
Ashlee Jollymore and Hongbin Li*

Department of Chemistry, The University of British Columbia, Vancouver, BC, Canada V6T 1Z1

Folding and unfolding are fundamental biological processes in cell and are important for the biological functions of proteins. Characterizing the folding and unfolding kinetics of proteins is important for understanding the energetic landscape leading to the active native conformations of these molecules. However, the thermal or chemical-induced unfolding of many proteins is irreversible in vitro, precluding characterization of the folding kinetics of such proteins, just as it is impossible to “un-boil” an egg. Irreversible unfolding often manifests as irreversible aggregation of unfolded polypeptide chains, which typically occurs between denatured protein molecules in response to the exposure of hydrophobic residues to solvent. An example of such a protein where thermal denaturation results in irreversible aggregation is the β-1,4 endoxylanase from Bacillus circulans (BCX). Here, we report the use of single-molecule atomic force microscopy to directly measure the folding kinetics of BCX in vitro. By mechanically unfolding BCX, we essentially allowed only one unfolded molecule to exist in solution at a given time, effectively eliminating the possibility for aggregation. We found that BCX can readily refold back to the native state, allowing us to measure its folding kinetics for the first time. Our results demonstrate that single-molecule force-spectroscopy-based methods can adequately tackle the challenge of “un-boiling eggs”, providing a general methodology to characterize the folding kinetics of many proteins that suffer from irreversible denaturation and thus cannot be characterized using traditional equilibrium methodologies.

Introduction

Folding is an essential process for proteins to acquire their unique three-dimensional structures and attain their biological functionality. Characterizing the folding and unfolding kinetics of proteins is important for understanding the energetic landscape leading to the active native conformations of these molecules. The reversible nature of the typically two-state conformational equilibrium between the folded and unfolded states of monomeric, single-domain proteins makes it possible to use classical methods, such as stopped-flow spectrofluorimetry, to characterize in vitro the folding kinetics of proteins by initiating folding reactions from their unfolded conformations. However, thermal or chemical-induced unfolding of many proteins, such as creatine kinase, acetylcholine esterases, α-amyloses, and Bacillus circulans xylanase (BCX), can be irreversible under in vitro experimental conditions. This precludes characterization of the folding kinetics of such proteins, just as it is impossible to “un-boil” an egg. Such irreversible denaturation of proteins often result from the subsequent aggregation of the unfolded proteins in question, which typically occurs between (partially) denatured molecules in response...
Fig. 1. Schematics of using single-molecule AFM to measure the folding kinetics of BCX, which cannot be measured using traditional methods. The initial experimental stage involves the adsorption of (GB1)_4-BCX-(GB1)_4 in a thin layer onto a clean glass surface (a). Stretching a single polyprotein (GB1)_4-BCX-(GB1)_4 results in the unfolding of BCX, while other BCXs remain folded (b). Since there is only one unfolded BCX molecule, unfolded BCX will not aggregate. Instead, upon relaxation, the unfolded BCX can refold to its native three-dimensional structure and regain its mechanical stability.

Measuring “Unmeasurable” Folding Kinetics by AFM

(a)

AFM cantilever and tip
Protein

(b)

: GB1

: BCX

glass substrate

(c)
to the exposure of their constituent hydrophobic residues to solvent.\textsuperscript{5-11} Characterizing the folding kinetics of such proteins has been a challenging task. BCX is a good example in this regard. BCX has been the subject of much previous study using a variety of techniques including X-ray crystallography, NMR spectroscopy, and mutational analysis, leading to a well-defined structure and enzymatic mechanism,\textsuperscript{12,13} including the identification of structural elements critical to cleavage of xylan substrates.\textsuperscript{14-15} Despite the extensive characterization of BCX, previous attempts to characterize its folding kinetics using thermal denaturation failed due to aggregation upon unfolding;\textsuperscript{4} attempts using chemical denaturation have encountered only limited success.\textsuperscript{17,19}

Because aggregation is highly dependent on protein concentration, refolding under dilute conditions has been used to alleviate such problems.\textsuperscript{5,20} In principle, if the concentration of unfolded proteins is sufficiently low, the rate of association between unfolded protein molecules (a second-order process) becomes slower than the rate of refolding (typically a first-order process), eliminating the possibility of aggregation. However, it can be challenging to realize this method in practice, as dilution reduces the signal-to-noise ratio for detection methods.

Using BCX as a model system, here we report the first characterization of folding kinetics of proteins that suffer from irreversible denaturation by employing single-molecule atomic force microscopy (AFM) techniques. Over the last decade, single-molecule AFM has evolved into a unique technique for characterizing the unfolding/folding kinetics of proteins at the single-molecule level.\textsuperscript{21-25} We reasoned that using AFM, we can unfold BCX such that only one unfolded polypeptide chain exists in the system at a given time, with the rest of BCX molecules remaining folded. By reaching the limit of essentially infinite dilution, we should be able to effectively prevent aggregation and measure the folding kinetics of unfolded BCX one molecule at a time. Figure 1 illustrates the experimental scheme for the proposed approach.

**Results and Discussion**

**BCX flanked by GB1 domains is biologically active**

To investigate the possibility of using single-molecule AFM to directly monitor the folding dynamics of BCX, we engineered a heteropolyprotein (GB1)\textsubscript{4}-BCX-(GB1)\textsubscript{4}, in which BCX is flanked by (GB1)\textsubscript{4} at its N- and C-termini. The construction of a polyprotein chimera is necessary, as nonspecific interactions between the AFM tip and the glass surface make it extremely challenging to unambiguously identify the mechanical unfolding event of the protein of interest. The mechanical properties of GB1 domains have been well characterized using AFM techniques.\textsuperscript{26-27} Thus, (GB1)\textsubscript{4} serves as a built-in fingerprint for identifying single-molecule stretching events and the mechanical unfolding signature of BCX in AFM experiments.

We found that the heteropolyprotein (GB1)\textsubscript{4}-BCX-(GB1)\textsubscript{4} expresses well in *Escherichia coli* in a soluble form with high yield. Since BCX is flanked by two (GB1)\textsubscript{4} in the heteropolyprotein, it is important to ensure that the flanking does not affect the folding/unfolding kinetics and biological functions of BCX. Since GB1 does not interact with BCX, we assumed that the folding kinetics of BCX is not affected by its neighboring GB1 domains. To confirm that the flanking GB1 domains are not detrimental to the biological activity of BCX, we carried out a substrate depletion assay and determined $k_{cat}/K_m$ to...
be 29.0 s\(^{-1}\)mM\(^{-1}\) for hydrolysis of 2,5-dinitrophenol β-xylobioside (Supplementary Information, Fig. S1), which is comparable to that of wild-type BCX (34.4 s\(^{-1}\)mM\(^{-1}\))\(^{15,26}\), indicating that the intrinsic catalytic activity of BCX is retained in the context of the heteropolyprotein.

**Mechanical unfolding signature of BCX measured at the single-molecule level**

Having established that (GB1)\(_4\)-BCX-(GB1)\(_4\) is catalytically active, we used single-molecule AFM to characterize the mechanical unfolding of the heteropolyprotein. The GB1 domains served as fingerprints for identifying force–extension curves arising from the stretching and unfolding of single (GB1)\(_4\)-BCX-(GB1)\(_4\) molecules and for discerning the unfolding signatures of BCX. The mechanical unfolding of GB1 domains is characterized by an unfolding force of ∼180 pN at a pulling speed of 400 nm/s and a contour length increment (ΔL\(_c\)) of 18 nm,\(^{26,27}\) as determined by fitting the worm-like chain (WLC) model of polymer elasticity to consecutive unfolding events.\(^{29}\) Stretching the polyprotein (GB1)\(_4\)-BCX-(GB1)\(_4\) results in force–extension curves with a characteristic sawtooth-like pattern, in which individual unfolding force peaks correspond to the mechanical unfolding of individual domains in the polypeptide (Fig. 2). The last peak corresponds to the stretching and subsequent detachment of the unfolded polypeptide chain from either the AFM tip or the glass surface. Unfolding events that tended to occur at high extension towards the end of the force–extension curves were characterized by unfolding forces of ∼180 pN with ΔL\(_c\) of ∼18 nm. These events can be readily recognized as the mechanical unfolding of GB1 domains flanking BCX in the heteropolyprotein (Fig. 2, blue WLC fits). Prior to the mechanical unfolding events of GB1, we also observed an unfolding event occurring at a lower force (∼50 pN) with a much larger ΔL\(_c\). Since BCX is flanked by (GB1)\(_4\) at both termini, we can be certain that the BCX domain has been stretched and unfolded and the force–extension curve should contain the signature of the mechanical unfolding of BCX if the force–extension curve contains at least five GB1 unfolding events. Therefore, the unfolding events with longer ΔL\(_c\) occurring prior to the unfolding of GB1 domains correspond to the unfolding of BCX domains (Fig. 2, curves a–c). Fitting the WLC model to the unfolding events of BCX measured an average ΔL\(_c\) of 64±8 nm (average±standard deviation), and the mechanical unfolding of BCX occurred at an average force of 55±30 pN (Supplementary Information, Fig. S2). Due to the presence of two cysteine residues engineered into the C-terminus of (GB1)\(_4\)-BCX-(GB1)\(_4\), (GB1)\(_4\)-BCX-(GB1)\(_4\) can be oxidized over time to form dimers, leading to the observation of more than eight GB1 unfolding events in a given force–extension curve and sometimes two unfolding events of BCX (see Fig. 2, curves d–f).

BCX is 185 residues long and the C\(^α\)–C\(^α\) distance between its salt-bridged amino- and carboxy-termini is 4.6 Å in its folded three-dimensional structure (Protein Data Bank code: 1HVO).\(^{15}\) The contour length of an unfolded and fully extended BCX is 66.6 nm (185 aa×0.36 nm/aa). Hence, the complete unfolding of a folded BCX domain will result in the extension of BCX by ∼66.1 nm (66.60 nm−0.46 nm). The experimentally measured ΔL\(_c\) (∼64 nm) is very close to this predicted value, suggesting that the unfolding events we observed indeed correspond to the unfolding of the initially native BCX domains. Moreover, it is of note that the width of the distribution of ΔL\(_c\) and unfolding force is quite broad. The origin of this broad distribution remains to be understood, but it is possible that this is the signature of static and dynamic heterogeneity in the unfolding pathway of BCX.

**The unfolding and folding reaction of BCX is fully reversible at the single-molecule level**

From a thermodynamic perspective, all protein folding should be reversible. Aggregation should be the origin for irreversible unfolding of BCX. To prove
that the folding of BCX is indeed reversible at the single-molecule level, we carried out stretching and relaxation experiments on the same BCX molecule. As shown in Fig. 3, after we stretched and unfolded a single (GB1)₄-BCX-(GB1)₄ molecule, we immediately relaxed the unfolded polypeptide chain back to zero extension before the unfolded molecule detaches from either the AFM tip or the glass surface. After waiting a few seconds, we stretched it again. We clearly observed the mechanical unfolding event of BCX again, which is characterized by a $\Delta L_c$ of $\sim 64$ nm, suggesting that the unfolded BCX molecule managed to refold back to its native state and regain its mechanical stability. This result clearly demonstrated that the unfolding and folding of BCX is intrinsically reversible, and the irreversible unfolding process observed macroscopically is most likely due to the aggregation of unfolded BCX molecules in bulk. Since the stretching and mechanical unfolding of BCX was carried out on individual molecules, only one unfolded BCX molecule existed at a given time. Thus, the aggregation between different unfolded BCX molecules is effectively eliminated in our experiments and the unfolding/folding reactions of BCX become reversible at the single-molecule level. Such reversibility makes it possible to characterize the folding kinetics of BCX using single-molecule force spectroscopy.

**Single-molecule force spectroscopy enables the measurement of folding kinetics of BCX for the first time**

Using the well-established double-pulse protocol in force spectroscopy, we directly measured the folding kinetics of BCX at zero force (Fig. 4a). A single molecule of (GB1)₄-BCX-(GB1)₄ was attached between the glass surface and silicon nitride cantilever tip. This molecule was then fully extended such that all constituent domains within the protein fragment attached between the substrate and tip was fully unfolded. The fully extended polyprotein chain was then allowed to relax to zero extension. The tip and surface were kept at zero extension for the time interval $\Delta t$, during which time BCX molecule had a certain probability to refold into its original three-dimensional conformation and to regain its mechanical stability. Determining whether BCX folded during $\Delta t$ was done by re-extending the molecule after this time interval $\Delta t$. The appearance of unfolding events with a $\Delta L_c$ of $\sim 64$ nm indicated the folding of the BCX domain.

**Fig. 4.** Folding kinetics of BCX at zero force. (a) The schematic of the double-pulse protocol used to measure the folding kinetics of BCX molecule at zero force. (b) Representative force–extension curves during refolding experiments with different waiting time, $\Delta t$. The folding behavior of BCX was determined using a refolding double-pulse method, in which a single molecule of BCX containing polyprotein was unfolded (trace 1), relaxed (trace 2), allowed to refold during the time $\Delta t$, and re-extended to determine whether BCX refolded during this time (trace 3). As the (GB1)₄-BCX-(GB1)₄ polyprotein contained only one BCX domain, statistics were generated by comparing refolding traces at a variety of $\Delta t$ values. (c) The folding kinetics of a single BCX molecule at zero force. The folding probability of BCX can be fitted using a single exponential distribution (continuous line) with a folding rate constant $\beta$ of $1.72 \pm 0.14$ s⁻¹.

Measuring “Unmeasurable” Folding Kinetics by AFM
after relaxation. As unfolding was done using a construct containing one BCX domain, the probability of observing refolding of BCX is either 0% or 100%, with the exception that a minority of curves from dimerized polyproteins exhibited two BCX unfolding events (Fig. 4a). By carrying out such stretching–relaxation experiments on different molecules, we compiled a large number of refolding curve pairs and determined the average folding probability \( N_{\text{refold}}/N_{\text{total}} \) of BCX within time \( \Delta t \), where \( N_{\text{total}} \) is the total number of BCX unfolding events observed in the first pulse and \( N_{\text{refold}} \) is the total number of BCX refolding events observed in the second pulse. By varying the time interval \( \Delta t \), we determined the refolding kinetics of BCX at zero force (Fig. 4b). We found that the folding kinetics of BCX can be best described by a first-order rate equation \( \frac{N_{\text{refold}}}{N_{\text{total}}} = A(1 - e^{-\beta t}) \), where \( N_{\text{refold}}/N_{\text{total}} \) is the folding probability of BCX domains at a relaxation time \( t \) and \( \beta \) refers to the folding rate constant, suggesting that the folding of BCX is likely to be a two-state process. Fitting the experimental data to the first-order rate equation measures a folding rate constant \( \beta \) of 1.72±0.14 s\(^{-1}\). This is the first experimentally measured folding kinetics of BCX, effectively solving the problem of irreversible denaturation commonly encountered with BCX. It is worth noting that the folding rate constant measured in single-molecule AFM experiments is a “mechanical” folding rate constant. As demonstrated before, the “mechanical” folding rate constant may be different from the chemical/thermal folding rate constant, as the protein may sample different folding pathways in two different experimental settings.\(^\text{30}\)

**Single-molecule AFM is a promising method for characterizing the folding kinetics of proteins that are otherwise difficult to characterize.**

Our results clearly indicate that by using single-molecule AFM techniques, it is possible to eliminate the irreversible aggregation of unfolded proteins in solution and enable the direct measurement of folding kinetics of BCX that are inaccessible by traditional methods such as thermal denaturation. Previously, Fernandez et al. showed that tandem repeating modular proteins can avoid aggregation during single-molecule force spectroscopy experiments\(^\text{30}\) but showed aggregation in ensemble studies possibly due to the identity of the neighboring domains.\(^\text{30}\) Our results on BCX not only are consistent with this previous observation but also generalize this observation on tandem repeating proteins to single-domain proteins. These studies demonstrate that single-molecule AFM provides a conceptually simple yet efficient approach to circumvent the almost impossible task of “un-boiling eggs” in quantifying the folding kinetics of proteins suffering from irreversible denaturation. We anticipate that this force-spectroscopy-based approach, including the use of AFM and optical tweezers, can be applied to a wide range of proteins whose folding kinetics were previously indeterminate by thermal/chemical denaturation methods due to irreversible unfolding caused by aggregation, providing an invaluable tool to view the folding dynamics of proteins that are otherwise inaccessible.

To apply force spectroscopy techniques to study the folding kinetics of these proteins, the only criterion is that the mechanical unfolding of the protein of interest be mechanically stable such that its unfolding results in a clear mechanical unfolding force peak in force-extension curves. The broad range of forces covered by AFM and optical tweezers will make it possible to study a wide range of proteins with diverse mechanical stability. Moreover, this method can potentially be used as a tool to study aggregation patterns of proteins,\(^\text{31,32}\) which should provide a unique perspective for understanding the causes of some of the so-called folding diseases, such as Alzheimer’s and Creutzfeldt–Jakob disease.

**Materials and Methods**

**Construction of the (GB1)\(^4\)-BCX-(GB1)\(^4\) polyprotein**

The heteropolyprotein construct (GB1)\(^2\)-BCX-(GB1)\(^4\) was engineered in a manner previously described.\(^\text{27}\) The plasmid containing a semi-synthetic gene encoding BCX was described previously.\(^\text{33}\) This plasmid was amplified and engineered to contain 5′ BamHI and 3′ KpnI and BglIII restriction sites using a polymerase chain reaction (PCR) with a 5′ primer of sequence CGC GGA TGC GCT AGC ACA GAC TAC TGG C and 3′ primer of sequence CGC GGT ACC GCA ACA AGA TCA TGG C and 3′ primer of sequence CGC GGT ACC GCA ACA AGA TCA TGG C. The BCX gene containing the requisite restriction sites was engineered in a manner previously described.\(^\text{27}\) The heteropolyprotein construct (GB1)\(^4\)-BCX-(GB1)\(^4\) polyprotein was constructed by ligating the sticky-ended insert (GB1)\(^4\)BCX, which was subsequently digested with T4 DNA ligase. The final plasmid construct pQE80L(G1B1)\(^4\)BCX was constructed by ligating the sticky ends of the (GB1)\(^4\) insert with the pQE80L (GB1)\(^4\)BCX vector. Expression of (GB1)\(^4\)BCX(GB1)\(^4\) was
done by over-expression within a DH5α strain of *E. coli*. The soluble protein was purified from the cell lysis supernatant by Co2+ affinity chromatography. The eluted protein was stored at 4 °C in a phosphate-buffered saline (PBS) solution at a concentration of 1.0 μg/ml.

**Single-molecule AFM**

Single-molecule AFM experiments were carried out on a custom-built AFM, which was constructed as described previously. Each individual AFM cantilever was calibrated using the equipartition theorem before and after each AFM experiment. Typical spring constant values for the cantilever ranged from 50 to 80 pN/nm. Approximately 1.0 μl of protein solution in PBS (1.0 μg/ml) was deposited onto a clean glass coverslip covered by PBS buffer (~50 μl). Protein solutions were allowed to adsorb for at least 5 min prior to beginning an experiment. The AFM tip was first brought into contact with the glass coverslip with a contact force of 50 to 80 pN/nm. Approximate 1.0 μl of protein solution in PBS (1.0 μg/ml) was deposited onto a clean glass coverslip covered by PBS buffer (~50 μl). Protein solutions were allowed to adsorb for at least 5 min prior to beginning an experiment. The AFM tip was first brought into contact with the glass coverslip with a contact force of ~5 nN in order to attach a single molecule between glass surface and silicon nitride cantilever tip. Upon withdrawing the AFM tip from the glass surface, polypeptide fragments can be attached to the cantilever tip through nonspecific interactions. Nonspecific interactions were often strong enough to allow for subsequent rounds of stretching and relaxing the polypeptide fragment.

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**Supplementary Data**

Supplementary data to this article can be found online at doi:10.1016/j.jmb.2010.07.059

**References**


