Published in final edited form as:

Proteins. 2010 August 15; 78(11): 2506–2522. doi:10.1002/prot.22759.

The catch bond mechanism between von Willebrand Factor and platelet surface receptors investigated by molecular dynamics simulations:

Catch bond between VWF and platelets

Gianluca Interlandi and Wendy Thomas*

Department of Bioengineering, University of Washington, Seattle, WA 98195, USA

Abstract

The multi-domain protein von Willebrand Factor is crucial in the blood coagulation process at high shear. The A1 domain binds to the platelet surface receptor glycoprotein Iba (GpIba) and this interaction is known to be strengthened by tensile force. The molecular mechanism behind this observation was investigated here by molecular dynamics simulations. The results suggest that the proteins unbind through two distinct pathways depending whether a high tensile force is applied or whether unbinding happens through thermal fluctuations. In the high force unbinding pathway the A1 domain was observed to rotate away from the C-terminus of GpIba. In contrast, during thermal unbinding the A1 domain rotated in the opposite direction as in the high force pathway and the distance between the termini of A1 and the GpIba C-terminus shortened. This shortening was reduced and the lifetime of the bond extended if a moderate tensile force was applied across the complex. This suggests that the thermal unbinding pathway is inhibited by a moderate tensile force which is in agreement with the catch bond property shown previously in single molecule experiments. A designed mutant of GpIba is suggested here in order to test *in vitro* the thermal unbinding pathway observed *in silico*.

1 Introduction

Blood platelets aggregate and adhere to sites of vascular injury. Elevated shear stress in rapidly flowing blood activates the multimeric protein von Willebrand Factor (VWF) which mediates binding between platelets and to collagen.¹ Each monomer of VWF consists of a number of repeated domains and monomers are linked to each other through disulfide bonds at the termini.² Because some bleeding disorders and thrombotic events have been found to be related to the function of VWF, its domains have been the target of intensive research. In particular, several studies have investigated the binding of the VWF A1 domain to the extracellular part of the platelet surface receptor glycoprotein Iba (GpIba).^{3–5} The bond between the isolated A1 domain and platelets has been shown by flow chamber experiments to be enhanced by shear stress.⁶ Recently, an atomic force microscopy study has indicated that the interaction between A1 and the isolated GpIba protein is a catch-bond, i.e., a tensile force across the complex extends the bond life time.⁷ This counter-intuitive behavior has

*Corresponding author: wendyt@u.washington.edu, Tel +1 (206) 616 3947, Fax +1 (206) 685 3300.

also been observed for other proteins such as selectins,⁸ the mannose binding FimH protein⁹ and the myosin-actin complex.¹⁰

Binding of VWF to GpIb α can be induced in the absence of shear by snake venoms such as botrocetin and bitiscetin¹¹ or by so called gain of function mutations. This type of mutations are believed to cause a minor bleeding disorder called type 2B von Willebrand disease where, because of enhanced binding to platelets, VWF multimers are cleared away in the plasma so that they are not present at bleeding sites. Recent single molecule experiments have shown that the mutation R543Q in A1, which is linked to type 2B von Willebrand disease,¹² and the mutation R687E increase binding at low shear and eliminate catch bond behavior⁷ (in the numbering scheme used in reference 7 the mutations are R1306Q and R1450E).

Shedding light onto the interaction between A1 and GpIb α at atomic level of detail can lead to the design of better drugs which prevent thrombus formation or therapeutics to establish normal VWF function in patients with von Willebrand disease. Several X-ray crystallographic studies have solved the structure of the A1 domain,¹³ GpIb α ,¹⁴ their complex,¹⁵ a mutant carrying the gain of function mutations R543Q in A1 and M239V in GpIb α ,^{14, 16} and the ternary complex A1/GpIb α /botrocetin¹⁷ (Figure 1). The A1 domain presents a mainly globular fold with a central parallel β sheet consisting of six strands surrounded by six α helices on either side.¹³ The N- and C-terminus of A1 are linked to each other by a disulfide bond between cysteines at positions 509 and 695.¹³ Superposition of unliganded A1 onto its complex with GpIb α shows little difference in the three dimensional fold apart from the loop between the first α helix and the second β strand (α_1 - β_2 loop). This loop is found in an extended conformation in all crystallographic structures except in the wild-type complex A1/GpIb α , where it adopts a bent conformation (Figure 1a). The fold of GpIb α presents an elongated structure consisting of 8 leucine rich repeats flanked with a β hairpin at its N-terminus (called here β finger) and a C-terminal flanking region at its C-terminus. This region presents a α helix, two disulfide bonds (Cys²⁰⁹-Cys²⁴⁸ and Cys²¹¹-Cys²⁶⁴) and a loop called β switch because it does not present any significant secondary structure in the unbound state but it switches to a β hairpin upon binding to A1^{14, 15} (Figure 1b). Despite the structural insights gained thanks to X-ray crystallography, the molecular mechanism behind the catch-bond behavior of this protein bond remains to be discovered.

In this study, the hypothesis is investigated that unbinding in the absence of force occurs through a different pathway than when pulling the proteins apart with a high tensile force. In the frame of this hypothesis, a moderate tensile force prolongs the lifetime of the bond by delaying unbinding which would occur due to thermal fluctuations in the total absence of force. The existence of a two-pathway model to explain the transition from slip to catch bond has been postulated in a theoretical work.¹⁸ Molecular dynamics (MD) simulations of the wild-type and of a gain of function mutant were performed here to shed light onto this hypothesis. The flexibility of the complex and the stabilizing interactions between A1 and GpIb α were investigated by room temperature simulations. The high force unbinding pathway was explored by pulling one of the A1 domain termini away from the C-terminus of GpIb α , which represents the physiological situation where shear stress acts to separate

the VWF A1 domain from the platelet. Unbinding due to thermal fluctuations was studied by both, high temperature simulations, as well as by increasing the distance between the centers of mass of both proteins. In order to validate the thermal unbinding pathway, a mutant of GpIb α was suggested and tested *in silico*.

2 Materials and Methods

Initial conformations

Table 1 lists the systems that were simulated in the present study. The simulations with the wild-type complex (labeled with “WT”) were started from the crystallographic structure with PDB code 1SQ0.¹⁵ The simulations with the mutant bearing the clinical gain of function mutations R543Q in A1 and M239V in GpIb α (labeled with “Mut”) were started from the X-ray structure with PDB code 1M10.¹⁴ One mutant was designed where a disulfide bond was modeled per homology into GpIb α by mutating Trp²³⁰ and Phe²⁰¹ into cysteines. The disulfide bond was modeled into the conformations obtained after 10 ns, 20 ns and 17.43 ns in the run WT300_1, which were used to start the simulations AddD_AS_1, AddD_AS_2 and AddD_AS_3, respectively. The run AddD_CV was started from the same conformation as AddD_AS_3 (Table 1). Throughout the manuscript, the amino acids were numbered using the same scheme as in the crystallographic studies.^{14, 15}

Simulations

The MD simulations were performed with the program NAMD¹⁹ using the CHARMM all-hydrogen force field (PARAM22)²⁰ and the TIP3P model of water. Initial conformations were minimized by performing 100 steps of steepest descent in vacuo and subsequent 500 steps of conjugate gradient minimization in a dielectric continuum with the CHARMM program.²¹ In the mutants where side chains were modeled per homology, the coordinates of all atoms were held fixed except for the mutated residues and additional 200 steps of steepest descent minimization in vacuo were performed. The proteins were then inserted into a cubic water box so that the distance between the protein and the boundary of the box was at least 12.5 Å. In the simulations where the N- or C-terminus of A1 is pulled at constant velocity, the water box was extended of additional 60 Å in the direction of pulling. The different simulations systems are summarized in Table 1. Chloride and sodium ions were added to neutralize the system and approximate a salt concentration of 150 mM. The water molecules overlapping with the protein or the ions were removed if the distance between the water oxygen and any atom of the protein or any ion was smaller than 3.1 Å. The number of water molecules ranged from 20333 to 42873, and the total number of atoms between 64512 and 136289. To avoid finite size effects, periodic boundary conditions were applied. Different initial random velocities were assigned whenever more than one simulation was performed with the same molecule. Electrostatic interactions were calculated within a cutoff of 12 Å, while long-range electrostatic effects were taken into account by the Particle Mesh Ewald summation method.²² Van der Waals interactions were truncated with the use of a switch function starting at 10 Å and turning off at 12 Å.

Before production runs, harmonic constraints were applied to the positions of all the atoms of the protein to equilibrate the system at 300 K or 400 K, respectively during a time length

of 0.2 ns. After this equilibration phase the harmonic constraints were released. In all simulations where no external force was applied and during the equilibration phase, the temperature was kept constant by using the Langevin thermostat²³ with a damping coefficient of 10 ps^{-1} , while the pressure was held constant at 1 atm by applying a pressure piston.²⁴ For the runs at 300 K with no external force, the first 10 ns of unconstrained simulation time were also considered part of the equilibration and were not used for the analysis. The dynamics were integrated with a time step of 2 fs. The covalent bonds involving hydrogens were rigidly constrained by means of the SHAKE algorithm with a tolerance of 10^{-8} . Snapshots were saved every 10 ps for trajectory analysis.

Constant velocity pulling—The simulations described in Section “Unbinding under high tensile force” were performed by pulling the N- or C-terminal C_α atom of the A1 domain away from the C-terminus of GpIb α . The atom to be pulled was attached through a virtual spring with a stiffness of $140 \text{ pN/\AA}$ to a dummy atom which was pulled at a constant velocity of $1 \text{ \AA/ns}$. Positional restraints were applied to the coordinates of the C_α atom of the GpIb α C-terminus. Pulling the dummy atom caused the virtual spring to extend and due to Hook’s law the resulting force ramped until the rupture force was reached.

Accelerated separation at room temperature—The simulations described in Section “Accelerated separation of the centers of mass” were performed by applying a total constant force of 400 pN to the C_α atoms of residues 509 to 701 of the A1 domain and 4 to 260 of GpIb α (forces were not applied to terminal amino acids to avoid restricting their natural fluctuations). The direction of force was chosen in such a way that the centers of mass of the proteins in the initial conformation were pulled away from each other. The magnitude and direction of force were kept constant throughout a simulation run.

Determination of native contacts

The conformations sampled at room temperature were used to determine native hydrogen bonds and salt bridges. To define a hydrogen bond, a H...O distance cutoff of $2.7 \text{ \AA}$ and a D-H...O angle cutoff of 120° was used, where a donor D could either be an oxygen or a nitrogen. For those side chains where a donor has more than one hydrogen (e.g., lysine and glutamine), no distinction was made as to which hydrogen was donated. Similarly, in the case of hydrogen bonds involving the side chain of glutamate or aspartate, no distinction was made as to which of the two oxygens was the acceptor. An interaction was defined as salt bridge if the atoms N_ζ of Lys or C_ζ of Arg were closer than $4 \text{ \AA}$ or $5 \text{ \AA}$, respectively, from either the C_γ of Asp or C_δ of Glu. All histidines were assumed neutral. Those hydrogen bonds and salt bridges present in at least 60% of the frames of one simulation at 300 K were selected as native contacts of a particular structure (two 300 K simulations were run with the wild-type complex, two with a mutant complex and one with the complex derived from the structure crystallized with botrocetin). A summary of all native contacts is presented in Table 2 and a detailed list is given in supplementary Table S1. Native inter-protein contacts were used to monitor the separation of the proteins from each other in the unbinding simulations.

Rotation axes and principal component analysis

In order to describe the unbinding pathways, two rotation axes were defined using the crystallographic structures (Figure 5). Both axes were chosen to go through the center of mass of GpIb α . The *parallel axis* (called like this because it is roughly parallel to the longest dimension of GpIb α) goes also through the C α atom of Cys²¹¹ and points towards the C-terminus of GpIb α . The *normal axis* is perpendicular to the plane defined by Cys²¹¹C α and the centers of mass of both proteins in the X-ray structure. Before calculating the rotation angles, all frames of the unbinding trajectories were aligned onto the X-ray structure from which the simulations were started in order to minimize the C α RMSD of GpIb α excluding the flexible β switch. The rotation of the center of mass of A1 around both axes was then calculated along the unbinding trajectories.

Principal component analysis of the trajectories was performed using the package GROMACS.²⁵ Using principal component analysis to determine the essential dynamics in molecular dynamics simulations was developed by Amadei et al.²⁶

3 Results

3.1 Fluctuations and stabilizing interactions at room temperature

Room temperature simulations were performed with the A1/GpIb α complex to study its conformational flexibility and to analyze stabilizing electrostatic interactions between the two proteins. The wild type complex is compared to a mutant carrying gain of function mutations. In order to obtain more sampling statistics, one simulation was started also from the wild-type complex crystallized in the presence of botrocetin (but only A1 and GpIb α are present). Two 300-K runs were performed with the wild-type,¹⁵ named here WT300_1 and WT300_2, two with the complex bearing the gain of function mutations R543Q in A1 and M239V in GpIb α ,¹⁴ called Mut300_1 and Mut300_2, and one with the wild-type crystallized in the presence of botrocetin,¹⁷ Bot300 (Table 1). The results obtained from the trajectories were compared with the published crystallographic data.

Backbone flexibility—The time series of the C α root mean square deviation (RMSD) and the plot of the C α atom fluctuations (Figure 2) show that the complex between A1 and GpIb α is stable during the 40-ns simulations at 300 K. Even if the A1/GpIb α complex is experimentally known to be short-lived in the absence of tensile force,⁷ separation of the proteins is not expected to occur in the time scale of a MD simulation at room temperature. The RMSD from the initial conformation generally remained below 2.5 Å for the whole complex and below 2 Å for each protein independently (Figure 2ac). The increase of the C α RMSD in the A1 domain is mainly accounted for by the fluctuations of the loops and to the rigid body motion of the helices, whereas the β strands were more rigid (Figure 2d–f and supplementary Figure S1a). In the protein GpIb α , the N-terminal β finger and the C-terminal flanking region are more flexible than the rest of the protein except for the rigid body motion of the leucine rich repeats relative to each other (Figure 2d–f and supplementary Figure S1b,c,d). The flexibility observed in the simulations is in general in good agreement with the X-ray studies, as it can be seen by comparing the C α root mean square fluctuations (RMSF) calculated from the simulations with those derived from the

crystallographic B-values (Figure 2d–f). There is a discrepancy for the 3_{10} helix including residues 629 to 631 which presents in the simulations higher fluctuations than the RMSF derived from the B-values. Interestingly, this 3_{10} helix is one of the docking sites for botrocetin and its flexibility observed in the simulations might be important to allow adaptation to botrocetin.

Inter-protein hydrogen bonds—The room temperature trajectories were screened for inter-protein hydrogen bonds and salt bridges. Those present in at least 60% of the frames of at least one simulation started from one of the three X-ray structures of the complex were defined as native inter-protein contacts of that structure (see “Materials and Methods”) and used to describe the unbinding pathways presented in this manuscript. Most inter-protein hydrogen bonds involve a region around the β switch of GpIb α (Figure 3). There is in general good agreement among the different simulations and with the X-ray structures concerning the hydrogen bonds which involve the β switch of GpIb α , e.g., there are four backbone hydrogen bonds involving Ser⁵⁶² and Ala⁵⁶⁴ of A1, and Lys²³⁷ and Met²³⁹ located on the β switch of GpIb α (Figures 3, 4 and supplementary Table S1). The β switch itself is also stabilized by intra-loop backbone hydrogen bonds most of which are stable in the simulations (Figure 3 and Supplementary Table S1). According to crystallographic studies, the β switch is very flexible in unliganded GpIb α but builds a stable β hairpin upon binding to A1 (Figure 1).

Inter-protein salt bridges—In contrast, there is a more severe discrepancy between the simulations and the X-ray structures concerning interactions between charged side chains. In the simulations with the wild-type there is a remarkably large number of native salt bridges (i.e., salt bridges formed in at least 60% of the frames of a trajectory) which are not observed in the X-ray structures (Figure 3a, Table 2, and supplementary Table S1). The binding sites of A1 and GpIb α present a large number of positively and negatively charged side chains, respectively. Several of these side chains are observed to form in the simulations a flexible network of inter-protein salt bridges (Figures 3 and 4). Some side chains form a stable salt bridge with the same partner in multiple independent simulations while others bond with different side chains in different simulations or alternate between different partners along the same trajectory (Figure 4 and supplementary Table S1).

It can be speculated that this flexible network of inter-protein salt bridges might be entropically favorable because charged side chains can engage in different salt bridges without losing degrees of freedom. Thus the large number of oppositely charged side chains at the binding interfaces might have two purposes: attract the binding sites of the proteins towards each other during binding and kinetically stabilize the bound state.

In summary, while most native hydrogen bonds involve a region of GpIb α which includes the β switch and are in general consistent in all simulations, most salt bridges involve the leucine reach repeats and the tip of the β switch and show a slightly different pattern in each simulation. All native inter-protein contacts observed in the 300 K simulations will be monitored along the trajectories in the unbinding simulations. This will be used to describe how the proteins separate in order to distinguish different unbinding pathways.

3.2 Unbinding under high tensile force

Recently, the complex between A1 and GpIb α was shown by single molecule force spectrometry to be a catch bond.⁷ The property of a catch bond is that a moderate tensile force increases its life time whereas a high tensile force converts the catch bond into a slip bond and causes a decrease of the life time. Here, the A1/GpIb α complex is hypothesized to dissociate via two distinct pathways, a slip pathway that is induced by force and a catch pathway that is inhibited by force. The force induced slip pathway was investigated by simulations where an external force is applied to pull at constant velocity the N- or C-terminus of A1 away from the C-terminus of GpIb α . Figure 5a,b illustrates the unbinding pathway observed in the pulling simulations.

Three simulations were performed with the wild-type where the N-terminus of A1 was pulled away from the C-terminus of GpIb α at constant velocity (see Materials and Methods and Figure 5a,b). These simulations are called WT_CV_n, where n=1,2,3. Three pulling simulations were performed with the complex bearing gain of function mutations R543Q in A1 and M239V in GpIb α to study whether these mutations alter the unbinding pathway under high tensile force (named Mut_CV_n). Furthermore, one simulation was performed where the C-terminus of A1 was pulled instead of the N-terminus to investigate whether unbinding depends upon which terminus is pulled (named WT_CV_cter). Because the A1 domain is covalently bound to the rest of VWF through both termini, pulling either of them mimics the physiological situation where, in the case of high shear, force is transmitted from the rest of the VWF multimer onto the A1 domain. On the other hand, GpIb α is attached to the rest of the platelet only through its C-terminus, thus the latter is restrained in the simulations. Time series showing conformational changes during pulling are shown in Figure 6 for three representative simulations (time series of replica simulations are presented in supplementary Figure S2).

Pulling the N-terminus of A1 with the wild-type complex—(Left column in Figure 6). In the simulations where the N-terminus of A1 was pulled at constant velocity (WT_CV), the force generally ramped up almost linearly until reaching the peak after which the force decreased sharply (Figure 6a). The sharp decrease of the force coincided with the complete rupture of all native inter-protein hydrogen bonds (Figure 6b), which can be referred to as the rate limiting step of the separation of the proteins under high tensile force. The dashed lines in the plots of Figure 6 indicate the first time point in the trajectory after which all native inter-protein hydrogen bonds are broken. Throughout this manuscript, the definition is used that the proteins have unbound from each other after all inter-protein hydrogen bonds are broken because in most high force pulling simulations none of the native inter-protein hydrogen bonds were seen to reform after the peak of the pulling force was reached (see Materials and Methods for the definition of native contacts). After the rate limiting step, the A1 domain rotated around an axis through the center of mass (CM) of GpIb α and normal to the plane defined by the CMs of the proteins and the C α of Cys²¹¹ (Figure 5b). The C α atom of Cys²¹¹ was chosen to define the axis because it is covalently linked to the C-terminus of GpIb α while it is less fluctuating than the C-terminal tail. Time series of the amount of rotation are plotted in Figure 6a. Most native salt bridges, which are mainly located in the concave region of GpIb α , remained formed during this rotation (Figures 1 and 6b). This is

likely the reason why the A1 domain is observed to rotate and cannot slide along the GpIb α surface as it is pulled away. During separation the A1 domain also rotated 25 degrees around an axis through the CM of GpIb α and the C α of Cys²¹¹ and thus parallel to the long dimension of GpIb α (data not shown, see Figure 5a for the location of the axis). A small rotation around the parallel axis is also observed in room temperature simulations indicating that this rotation is one of the degrees of freedom of the A1/GpIb α complex. After unbinding, the distance between the N-terminus of A1 and the C-terminus of GpIb α extended and the centers of mass separated (Figure 6c). The C α RMSD from the starting structure for each protein individually remained in general below 3 Å indicating that the proteins undergo only moderate conformational changes during pulling (Figure 6d). In most simulations, soon after unbinding, the β switch moved away from the rest of the protein as a rigid body while retaining its secondary structure (Figure 6e) suggesting that the β switch is a structurally flexible element of GpIb α .

Pulling the N-terminus of A1 with the mutant complex—(Middle column in Figure 6). A similar unbinding pathway and rupture force were observed in the pulling simulations with the gain of function mutant (Mut_CV) as in the runs with the wild-type (Figures 6 and 7a) indicating that the gain of function mutations R543Q in A1 and M239V in GpIb α might not influence the high tensile force unbinding pathway. In the pulling runs with the mutant unbinding was observed on average 10 ns later than in the pulling runs with the wild-type and yet at a lower force. This is probably due to the fact that the X-ray structure of the mutant used in the simulations has one additional amino acid at the N-terminus of A1 and two additional amino acids at the C-terminus of GpIb α compared to the X-ray structure of the wild-type complex which delayed the onset of force.

Pulling the C-terminus of A1 with the wild-type complex—(Right column in Figure 6). The time series of the force in the simulation where the C-terminus of A1 was pulled (WT_CV_cter) presents a slightly different slope than in the runs where the N-terminus was pulled (right column in Figure 6a). One peak of the force is observed after 33.9 ns which corresponds to a shift of the C-terminal helix towards the pulling direction of about 10 Å. The major peak of the force is observed after 46 ns and corresponds to the uncoiling of the C-terminal helix. It should be noted here that uncoiling of the C-terminal helix happens while the disulfide bond near the terminii of A1 is still formed (breaking and forming of disulfide bonds cannot be simulated in MD runs). After 51 ns all inter-protein hydrogen bonds were lost and the force decreased (Figure 6a,b). Despite the denaturation of the C-terminal helix, the overall picture of the unbinding pathway is similar to the simulations where the C-terminus was pulled. This is probably due to the fact that the force is transmitted through the disulfide bond between Cys⁵⁰⁹ and Cys⁶⁹⁵.

3.3 Unbinding at high temperature

To explore the unbinding pathway of A1/GpIb α in the absence of force, i.e., due to thermal fluctuations, one 140-ns simulation was performed at 400 K (it is called here WT_400K). High temperature simulations have been used to study the conformational stability and unfolding pathways of medium sized proteins.^{27, 28} Here, high temperature is used to investigate unbinding pathways. Furthermore, to investigate how a moderate tensile force

might alter this unbinding pathway, one 100-ns long simulation was performed where the N-terminus of A1 and the C-terminus of GpIb α were pulled away from each other with a constant force of 50 pN (WT_400K_CF). Time series of conformational changes during both simulations are plotted in Figure 8.

High temperature simulation—Interestingly, in the run WT_400K unbinding of the proteins was preceded by a rotation of the A1 domain around the normal axis (Figure 5c) but in the opposite direction as in the pulling simulations (Figure 5b). Principal component analysis (see Materials and Methods) shows that this rotation correlates with the projection along the first eigenvector of motion (Figure 8a). Principal component analysis was performed also for the high force pulling simulations and the rotation around the normal axis observed there is described by a similar eigenvector (Table 3). A rotation of up to 25 degrees is observed also around the parallel axis similarly as in the high force pulling simulations (Figure 5). This rotation corresponds in most simulations to the second eigenvector of the principal component analysis and it is observed also in the 300-K runs (data not shown). While the A1 domain rotates around the normal axis, the salt bridges near the N-terminus of GpIb α were observed to break (Figure 8b). After 76 ns of simulation all native inter-protein hydrogen bonds ruptured (Figure 8b and insert). Similarly to the high force pulling simulations, the time point after which all native inter-protein hydrogen bonds are broken is defined as the unbinding time of the proteins from each other even if breaking and reforming of some of the hydrogen bonds was observed prior to this time point (Figure 8b). The rupture of the hydrogen bonds was also preceded by a shortening of the distance between two cysteines located near the C-terminus of each protein, i.e., A1 Cys⁶⁹⁵ and GpIb α Cys²¹¹ (Figure 8c) of about 7.7 Å (this number was obtained by subtracting the minimum value observed during the entire 140 ns of simulation from the average value measured in the two 300 K runs, i.e., 61 ± 0.81 Å). The distance between these two cysteines is of interest because physiologically a tensile force is expected to pull the termini of A1 away from the C-terminus of GpIb α . The fact that this distance is observed to shorten in the run WT_400K might support the hypothesis that a moderate tensile force could inhibit this unbinding pathway by preventing the shortening of this distance. The distance was measured between A1 Cys⁶⁹⁵ and GpIb α Cys²¹¹ and not between Cys⁵⁹⁶ and Cys²⁶⁴, to which they are connected through disulfide bonds, because the last two are closer to the surface of the proteins and thus they are more fluctuating. After the rupture of the hydrogen bonds the distance between the centers of mass of the two proteins increased (Figure 8c). Several partial rebinding events were observed in the time window between 76 ns and 115 ns where native hydrogen bonds between the β switch of GpIb α and A1 reformed and broke several times. After in total 115 ns of simulation time, all native inter-protein salt bridges were lost, the distance between the centers of mass increased (Figure 8b) and the A1 domain separated from GpIb α by rotating around the normal axis in the same direction as in the pulling simulations. It is important to note that this final rotation could be due to random diffusion of the protein because it happened after all native inter-protein contacts were broken. Despite the high temperature of 400 K the total C α RMSD of the A1 domain remained below 3.5 Å during the entire simulation. However, the C α RMSD of GpIb α measured less than 4 Å before the rupture of hydrogen bonds but later in the simulation it increased to values up to 10 Å (Figure 8d). This increase was mainly due to distortions in the C-terminal flanking

region of GpIb α due to the elevated temperature (supplementary Figure S3). Furthermore, the β switch was observed to swing out away as a rigid body from the rest of GpIb α while A1 rotated around the normal axis before rupture of all inter-protein hydrogen bonds (Figure 8e). This observation was considered when designing a mutant to experimentally test this unbinding pathway.

High temperature simulation with moderate tensile force—In the simulation WT_400_CF, where a constant tensile force of 50 pN was applied, no unbinding event was observed and the centers of mass between the proteins did not separate during the total simulation time of 100 ns. The simulation was stopped after 100 ns because of large distortions in the C-terminal flanking region due to the elevated temperature. The A1 domain was not observed to rotate around the normal axis (Figure 8a). The distance between A1 Cys⁶⁹⁵ and GpIb α Cys²¹¹ shortened during the simulation of a value of 6.53 Å which is smaller than in the run WT_400K (Figure 8c). The distance between the centers of mass remained almost constant (Figure 8c) and most inter-protein hydrogen bonds were still formed during the 100 ns of simulation time (Figure 8b).

3.4 Accelerated separation at room temperature

The observations made in Section “Unbinding at high temperature” suggest that unbinding due to thermal fluctuations involves a rotation of A1 towards the C-terminus of GpIb α causing a shortening of the distance between the termini of A1 and the C-terminal part of GpIb α . This shortening might be counteracted by a tensile force which pulls the A1 domain away from GpIb α which is anchored to the rest of the platelet through its C-terminus. Although the high temperature simulations provide a qualitative description of the unbinding pathway in the absence of force, repeating them several times to obtain better statistical sampling is computationally expensive and heating the system to an even higher temperature might cause the proteins to unfold before unbinding is observed. On the other hand, spontaneous unbinding would not be observed in the time scale of a simulation at 300 K because it would require extremely long simulation times which is computationally prohibitive. For this reason, a method is required to accelerate sampling of the spontaneous separation of the proteins at room temperature such that possible artefacts of high temperature simulations can be avoided and enough statistical sampling can be achieved with shorter simulations. This was done here by inducing the proteins to separate by applying constant forces which pulled the centers of mass away from each other. The assumption was made that if this is done gently enough, then the proteins will unbind through the lowest energy pathway which might correspond to the same pathway through which unbinding occurs in the total absence of force and due to thermal fluctuations.

Accelerated separation of the wild-type complex—In total, three accelerated separation simulations were performed with the wild-type (WT_AS_n, n=1,2,3), three with the mutant bearing the gain of function mutations R543Q in A1 and M239V in GpIb α (Mut_AS_n), three with the wild-type where a constant tensile force of 50 pN was applied across the complex (WT_AS_CF_n) and three with a designed mutant (AddD_AS_n). The runs were started from different snapshots taken from the 300 K simulations (Table 1 and “Materials and Methods”). Time series calculated for representative simulations are

displayed in Figure 9 whereas the time series of the replicated runs can be found in supplementary Figures S4 and S5. Similarly to the high temperature simulation, rupture of all native inter-protein hydrogen bonds was preceded by a rotation of A1 around the normal axis towards the C-terminus of GpIb α and shortening of the distance between the termini of A1 and the C-terminus of GpIb α (Figures 5c and 9). Furthermore, the rotation of A1 around the normal axis also correlated with the projection along the first eigenvector of motion (Figure 9a). This rotation is described by similar eigenvectors in all unbinding simulations (Table 3). In the run WT_AS_3 before rupture of the two inter-protein backbone hydrogen bonds proximal to the turn of the β switch, the A1 domain rotated back to its position as in the native state (although the two hydrogen bonds distal to the turn of the β switch did not reform) and unbound from GpIb α by rotating around the normal axis away from the C-terminus of GpIb α (supplementary Figure S5). This observation made only in the run WT_AS_3 suggests that after rupture of most inter-protein native contacts unbinding can continue through more than one pathway. However, the limited statistics does not allow to draw strong conclusions about the presence of multiple thermal unbinding pathways.

Accelerated separation of the mutant complex—The forced separation simulations with the gain of function mutant present a qualitatively similar unbinding pathway and on average a slightly shorter unbinding time as the simulations with the wild-type (Figure 7c), although the difference is not statistically significant. Furthermore, the shortening of the distance between the termini of A1 and the C-terminus of GpIb α was much less pronounced. These observations are in contrast with single molecule spectroscopy which showed that the mutation R543Q induces a gain of function.⁷

Accelerated separation of the wild-type complex with added tensile force—In the runs where a constant force of 50 pN was applied to the N-terminus of A1 and the C-terminus of GpIb α , in addition to pulling the centers of mass away from each other, unbinding occurred on average later than in the runs where a constant force was applied only to the centers of mass (third column in Figure 9 and Figure 7b). The correlation value calculated from a single tailed paired student t-test is 0.02. It makes sense to use here the paired versus the unpaired student t test because the unbinding time correlates with the starting conformation (for each of the three initial conformations, one run was performed without and one run with a tensile force of 50 pN, see Table 1). Furthermore, shortening of the distance between the N-terminus of A1 and the C-terminus of GpIb α was reduced (Figures 9c and 7c). The t-test gives a correlation of 0.01 indicating that the difference is statistically significant.

Mutant designed to increase the lifetime during thermal unbinding—A mutant of GpIb α was designed in order to validate the thermal unbinding pathway *in vitro*. In almost all simulations where the thermal unbinding pathway was simulated, the β switch moved as a rigid body away from the rest of GpIb α before the rupture of all inter-protein hydrogen bonds. This movement correlated with the rotation of A1 around the normal axis and it was probably due to the presence of backbone hydrogen bonds between the β switch and one strand of A1. This observation lead to the hypothesis that constraining the β switch delays unbinding. To test this, a disulfide bond was modeled per homology between the β

switch and the rest of GpIb α through the mutations F201C and W230C. In accelerated separation simulations performed with this designed mutant, AddD_AS_n (n=1,2,3), unbinding occurred on average later than with the wild-type (fourth column in Figure 9 and Figure 7b,c). The correlation value obtained from the paired student t test is 0.05 indicating that the difference is statistically significant. These mutations are not likely to alter the high force unbinding pathway because in a constant velocity pulling run the complex ruptured at a similar force as the wild-type (Figure 7a). This mutant is expected to bind more strongly than the wild-type at low shear in flow chamber experiments.

4 Discussion

Binding between the A1 domain of VWF and platelet surface receptor GpIb α is known to be enhanced by a tensile force.^{6, 7} However, the underlying molecular mechanisms for these two types of activation have not been thoroughly elucidated yet. In the present study, multiple molecular dynamics simulations have been performed to investigate the stabilizing interactions between A1 and GpIb α at room temperature and their unbinding pathways. The results suggest a model according to which A1 and GpIb α unbind from each other through two distinct pathways depending whether a high tensile force is applied or whether unbinding occurs due to thermal fluctuations. Furthermore, the thermal unbinding pathway is a catch pathway because it is inhibited by a moderate tensile force. The suggested model provides an explanation for the experimentally observed force activation of the bond between A1 and GpIb α .

The high force unbinding pathway was simulated by pulling either terminus of A1 away from the C-terminus of GpIb α at constant velocity until the proteins separated. The A1 domain was observed to unbind by rotating away from the C-terminal region of GpIb α (Figure 5b). The hinge point of this rotation was the N-terminal region of GpIb α which presents a large number of inter-protein salt bridges with A1 (Figure 3a). The presence of these salt bridges is likely to prevent the proteins from sliding along their surfaces causing the observed rotation.

In order to study the thermal unbinding pathway, separation of the proteins was accelerated by either simulating the complex at high temperature or by forcing the distance between the centers of mass to increase. In both types of simulations the proteins unbound through a qualitatively similar pathway. The A1 domain separated from GpIb α by rotating in the opposite direction as in the high force pulls, i.e., towards the C-terminal region of GpIb α (Figure 5c). During this rotation, the distance between the N-terminus of A1 and the C-terminus of GpIb α was observed to shorten. The shortening of this distance might be linked to the physiological tensile force activation of the A1/GpIb α bond because GpIb α is anchored to the rest of the platelet through its C-terminus and A1 is covalently linked to the rest of VWF through both termini. Thus, it can be hypothesized that a tensile force, which is generated for example when VWF and platelets are pulled away from each other in the presence of shear flow, might inhibit this distance to shorten and thus prevent the rotation and unbinding of the proteins. This hypothesis was tested by applying a force of 50 pN to the N-terminus of A1 and to the C-terminus of GpIb α in opposite directions, mimicking the physiological situation existing in shear flow. This was done in both types of simulations,

high temperature and simulations where the distance between the centers of mass of the proteins was forced to increase. Strikingly, the shortening of the distance between the A1 N-terminus and GpIb α C-terminus was reduced and unbinding was delayed when compared to the simulations where no tensile force was applied across the complex (Figure 7b,c). This is the first observation in molecular dynamics simulations of a catch bond behavior, defined as longer life time under tensile force.

The observation of two distinct unbinding pathways and the force inhibition of one of them can be explained by the particular geometry of the complex formed by A1 and GpIb α . The A1 domain is a globular protein with its termini close to each other and linked by a disulfide bond. On the other hand, GpIb α is an elongated protein presenting a concave surface which contacts A1 through stable backbone hydrogen bonds involving the β switch (a β hairpin located near its C-terminus) and a flexible network of salt bridges involving the N-terminal half of GpIb α (Figures 3 and 4). Because these salt bridges act like a hinge, a high tensile force applied across the complex induces a rotation of A1 away from GpIb α instead of a sliding along its surface. On the other hand, when unbinding is caused by thermal fluctuations, in the absence of force, the relatively less stable salt bridges near the N-terminus of GpIb α break before the more stable backbone hydrogen bonds located near the C-terminus causing the observed rotation of A1 towards the C-terminal region of GpIb α . Because of this partial separation water can penetrate further into the contact surface between the two proteins and hydrate the β switch thus destabilizing it and causing the backbone hydrogen bonds to rupture as well. This pathway can be described as a catch pathway because it is delayed in the simulations when a moderate tensile force is applied. However, when force is increased to higher values the proteins unbind from each other through the slip pathway described above where the A1 domain rotates in the opposite direction as in the catch pathway.

The simulation results presented here deliver a tentative explanation for the catch bond behavior observed in recent single molecule experiments where the A1 domain and GpIb α were pulled away from each other by means of an atomic force microscope.⁷ There, no significant or very low binding was observed under low force, which corresponds to the situation where the proteins unbind through the thermal or catch pathway. As force is increased above 20 pN, an increase in bond life time is observed which can be explained by the inhibition of the catch pathway through the applied tensile force. A further increase in tensile force applied through the microscope causes a decrease in bond life time, which can be interpreted as the proteins unbinding through the slip pathway.

Interestingly, the two-pathway theory discussed here, where one of the pathways is inhibited by moderate force, can be applied in certain cases also when studying the force induced unfolding of proteins. In fact, using a type of coarse grained model called G β model,²⁹ a study found for two proteins that the life time of their folded state was extended when a constant force was applied to the termini.³⁰ These two proteins also presented a transition state very similar to the native state pointing at their high force resistance against unfolding. This is similar to the A1/GpIb α bond studied here where the rate limiting step of unbinding is very close to the bound state explaining the high resistance of this bond against forced unbinding.³¹ It might be possible to extend this study using a G β model in order to estimate

the life time of the A1/GpIb α bond as a function of the applied tensile force, although G models take only the topology of the native state into account.

In order to make it possible to test *in vitro* the thermal unbinding pathway observed in the simulations, one mutant was designed where a disulfide bond was introduced between the β switch of GpIb α and the rest of the protein. The simulations predict that, at low shear, the A1 domain will bind stronger to this mutant than the wild-type (Figure 7b).

An alternative explanation for the A1/GpIb α catch bond was previously suggested based on MD simulations of the unbinding at high force, where a similar unbinding pathway was observed as described here. The authors observed the formation of a transient non-native inter-protein salt bridge while the A1 rotated and claimed that this salt bridge might induce A1 to rotate back into the bound state.⁷ However, rebinding was not observed in the simulations and formation of this salt bridge was seen only after the hydrogen bonds involving the β switch were broken, i.e., after the rate limiting step, and thus it is unlikely to cause a rebinding. Thus it can be concluded that understanding the catch bond behavior of this complex is not possible by analyzing only the high force slip pathway, but rather requires exploring also the thermal catch pathway described in this manuscript.

Several other proteins have been observed to exhibit catch bond behavior, for example the FimH protein at the tip of bacterial fimbria,^{9, 32} L-selectins,³³ the actin/myosin complex¹⁰ and integrins.³⁴ However, a different catch-bond mechanism has been presented for some of these proteins which differs from the one proposed here for the A1/GpIb α bond. The protein FimH and L-selectins are known to be regulated allosterically as they consist of a binding and a regulatory domain.³⁵ In the absence of tensile force, the two domains are hinged with respect to each other and the proteins are in a low affinity state for binding to a target. If the hinge angle between the regulatory and the binding domain is straightened by either mutations or tensile force, the proteins switch to a high affinity binding state.^{8, 36}

The study presented here does not provide an explanation for the gain of function mutations R543Q in A1 and M239V in GpIb α . Both mutations are clinically linked to von Willebrand disease because excessive binding to platelets causes VWF multimers to be cleared away from the plasma and not be present where they are needed. The mutation M239V in GpIb α was shown by optical tweezers experiments to cause an increase of the rupture force.³⁷ The fact that the same observation was not made in the pulling runs presented here could be due to the much higher force used in simulations and to the resulting shorter unbinding time. The mutation of a methionine into a valine might be entropically favorable for binding, because a smaller surface area must be buried and valine has a higher β sheet propensity than methionine.³⁸ The role of side chain entropy in binding has been investigated also in the case of small molecules used as drug targets.³⁹

The mutation R543Q in A1 was shown by single molecule experiments to increase binding at low force so that catch-bond behavior was not measured.⁷ However, in the experiments performed therein the A1 domain contained a longer N-terminal chain than in the crystallographic structures used to start the simulations here. Precisely, 32 amino acids, which connect the A1 domain to the neighboring domain of VWF, are missing from the X-

ray structure of the complex. It is not clear how this long N-terminal chain might influence binding between A1 and GpIb α and cause effects which cannot be detected by the simulations presented here because the currently available crystallographic structure of the complex lacks these additional amino acids.

In summary, this study presents a mechanism whereby a tensile force extends the life time of a bond, for the first time corroborated by multiple molecular dynamics simulations which totaled over 1 μ s. The atomistic view presented here of the unbinding pathways of the VWF A1 domain from GpIb α and how tensile force affects them can help to design drugs which inhibit binding between the two proteins in order to prevent thrombosis, or substitutes of VWF for patients suffering of von Willebrand disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. B. Kidd for helpful and interesting discussions. This research was supported by an allocation of advanced computing resources supported by the National Science Foundation. The computations were performed on the Abe supercomputer at the National Center for Supercomputing Applications. This research was financially supported by a postdoctoral fellowship from the Swiss Science National Foundation number PBZHA-112719 and from the American Heart Association number 0820107Z both to GI, an award from the University of Washington Royal Research Fund and an American Heart Association National Scientist Development Grant number 0735337N to WT.

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Figure 1.

(a) Superposition of three X-ray structures of the A1/GpIb α complex and one structure of unliganded A1. Red: wild-type (PDB code 1SQ0), violet: crystallized with botrocetin (PDB code 1U0N), green: gain of function mutations R543Q in A1 and M239V in GpIb α (PDB code 1M10), cyan: unliganded A1 (PDB code 1AUQ). The β switch and the α_1 - β_2 loop are presented enlarged on the right. (b) Superposition of three X-ray structures of unliganded GpIb α (PDB codes 1M0Z (orange) 1P9A (lime) 1QYY (violet)). Unless GpIb α is bound to A1, the β switch (highlighted by a blue ellipse) does not contain any significant secondary structure elements although it is found in similar conformations in all three crystallographic structures.

Figure 2.

(a)–(c) Time series of the C_{α} RMSD during the 300 K simulations with wild-type (two runs, PDB code 1SQ0), gain of function mutant (PDB code 1M10, two runs) and wild-type crystallized with botrocetin (PDB code 1U0N, one run), respectively. Although displayed, the first 10 ns of each run have been considered equilibration and not used for the analysis. (d)–(f) C_{α} RMSF calculated using the last 30 ns of the in total 40 ns long 300 K simulations (for those structures where two room temperature runs were performed the calculation was averaged over a total of 60 ns). The values for the A1 domain are in the plots on the left column, those for GpIb α are displayed in the right column. Solid red, green and violet circles indicate C_{α} atoms contained in α helices, β strands and 3_{10} helices, respectively. Values of RMSF derived from crystallographic B factors of the C_{α} atoms (displayed in

magenta) were calculated using the formula $\text{RMSF}_i^{\text{exp}} = \sqrt{\frac{3}{8\pi^2} B_i}$, where B_i is the B factor of C_α of residue i .⁴⁰

Figure 3.

Persistent inter-protein and intra- β switch hydrogen bonds observed in two runs with wild-type A1/GpIb α (a) and in two runs with mutant A1/GpIb α (mutations R543Q in A1 and M239V in GpIb α) (b). The structure represented in (a) is a snapshot saved after 36 ns in the run WT300_1, whereas the structure in (b) was saved after 20 ns from the run Mut300_1. Side chains involved in persistent contacts are shown in the ball and stick representation and colored according to atom type (red for oxygen, blue for nitrogen and grey for carbon). Hydrogen bonds and salt bridges are indicated by blue and red dashed lines, respectively. Salt bridges which are persistent in only one of the two runs are indicated by green dashed lines. In (b) the backbone of the gain of function mutation sites is colored green and the respective side chains are shown in the ball and stick representation.

Figure 4.

Time series of the formation of native inter-protein hydrogen bonds (blue) and salt bridges (red) during five room temperature simulations (two with the wild-type structure, two with a gain of function mutant and one with the wild-type crystallized in the presence of botrocetin). A hydrogen bond or salt bridge is considered a native contact if it is formed in at least 60% of the simulation frames during the last 30 ns of a 300 K simulation (see Materials and Methods for more details). A list of native contacts is presented also in Table 2 and supplementary Table S1. Time series of native contacts are also used to monitor unbinding in high temperature and pulling simulations, except the two salt bridges in square brackets which are never observed to be formed in any of the unbinding simulations. The first 10 ns of each simulation (indicated by a cyan dashed line) were considered equilibration and not used for the determination of native contacts.

Figure 5. Rotation of the A1 domain during unbinding due to high force or thermal fluctuations (stereoview). The rotation axis (blue) is normal to the plane defined by the centers of mass (CM) of each protein and the C α atom of GpIb α Cys²¹¹ and going through the CM of GpIb α . A smaller rotation is also observed around an axis (green) parallel to the long dimension of GpIb α . The red dashed line indicates the distance between Cys⁶⁹⁵ of A1 and Cys²¹¹ of GpIb α monitored in the unbinding simulations. **(a)** Native state. **(b)** Conformation reached after 24.5 ns during the pulling simulation WT_CV_1 illustrating the high force unbinding pathway. **(c)** Conformation reached after 81.59 ns during the 400 K run WT400 illustrating the thermal unbinding pathway.

Figure 6.

Time series of conformational changes during three representative pulling simulations (see Table 1). The coordinates of the C-terminus of GpIb α were fixed and either the N-terminus (runs WT_CV_1 and Mut_CV_3) or the C-terminus (run WT_CV_ter) of A1 was attached to a virtual spring which was pulled at constant velocity away from the C-terminus of GpIb α . The vertical dashed lines indicate the time point after which all inter-protein hydrogen bonds are broken. All plots are time averages over a 200 ps time window. **(a)** The force applied onto the pulled A1 terminus calculated from Hook's law (abscise on the right) and the amount of rotation around the normal axis (Figure 5). **(b)** Number of native hydrogen bonds and salt bridges (see Materials and Methods for the definition of native contacts). **(c)** Distance between the C α atoms of A1 Cys⁶⁹⁵ and GpIb α Cys²¹¹, and between the centers of mass (abscise on the right). **(d)** C α RMSD of the A1 domain excluding the fluctuating tails and of GpIb α . **(e)** C α RMSD of the β switch. The rigid body motion of the β switch was monitored by aligning GpIb α onto the X-ray structure excluding the β switch (green line).

Figure 7.

Comparison between different unbinding simulations. The presented values are averages and standard errors of the mean of three independent simulations except AddD CV where only one run was performed. Table 1 lists the simulations performed. **(a)** Maximum force observed in simulations where the N-terminus of A1 was pulled away from the C-terminus of GpIbα at constant velocity. **(b)** Time until all inter-protein backbone hydrogen bonds were broken in simulations where the CM of the proteins were pulled apart. **(c)** Difference between the average value of the distance between A1 Cys⁶⁹⁵C_α and GpIbα Cys²¹¹C_α and the smallest value measured in simulations where the CM of the proteins were pulled apart at constant force.

Figure 8.

Time series of conformational changes during two 400 K simulations. Run WT_400K was performed in the absence of tensile forces, whereas in run WT_400K_CF the N-terminus of A1 and the C-terminus of GpIbα were pulled away from each other with a constant force of 50 pN (see Table 1). The vertical dashed lines indicate the time point after which all inter-protein hydrogen bonds are broken for the first time in the simulation (not observed in WT_400K_CF). All plots are time averages over a 200 ps time window except for the insert in (b). (a) The amount of rotation around the normal axis (Figure 5) and the principal component along the first eigenvector of motion. (b) Number of native hydrogen bonds and salt bridges (see Materials and Methods for the definition of native contacts). (c) Distance between the C_α atoms of A1 Cys⁶⁹⁵ and GpIbα Cys²¹¹, and between the centers of mass (abscise on the right). (d) C_α RMSD of the A1 domain excluding the fluctuating tails and of GpIbα. (e) C_α RMSD of the β switch. The rigid body motion of the β switch was monitored by aligning GpIbα onto the X-ray structure excluding the β switch (green line).

Figure 9.

Time series of conformational changes during four representative forced separation simulations (see Table 1). Replicas of these simulations can be found in Supplementary materials. The proteins were forced to separate from each other by pulling the centers of mass with a constant force of 400 pN. WT_AS_1 and WT_Mut_1 denote the run performed with the wild-type and the mutant, respectively. In the run WT_AS_CF_1, in addition a constant force of 50 pN was applied to the N-terminus of A1 and the C-terminus of GpIb α . The run AddD_AS_1 was done with a designed mutant where a disulfide bond was added between the β switch and the rest of GpIb α . The vertical dashed lines indicate the time point after which all inter-protein hydrogen bonds are broken. All plots are time averages over a 200 ps time window. **(a)** The amount of rotation around the normal axis (Figure 5) and the principal component along the first eigenvector of motion. **(b)** Number of native hydrogen bonds and salt bridges (see Materials and Methods for the definition of native contacts). **(c)** Distance between the C_{α} atoms of A1 Cys⁶⁹⁵ and GpIb α Cys²¹¹, and between the centers of mass (abscise on the right). **(d)** C_{α} RMSD of the A1 domain excluding the fluctuating tails and of GpIb α . **(e)** C_{α} RMSD of the β switch. The rigid body motion of the β switch was monitored by aligning GpIb α onto the X-ray structure excluding the β switch (green line).

Table 1

Simulation systems

Name	Start structure	Temp [K]	Force ^d	Duration [ns]	Unbinding ^e [ns]	Distance ^f N-C [Å]
WT300_1/2 ^a	A1/Gplbα (1SQ0)	300		40+40		61 ± 0.82
Mut300_1/2 ^a	A1-R543Q/Gplbα-M239V (1M10)	300		40+40		59 ± 0.54
Bot300 ^a	A1/Gplbα (1U0N)	300		40		66 ± 0.80
WT_CV_1	10 ns WT300_1 ^b	300	CV N-C max: 1067 pN	33	24.20	
WT_CV_2	20 ns WT300_1 ^b	300	CV N-C max: 945 pN	30	25.31	
WT_CV_3	10 ns WT300_2 ^b	300	CV N-C max: 982 pN	30	27.46	
WT_CV-cter	10 ns WT300_1 ^b	300	CV C-C max: 846 pN	55	50.83	
Mut_CV_1	10 ns Mut300_1 ^b	300	CV N-C max: 730 pN	44	38.17	
Mut_CV_2	20 ns Mut300_1 ^b	300	CV N-C max: 839 pN	40	33.51	
Mut_CV_3	10 ns Mut300_2 ^b	300	CV N-C max: 942 pN	40	38.78	
WT400	A1/Gplbα (1SQ0)	400		140	75.61	7.74
WT400_CF	A1/Gplbα (1SQ0)	400	CF N-C 50 pN	100	> 100	6.53
WT_AS_1	10 ns WT300_1 ^b	300	CM 400 pN	11	10.69	3.14
WT_AS_2	20 ns WT300_1 ^b	300	CM 400 pN	23	22.66	3.66
WT_AS_3	17.43 ns WT300_1 ^b	300	CM 400 pN	26	25.31	2.77
WT_AS_CF_1	10 ns WT300_1 ^b	300	CM 400 pN + CF N-C 50 pN	23	21.17	2.13
WT_AS_CF_2	20 ns WT300_1 ^b	300	CM 400 pN + CF N-C 50 pN	23	22.77	1.68
WT_AS_CF_3	17.43 ns WT300_1 ^b	300	CM 400 pN + CF N-C 50 pN	36	35.58	1.32
Mut_AS_1	10 ns Mut300_1 ^b	300	CM 400 pN	7	7.03	2.11
Mut_AS_2	20 ns Mut300_1 ^b	300	CM 400 pN	6	5.53	1.46
Mut_AS_3	10 ns Mut300_2 ^b	300	CM 400 pN	23	22.44	1.34
AddD_AS_1	Added disulfide 10 ns ^c	300	CM 400 pN	32.5	32.06	2.17
AddD_AS_2	Added disulfide 20 ns ^c	300	CM 400 pN	27	25.60	2.41
AddD_AS_3	Added disulfide 17 ns ^c	300	CM 400 pN	31	29.76	1.35

Name	Start structure	Temp [k]	Force ^d	Duration [ns]	Unbinding ^e [ns]	Distance ^f N-C [Å]
AddD_CV	Added disulfide 17 ns ^c	300	CV N-C max: 1022 pN	28	26.64	

^a300 K simulations (the PDB codes of the starting structures are given in parenthesis). The total simulation time includes the first 10 ns which were not used for the analysis.

^bRuns started from snapshots sampled during the 300-K simulations.

^cAn additional disulfide bond was introduced through the mutations W230C and F201C.

^dCV stands for constant velocity (rupture force indicated by the keyword “max:”), CF for constant force and CM for pulling the centers of mass with a constant force of 400 pN. “N-C” indicates that the N-terminus of A1 was pulled away from the C-terminus of Gplba.

^eSimulation time after which the proteins unbind, defined as the first time point in the trajectory after which all inter-protein hydrogen bonds are broken.

^fDistance between Cys⁶⁹⁵ near the N-terminus of A1 and Cys²¹¹ near the C-terminus of Gplba.

For the 300-K runs an average is reported together with the standard deviation; in the high temperature and accelerated separation simulations the reported value is the difference between the average measured along the room temperature runs and the minimum value measured along the unbinding trajectory.

Table 2

Number of persistent inter-protein and intra- β switch contacts

	WT X-ray ^a	WT300 ^b	Mut X-ray ^c	Mut300 ^d	Bot X-ray ^e	Bot300 ^f
Inter-protein hbonds ^g	9	7(7)/7(7)	9	8(6)/7(6)	11	7(6)
Intra β switch hbonds ^g	6	6(5)/5(4)	7	4(4)/7(7)	7	3(3)
Inter-protein salt bridges	3	8(2)/5(1)	1	4(1)/3(1)	2	3(2)

The numbers in parentheses indicate the number of native contacts which are also observed in the X-ray structure (a more detailed overview is given in Supplementary Table S1.)

^aX-ray structure of wild-type (PDB code 1SQ0).

^b300 K runs with wild-type WT300_1/WT300_2.

^cX-ray structure of mutant A1-R543Q/Gplb α -M239V (PDB code 1M10).

^d300 K runs with mutant Mut300_1 and Mut300_2.

^eX-ray structure of wild-type in the presence of botrocetin (PDB code 1U0N).

^f300 K run started with wild-type crystallized in the presence of botrocetin.

^ghbonds stands for hydrogen bonds.

Table 3

Comparison between eigenvectors describing the rotation around the normal axis

Simulation	Scalar product	Simulation	Scalar product
WT_CV_1	1.00(1stEV)	WT_AS_1	0.73(1stEV)
WT_CV_2	0.96(1stEV)	WT_AS_2	0.79(1stEV)
WT_CV_3	0.96(1stEV)	WT_AS_3	0.76(2ndEV)
WT_400	0.77 (1st EV)	WT_AS_CF1	0.49(1stEV)
WT_400_CF	0.56 (3rd EV)	WT_AS_CF2	0.80(1stEV)
		WT_AS_CF3	0.77(1stEV)

The rotation around the normal axis during separation of the proteins was observed in every unbinding simulation to correlate with the projection along one of the eigenvectors of motion. In order to evaluate how similar the eigenvectors are, the scalar product was calculated between the eigenvector calculated from a simulation and the first eigenvector of WT_CV_1. The more similar two eigenvectors are, the closer their scalar product is to 1. The eigenvector which best describes the rotation (i.e., the time series of the projection along the trajectory correlates with the rotation angle) is indicated in parenthesis.