amount of work performed is determined by the folding probability. This work of protein folding supplies a pulling force from the periplasm that can be harnessed to lower the number of ATP consumed by SecA for translocation. Without DsbA in the periplasm, folding of protein L can only generate 17.1 zJ of energy (9 pN * 9.5 nm *0.2 folding probability - see Figure 2B). However, the presence of DsbA increases the work done by folding to 56 zJ (9 pN * 9.5 nm * 0.66 folding probability). Thus the work of protein folding, assisted by the DsbA chaperone can supply one-third of the energy needed for protein L translocation, lowering the ATP consumption to only 2 molecules per protein L polypeptide translocated. If the SecA motor has even lower efficiency, as suggested by certain studies demonstrating the requirement of 1,000 ATP molecules per proOmpA transported, then the mechanical work done by DsbA is of even greater importance (*50*).

The evidence is beginning to suggest that all chaperone assisted folding is mechanical. Trigger Factor increases the transmission force of the polypeptide through the ribosomal tunnel by assisting protein folding at higher forces (*28*, *52*). The results from the present study show how the chaperone activity of DsbA could assist the protein to translocate from the cytoplasmic end to the periplasmic end. Besides DsbA, other chaperones like HSP70 and Bip are known to assist the translocation process (*21*, *22*). It has even been demonstrated that high stability titin domains

are transported into the lumen of the mitochondria with the help of mtHSP70 (*23*). Another common chaperonin, GroEL, mechanically unfolds the misfolded proteins on its apical domain (*3*). Thus, a similar mechanical chaperone activity by all these chaperones indicates a broad mechanical role of chaperones which might be required in many biological process such as rescuing of stalled polypeptide chains in the translation process, accelerating protein folding in elastic tissues, or biasing translocation of polypeptides from one cellular compartment to another.

Our characterization of mechanical chaperone activity at the catalytic hydrophobic groove offers a new insight into ongoing antibacterial drug development efforts targeting DsbA. Several small molecules and peptides have been developed to inhibit DsbA's oxidase activity. We have found that one such peptide, PWATCDS, also blocks the DsbA mechanical foldase. As a factor involved in the maturation of a diverse set of bacterial exotoxins, adhesins, and secretion machinery (*35*), DsbA affords a single attractive target for attenuating a range of virulence phenotypes (*53*). Deletions of DsbA have been shown to alter the course of infection in animal models of *E. coli* (*13*) and *P. mirabilis* urinary tract infection (*54*) and *F. tularensis* tularemia (*15*), among others. Moreover, the loss of DsbA does not appreciably alter cell viability, lessening the selective drive for antibiotic resistance. For example, a small molecule phenylthiazole analogue developed to bind at the catalytic hydrophobic groove and inhibit DsbA's oxidative function will also reduce an *E. coli* swarming motility phenotype of DsbA-dependent flagellar assembly(*55*). However, this candidate drug does not alter growth in liquid culture. Anti-DsbA therapeutic development has largely focused on inhibiting its oxidoreductase activity localized to the catalytic hydrophobic groove (*10*, *11*, *55*, *56*). Recent efforts have also explored potential small molecule and peptide binders of the non-catalytic groove on the opposite face, thought to be involved in protein-protein interactions (*12*, *57*). In this context, it is noteworthy that the PWATCDS peptide which binds at the catalytic hydrophobic groove and inhibits the DsbA oxidase also inhibits the mechanical foldase. In a related drug development program, a class of small molecules termed 'pilicides' are capable of preventing pilus formation by disrupting chaperone-mediated folding and assembly of the Chaperone-Usher system(*58–60*). Along a similar vein, we propose that the DsbA chaperone activities afford a novel avenue for anti-virulence antibiotic development.

Methods

Protein expression and purification – DsbA was purified from *E. coli* as described previously(*42*). The DsbA was used fresh after purification. Oxidized DsbA was generated by incubating the enzyme in 10 mM oxidized glutathione overnight at 4° C. Reduced DsbA was made by incubation with 100 µM TCEP overnight. The GSSG or TCEP was then removed by >1,000 fold dilution into phosphate buffered saline (PBS) and centrifugal concentration. Oxidation state and concentration of the DsbA was determined with a DTNB assay and absorbance at 412 nm and 280 nm respectively. Octamer protein L construct along with AviTag at C-terminus and HaloTag enzyme at N-terminus was expressed and purified as described before(*29*). PWATCDS peptide (99.3%) was purchased from Biomatik. Paramagnetic Dynabeads (M-270) coated with streptavidin were purchased from Invitrogen. All the experiments were done PBS.

Magnetic tweezers instrumentation – Magnetic tweezers experiments were accomplished in our custom-made magnetic tweezers setups, where an inverted microscope (Zeiss Axiovert S100) mounted on a nanofocusing piezo actuator (P-725 PIFOC, Physik Intrumente), a magnet-positioning voice coil (LFA-2010, Equipment Solutions), and a high-speed camera (xiQ CMOS, Ximea GmbH). Fluid chambers are made of two cover slips sandwiched by two strips of parafilm. Both the top and bottom cover slips were washed by sonicating them separately for 30 min in 1 % Hellmanex solution, 30 min in acetone, and 30 min in ethanol. The bottom slides are silanized by dipping them in a solution of (3-aminopropyl)- trimethoxysilane 0.1% v/v for 30 minutes in ethanol. After salinization the bottom glasses are then dried at 100° C for an hour and then sandwiched with washed coverslips by using two strips of parafilm at two sides with a gentle heating at 85° C for 1 minutes. Then the chamber was functionalized with glutaraldehyde, nonmagnetic polystyrene beads (3 µm) and HaloTag amine ligand respectively as described previously(*29*). Recombinant octamer protein was tethered using HaloTag chemistry (N-terminal) and the other end of the protein was decorated with a single paramagnetic bead through biotin-streptavidin chemistry (AviTag). Force is applied to the protein by changing the position of the permanent magnets, which was controlled with a linear voice-coil with a speed of ~0.7 m/s speed and 150 nm position resolution. Experiments with DsbA are performed after >15

minute equilibration with Protein L in the fluid chamber. Similarly, PWATCDS peptide are incubated for > 30 minutes with DsbA before adding to the fluid chamber.

Data analysis – Only proteins showing eight unfolding events in the high force (45 pN) fingerprint pulse are considered for analysis. After unfolding the protein, the force is relaxed to achieve an equilibrium between the folding and unfolding states to calculate the folding probability. We measure this by calculating the probability of finding the system in a particular state, considering that t_i is the total time the system spends in state i (where i equals the number of folded domains, $i = 0, 1, \ldots, N$) and t_t the total time of experiment, $t_t = \Sigma_i t_i$. Then, the folding probability is $P_f = \Sigma_i i\pi_i/N$, where $\pi_i = t_i/t_t$.

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Author Contributions

S.H., E.C.E., D. J. E. and J.M.F. designed the project, S.H., S.H., E.C.E., D. J. E. and J.A.R-P performed the experiments and analyzed the data, S.H., R.T.-R., E.C.E., and J.M.F. wrote the paper.

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Figures

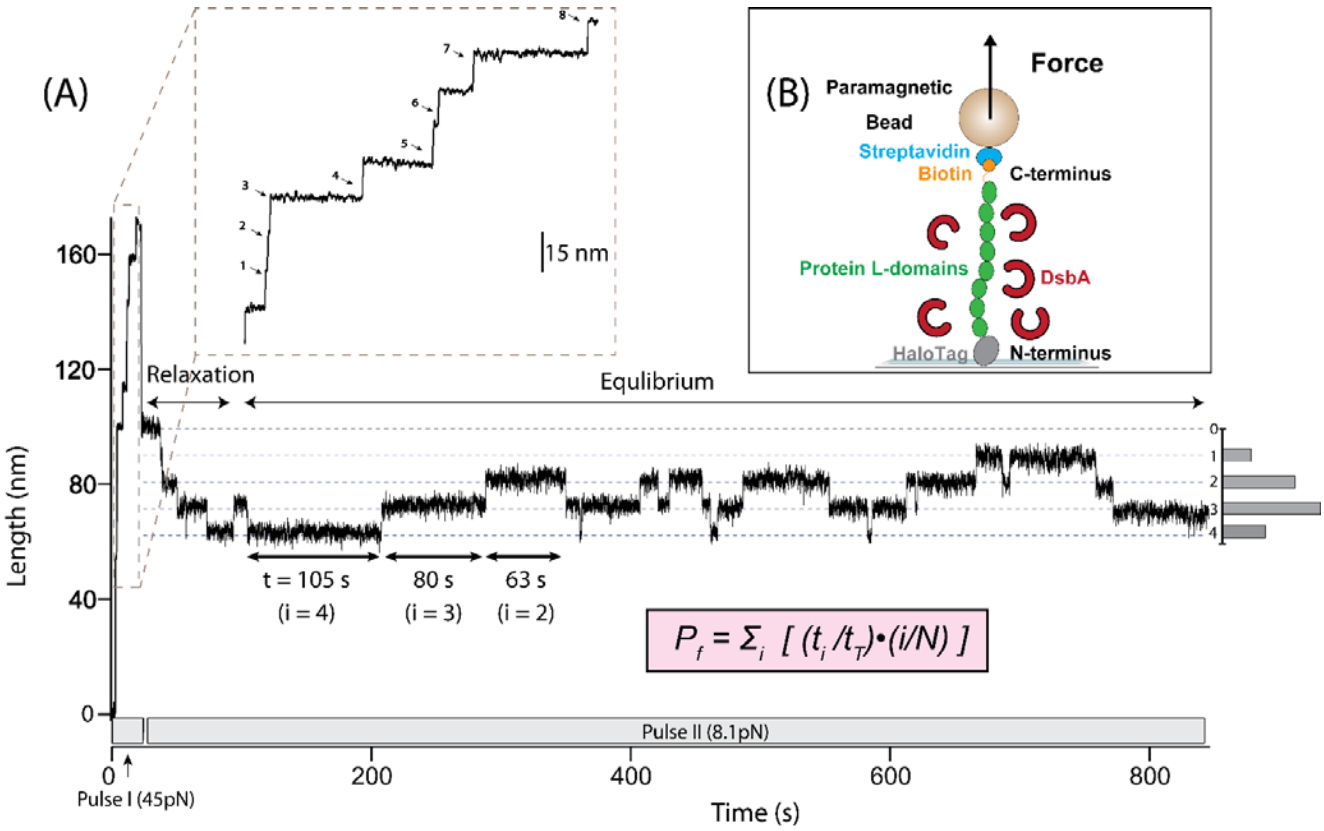

Figure 1. Folding probability under force measured by magnetic tweezers (A) Representative magnetic tweezers demonstrating unfolding and refolding transitions of an eight-repeat (N=8) construct of the protein L domain. The protein is first unfolded at a constant force of 45 pN (Pulse I) resulting in eight consecutive unfolding (upwards-step) transitions of 15 nm each (see inset for magnified). The force is then quenched to 8.1 pN (Pulse II) resulting in entropic recoil of the protein followed by relaxation to an equilibrium between folding (downwards-step) and unfolding transitions. The state i of the protein at any point during the recording is equal to the number of folded domains. The total residence time (t_i) of each folded state during the equilibrium phase is shown by the gray histogram on the right and the sum of all residence times (t_T) is equal to the duration of the equilibrium phase. After calculating the states i and the residence times t_i the folding probability at a force of 8.1 pN is calculated according the presented equation (pink inset). **(B)** Schematic of the magnetic tweezers experiment: One end of octamer of protein L is attached to the glass coverslip via HaloTag covalent chemistry and the other end is tethered to a paramagnetic bead via biotin-streptavidin binding. A precise pulling force is applied by positioning a pair of permanent magnets above the tethered paramagnetic bead with sub-micron resolution. DsbA (red curls) can be washed into or out of the flow cell during the course of an experiment.

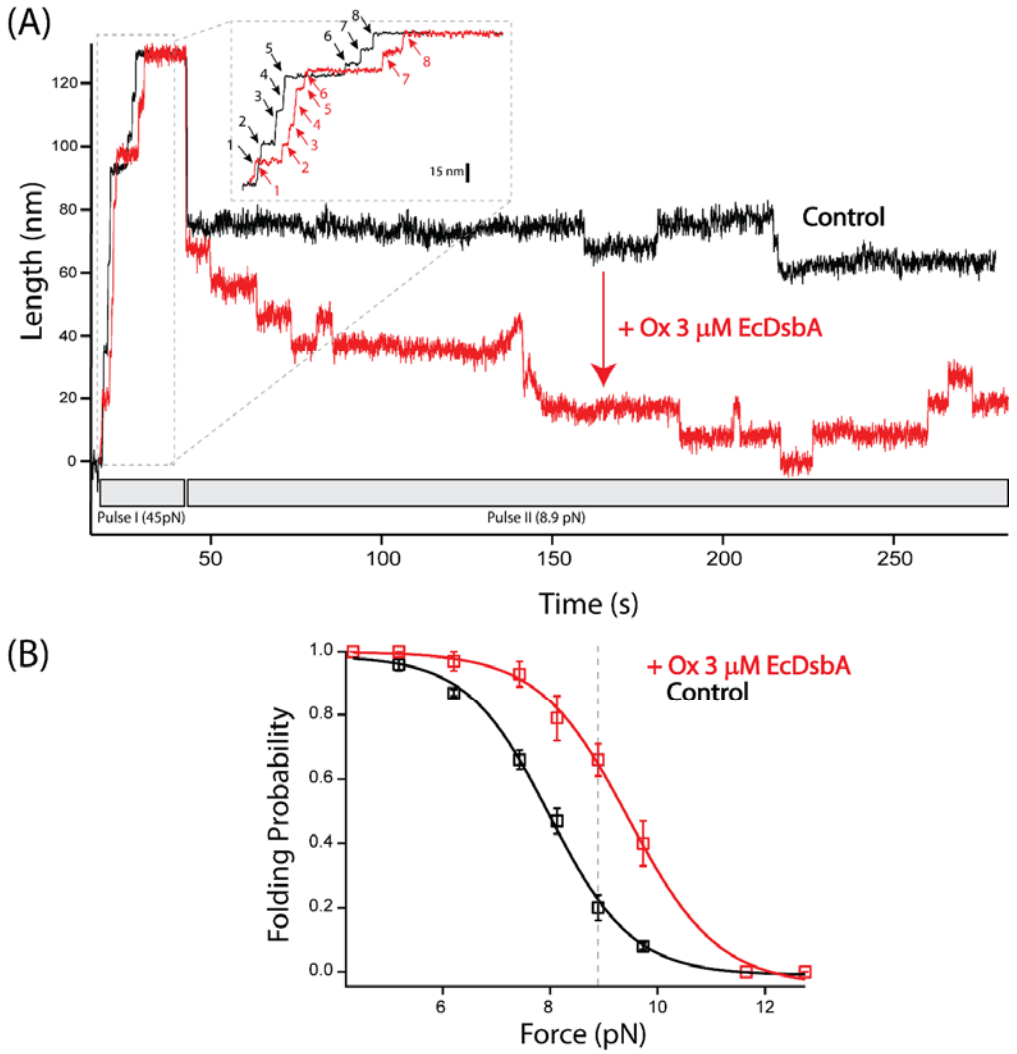

Figure 2. DsbA acts as a chaperone. (A) Magnetic tweezers force-clamp trajectory of an protein L octamer in the presence (red) and absence (black) of 3 µM oxidized DsbA at 8.9 pN. In Pulse I, the protein is unfolded at 45 pN, where the 8 protein L unfolding events are observed as steps of ~15 nm (see inset for horizontally magnified trace). In Pulse II, force is quenched to 8.9 pN, where an initial relaxation refolding is observed as downward steps of ~9.5 nm, followed by equilibrium unfolding/refolding steps. The refolding trajectory in the presence of oxidized DsbA is markedly accelerated and achieves an equilibrium where more domains are folded **(B)** Folding probability as a function of force in the presence (red) and absence (black) of DsbA. The rightward shift of the curve in the presence of DsbA indicates mechanical chaperone activity of DsbA that enhances folding of protein L in the force range from 4-11 pN. The maximum difference in the folding probabilities, marked by the gray line, occurs at 8.9 pN, the force demonstrated in panel (A). Error bars represent the standard errors of the mean of ≥ 5 independent molecules.

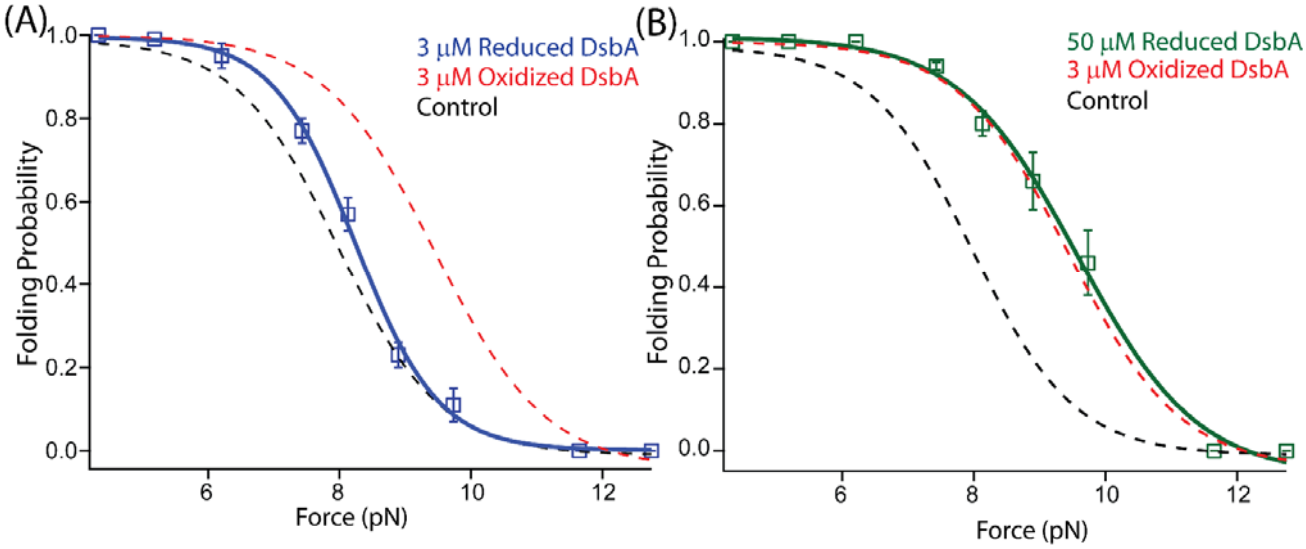

Figure 3: A redox switch controls the chaperone activity of DsbA: (A) The folding probability of 3 μM reduced DsbA (blue squares) has no significant effect on the folding probability of protein L, coinciding with the control experiment (black dotted line). **(B)** Increasing the concentration of reduced DsbA to 50 μM restores the mechanical chaperone activity of DsbA. The folding probability of protein L as a function of force in the presence of 50 μM reduced DsbA (green squares) coincides with the folding probability curve of the 3 μM oxidized DsbA (red dotted line). The fittings in the absence (black dotted line) and in the presence of 3 μM oxidized DsbA (red dotted line) is plotted as control.

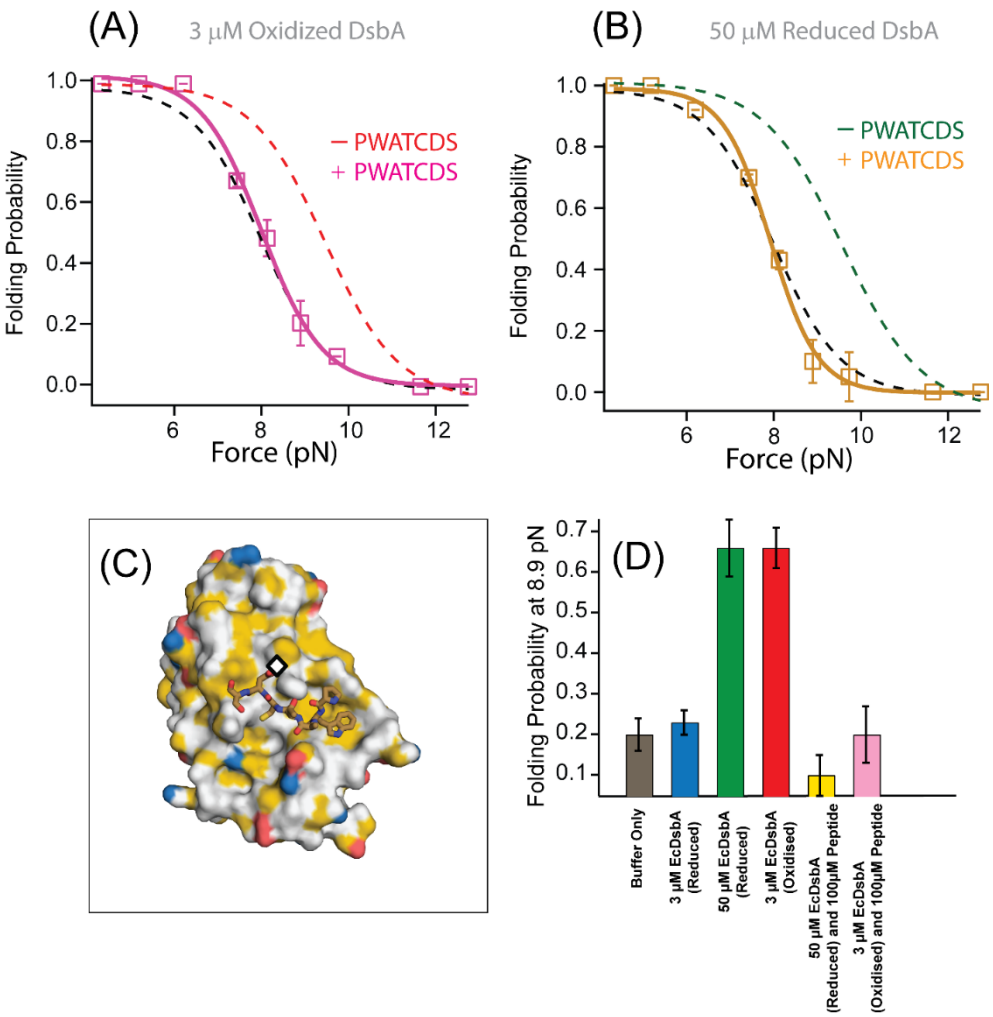

Figure 4: Peptide inhibitor of DsbA maps the chaperone activity to the groove surrounding the catalytic site (A) The PWATCDS peptide was previously found to inhibit the oxidoreductase activity of DsbA by binding to and occupying the hydrophobic groove adjacent to the catalytic *CXXC* motif of DsbA. The folding probability of protein L in the presence of 3 μM oxidizied DsbA and 100 μM PWATCDS peptide (pink squares) is shifted back to the folding probability of the control experiment (no DsbA or peptide, black dotted line), indicating inhibition of the mechanical chaperone activity of oxidized DsbA (red dotted line fittings). **(B)** The mechanical chaperone activity of 50 μM reduced DsbA is also inhibited in the presence of 100 μM peptide (gold squares), aligning with the folding probability in the absence of DsbA (black dotted line fittings). **(C)** Structure of *Proteus mirabilis* DsbA with PWATCDS peptide bound. The active site *CXXC* motif is marked with a black diamond. The surface surrounding the active site is largely hydrophobic, as colored according to the schema of Hagemans *et al.* whereby any carbon not bound to a heteroatom is colored yellow. **(D)** The lower insert shows the folding probability at 8.9 pN in absence (gray) and presence of 3 μM reduced DsbA (blue), 50 μM reduced DsbA (green), 3 μM oxidized DsbA (red), 50 μM reduced DsbA with 100 μM PWATCDS peptide (yellow) and 3 μM oxidized DsbA with 100 μM PWATCDS peptide (pink).

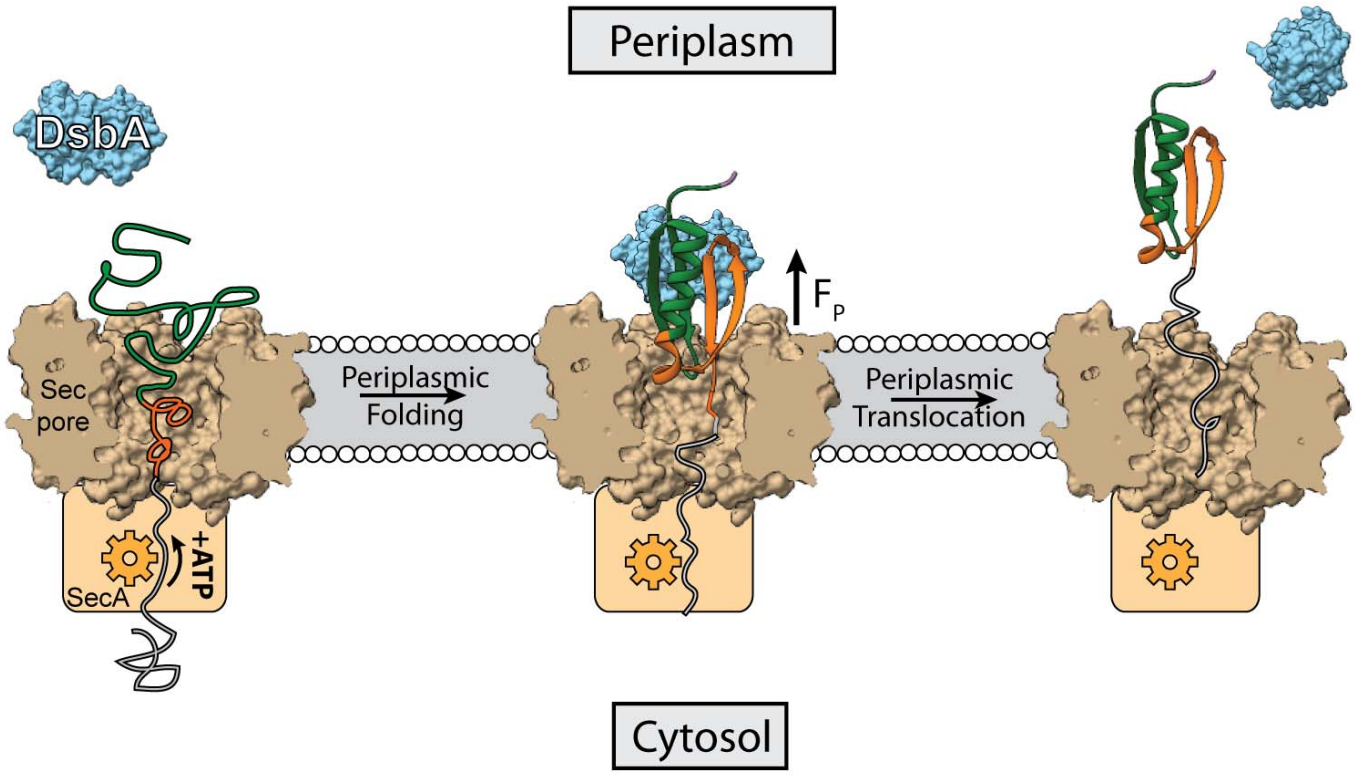

Figure 5: DsbA accelerated transport across the translocon pore. Translocation of peptides into the periplasm take place with the help of Sec machinery, where SecA (orange) translocates the peptide with the help of ATP hydrolysis. Here we show the portion of the translocated polypeptide that is exposed to the periplasm (green polymer) may interact with the high concentration of oxidized DsbA (light blue) and fold on the mouth of the translocon pore, generating a pulling force (F_P) of several piconewtons. The pulling force strains the remaining polypeptide in the translocon pore (grey polypeptide) and overcoming the friction of the tunnel, pulls it through to the periplasmic side. The work of protein folding therefore reduces the number of rounds of ATP hydrolysis that the SecA motor must undergo to push the polypeptide through the translocon pore.