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Single molecule force spectroscopy reveals the effect of BiP chaperone on protein folding

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Received 1 November 2016; Accepted 3 February 2017
DOI: 10.1002/pro.3137
Published online 8 February 2017 proteinscience.org

Abstract: BiP (Immunoglobulin Binding Protein) is a member of the Hsp70 chaperones that participates in protein folding in the endoplasmic reticulum. The function of BiP relies on cycles of ATP hydrolysis driving the binding and release of its substrate proteins. It still remains unknown how BiP affects the protein folding pathway and there has been no direct demonstration showing which folding state of the substrate protein is bound by BiP, as previous work has used only peptides. Here, we employ optical tweezers for single molecule force spectroscopy experiments to investigate how BiP affects the folding mechanism of a complete protein and how this effect depends on nucleotides. Using the protein MJ0366 as the substrate for BiP, we performed pulling and relaxing cycles at constant velocity to unfold and refold the substrate. In the absence of BiP, MJ0366 unfolded and refolded in every cycle. However, when BiP was added, the frequency of folding events of MJ0366 significantly decreased, and the loss of folding always occurred after a successful unfolding event. This process was dependent on ATP and ADP, since when either ATP was decreased or ADP was added, the duration of periods without folding events increased. Our results show that the affinity of BiP for the substrate protein increased in these conditions, which correlates with previous studies in bulk. Therefore, we conclude that BiP binds to the unfolded state of MJ0366 and prevents its refolding, and that this effect is dependent on both the type and concentration of nucleotides.

Keywords: BiP chaperone; optical tweezers; force spectroscopy; binding parameters; nucleotides dependence

Abbreviations: BiP, immunoglobulin binding protein; DTDP, 2,2'-dithiodipyridine; ER, endoplasmic reticulum; Hsp70, heat shock protein 70; NBD, nucleotide binding domain; SBD, substrate binding domain.

Additional Supporting Information may be found in the online version of this article.

María Paz Ramírez and Maira Rivera contributed equally to this paper

Grant sponsor: Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt), Chile; Grant numbers: Project 11130263 (CW), Project 1151274 (MB), Project 11110534 (MB), Project 3160645 (DQ); Grant sponsor: Project CONICYT + NERC + Programa de Colaboración Internacional; Grant number: PCI-PII20150073 (CW); Grant sponsor: U-inicia from the Vicerrectoría de Investigación Universidad de Chile (CW); Grant sponsor: CONICYT-PCHA/MagísterNacional; Grant number: 2015-22151448 (MPR); Grant sponsors: Aid for short stays for research abroad, International Engagement, Vice-Presidency of Academic Affairs, University of Chile (MPR); Grant sponsor: CONICYT-PCHA/DoctoradoNacional; Grant number: 2013-21130254 (MR); Grant sponsor: CONICYT-PCHA/DoctoradoNacional; Grant number: 2014-21150966 (AB).

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Introduction

There are many types of chaperones in the cell. One of them, the Hsp70 family, is a group of conserved chaperones with ATPase activity that is essential for protein folding.[1] All Hsp70 family members are considered to be molecular motors and are mostly found as monomers. These chaperones are present in prokaryotes, eukaryotes, and archaea, and exhibit a conserved action mechanism.[2] One of the most extensively studied members of the Hsp70 family is the Immunoglobulin Binding Protein (BiP, Kar2p in yeast). BiP resides inside the endoplasmic reticulum (ER) and is considered to be the master regulator of the ER.[3,4] This chaperone plays a role in assisting protein translocation into the ER by binding to nascent proteins, by participating in the activation of the Ire1-α pathway in the unfolded protein response (UPR) and in the retrograde transport of aberrant proteins destined for proteasome dependent degradation across the ER membrane.[5,6]

BiP has two domains, an N-terminal ATPase domain, known as the nucleotide binding domain (NBD), and a C-terminal substrate binding domain (SBD). The NBD hydrolyzes ATP to ADP and inorganic phosphate (Pi), and the SBD binds to polypeptides with diverse sequences, allowing BiP to interact with a wide variety of unrelated nascent polypeptides. In particular, it binds to a heptameric motif, Hy(WX)HyXHyXHy, where Hy is a bulky aromatic or hydrophobic residue, W is tryptophan, and X is any amino acid.[7] The SBD has two subdomains, α and β. The β subdomain has a binding pocket for polypeptides and the α subdomain acts as a lid that covers it.[8] BiP's activity depends on an ATPase cycle, where ATP binding regulates the affinity and kinetics of substrate binding to the SBD. When ATP is bound to BiP, the lid is open and decreases the affinity for the substrate peptide. However, when ATP is hydrolyzed to ADP the lid closes and increases the affinity for the polypeptide.[9,10] The exchange of ADP to ATP finally leads to the release of the peptide.[9]

Despite the key role of BiP as a chaperone, some important features still remain unknown, including the binding mechanism to its substrate. Although structural and functional studies throughout the years revealed valuable information about where BiP binds and how it affects protein folding pathways, it has not been directly demonstrated yet whether BiP binds to folded or to unfolded protein substrates. BiP recognizes linear motifs enriched in hydrophobic residues,[7] which due to their hydrophobic nature are most likely located in the interior of a folded protein. However, since BiP interacts with early and less compactly folded intermediates,[11] it has been proposed that BiP binds to the unfolded state of proteins. At the same time, there are several intrinsically disordered proteins that expose hydrophobic patches,[12] so this assumption cannot be generalized to all proteins. Also, studies to explore BiP's binding kinetics and affinities have mostly been done using peptides rather than complete proteins.[13,14] No direct observation has been carried out so far, which is partially due to the experimental challenge to prepare protein substrates that can be driven to an unfolded state in a controlled and non-interfering way. In addition, most investigations on the mechanism of the BiP chaperone are based on bulk experiments, which often mask the heterogeneity inherent to the populations of macromolecules. Single molecule approaches, especially single molecule force spectroscopy experiments, thus present an ideal approach to study the mechanism of BiP, as they allow for both reversibly driving a protein substrate to an unfolded state and detecting the binding and folding events of individual proteins.

Previous studies at the single molecule level have proven useful to probe the function and conformational changes in chaperones. Studies of Hsp70 family members using single molecule smFRET (single molecule Förster Resonance Energy Transfer), for instance, have shown the relationship between domain movements and nucleotide binding in the yeast BiP chaperone.[15] A different study by Bechtluft *et al*.[16] using optical tweezers showed a clear effect on the folding of maltose binding protein upon binding of the chaperone SecB. It was shown that SecB binds to the unfolded protein and prevents it from completely refolding.

Here, we employ single molecule force spectroscopy with optical tweezers to study BiP's function as a chaperone, and to determine how it affects protein folding *in singulo*. The substrate protein selected for BiP was the well characterized[17] protein MJ0366, a hypothetical cell-expressed knotted protein from *Methanocaldococcus jannaschii*, which was used for several reasons. First, MJ0366 was found to be easily modified with DNA handles for optical tweezers experiments. Second, its mechanical unfolding and refolding behavior has been well characterized in our lab at the single molecule level and exhibits clearly detectable folding and unfolding events [Fig. 1(B)], which is crucial for this study. Third, MJ0366 has most likely just one putative binding site for BiP (Supporting Information Fig. S3) allowing a simpler analysis and interpretation of the data. Our results show that binding of BiP prevents the formation of tertiary contacts within MJ0366 and therefore, the refolding back to its native state. Furthermore, we show that this effect is dependent on the concentration and type of nucleotide present in solution, which correlates with previous bulk experiments. This is the first study, to our knowledge, done using a complete protein to

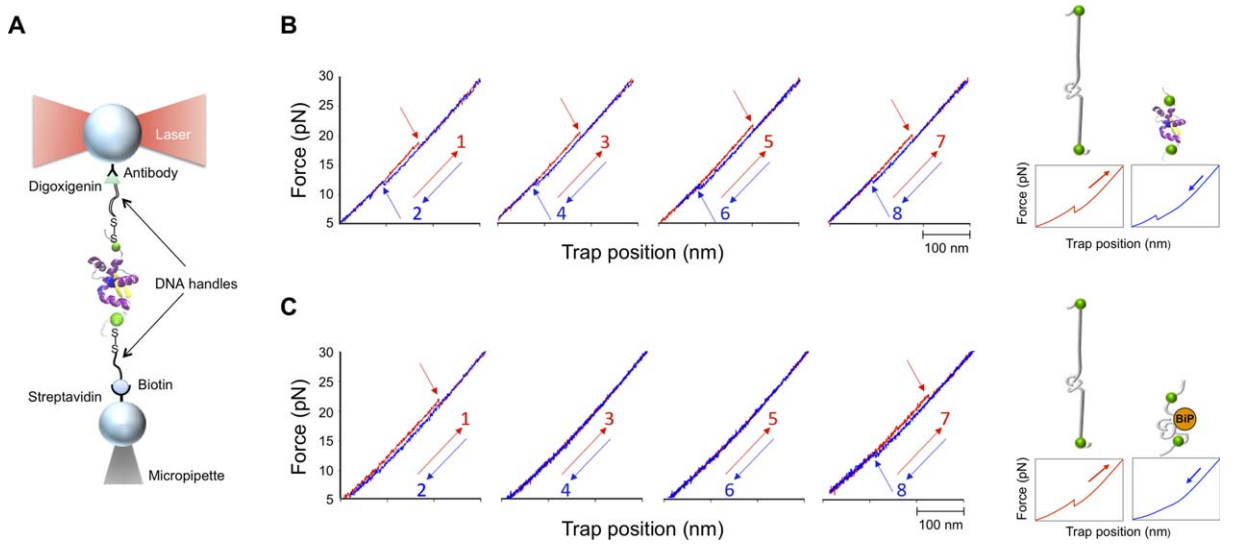

Figure 1. Experimental optical tweezer setup and the effect of BiP binding on MJ0336 folding and unfolding. A: The substrate protein MJ0336 is tethered between two dsDNA handles through disulfide bonds at its N and C terminus. These handles are modified at the 5′ ends with digoxigenin and biotin to bind the respective polystyrene beads coated with anti-digoxigenin and streptavidin, respectively. The anti-digoxigenin bead is held in an optical trap and the streptavidin bead is attached to a micropipette by suction. B: At the left, force-extension curves showing the consecutive pulling cycles of MJ0336 at a constant speed of 100 nm/s in the absence of BiP. At the right, a schematic representation of the mechanical unfolding and refolding of MJ0366, with the respective unfolding and refolding events. C: At the left, force-extension curves of MJ0366 in the presence of BiP. At the right, a schematic representation of the mechanical unfolding and inhibition of refolding of MJ0366 due to the binding of BiP (orange) to the unfolded state, with their respective unfolding and lost refolding event. Pulling traces where unfolding occurs at high forces are shown in red and relaxation traces with refolding events at lower forces are shown in blue. In the upper image (B), the force spectroscopy experiments without BiP and with 2 m*M* ATP show that the pulling of the protein leads to its unfolding and later refolding in all cycles. In the lower image (C), experiments were performed with 1 μ*M* BiP and 2 m*M* ATP, and unfolding and refolding events were lost (second and third force-extension curves), and regained after some time (fourth force-extension curve).

directly explore BiP's binding mechanism with single molecule manipulation technique.

Results

BiP purification

Due to the large quantity of active protein required to perform the optical tweezers experiments, we developed the BiP purification protocol described in the material and methods section. With this protocol ~3 mg of purified BiP per liter of culture were obtained. The final purified product was assessed by a 10% SDS-PAGE (Supporting Information Fig. S1). Finally, to confirm that the purified BiP was active after the final purification steps, an ATPase assay was performed with a spectrophotometrical coupled assay measuring the formation of a formazan blue precipitate (Material and Methods).

Robust detection of reversible MJ0336 unfolding and folding cycles in force extension traces

In the single molecule force spectroscopy experiments, the stretching of a protein first results in a rise in force caused by the entropic elasticity of the linker molecules. Once the force is high enough, a protein domain or the entire protein unfolds, which results in a drop in force and in a sudden increase in the molecule's extension. If further stretched, the force again rises due to the combined entropic elasticity of the linker molecules and the unfolded polypeptide chain. Both the rupture force as well as the increase in contour length (ΔL_c) upon unfolding have characteristic values that depend on the energy barrier between the folded and unfolded state and the number of unfolded amino acids, respectively. Conversely, the refolding of a protein, which usually occurs at lower forces due to the non-equilibrium conditions of the experiment, results in an increase in the force and a decrease in the extension.

As shown in Figure 1(B), we were able to perform multiple unfolding and refolding cycles with a single MJ0336 protein molecule with up to 100 repeats. In each cycle, the unfolding as well as refolding of MJ0336 resulted in a clear and quantifiable fingerprint with an average unfolding and refolding force of 19.5 ± 3.0 pN and 10.8 ± 0.9 pN, respectively, and a ΔL_c of 25.4 ± 1.5 nm, which correlates well with the theoretical value of 24.8 nm. Importantly, in all of the traces both unfolding and refolding were observed, which demonstrates that the robust refolding of MJ0336 is detected with 100% efficiency [Figs. 1(B) and Fig. 2].

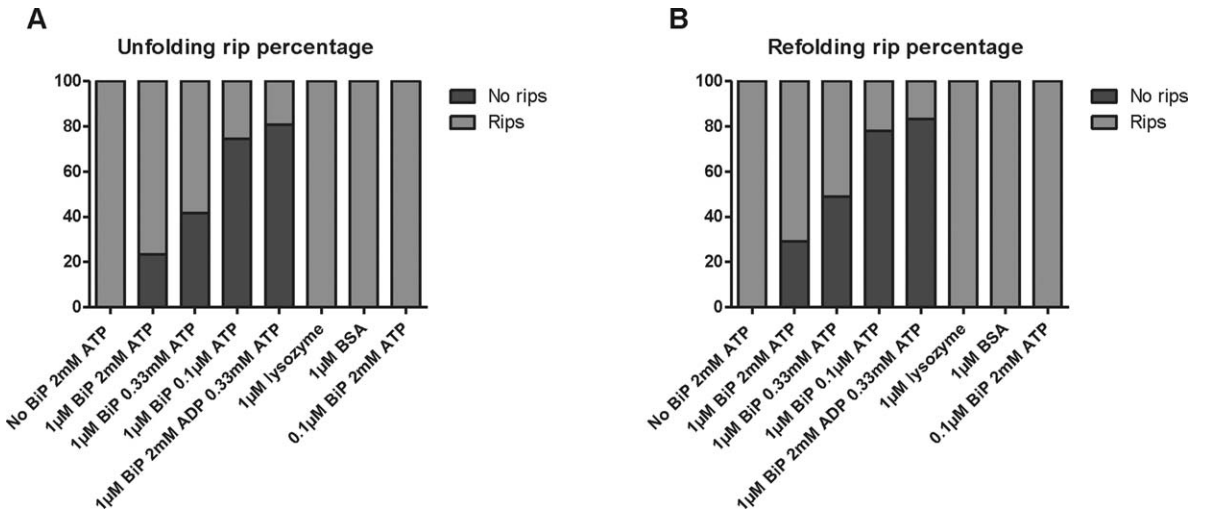

Figure 2. Frequency of unfolding and refolding events at different conditions. For all eight conditions, the frequency of unfolding and refolding events ("rips") was quantified, as well as the loss of these events. A: The frequency of unfolding and (B) refolding events was compared at different buffer conditions.

The chaperone BiP specifically binds to the unfolded substrate MJ0336 and inhibits its refolding

Having established a robust assay to observe reversible unfolding and refolding cycles of the protein substrate MJ0336, we investigated the effect of the chaperone BiP upon binding. In the presence of 1 μ*M* BiP we observed a decrease of the unfolding and refolding events [Fig. 1(C)], which is in contrast to experiments in the absence of BiP. In the presence of BiP, the percentage of observable unfolding and refolding events during the stretching and relaxing of the protein was 76.6% and 70.9%, respectively (Fig. 2). During these stretching and relaxing cycles to mechanically unfold and refold MJ0366, events were lost for periods of time. The loss of these events occurred almost exclusively after an observable unfolding event (Supporting Information Table S2), as shown in Figure 1(C) where it is the refolding event that is lost first, meaning that BiP bound to MJ0366 after it unfolded. Therefore, we conclude that BiP inhibits the refolding of the protein substrate unfolded with optical tweezers. The fact that no changes in the force distributions of the observed events occurred supports the proposal that BiP preferentially binds to the unfolded state of the protein substrate (Supporting Information Table S4). In order to rule out nonspecific effects of BiP the mechanical unfolding of MJ0366 was assayed in the presence of 1 μ*M* lysozyme and 1 μ*M* BSA, which do not have binding sites for MJ0336. With each of these proteins no unfolding or refolding events were lost (Fig. 2, Supporting Information Table S1), confirming that the interaction between BiP and MJ0336 is specific. In both control experiments, the unfolding forces were measured to be 16 ± 3 pN and 15 ± 2 pN, which agrees with the unfolding forces in the absence of protein in solution within the experimental error. The refolding forces also remained the same (Supporting Information Table S4).

The dissociation kinetics of BiP is dependent on the type and concentration of nucleotides

The chaperone activity of BiP requires cycles of ATP hydrolysis to drive the binding and release of its substrate proteins. When ATP is bound to the NBD, BiP is in a low affinity conformation for its substrates with high on and off rates,[18] while the converse occurs in the presence of ADP. To investigate the effect of nucleotides on the affinity between BiP and MJ0366, decreasing concentrations of ATP were used (0.33 m*M* and 0.1 μ*M*) in addition to the original concentration of 2 m*M*. The length of time that BiP remained bound to MJ0366 was reflected in the loss of refolding and unfolding events, and was directly quantified from the extension vs. time traces. As the ATP concentration decreased (2 m*M*, 0.33 m*M*, and 0.1 μ*M*) BiP would stay bound to MJ0336 for longer periods of time (49.40 ± 8.03, 63.75 ± 17.48, and 111.44 ± 35.40 sec, respectively), which in turn lead to a decrease in the overall frequency of unfolding and refolding events (76.6%, 58.4%, and 25.6%; 70.9%, 51.0%, and 22.1%, respectively) (Fig. 2, Supporting Information Table S1). Therefore, we conclude that the presence of ATP promotes an open conformation of the SBD of BiP, which in turn leads to a decreased affinity for the protein substrate. Also, when 2 m*M* ADP was added to a solution with 1 μ*M* BiP and 0.33 m*M* ATP as a competitor, BiP stayed bound to MJ0336 even longer (139.12 ± 43.64 sec) and the loss of unfolding and refolding events was even greater (19.2%; 16.8%) (Fig. 2, Supporting Information Table S1). The

Table I. *Kinetic Parameters for BiP in Different Conditions*

Condition	app K_D (μM)[a]	app K_D (μM)[b]	k_{on} (μM^{-1} sec^{-1})	k_{off} (sec^{-1})
1 μM BiP 2 mM ATP	3.37 ± 0.70	1.75 ± 0.43	0.012 ± 0.0022	0.021 ± 0.0034
1 μM BiP 0.33 mM ATP	1.43 ± 0.25	1.31 ± 0.50	0.013 ± 0.0035	0.017 ± 0.0046
1 μM BiP 0.1 μM ATP	0.35 ± 0.06	0.33 ± 0.15	0.030 ± 0.0098	0.0099 ± 0.0032
1 μM BiP 2 mM ADP 0.33 mM ATP	0.24 ± 0.05	0.28 ± 0.12	0.029 ± 0.0082	0.0080 ± 0.0025

[a] app K_D calculated with Eq. (2).
[b] app $K_D = k_{off}/k_{on}$.

average unfolding and refolding forces were not affected by the concentration of nucleotides.

We further analyzed the binding and unbinding of BiP by assuming a first order reaction and by calculating the dissociation (k_{off}) and association rates (k_{on}) constants from the mean absence and residence times. The off rate k_{off} (0.021 ± 0.0034, 0.017 ± 0.0046, 0.0099 ± 0.0032 sec^{-1}) decreased with the decrease in ATP concentration, being lowest in the presence of ADP (0.0080 ± 0.0025 sec^{-1}). Using the ratio between the k_{off} and k_{on}, the apparent K_D was calculated (Table I), which confirmed that at lower concentrations of ATP or in the presence of ADP the affinity for the substrate protein increased. Also, we were able to calculate the apparent K_D (Table I) from the unfolding and refolding event probabilities (Fig. 2, Supporting Information Table S1), assuming one binding site for BiP. This provides evidence of a direct relationship between the NBD and SBD, since the conformation that the NDB adopts in the presence of a certain nucleotide leads to a conformational change in the SBD that affects its affinity for the substrate protein. Taken together this data shows at a single molecule level that the affinity between BiP and MJ0336 changes with different types of nucleotides and their concentration.

Discussion

Our results show that BiP binds to the unfolded state of MJ0366 in a reversible manner, and that its affinity is dependent on the type of nucleotide bound to the NBD and its concentration. When the chaperone was added, the unfolding and refolding events of MJ0366 were lost for a period of time, which is in agreement with the results of Betchluf *et al*.[16] With these results and the observation that loss in folding always occurs after successful unfolding [as in Fig. 1(C)], we conclude that BiP binds to the unfolded state of MJ0366. In addition, if BiP were to interact with the folded state, the unfolding forces would most likely change in its presence,[19–21] which is not observed in our experiments. Our conclusion is consistent with the fact that BiP, as a chaperone, binds to unfolded proteins stabilizing their unfolded state until they spontaneously fold. BiP binds to a heptameric motif of hydrophobic/aromatic amino acids, which are most likely hidden while MJ0366 is folded and are exposed once it unfolds. This allows BiP to interact with the substrate protein after the unfolding process and to hinder the formation of tertiary contacts, which is thought to be its primary role as a chaperone.

When the concentration of ATP was decreased, the binding frequency of BiP increased. This resulted in a decrease in the dissociation rate and in the frequency of unfolding and refolding events, meaning that the affinity for the protein increased. When ADP was added to the solution its effect was similar to that of 0.1 μM ATP. At a single molecule level this shows that as we decrease the concentration of ATP, the conformational state of the SBD becomes more and more similar to the one of BiP with ADP bound to the NBD, which is supported by previous studies by Marcinowsky *et al*.,[15] using smFRET. The calculated k_{off} in the different experimental conditions are comparable to the ones determined by other studies.[14] The calculated K_D is an apparent K_D, as it may be convoluted by two factors, which are the number of binding sites and the force. In our analyses a 1:1 stoichiometry was assumed between BiP and MJ0366 based on the sequence analysis of MJ0366 for possible binding sites and the exponential decay distribution obtained for the residence times (Supporting Information Fig. S4). The analysis shows that there is only one site that perfectly fits the motif. Consequently, the other potential binding sites would have lower affinity, and given the size of BiP (it has a 10–8 nm diameter in its open conformation[13]) and how close the other sites are to each other and to the proposed binding site, there would only be enough room for BiP to bind at a single location due to steric hindrance. Regarding the effect of the force on the K_D, previous studies with peptides have shown that BiP binds to its substrates in an extended conformation.[13] The force applied to the protein substrate will extend it, so this should not affect BiP's binding. At the low forces accessible with optical traps, the unfolded substrate still behaves as a stretched random coil according to the worm-like chain model. Only at forces above 500 pN–1 nN achievable with atomic force microscopy, the structure of the polypeptide backbone and spacing between amino acids comes into play.[22] Since our calculation of apparent K_D is averaged over a 10–20 pN force range, the force is not likely to be a significant factor in our calculation.

Through single molecule manipulation of a substrate protein for BiP, we have determined that this

chaperone binds to the unfolded state of a protein, which other studies had indirectly shown but had not directly proven as in this study. Using optical tweezers, we were further able to measure the affinity, association, and dissociation rates, and to compare the results with previous studies using smFRET.[15]

Materials and Methods

MJ0366 construct preparation

An *E. coli* codon optimized synthetic gene was synthesized corresponding to the coding sequence of MJ0366 (GenScript, USA) and was subcloned into a modified pET-21b vector between NcoI and EcoRI sites, resulting in a fusion with the MJ0366 reading frame, followed by a TEV protease cleavage site and His-tag at the C-terminus. Phe6 and Gly89 were mutated to cysteine and the wild type Cys81 was mutated to alanine, using the QuikChange site-directed mutagenesis kit (Invitrogen, USA).

MJ0366 expression and purification

The plasmid was transformed into a BL21 *E. coli* strain. Protein overexpression was induced at an OD_{600} of 0.5–0.7 by incubation with 0.5 m*M* isopropyl-β-D−1-thiogalactopyranoside (IPTG) for 3 h. The cells were harvested by centrifugation for 10 min at 5000 rpm at 4°C using a Sorvall RC-5B centrifuge with a GSA rotor. For the protein purification, cells were resuspended in lysis buffer (10 m*M* Tris-HCl pH 8.0; 8*M* urea; 50 m*M* Na_2HPO_4 pH 8.0; 40 m*M* imidazole; 10 m*M* β-mercaptoethanol; 400 m*M* NaCl) and incubated 1 h at room temperature (RT) with constant agitation at 200 rpm. Suspended cells were disrupted by sonication at 40% power and 50% rest time for 10 min (Sonic ruptor 250, Omni international, USA) and the cell debris and supernatant were separated by a second centrifugation step at 11,000 rpm for 20 min at 4°C using a Sorvall SS-34 rotor. The supernatant was loaded onto a prepacked 5 mL His-Trap HP column (GE Healthcare Life Science, USA) previously equilibrated with lysis buffer. To remove protein impurities, the loaded column was washed with 20 volumes of washing buffer (10 m*M* Tris-HCl pH 8.0; 8*M* urea; 40 m*M* imidazole; 10 m*M* β-mercaptoethanol; 500 m*M* NaCl). Finally, the protein was eluted with elution buffer (8*M* urea; 10 m*M* β-mercaptoethanol; 500 m*M* NaCl; 200 m*M* acetic acid). The eluted fractions were pooled and incubated with TEV protease in cutting buffer (10 m*M* Tris-HCl pH 8.0; 2*M* urea; 10 m*M* β-mercaptoethanol; 500 m*M* NaCl) overnight. A second His-tag based affinity purification step was carried out to separate TEV protease from MJ0366. Protein was then dialysated in a bag of 3.5 kDa molecular mass cutoff for 4 h in dialysis buffer (50 m*M* Na_2HPO_4 pH 8.0; 10 m*M* β-mercaptoethanol; 500 m*M* NaCl; 10% glycerol).

Protein activation for covalent modification with DNA handles

An aliquot of the purified protein (around 200 μ*M*) was incubated overnight with 50 m*M* dithiothreitol (DTT) at 4°C, then the protein was purified by gel filtration using a Superdex™ 75/300 GL column (General Electric, USA) previously equilibrated in 50 m*M* Na_2HPO_4 pH 8.0 and 500 m*M* NaCl. The eluted protein was immediately incubated with a 70-fold excess of 2,2′-dithiodipyridine (DTDP) and the reaction was followed by absorbance at 343 nm.[23] When the reaction was complete, the excess of DTDP was removed using two Micro Bio-Spin (Bio-Rad, USA) columns equilibrated with 50 m*M* Na_2HPO_4 pH 8.0 and 500 m*M* NaCl and 0.001% Tween-20. The binding of DTDP was confirmed by Matrix-Assisted Desorption/Ionization Mass Spectrometry (MALDI-TOF MS).[24]

Protein DNA handles attachment

Each DNA handle of 558 bp length was synthesized by PCR reaction using biotinylated and digoxigeninylated forward DNA oligomers as well as SH-modified reverse DNA oligomers as introduced by Cecconi *et al*.[25] A solution of 18–25 μ*M* SH-DNA handle in 10 m*M* Tris pH 8.5 was reduced with 20 m*M* DTT at RT for 2 h and then buffer exchanged into 50 m*M* Na_2HPO_4 pH 8.0, 500 m*M* NaCl, 0.001% Tween-20 using two Micro Bio-Spin (Bio-Rad, USA) columns. The reduced DNA solution was immediately mixed with the DTDP-activated proteins in a 6:1 (DNA:Protein) molar ratio overnight at RT. The hybridization products were observed in 6% polyacrylamide gels (without SDS) run at 100 V for 1.5 h in 250 m*M* Tris and 1.92*M* Glycine, and stained with GelRed™ (Biotium, USA). The DNA–Protein–DNA complexes were purified by electroelution. The band was cut and introduced into a dialysis bag of 3.5 kDa molecular mass cutoff, which was placed into a horizontal electrophoresis chamber. The electric field of 3 V/cm was applied for 1 h, followed by an inversion of the charge poles and application of an electric field of 5 V/cm for 1 min. The DNA–protein–DNA complex was then dialyzed for 1 h in 50 m*M* Na_2HPO_4 pH 8.0, 500 m*M* NaCl and 2 m*M* EDTA in a 1:5000 ratio. Finally, the sample was supplemented with 50% glycerol and stored at −80°C.

BiP expression and purification

The RR1 *E. coli* strain, containing the expression plasmid for mature yeast BiP (without signal sequence) with an N-terminal fusion to a His-tag, was kindly provided by Jeffrey Brodsky (Pittsburgh University). Overexpression of the protein construct

was induced for 4 h with 0.5 m*M* IPTG at 26°C at a cell density of $OD_{600} = 0.6$. BiP was purified by combining and modifying two previously published protocols of two affinity chromatographies, in order to obtain BiP in purer form and in higher yield. First a nickel affinity column purification (1 mL His Trap HP, General Electric, USA)[26] was used, followed by a 10 mL ATP-agarose affinity column purification (Sigma, USA).[27] The first purification was modified regarding the sequential washing steps, where 5% glycerol was not added to the fourth and fifth step. The second purification was modified by not adding NaOH to the buffer C and by adding 2 m*M* ATP to the elution buffer.[22] The elution fractions, which contained the protein, were assessed by Bradford assay.[28] A final 10% SDS-PAGE was performed to confirm the purity of the pooled fractions (Supporting Information Fig. S1).

ATPase assay

To measure the activity of purified BiP, a spectrophotometrical coupled assay was performed measuring the formation of a formazan blue precipitate. The coupled enzymes used were *Thermococcus litoralis* glucokinase[29] and glucose 6 phosphate dehydrogenase (Sigma, USA). The reaction buffer contained 100 m*M* HEPES pH 7.0, 2 m*M* ATP, 1 m*M* NAD^+, 7 m*M* $MgCl_2$, 9 m*M* glucose, 25 m*M* KCl, 0.5 m*M* DTT, 0.2 m*M* nitroblue tetrazolium, 0.4 m*M* phenazine methosulfate, 10 U *Thermococcus litoralis* glucokinase, and 25 U glucose 6 phosphate dehydrogenase. The reaction was started by addition of the BiP enzyme solution to a final concentration of 4 μ*M* and then left at RT in darkness for 1 h. After this time the reaction was terminated by the addition of 2 volumes of 0.1*M* HCl. The formazan product was determined by the change in absorbance at 550 nm, $\varepsilon_{formazan} = 0.795$ mM^{-1} cm^{-1}. A non-enzymatic control was also performed. One unit of enzyme activity was defined as in the work by Koga *et al*., as the amount of enzyme required to convert 1 μmol of glucose per min.[30]

Preparation of MJ0366 samples for optical tweezers

The electroeluted and purified DNA–protein–DNA constructs were first incubated with anti-digoxigenin coated polystyrene beads (dig-beads) for 15 min at RT. After this time the optical tweezers buffer (20 m*M* HEPES pH 7.0, 2 m*M* ATP, 2 m*M* $MgCl_2$, 15 m*M* Na_2HPO_4 pH 7.6, 0.1 m*M* EDTA, 25 m*M* KCl, 0.5 m*M* DTT) was added to a final volume of 500 μL. The dilution of the DNA–protein–DNA constructs was optimized to yield the best density for performing optical tweezers experiments. Finally, this solution was injected into the optical tweezers chamber and a DNA–protein–DNA construct bound to a dig-bead was placed in the optical trap and then brought in close proximity to a 2.10 μm streptavidin-coated bead (Spherotech, USA) which was held in place at the end of a pipette by suction, until a tether between the two beads was attained.[25] Dig-beads were generated by coupling anti-digoxigenin antibodies (Roche, Switzerland) to 3.2 μm proteinG-coated beads (Spherotech, USA).[25]

Optical tweezers experiments

The pulling experiments were conducted at a constant velocity, 100 nm/s, and were performed using a simple trap optical tweezers instrument called "miniTweezers".[31] The trap was calibrated as described by Bustamante and Smith,[31] using a stiffness of 0.1 pN/nm. Individual MJ0366 molecules were attached to two polystyrene beads through modified 558bp dsDNA linkers as shown in Figure 1(A), where MJ0366 is tethered to them by means of disulfide bonds. The tethered protein was pulled and relaxed by its N-and C-terminus to mechanically unfold and refold, respectively, while recording the force and trap position. This was done without BiP in the optical tweezers buffer, with 1 μ*M* BiP present in solution with different concentrations of ATP (2 m*M*, 0.33 m*M*, 0.1 μ*M*) or ADP (2 m*M*), or with control proteins, 1 μ*M* lysozyme or bovine serum albumin (BSA). The experiments were carried out either until at least 100 pulling events to stretch and relax the protein were recorded, or until the tether ruptured. In each experimental condition data of at least three different pairs of beads was recorded.

Optical tweezers data processing and analysis

The data processing was performed with MATLAB using the "Tweezer Analysis" program provided by Jesse Dill. For the analysis of the pulling data at constant velocity, the force at which protein unfolding/refolding occurs was determined as well as the distance of this event. In order to determine the latter, the curve was adjusted to a line before and after the unfolding/refolding and the distance between these lines was determined.

Worm-like chain fit

The equation of the worm-like chain shows the dependence of the force on the extension of a flexible polymer in a thermal bath. A long polymer tends to contract as thermal forces try to randomize any alignment of its chain segments. This force has an entropic origin and its magnitude is given by[32]:

$$F = \frac{k_B T}{p}\left[\frac{1}{4}\left(1-\frac{x}{L_C}\right)^{-2} - \frac{1}{4} + \frac{x}{L_C}\right] \quad (1)$$

where k_B is the Boltzman constant (pNnm/K), p is the persistent length of the chain (a value of

0.65 nm is used[33]); x is the end-to-end extension, L_c is the contour length, calculated by multiplying the number of amino acids by 0.36 nm/aa. The measured folded extension was subtracted from the contour length to determine the distance between the attachment points of the DNA handles in the absence of force. For each "rip" (sudden change in extension under application of force) for mechanical unfolding in the force-extension traces, the length of the rip is determined and is placed on a scatter plot of the rip length versus force. A best fit to the worm-like chain values is made from these data by using the "Tweezer Analysis" program.

Quantification of total unfolding and refolding events

Pulling events with unfolding and refolding rips were clearly distinguishable from those without them, so the determination of a pulling event with or without a rip was performed manually. The total number of pulling events refers to the number of cycles stretching and relaxing the protein. In Figure 1(B) for example, four unfolding and four refolding cycles are shown, which all exhibit unfolding and refolding events. Therefore, the frequency for unfolding and refolding is 100%. The four unfolding and refolding cycles shown in Figure 1(C) exhibit only two unfolding events (pulling 1 and 7) and only one refolding event (pulling 8). The frequency of unfolding and refolding in this example is 50% and 25%, respectively. The entire statistics of this work includes around 300 unfolding and refolding cycles for each condition, which add up to a total number of 2491 unfolding and refolding traces. The errors for the frequencies of unfolding and refolding events due to the limited number of pulling cycles were calculated using the inverse beta function and a 95% confidence interval as in the work by Puchner *et al.*[34] These errors mean that the true probability for the binomial unfolding and refolding distribution will be with 95% probability within the cited errors.

Determination of BiP's dissociation constants and kinetics

As we show in our results, the binding of BiP to the unfolded protein substrate can be detected by the loss of folding- and unfolding events following binding. Therefore, it is possible to calculate the dissociation constant K_D of BiP for the used substrate according to:

$$K_D = \frac{[S][BiP]}{[S:BiP]} = [BiP]\frac{P_{rip}}{P_{norip}} = [BiP]\frac{P_{rip}}{1-P_{rip}} \qquad (2)$$

[S] is the concentration of the substrate protein [S:BiP] is the concentration of the BiP–substrate complex and P_{rip} and P_{norip} are the probabilities for observing rips and no rips. Again, the errors due to the limited statistics were calculated using the inverse beta function and a 95% confidence interval.[34]

In our data, binding of BiP to the unfolded protein substrate is detected by a loss in re- and unfolding events and conversely unbinding of BiP is observed by the reappearance of re- and unfolding events. Therefore, we can directly determine the time intervals that BiP stayed bound and unbound to the protein substrate and calculate the corresponding on- and off rate constants. By assuming an exponential distribution for the on- and off times, we calculated the on- and off rate constants from the inverse average of the on- and off times. For each experimental condition, we determined the error of the average times due to the limited statistics by bootstrapping: for each data set 10,000 random samples were drawn and from the resulting distribution of average times the variance was determined. This variance which represents the uncertainty due to the limited statistics was used as an error for on- and off times and the corresponding rates.

Acknowledgement

Authors thank Andreas Tapia for helpful discussions, Robert Lesch for revising the manuscript, and Steven B. Smith for help in minitweezers construction.

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