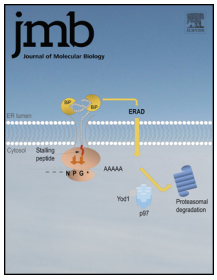

Calcium-mediated Protein Folding and Stabilization of *Salmonella* Biofilm-associated Protein A

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Abstract

Biofilm-associated proteins (BAPs) are important for early biofilm formation (adhesion) by bacteria and are also found in mature biofilms. BapA from *Salmonella* is a ~386-kDa surface protein, comprising 27 tandem repeats predicted to be bacterial Ig-like (BIg) domains. Such tandem repeats are conserved for BAPs across different bacterial species, but the function of these domains is not completely understood. In this work, we report the first study of the mechanical stability of the BapA protein. Using magnetic tweezers, we show that the folding of BapA BIg domains requires calcium binding and the folded domains have differential mechanical stabilities. Importantly, we identify that >100 nM concentration of calcium is needed for folding of the BIg domains, and the stability of the folded BIg domains is regulated by calcium over a wide concentration range from sub-micromolar (μM) to millimolar (mM). Only at mM calcium concentrations, as found in the extracellular environment, do the BIg domains have the saturated mechanical stability. BapA has been suggested to be involved in *Salmonella* invasion, and it is likely a crucial mechanical component of biofilms. Therefore, our results provide new insights into the potential roles of BapA as a structural maintenance component of *Salmonella* biofilm and also *Salmonella* invasion.

Introduction

Bacteria have an amazing capability to adapt and survive a wide range of environmental conditions. A bacterial cell can live on its own, the planktonic lifestyle, or be a part of a multicellular structured community, the biofilm [1,2]. Biofilm is formed by adhesion of communities of bacteria to various surfaces and to each other, which involves surface-sensing and molecular transmission events that alter their genetic program [3]. Biofilm formation is promoted under various mechanical, osmotic, and chemical stresses [1,4] and is beneficial for bacterial survival. For example, bacterial biofilms result in increased resistance toward antibiotic treatment [1,5] and disinfectants [6].

Most bacterial species have the capacity to form biofilms [7]. The major components of these biofilms are water, bacteria cells, and extracellular polymeric substances. Extracellular polymeric substances are mainly polysaccharides, extracellular nucleic acids, proteins, and lipids. Biofilm associated proteins were first reported in *Staphylococcus aureus* and proteins that have homology to these are termed biofilm-

associated proteins (BAPs). BAPs are conserved across the bacterial kingdom, in proteins such as Mus-20 (*Pseudomonas putida*), BapA (*Salmonella enteritidis*), Bap (*Burkholderia cepacia*), Espfm (*Enterococcus faecium*), Esp (*Enterococcus faecalis*), and LapA (*Pseudomonas fluorescens*) [8]. Although the function of these proteins is not fully understood, they have been suggested to be involved in adherence to external substrates, other bacterial cells, and host cells [9,10]. Bioinformatics suggests that they contain Ig-like domains called bacterial immunoglobulin-like (Big) domains. The Big domains are part of the E-set clade of the Ig-like fold superfamily [11,12].

Salmonella enterica serovar Typhimurium is a gram-negative bacterium, having a rod-shaped structure. It can infect humans causing foodborne illness, mainly typhoid fever. BapA from *S.* Typhi can be divided into three regions [10]: regions A (aa 1–158), B (aa 159–3003), and C (aa 3004–3824) (Fig. 1a). Region A contains a largely unstructured region and likely positions BapA away from the cell wall. Region C contains three Vibrio–Colwellia–Bradyrhizobium–Shewanella domains (3117–3214, 3371–3466, and 3626–3724), suggesting a potential role in cell adhesion (TIGRFAMs database). Region C also has a secretion domain (3741–3821) bearing a signaling peptide, which is recognized by the ABC transporter and allows type 1 (T1SS) secretion [10].

Region B comprises ~27 Big repeats. Importantly, all the domains in region B contain RTX motifs [10]. Such RTX motifs contain glycine–aspartate-rich sequences that have been shown to be calcium sensitive [13,14]. Interestingly, many BAPs such as SiiE from *Salmonella* [15], Bap from *S. aureus* [16], LapF from *P. putida* [17], LapA from *P. fluorescens* [8], *Vibrio cholerae* FrhA [18], *Shewanella oneidensis* BpfA [19], *Legionella pneumophila* RtxA [20], Lig protein in *Leptospira* [21], and many more [22] share RTX homology in their Big domains. Since RTX motifs are calcium-binding sites; therefore, the stability of the RTX domains is calcium dependent.

A recent study showed that LapA from *P. fluorescens* Pf01 is secreted via a two-step mechanism, involving an intermediate step where the secretion substrate is tethered to the bacterial surface [23]. This example suggests that the secreted BAPs have two fractions, one remains attached to the cell surface and one is

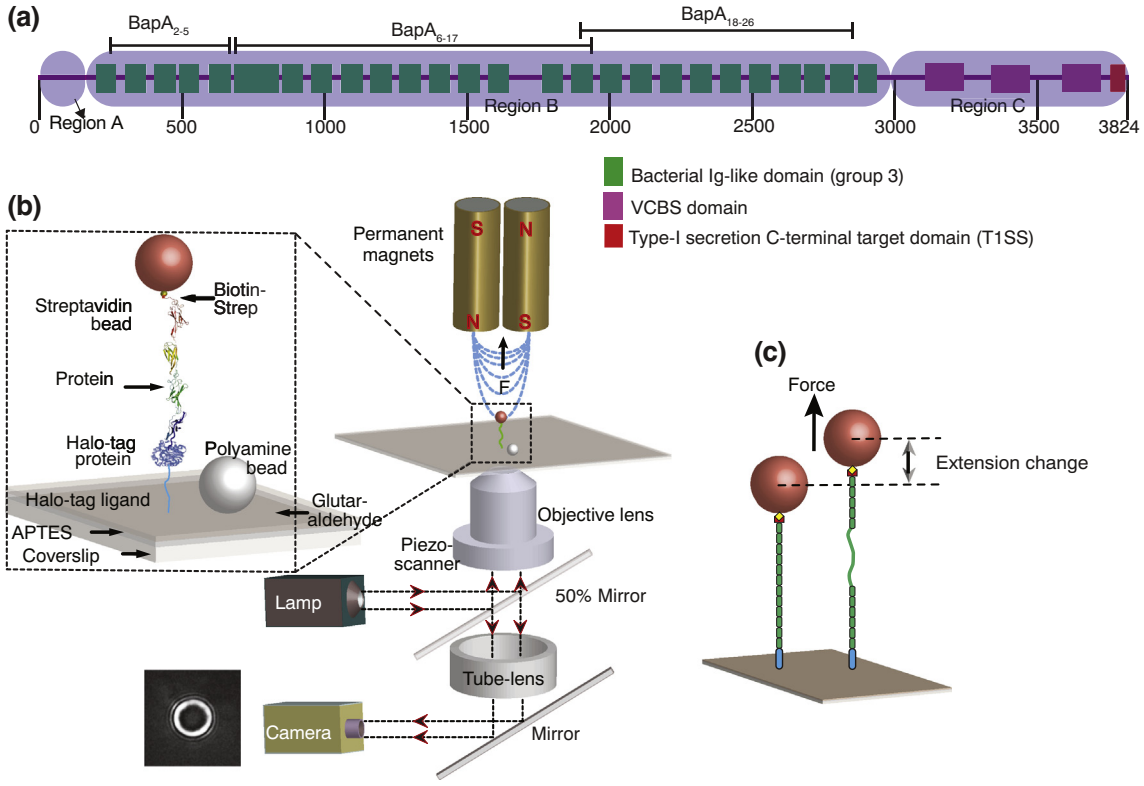

Fig. 1. Experimental design. (a) Schematic structure of BapA. The 27 Big domains (green), the three Vibrio–Colwellia–Bradyrhizobium–Shewanella (VCBS) domains (magenta), and the T1SS domain (red) are shown. (b) Schematic representation of magnetic tweezers setup. The protein is tethered via its two termini between a coverslip and a superparamagnetic bead. (c) Schematic representation of unfolding of protein domains that can be detected by the extension change.

released from the cell surface. Although the function of the largest region (region B) of BapA is unclear, it contains multiple protein domains that may act as a spacer between the cell and neighboring cells or other extracellular surfaces. Considering that the biofilm may be subject to complex mechanical perturbations, we reason that region B may also serve as a mechanical element to regulate cell adhesion and the biofilm mechanical property.

Several aspects related to the BAPs secretion and potential functions still remain unclear. To name a few: (1) whether the intracellular RTX BIg domains indeed exist in an unfolded conformation is unclear; (2) the mechanical stability of the BIg domains at physiologically relevant loading rate has yet to be quantified; and (3) how the stability of the BIg domains depends on calcium concentrations largely remains unknown. In this study, we addressed these questions at the single-molecule level using an in-house constructed magnetic tweezers setup [24,25]. We show that (1) the intracellular calcium concentration is sufficient to fold the majority of the BIg domains in region B, (2) the calcium-binding sites on BapA BIg domains interact with calcium over a wide range of concentration of sub-μM–mM, and (3) increasing calcium concentration over this range results in increased mechanical stability of the BIg domains. Together, the differential calcium-sensing capability of the calcium-binding sites on the BapA BIg domains ensures that BapA can respond to changes in its environmental calcium level. We propose that it is only when the BapA protein is exposed to the elevated calcium concentration of the extracellular environment does the formation of the highly stable, rigid, conformation of the BapA protein occur. At intracellular calcium concentration, the BapA domains are folded but more flexible relative to each other, facilitating the secretion of the entire protein.

Results

Purified BapA segments in a calcium-free buffer solution

Simple Modular Architecture Research Tool (SMART database) sequence analysis predicts that region B of BapA contains 27 repeats of BIg domains (Fig. 1a). The sequence similarities and conserved domains are shown in Fig. S1. Due to the challenge of expressing the full-length region B, we divided the 27 tandem repeats into three segments (Fig. 1a). The first segment contains domains 2 to 5 ($BapA_{2–5}$), the second contains domains 6 to 17 ($BapA_{6–17}$), and the third contains domains 18 to 26 ($BapA_{18–26}$), each of these three segments expressed well as recombinant proteins.

The purified segments were covalently attached to a coverslip surface coated with Halo-tag ligand using Halo-tag chemistry [26–28]. Following attachment, streptavidin coated 2.8-μm-diameter superparamagnetic beads were introduced to bind the C-term AviTag and form protein tethers between the bead and the surface (see the Materials and Methods section) (Fig. 1b). A pair of magnets was used to apply force to the bead, and the amount of force applied was adjusted by changing the distance between the magnets and the bead. Unfolding of the BIg domains will cause extension change of the tether, resulting in a change of the bead height from the surface which is monitored in real time with nanometre resolution (Fig. 1c). During force change, the bead height change is affected by both bead rotation and extension change of the molecule, while at the same force, the bead height change equals the extension change [25]. In all the mechanical unfolding experiments in this work, the force was increased from <1 to 120 pN at a constant loading rate of 1.8 ± 0.2 pN/s. The technical details of the force control and the detection resolution of our magnetic tweezers can be found in our recent review [25].

Figure 2a shows three representative force–height curves for each segment recorded during the force-increase process. Stepwise extension changes were observed for each segment indicating mechanical unfolding of the BIg domains. During each stepwise extension change, the force remains the same before and after the transition; therefore, the step size is the extension change of the molecule. The maximum numbers of such unfolding steps were 4, 12, and 9 for $BapA_{2–5}$ (4 domains), $BapA_{6–17}$ (12 domains), and $BapA_{18–26}$ (9 domains), respectively. These numbers are consistent with the numbers of domains in the respective segments predicted in the SMART database, confirming the domain structure and revealing that all the domains in these constructs are folded. Here note that all experiments in Fig. 2 were done with purified protein in calcium-free buffer. In addition, no calcium was added during the purification process (see the Materials and Methods section). Therefore, these results suggest that either the intracellular level of calcium is sufficient to fold the domains, or the folding of the domains does not depend on calcium.

In 100 mM KCl, at our loading rate of 1.8 ± 0.2 pN/s, the domains in the segments unfolded over a wide range of forces (Fig. 2b). The majority of unfolding forces for $BapA_{2–5}$ are distributed over a range of 5–50 pN, with a rather flattened distribution between 5 and 40 pN. The unfolding forces of $BapA_{6–17}$ and $BapA_{18–26}$ domains exhibit a peaked distribution in the range of 5–80 pN. These results show that the BapA BIg domains have differential mechanical stabilities.

Unfolding of protein domains results in an unfolded polypeptide chain under force. It is known that the force extension curve of a polypeptide chain can be described by the worm-like chain polymer model, with a persistence length of ~0.8 nm [28–30]. From

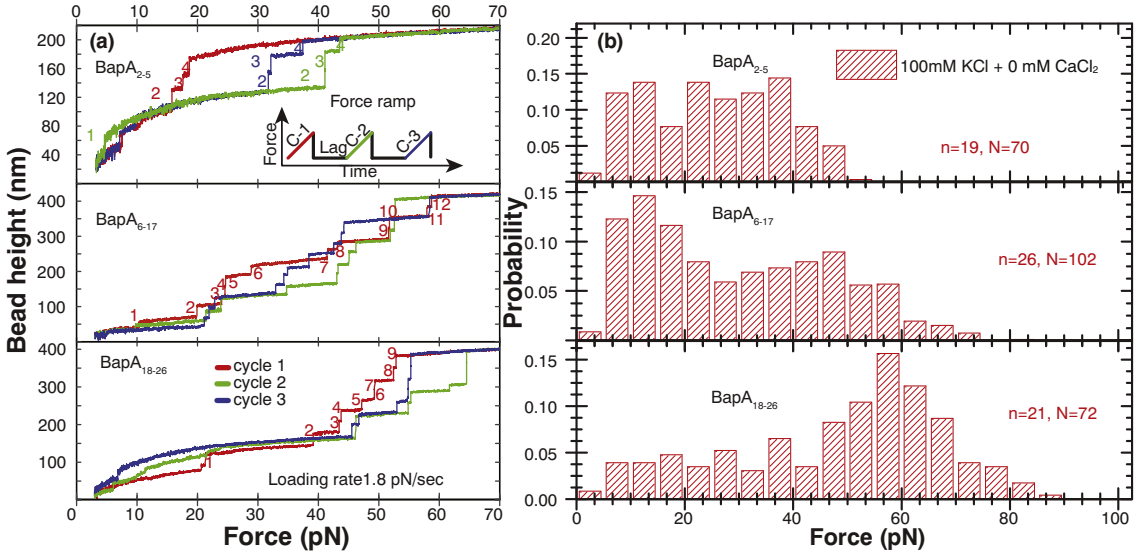

Fig. 2. Mechanical unfolding of BapA domains. Top, middle, and bottom panels show $BapA_{2–5}$, $BapA_{6–17}$, and $BapA_{18–26}$ protein segments, respectively. (a) Representative force–bead height curves during force-increase scans at a loading rate of 1.8 ± 0.2 pN/s. Data in different colors were obtained from different cycles of the same protein tether (see inset schematic of the experiment). Unfolding steps are labeled with numbers. (b) The unfolding force histogram. Top, middle, and bottom panels show corresponding histograms of forces at which unfolding steps were observed. Where n is the number of independent protein tethers and N is the number of cycles.

the force–extension curve calculated using the Marko–Siggia's formula [31], the unfolding step sizes can be converted into contour lengths released during unfolding. The histograms of the contour lengths exhibit a Gaussian-like distribution over a range of 20–40 nm (Fig. S2, red color histogram and red color Gaussian-like distribution) peaked at ~30 nm spread over a range from ~22 to ~38 nm (full width at half maximum range ~16 nm). The range of peptide contour lengths corresponds to unfolding of 58–100 amino acids, and the domain size range predicted from the SMART database is 66–82 amino acids, which is in good agreement with our experimentally defined size.

Calcium-binding sites in BapA

To obtain further insights into how calcium binds to the BapA domains, we performed server-based protein structure and function prediction for $BapA_{2–5}$ using RaptorX server (http://raptorx.uchicago.edu) [32]. As expected, the server predicted Ig-like domain structures for BIg domains in all segments (Fig. 3 shows the predicted domain structures for $BapA_{2–5}$ as an example). A prediction by Griessl *et al.* [15] suggested calcium-binding sites throughout the BIg domains of BapA based on sequence homology between BIg domains of SiiE and BapA (Fig. S3), which is consistent with the calcium-binding prediction done by the RaptorX server (Fig. 3). Both the predicted structures and the crystal structure of the triple BIg50:52 segment from SiiE [16] show that calcium may bind at the interface between two BIg domains inter-locking the β-strands (Figs. 3 and S3) or in folded BIg domains via RTX-motif interactions. As a test of the structure prediction software, we also performed the prediction for filamin A (FLNa) rod domain 1–8 and titin I27 that have known structures, and found that the server correctly predicted their structures. In addition, no calcium-binding sites were

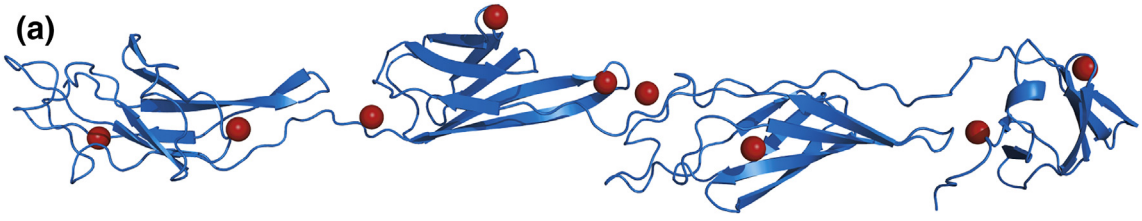

Fig. 3. Calcium-binding site prediction. Representative $BapA_{2–5}$ segment predicted structure by RaptorX server (http://raptorx.uchicago.edu). Red color spheres are the calcium ions, which are bound to the protein.

predicted for FLNa and I27 (Table. S1 and Fig. S8). These results suggest that the calcium-binding sites on the BapA Big domains are differentially exposed to calcium binding, which are likely associated with different calcium binding affinities.

Calcium binding is needed for stable Big domain folding

Whilst the results described in Fig. 2 were obtained from purified protein in calcium-free buffer solution, the Big domains were expressed and purified from *Escherichia coli* where the intracellular calcium concentration is ~100 nM [33]. There is a possibility that the folding of the Big domains was mediated by this level of calcium concentration in cells and that bound calcium ions remained bound in calcium-free buffer solution. To explore this possibility, we repeated the experiments in the presence of 10 mM EGTA in order to remove any potential bound calcium ions (Fig. 4; N.B. The experimental design throughout Fig. 4 is shown schematically in the inset). Figure 4a shows the representative force–height curves of $BapA_{18–26}$ in 100 mM KCl before introducing EGTA (force cycles C-1 and C-2, equivalent to Fig. 2a) and after introduction of 10 mM EGTA (force cycles C-3 and C-4). These experiments reveal a striking result that after the addition of EGTA (force cycles C-3 and C-4), the Big domains are no longer able to refold into stable structures upon release of force, indicated by the disappearance of unfolding steps at forces above 10 pN and a much longer extension at forces below 40 pN compared to the data from force cycles in C1 and C2. This is markedly different behavior from what was seen in Fig. 2, where repeated cycles of unfolding–refolding gave similar unfolding profiles. Some steps are observed below 10 pN, which indicates that the BapA rod domains may exist in a partially folded unstable conformation.

Following removal of EGTA, the force–bead height curves (force cycles C-5 and C-6) remained similar to those in force cycles C-3 and C-4 in the presence of EGTA, indicating that the loss of the stabilization of the Big domains is not caused by any potential interaction between EGTA and the unfolded polypeptide. These results suggest that (1) calcium binding is needed for the stabilization of the Big domains, and (2) these calcium ions can be rapidly removed by the chelating agent EGTA in the unfolded conformation of the Big domains (as

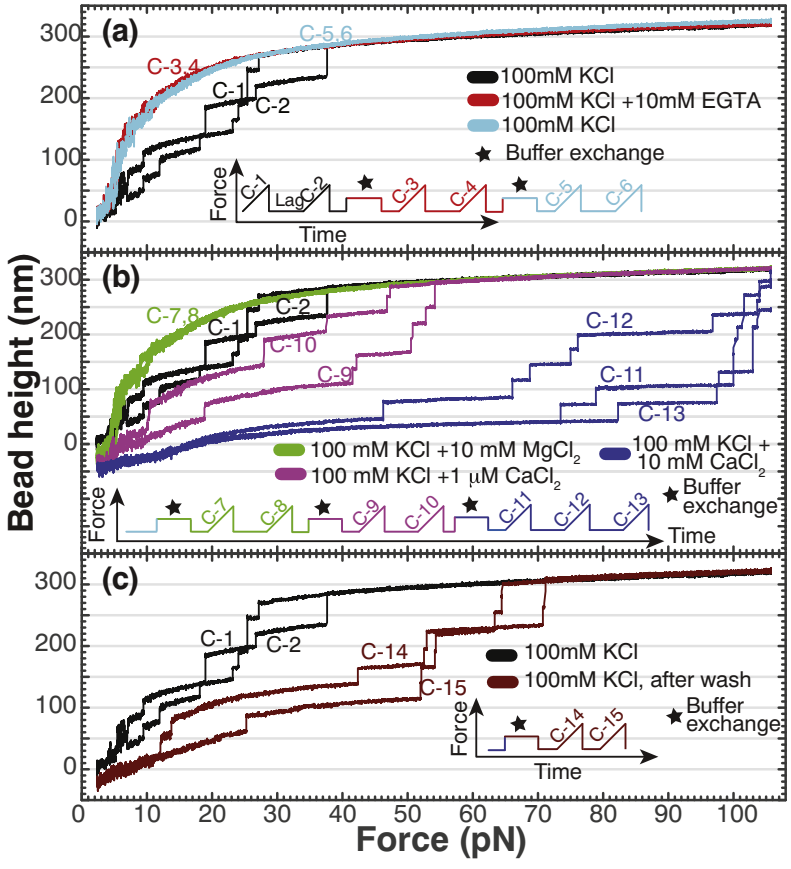

Fig. 4. Calcium-dependent $BapA_{18–26}$ domain folding. All data in this figure are taken from the same molecule, subjected to a series of different buffer exchanges. Inset: The experimental design shown as a schematic. Force–bead height curves are sequentially labeled C-1, C-2, … C15. (a) Domain folding is calcium dependent. Data obtained first in control buffer (black, C1 and C2), then switched to 10 mM EGTA (red, C-3 and C-4) and then back to control buffer (cyan, C-5 and C-6). The protein shows an unfolding profile in C-1 and C-2 but loses its unfolding steps following the addition of 10 mM EGTA (C-3,4) and remains unfolded when subsequently returned to control buffer (C-5,6). (b) Magnesium does not enable protein refolding. $MgCl_2$ buffer (10 mM; green, C-7 and C-8) as compared with control buffer (black, C-1 and C-2). Calcium-mediated protein folding going from green to magenta (1 μM $CaCl_2$, C-9 and C-10) and further stabilized by the addition of 10 mM $CaCl_2$ (blue, C-11 to C-13). (c) Calcium ions stay associated with the protein after washing with calcium-free control buffer (data shown in wine color (C-14 and C-15) as compared with data in black color)

shown in Fig. 4b). Thus, in the absence of calcium, the BIg domains exist in a partially folded conformation or disordered peptide conformation. Since calcium is needed for stable folding of the BIg domains, the fact that we observed stably folded domains for purified BapA rod segments in calcium-free buffer solution implies that the purified proteins have calcium ions bound to the BIg domains, which are retained during series of unfolding and folding cycles (Fig. 2).

Figure 4b shows that the loss of domain refolding in $BapA_{18–26}$ after treatment of EGTA could be rescued by reintroducing calcium (force cycles C-9 to C-13), but not magnesium (force cycles C-7 and C-8). This result suggests that the domain stabilization may specifically depend on calcium ions. At a low concentration (1 μM) of $CaCl_2$ (force cycles C-9 and C-10), the refolded domains unfold at comparable forces to that obtained from purified protein in calcium-free buffer solution (force cycles C-1 and C-2). Following this recapitulation of the unfolding response of the BIg domains by addition of calcium, we tested what would be the effect of adding additional calcium. Strikingly, at higher calcium concentration (10 mM $CaCl_2$), the mechanical stability of the domains is significantly enhanced, and the same numbers of unfolding steps are present but they unfold at much higher forces (force cycles C-11 to C-13). Similar effects of calcium were also observed for $BapA_{2–5}$ and $BapA_{6–17}$ (Fig. S4). Notably, we found that the folding of the BIg domains could be folded at concentrations as low as 100 nM (Fig. S5). These results reveal that the calcium-binding sites on the BIg domains have a wide range of affinities to calcium, allowing them to respond to different level of calcium concentrations.

Bound calcium is retained during unfolding/refolding cycles in calcium-free buffer solution

We have shown that calcium binding is needed for stable BIg domain folding, based on which we have inferred that the purified BapA rod segments have calcium ions bound to the BIg domains, which are retained during series of unfolding and folding cycles. To directly show that calcium ions can be retained after domain unfolding, after the force cycle C-13, free $CaCl_2$ in solution was removed by washing the channel (volume <100 μl) with a large volume (500 μl) of 100 mM KCl solution. In the absence of free calcium, the BIg domains could still refold in multiple unfolding/refolding force cycles (data from the first two cycles, C-14 and C-15, are shown in Fig. 4c). The unfolding forces (C-14 and C-15) of the refolded domains become smaller than in 10 mM $CaCl_2$ (C-11 to C-13) but higher than the purified proteins in calcium-free buffer solution containing 100 mM KCl (C-1 and C-2). This result confirms that BIg domains can retain calcium through multiple rounds of unfolding and refolding cycles after free calcium is removed from solution.

Overall, these results indicate that there are multiple calcium-mediated structural processes involved in the BIg domain folding and stabilization. Some of the high-affinity calcium-binding sites are required for BIg domain folding at low calcium concentrations, while some lower-affinity sites bind calcium at high calcium concentration that significantly increases the mechanical stability of the domains. Consistently, circular dichroism of $BapA_{2–5}$ confirms that the purified protein is already folded (Fig. S6), and addition of 5 mM $CaCl_2$ has little effect on domain folding as no additional secondary structure was observed. As the same numbers of unfolding steps are present (and no additional increase of secondary structure is seen via circular dichroism), we suggest that the additional increase in mechanical stability arises from binding of calcium ions to some lower-affinity calcium-binding sites.

High calcium concentration enhances the mechanical stability of the BapA rod domains

Our data show that BapA segments contain calcium-dependent folded domains that retain calcium throughout the purification process and even following unfolding of the tethered protein. Furthermore, as seen in Fig. 4b, further mechanical stabilization occurs upon exposure to high, millimolar, concentrations of calcium, indicated by much greater forces needed to unfold the domains compared to the same domains at μM calcium concentrations. The RaptorX structure and binding sites prediction server predicted different types of calcium-binding sites (Fig. 3). Since the mechanical stability of the BIg domains is dramatically enhanced when the calcium concentration increases from μM to mM, we reason that the lower-affinity calcium-binding sites are responsible for this enhanced mechanical.

To further explore this stabilization effect at high calcium levels, we tested the effect of 3 mM calcium on the three BapA segments. Representative force–bead height curves of $BapA_{2–5}$, $BapA_{6–17}$, and $BapA_{18–26}$ segments before (red) and after adding 3 mM $CaCl_2$ (blue) are shown in Fig. 5a. For the $BapA_{2–5}$, $BapA_{6–17}$, and $BapA_{18–26}$ segments, 4, 12, and 9 unfolding steps were observed, respectively. The addition of millimolar calcium to the chamber results in a marked mechanical stabilization of the BIg domains in all three segments. For $BapA_{2–5}$, the unfolding forces are shifted from 5 to 50 pN to 40–90 pN, whereas for $BapA_{6–17}$ and $BapA_{18–26}$, the unfolding forces are shifted from 40 to 80 pN to 80–120 pN. In all cases, the unfolding forces significantly shift to higher range after adding calcium. Strikingly, no additional unfolding steps are observed and there is no change of contour length after addition of 3 mM $CaCl_2$ (Fig. S4).

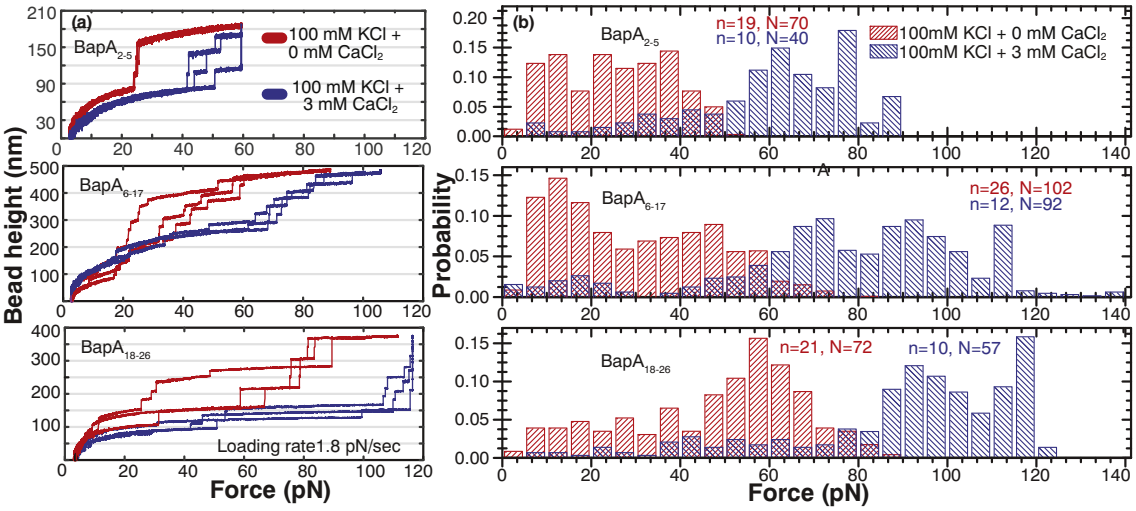

Fig. 5. The effect of calcium on the mechanical stability of the BapA domains. Top, middle, and bottom panels show $BapA_{2–5}$, $BapA_{6–17}$, and $BapA_{18–26}$ protein segments, respectively. (a) Representative force–bead height curves before (red) and after (blue) introduction of 3 mM $CaCl_2$. (b) Probability distribution of unfolding forces (i.e., histograms of unfolding forces normalised by the total number of unfolding events), before (red) and after (blue) introduction of 3 mM $CaCl_2$. Where n is the number of independent protein tether and N is the number of cycles.

Discussion

Bacterial cells adhere to surfaces and to cells, and are subject to mechanical constraints (e.g. shear force applied by body fluid or shear force by the environment). In these conditions, when attached, bacterial adhesion proteins will be under mechanical force. Therefore, understanding the mechanical properties of BAPs is important to understand their adhesive functions.

It has been suggested that BapA and similar surface proteins are involved in bacterial adhesion [8]. BapA is classified as a non-fimbrial adhesion protein secreted by the type 1 secretion systems (T1SS) in *Salmonella* [10]. It has been shown that disruption of a similar surface protein Bap (*S. aureus*) by proteinase can impede the initial bacterial adhesion, as well as biofilm dispersal [34]. Over-expression of BapA (*S. enteritidis*) causes pellicle formation, and disruption of BapA causes inability to form biofilm in LB medium [10].

Several interesting findings were revealed from this study, namely, (1) calcium is needed for the folding of the BIg domains, (2) the intracellular calcium level is sufficient for folding of the BIg domains, and (3) increasing concentration of the calcium enhances the mechanical stability of the BIg domains, with millimolar calcium concentrations being required for maximal stability. Unexpectedly, bound calcium can be retained during cycles of unfolding and refolding transitions. These findings collectively underscore the importance of calcium in regulating the folding/stability of BapA BIg domains. Since the BAP domains share strong sequence identity and similarity, the calcium-dependent folding and stabilization may be a shared property across the bacterial kingdom.

The finding that the BapA BIg domains requires sub-μM calcium concentration for folding and higher concentration for enhanced stabilization implies the existence of multiple types of calcium-binding sites with different functions—the higher-affinity ones mediate structural folding of the domains and the lower-affinity sites regulate the stability.

Our results suggest that the BapA BIg domains have a wide range of mechanical stabilities. Although the BIg domains are predicted to have similar ternary structures, they still have significant differences in sequence—they share only about 30% of sequence identity (Fig. S1). They also differ in the number of residues in the domains. These factors could affect the exact unfolding transition pathways and the unfolding energy barrier, resulting in a variety of mechanical stability of the domains.

Our data suggest that the BapA BIg domains are likely folded intracellularly. It was previously suggested that the intracellular calcium concentration is ~100 nM [13,35,36]. Consistently, we found that calcium at concentrations as low as 100 nM is sufficient to fold the BIg domains (Fig. S5). Such calcium-mediated protein folding and mechanical stabilization is not observed for magnesium (Fig. S7), indicating selectivity of calcium over magnesium and perhaps other divalent cations.

Interestingly, a recent publication reported that the ice-binding surface protein mpAFP from *Marinomonas* primoryensis contains calcium-binding sites and that calcium binding is needed for mechanical stability of the

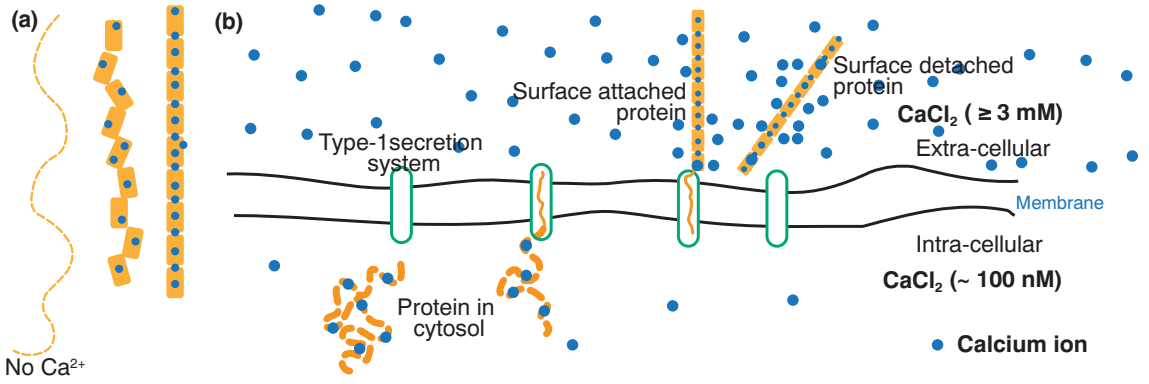

Fig. 6. BapA protein secretion through the membrane. (a) Cartoon representation of the three states of BapA. Left: In the absence of calcium BapA is unstructured. Middle: At ~100 nM calcium concentration, the BIg domains fold but are joined by flexible linkers. Right: At 3 mM calcium concentration, the domains interact to form the rigid, mechanically strong, rod-like structure. (b) BapA exists as a series of domain-like beads on a string in the cytosol or less stable folded domains, where the calcium ion concentration is lower than ≤100 nM. Following translocation across the membrane into the extracellular environment, where the calcium concentration is ≥3 mM, the BapA adopts its mechanically stable conformation either tethered to the bacteria or, following cleavage or complete secretion, as a surface detached protein that forms part of the biofilm matrix.

mpAFP rod domain [37,38]. In contrast to the BapA rod domain studied in this work that contains heterogeneous tandem repeats of Ig-like structures, the mpAFP rod domain consists of identical repeats of 104 amino acids that form a continuous β-solenoid structure. In addition, previous studies on the rod domains of SiiE from *Salmonella* [15] and Bap from *S. aureus* [16] also revealed the importance of the calcium binding on the stability of the domains. Together with these previous studies, our results suggest that calcium-assisted stabilization of secreted bacterial surface protein could be a generic phenomenon.

Our results indicate that the purified BIg domains retain calcium from *E. coli* that maintain the folded domains. Consistently, we found that the BapA BIg domains could be folded at intracellular calcium concentrations of ~100 nM [33]. Strikingly, our results reveal a dramatic increase in mechanical stability of the domains when the calcium concentration is further increased to 3 mM, levels that resemble the conditions of the extracellular environment. This result suggests that on a partially translocated BapA, the extracellular BIg domains are more stable than the intracellular BIg domains. This also suggests that maximal stability, and likely rigidity, of the BapA protein is only achieved extracellularly. Therefore, translocation of the BapA could result in reduction of the free energy, driving the directional translocation of the BapA protein (Fig. 6). Interestingly, BapA contains a signaling peptide at the C-terminal that can be recognized by the ABC transporter [10], an ATPase associated with the T1SS system that is known to transport long multidomain peptides. Therefore, it is possible that the translocation of the protein domains may also be powered by the energy released from the ATP hydrolysis of ABC transporter.

We observed that calcium ions bound on the BIg domains could remain bound for a long time in solution without free calcium. Our experiments in calcium-free buffer (Fig. 2a) demonstrate that BIg domains retain calcium from expression in *E. coli*. Even following unfolding by mechanical force, these domains can retain the calcium ions to facilitate refolding. While the slow dissociation of calcium ions in calcium-free buffer solution seems surprising, it is consistent with a series of recent studies revealing that the dissociation rate of a bound ligand on a substrate in the absence of free competitive ligand/substrate in solution can be a few orders of magnitude slower than that in the presence of competition [39]. The mechanisms underlying these observations are still unclear and are currently a hot topic of theoretical study [40–42].

Adhered bacterial cells are likely subject to external mechanical perturbation and as a result large conformational deformation. Our results show that under force, the BapA domains will undergo unfolding transitions. BapA is a large protein and comprises 3824 amino acids, and a fully unfolded BapA could release a contour length of ~1.3 μm. A recent study has suggested that any force-transmitting protein containing an array of modular domains has the capacity to act as a molecular mechanical absorber, buffering force within a certain range over a large range of strain change [43,44]. The large multi-domain composition of BapA suggests that it may also serve as a mechanical absorber. This may provide flexibility within the biofilm and at the interface between

Salmonella and substrates, which may be beneficial for biofilm to resist mechanical perturbations [43].

Interestingly, force has been reported to play a crucial role in the formation and maintenance of biofilm. For example, force increases the bacterial adhesion to surfaces [45] and to cells [46,47]. These results suggest that components in biofilms can sense and convert mechanical cues to cellular responses. It remains unclear which biofilm components are responsible for biofilm mechano-sensing. We reason that BapA and similar surface proteins with Ig-like domains may take a part in this process, as Ig domains often provide binding sites for other partners. The conformational change of BapA induced by mechanical perturbation observed in our experiments suggests possible force-dependent interactions between BapA and other binding partners.

Materials and Methods

Protein purification

Plasmid pFN18A (Promega) was used to construct the vectors and co-expression with biotin holoenzyme synthetase plasmid BirA. Briefly, the relevant domain fragments were amplified from *S.* Typhi (str. 14028S) using PCR. An AviTag and 6x His-tag coding sequence was added at the C-terminal of the protein segments. The primers used to construct these segments are listed in Appendix Table S2. pFN18A plasmid was cut at PmeI and SgfI restriction sites followed by ligation with PCR products of BapA subdomains. DH5α cells were used for cloning of $BapA_{2–5}$, and BL21(DE3) competent cells were used for expression. Single-step competent cells KRX (Promega) were used for cloning and expression for $BapA_{6–17}$ and $BapA_{18–26}$. Bacteria were grown in LB medium at 37 °C in the presence of ampicillin (100 μg/ml) with orbital shaking. When the OD_{600} of the culture reached 0.5, the temperature was reduced to 21 °C. The culture was supplemented with the addition of biotin (final concentration 50 μg/ml). The cells were induced overnight by the addition of 0.5 mM IPTG. Harvested bacteria were resuspended in 10 mM Tris (pH 7.4), 250 mM KCl, and 0.5 mM PMSF and disrupted by French press. Protein purification was done with Ni^{2+}-NTA column followed by gel filtration. The quality and purity of the protein were confirmed by 4%–12% SDS-PAGE and mass spectrometry.

Force calibration

Force calibration was carried out using the method detailed in our previous publication [24] and has an intrinsic relative error of $<15\%$ (due to heterogeneous bead size). The loading rate control was achieved by programming the movement of the magnet in such a way that the loading rate is a constant. Briefly, in magnetic tweezers experiments, force is a function $F(d)$ of bead–magnet distance d. Therefore, one can change the force by changing d. A time varying force can be applied by changing d with time. In a loading rate experiment, the force is changed linearly with time with a slope of r, which is called the loading rate. In a typical loading rate experiment, force was increased from an initial value F_0 through a time trajectory $g(t) = F_0 + rt$. In order to achieve loading rate control, one can correspondingly program the movement of the magnets through a trajectory of $d(t) = F^{-1}(g(t))$, where F^{-1} is the inverse function of $F(d)$. In our instrument, $F(d)$ has been calibrated (see Chen *et al.* [24] for details), based on which the constant loading rate control was achieved using LabVIEW program to move the magnets attached on a linear translational motor.

Single-molecule manipulation

Experimental setup was as described elsewhere [48]. Briefly, coverslips are cleaned with detergent, acetone, and isopropanol for 30 min each followed by plasma treatment. Coverslips are then incubated with 1% APTES in methanol and acetone solution for 20 min; after washing, the coverslips are cured at 110 °C for 1 h. Channels were prepared as described previously and have a volume ~40 μl [48]. Flow channels are treated with 0.1% glutaraldehyde for 1 h followed by incubation with 3-μm polyamine beads and then halo-tag ligand (Promega). The channels were blocked with 25 mM Tris (pH 7.4), 100 mM KCl, 1% BSA, and 0.02% T-20 (control buffer). Protein segments were introduced into the flow channel in control buffer and incubated for 15 min. Free protein was washed from the channel and then 2.8-μm streptavidin-coated super-paramagnetic beads (M-270; Invitrogen) were added into the channel. Force was calibrated for each tether before the experiment. A constant loading rate of ~1.8 pN/s was applied in all experiments [24]. Note: The magnetic tweezers detect the height change of bead, which has an offset to the actual extension change of the molecule. In experiments, we set the extension to be zero at forces below 1 pN. The uncertainty of the offset does not affect the measurement of protein unfolding.

$CaCl_2$ (1 μM, 3 mM, or 10 mM) was added to control buffer for calcium experiments as required. For buffer switch experiments (Fig. 4), protein was held at a force ~12 pN and buffer of 300–500 μl was applied depending on buffer condition and concentration.

CRediT authorship contribution statement

Durgarao Guttula: Investigation, Formal analysis, Writing - original draft. **Mingxi Yao:** Investigation.

Karen Baker: Investigation. **Liang Yang:** Conceptualization. **Benjamin T. Goult:** Formal analysis, Investigation, Writing - original draft. **Patrick S. Doyle:** Conceptualization, Supervision, Writing - original draft. **Jie Yan:** Conceptualization, Formal analysis, Supervision, Writing - original draft.

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Appendix A. Supplementary figures and tables

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Abbreviations used:
FLNa, filamin A; BAPs, Biofilm-associated proteins; BIg, bacterial Ig-like domain; T1SS, type 1 secretion system.

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