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Mapping mechanical force propagation through biomolecular complexes

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Abstract

Here we employed single-molecule force spectroscopy with an atomic force microscope (AFM) and steered molecular dynamics (SMD) simulations to reveal force propagation pathways through a mechanically ultrastable multi-domain cellulosome protein complex. We demonstrate a new combination of network-based correlation analysis supported by AFM directional pulling experiments, which allowed us to visualize stiff and soft paths through the protein complex along which force was transmitted. The results implicate specific force-propagation architectures non-parallel to the pulling axis that are advantageous for achieving high dissociation forces.

Keywords

force propagation; single molecule force spectroscopy; steered molecular dynamics; network analysis; cohesin-dockerin

Mechanical forces play a fundamental role in biological systems. Cells are able to sense and respond to mechanical cues in their environment by, for example, modulating gene expression patterns,¹ reshaping the extracellular matrix,² or exhibiting differential

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Supporting Information Available

Details of experimental materials and methods, steered molecular dynamics simulations, and additional theoretical discussion This material is available free of charge via the Internet at <http://pubs.acs.org/>.

biochemical activities.³ At the molecular level, these behaviors are governed by mechanically active proteins. Such proteins are able to sense and respond to force by undergoing conformational changes,⁴ exposing cryptic binding sequences,⁵ acting synergistically with ion channels,⁶ or modulating their function in a variety of ways.^{7–9}

Experimental methods including AFM single-molecule force spectroscopy (SMFS) allow direct measurement of molecular mechanical properties. These studies have demonstrated the importance of the shear topology involving parallel breakage of hydrogen bonds in providing mechanical stability to protein folds.^{10,11} Many globular domains and protein complexes also exhibit a directional dependence in unfolding mechanics, consisting of stiff and soft axes.^{12–18} Pulling geometry can be defined by controlling the positions of the chemical linkages between protein monomer units through a variety of bioconjugate techniques.

Primary sequences of mechanically active proteins are extremely diverse, essentially rendering them undetectable by conventional bioinformatics approaches. Yet, another computational approach, namely molecular dynamics (MD), allow sampling of structural conformations of large and frequently mechanostable protein complexes.^{19,20} Analysis of these conformations from MD trajectories have recently led to the development of network-based correlation methods for investigating signal transmission and allosteric regulation in proteins.^{21–23} In network models, local correlations of positional fluctuations in a protein are represented as a web of inter-residue connections. Within such a network, the behavior of nodes that are highly correlated and within close physical proximity can be analyzed to obtain the shortest path between two network nodes (i.e., amino acids). This analysis helps to identify which connecting residues are most important for intramolecular communication.^{23–25} Examination of multiple pathways, also known as suboptimal paths, within an acceptable deviation from the optimal path helps to detect the web of nodes critical for transmission of information.

Among MD methods, steered molecular dynamics (SMD) simulations, in which external forces are used to explore the response and function of proteins, have become a powerful tool especially when combined with SMFS.⁶ SMD has been successfully employed in a wide range of biological systems, from the investigation of protein mechanotransduction,^{5,26} to permeability of membrane channels,^{27,28} and the characterization of protein-receptor interactions.²⁹ SMD simulations have also been used to study force propagation through proteins by employing force distribution analysis (FDA).^{30,31} In FDA, all pair-wise forces, which are usually calculated in MD simulations, are stored in $N \times N$ matrices, where N is the number of atoms.³² These pair-wise forces can then be used to assess a protein's response to a mechanical or allosteric signal.³³ In the FDA approach, atoms under mechanical strain are identified by subtracting forces of both loaded and unloaded states for each pair of interacting atoms.³¹ However, to achieve a sufficient signal to noise ratio, FDA will often require exhaustive sampling of the conformational space.^{32,34} FDA therefore, requires more computational resources than usual SMD studies, which are frequently already computationally demanding. There is therefore a clear need for new analysis methods that enable visualization of force propagation pathways from a single SMD trajectory.

Here we implemented a novel combination of SMD, network-based correlation analysis, and thermodynamic fluctuation theory, supported by AFM-SMFS experiments to study force propagation through a protein complex subjected to different pulling geometries. We chose an ultrastable receptor-ligand interaction as a model system because of its remarkably high mechanical stability,²⁹ which effectively improves the signal-to-noise ratio. This complex consists of two interacting protein domains called cohesin (Coh) and dockerin (Doc) that maintain bacterial adhesion of *Ruminococcus flavefaciens* (Rf) to cellulosic substrates. Doc is found within the same polypeptide chain as a stabilizing ancillary domain called X-module (XMod), located N-terminally of Doc. Based on its position with the Rf cellulosomal network, Coh is mechanically anchored *in vivo* at its C-terminal end to the cell surface. Our prior work demonstrated that, when force is applied to the complex in the native configuration (i.e., C-terminal Coh, N-terminal XMod-Doc anchor points), the complex is extremely stable, exhibiting high rupture forces of 600 – 750 pN at loading rates from 1 – 100 nN s⁻¹.²⁹ Since the bulk equilibrium affinity of the complex is an unremarkable 20 nM,³⁵ we hypothesized that the high mechanostability was explained by a catch bond mechanism. AFM rupture force data and SMD simulations supported this prediction, where it was observed that the contact surface area of the two proteins increased as mechanical force was applied.

To characterize the mechanisms behind Coh:Doc high stability, here we additionally pulled the complex apart in a non-native configuration (i.e., N-terminal Coh, N-terminal XMod-Doc anchor points). In the non-native pulling configuration, we found that the complex dissociated along two competing pathways with very different mechanical characteristics.

Our new dynamic network analysis protocol reveals how different mechanical behaviors are attributable to differences in the direction of force transmission across the binding interface. Together, the experiments and simulations depict a simple physical mechanism for achieving high complex rupture forces: the complex directs force along pathways orthogonal to the pulling axis.

Single-molecule pulling experiments and SMD

For SMFS experiments, XMod-Doc was produced as a fusion protein with an N-terminal Xylanase (Xyn) domain. Coh was produced as either an N- or C-terminal fusion domain with a carbohydrate binding module (CBM). These fusion domains were used for site specific immobilization to a glass surface and AFM cantilever to achieve the two loading configurations shown in Fig. 1A and further served as marker domains with known unfolding length increments to validate single-molecule interactions and sort SMFS data traces.³⁶

For the native pulling configuration found *in vivo*, the CBM and XMod-Doc are loaded from their C- and N-termini, respectively (Fig. 1A). A representative unbinding trace for the native pulling configuration is shown in Fig. 1B. We measured the loading rate dependence of complex rupture using both experimental and SMD datasets (unbinding trace from SMD shown in Fig. 3A), and plotted them on a combined dynamic force spectrum (Fig. 1E). The linear Bell model produced fit parameters for the effective distance to the transition state x

$= 0.13$ nm, and the zero-force off rate $k_{off} = 4.7 \times 10^4 \text{ s}^{-1}$. Both experimental and simulation data were well described by a single Bell expression, despite the differences in loading rates between experiments and simulation. This observation suggests that the application of force does not significantly change x for this particular configuration.

To test the influence of pulling geometry on mechanical stability, we performed SMFS and SMD on the system where Coh was pulled from the opposite terminus (i.e., non-native N-terminus, *cf.* Fig. 1A). Unlike the native pulling geometry, this configuration exhibited two clearly distinct unbinding pathways that were characterized by different force ranges (high or low) at which the complex dissociated. We refer to these pathways as non-native high force (HF) (Fig. 1C) and non-native low force (LF) (Fig. 1D).

AFM data traces classified as non-native HF showed similar characteristics as those in the native pulling configuration (*cf.* Fig. 1B,C,F). The non-native LF traces, however, exhibited a markedly different unfolding behavior (Fig. 1D). Xyn unfolding (highlighted in orange) was regularly observed, but CBM unfolding was only very rarely observed. The complex usually did not withstand forces high enough to unfold CBM when rupturing along the non-native LF path. Among non-native LF curves, we regularly found an additional contour length increment of 17 – 19 nm consistent with unfolding of ~60 amino acids located at the N-terminus of Coh. This unfolding occurred immediately following Xyn unfolding (Fig. 1D, red), or alternatively prior to Xyn unfolding, or with a substep (Supporting Fig. S1). Taken together, it appears that partial Coh unfolding from the N-terminus destabilizes the complex, causing lower rupture forces (Fig. 1G).

The experimental rupture forces from the non-native HF class were indistinguishable from those arising in the native configuration. To confirm this, we performed additional measurements where both Coh configurations were alternately probed with the same Xyn-XMod-Doc functionalized cantilever (Supporting Fig. S2), eliminating inaccuracies introduced through multiple cantilever calibration. Most probable rupture forces at a pulling speed of 800 nm s^{-1} of 606 pN and 597 pN for the native configuration and non-native HF class respectively were determined in the Bell Evans model (Fig. 1F, Supporting Eq. S2), demonstrating that the native and non-native HF classes are experimentally indistinguishable.

For the LF class, we analyzed the final complex rupture event and plotted the combined dynamic force spectrum (Fig. 1G). Here, simulated and experimentally observed data were not well described by a single Bell expression. In such cases non-linear models have been developed to obtain kinetic and energetic information from dynamic force spectra.^{37,38} To fit the combined data, we used the non-linear Dudko-Hummer-Szabo (DHS) model (Supporting Eq. S3) and obtained values of $x = 0.42$ nm and $k_{off} = 0.005$ s. The DHS model further provides the free energy difference G between the bound state and the transition state as a fit parameter, which was found to be $G = 129 k_B T$. The model fit produced a distance to transition that was much longer than observed for the native configuration. Independent SMD simulations for the non-native pulling configuration were found to also lead to HF and LF unbinding scenarios (see below, Fig. 4A and D, respectively).

The differential solvent contact area was calculated from SMD simulations to estimate the intermolecular contact area in the Doc:Coh complex. In the native configuration, the simulated Doc:Coh contact area increased by 14% and 9% for Coh and Doc, respectively (Fig. 1H). For the non-native HF class, the contact area increased by 11% and 12% for Coh and Doc, respectively (Fig. 1I). In the non-native LF class, the contact area increased by only 7% for Coh, and decreased by 3% for Doc (Fig. 1J). Evidently, an increased surface contact area for Doc in the native and non-native HF pathways correlated with high mechanostability of the system.

Force propagation theory - a simple model

To further understand the observed unbinding pathways, we sought to identify paths through the molecule along which the externally applied load propagates. From thermodynamic fluctuation theory,^{39,40} it is known that the correlation of fluctuations of atoms i and j and the force $\mathbf{F}_i$ on atom i are related through:

$$\langle \Delta \mathbf{r}_i \Delta \mathbf{r}_j^T \rangle = k_B T \frac{\partial \mathbf{r}_j}{\partial \mathbf{F}_i} \quad (1)$$

where $\mathbf{r}_i = \mathbf{r}_i(t) - \langle \mathbf{r}_i(t) \rangle$ and $\mathbf{r}_i$ is the position of atom i . The derivative on the right hand side of Eq. (1) states that neighboring atoms i and j will move with high correlation due to an external force $\mathbf{F}_i$ acting on atom i if the coupling between them is strong. Hence, a given element of a correlation matrix $M_{ij} = \langle \Delta \mathbf{r}_i \Delta \mathbf{r}_j^T \rangle$ will be large in the case of a strong interaction potential between i and j . When force is propagated through a molecule, soft degrees of freedom will be stretched out along the path of force propagation, while stiff degrees become more important for the dynamics of the system.

Consequently, paths with high correlation of motion describe the paths along which force propagates through the system. To illustrate this behavior for a toy system, we employed the NAMD⁴¹ SMD⁴² constant velocity protocol to a test pattern of identical spheres connected with harmonic springs of different stiffness (Fig. 2A). The position of one sphere was fixed during the simulation while another sphere on the opposite side of the structure was withdrawn at constant velocity. The strained structure at the end of the simulation is shown in Fig. 2B. We assigned weights to the lines between spheres according to the Pearson correlation coefficient C_{ij} (Supporting Eq. S4) between those network nodes (Fig. 2C). The Pearson correlation coefficient differs from the left hand side of Eq. (1) by a normalization

factor $(\langle \Delta \mathbf{r}_i^2(t) \rangle \langle \Delta \mathbf{r}_j^2(t) \rangle)^{-\frac{1}{2}}$ and was chosen to make our analysis mathematically more tractable. For a detailed discussion on this choice of correlation measure, see Supporting Information. In a harmonic potential approximation, the equipartition theorem can be applied to this normalization factor resulting in the following expression for C_{ij} :

$$C_{ij} = \frac{\partial \mathbf{r}_j}{\partial \mathbf{F}_i} \cdot \sqrt{k_{i,eff} \cdot k_{j,eff}} \quad (2)$$

where $k_{i,eff} = \left(\frac{1}{k_{x_i}} + \frac{1}{k_{y_i}} + \frac{1}{k_{z_i}} \right)^{-1}$ and k_{x_i} is the curvature of the potential on atom i in the x direction. For a full derivation, see Supporting Information. Eq. (2) illustrates how Pearson correlation is a suitable measure to identify the stiff paths in our simple model. We then used dynamical network analysis⁴³ to find the path of highest correlation (Fig. 2D). As expected from Eq. (1), we found this path to be the one connected by the stiff springs.

Force propagation through XMod-Doc:Coh Complex

The simple pattern of spheres validated our general approach of using local correlations to identify load-bearing pathways through networks. We next employed dynamical network analysis to understand force propagation through the XMod-Doc:Coh complex.

The dynamic networks for the native configuration (unloaded and loaded) are shown in Fig. 3B and C, respectively. While the network shows multiple suboptimal paths in the unloaded scenario, the loaded case exhibits a well defined main path along which force propagates through the system. Interestingly, in the loaded configuration, force propagates through both binding helices of Doc, which results in a force path with large normal components to the unbinding axis close to the binding interface as illustrated in Fig. 3D. It was shown for another ultrastable protein, namely silk crystalline units, that curving force paths distribute tension through the entire system.³¹ A strategy that assumes an indirect path would therefore allow the system to have more time to absorb the tension from the applied force. The result here supports the view that directing the force along a path with significant perpendicular components to the pulling axis leads to high mechanical stability. In a simple mechanical picture, a certain amount of mechanical work $dW = \mathbf{F} \cdot d\mathbf{s}$ is required to separate the two binding interfaces by a distance z and break the interaction. In this simplified picture, $d\mathbf{s}$ points along the unbinding axis, whereas the force $\mathbf{F}$ is locally largely perpendicular to this direction. Consequently, a larger force is required to break the interaction than in a scenario where the force path would point along the unbinding axis.

To validate this picture, we repeated the same analysis for the non-native HF and non-native LF pathways. The HF simulation (Fig. 4A) exhibited only a small stretching of the flexible N-terminal region of Coh and complex dissociation at approximately 800 pN and a pulling distance around 10 nm. However, the LF case shown in Fig. 4D exhibited a stepwise N-terminal Coh unfolding, dissociating at a force of about 480 pN at a pulling distance of about 25 nm. This behavior confirmed our assignment of the experimentally observed 17–19 nm contour length increment to Coh unfolding up to residue 62 in PDB 4IU3.

While the experimental data did not show a detectable difference between the native configuration and the non-native HF class, the propagation of force takes place along a different pathway (Fig. 4B). For N-terminal Coh pulling, helix 3 of Doc is not involved in the propagation of force as it is for the native geometry. In the native configuration, force propagated through the center of Coh, while for non-native HF the path is shifted towards the side of the molecule. Despite these differences, there is a common feature between the native and non-native HF pathways. At the binding interface, the pathway again shows pronounced normal components to the unbinding axis (cf. Fig. 4C), suggesting that this

feature is indeed responsible for the exceptional mechanical strength observed for these two unbinding pathways.

Fig. 4E shows the force propagation pathway for the non-native LF class prior to rupture. Due to the unfolding of the N-terminal Coh segment, the propagation of force is shifted even further away from the central portion of Coh than for the non-native HF class. Interestingly, force is propagated through the small helical segment of Coh (ALA167-GLN179), a portion of the molecule that is not involved in force propagation for any of the other analyzed trajectories. Unlike in the aforementioned scenarios, there is no pronounced tendency for normal force components at the binding interface for the non-native LF class. In fact, the force is propagated along a path largely parallel to the pulling axis (cf. Fig. 4F). In cases where force propagation occurs parallel to the pulling axis, as in Fig. 4E, low mechanical stability was observed.

The aforementioned force propagation architecture along with the effect of increasing contact surface area upon mechanical loading combine for elevated mechanostability of the system. In cases where we observed an N-terminal Coh unfolding of 62 amino acids in the non-native geometry, the system was no longer able to summon this mechanism, causing dissociation at much lower forces.

Previously, our groups have reported on a family of mechanically stable protein ligand receptor complexes that are key building blocks of cellulosomes,^{29,44–46} the multi-enzyme complexes used by select anaerobic bacteria to digest lignocellulose. However, the molecular origins of the stability of these complexes remained largely unclear. An initial clue was obtained when, in a previous work, we were able to show that contact surface area of the two proteins increased as mechanical force was applied.²⁹ In a different study,⁴⁷ coarse-grained MD simulations showed much smaller rupture forces at similar loading rates both for native and non-native pulling than we report here. This disagreement is likely due to the inability of the coarse-grained model to capture the rearrangement of amino acid side chains observed here. As we demonstrated, force propagation calculation from network-based correlation analysis helped in investigating the dramatic effect on the mechanical stability of the Doc:Coh interaction when different pulling geometries are applied. Our methodological approach, to the best of our knowledge, has never been applied even though network analysis of SMD trajectories was performed before to probe the mechanism of allosteric regulation in imidazole glycerol phosphate synthase.⁴⁸

In summary, for both unbinding cases where we observed high mechanostability, we found that across the binding interface, force propagated along paths with strong normal components to the pulling direction. Such a behavior was not observed for the non-native LF class, where, presumably due to N-terminal Coh unfolding, the system was no longer able to direct the force across the binding interface at high angles. From these findings, we conclude that the ultrastable complex formed by Coh and Doc achieves its remarkable mechanostability by actively directing an externally applied force toward an unfavorable angle of attack at the binding interface, consequently requiring more force to achieve a given amount of separation along the pulling direction. Our results show that this mechanically stable complex uses an architecture that exploits simple geometrical and physical concepts

from Newtonian mechanics to achieve high stability against external forces. The analytical framework derived here provides a basis for developing a deeper understanding of the functioning of various mechanoactive proteins that are crucial for physiologically relevant processes such as mechanotransduction, cellular mechanosensing, and pathogenesis. Additionally, it could provide a design platform for development of artificial mechanoactive systems with applications as tissue engineering scaffolds or components in engineered nanomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Single molecule force spectroscopy and steered molecular dynamics of XMod-Doc:Coh in two pulling configurations. (A) Crystal structure of the XMod-Doc:Coh complex (PDB 4IU3) with orange spheres marking the termini where force was applied. (B) Experimental unfolding trace for the native pulling configuration at a pulling speed of 1600 nm s^{-1} . The inset shows a schematic of the pulling geometry. Unfolding signatures of the Xyn and CBM marker domains are marked in orange and green, respectively. (C) Experimental unfolding trace for the non-native high force class obtained at a pulling speed of 700 nm s^{-1} . (D) Experimental unfolding trace for the non-native low force class obtained at a pulling speed of 700 nm s^{-1} . The additional 17–19 nm contour length increment attributed to N-terminal Coh unfolding is shown in red. (E) Dynamic force spectrum for XMod-Doc:Coh unbinding in the native geometry obtained from experiment and simulation. Gray points and squares represent the rupture force/loading rate pairs obtained from experiment and simulation, respectively. Black circles represent the most probable rupture force/loading rate obtained from Gaussian fits to the experimental data at 6 pulling speeds. The black square shows the mean rupture force and loading rate for the simulated rupture events. (F) Rupture force histograms obtained at a pulling speed of 800 nm s^{-1} for the native (gray, $n = 46$) and non-native high force class (red, $n = 48$). Fitted probability densities $p(F)$ are shown as solid black and red lines. Data for both pulling configurations were obtained with the same cantilever to minimize calibration errors. (G) Dynamic force spectrum for XMod-Doc:Coh unbinding in the non-native low force class obtained from experiments and simulation. The same representation as in (E) is used. (H, I, J) Unloaded and loaded surface contact areas for the different pulling geometries ((H) native, (I) non-native high force class, (J) non-native low force class)

Figure 2.

Network analysis test simulation. (A) Simulated pattern of atoms depicted by spheres. Connecting lines between atoms represent harmonic springs with different stiffnesses (red: k , blue: $5k$, yellow: $7.5k$, black: $10k$). The green atom was fixed (anchor), while a second green atom was withdrawn at constant speed (arrow). Black and yellow atoms and their adjacent springs were introduced to maintain the general shape of the pattern. (B) Deformed sphere pattern at the end of the simulation. (C) Edges between nodes are weighted by the corresponding correlation matrix elements. (D) The path with highest correlation of motion is shown in red.

Figure 3.

Force propagation through XMod-Doc:Coh in the native pulling configuration. (A) Unbinding trace of XMod-Doc:Coh obtained from SMD at a pulling speed of $0.25 \text{ \AA ns}^{-1}$. The full trajectory is shown in gray. The black line represents a moving average with a box size of 500 steps. The highlighted red areas denote the windows where dynamic networks and contact areas were calculated. (B) Network paths for the unloaded system. The thickness of the orange tube represents the number of suboptimal correlation paths passing between two nodes. (C) Network paths for the loaded system. A detailed 2D representation of the pathway, highlighting the amino acids present in the pathway, is shown in Fig. S5. (D) Schematic model of force propagation across the Coh:Doc binding interface. Force takes a path across the binding interface with large normal components to the unbinding axis.

Figure 4.

Force propagation through XMod-Doc:Coh in the non-native pulling configuration. (A) Unbinding trace of XMod-Doc:Coh in the non-native pulling configuration obtained from SMD at a pulling speed of $0.25 \text{ \AA ns}^{-1}$. The full trajectory is shown in gray, the black line represents a moving average with a box size of 500. Note that this computational pulling experiment revealed a high-force behavior. (B) Network pathways calculated from dynamical network analysis for the non-native HF trajectory. A detailed 2D representation of the pathway, presenting amino acid identification, is shown in Supporting Fig S6. (C) Schematic model of force propagation across the Coh:Doc binding interface. Force takes a path across the binding interface with large normal components to the unbinding axis. (D) Unbinding trace of XMod-Doc:Coh in the non-native pulling configuration obtained from SMD at a pulling speed of $0.25 \text{ \AA ns}^{-1}$. The full trajectory is shown in gray, the black line represents a moving average with a box size of 500. This computational pulling experiment revealed partial Coh unfolding that led to LF behavior. (E) Network pathways for the non-native LF scenario. A detailed 2D representation of the pathway, presenting amino acid identification, is shown in Supporting Fig S7. (F) Schematic model of force propagation across the Coh:Doc binding interface. Unlike in both HF scenarios, force propagates across the binding interface mostly along the unbinding axis.