

Calcium stabilizes the von Willebrand factor A2 domain by promoting refolding

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Von Willebrand factor (VWF) is a large, multimeric plasma glycoprotein that critically mediates hemostasis at sites of vascular injury. Very large VWF multimers have the greatest thrombogenic activity, which is attenuated by cleavage in the A2 domain by the metalloproteinase ADAMTS13. ADAMTS13 proteolysis requires mechanical force to expose the scissile bond and is regulated by a calcium-binding site within A2. In this study, we characterized the interaction between VWF A2 and calcium by examining the effect of calcium on VWF A2 stability and mechanical unfolding and refolding. Isothermal calorimetry yielded a calcium binding $K_d = 3.8 \pm 1.0 \mu\text{M}$ and reversible thermal denaturation showed that 5 mM calcium stabilized the unfolding transition from 56.7 ± 0.1 to $69.1 \pm 0.1^\circ\text{C}$. Using optical tweezers to apply tensile force to single domains, we found that calcium did not affect VWF A2 unfolding, but rather enhanced refolding kinetics fivefold, resulting in a 0.9 kcal/mol stabilization in the folding activation energy in the presence of calcium. Taken together, our data demonstrate that VWF binds calcium at physiologic calcium concentrations and that calcium stabilizes VWF A2 by accelerating refolding.

Von Willebrand factor (VWF) is a large plasma glycoprotein that critically mediates hemostasis at sites of vascular injury. Secreted by endothelial cells and platelets in response to thrombogenic stimuli, VWF circulates as ultralong, disulfide-bonded concatamers (1) reaching >12 MDa and >15 μm in size (2, 3). Each VWF monomer is comprised of multiple domains, including domains that bind clotting factor VIII, collagen, platelets, and integrins (1). The A2 domain contains a cleavage site recognized by the enzyme ADAMTS13 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13), which regulates VWF length. The largest VWF concatamers mediate the formation of a platelet plug through multivalent binding to collagen and platelet membrane glycoprotein Ib. Mutations within VWF cause von Willebrand's disease (VWD), a heterogeneous set of bleeding disorders that underscore VWF function in normal physiology (4).

VWF function is critically determined by concatamer length. Healthy individuals exhibit a heterogeneous distribution of VWF concatamers, whose size is regulated through specific cleavage by ADAMTS13. Absence of ADAMTS13 function causes thrombocytopenic purpura (TTP), a life-threatening condition characterized by extensive microvascular thromboses that result in tissue ischemia and infarction. Neurologic symptoms and renal failure are often described, and the condition resolves upon replenishment of ADAMTS13 with plasma exchange (5). In contrast, genetic mutations that shift the VWF size distribution to smaller multimers, comprising type 2 VWD, present with increased bleeding due to reduced VWF thrombogenicity (6). Subtype 2A VWD mutations cluster within the A2 domain, which bears the ADAMTS13 cleavage site, and whose structure illuminates mechanisms that regulate VWF cleavage (7).

Fluid shear stress is critical for VWF size regulation (8) and VWF A2 is remarkable for several structural features that render the domain sensitive to hydrodynamic forces exerted on VWF concatamers in the circulation. The ADAMTS13 scissile bond is buried in a central β 4-strand within A2 that is inaccessible

in the native state (Fig. 1A). Compared to neighboring domains, VWF A2 lacks a long-range disulfide bond to protect the domain against unfolding by elongational (tensile) force exerted on VWF concatamers. Single molecule studies have demonstrated that ADAMTS13 cleavage requires unfolding of the A2 domain (7, 9) to expose the scissile bond. Furthermore, in place of an α 4-helix, VWF A2 contains an "alpha-less" loop that is thought to slow refolding to promote cleavage site accessibility (7). Thus, structural specializations within VWF A2 predict that reversible unfolding and refolding regulate VWF cleavage via dynamic accessibility of a buried scissile bond.

Recent studies have described a calcium-binding site that is unique amongst VWF A domains, and likely another structural specialization that regulates VWF A2 force sensing. VWF coordinates calcium by four residues from the α 3- β 4 loop and a single residue from the N-terminal β 1-strand (10, 11) (Fig. 1B). Calcium binding was not seen in an earlier structure due to a lattice contact that enforced a conformation of the α 3- β 4 loop that precluded calcium binding (7, 10). Unfolding of A2 is predicted to proceed from the C terminus because, whereas the N-terminal β 1-strand is clamped by hydrogen bonds in the center of the β -sheet, the C-terminal α 6-helix is exposed and only locally hydrogen bonded (Fig. 1A). Furthermore, unfolding from the C terminus of secondary structure elements in the sequence of α 6, β 6, α 5, β 5, α 4-less loop, and β 4 exposes the minimal portion of the A2 domain required for maximal ADAMTS13 cleavage, as shown in peptide substrates (12). VWF A domains feature a "doubly wound" topology that juxtaposes two regions of sequentially arranged secondary structures. The α 3- β 4 loop lies at the junction between these two windings (Fig. 1A) and is thus in a strategic position to regulate A2 folding. In the sequential process of unfolding, the β 4-strand bearing the scissile bond immediately precedes the α 3- β 4 loop. Cleavage assays of isolated A2 demonstrate that calcium binding protects the domain from cleavage by ADAMTS13 (10, 11), and protection against unfolding has also been reported (11). However, the quantitative details of how calcium stabilizes A2 remain to be defined.

In this study, we characterized the mechanisms underlying calcium stabilization of VWF A2. We derived thermodynamic parameters to quantitatively describe the interaction of calcium with VWF A2 and establish physiologic relevance. In parallel, we applied single molecule optical tweezers to functionally characterize the effect of calcium on A2 conformational dynamics. Combining such bulk and single molecule approaches, we gain a quantitative understanding of the conformational dynamics that regulate VWF cleavage.

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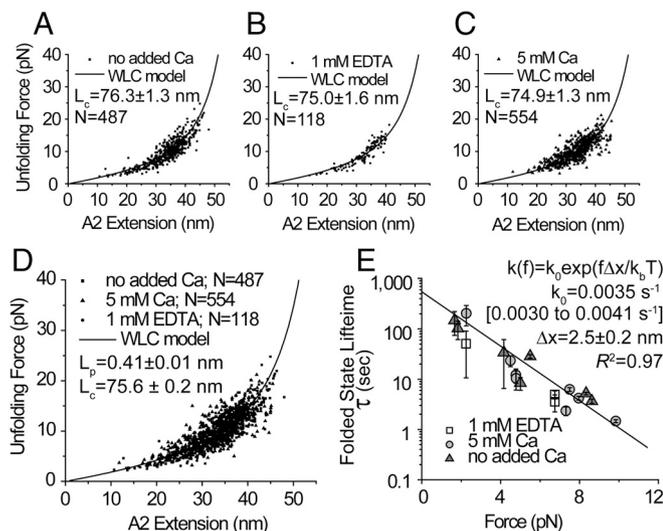


Fig. 5. Lack of effect of calcium on A2 unfolding. (A–C) Lack of effect of calcium on the increase in extension upon A2 unfolding. Independent WLC fits for data collected in the absence of calcium (A), in the presence of calcium (B), and under calcium chelating conditions (C) demonstrate that calcium does not affect A2 compliance in the unfolded state and that A2 exhibits full unfolding behavior under all three conditions. (D) Force-extension discontinuities from all experiments fit to a single WLC model. Individual fits agreed within 4% ($p < 0.05$). (E) Calcium does not affect kinetics of A2 unfolding. The force dependence of unfolding was derived from rupture-force histograms obtained over a range of pulling speeds according to the Hummer–Szabo–Dudko method (21). Errors shown are based on Poisson noise.

(7). Thus, our data predict a 0.4 nm contour length per residue ($75.6 + 1 = 76.6$ nm divided by 177 residues), which agrees well with reported values (16), confirming the force-extension discontinuities observed as full A2 unfolding under all calcium conditions.

Unfolding kinetics were determined by examining the forces at which A2 unfolding was observed. The force dependence of unfolding was extracted from rupture-force histograms obtained over a range of pulling speeds (20–160 nm/s), corresponding to force application rates of 0.1–10 pN/s, according to the Dudko–Hummer–Szabo method (17). Data were fit to a single-barrier kinetic model, $k = k_0 \exp(f\Delta x/k_b T)$, where the unfolding rate is determined by the unfolding rate at zero force, k_0 , and an exponential dependence on force, f , multiplied by the distance to the transition state, Δx (18). Fit independently, the intrinsic unfolding rates at zero force, k_0 , were 0.0034 s^{-1} (95% confidence interval 0.0032 to 0.0037 s^{-1}) and 0.0036 s^{-1} (0.0032 to 0.0042 s^{-1}) in the presence and absence of calcium, respectively, agreeing with statistical significance within 20% ($p < 0.05$). A fit to all data in the presence and absence of calcium yielded $k_0 = 0.0035 \text{ s}^{-1}$ (0.0030 to 0.0041 s^{-1}) with a barrier to activation $\Delta x = 2.5 \pm 0.2 \text{ nm}$ ($R^2 = 0.97$, Fig. 5E).

Calcium Enhances VWF Refolding. Refolding kinetics were assessed by monitoring the probability of refolding across consecutive pulling cycles on the same construct (Fig. 4C). Single tethers were pulled then relaxed with loading rates of 0.1–10 pN/s. Once returned to a low force, the construct was held in constant position, corresponding to a constant force, to allow refolding (Fig. 4C and D). Protein refolding was monitored by force-extension profiles (Fig. 4B) and the presence or absence of unfolding in a subsequent pull (Fig. 4C).

Observations of A2 refolding over many cycles ($N = 786$) demonstrated that A2 refolding was sensitive to force, time, and calcium. The probability of refolding significantly increased with decreased force (Fig. 6A) and increased time (Fig. 6B). Calcium chelation significantly decreased the probability of refolding (Fig. 6C). Both in the presence and absence of calcium, the

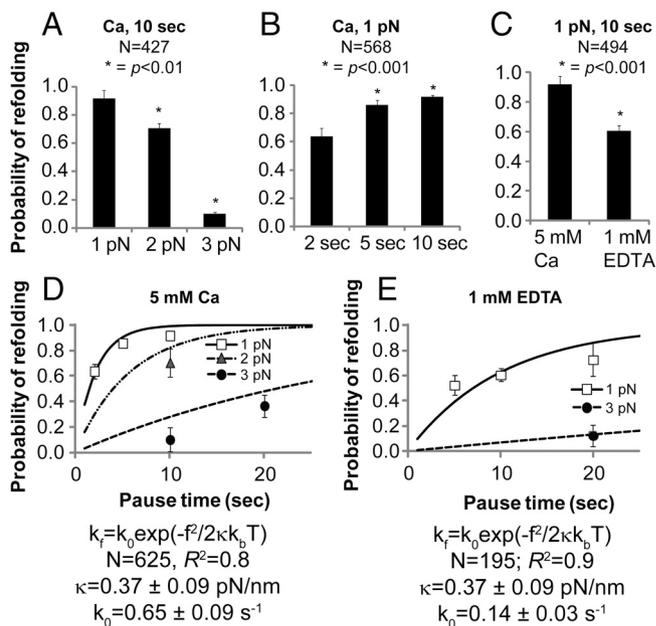


Fig. 6. Calcium enhances kinetics of VWF A2 refolding. (A–C) Binary refolding statistics demonstrate that probability of refolding was sensitive to force (A), time (B), and calcium (C). (D–E) Force dependence of refolding kinetics. Refolding probabilities fit to a single exponential dependence of refolding kinetics on the square of applied force (18).

kinetics of refolding, k_f , were well fit by a single exponential dependence on the refolding rate at zero force, k_0 , the square of applied force, f^2 , and the compliance of the unfolded state, κ (18) (Fig. 6D and E). Under each calcium condition, data obtained at 1 pN, 2 pN, and 3 pN were globally fit to a single set of parameters. The intrinsic kinetics of refolding at zero force, k_0 , in the presence and absence of calcium were $0.65 \pm 0.09 \text{ s}^{-1}$ and $0.14 \pm 0.03 \text{ s}^{-1}$, respectively. This fivefold difference in refolding kinetics yields a 0.9 kcal/mol difference in the folding activation energy.

At moderate force, refolding was observed directly (Fig. 4D). In the presence of calcium at 3 pN, the lifetime of the unfolded state was 41 s (95% confidence interval 32 to 101 s, $N = 40$, 12 events observed directly), agreeing well with the unfolded state lifetime of 31 s expected from the kinetic model. In the absence of calcium, the 3 pN unfolded state lifetime was 96 s [(47 to 296 s), $N = 23$, five events observed directly], compared with 144 s predicted by the kinetic model. Thus, our direct observations of refolding agree well with the kinetic fits obtained from the much larger dataset of refolding probabilities.

Discussion

In this study, we report quantitative bulk measurements of calcium binding and folding thermodynamics, and single molecule measurements of unfolding and refolding kinetics, to understand the mechanisms underlying calcium stabilization of VWF A2 against cleavage. Isothermal calorimetry yielded a 3.8 μM binding affinity between A2 and calcium. The approximately 1.1–1.3 mM free calcium in healthy human blood is greatly above this measured K_d . Thus, the calcium-binding site is predicted to be saturated, establishing the relevance of calcium binding in VWF A2 under physiologic concentrations. Reversible thermal denaturation monitored using CD showed that calcium stabilized VWF A2 in the native state. We measured a thermodynamic stability shift of 12 °C in 5 mM calcium, which is consistent with the apparent 19 °C shift reported using fluorescent dye binding (11). Because our data were obtained under reversible conditions, we were able to estimate a folding free energy of $-2.3 \pm 0.1 \text{ kcal/mol}$ in the absence of calcium and $-3.6 \pm 0.8 \text{ kcal/mol}$

in 5 mM calcium. The difference between these A2 unfolding energies predicts that 5 mM calcium stabilized the folded state by 1.3 ± 0.8 kcal/mol at 25 °C. Single molecule measurements of A2 unfolding and refolding demonstrated that calcium stabilizes A2 by promoting refolding. Unfolding measurements revealed that calcium had little if any effect on unfolding behavior. In contrast, calcium accelerated refolding approximately fivefold, lowering the activation barrier to refolding by 0.9 kcal/mol. Thus, we conclude that calcium stabilizes VWF A2 by promoting refolding.

Our thermodynamic and single molecule data allow us to postulate a free energy landscape for A2 unfolding (Fig. 7). Folding free energies determined by CD thermal denaturation indicate that native A2 (N) is stabilized by calcium (Fig. 7, gray). Similar unfolding kinetics in the presence and absence of calcium dictate similar activation energies from N to the unfolding transition state (TS 1). As a result, calcium stabilizes TS 1 to the same extent as N. Similar energetics are a consequence of similar structural environments (19). Thus, our results suggest that calcium occupies similar structural environments within TS 1 and N, indicating that TS 1 binds calcium with native-like coordination.

In the refolding direction, we assume that A2 does not bind calcium in the unfolded state (U), consistent with coordination of calcium by residues far apart in sequence (from residue 1498 to 1602) and with the similar contour lengths measured in the presence and absence of calcium (Fig. 5). Going from calcium-free U to calcium-bound N, the significant binding energy between calcium and A2 measured by isothermal calorimetry precludes the calcium-binding event from occurring during a transition state. Given that TS 1 binds calcium, such a calcium-free transition state in the refolding direction (TS 2) requires a calcium-binding intermediate (I). Indeed, an unfolding intermediate has been previously described at 1,000 Hz with a 1.2 ms lifetime (9), too transient to be characterized by our data acquired at 500 Hz but consistent with an intermediate structure with high energy relative to N and U (Fig. 7). Further insight into TS 2 is gleaned from the dependence of refolding on calcium. Our single molecule data demonstrate that calcium accelerates refolding. Thus, the rate-limiting transition state from calcium-free U to calcium-bound N must also bind calcium, and cannot be TS 2. Instead, TS 1 functions as the kinetically relevant transition state by occupying a higher energy level than TS 2 in the refolding pathway. In our model, the disparate effects of calcium on the unfolding and refolding kinetics are accounted for by an unfolding transition state, TS 1, that binds calcium with native-like coordination, and that is rate limiting in both unfolding and refolding pathways.

The known three-dimensional structure of A2 (Fig. 1) places some limits on the structure of calcium-binding TS 1. As previously introduced, calcium binds A2 at the junction between two topologically defined windings (Fig. 1A). Both windings begin near the center of the β -sheet but proceed in opposite directions.

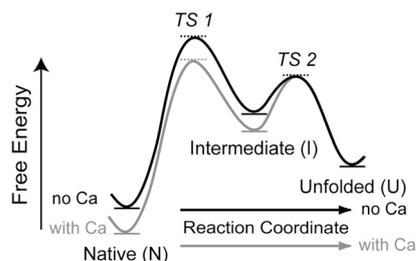


Fig. 7. Proposed A2 energy landscape. Our proposed free energy landscape describing calcium stabilization of A2 features a calcium-binding native state (N); a calcium-binding transition state (TS 1) that binds calcium with native-like coordination and is rate-limiting in both the unfolding and refolding directions; a high energy, calcium-binding intermediate (I); a calcium-free transition state (TS 2) in the refolding pathway; and a non-calcium-binding unfolded state (U).

Winding 1 proceeds from the central β 1-strand to the α 3-helix, whereas winding 2 proceeds from the central β 4-strand to the α 6-helix (Fig. 1A). The calcium-binding site includes Asp-1498 in the β 1-strand, residues 1596, 1597, and 1600 in the α 3- β 4 loop, and Asn-1602 in the β 4-strand (Fig. 1B). Similar unfolding kinetics implicate native-like coordination of calcium within the unfolding transition state and locate the boundary between native-like and unfolded regions of A2 between the β 4-strand and the C terminus. This region includes the α 4-less loop that, like the calcium-binding site, might be important in regulating the kinetics of A2 refolding. Such a model is also consistent with the idea that unpoping of the rigid C-terminal vicinal disulfide plug could be rate limiting for unfolding, as previously proposed (1).

A very recent study used optical tweezers to report that A2 unfolding requires higher forces in the presence of calcium and primarily proceeds through a mechanically stable intermediate (11). Force-extension behavior in this study was measured at 50 Hz and exhibited slow unfolding and refolding (on the order of seconds) with unfolding intermediates lasting 1–3 s. This behavior clearly differs from the highly cooperative unfolding and refolding (within milliseconds) previously reported (9) and observed in our study, as well as the 1.2 ms lifetimes previously reported for A2 unfolding intermediates (9). Because we did not observe such slow phenomena in our thousands of characterized events, we think it is unlikely that A2 follows an alternative unfolding pathway, but rather attribute these discrepancies to different methods for suspending single molecules of A2 between beads. Whereas we attached DNA handles to both ends of A2, holding A2 approximately 260 nm away from each bead and preventing nonspecific interactions, the other study attached DNA at only one end of the A2 construct. Their A2 N terminus contained a (c-myc)₄ tag that directly bound an anti-c-myc antibody-coated bead, placing A2 approximately 840 nm away from the DNA-bound bead but only 0–30 nm away from the (c-myc)₄ tag-bound bead [depending on whether the antibody coupled through its Fab or Fc region to the bead, which of the four c-myc tags the antibody (20) bound to, and fluctuations in the flexible c-myc tags and antibody at low force]. DNA handles are not only much longer but also much stiffer than the c-myc tags. We speculate that direct physisorption of the A2 protein to the anti-c-myc bead could have occurred, and that sequential force discontinuities summing to the overall contour length of the polypeptide could have been convoluted with A2 unfolding and refolding. Robustness of the 28 measured events (11) was not tested by fits to biophysical models that predict exponential dependence of unfolding and refolding rates on applied force (17, 21). In contrast, our 1,881 events were fit to such models, and we have reported estimates of k_0 and Δx for unfolding, and k_0 and κ for refolding.

VWF is a multidomain protein and interdomain interactions are likely important for regulating function. Thus, our study is limited in characterizing conformational dynamics within isolated VWF A2. However, studies on the A1-A2-A3 tridomain have demonstrated that force-induced A2 unfolding is required for cleavage in the presence of neighboring domains as well (22, 23), and that calcium mediated stabilization against thermal denaturation is exclusive to the A2 domain (11), affirming the relevance of understanding unfolding dynamics within the isolated A2 domain.

ADAMTS13 circulates in the body as a constitutively active enzyme, with cleavage regulated by conformational changes within its substrate, VWF. Much attention on A2 conformational regulation has focused exclusively on unfolding (22, 23). However, given the slow kinetics of refolding, at equilibrium, VWF A2 is predicted to be fully unfolded at forces above 5 pN (Fig. S1), significantly lower than the 10 pN peak force estimated to be experienced by a 200-mer VWF at the highest shear stress measured in arteriolar flow (9). The concentration of ADAMTS13 in vivo is estimated at 6 nM (24), well below the estimated K_m of 160 nM (9) required for saturation binding to its unfolded substrate.

Therefore, the time required for refolding regulates the availability of unfolded A2 for cleavage. Our data suggest that refolding plays a critical role in regulating VWF proteolysis and highlights the dynamic nature of A2 conformational change in dictating VWF function.

Materials and Methods

CD. CD spectra were obtained on an Aviv 62 DS spectropolarimeter equipped with a Peltier temperature control unit (Aviv Associates). A2 protein (2 μ M) in 20 mM Tris (pH 7.50), 30 or 150 mM NaCl, and varying concentrations of EDTA or CaCl₂ was monitored within a 1.0 cm pathlength quartz cuvette with a magnetic stirrer. Baseline spectra of the folded and unfolded states were obtained at 4 °C and 90 °C, respectively, with five accumulations scanned from 260 to 195 nm at 1.0 nm intervals. Thermal denaturation was monitored at 222 nm from 4 to 90 °C at 1 °C intervals, equilibrating for 2 min at each temperature before signal averaging for 1 min. Spectra shown are corrected for background contribution of buffer. Thermal unfolding curves were derived from linear fits to thermal denaturation signal before and after the unfolding transition. Single wavelength CD temperature melts were fit to a Gibbs–Helmholtz equation as described by Greenfield (15).

Isothermal Calorimetry. Isothermal calorimetry was conducted on an iTC₂₀₀ (Microcal) in a 200 μ L reaction cell loaded with 140 μ M protein at 25 °C. To ensure complete removal of calcium, protein was dialyzed for 3 d against 5 mM EDTA, which was then removed via gel filtration on a Superdex 75 10/300 column (GE Lifesciences) immediately before the experiment. Titration was performed using 20 \times 2 μ L injections of 1.4 mM CaCl₂ in the same buffer (20 mM Tris, pH 7.5, 30 mM NaCl) at 180 s intervals, stirring at 1,000 rpm. Binding isotherms were analyzed using the Microcal Origin version 7 software package and fit to a single site binding model.

Protein Expression and Purification. VWF A2 domain was prepared as previously described (9).

Single Molecule Sample Preparation. DNA handles (802 base pairs) were PCR-amplified using forward primers with a 5' thiol group and reverse primers with either 5' biotin or 5' digoxigenin and activated with 2,2'-dithio-dipyridine (DTDP) as described previously (9). For protein–DNA coupling, 50 μ L of 12 μ M protein was reduced with 1 mM DTT for 1 h at room temperature, followed by three sequential passages through 0.5 mL Zeba desalting columns (Pierce). Freshly reduced A2 (5 μ M) was reacted with 3 μ M DTDP activated handles in 20 μ L 0.2 M sodium acetate (pH 5), 1 M NaCl, 1 mM EDTA. Reaction components were quickly mixed before incubating under argon in a

1.5 mL Eppendorf for 16 h at room temperature. In our hands, coupling was tricky and highly sensitive to DNA:protein ratios. Due to small volumes and rapid oxidation, precise concentrations were difficult to predict and thus coupling was performed in parallel across a range of DNA:protein ratios (Fig. S2B). Different handle preparations varied in coupling efficiency and the preparations were selected as described below and used for experiments. Coupling efficiency was assayed by running reaction products on 4–20% polyacrylamide gels. Ethidium bromide staining reported shifts in DNA handles upon successful coupling to A2, which was specifically verified via Western blot analysis on the same gel (Fig. S2A). As final verification, constructs were tested on single molecule optical tweezers. For constructs with poor coupling efficiency, only DNA handle dimers or nonspecific extension lengths were observed with great effort. Successful coupling yielded constructs with reliable observations of single A2 unfolding events (>50% of tethers). Verified DNA/protein coupled constructs were aliquoted and stored at –80 °C.

Single Molecule Force Experiments. Polystyrene beads (4.2 μ m, Spherotech) functionalized with antidigoxigenin Fab (Roche) were incubated with 10 pM protein–DNA constructs for 30 min at room temperature (optimized for single tethers). Single beads were then trapped in the laser tweezers and brought close to a streptavidin-functionalized polystyrene bead (2.1 μ m) suctioned onto the tip of a micropipette. Single tether formation was verified by overstretching the construct past 65 pN to confirm the signature force–extension curve plateau corresponding to the B to S form DNA transition (25). The average tether lasted 80 cycles with force–extension discontinuities observed in 75% of pulls. All pulls from each tether were used for analysis. The instrument used for optical trapping has been previously described (26). Experiments were conducted in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.02% Tween 20. Experiments were conducted with no added calcium [buffer confirmed as <20 μ M Ca via a QuantiChrom Calcium Assay Kit (Bioassay Systems)], with 5 mM CaCl₂, and with 1 mM EDTA.

Statistical Analysis. Errors are estimated errors based on χ^2 minimized fits using Origin 6.1 (OriginLab Corporation). Data were noted to be statistically similar by comparing the maximal spread of 95% confidence intervals.

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Supporting Information

Xu and Springer 10.1073/pnas.1121261109

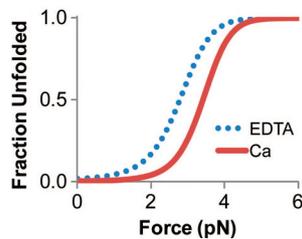


Fig. S1. Fraction of unfolded von Willebrand factor under applied force. The population of unfolded A2 under equilibrium predicted from the ratio of unfolding to refolding kinetics was calculated using the biophysical constants determined here and the biophysical equations of force dependence to which they were fit. In the absence of calcium, slowed refolding kinetics account for the increased fraction of unfolded A2. Our quantitative parameters predict that A2 is fully unfolded at forces above 5 pN.

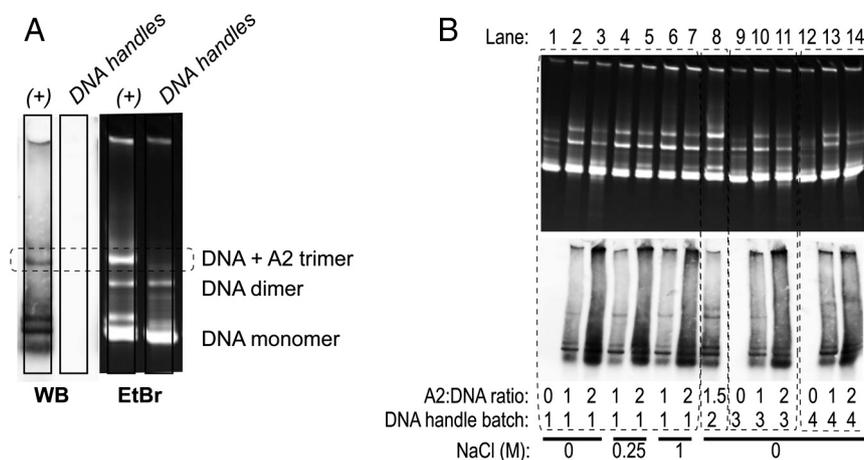


Fig. S2. Coupling of von Willebrand factor (VWF) A2 to DNA handles. (A) Trimolecular construct verified by running the reaction on a 4–20% polyacrylamide gel and staining for DNA and A2 in parallel. Following ethidium bromide (EtBr) staining, the same gel was subjected to Western blotting (WB) against the A2 histidine tag. (B) Full view of gels highlighted in A. Coupling efficiency, assessed via the presence of a trimolecular construct, was sensitive to DNA/protein ratio. Both too much protein (lane 2) and too much DNA (lane 3) resulted in poor yields of the trimolecular construct. Coupling efficiency varied with DNA handle preparations (four batches shown) and did not seem to be affected by NaCl concentrations (lanes 1–7, black bar). The best coupling (lane 8), visualized by the presence of a trimolecular construct, translated to reliable observations of unfolding events using optical tweezers.