DsbA is a switchable mechanical chaperone

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Abstract:

In bacteria, many toxins and virulence factors pass through a translocon pore as unfolded polypeptides en route to the periplasm where they first encounter DsbA, a ubiquitous bacterial oxidoreductase enzyme that introduces disulphide bonds into nascent proteins. Here, using magnetic tweezers based single molecule force spectroscopy, we demonstrate for the first time that DsbA can also accelerate folding of cysteine-free proteins by using the globular domain of the protein L super-antigen as a substrate. 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 foldase activity. 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, focal adhesion formation, or protein translocation [1–3]. 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 [4,5]. 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 [6]. As the nascent chains emerge, they are greeted by one of several periplasmic chaperones that assist with folding, transport, or membrane insertion[7]. As many extracellular proteins contain disulfide bonds, they often encounter the prototypical oxidoreductase enzyme DsbA [8], which is necessary for the maturation of a range of virulence factors including flagellar motors, pilus adhesins, Type III secretion systems, and heat-labile or heat-stable enterotoxins [9–12]. While it is well known that DsbA is required in many cases for the secretion of disulfide containing proteins[4], it has also been demonstrated that certain proteins lacking disulfides need DsbA for efficient transport into the periplasm[13]. This is reminiscent of translocation of eukaryotic peptides into the endoplasmic reticulum (ER) or mitochondria by the chaperones BiP and Hsp70 respectively [14,15], which are thought to cause biased transport across cellular membranes through a Brownian ratchet mechanism [16,17]. It is plausible that DsbA has some chaperone activity independent from its oxidoreductase activity given that other members of the thioredoxin fold family, such as human protein disulfide isomerase (PDI) and *E. coli* DsbC, are able to reactivate denatured enzymes that lack disulfide bonds [18,19].

There are few investigations of the role of DsbA in folding of cysteine free peptides, much less a systematic study of DsbA interaction with peptides under an external force [4,13]. Here using single molecule magnetic tweezers, we aim to mimic the stretching forces experienced by polypeptides emerging from 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. Notably, we found that addition of a short peptide knocks out DsbA mechanical chaperone activity, likely through competition for the hydrophobic groove surrounding the catalytic site. These results suggest that DsbA is a mechanical foldase that drives protein folding at higher forces, which may provide an important energy source to accelerate peptide transport across the translocon pore.

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 certain limitations of bulk studies of chaperones [20]. In magnetic tweezers-based force spectroscopy, proteins are tethered between a glass surface and a microscopic paramagnetic bead while a pair of permanent magnets generates a passive force clamp that can apply a broad range of forces up to ~100 pN [21] . The model substrate used here is the B1 antibody-binding domain of protein L from *Finegoldia magna*, 62 residues in length with a simple α/β fold [22], and extensively characterized by bulk biophysical and single-molecule force spectroscopy techniques [21,23,24]. In all studies herein, we employ an octameric tandem modular protein L, flanked with N-terminal HaloTag and C-terminal biotin for tethering so a streptavidin coated bead (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 SI) [20]. 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 [20].

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). 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 proteins have multiple binding interfaces that are targets of drug development: a hydrophobic patch immediately

adjacent to the catalytic *CXXC* motif, and a non-catalytic groove on the opposite face involved in protein-protein interactions [11,25]. The hydrophobic patch has been shown to have a high affinity for a DsbB derived seven residue peptide, PWATCDS (Fig 4C), which was demonstrated to effectively block any DsbA oxidoreductase activity [10] . 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

The mechanisms by which ATP independent chaperones, such as DsbA, can accelerate refolding remain unclear [7]. Here we have provided an empiric description and measurement of a chaperone-like behavior of *E. coli* DsbA, which acts as a mechanical chaperone to accelerate refolding to the native state and to shift the folding probability under force. Previous studies on a disulfide containing protein using AFM based force spectroscopy demonstrated that DsbA-catalyzed oxidative folding occurs ~3-fold faster than folding of the reduced protein [26]. This acceleration in the folding rate with DsbA versus reduced substrate is likely an indirect indication of the chaperone activity of the enzyme, however these studies by AFM lacked sensitivity required to resolve folding events at low force, which we can now directly observe in the magnetic tweezers. 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. There are several other reported cases (peroxiredoxins and thioredoxins) in which oxidative or nitrosylative modification of thiols acts as a "redox switch" for chaperone activity [27–29].

Perhaps this high affinity of DsbA for unfolded polypeptides could be harnessed to accelerate transport into the periplasm. Here we propose a new mechanism by which 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 (Figure 5). Prior to translocation, a protein is 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 [30]. 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 [31], so a single protein L domain with 60 residues participating in the fold would require 3 ATP molecules for translocation. Assuming a mechano-chemical coupling efficiency of ~50% for the SecA motor [32], hydrolysis of 3 ATP molecules would generate 150 zJ of mechanical work (100 zJ per ATP * 50% efficiency * 3 molecules ATP)[33]. Although there are no single molecule measurements of the forces generated by the SecA motor, it is thought to operate optimally over the force range of 5 – 11 pN [33], stalling at the higher forces, which holds true for most protein translocating motors [3,34]. 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 substrate polypeptide transported, then the mechanical work done by DsbA is of even greater importance [33]. This adds to the evidence that many molecular chaperones are involved in mechanically gated processes: Trigger factor sits at the mouth of the ribosome, coupling protein folding to translational synthesis[20,35]; mtHsp70 couples mechanical unfolding of proteins to transport into the mitochondrial lumen[15]; the GroEL chaperonin pulls apart misfolded polypeptides via its apical domain[36].

As a factor involved in the maturation of a diverse set of bacterial exotoxins, adhesins, and secretion machinery [37], DsbA affords a single attractive target for attenuating a range of virulence phenotypes [38]. Indeed, DsbA is viewed as a viable target for drug development due to its direct relevance in urinary tract infections, nosocomial infections, and drug-resistant pneumonias [12,39–41]. While current therapeutic developments typically identify drugs by measuring their ability to knock out DsbA oxidoreductase activity, the mechanical chaperone activity at the catalytic groove offers a new target for antibiotic development. We believe that interfering with the foldase activity of DsbA and other periplasmic chaperones, Skp, Spy, SurA, likely involved in translocation, provides a viable path to a new class of antibiotic compounds.

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Author Contributions

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

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Figures

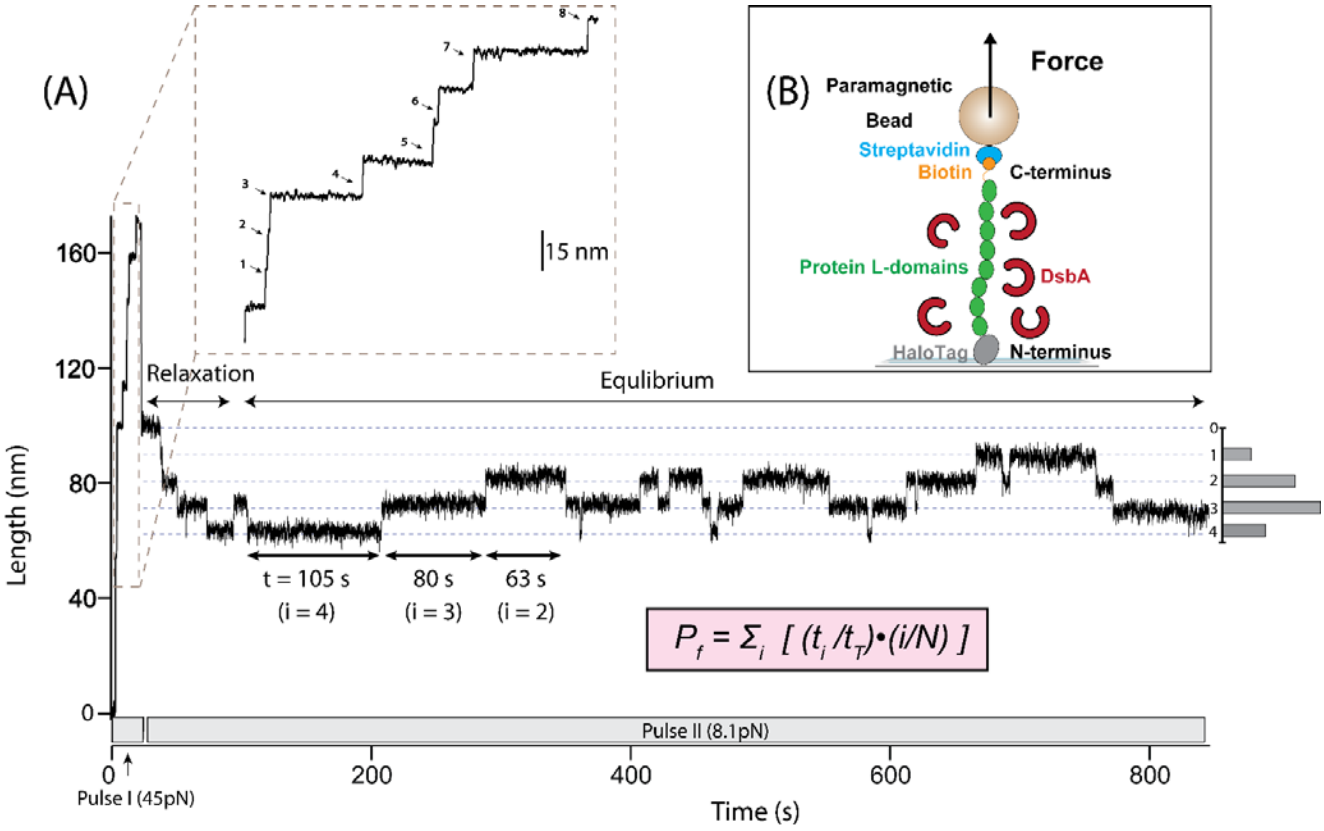

Figure 1. Folding probability under force measured by magnetic tweezers (A) Representative recording demonstrating unfolding and refolding transitions of an eight-repeat (*N*=8) construct of the protein L domain. The protein is first unfolded at a constant force of 45 pN (Pulse I) resulting in eight 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 folding probability at a force of 8.1 pN is calculated from the residence times t_i in each state *i* according the presented equation (pink inset and supporting information). **(B)** The protein L construct is tethered to the glass and paramagnetic bead through HaloTag and biotin-streptavidin chemistry respectively. 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.

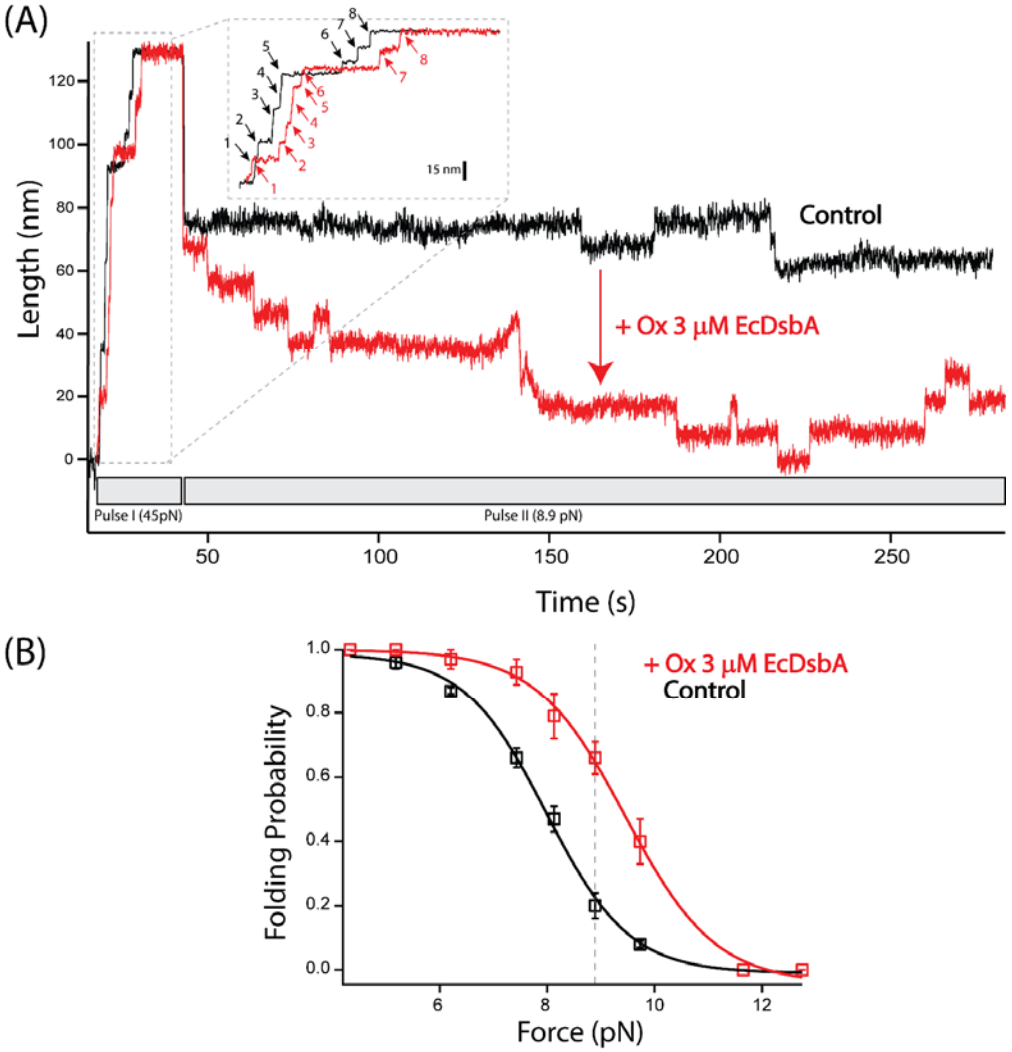

Figure 2. DsbA acts as a chaperone. (A) Magnetic tweezers force-clamp trajectory of a protein L octamer in the presence (red) and absence (black) of 3 µM oxidized DsbA at 8.9 pN. The refolding trajectory in the presence of oxidized DsbA is markedly accelerated and achieves an equilibrium where more domains are folded **(B)** Folding probability as a function of force in the presence (red) and absence (black) of DsbA indicates mechanical chaperone activity. The maximum difference in the folding probabilities, marked by the gray line, occurs at 8.9 pN.

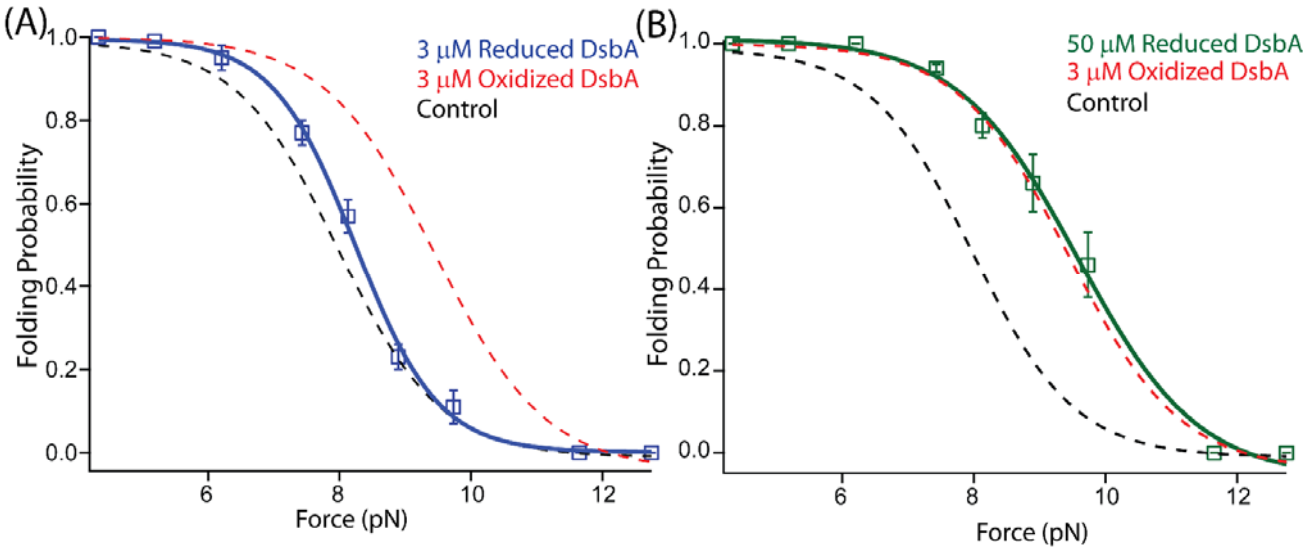

Figure 3: A redox switch controls the chaperone activity of DsbA: (A) The folding probability of 3 μM reduced DsbA (blue squares) has no significant effect on the folding probability of protein L, coinciding with the control experiment (black dotted line). **(B)** Increasing the concentration of reduced 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.

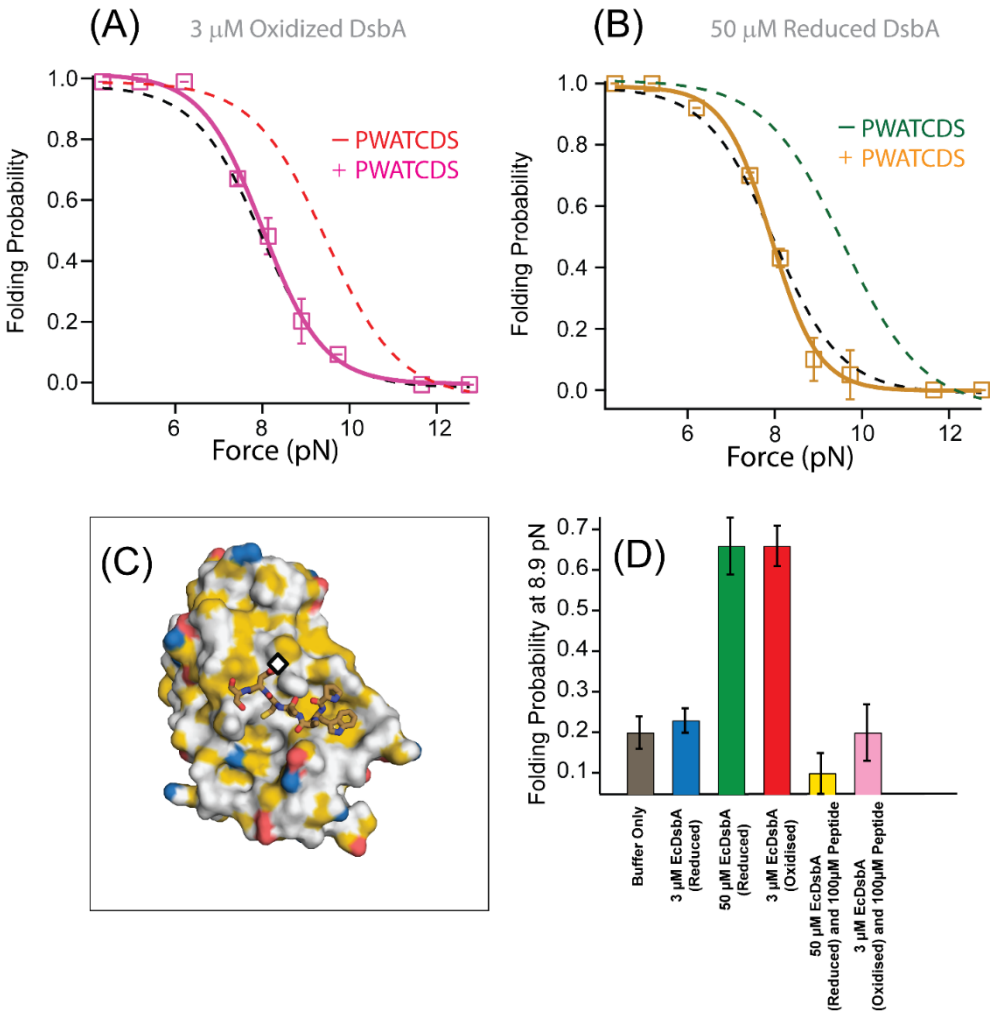

Figure 4: Peptide inhibitor of DsbA maps the chaperone activity to the groove surrounding the catalytic site (A) The PWATCDS peptide was previously found to inhibit the oxidoreductase activity of DsbA by binding to and occupying the hydrophobic groove adjacent to the catalytic *CXXC* motif of DsbA. The 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 [10]. The active site *CXXC* motif is marked with a black diamond and hydrophobic regions are colored yellow. **(D)** The lower insert shows the folding probability at 8.9 pN under all conditions probed in this study.

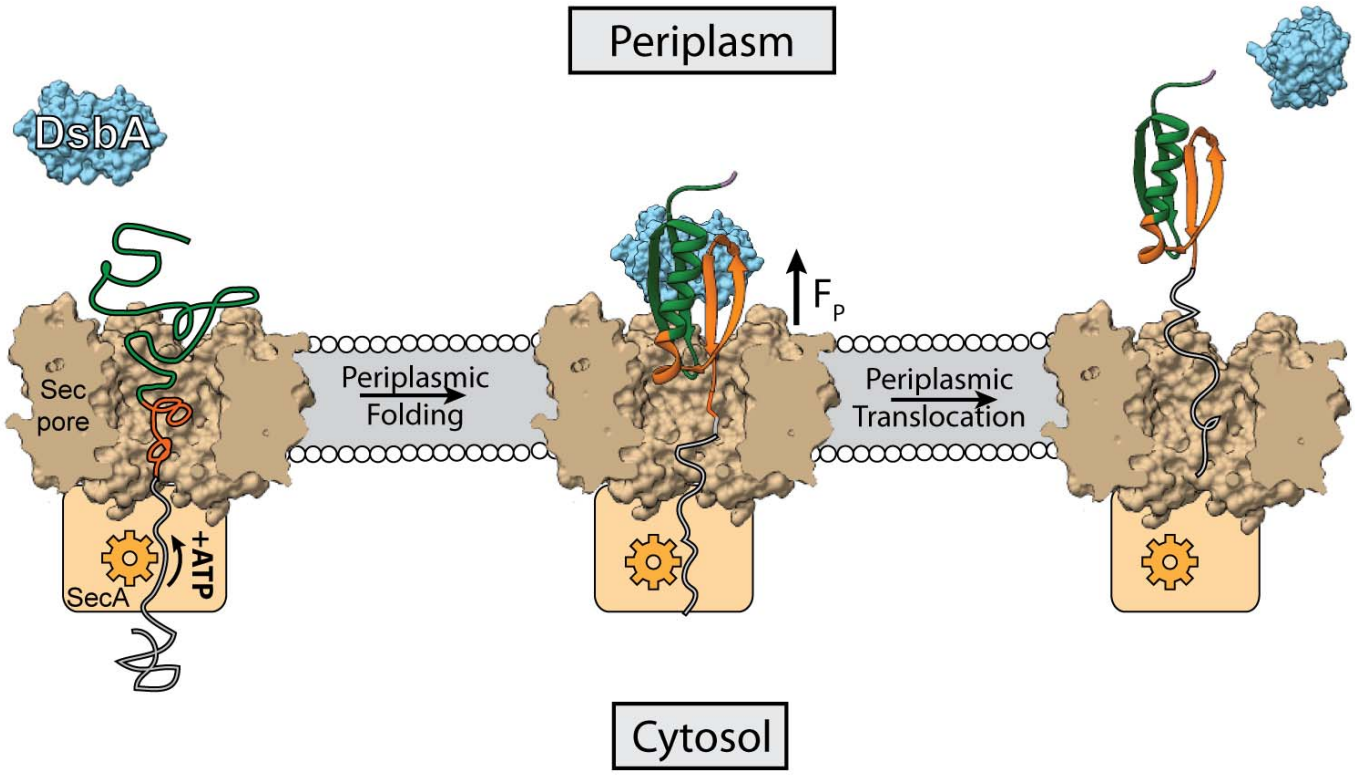

Figure 5: DsbA accelerated transport across the translocon pore. Translocation of peptides into the periplasm take place with the help of Sec machinery, where SecA (orange) translocates the peptide with the help of ATP hydrolysis. Here we show the portion of the translocated polypeptide that is exposed 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.