Heterogeneous and rate-dependent streptavidin–biotin unbinding revealed by high-speed force spectroscopy and atomistic simulations

Felix Rico\textsuperscript{a,1,2}, Andreas Russe\textsuperscript{b,1}, Laura González\textsuperscript{2}, Helmut Grubmüller\textsuperscript{b,2}, and Simon Scheuring\textsuperscript{a,5}

\textsuperscript{a}Laboratoire Adhésion et Inflammation (LAI), Aix-Marseille Université, CNRS, INSERM, 13009 Marseille, France; \textsuperscript{b}Department of Theoretical and Computational Biophysics, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany; \textsuperscript{c}Department of Electronics, Universitat de Barcelona, 08028 Barcelona, Spain; \textsuperscript{d}Department of Anesthesiology, Weill Cornell Medical College, New York, NY 10065; and \textsuperscript{e}Department of Physiology and Biophysics, Weill Cornell Medical College, New York, NY 10065

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Receptor–ligand interactions are essential for biological function and their binding strength is commonly explained in terms of static lock-and-key models based on molecular complementarity. However, detailed information on the full unbinding pathway is often lacking due, in part, to the static nature of atomic structures and ensemble averaging inherent to bulk biophysics approaches. Here we combine molecular dynamics and high-speed force spectroscopy on the streptavidin–biotin complex to determine the binding strength and unbinding pathways over the widest dynamic range. Experiment and simulation show excellent agreement at overlapping velocities and provided evidence of the unbinding mechanisms. During unbinding, biotin crosses multiple energy barriers and visits various intermediate states far from the binding pocket, while streptavidin undergoes transient induced fits, all varying with loading rate. This multistate process slows down the transition to the unbound state and favors re-binding, thus explaining the long lifetime of the complex. We provide an atomistic, dynamic picture of the unbinding process, replacing a simple two-state picture with one that involves many routes to the lock and rate-dependent induced-fit motions for intermediates, which might be relevant for other receptor–ligand bonds.

Significance

Protein–ligand interactions are commonly described in terms of a two-state or a lock-and-key mechanism. To provide a more detailed and dynamic description of receptor–ligand bonds and their (un)binding path, we combined high-speed force spectroscopy and molecular dynamics simulations to probe the prototypical streptavidin–biotin complex. The excellent agreement observed, never used for force-field refinement, provides the most direct test of the “computational microscope.” The so-far largest dynamic range of loading rates explored (11 decades) enabled accurate reconstruction of the free-energy landscape. We revealed velocity-dependent unbinding pathways and intermediate states that enhance re-binding, explaining the long lifetime of the bond. We expect similar behavior in most receptor–ligand complexes, implying unbinding pathways governed by transient, timescale-dependent induced fits.


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1F.R. and A.R. contributed equally to this work.

2To whom correspondence may be addressed. Email: felix.rico@inserm.fr, hgrubmu@gwdg.de, or sis2019@med.cornell.edu.

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Therefore, most structural knowledge on unbinding/unfolding processes has been derived from atomistic simulations that were often limited to short timescales inaccessible to experiments and therefore not rigorously validated (24–28). Thus, today, a direct relationship between the energy landscape and the dynamic structural details of these seemingly simple biomolecular processes is missing. As a result, it is still unclear (i) how b precisely outlives days and unbinds under load, (ii) how and where b is located at the point of rupture and how the respective intermediates are stabilized, (iii) if there is only one or possibly several unbinding pathways, and if so, (iv) to what extent the unbinding paths change with loading rate. Here we address these questions by combining high-speed force spectroscopy (HS-FS) and fully atomistic simulations to observe b unbinding from SA over 11 decades of loading rates.

For the HS-FS experiments we used microcantilevers functionalized with b to probe the force required to rupture individual SA–b bonds at various loading rates (Fig. L4, Left). The use of microcantilevers with response time of ∼0.5 μs and reading out the reflected laser beam at 0.05 μs (high sampling rates up to 20 million samples per s) allowed tracking the cantilever position while pulling at velocities up to ∼30,000 μm/s, almost an order of magnitude faster than previous HS-FS measurements and about 1,000 times faster than conventional AFM FS measurements (11, 19, 29). All atom steered molecular dynamics (SMD) simulations precisely mimicked the experimental setup by using the fully solvated tetrameric structure of SA [Protein Data Bank (PDB) ID code 3RY2 (4)] and by pulling b using two springs in series, using the worm-like chain (WLC) model describing the PEG linker and a linear spring for the cantilever, whose end was moved at constant velocity (Fig. L4, Right and Movies S1–S3). The overall applied pulling velocities ranged from 0.05 μm/s to 30,000 μm/s in HS-FS experiments (Fig. 1B) and from 1,000 μm/s to 5 × 10^13 μm/s in SMD simulations (Fig. 1C), resulting in a combined range of loading rates from ∼100 pN/s to ∼10^13 pN/s, covering 11 decades.

The measured force curves showed a characteristic curve of increasing force due to stretching of the flexible PEG linker (Fig. 1), signature of the specificity of the interaction (32) (Materials and Methods). Fig. 2 shows the dynamic force spectrum obtained both from the most probable rupture forces at each loading rate (in piconewtons per second) in the experiments (circles) as well as from the average rupture force of 10 to 20 simulations per loading rate (triangles). At the overlapping loading rates, rupture forces from experiments and simulations agreed very well, thereby providing independent validation for the MD simulations. At the lowest loading rates, from ∼10 pN/s up to ∼10^3 pN/s (∼10^13 pN/s pulling velocity), rupture forces increased almost linearly with the logarithm of the loading rate, indicating one single dominant barrier in this loading rate regime. At faster loading rates, steeper slopes are observed. This behavior has been interpreted before in several ways: (i) multiple transition barriers along the 1D energy landscape, inner barriers becoming dominant at high loading rates and outer barriers, at low rates (10, 19); (ii) force-induced shortening of the distance to the transition state (14, 33); and (iii) a transition from a thermally activated to a so-called deterministic regime (15, 24, 34). Another possible cause (iv) might be that the cantilever response affects the force spectrum for pulling timescales shorter than the cantilever response time (35, 36).

Actually, previous experiments using devices.
with a dynamic response slower than HS-FS cantilevers (response time \( \tau \sim 50 \) to 500 \( \mu \)s, effective diffusion constant \( D_p \sim 10^7 \) to \( 10^8 \) \( \text{nm}^2/\text{s} \), compared with \( \tau \sim 0.5 \) \( \mu \)s, \( D_p \sim 10^6 \) \( \text{nm}^2/\text{s} \)) have reported a marked slope increase at loading rates \( \sim 10^9 \) \( \text{pN/s} \) (10, 16, 19, 29), while our first slope increase occurred at \( \sim 10^7 \) \( \text{pN/s} \). This suggests that this possible effect (iv) is reduced using HS-FS microcantilevers, which allows orders of magnitude faster loading rates before this possible effect may appear.

By virtue of the broad range of loading rates covered here, the combined dynamic force spectrum contains more information on the free-energy landscape of unbinding than has been accessible before. As detailed below, all three possible explanations (i–iii) seem to contribute to the shape of the energy landscape. In particular, single-barrier models did not describe the entire dynamic force spectrum satisfactorily, supporting the presence of a more complex energy landscape with multiple barriers (14, 15, 34, 36, 37). To avoid approximations inherent to analytic theories, we instead performed Brownian dynamics simulations using a more complex energy landscape with two barriers and varied the shape and height of these barriers (Fig. 2, Inset; see also SI Appendix) until the best agreement with the dynamic force spectrum (blue line in Fig. 2) was obtained. Importantly, this free-energy landscape explains both experiment and simulation over the whole 11 decades of loading rates. The energy landscape has a first (inner) \( \sim 17 \) k\( \text{BT} \) barrier at 0.19 nm, which determines the force spectrum slope at loading rates faster than \( 10^9 \) \( \text{pN/s} \), and a second \( \sim 21 \) k\( \text{BT} \) unbinding barrier further out at 0.44 nm (Fig. 2, Inset, blue line). The longer rupture length of the second barrier, which becomes rate-limiting only at lower unbinding rates, gives rise to the shallower slope at loading rates below \( 10^9 \) \( \text{pN/s} \). We note that the MD simulations suggest intermediate states (i.e., a well between these two barriers, sketched as a dashed blue line in the inset), which, however, cannot be extracted (or ruled out) by analyzing the force spectrum alone (33). Also, a third unbinding barrier further out at distances larger than 0.44 nm (indicated by the dashed red line in the inset) is seen in our experimental force curves and MD simulations and will be discussed below. However, in the force spectrum this barrier would only become kinetically dominant at lower loading rates than probed in our HS-FS experiments and is therefore not seen.

According to the Bell–Evans theory, one would expect two distinct slopes in the force spectrum, corresponding to the two kinetically relevant unbinding barriers (13). In our dynamic force spectrum, we observe, however, a rather continuous slope increase up to \( 10^{11} \) \( \text{pN/s} \) attributed to a force-induced shortening of the rupture length of these two barriers, as predicted by theories that take the shape of the barriers into account (14, 36). Finally, the slightly deviation of the Brownian dynamics (BD) curve at very fast loading rates \( > 10^{11} \) \( \text{pN/s} \) may indicate a transition from a diffusion-dominated (Bell–Evans regime) to a deterministic regime (15, 34). For SA–b, this critical loading rate \( (F_c \gg F_D x_b^{-2}) \sim 10^{11} \) \( \text{pN/s} \) (or \( \sim 10^{10} \) \( \text{nm/s} \)) is orders of magnitude faster than that observed in previous HS-FS experiments of titin unfolding (\( \sim 10^{7} \) \( \text{pN/nm} \), \( \sim 10^{8} \) \( \text{nm/s} \)) (24). This was expected since the transition is supposed to emerge when the pulling rate is faster than the intrinsic time required for the complex to explore its energy landscape. This intrinsic time was \( (x_b^2/D) \sim 0.2 \) ms for titin 191 but much shorter (\( \sim 1 \) ms) for SA/b, which is reasonable given the less pronounced structural changes of the b molecule compared with partial titin domain unfolding.

The MD unbinding simulations (>300 in total) provided structural information on the loading-rate-dependent unbinding paths. Fig. 3A shows the distribution of center of mass (COM) distances from all MD trajectories between the SA binding pocket, defined as the set of amino acids that interact with the bound b in the static crystal structure, and the b molecule. The peak at 0 nm represents the bound state followed by two consecutive minima at \( \sim 0.25 \) nm and \( \sim 0.5 \) nm. These values are slightly left of the positions of the two barriers (\( \sim 0.19 \) nm and \( \sim 0.44 \) nm) obtained from the BD fit of the force spectrum and suggest the COM distance as an appropriate reaction coordinate of unbinding. Interestingly, the peak between and to the right of these two minima suggests that, at least upon force application, one or several metastable states appear, as a result of the tilted energy landscape. Importantly, these metastable states will favor rebinding at sufficiently slow pulling rates (37, 38). The simulations show that most of the H-bonds between b and the SA binding pocket remain intact until b has moved \( \sim 0.15 \) nm toward the outside of the SA binding pocket. At the distance corresponding to the first barrier, the H-bonds between residues Ser27, Tyr43, Asn49, and Asp128 and the b, rather parallel to the pulling direction, rupture. Escape from the binding pocket occurs only after the second barrier at \( \sim 0.5 \) nm, where most of the remaining H-bonds between b and SA (mainly with residues Asn49, Tyr54, and Arg84) rupture (Fig. 4B and SI Appendix, Fig. S9). Notably, these H-bonds are nearly perpendicular to the unbinding direction (long axis of the binding pocket), which implies a shear force, and only simultaneous failure of all H-bonds lead to dissociation, with subsequent transient formation of a different H-bond network (SI Appendix, Fig. S9). A similar mechanism has been observed before as key to providing stability against forced protein unfolding (39, 40). Overall, the geometry of rupture and reconfiguration of the H-bond network between b and adjacent amino acids of the binding pocket seems to represent the main determinants of the dynamic force spectrum in Fig. 2, described by the energy landscape with two barriers.
One might assume that linear extrapolation of the force spectrum to zero force should yield a time scale similar to the spontaneous SA–b unbinding off-rate $k_{off} \sim 10^{-6}$ s$^{-1}$ obtained from bulk equilibrium experiments (7). However, as in previous single-molecule force experiments (10, 19) (SI Appendix), Fig. 2 would suggest a much faster off-rate of $\sim$1 s$^{-1}$. Notably, also the 21 k$_B$T barrier (blue line in Fig. 2, Inset) is located outside the energy landscape is $\sim$19 k$_B$T lower than the calorimetric SA–b binding free energy of $\sim$40 k$_B$T (7). Further, a Kramers estimate using an attempt frequency of 10$^{12}$ s$^{-1}$ would also predict a $\sim$1 s$^{-1}$ off-rate. Whereas the end states of enforced and spontaneous unbinding are not the same and, hence, the respective (un)binding free-energy differences are not expected to fully agree, such a large discrepancy is unexpected. To reconcile forced and equilibrium unbinding, a third barrier located further out on the unbinding pathway should be present (as depicted in Fig. 2, Inset, red dashed line), as was speculated before from indirect evidence (10, 21). Such a barrier would show up in a dynamic force spectrum at loading rates much lower than accessible to experiments (and certainly to simulations) and, therefore, remained so far unobserved. One would, however, expect to observe interactions between SA and b farther out of the binding pocket, as structural determinants of this barrier.

Indeed, our MD simulations revealed such interactions and corresponding intermediate states. In particular, the distribution of COM distances between the SA binding pocket and the b molecule displays a pronounced peak after the second minimum followed by smaller peaks at distances up to 1.5 nm (Fig. 3A and SI Appendix). Detailed inspection of the individual MD trajectories showed as many as eight transient H-bonds (mainly to ASN49, GLU51, and TYR54) formed with $\sim$1 nm away from the binding pocket (Fig. 3A). Importantly, the force profiles (Fig. 3B) from these trajectories ($\sim$15% of all trajectories) displayed adhesive interactions after and at a lower value than the main force peak and, therefore, these states are not seen in the dynamic force spectrum. In these events, the force applied to b displays a drop due to the exit from the binding pocket and a subsequent intermediate force plateau with a final drop due to complete detachment. At the slowest MD velocities, this transient unbinding state lasted up to several hundred nanoseconds, such that it should also be detectable using HS-AFM micro-cantilevers with submicrosecond resolution.

To test this hypothesis, we analyzed in further detail the individual experimental force curves. Remarkably, we observed a similar signature in about 5% of the HS-FS unbinding events with a transient force plateau during the snap off of the cantilever (Fig. 3C and SI Appendix, Figs. S5 and S6). These transient, microsecond-long events were observed over the full range of experimental loading rates and provide an experimental signature of the transient outer states. The low occurrence of these events in HS-FS curves may be due to their short lifetime. Moreover, the distance from the force peak to the transient binding had an average value $\sim$1 nm extending up to 3 nm (Fig. 3D), similar to the distances seen in the MD trajectories (Fig. 3A). Therefore, the combination of HS-FS and MD simulations of SA/b forced unbinding provided first direct experimental evidence and a structural description of an outer binding state that may be at the basis of the SA/b sturdiness.

The large number of experiments and simulations allowed us to characterize the average lifetime $\tau$ of these outer binding states. Fig. 3E shows that $\tau$ ranges from 0.001 μs to 100 μs for forces $F_0$ between 500 pN down to 20 pN (including MD and HS-FS data). Hence, excellent agreement between experiments and simulations is seen also in the time domain. The average lifetime decayed exponentially with force and can be described by a single barrier (14) of 12 k$_B$T additional height with a rupture length of 0.16 nm and, notably, of $\sim$16-μs lifetime at zero force (Fig. 3D). This third barrier (red dashed line in Fig. 2, Inset) is located outside the second barrier (blue line), at a distance of 0.60 nm from the bound state (red line in Fig. 2, Inset) and adds a further step upward to the energy landscape toward the fully unbound state. This distance correlates with a minimum in the COM distance at $\sim$0.7 nm. Although the exact minimum preceding this outer barrier is difficult to pinpoint, metastable states before this barrier are expected upon force application, as reflected from the peak in the COM distribution at $\sim$0.6 nm. This barrier further slows down b unbinding by several orders of magnitude, thereby reconciling it with the observed slow equilibrium off-rate. The respective interactions between b and the outside of the binding pocket should favor rebinding events, in particular at low and zero forces. Although rare, back-and-forth fluctuations between intermediate states were actually observed in some of the MD trajectories (Fig. 4).

As shown in Fig. 3D, the experimental distance to the outer binding increased with the pulling velocity, suggesting that shorter jumps occur more often at slow pulling. This suggests that, although
effectively described by a single barrier, the outer barrier may involve not only one but several intermediate states, not directly resolved experimentally and with varying occupancies that depend on the pulling velocity. This notion is further supported by the various peaks observed in the COM distance outside of the binding pocket, which allowed characterizing the four most populated intermediate states (Int 1 through Int 4, Fig. 4A). Importantly, various unbinding paths were seen in the trajectories. The large number of atomistic simulations for each loading rate allowed us, finally, to study to what extent the observed unbinding pathways change with loading rate. Under high load, mainly two intermediate binding states (Int 1 and Int 2) are visited along the unbinding pathway. At lower loading rates, states Int 3 and Int 4, farther out, are also visited. Likely these, and even intermediates lying farther outside, provide a rugged funnel (41–43) for rebinding under equilibrium conditions.

Calculating the average energy of the H-bonds between b and individual amino acids in the binding pocket from all MD trajectories as a function of the b position (Fig. 4B and SI Appendix, Fig. S9) allowed us to extract structural snapshots of each intermediate state (Fig. 4C). Whereas the inner intermediate states showed strongest interactions between the ureido moiety of b and residues Ser27, Tyr43, and Asp128, respectively, at later stages of unbinding other bonds, largely overlooked so far, become relevant, such as Arg64, Glu51, and Tyr54, at COM distances of up to 1.5 nm from the bound state (Fig. 4B). While SA modifications of these three residues have reported lower b affinity (6, 44, 45), they have not been expected to be involved in b binding due to their large separation from the binding pocket and their little contribution to the bound state, underscoring the impact of features along the unbinding path on binding kinetics.

One might speculate that SA has evolved in tetrameric form because it allows for even larger binding affinity due to intermonomeric stabilization, with the 7–8 loop providing the key interprotomer interaction (5). To test this idea, we repeated our steered MD simulations using monomeric SA—which is difficult experimentally. As suggested before from high-load-rate simulations on avidin–b unbinding (27), rupture forces of the monomer were systematically 10 to 20% lower than for the tetramer over the whole loading-rate range (SI Appendix, Fig. S8 and Movies S4–S6), thus further supporting this hypothesis. Closer structural analysis of the unbinding paths suggests that these differences in rupture forces are due to (i) lacking intermonomeric interactions [specifically to the 7–8 loops of the adjacent protomer (27)] and (ii) an increased heterogeneity of unbinding paths, the larger entropy of which further reduces the unbinding barrier.

Strikingly, the unbinding-rate-dependent heterogeneity and occupancies of intermediate states are accompanied by rate-dependent, nonequilibrium dynamics of the SA structure while b moves toward the unbound state. We observed induced-fit motions of the binding pocket and adjacent loops outside the fully bound state, along the (un)binding path. As an example, loop 3–4 switches between an open and a closed conformation during unbinding, depending on the distance between b and the binding pocket (SI Appendix, Fig. S10). These nonequilibrium conformational changes are more pronounced at slower loading rates, for which the loop has more time to fluctuate and equilibrate (SI Appendix, Fig. S10). Therefore, it should also occur in the AFM experiments as well as during spontaneous unbinding. This finding suggests that “induced fit” and other nonequilibrium conformational changes of SA control not only the bound state but also the transient energetics and kinetics along the binding and unbinding paths, and in a loading-rate-dependent manner.

The combination of HS-FS and MD simulations at overlapping loading rates allowed us to obtain a dynamic and atomistic description of a receptor–ligand unbinding process. Characterizing enforced SA/b unbinding over an unprecedentedly large range of loading rates enabled us to characterize large portions of the underlying energy landscape, which would not have been accessible by one of the two methods alone. Notably, it also allowed for a most direct comparison between AFM experiment and MD simulation. The excellent agreement of rupture forces at overlapping loading rates—an observable that has never been used for force field parameterization—underscores the predictive power of atomistic MD simulations.

Single-barrier theoretical models successfully describe the spectrum over a wide range at high loading rates and serve to interpret our results (SI Appendix, Fig. S7). The fitted Bullerjahn–Sturn–Kroy (BSK) model predicts a transition toward the deterministic regime at rates ~10^11 pN/s only reached by MD simulations, that may explain the change in slope at this loading rate (15). The recently developed Cossio–Hummer–Szabo (CHS) model, which described remarkably well the dynamic force spectroscopy, predicts instead a kinetically ductile regime described as gradual stretching and shortening of the distance to the transition state under force before unbinding, which helps understand the curvature observed at high loading rates (Fig. 2 and SI Appendix, Fig. S7). The low value, close to zero, of the unitless kinetic brittleness [μ ~ 10^-6; SI Appendix, Fig. S7 (36)] found for our spectrum is consistent with a ductile behavior for SA/b. Inclusion of the low-loading-rate regime requires multiple barriers (38), and the full dynamic force spectrum over 11 decades is only accurately described by BD simulations. Finally, outer barriers at distances beyond the energy landscape of Fig. 2 (Inset, blue line) would enhance rebinding and likely emerge in the force spectrum at loading rates lower than the ones explored in our work (but were detected as transient binding events in both HS-FS and SMD). Therefore, combinations of available analytical theories seem to be required to fully explain the force spectrum over the whole dynamic range, including a more refined description taking into account the dynamic nature of the energy landscape.

Our concerted approach revealed multiple unbinding pathways and nonequilibrium conformational changes of the SA binding pocket dependent on loading rate, with a detailed description of the pathway fluxes. In particular, outer intermediates were found that affect binding energetics and kinetics. Future combination of HS-FS and MD simulations will answer whether these proposed mechanisms found for SA/b are specific to this bond or—in our view more likely than not—a common feature of many receptor/ligand complexes and thus a general mechanism of regulating binding kinetics. If this were the case, the study of intermediates and their mechanism would be instrumental in improving the binding kinetics and specificity of drug-like compounds. Taken together, our results suggest that the current static picture based on the bound state may need to be extended in terms of many routes to (un)binding as well as multiple, transient, and nonequilibrium-induced fits, resembling a combination lock, where several intermediate positions have to be visited for final release. The development of theoretical models taking into account the dynamic nature of the energy landscape will help us better understand biological bonds.

Materials and Methods

HS-AFM Tips and Sample Preparation. SA-coated 4% agarose beads (Sigma) were immobilized on the sample surface by embedding them in a thin agarose layer. b was covalently attached to the cantilever through a PEG linker (stretched length ~10 nm; Fig. 1A). Briefly, HS-AFM (AC10DS and AC7) cantilevers (Olympus) were rinsed with acetone for 10 min, plasma-cleaned for 5 min in O2, and immersed in a solution of 10 to 20 mg/mL silane-PEG-biotin (1 kDa; Nanocs Inc.) in ethanol/water (95/5). After 2 h incubation, cantilevers were rinsed with ultrapure water and stored at 4 °C until use.

HS-FS Measurements. HS-FS measurements were carried out on an HS-AFM (RIBM) featuring a high-speed acquisition board system (PKI; National Instruments) to control the z-movement and acquire force curves at sampling rates up to 20 Mz/s. Two types of cantilevers with submicrosecond time response
and small viscous damping were used: AC10 cantilevers with 600-kHz resonance frequency in liquid, 0.1 Nm spring constant, quality factor of 0.9, and 0.09 pN/\(\text{nm}^2\) viscous coefficient; and shorter AC7 cantilevers, with 1.3-MHz resonance frequency in liquid, 0.6 Nm spring constant, quality factor of 0.6, and 0.05 pN/\(\text{nm}^2\) viscous coefficient. The spring constant of the cantilevers was determined in air using the Sader method (46). The optical lever sensitivity was then determined in liquid from the thermal spectrum and the known spring constant (24, 47). Short HS-AMF cantilevers were placed on the cantilever holder immersed in the fluid cell with PBS buffer and placed on the HS-AMF. The SA agarose functionalized sample-stage was then mounted onto the fluid cell to achieve a lower binding frequency of 

\[
\omega_\text{SA} = \frac{1}{2\pi} \sqrt{\frac{k_B T}{m}},
\]

with a persistence length \(l_p = 1.4\) nm and a contour length \(l_c = 10\) nm, similar to the experimental values (Fig. 1, simulation setup), which was added to GROMACS as a tabulated bonded potential (54). To keep the simulation box size small we shifted the WLC potential by a constant \(k_{\text{start}} = 7\) nm, nevertheless making sure that the forces required to stretch the linker were lower than those to rupture the complex. All simulations were performed at a sodium chloride concentration of 150 mM. The simulation box sizes perpendicular to the pulling direction were chosen to be 8.5 nm, which is a minimal distance of 0.7 nm between the solute and the periodic boundaries, leading to a minimum distance between SA and its periodic image of more than 1.4 nm. The box length in the pulling direction varied between 18 nm for the faster and 13 nm for the slowest simulations.

The starting structure for the simulations was based on the tetramer PDB ID code 3RY2 (4). In case of the tetramer, only one b was pulled out of the binding pocket to ensure single unbinding events.

Rupture forces and loading rates were determined for each individual force curve. As for experiments, the loading rate was also determined from the slope of the force–time curve before rupture. We calculated the mean and SD of the rupture forces for each loading rate.

**Brownian Dynamics Simulations.** To extract information on the underlying free-energy landscape along the unbinding reaction coordinate, and in addition to the application of the simple Bell model and more sophisticated theoretical approaches [BSK (15), Fridle-Noy-deYoreo (FNdY) (37), Dudko-Hummer-Szabo (DHS) (14), and CHS (36)], numerical simulations of enforced unbinding with one-dimensional energy landscapes were carried out. To that end, the Smoluchowsky equation,

\[
\partial_t \rho(x, t) = \mathcal{V} \rho(x, t) - \langle \nabla \mathcal{V} \rangle \cdot \mathbf{v}(x, t),
\]

was solved numerically for a time-dependent potential \(\mathcal{V}(x, t) = V_0(x) - F t\), where \(\beta = 1/k_B T\) and \(F\) is the applied loading rate. As underlying unbinding potential, a double-barrier potential \(V_{\text{double}}(x) = 2\Delta G_{\text{start}}(x)(1.5 - 0.9y(x))\) for \(x < x_{\text{start}}\) was chosen, with barrier height \(\Delta G_{\text{start}}\) of the first barrier. The function \(y(x) = (c_1 + c_2 + \frac{x}{x_{\text{start}}})\) was with the constants \(c_1 = w_1 + w_2 - 2\) and \(c_2 = 3 - 2\text{min}(w_1, w_2)\) served to control the shape of the first barrier, particularly the curvatures on \(w_1\) and on \(w_2\) of the well and, respectively, that at the first barrier top. The above function also serves to control the rupture length \(x_{\text{rupt}}\), here defined as the distance between first barrier top and minimum of the well of the bound state.

For \(x_{\text{start}} < x < x_{\text{rupt}}\) the potential becomes

\[
V(x) = V_0(x) + 2\Delta G_1 - \Delta G_1 y(x)^2 (1.5 - 0.9y(x))
\]

with \(y(x) = \frac{x_{\text{rupt}}}{x_{\text{rupt}} - x}\) and the position of the second barrier \(x_{\text{rupt}}\). For all \(x > x_{\text{rupt}}\) the potential is set to \(\Delta G_{\text{start}}\), the height of the second barrier.

Trajectories were generated starting from a Boltzmann ensemble \(\rho_0(x)\) \(\exp(-\mathcal{V}(x))\) within the well of the bound state. Positions were updated according to the solution of the Smoluchowski equation for linear potential,

\[
x_{\text{new}} = x_{\text{start}} + \frac{F\Delta t}{k_B T} \mathcal{V}(x, t) + \frac{1}{\sqrt{2D\Delta t}} \xi(t),
\]

with an integration time step of 0.5 ps, which ensured that less than 5% of the integration steps were larger than 0.05 \(x_{\text{rupt}}\). A Gaussian distributed (variance one) random force \(\xi(t)\) was used. A diffusion constant \(D = k_B T/(\gamma \cdot 4 \pi \cdot 10^{-11} \text{m}^2/\text{s})\) was found to provide the best force spectrum.

To reduce computational effort and thus to facilitate the generation of trajectories even for the slowest loading rates, an appropriate biostatic potential

\[
V(x) = \kappa_l |x|,\quad \kappa_l = \frac{1}{2k_B T/\gamma}.
\]
\[
\nu(t) = a \left( \frac{n}{n-1} \right) + v(t)
\]
was added whenever the (actual) barrier height exceeded 5 k\text{B}T, with

and \(v\) adapted such that the barrier height of the resulting total potential \(V(x, t) + V_{\text{bias}}(x, t)\) remained between 5 and 7 k\text{B}T at all times. To compensate for the reduced barrier height due to the biasing potential and the increased barrier transition probability per integration step, the integration step size was dynamically rescaled by an acceleration factor \(\frac{\Delta Z_\text{dot}(t)}{Z_\text{dot}(t)}\) where \(Z_\text{dot}\) are the partition functions of the unperturbed and, respectively, perturbed bound states:

\[
Z_\text{dot}(t) = \int \exp(-\beta V(x, t) + V_{\text{bias}}(x, t)) \text{d}x.
\]

For each of the loading rates for which experimental or MD simulation derived rupture forces were obtained (Fig. 2), 1,000 trajectories were generated and the resulting individual force at the point of barrier crossing was averaged. The fitting parameters \(\Delta G_\text{dot}, \chi_\text{fl}, \beta, \chi_{\text{bias}}, \text{and } \omega_{\text{bias}}\) were varied using simple line-scanning until the \(\chi^2\) (weighted by the SEM) relative to the 37 experimental/simulation averaged rupture forces was minimal. Uncertainties of the six fitting parameters (given in the caption of SI Appendix, Fig. 57) were estimated conservatively via nonparametric bootstrapping with 80 replica datasets, each of which was generated by randomly drawing 37 from the 37 rupture forces (allowing for multiple draws of the same data point). Parametric bootstrapping using a Gaussian error model and the SEMs rupture forces yielded slightly smaller uncertainties.

**COM Distributions.** To calculate the distributions of the COM distance of each MD trajectory, we calculated the distance between the COM of the binding-pocket-forming residues (L25, S27, Y43, S45, V47, G48, A50, W79, R84, A86, 588, T90, W92, W108, L110, and D128) and the COM of b. This was done starting shortly before rupture and ending when the distance between b and the binding pocket was larger 4 nm.

As we do not see any binding patterns further away than 2 nm we reduced the plotted data to a maximal distance of 2 nm. The COM distances were then binned into 200 equally spaced bins and normalized by the total amount of data points.

**MD Intermediate States and Transition Plots.** To determine the intermediate states, we split the COM distance distributions according to the dominant peaks, such that each peak represents a different intermediate state. We then calculated the transition rates by counting the transitions from state \(s\) to state \(s'\) and subtracted the amount of back-transitions (\(s\) to \(s'\)) for each combination, resulting in a net transition of \(-1, 0, or 1\). Finally, each rate was averaged for each velocity. The probabilities for being in an intermediate state were calculated by counting the total time that an intermediate state is visited normalized by the total time spent in all intermediate states. The time spent in the ground state and the unbound state were not taken into account to provide comparability, as the time spent in either of them is arbitrary and depends only on the chosen starting and end point of the rupture event.

**Principal Component Analysis of Loop 3–4.** To understand the different unbinding pathways, we analyzed the motion of loop 3–4 by performing a principal component analysis on the backbone atoms of residues 44–54. Therefore, a representative simulation was used to calculate the characteristic eigenvectors of the loop 3–4 motion going from a closed conformation (negative values) to an open conformation (positive values). All other simulations were projected onto the first eigenvector and analyzed depending on the applied loading rate regime. The time-resolved projections were finally binned along the COM distance and the average projection on the first eigenvector for each loading rate regime was calculated (SI Appendix, Fig. 510).

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