Mutational constraints on local unfolding inhibit the rheological adaptation of von Willebrand factor

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*Running Title: Restrained intradomain flexibility of VWF in type 2M VWD

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ABSTRACT:

Unusually large von Willebrand factor (ULVWF), the first responder to vascular injury in primary hemostasis, is designed to capture platelets under the high shear stress of rheological blood flow. In type 2M von Willebrand disease (VWD), two rare mutations (G1324A and G1324S) within the platelet GPIbα binding interface of the VWF A1 domain impair the hemostatic function of VWF. We investigate structural and conformational effects of these mutations on the A1 domain’s efficacy to bind collagen and adhere platelets under shear flow. These mutations enhance the thermodynamic stability, reduce the rate of unfolding, and enhance the A1 domain’s resistance to limited proteolysis. Collagen binding affinity is not significantly affected indicating that the primary stabilizing effect of these mutations is to diminish the platelet binding efficiency under shear flow. The enhanced stability stems from the steric consequences of adding a side chain (G1324A) and additionally a hydrogen bond (G1324S) to H1322 across the β2-β3 hairpin in the GPIbα binding interface which restraints the conformational degrees of freedom and the overall flexibility of the native state. These studies reveal a novel rheological strategy in which the incorporation of a single glycine within the GPIbα binding interface of normal VWF enhances the probability of local unfolding that enables the A1 domain to conformationally adapt to shear flow while maintaining its overall native structure.

The Von Willebrand factor (VWF) A1 domain mediates platelet adhesion to exposed subendothelial connective tissue and contributes to the arrest of bleeding during primary hemostasis. An early clinical study of a patient with a new variant form of Von Willebrand Disease (VWD) more than four decades ago distinguished a functional phenotype separate from the more common quantitative deficiencies of Von Willebrand Factor [1, 2]. Studies of this unique abnormality established that plasma VWF was undetectable via ristocetin induced platelet agglutination (RIPA) while platelet agglutination induced by the botrocetin snake venom (BIPA) confirmed the presence of VWF in plasma [3, 4]. Two decades following the initial presentation, the G1324S mutation, now classified as a type 2M loss of function VWD phenotype, was sequenced.
and complementary studies on recombinant VWF harboring this mutation within the A1 domain reproduced the patient VWF functional characteristics [5]. Its sister mutation, G1324A, was later identified having similar loss of function characteristics [6,7].

These early studies were important for two reasons. First, a cognizance of functional deficiencies of VWF became apparent at a time when VWD cases were thought to be due only to a quantitative lack of a blood clotting factor associated with FVIII. Ristocetin is quite effective at detecting a loss of VWF activity via the absence of RIPA [8] although its current efficacy in diagnosing functional abnormalities associated with type 2 VWD is debatable [9,10]. Secondly, the functional activity of VWF and our ability to detect it with ristocetin could be abrogated by replacing a glycine with the simplest single carbon sidechain containing amino acids, alanine and serine.

Over the last two decades, an extraordinary effort has been placed on identifying a structural basis for A1 domain high affinity platelet adhesion. 15 structures have been determined of wild-type [11] and type 2B VWD variants of the A1 domain (R1306Q & I1309V) [12, 13], various complexes of A1 with platelet GPIbα [14, 15], function blocking monoclonal antibodies [16], DNA aptamers [17], and activating snake venoms [18,19]. Yet despite these efforts to identify a high affinity conformation that would provide a structural explanation for type 2B VWD, the native fold is retained among all the structures with the backbone RMSD < 2 Å over much of the sequence within the disulfide loop [21]. Here we provide the first crystallographic structure of the A1 domain with the loss of function type 2M VWD G1324S mutation. The structural identity of G1324S to all previously published structures emphasizes that even the structures containing type 2B mutations do not depict high affinity conformations and co-crystal complexes with GPIbα are not representative of the high-strength interactions responsible for platelet adhesion to VWF under shear flow.

A significant number of inherited type 2M and 2B VWD mutations induce misfolding of the A1 domain leading to both "off-pathway” loss-of-function states and rheologically favored "on-pathway” gain-of-function states [10]. They represent extreme examples of the A1 domain’s propensity to populate locally unfolded molten globule conformations. Here we illustrate that this propensity for local unfolding exists even in the native state of the A1 domain and that the G1324A and G1324S disease mutations suppress these conformational fluctuations in the GPIbα binding interface resulting in a substantially reduced efficacy to capture platelets under shear flow. The evidence for reduced flexibility of the GPIbα binding interface is supported by an increase in thermodynamic stability concomitant with an increased cooperativity of unfolding, a reduction in the rate of urea-induced unfolding, and a reduced susceptibility to limited proteolysis by trypsin.

These results highlight yet another example of how conformational fluctuations in the native state enable biological function in the presence of an environmental stress. Natural selection of conformational flexibility in proteins via the incorporation of glycine is a common genetic mechanism for cold adaptation of enzymes [24]. Similarly, placing a glycine at position 1324 in the A1 domain GPIbα binding interface of VWF is a strategy for rheological adaptation to the shear stress of blood flow. Restraining the flexibility of this structural region by mutation impairs VWF-dependent primary hemostasis causing bleeding in Type 2M Von Willebrand disease.

METHODS
A1 expression, purification quantification and quality control. Wildtype vWF A1 (amino acids Q1238 - P1471) and its mutants G1324A and G1324S were expressed in E. coli M15 cells as fusion proteins containing a N-terminal 6xHis-Tag. After the isolation of the inclusion bodies, all proteins were solubilized in 6M GdnHCl, 25 mM TrisHCl, pH = 7.5, refolded in 4L of cold buffer containing 50 mM TrisHCl, pH 7.5, refolded in 4L of cold buffer containing 50 mM TrisHCl, 1M NaCl, 0.5% Tween 20, pH 7.5 and purified by affinity chromatography using a Ni2+-NTA column followed by purification via Heparin Sepharose. Proteins were stored on water ice at 0°C in PGA-buffer (10mM sodium phosphate, 10mM sodium...
acetate, 10mM Glycine, 150mM NaCl, pH = 8) or TBS for a maximum of two weeks. Prior to all experiments, protein solutions were centrifuged for at least 10min at 4°C and 60000g to remove any potential protein aggregates. Protein concentrations were determined spectrophotometrically with a Shimadzu UV2101PC spectrophotometer from the absorption at 280nm minus twice the absorption at 333nm as a correction for light scattering. Extinction coefficient = 15,350 L mol⁻¹ cm⁻¹. Analytical size exclusion chromatography was performed as a quality control step using an analytical Phenomenex SEC-S3000 on a Beckman System Gold HPLC (Pump model 125, UV-detector model 166). PGA-buffer containing 1M NaCl was used as mobile phase. The flow rate was 0.5 mL/min and the absorption was monitored at 280nm. The molecular weight calibration curve was determined from the elution times of Ferritin (440 kDa), Aldolase (158 kDa), BSA (67 kDa), Ovalbumin (43 kDa), Ribonuclease A (13.7 kDa) and Vitamin B12 (1.35 kDa).

**G1324S crystallization, data processing, and refinement.** A1 G1324S was filtered through a 0.22 μm filter, concentrated to 20 mg/mL, mixed 1:1 with 30% v/v PEG 400 100mM CAPS/NaOH, pH=10.5, and set up as hanging drops at 4°C. Crystals grew within 5 days and were flash frozen at 280nm. The molecular weight calibration curve was determined from the elution times of Ferritin (440 kDa), Aldolase (158 kDa), BSA (67 kDa), Ovalbumin (43 kDa), Ribonuclease A (13.7 kDa) and Vitamin B12 (1.35 kDa).

**Platelet flow assay and analysis.** Parallel plate flow chamber studies were performed as previously described [10,27] using Cellix Vena8 CGS biochips on a Zeiss Axio Observer-A1 microscope operated by the Zen2012 Software. Platelet movies were recorded in phase contrast using a PCO edge camera at 25 frames per second. Citrated whole blood was perfused over the surface of immobilized Cu²⁺ chelated A1 domain.

The analysis and the determination of platelet pause times and of the survival fractions was performed as previously described [21,27]. After immobilization of 5μM A1 domain in the flow chamber, citrated whole blood was perfused at a shear rate of 800s⁻¹. This perfusion was followed for ~3-4min by TBS buffer to remove red blood cells from the channel. Subsequently, the shear was either decreased to 100s⁻¹ or increased to 9000s⁻¹ at logarithmic intervals of the shear rate every 2min. After ~50s at a given shear rate, a 60s video at 25 frames/second was recorded and analyzed. This procedure works well for the WT A1 domain and type 2B A1 domain variants, but shear-induced platelet interactions with G1324A and G1324S variants were significantly weaker than with WT A1 resulting in no observable interactions following the removal of red cells from the flow chamber. We improved the microscope focus in the filming of platelet interactions with G1324A and G1324S so that pause times for the adhesion can be acquired during the initial perfusion of whole blood with red cells still flowing through the chamber.

**Collagen Binding via surface plasmon resonance.** SPR kinetic experiments were performed using a Biacore T-100. The acid soluble collagen type III from human placenta (Sigma-Aldrich) was covalently coupled via primary amines to the active channel of a CM5 chip resulting in ~ 3000 response units, or ~ 30 ng/mm² collagen. The reference channel lacking collagen was subtracted as background. All the kinetic experiments were performed at 25°C at a flow rate of 30 μL/min with 500s of both association and dissociation time. Regeneration of the biosensor was performed by injection of 1mM EDTA, 2M NaCl, 0.1M sodium citrate, pH=5 for 60s at 10 μL/min and followed by injection of 0.1M H₃PO₄ for 30s at 30 μL/min. Due to the heterogeneity of collagen, the simple models using mono- or bi-exponential fits for the association and dissociation phases provided by the Biacore evaluation software were not able to yield a satisfying fit quality. The sensorgrams were exported to SigmaPlot 11 and the association and the dissociation phases were fit independently using tri-exponential functions by non-linear least
squares fitting routines to obtain apparent values of the rates and affinity constants. While not ideal for elucidating mechanisms of binding, these procedures are acceptable within the context of comparing the A1 domain variants. The association phase was fit to the following tri-exponential rise function.

\[ \text{RUass}[t] = A \cdot (1 - \exp(-k_{a1}t)) + B \cdot (1 - \exp(-k_{a2}t)) + C \cdot (1 - \exp(-k_{a3}t)) \]

The dissociation phase was fit to a tri-exponential decay function.

\[ \text{RUdiss}[t] = A \cdot \exp(-k_{d1}t) + B \cdot \exp(-k_{d2}t) + C \cdot \exp(-k_{d3}t) \]

The parameters \( A, B \) and \( C \) are pre-exponential amplitudes. The apparent rate constants for association are \( k_{a1}, k_{a2} \) and \( k_{a3} \) and those for dissociation are \( k_{d1}, k_{d2} \) and \( k_{d3} \). In all cases the fit quality gave an \( R^2 > 0.99 \).

CD and Fluorescence spectroscopy. As previously described [10, 21, 27], circular dichroism measurements were performed on an Aviv Biomedical Model 420SF circular dichroism spectrometer. Fluorescence measurements were performed on a Horiba Jobin-Yvon Fluorolog 3 spectrofluorometer equipped with a Wavelength Electronics LF1-3751 temperature controller. Far UV CD Spectra of the A1 domains were recorded between 190nm and 260nm in a 0.1 mm quartz cell at 20\(^\circ\)C. Near UV CD spectra were recorded at room temperature between 260nm and 360nm using a 5 or 10cm cylindrical quartz cell. The step width for all CD spectra was 1nm, the integration time 60s. All spectra were corrected for the signal of the corresponding buffer and converted to mean ellipticity per amino acid residue.

A1 Unfolding. Isothermal urea induced unfolding of WT A1 and of the two mutants was monitored at 222 nm using 0.2cm quartz cells and defined protein concentrations between 3-5\( \mu \)M. All samples were equilibrated overnight in their urea containing buffer at temperatures of 5, 15, 25, and 35\(^\circ\)C. CD signal was collected for 5 to 10min using an integration time of 1s. Isothermal unfolding of the A1 domain and its mutants was followed either by CD at 222nm or intrinsic protein fluorescence using wavelengths of 280nm and 359nm for excitation and emission respectively. In both cases, 1\( \mu \)M protein was equilibrated at 20\(^\circ\)C for at least 10min under slight stirring in 1cm quartz cells. The temperature was increased slowly up to 95\(^\circ\)C at various scan rates between 0.4 and 2.0C/min. Kinetics of urea unfolding of WT A1, G1324A, G1324S, I1309V and R1306Q VWD variants were followed by intrinsic protein fluorescence (280nm excitation; 359nm emission) at 20\(^\circ\)C after dilution of A1 domain into defined concentrations of buffered urea solutions to a final protein concentration of 1\( \mu \)M.

Thermodynamic Analysis via the Phase Diagram Method. All non-linear least squares fitting routines were performed with gnuplot version 4. Isothermal urea unfolding of each domain was analyzed using a three state reversible model \((N \leftrightarrow I \leftrightarrow D)\) as previously described [20, 22]. Though kinetically irreversible even in presence of urea, all iso-urea thermal unfolding curves were treated as reversible in order to obtain the midpoints of the transition curves for the scan rate dependencies and the extrapolation to 0C/min scan rate. The \( c_{1/2} \) urea denaturation midpoints and the extrapolated apparent \( T_M \)s were fit using the phase diagram method as previously described by Tischer & Auton [26] using the equation …

\[ c_{1/2}[T] = -\ln K[T] / \partial \ln K/\partial_{\text{c urea}}[T] \]

where \( \ln K[T] \) and \( \partial \ln K/\partial_{\text{c urea}}[T] \) as a function of temperature are

\[ \ln K[T] = -\Delta G^0/(RT) \]
\[ = (\partial \ln K/\partial \beta)^* \Delta \beta + (\partial^2 \ln K/\partial \beta^2)^* \Delta \beta^2/2 \]
\[ = -\Delta H^0* \Delta \beta + RT^2* \Delta C_P^0* \Delta \beta^2/2 \]

\[ \partial \ln K/\partial_{\text{c urea}}[T] = (\partial \ln K/\partial_{\text{c urea}}) + \]
\[ (\partial^2 \ln K/\partial_{\text{c urea}} \partial \beta)^* \Delta \beta + \]
\[ (\partial^3 \ln K/\partial_{\text{c urea}} \partial \beta^2)^* \Delta \beta^2/2 \]
\[ = -m/RT - (\partial \Delta H^0/\partial_{\text{c urea}})* \Delta \beta + \]
\[ RT^2*(\partial \Delta C_P^0/\partial_{\text{c urea}})* \Delta \beta^2/2 \]
Δβ = (1/(RT) - 1/(RTM)). K is the equilibrium constant for unfolding, ΔG is the free energy of unfolding, ΔH is the enthalpy of unfolding, and ΔCp is the heat capacity of unfolding. Likewise, m is the cooperativity of urea denaturation and ∂ΔH/∂curea and ∂ΔCp/∂curea are the urea concentration dependencies of the unfolding enthalpy and heat capacity.

Limited proteolysis. 2mL of 7μM protein solutions were mixed with 5μL of 1mg/mL Trypsin solution to yield an A1 to Trypsin ratio of 1:80. All three A1domain/Trypsin mixtures were incubated at 37°C and at certain time points (0-18h), 200μL aliquots were taken from each solution and the proteolysis was quenched by a drop in pH via addition of 10μL 4%v/v trifluoroacetic acid. Quenched reactions were frozen on dry ice and stored at -80°C. Proteolysis samples were analyzed in the Mayo Clinic Medical Genome Facility Proteomics Core using reverse phase HPLC followed by positive mode ESI mass spectrometry using an Agilent 1200 HPLC System coupled to an Agilent 6224 TOF mass spectrometer. Water with 0.1% formic acid was used as solvent A and acetonitrile with 0.1% formic acid was used as solvent B. The flow rate on the system was 300uL/min and the injection volume was 5uL. Samples were separated on a Agilent Zorbax 300SB C18 column prior to positive mode ESI mass spectrometry analysis. The obtained data were correlated to the A1-domain amino acid sequence based on molecular mass with an accuracy of 10 ppm using Agilent Mass Hunter Qualitative Analysis/BioConfirm Software.

RESULTS
Structure of the type 2M G1324S A1 domain. The structure of the A1 domain with the type 2M mutation G1324S was solved at 1.59Å resolution. The protein crystallized as needles in the P61 space group. The overall fold containing a central β-sheet flanked on two sides by amphipathic helices (Fig. 1A) was identical to the WT crystal structure published by Emsley et al. [11] within an RMSD of ±1Å as demonstrated by comparing α carbon distance difference matrices (Fig. 1B). Fig. 1C illustrates a potential hydrogen bond formed between the histidine 1322 and serine 1324 residues that contributes to the G1324S variant’s enhanced thermodynamic stability [22]. Within this region, the histidines 1322 and 1326 were previously chelated by the excipient cation, Cd⁴⁺, in the original published structure of the wild type A1 domain [11].

In solution, each of the three A1 domain variants show identical far-UV and near-UV circular dichroism spectra indicating that the secondary structure content and tertiary structure environment of the aromatic Tyr and Trp residues are identical (Fig. 1D). Analytical size exclusion chromatography (Fig. 1E) confirmed that the hydrodynamic radii of all three proteins are identical and that the A1 domain exists as a globular domain in solution as its retention time correlated to its molecular weight as compared to a set of standard proteins. Taken together with the crystal structures of WT and G1324S, these spectroscopic and chromatographic metrics confirm that G1324A and G1324S do not change the native fold or the predominant solution conformation of the A1 domain in the absence of shear stress.

Platelet adhesion under shear flow. The interaction of WT A1, G1324A and G1324S with platelets was studied using a parallel plate flow chamber in which platelet pause times are determined as a function of shear rate as previously described [10]. In Fig. 2A, G1324A and G1324S exhibited mean platelet pause times below 0.1s at all tested shear rates, significantly lower than WT A1. The pause time survival fractions obtained from the cumulative average of the pause time distribution, Fig. 2B demonstrate that G1324A and G1324S can only interact weakly with platelets as they did not exceed 0.4s for G1324A or 0.2s for G1324S. On average, the platelet dissociation rates were 1.06 ± 0.58s⁻¹ for WT A1, 17.23 ± 5.20s⁻¹ for G1324A and 22.96 ± 3.24s⁻¹ for G1324S. Movies of platelets translocating on surface-immobilized A1 domains at a shear rate of 1500s⁻¹ can be found as separate attachments in the supporting information.
Collagen binding. The A1 domain also binds type III collagen and assists the A3 domain in recruiting platelets to subendothelial exposed collagen [25]. Fig. 3A shows the responses for the binding of WT, G1324A, and G1324S to immobilized collagen at varying concentrations ranging from 1 to 30μM. The apparent binding constants were derived from the fitting of association and dissociation phases independently using triexponential functions as described in the methods. Three sets of apparent rate constants for each association and dissociation phase are plotted as a function of protein concentrations (Fig. 3B). These rate constants are typically insensitive to A1 concentration due to the heterogeneity of the collagen surface. Although, the association rate constants for G1324A and G1324S are similar to WT A1 within experimental error and ±2 standard deviations of WT, the rates of dissociation of G1324A and G1324S are slightly faster than WT and outside of ±2 standard deviations of WT. As a result, the apparent binding affinities for G1324A and G1324S calculated from the ratio of apparent rate constants are slightly weaker (Fig. 3C), but within experimental error, the binding affinities of these three variants of the A1 domain to collagen are quite similar.

Thermodynamic stability. We have used the phase diagram method to assess the thermodynamic stability of the native to intermediate (N ⇌ I) unfolding transition. The WT A1, G1324A, and G1324S domain variants were denatured isothermally with urea (Fig. 4A-C) and thermally denatured at constant urea concentration (Fig. 4D). These denaturation data illustrate several important points. 1) As previously observed, the urea unfolding transitions have three state character with both low and high urea transitions [22]. Although urea denaturation is reversible, the thermal unfolding is kinetically controlled with a scan rate dependent apparent TM from which the equilibrium Tm,eq is obtained by extrapolation to 0 °C/min [21]. This scan rate dependency persists even in the presence of urea for all variants (Fig. 4E). 2) The secondary structure content of the intermediate baseline from ~−4 to −6 at 6M urea in Fig. 4A-C. This intermediate conformation also has significant secondary structure content at temperatures above the thermal unfolding transition (mean residue ellipticities = −6) indicating that the intermediate state is only partially disordered.

The urea-temperature phase diagram of the N ⇌ I transition midpoints obtained from fitting the data in Fig. 4A defines urea concentrations and temperatures where the proteins exist as a mixture of 50% populations of native and intermediate conformations. This phase diagram illustrates that 3) G1324A and G1324S both unfold at higher concentrations of urea and higher temperatures than WT A1 (Fig. 4E), and 4) G1324A and G1324S are thermodynamically more stable (∆G) than WT A1 (Fig. 4F). 5) The cooperativity of both urea and thermal unfolding, as given by the m-value (−m/RT), enthalpy (∆H) and heat capacity (∆Cp), is also greater indicating that the native state structure in G1324A and G1324S is less dynamic than WT A1 (Fig. 4F and Table 2).

Unfolding Kinetics. In addition to the thermodynamic experiments, the urea-induced denaturation of WT A1, G1324A and G1324S was also studied by following the kinetics of unfolding by monitoring the intrinsic protein fluorescence (Fig. 5A). Each A1 variant was titrated into a 1 cm cuvette containing a buffered urea solution and the change in signal was recorded at λ = 359nm. The resulting kinetic traces were fit using mono-exponential functions to obtain the rate constants as a function of urea are given in Fig. 5B. Unfolding kinetics of the type 2B R1306Q and I1309V variants are also included as a control.

In agreement with the stability, the unfolding rates of G1324A and G1324S were significantly slower than WT A1 at all urea concentrations and I1309V was faster [22]. The slope of the unfolding rate with respect to urea concentration was identical for all variants indicating the diminished rates of unfolding caused by the mutations were only due to the stability of the domain rather than alternate unfolding pathways. The extrapolation of the rates of unfolding towards the absence of urea resulted
in apparent rate constants of $7.58 \pm 1.62 \times 10^{-5} \text{s}^{-1}$ for WT A1, $2.10 \pm 0.45 \times 10^{-5} \text{s}^{-1}$ for G1324A, $1.89 \pm 0.41 \times 10^{-5} \text{s}^{-1}$ or G1324S, $1.70 \pm 0.30 \times 10^{-4} \text{s}^{-1}$ for I1309V, and $8.50 \pm 2.25 \times 10^{-5} \text{s}^{-1}$ for R1306Q. In absence of urea, WT A1 unfolds ~ 4x faster than G1324A and G1324S and I1309V unfolds ~ 2x faster than WT A1.

**Limited proteolysis.** The above thermodynamic and kinetic experiments show that the addition of a sidechain to position 1324 stabilizes the domain to a more rigid structure. Combined with the reduced platelet function of G1324A and G1324S, these results demonstrate that conformational flexibility in this structural region is required for normal platelet adhesion under shear stress. To probe the flexibility of these domain variants, time dependent limited trypsinolysis was followed with mass spectrometry. Of the 30 predicted theoretical fragments in Table S3, 8 were well resolved in the chromatogram (Fig. 6A). Peak areas for the uncleaved domains and the resolved fragments T4, T5, T9 (containing G1324), T15, T16, T18, T25 and the N- and C-terminal fragments containing the disulfide bond, T2-S-S-T30, T3, and T29 were monitored over time. The N- and C-terminal fragments did not elute from the C18 column separately and were therefore treated as a single fragment (disulfide fragment - DF).

The kinetics of proteolysis demonstrate that the rate of accumulation of the T25 fragment containing $\beta$5 strand and N-terminal part of the $\alpha$5 helix was the fastest (Fig. 6B). Accumulation of T9 containing position 1324 and the disulfide fragment (DF) containing the $\alpha$6 helix and $\beta$1, T15 and T16 in $\alpha$3, and T18 containing the protein core $\beta$4 strand followed. The T4 and T5 fragments of $\alpha$1 were the most resistant to proteolysis. WT A1 was digested to near completion after 18h of incubation at 37°C, but G1324A and G1324S had ~ 80% of uncleaved protein remaining. Consequently, the chromatographic fragment populations were greatly reduced for G1324A and G1324S relative to WT. The kinetics of fragment accumulation estimated from the initial slopes (Fig. 6C) quantify the slower proteolytic cleavage for G1324A and G1324S variants relative to the WT A1 domain and support the thermodynamic evidence for reduced conformational flexibility.

**DISCUSSION**

At the outset of vascular injury, unusually large (UL) multimeric strings of VWF secreted into the blood by vascular endothelial cells function in concert with untethered plasma VWF to capture, sequester and deposit free flowing platelets to plug the wound and stop the bleeding [28,29]. Functioning like multiple hooks on fishing lines, each A1 domain per monomeric unit of the VWF multimer must be conformationally malleable and responsive to the rheological stress of flowing blood in order to effectively bind platelet GPIIb-IIIa. Too much flexibility can either result in enhanced affinity of the native state [22] or lead to local misfolding of the A1 domain which can induce both gain and loss of function types 2B and 2M Von Willebrand disease (VWD) depending on the structural location of the mutation. [10]. Too much rigidity in the A1 domain prevents stable platelet attachment to ULVWF strings leading to type 2M VWD. It’s a “Goldilocks” predicament in that the conformational dynamics of the A1 domain must be “just right” for efficient VWF-mediated primary hemostasis.

At the opposite extreme from misfolding, the G1324A and G1324S VWD mutations restrict the conformational degrees of freedom of the $\beta$-turn between $\beta$ strands 2 and 3 within the native state. Two forces are at work here that result in the overall stabilization of the A1 domain structure. Substitution of a side chain containing amino acid sterically hinders the $\phi$-$\psi$ conformational space of the peptide backbone introducing rigidity into this region of structure. This reduction of flexibility by the presence of a side chain occurs when glycine is replaced by either alanine or serine, but the serine hydroxyl provides additional structural stability through the formation of an additional hydrogen bond to the histidine sidechain at position 1322. These forces result in a reduced probability of populating locally unfolded conformations that is observed as a stabilization of the native to intermediate ($N \rightleftharpoons I$) urea unfolding transition, a decreased rate of unfolding, and a resistance to limited proteolysis by trypsin.
These stabilizing forces have a profound effect on the ability of A1 to efficiently capture platelets under shear flow. Pause times of platelet translocations on A1 are diminished 8-fold down to near the limit of detection at video frame rates of 25s$^{-1}$, and pause time survival fractions rarely exceed 0.2s. Since pause times are proportional to the strength of the adhesion, G1324A and G1324S weaken the interaction of VWF with GPIb$\alpha$. In contrast to platelet adhesion under shear flow, these stabilizing forces do not result in very large changes in collagen affinity. The apparent rates of association and dissociation of G1324A and G1324S from collagen are very similar yielding binding affinities that are at most 2-3 fold different in KD than WT A1 and still within the experimental error. Contrary to previous reports which claim that collagen induces a conformational change in A1 upon binding [30] and that G1324S impairs this conformational change [31], we do not observe significant differences in the dissociation rates of these type 2M variants relative to WT A1 that would result from conformation dependent collagen binding interactions. The increase in thermodynamic stability caused by G1324A and G1324S significantly reduces the probability of populating intermediate conformations that would have altered affinities for collagen. As such, one might expect that dissociation of these type 2M variants from collagen would be significantly faster than WT A1 and commensurate with the enhanced stability. Within the native state, however, decreasing the probability for local unfolding has little effect on the binding to collagen, but the effect on platelet adhesion is substantial.

The most direct evidence of restrained conformational fluctuations in the A1 domain are the reduced rates of proteolysis of the G1324A and G1324S variants. Using trypsinolysis as a metric for assessing the conformational dynamics of the native state, we find that the rate of appearance of all proteolytic fragments is proportionally slower for the type 2M variants than for WT A1. In agreement with the thermodynamics, the local effects of these mutations on conformational dynamics also extend globally throughout the structure of A1, but the rank order of the rates of accumulation of these fragments indicate that different regions of the A1 domain structure have distinct local stabilities and propensities to populate locally unfolded excursions from the native state. The three fragments that accumulate early in the proteolysis by trypsin are T25 in the loop N-terminal to $\alpha_5$, the disulfide fragment (DF) containing $\alpha_6$, and T9 containing position 1324. These fragments encompass regions of secondary structure that have been implicated as locally dynamic where mutations either destabilize the N $\rightleftharpoons$ I transition or induce molten globule conformations [32]. Accumulation of T9 in the binding interface requires a cut at R1315 directly behind the $\alpha_2$ helix and at K1332 in the loop immediately prior to the $\alpha_2$ helix. This loop is locally stabilized by the G1324A and G1324S mutations putting restraints on the conformational dynamics of the $\alpha_2$ helix which inhibits trypsinolysis at R1315. Following the accumulation of T9, the lysine and arginine rich $\alpha_2$ helix is cleaved quickly into small peptides after which proteolysis of the $\alpha_3$ helix generates the T14 and T15 fragments. Based on the higher rates of accumulation of these particular fragments of the A1 domain structure relative to other fragments that are within more stable secondary structures (T4 and T5 in $\alpha_1$) or the hydrophobic core of the domain (T18 in $\beta_4$), we deduce that these regions of structure are responsible for the intermediate conformations observed in urea denaturation. In addition to the thermodynamic consequences of the type 2M mutations presented above, this deduction is also supported by our previous observations of mutation induced misfolding in these regions of structure [10].

The effects of G1324A and G1324S on local unfolding in the binding interface also manifest in the rates of A1 unfolding. Given that the A1 GPIb$\alpha$ interaction is shear rate dependent, we found that the rate of platelet dissociation from A1 is logarithmically proportional to the rate of unfolding obtained from urea denaturation kinetics (Fig. 7). This kinetic relationship provides further evidence that the local unfolding occurring under shear stressed platelet adhesion and in the presence of urea are within the same regions of A1 structure [32]. Inclusion of R1306Q and I1309V in this correlation demonstrates that these principles
are universal and apply for type 2B as well as type 2M VWD.

The conservation of protein flexibility in enzymology [33] is a general strategy of adaptation of cells and organisms that must survive under stressful environmental conditions. In particular, conformational flexibility in cold adapted and thermophilic enzymes are finely tuned to the environmental temperature of extremophiles such that the ground state structure of the native fold is maintained while the dynamics modulate the catalysis [24,34]. Protein solvation by low concentrations of denaturants also increases enzymatic activity [35, 36] and strategically placed glycine mutations at residues allosteric to binding sites can modulate affinities by enabling the native fold to transiently sample locally disordered conformations [37]. The vascular environment is extremely high stress where the dynamics of protein interactions are dependent on flexibility in order to regulate the strength of cell adhesion under the physical forces of rheological blood flow. Flexibility in the context of platelet adhesion to VWF has previously been limited to the context of polymer physics as it relates to shear stress effects on the unfolding of VWF multimeric strings to expose the A1 domains [38]. Once ULVWF multimeric strings are unfurled under shear flow, conformational dynamics in the traditional thermodynamic context is critical for platelet adhesion to the exposed A1 domains. Restraining these fluctuations by decreasing the probability of local unfolding in response to shear stress results in bleeding due to the inability of A1 to conformationally adapt to the blood flow.

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The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
AT expressed and purified the proteins, performed the spectroscopy, chromatography, limited proteolysis, thermodynamics and kinetics experiments, flow assay and the phase diagram analysis. JCC, BS and CK did the crystallization and the refinement of the structure. VRM performed and analyzed the SPR experiments. LMT performed the mutagenesis and cloning. LMB performed the mass-spectrometry for the limited proteolysis. AT, VRM and MA wrote the manuscript. MA designed the research. The authors have no conflict of interest.
REFERENCES
1. Firkin B., Firkin F. & Scott L. (1973). Von Willebrand’s disease Type B: A newly defined bleeding
diathesis. Aust NZ J Med 3:225-229.
2. Holmberg, L. & Nilsson, I.M. (1972). Genetic variants of von Willebrand’s disease. Brit Med J
3:317-320.
3. Howard M.A., Salem H.H., Thomas K.B., Hau L., Perkin J., Coghlan, M. & Firkin, B. G. (1982).
Variant von Willebrand’s Disease Type B - Revisited. Blood 60(6):1420-1428.
4. Howard M.A., Perkin J., Salem H.H., & Firkin, BG. (1984). The agglutination of human platelets by
botrocetin: evidence that botrocetin and ristocetin act at different sites on the factor VIII molecule and
platelet membrane. Br J Haematol 57:25-35.
5. Rabinowitz I., Tuley E.A., Mancuso D.J., Randi A.M., Firkin B.G., Howard M.A., & Sadler J.E.
(1992). von Willebrand disease type B: a missense mutation selectively abolishes ristocetin-induced
von Willebrand factor binding to platelet glycoprotein Ib. Proc Natl Acad Sci U S A, 89(20),
9846-9849.
6. Ajzenberg N., Ribba A.S., Rastegar-Lari G., Meyer D., & Baruch D. (2000). Effect of recombinant von
Willebrand factor reproducing type 2B or type 2M mutations on shear-induced platelet aggregation.
Blood, 95(12), 3796-3803.
7. Hilbert L., Fressinaud E., Ribba A., Meyer D. & Mazurier C. (2002) Identification of a new type 2M
von Willebrand disease mutation also at position 1324 of von Willebrand factor. Thromb Haemost
87:635-640.
8. Weiss H.J., Hoyer L.W., Rickles F.R., Varma A. & Rogers J. 1973. Quantitative assay of a plasma
factor deficient in von Willebrands disease that is necessary for platelet aggregation. Relationship to
factor VIII procoagulant activity and antigen content. J Clin Invest 52, 2708-2716.
9. Kitchen S., Jennings I., Woods T.A.L., Kitchen D.P., Walker I.D. & Preston F.E. 2006. Laboratory tests
for measurement of von Willebrand factor show poor agreement among different centers: results from
the United Kingdom National External Quality Assessment Scheme for Blood Coagulation. Semin
Thromb Hemost 32, 492-498.
10. Tischer, A., Madde, P., Moon-Tasson, L., Auton, M. (2014). Misfolding of vWF to pathologically
disordered conformations impacts the severity of von Willebrand disease. Biophys J, 107(5),
1185-1195.
11. Emsley, J., Cruz, M., Handin, R., Liddington, R. (1998). Crystal structure of the von Willebrand
Factor A1 domain and implications for the binding of platelet glycoprotein Ib. The J Biol Chem,
273(17), 10396-401.
12. Fukuda K., Doggett T.A., Bankston L.A., Cruz M.A., Diacovo T.G. & Liddington R.C. Structural
Basis of von Willebrand Factor Activation by the Snake Toxin Botrocetin. (2002) Structure, 10,
943-950.
13. Huizinga E.G., Tsuji S., Romijn R.A., Schiphorst M.E., de Groot P.G., Sixma J.J., & Gros P. (2002).
Structures of glycoprotein Ibα and its complex with von Willebrand factor A1 domain. Science,
297(5584), 1176-1179.
14. Dumas JJ., Kumar R., McDonagh T., Sullivan F., Stahl M.L., Somers W.S., & Mosyak L. (2004).
Crystal structure of the wild-type von Willebrand factor A1-glycoprotein Ibα complex reveals
conformation differences with a complex bearing von Willebrand disease mutations. J Biol Chem,
279(22), 23327-23334.
15. Blenner MA., Dong X., & Springer T.A. (2014). Structural basis of regulation of von Willebrand
factor binding to glycoprotein Ib. J Biol Chem, 289(9), 5565-5579.
16. Celikel R., K. Varughese, Madhusudan, A. Yoshioka, J. Ware, and Z. Ruggeri. (1998) Crystal structure
of the von Willebrand factor A1 domain in complex with the function blocking NMC-4 Fab. Nat
Struct Biol 5:189-194.
17. Huang R., Fremont D.H., Diener J.L. Schaub R.G. & Sadler J.E. A structural explanation for the antithrombotic activity of ARC1172, a DNA aptamer that binds von Willebrand factor domain A1. Structure, 17, 1476-1484.

18. Fukuda, K., Doggett, T., Laurenzi, I. J., Liddington, R. C., & Diacovo, T. G. (2005). The snake venom protein botrocetin acts as a biological brace to promote dysfunctional platelet aggregation. Nat Struct Mol Biol, 12(2), 152-159.

19. Maita, N., Nishio K., Nishimoto E., Matsui T., Shimamoto Y., Morita T., Sadler J.E., and Mizuno H. (2003) Crystal structure of von Willebrand factor A1 domain complexed with snake venom, bitiscetin: insight into glycoprotein Ibα binding mechanism induced by snake venom proteins. J Biol Chem 278:37777-37781.

20. Auton, M., Cruz, M. a, Moake, J. (2007). Conformational stability and domain unfolding of the Von Willebrand factor A domains. Journal of Molecular Biology, 366(3), 986-1000.

21. Tischer A., Cruz M.A., & Auton, M. (2013). The linker between the D3 and A1 domains of vWF suppresses A1-GPIbα catch bonds by site-specific binding to the A1 domain. Protein Sci, 22(8), 1049-1059.

22. Auton M, Sedlak E, Marek J, Wu T, Zhu C, Cruz MA (2009) Changes in thermodynamic stability of von willebrand factor differentially affect the force-dependent binding to platelet GPIbα. Biophys. J. 97:618-627.

23. Auton M., Zhu C. & Cruz M.A. 2010. The mechanism of vWF-mediated platelet GPIbα binding. Biophys J 99:1192-1201.

24. Fields P.A., Somero G.N. (1998) Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. Proc Natl Acad Sci USA 95:11476-11481.

25. Bonnefoy, A., Romijn R., Vandervoort P., Van Rompaey I., Vermylen J., & Hoylaerts M. (2006) Von Willebrand factor A1 domain can adequately substitute for A3 domain in recruitment of flowing platelets to collagen. J Thromb Haemost 4:2151-2161.

26. Tischer A. & Auton M. 2013. Urea-temperature phase diagrams capture the thermodynamics of denatured state expansion that accompany protein unfolding. Protein Sci 22:1147-1160.

27. Tischer, A., Madde, P., Blancas-Meja, L. M., Auton, Blancas-Mejia, L. M., Auton, M. (2014). A molten globule intermediate of the Von Willebrand factor A1 domain firmly tethers platelets under shear flow. Proteins 82: 867-878.

28. Lopez J., & Dong J.F. (2005) Shear stress and the role of high molecular weight von Willebrand factor multimers in thrombus formation. Blood Coagul Fibrinolysis 16 Suppl 1:S11-16.

29. Arya M., Anvari B., Romo G.M., Cruz M.A., Dong J.F., McIntire L.V., Moake J.L., & Lopez J.A. (2002) Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood 99:3971-3977.

30. Ju L., Chen Y, Zhou F., Lu H. Cruz M.A, & Zhu C. (2015) Von Willebrand factor-A1 domain binds platelet glycoprotein Ibα in multiple states with distinctive force-dependent dissociation kinetics. Thromb. Res. 136:606-612.

31. Morales L., Martin C., & Cruz M.A. (2006) The interaction of von Willebrand factor A1 domain with collagen: mutation G1324S (type 2M von Willebrand disease) impairs the conformational change in A1 domain induced by collagen. J Thromb Haemost 4:417-425.

32. Zimmermann M.T., Tischer A., Whitten S.T. & Auton M. (2015) Structural Origins of Misfolding Propensity in the Platelet Adhesive Von Willebrand Factor A1 Domain. Biophys J 109:398-406.

33. Koshland D.E. Jr. (1959) Enzyme flexibility and enzyme action. J Cell Comp Phys 54(S1):245-258.

34. Zavodszky P., Kardos J., Svingor A. & Petsko G.A. (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. Proc Natl Acad Sci USA 95:7406-7411.
35. Zhang H.J., Sheng X.R., Pan X.M. & Zhou J.M. (1997) Activation of adenylate kinase by denaturants is due to the increasing conformational flexibility at its active sites. Biochem Biophys Res Commun 238:382-386.

36. Zoldak G., Sprinzl M. & Sedlak E. (2003) Modulation of activity of NADH oxidase from Thermus thermophilus through change in flexibility in the enzyme active site induced by Hofmeister series anions. Eur J Biochem 271:48-57.

37. Schrank T., Bolen D., and Hilser V. (2009) Rational modulation of conformational fluctuations in adenylate kinase reveals a local unfolding mechanism for allosteric and functional adaptation in proteins. Proc Natl Acad Sci USA 106:16984-16989.

38. Schneider S., Nuschele S., Wixforth A., Gorzelanny C., Alexander-Katz A., Netz R. & Schneider M. (2007) Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. Proc Natl Acad Sci USA 104:7899-7903.

39. Winn M.D., Ballard C.C., Cowtan K.D., Dodson E.J., Emsley P., Evans P.R., Keegan R.M., Krissinel E.B., Leslie A.G.W., McCoy A., McNicholas S.J., Murshudov G.N., Pannu N.S., Potterton E.A., Powell H.R., Read R.J., Vagin A., and Wilson K.S. (2011) Overview of the CCP4 suite and current developments. Acta Crystallogr D 67:235-242.

40. Diederichs K. & Karplus P.A. (2013) Better models by discarding data? Acta Crystallogr D Biol Crystallogr 69:1215-1222.

41. Afonine P.V., Grosse-Kunstleve R.W., Echols N., Headd J.J., Moriarty N.W., Mustyakimov M., Terwilliger T.C., Urzhumtsev A., Zwart P. & Adams P.D. (2012) Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr D Biol Crystallogr 68:352-367.

42. Cowtan K. & Emsley P. (2004) COOT: model building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 60:2126-2132.
TABLE LEGENDS

Table 1: Parameters obtained for the crystal structure of G1324S (5BV8.pdb).

Table 2: Thermodynamic parameters defining the urea-temperature phase diagrams for the N ⇔ I and I ⇔ D transitions for wild-type A1, G1324A and G1324S.
FIGURE LEGENDS

Figure 1: A) Overlay of WT A1 and G1324S structures. The parameters obtained from the crystallographic analysis are summarized in Table 1. B) Distance difference matrix of amino acid residue α-carbons within ±1Å. C) Hydrogen bond between H1322 and S1324. D) Far UV and Near UV CD (inset) spectra of WT A1, G1324A, and G1324S. E) Size exclusion chromatograms of WT A1, G1324A, and G1324S. The inset shows the retention time of all three proteins relative to a standard curve obtained from the retention times of Ferritin (440 kDa), Aldolase (158 kDa), BSA (67 kDa), Ovalbumin (43 kDa), Ribonuclease A (13.7 kDa) and Vitamin B12 (1.35 kDa).

Figure 2: A) Platelet translocation pause times on WT A1 domain, G1324A, and G1324S as a function of shear rate. B) Survival fraction decay functions of platelet pause times at various shear rates for WT A1, G1324A and G1324S (left to right). A1 was chelated to the surface via the 6xHis tag at a total concentration of 5 μM.

Figure 3: Collagen III binding. A) Sensorgrams for WT A1, G1324A and G1324S. The inset in the WT A1 panel shows typical fit residuals that lie within 0.1-1% of the signal amplitude for all domain variants. B) Apparent rate constants for association and dissociation as a function of the protein concentration. Average rates for WT A1 are represented by black solid lines; the 1SD and 2 SD range are shown by dark gray and light gray dashed lines respectively. C) Apparent affinities obtained from the rates shown in panel B.

Figure 4: Isothermal urea denaturation of WT A1 (A), G1324A (B) and G1324S (C) at 5, 15, 25, and 35 °C monitored by CD at 222nm. D) Thermal unfolding of WT A1, G1324A and G1324S at 2 °C/min measured with CD. Inset shows the scan rate dependency of the transition midpoint for each variant caused by the kinetic irreversibility of the thermal unfolding monitored both by CD and intrinsic protein fluorescence (λEx = 280nm; λEm = 359nm) [21]. Tm,eq is obtained by extrapolation to 0 °C/min. E) Phase diagrams for WT A1 (closed circles), G1324A (closed squares) and G1324S (open circles) obtained from the isothermal urea induced unfolding curves and from iso-urea thermal denaturation. Lines represent boundaries where all proteins are 50% native. Phase diagrams of R1306Q and I1309V are published in [22]. F) Gibbs free energy, m-value and scan rate dependency of the thermal unfolding of G1324S in presence of 0, 1, 2, and 3 molar urea. Fitting parameters are summarized in Table 2.

Figure 5: A) Kinetics of A1 unfolding in 5.2M urea monitored via intrinsic protein fluorescence (280nm excitation; 359nm emission) at 20°C. B) Urea dependence of the unfolding rate constants. Error bars are the result of 3 independent measurements.

Figure 6: Limited proteolysis. A) Chromatogram of WT A1 after 18 h of incubation. Peaks are labeled with tryptic fragments as identified by mass spectrometry. See Figs. S4-S6 for chromatograms for all three A1 variants at all time-points. B) Time dependent accumulation of tryptic fragments of WT A1, G1324A, G1324S and digestion of the native protein (left to right). C) Rate constants obtained from fitting the initial slopes of the time courses. WT A1 - black, G1324A - light gray and G1324S - dark gray bars. D) Structural positions of tryptic fragments.

Figure 7: Correlation of the unfolding rates in absence of urea (Fig. 5B) and of the pause time survival fraction dissociation rates from platelets (Fig. 2). Fit function: y(x)=(4.13×10^−9) x^{−2.05} , R^2 =0.993.
**Table 1:** Parameters obtained for the crystal structure of G1324S (5BV8.pdb).

| Data collection |                |
|-----------------|----------------|
| Wavelength (Å)  | 0.97741        |
| Space group     | P61            |

| Cell dimensions |                |
|-----------------|----------------|
| a; b; c (Å)      | 86.45; 86.45; 68.16 |
| α; β; δ(°)       | 90; 90; 120     |
| Resolution Å     | 68.16 – 1.59   |
| R_{merge}        | 0.081(0.986)   |
| CC_{1/2} †       | 0.999(0.651)   |
| CC*            | 0.999(0.888)   |
| I/σI            | 14(1.5)        |
| Completeness (%) | 99.9(98.9)     |
| Redundancy      | 6.5(5.7)       |

| Refinement |                |
|------------|----------------|
| Resolution Å | 43.23 – 1.59  |
| Number of reflections | 38781          |
| R_{work}/R_{free} † | 0.164/0.180   |

| Ramachandran Angles |                |
|---------------------|----------------|
| Favored Region %    | 97             |
| Allowed Region %     | 3              |
| Outlier Region %     | 0              |

| Number of Atoms |                |
|-----------------|----------------|
| Protein         | 1651           |
| Ligand / ion    | 5              |
| Water           | 232            |

| B-factors |                |
|-----------|----------------|
| Protein   | 22.699         |
| Ligand / ion | 55.408       |
| Water     | 35.498         |

| R.m.s. deviations |                |
|-------------------|----------------|
| Bond lengths (Å)  | 0.006          |
| Bond angles (°)   | 1.109          |

†: CC_{1/2} is defined as the correlation coefficient between two random half data sets.
†: 5% of data were used for validation and were excluded from refinement.
Table 2: Thermodynamic parameters defining the urea-temperature phase diagrams for the $N \leftrightarrow I$ and $I \leftrightarrow D$ transitions for wild-type A1, G1324A and G1324S.

|                      | Taylor Expansion Thermodynamic Parameters | Classical Thermodynamic Parameters |
|----------------------|-------------------------------------------|-----------------------------------|
|                      | Wild-type A1 $N \leftrightarrow I$ transition |                                    |
| $T_m$                | $50.2 \pm 1.0^\circ$C                      | $285 \pm 22$ kJ/mol               |
| $\ln K / \partial \beta$ | $-285 \pm 12$ kJ/mol                      | $\Delta H^0$                      |
| $\partial^2 \ln K / \partial \beta^2$ | $(6.02 \pm 0.44) \times 10^3$ (kJ/mol)$^2$ | $\Delta C_P^0$                    |
| $\partial \ln K / \partial c$       | $1.62 \pm 0.04$ M$^{-1}$                  | $m$                              |
| $\partial^2 \ln K / \partial c \partial \beta$ | $16 \pm 2$ kJ/mol M$^{-1}$              | $\partial \Delta H^0 / \partial c$ |
| $\partial^3 \ln K / \partial c \partial \beta^2$ | $(-0.15 \pm 0.05) \times 10^3$ (kJ/mol)$^2$ M$^{-1}$ | $\partial \Delta C_P^0 / \partial c$ |
|                      | G1324A $N \leftrightarrow I$ transition  |                                    |
| $T_m$                | $53.85 \pm 0.68^\circ$C                   | $413 \pm 14$ kJ/mol               |
| $\ln K / \partial \beta$ | $-413 \pm 14$ kJ/mol                      | $\Delta H^0$                      |
| $\partial^2 \ln K / \partial \beta^2$ | $(8.39 \pm 0.53) \times 10^3$ (kJ/mol)$^2$ | $\Delta C_P^0$                    |
| $\partial \ln K / \partial c$       | $1.32 \pm 0.12$ M$^{-1}$                  | $m$                              |
| $\partial^2 \ln K / \partial c \partial \beta$ | $33 \pm 8$ kJ/mol M$^{-1}$              | $\partial \Delta H^0 / \partial c$ |
| $\partial^3 \ln K / \partial c \partial \beta^2$ | $(-0.31 \pm 0.21) \times 10^3$ (kJ/mol)$^2$ M$^{-1}$ | $\partial \Delta C_P^0 / \partial c$ |
|                      | G1324S $N \leftrightarrow I$ transition  |                                    |
| $T_m$                | $55.5 \pm 0.3^\circ$C                     | $481 \pm 10$ kJ/mol               |
| $\ln K / \partial \beta$ | $-481 \pm 10$ kJ/mol                      | $\Delta H^0$                      |
| $\partial^2 \ln K / \partial \beta^2$ | $(9.74 \pm 0.4) \times 10^3$ (kJ/mol)$^2$ | $\Delta C_P^0$                    |
| $\partial \ln K / \partial c$       | $1.49 \pm 0.10$ M$^{-1}$                  | $m$                              |
| $\partial^2 \ln K / \partial c \partial \beta$ | $34 \pm 7$ kJ/mol M$^{-1}$              | $\partial \Delta H^0 / \partial c$ |
| $\partial^3 \ln K / \partial c \partial \beta^2$ | $(-0.33 \pm 0.19) \times 10^3$ (kJ/mol)$^2$ M$^{-1}$ | $\partial \Delta C_P^0 / \partial c$ |
|                      | $I \Rightarrow D$ transition for all three proteins |                                    |
| $T_m$                | $135 \pm 10^\circ$C                       | $335 \pm 27$ kJ/mol               |
| $\ln K / \partial \beta$ | $-335 \pm 27$ kJ/mol                      | $\Delta H^0$                      |
| $\partial^2 \ln K / \partial \beta^2$ | $(1.99 \pm 0.35) \times 10^3$ (kJ/mol)$^2$ | $\Delta C_P^0$                    |
| $\partial \ln K / \partial c$       | $1.07 \pm 0.04$ M$^{-1}$                  | $m$                              |
| $\partial^2 \ln K / \partial c \partial \beta$ | $14.9 \pm 0.3$ kJ/mol M$^{-1}$          | $\partial \Delta H^0 / \partial c$ |

All parameters are referenced to the indicated $T_m$ in the absence of urea where ln $K = 0$. 
FIGURES

Figure 1
Figure 2

A

Pause time (s)

WT
G1324A
G1324S

Shear rate (s⁻¹)

B

Pause time (sec)

WT
G1324A
G1324S

increasing shear rate
Figure 3

A

B

C

Figure Caption:

A: Time courses of response units for WT, G1324A, and G1324S proteins. (Inset) Residuals for each condition.

B: Graph showing association and dissociation phase rates for WT, G1324A, and G1324S proteins as a function of protein concentration.

C: Bar graph showing apparent dissociation constants for G1324A, G1324S, and WT proteins. The affinity is calculated using $K_{D, \text{app}} = k_d/k_a$. 

Protein Concentration (µM)

$K_{D, \text{app}}$ (s⁻¹)

$K_{D, \text{app}}$ (µM)

$K_{D, \text{app}}$ (s⁻¹)

$K_{D, \text{app}}$ (µM)

Affinity

WT  G1324A  G1324S

0.1

0.01

0.01

0.01

0.1
Figure 4

A

WT

B

G1324A

C

G1324S

D

E

Isothermal

Isourea

F

WT A1

G1324A

G1324S

-8

-6

-4

-2

0

0

2

4

6

8

Urea (M)

Urea (M)

Urea (M)

Temperature (°C)

Isothermal

Isourea

Temperature (°C)

Temperature (°C)

Temperature (°C)

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Temperature (°C)
Figure 5

The graph shows the time course of unfolding (left) and the rate of unfolding (right) for different mutants. The mutants include WT, I1309V, R1306Q, G1324A, and G1324S. The x-axis represents time (s) and urea (M), while the y-axis represents fluorescence at 359 nm (cps * 10^6) and the rate of unfolding (s^-1).
Figure 7

Average rate constants from platelet survival fraction (s⁻¹)

100

10

1

0.1

1e-5

1e-4

Apparent rate of unfolding at 0 M urea (s⁻¹)

- WT A1
- G1324A
- G1324S
- I1309V
- R1306Q
