Von Willebrand factor A1 domain affinity for GPIb α and stability are differentially regulated by its O-glycosylated N-linker and C-linker

Running title: Biophysical regulation of VWF by O-glycosylated linkers

Klaus Bonazza¹, Roxana E. Iacob², Nathan E. Hudson³, Jing Li¹, Chafen Lu¹, John R. Engen² and Timothy A. Springer^{1*}

¹Children's Hospital Boston and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA.

²Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115, USA

³Department of Physics, East Carolina University, Greenville, NC 27858

*Correspondence:

Timothy A Springer
Center for Life Science - 03103
3 Blackfan Circle, Boston MA 02115
springer@crystal.harvard.edu
P 617.919.6935 | F 617.730.4731

Keywords: von Willebrand factor, mucin, glycoprotein, intrinsically disordered protein, protein stability, conformational change

Impact Statement. Both the polypeptide and attached O-glycans N-terminal to the A1 domain in von Willebrand factor lower its affinity for its ligand GPIb α on platelets, its stability, and structural dynamics and decrease population of a high-affinity, intermediate state in unfolding.

Key points:

- Ligand-binding affinity of A1 and the stability of its native state is regulated by an N-terminal inter-domain, O-glycosylated linker

Abstract. (241 /250)

Hemostasis in the arterial circulation is mediated by binding of the A1 domain of the ultralong protein von Willebrand factor to GPIb α on platelets to form a platelet plug. A1 is activated by tensile force on VWF concatemers imparted by hydrodynamic drag force. The A1 core is protected from force-induced unfolding by a long-range disulfide that links cysteines near its N and C-termini. The O-glycosylated linkers between A1 and its neighboring domains, which transmit tensile force to A1, are reported to regulate A1 activation for binding to GPIb, but the mechanism is controversial and incompletely defined. Here, we study how these linkers, and their polypeptide and O-glycan moieties, regulate A1 affinity by measuring affinity, kinetics, thermodynamics, hydrogen deuterium exchange (HDX), and unfolding by temperature and urea. The N-linker lowers A1 affinity 40-fold with a stronger contribution from its O-glycan than polypeptide moiety. The N-linker also decreases HDX in specific regions of A1 and increases thermal stability and the energy gap between its native state and an intermediate state, which is observed in urea-induced unfolding. The C-linker also decreases affinity of A1 for GPIb α , but in contrast to the N-linker, has no significant effect on HDX or A1 stability. Among different models for A1 activation, our data are consistent with the model that the intermediate state has high affinity for GPIb α , which is induced by tensile force physiologically and regulated allosterically by the N-linker.

INTRODUCTION

The ultra-long length of the blood plasma protein von Willebrand factor (VWF) enables its activation by hydrodynamic forces at sites of hemorrhage (Springer, 2014) (Fig. 1A and B). VWF, a long concatemer of monomers linked head-to-head and tail-to-tail, undergoes a change of its multimeric superstructure from a compact, random coil form (Parker & Lollar, 2021) to a thread-like, extended form when exposed to elongational flow (Bonazza et al., 2015; Fu et al., 2017). Hydrodynamic flow activates VWF to bind to Glycoprotein Ib (GPIb) on platelets, the essential interaction required for hemostasis and thrombosis in the arteriolar circulation. Fluorescent imaging of single VWF concatemers tethered in a flow cell showed that binding of GPIb α not only requires elongation of VWF but also a half-maximal tensile force of ~ 20 pN transmitted through the backbone of VWF concatemers (Fu et al., 2017). Activation could be well fit to a model of transition from a low-affinity state 1 to a high affinity state 2 (Figure 1B). Measuring A1-GPIb α binding and unbinding with laser tweezers also revealed two states, with a force dependent switch between two states with different on and off-rates (Kim et al., 2015; Kim et al., 2010). In addition to such flex-bond measurements, A1 and GPIb α are also reported to form catch-bonds, although these measurements lack single molecule fiduciary markers or fits to models from which on and off-rates could be extracted (Ju

Figure 1. VWF, hydrodynamic flow, A1 activation, and A1 linkers. A) N and C-terminal linkage and domain organization of a VWF monomer in a VWF concatemer. Monomers are connected head-to-head and tail-to-tail. B) When exposed to elongational flow and tethered (black triangle) on a vessel wall, VWF concatemers extend to a linear shape. At higher elongational flow, which exerts higher mechanical tension along the length of the concatemer (highest at the tether point and zero at the downstream end) A1 undergoes transition from a low to a high affinity state as a consequence of tensile forces transmitted to it through its linkers. The disulfide bond between Cys1272 and Cys1458 is indicated with red S-S. C) A1 protein constructs studied here that differ in length of linkers and were expressed in E coli (not glycosylated) or in mammalian cells (O-glycosylated) (lollipop). The long-range disulfide bond is schematized in red. D) A1 constructs were subjected to SDS-PAGE and stained with Coomassie Blue.

et al., 2013). Despite multiple crystal structures of the A1 domain bound to GPIIb, distinct conformational states of A1 that explain force-dependent regulation of A1 affinity for GPIIb have yet to emerge (Blenner et al., 2014; Dumas et al., 2004).

The force-responsive A1 domain is unusual among protein domains in being linked to neighboring domains through O-glycosylated, mucin-like segments (Fig. 1A, blue hashed lines). Tensile force exerted on A1 is transmitted through these linkers. It has been suggested that A1-flanking sequences (Auton et al., 2012; Interlandi et al., 2017; Ju et al., 2013; Miyata & Ruggeri, 1999; Sugimoto et al., 1993) or domains (Aponte-Santamaria et al., 2015; Ulrichs et al., 2006) may shield the GPIIb binding epitope and thereby inhibit otherwise tight binding to GPIIb. It was also proposed that the N- and C-linkers interact with each other and form an auto-inhibitory module that masks A1 (Deng et al., 2017). A force-dependent signature for breakage of this module has been reported that correlates with the combined expected extension of the N and C-linkers (Arce et al., 2021). An alternative mechanism has also been proposed by which the N-linker could regulate A1 affinity, i.e., by regulating the relative stability of the A1 native and intermediate states. A1 has been found to have three states, native (N), intermediate (I), and denatured (D), while the homologous A2 and A3 domains in VWF have only two states, native and denatured (Auton et al., 2007). Lower stability of the A1 native state correlates with higher affinity (Auton et al., 2012; Auton, Sowa, et al., 2010). This anti-correlation between A1 affinity and stability is also seen with von Willebrand disease type 2B gain-of-function mutations, which increase A1 affinity and decrease stability (Auton, Zhu, et al., 2010; Tischer et al., 2017). Conversely, the G1342S decrease-in-function type 2M von Willebrand disease mutation decreases A1 affinity for GPIIb and increases A1 stability (Auton et al., 2009).

Here, to understand how binding of the VWF A1 domain to platelet GPIIb is regulated by its N- and C-terminal mucin-like linkers, we have made fundamental measurements of the effects of both the glycan and polypeptide moieties of these linkers on stability, thermodynamics, and ligand-binding affinity and kinetics of A1. Mucin-like regions in proteins have unique characteristics distinct from both intrinsically disordered polypeptide segments and folded domains. The bulky O-linked glycans which include sialic acid that are attached to threonine and serine residues in mucins are highly solvated, repel one another, and make mucins extended, with an average length of ~ 2 Å/residue (Clemetson, 1983; Woollett et al., 1985). Electron microscopy showed that D3 and A1 in

VWF, which are connected by the N-linker, have many possible orientations, and an interdomain distance as long as ~7 nm (Zhou et al., 2011). A1 is mechanically stabilized by a long-range disulfide bond between Cys1272 and Cys1458 (Figure 1B,C). Under tension, the core of A1 between these two cysteines, residues 1273-1457, is protected from complete unfolding. Only the portions of A1 external to this disulfide bond, residues 1238-1272 and 1458-1489, are directly subjected to the mechanical force transmitted through the spine of the VWF concatemer. Some folded portions of A1, including the C-terminal portion of the α 6-helix, are external to the disulfide bond yet are in hydrogen bond communication with the folded portion internal to the disulfide. Here, we focus on the portions of the N and C-linkers that lack well-defined structures and include the multiple serine and threonine residues that were found to be O-glycosylated during protein sequencing of native plasma VWF, including Thr-1248, Thr-1255, Thr-1256, and Ser-1263 in the N-linker (Titani et al., 1986). Mass spectrometry verified these assignments and showed a total of 8 sialic acids over the four sites (Solecka et al., 2016). We show that both the N and C-linkers affect affinity, that both their polypeptide and O-glycan moieties are important, and that the N-linker affects stability and HDX of A1 including in specific regions internal to its disulfide.

RESULTS

Proteins. We compared the effect of the linkers N-terminal and C-terminal to A1, which separate A1 from D3 and A2, respectively, and which contain O-glycosylation sites that have been chemically identified in native VWF (Solecka et al., 2016; Titani et al., 1986) (Fig. 1). Because the linkers are O-glycosylated in mammalian Expi293 cells and not in *E. coli*, we expressed A1 protein constructs in both cell types in order to test the effect in some assays of both polypeptide and O-glycan components of the linkers (Fig. 1C). Slower migration in SDS-PAGE of the mammalian than corresponding bacterial A1 constructs confirmed O-glycosylation (Figure 1D). Furthermore, faster migration of all seven A1 constructs in non-reducing than reducing SDS-PAGE confirmed that all contained the long-range disulfide bond.

A1-GPIIb α binding kinetics and thermodynamics. Using bio-layer interferometry (BLI), we measured A1 association to and dissociation from GPIIb α immobilized through a biotinylated C-terminal avitag to BLI sensor tips. A1 preparations were subjected to S200 gel filtration the day of each affinity measurement to remove

117 aggregates. Global fits at all analyte concentrations to a single on and off-rate for each A1 construct were good
118 (Fig. 2). In contrast, when gel filtration was omitted, data could not be fit to a single on and off-rate. Differences
119 among all seven A1 constructs in 150 mM NaCl showed that the linkers were of great importance in regulating

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Figure 2. Effect of A1 linkers, their glycosylation, and ionic strength on GPIIb/IIIa binding kinetics. A) BLI traces in grey are shown for the indicated concentrations of the indicated A1 construct at the indicated NaCl concentration. In each panel, all A1 concentrations were fit globally to a one-site binding model to obtain a single k_{on} and k_{off} . Thin black lines show fits. Vertical dashed lines mark the beginning of the dissociation phase. B) Kinetic constants and derived K_D . Errors show difference from mean of $n=2$ independent experiments.

A1 affinity for GPIb α (Fig. 2). Comparisons to A1 short showed that non-glycosylated linkers in A1+N+C lowered affinity by 10-fold while the presence of glycosylated linkers in A1+OGly+N+C lowered affinity by 50-fold. The N-linker in A1+N lowered affinity by 4.3-fold while the C-linker in A1+C lowered affinity by 2.5-fold. Glycosylation of the N-linker was also more important than the C-linker; the affinity of A1+OGly+N was 10-fold lower than A1+N while the affinity of A1+OGly+C was only 2-fold lower than A1+C. O-glycosylated linkers consistently lowered on-rates (Fig. 2B). Overall, the results showed that both the polypeptide linker moiety and the O-glycan moiety of A1 linkers contributed to lowering A1 affinity for GPIb α , that the N-linker was more important than the C-linker in lowering affinity, and that the combined effect was very large at 50-fold.

To test electrostatic contribution to binding affinity and on-rates, binding of A1 short and the three glycosylated constructs was additionally measured in 30 mM and 10 mM NaCl (Fig. 2). Lowering NaCl concentration from 150 mM to 10 mM increased on-rates by an average of 17-fold for the four constructs. The effect of NaCl was predominantly on on-rate, since on-rate and off-rate together resulted in only a somewhat greater average increase in affinity of 23-fold (Fig. 2B). Our results demonstrate that electrostatic complementarity between the binding interfaces in the basic A1 (pI 9.2) and acidic GPIb α (pI 5.5) domains (Huizinga et al., 2002) gives rise to electrostatic steering that speeds association kinetics.

To independently test affinity, and obtain binding thermodynamics, we used isothermal titration calorimetry (ITC). Although the GPIb α protein used in ITC lacked the avitag and its biotinylation, K_D measurements in BLI and ITC were on average within 1.3-fold of one another and showed the same trends (Fig. 3). A1 short showed a 39-fold increase in affinity compared to A1+OGly+ N+C, the N-linker was more important than the C-linker, and the O-glycan moiety was more important than the polypeptide moiety in regulating affinity. Binding of all A1 protein constructs to GPIb α releases heats that showed the reaction was endothermic, i.e., entropically driven (Fig. 3B). The thermodynamics of A1- GPIb α binding showed entropy-enthalpy compensation: the higher the entropic contribution to binding, the higher the enthalpic cost, with all seven constructs following the same rank order for the entropic and enthalpic terms. Thus, the linkers affected both the entropic and enthalpic components of GPIb α binding.

Figure 3. Effect of A1 linkers and their glycosylation on GPIIb binding thermodynamics. A) Panels show ITC traces of heat absorption after each injection (above) and fit of enthalpy to the binding isotherm (below) in 150mM NaCl at 22 °C. B) Table summarizing the reaction Gibbs free energies (ΔG) and the enthalpic (ΔH) and entropic ($T\Delta S$) contributions. Errors show difference from mean of $n=2$ independent experiments, except for for A1+N+C which shows the fitting error for one experiment.

B	K_D nM	ΔG kcal mol ⁻¹	ΔH kcal mol ⁻¹	$-T\Delta S$ kcal mol ⁻¹
A1short	290±3	-8.8±0.0	5.9±0.6	-14.7±0.6
A1+C	730±50	-8.3±0.0	5.8±0.9	-14.1±0.9
A1+OGly+C	1150±50	-8.0±0.1	4.9±0.3	-13.0±0.3
A1+N	750±60	-8.3±0.5	5.1±1.6	-13.4±1.6
A1+OGly+N	7900±600	-6.9±0.1	3.2±0.2	-10.1±1.5
A1+N+C	1400±200	-7.9±0.1	3.7±0.2	-11.6±0.3
A1+OGly+N+C	11300±2200	-6.7±0.1	1.5±0.3	-8.2±0.1

A1 stability and the thermodynamics of the intermediate state. The relative stabilities of A1 short and the three O-glycosylated A1 constructs to denaturation by heat or urea were measured by tryptophan fluorescence, whose emission maximum shifts to higher wavelength upon exposure to solvent during unfolding. The fluorescence intensity ratio (FIR), I_{350}/I_{330} , showed that constructs had three states, i.e., exhibited two unfolding transitions (Fig. 4A, B, and D). Constructs containing the N-linker, i.e. A1+OGly+N+C and A1+OGly+N, were more stable to heat (T_m of 53.2 and 53.3°C for the first transition, respectively) than constructs lacking the N-linker, i.e. A1+OGly+C and A1 short (T_m of 49.9 and 50.7°C for the first transition, respectively) (Fig. 4A and

154 B). These results suggest that the N-linker stabilizes the native state of A1 relative to the intermediate state.
 155 Furthermore, all 4 constructs showed a second inflection point between 60 and 65° (second peak or shoulder of
 156 the derivative curve, Fig. 4B). These results suggested two-step unfolding behavior and thus that A1 has three
 157 states, native (N), intermediate (I), and D (denatured) as previously observed (Auton et al., 2007).

Figure 4. Stability of A1 constructs to heat and urea and free energy difference between the native and intermediate A1 states. A-C) Effect of heating from 30° to 85 °C on A1 stability and aggregation. A) Unfolding was measured by excitation of tryptophan at 275 nm and measuring the ratio of the fluorescence intensity at 350 and 330 nm ($FIR = I_{350}/I_{330}$). B) The derivative of data in A ($dFIR/dT$) shows two unfolding transitions for each A1 construct and provides evidence for three states of A1: N, I, and U as described in Results. C) Backscattered light intensity as a measure of A1 aggregation. D) Unfolding of A1 by urea measured at 20 °C by FIR as in panel A. Lines show fits to the three-state model of A1 unfolding as described in Methods and Results (see also: Source Data). E) Tabulation of the melting temperature of the native state (T_m) from panels A and B and the free energy difference between the native and intermediate states of A1 (ΔG_{I-N}), the concentration of urea at which the I and N states are equally populated ($C_{urea\ I-N}$), and m , from the fits in panel D.

A1 aggregation by heat was measured by backscattered light intensity. After the inflection point for the intermediate state, A1 short began to aggregate (Fig. 4C compared to 4B). A1+OGlyc+C required higher temperature for aggregation, which began to occur after the inflection point for the denatured state (Fig. 4C compared to 4B). Strikingly, the O-glycosylated N-linker completely protected A1+OGly+N and A1+OGly+N+C from aggregation at temperatures up to 85°C.

Denaturation in urea confirmed the existence of an intermediate state and was used to estimate the free energy of the intermediate state relative to the native state, i.e., ΔG_{I-N} . The A1 constructs showed two separate unfolding transitions, one between 1 to 4 M Urea, and another above 5 M urea (Fig. 4D). All constructs showed a plateau in between, or in the case of A1+OGly+C, followed a similar trend, but with a decrease in slope instead of a clear plateau. Similar 3-state unfolding curves were previously seen for a construct similar to A1+N+C using circular dichroism (Auton et al., 2007). We fit the unfolding data to a three-state model. We assumed that all four constructs had the same m value (a parameter that defines the cooperativity of transition) and that each state had a unique baseline FIR slope as a function of urea concentration that was shared among the four constructs (Methods). We thus determined the difference in energy between the I and N states (ΔG_{I-N}), the concentration of urea at which the I and N states are equally populated ($C_{urea\ I-N}$), and m , a value that is related to the size of folded domains (Ghosh & Dill, 2009; Santoro & Bolen, 1988) (Fig. 4E). The results showed that the O-glycosylated N-linker markedly increased the stability of the native state and thus the gap in energy between the native and intermediate state, with higher ΔG_{I-N} values and transitions at higher urea concentrations for A1+OGly+N and A1+OGly+N+C than for A1 short and A1+OGly+C (Fig. 4E).

Changes in HDX dynamics among A1 constructs correlate with affinity and stability differences.

HDX-MS measures solvent accessibility and H-bonding and reports on the dynamics and stability of proteins (Wales & Engen, 2006). The O-glycosylated A1 constructs and A1 short were allowed to exchange their backbone amide hydrogens for deuterium in D₂O for varying periods of time, digested with pepsin, and the kinetics of deuterium exchange were measured for 108 A1 peptides (91% coverage, 5.8x redundancy, Fig. 5 and Supplemental Figs. 1-5). HDX as a function of secondary structure and sequence position trended similarly for all four constructs studied (shown for A1 short in Fig. 5A and for all in Supplementary Figs. 1-2). Fig. 5A shows

exchange for all 108 peptides at all time points, from 10 s to 4 h. Over the A1 sequence from N to C-terminal, the least exchange was seen for the β 1-strand, the α 1-helix, the β 2 and β 3-strands, the α 3-helix, and the α 6-helix, which all had at least one peptide with less than 20% deuteration at 4 h. These slowly exchanging secondary structural elements neighbor one another and the GPIIb α binding site on A1 (Fig. 5D).

Figure 5. The N-linker decreases A1 dynamics measured by HDX. A) Relative deuterium exchange at all timepoints for A1 short as % of the available amide backbone H atoms in each peptide, colored according to the key. Figure Supplements 1 and 2 show data for all constructs. B) Difference (Δ) in HDX at all time-points of A1 short minus HDX for the other three constructs as indicated. In A) and B), residues with VWD type 2B mutations are shown below sequence numbers as red circles. C) Full HDX kinetics for selected peptides. Figure Supplements 3, 4, and 5 show data for all peptides. D) Structure of A1 bound to GPIIb α (PDB 1SQ0). A1 is colored rainbow from N (blue) to C-terminus (red). Residues with VWD type 2B mutations are shown as C α atom spheres. Labeled residues are shown in stick. GPIIb α is shown in silver, from the β -finger to the β -switch.

Consistent differences in the amount of HDX were found in specific regions among the four A1 constructs (Fig. 5B-C and Supplemental Figs. 3-5). The A1+OGly+N+C and A1+OGly+N constructs showed significantly less deuterium exchange (> 0.7 Da) at almost all timepoints in peptides that were centered on the $\alpha 1$ - $\beta 2$ loop and the $\beta 3$ - $\alpha 2$ loop. These loops locate near to GPIIb α (Fig. 5B-D). We also observed reduced exchange in the $\alpha 2$ - $\alpha 3$ loop, but the most meaningful difference was only seen at the 4 h timepoint. The $\alpha 1$ - $\beta 2$ loop is nearby the long-range disulfide and in a region with multiple gain of function VWD type 2B mutations (Fig. 5D). The $\beta 3$ - $\alpha 2$ loop is near to GPIIb α and has four basic residues but they are not close enough for optimal interaction with acidic residues in the GPIIb α leucine-rich repeats.

The HDX data showed no significant HDX differences between A1 short and A1+OGly+C. Further, the magnitude of HDX differences between A1 short and both A1+OGly+N+C and A1+OGly+N was very similar. However, while A1+OGly+N+C and A1+OGly+N were similar to one another in HDX, they both exchanged less deuterium than A1 short and A1+OGly+C, indicating that both A1 short and A1+OGly+C were more dynamic than A1+OGly+N+C and A1+OGly+N. These HDX findings resembled the measurements of the stability of the native states relative to the intermediate states (Figure 4), which showed that A1+OGly+N+C and A1+OGly+N were similarly stable and more stable than A1 short and A1+OGly+C, which were also similar in stability.

DISCUSSION

Among domains in proteins, the A1 domain of VWF is highly unusual in being separated from neighboring domains by mucin-like linkers and being activated by tensile force transmitted through those linkers that originates from hydrodynamic force on VWF concatemers and bound platelets. Here, we have investigated the effects of these linkers on A1 thermodynamics and function in binding to its ligand, GPIIb α . Previous studies have examined the effect of the N-terminal linker or both linkers on various types of quantitative or non-quantitative binding assays and thermodynamics and found inhibitory effects on GPIIb α binding from adding the N-linker or both linkers to A1 (Arce et al., 2021; Auton et al., 2012; Deng et al., 2018; Deng et al., 2017; Interlandi et al., 2017; Ju et al., 2013; Miyata & Ruggeri, 1999; Nakayama et al., 2002; Tischer et al., 2017; Tischer et al., 2014). We found that both the N and C-linker decreased A1 affinity for GPIIb α and that for both the

N and C-linker, O-glycosylated linkers decreased affinity more than polypeptide linkers. For the C-linker, both the O-glycan and polypeptide moieties were similar in importance, giving ~2.2-fold decreases in affinity each, while for the N-linker, the O-glycan component was more influential (9.5-fold) than the polypeptide alone (4.3-fold). While adding O-glycosylated linkers on the N and C-terminal ends of A1 lowered affinity substantially (51-fold and 6-fold, respectively), the presence of both O-glycosylated linkers lowered binding little compared to the presence of the N-linker alone (1.2-fold). The separate contributions of the polypeptide and glycan moieties of the A1 N and C-linkers had not previously been examined; however, O-glycosylation of both linkers together was reported to decrease affinity from 3.9 μ M to 32 μ M (Tischer et al., 2020). While these reported affinities differ from ours by 2 to 3-fold, they agree in terms of the affinity difference caused by O-glycosylation. Kinetic measurements showed that the effect of both the polypeptide and O-glycan components of the linkers was primarily on-rate driven. Overall, among the seven constructs, on-rates varied by up to 80-fold while off-rates varied up to 10-fold.

Rapid binding is especially important for proteins that function in hemostasis; electrostatics can help orient proteins for proper binding before they contact one another, as previously demonstrated using the ionic strength dependence of binding of soluble GPIIb α to VWF tethered to the wall of a flow chamber (Fu et al., 2017). Our kinetic measurements in 10 mM compared to 150 mM NaCl showed a 20-fold increase in affinity for A1+OGly+N+C largely driven by a 7-fold increase in k_{on} , while VWF tethered to a flow chamber showed a 130-fold increase in affinity driven by an 11-fold increase in k_{on} .

In 150 mM NaCl, the high affinity state of tethered, mechanically tensioned VWF had an affinity of 80 nM for GPIIb α , compared to the K_d values of 180 nM for A1 short and 9,200 nM for A1+OGly+N+C measured here. Introduction of two VWD mutations into A1 was shown to increase affinity for GPIIb α by 10-fold (Blenner et al., 2014). However, even though such a mutant would be expected to increase the affinity of the A1 short construct to a higher level than found for mechanically-tensioned A1 in intact VWF, a crystal structure of A1 short with two VWD mutations bound to GPIIb α revealed only minor differences from a WT-GPIIb α crystal structure (Blenner et al., 2014). Mechanical tension on one hand and introduction of VWD mutations in the absence of tension and removal of the N and C-linkers on the other hand may have different effects on the

structure of the A1 domain, consistent with the hypothesis that A1 short with two VWD mutations bound to GPIb α did not reveal an A1 high affinity state, despite an affinity for GPIb α comparable to that seen with mechanically-tensioned VWF (Blenner et al., 2014).

We buttressed BLI affinity measurements here with ITC measurements of affinity and binding energies. While affinity measurements in the literature on essentially identical A1 fragments can differ up to 15-fold from those reported here (Huizinga et al., 2002), affinity measurements here on seven different A1 constructs by BLI and ITC were all within 1.6 to 1.04-fold of one another. We found it was necessary to remove aggregates from A1 preparations by gel filtration immediately prior to BLI measurements to avoid heterogeneous kinetics that required two on and off-rates to fit, which appear in some of the literature on A1 binding to GPIb α (Arce et al., 2021; Tischer et al., 2017). In general, measurements in the liquid phase are more accurate than those in solid/liquid phases such as SPR and BLI, where a small amount of an avid aggregate can dominate binding kinetics.

We are unaware of previous thermodynamic measurements of A1 and GPIb α binding. ITC results show that the interaction of GPIb α with all seven A1 constructs was endothermic with a large entropic term that ranged from -8.2 to -14.7 kcal/mol at 22°C. Among all seven A1 constructs, the enthalpy and entropy terms were highly correlated, so that their rank orders were identical. Entropy-driven binding of proteins is usually attributed to the increase in water entropy when waters are released from hydrophobic binding interfaces (Richards, 1977).

Our HDX studies revealed that the O-glycosylated N-linker has a significant effect in stabilizing the α 1- β 2, β 3- α 2, and the α 2- α 3 loops, as shown by less deuterium incorporation. Previous HDX studies have shown that destabilizing A1 by reducing and alkylating its long-range disulfide, introducing VWD Type 2B mutations, or omitting N-linker residues 1238-1260 increase HDX in the same regions (Deng et al., 2018; Deng et al., 2017; Tischer et al., 2017; Tischer et al., 2014). The finding that stands out from previous reports, which did not study the C-linker individually in HDX, is that in contrast to the N-linker, the C-linker did not significantly affect HDX in any of these regions.

We directly measured the stability of A1 by measuring its unfolding by heat and urea. We found evidence for three states by both techniques, consistent with a previous finding of an intermediate state that is hypothesized

to represent a state with high affinity for GPIIb α (Auton, Sowa, et al., 2010; Tischer et al., 2017; Tischer et al., 2014). A plateau between the first and second transition clearly demonstrated the existence of the intermediate state for three of our four constructs, in contrast to the decrease in slope seen previously. This difference was likely due to measurement here of the fluorescence of the single tryptophan residue, Trp-1313 in A1, as opposed to circular dichroism, which measures contributions from all A1 secondary structures. Trp-1313 is in the α 1- β 2 loop that has HDX dynamics that are sensitive to the presence of the N-linker, locates near the GPIIb α β -finger, is mutated in VWD type 2B, and appears to be a more discrete reporter of A1 conformational state than circular dichroism. Heat and urea denaturation had similar linker-dependent effects on stability of A1. A1-Ogly+N and A1-Ogly+N+C were each $\sim 3^\circ\text{C}$ more stable than A1short and A1-Ogly+C. Likewise, A1-Ogly+N and A1-Ogly+N+C each had native (N) states that were relatively more stable than their intermediate (I) states, by ~ 2 kcal/mol, than found for A1 short and A1+Ogly+C. Additionally, the N-linker was sufficient to completely protect A1 against heat-induced aggregation up to 85°C . Since physiological tensile force on A1 may induce the I state, which could make A1 and with it an entire VWF concatemer susceptible to aggregation, one of the several important physiological roles of the N-linker and its O-glycosylation may be to protect it from aggregation. The neighboring A2 domain in VWF can be completely and reversibly unfolded by tensile force and can also be reversibly heated to 90°C without aggregation in the presence of its O-linked N-linker (the C-linker of A1) and its C-linker (Xu & Springer, 2013; Zhang et al., 2009), which may have a similar protective role physiologically to prevent aggregation during physiological partial (A1) and full (A2) unfolding.

Our results on the effects of the O-linked N and C-linkers on affinity and stability differ. The C-linker decreased A1 affinity for GPIIb α in two different measures, BLI and ITC, but had no effect on A1 stability as measured by HDX, melting temperature, and the relative stability of the A1 N and I states in urea denaturation experiments. These results suggest that the C-linker decreases affinity by direct mechanisms such as by steric or electrostatic repulsion. O-glycans are decorated with sialic acid, which would repel negatively charged GPIIb α . In contrast to the C-linker, the N-linker not only decreased affinity but also increased A1 stability as shown by significantly decreased HDX, increased melting temperature, and increased stability of the N state relative to the I state. The ~ 2 kcal/mol difference in ΔG between the N and I state for A1+Ogly+N compared to A1 short

corresponds to a ~30-fold difference in population of the I state. Thus, if the I state was the high affinity state, much of the 40-fold difference in affinity between the I and N states could be accounted for by the shift in equilibrium toward the native state caused by the N-linker, and the remainder could be caused by steric and electrostatic repulsion of GPIb α . In VWF concatemers, the ΔG required for activation by mechanical tension of the high affinity state was measured as 1.9 kcal/mol (Fu et al., 2017), considerably lower than the difference in energy between the I and N states measured for A1+Ogly+N+C here of 4.7 kcal/mol. These differences suggest that the directional nature of energy input by mechanical tension allows for more efficient activation of A1 than urea denaturation and may induce a distinct and less disordered intermediate state.

Three different mechanisms have been proposed for how the linkers affect A1 binding to GPIb α . The proposal that the N-linker and C-linker associate with one another to form an autoinhibitory module (Deng et al., 2018; Deng et al., 2017) is not supported by our data that show that the N-linker alone is sufficient to inhibit binding of A1 to GPIb α . Furthermore, we find that the N-linker and C-linker are not synergistic and to the contrary, have much less than additive effects on lowering binding affinity. It also has been proposed that residue Asp-1261 in the N-linker interacts with basic residues in the A1 domain to stabilize a low affinity state of A1 (Interlandi et al., 2017). While our results do not bear on this mechanism, they do demonstrate that the O-linked glycans within the N-linker are sufficient to strongly inhibit binding of A1 to GPIb α . Finally, Auton and colleagues have described an intermediate state of A1 that is proposed to correspond to an activated high affinity state of A1 (Auton et al., 2007; Auton et al., 2012; Tischer et al., 2014). Our data are consistent with and provide further evidence in support of this hypothesis.

The association of the N-linker but not the C-linker with A1 stability is consistent with structural analysis of A1 complexes with GPIb α that demonstrate that their association strains the N-terminal portion of A1 (reviewed in (Blenner et al., 2014)). The main site of GPIb α association is at the β -switch in its C-terminal cap. The flexible β -finger in the N-terminal cap of GPIb α associates with A1 over a smaller region, including the α 1- β 2 loop, with few specific contacts that vary among GPIb α -complexes (Fig 5D). Strain in A1 upon binding GPIb α causes shifts in some residues in the α 1- β 2 loop and Cys-1272 away from GPIb α . Furthermore, the hydrogen bond between the backbone of Cys-1272 and the backbone of Arg-1308 (a residue often mutated in VWD), which

is conserved in all structures of the isolated A1 domain, is lost in all A1-GPIb α complex structures. This loss admits a water molecule to the hydrophobic core of A1. These structural observations begin to define a pathway for conformational communication between the N-linker and regions of A1 that change upon GPIb α -binding, including the α 1- β 2 loop, which showed increased HDX dynamics in the absence of the N-linker. Crystal structures of A1 diverge prior to residue 1273, suggesting that the O-glycosylated N-linker of A1 (defined in our experiments as residues 1238-1263) is natively disordered. It is now well established that natively disordered regions in proteins frequently have important roles in regulating protein allostery (Motlagh et al., 2014). We speculate that in the intermediate state, A1 would reshape to enable closer approach to the concave, acidic surface of GPIb α of more A1 basic residues, such as those in its β 3- α 2 loop shown in stick in Fig. 5D.

In summary, our results show that the A1 N-linker, but not the C-linker, increases the stability of the A1 native state relative to the intermediate state, thus regulating conformational change. The previous paragraph discussed structural evidence for a pathway for communication from the N-linker to regions that are known to change structurally upon GPIb α binding and also have backbone motions that are influenced by the N-linker as shown by HDX. Additionally, our results suggest that the C-linker influences affinity for GPIb α by steric and possibly charge repulsion. The N-linker may repel GPIb α as well, in addition to regulating A1 conformation.

MATERIALS AND METHODS

Glycosylated proteins. Human VWF A1 domain constructs as shown in Figure 1C (pre-pro-VWF amino acid residue numbering), beginning with residue 1238 or 1264 and ending with residue 1469 or 1489 and followed with a C-terminal 6xHis tag were cloned into a bicistronic IRES-GFP expression vector, ET8 (Zhou & Springer, 2014) and transfected into Expi293F cells using lipofectamine reagent (Invitrogen). Stable transfectants were selected in the presence of 500 μ g/mL G418 (Geneticin) and subjected to 2 rounds of fluorescence-activated cell sorting. In each sort, the 8% most fluorescent cells were expanded in Expi293 expression medium. Culture supernatants were harvested after 5 days, supplemented with 20 mM HEPES pH 7.4 and 0.2 mM NiCl (final concentration), and loaded onto a Ni-NTA agarose column by gravity (2 mL Ni-NTA agarose beads per 500 mL of culture supernatant). The column was pre-equilibrated in Washing Buffer (20 mM HEPES pH 7.4, 150 mM

NaCl, 0.2 mM NiCl). After loading the supernatant, the column was washed with 5 column volumes of Washing Buffer followed by 10 column volumes of 20 mM HEPES, 1M NaCl, 16 mM imidazole, 0.2 mM NiCl. Protein was eluted with 20 mM HEPES, 150 mM NaCl and 300 mM imidazole, and further purified by Superdex 200 (GE Healthcare) size-exclusion chromatography in 20 mM HEPES, 150 mM NaCl, pH 7.5.

Wild-type GPIIb α , residues 1-290 of the mature protein with a C-terminal His₆ tag, with or without an intervening Avi-tag sequence (GLNDIFEAQKIEWHE), was purified from culture supernatant of 293 stable transfectants (Blenner et al., 2014) by Ni-NTA affinity chromatography and Superdex 200 size-exclusion as above.

Non-glycosylated proteins. The cDNA sequence encoding A1 beginning with residue 1238 or 1264 and ending with residue 1469 or 1486 (Figure 1C) was cloned into the pET32a vector with a thioredoxin fusion protein (LaVallie et al., 1993) and a TEV cleavage site at its N-terminus and a 6xHis tag at its C-terminus. Proteins were expressed in SHuffle cells (Lobstein et al., 2012), which were grown at 37 °C in LB medium, induced at an A₆₀₀ of 0.8 by the addition of 1mM IPTG at 25 °C, and collected after 20 h by centrifugation. Cells were lysed in a French press at 4 °C in 20 mM HEPES (pH 7.4), 150 mM NaCl. The lysate was centrifuged at 17,000xg at 4 °C for 40 min. Ni-NTA agarose beads were added to the supernatant containing soluble A1 and shaken overnight at 4 °C in 40 mL Falcon tubes (1.5 mL beads / 40 mL supernatant). Beads were centrifuged at 2500 RPM for 5 min and re-suspended in 10mL buffer (20 mM HEPES, 150 mM NaCl, pH7.4) per tube and vortexed for 2 min (1st washing step). After two more washing steps, 5 cleaning steps were performed following the same procedure but with 20 mM HEPES, 1M NaCl and 16 mM imidazole. The beads were then packed into a column and eluted with 20 mM HEPES, 150 mM NaCl, 300mM imidazole by gravity. The eluate was mixed with Tobacco Etch Virus (TEV) protease (A1:TEV mass ratio of 10:1) and dialysed against 20 mM HEPES, 150 mM NaCl overnight at 4°C. The thioredoxin-cleaved A1 was centrifuged for 10 min at 13,000 rpm to remove precipitate. The supernatant was further purified by heparin column (HiTrap Heparin HP, GE Healthcare), washed with 20 mM HEPES, 350 mM NaCl and eluted with 20 mM HEPES, 800 mM NaCl. Finally, the protein was subjected to Superdex 200 size-exclusion chromatography as above.

Storage and re-purification. All proteins were stored at -80°C and subjected to a second round of size exclusion chromatography on the day of measurements. This was essential to prevent protein aggregates from contributing to affinity and kinetics measurements, i.e., to obtain data that can be reliably fitted with a 1:1 binding model.

Kinetic binding measurements. The GPIIb α used for kinetic measurements contained an N-terminal Avi-tag which was biotinylated using a BirA biotin-protein ligase kit (Cat #BirA500, Avidity, Aurora, CO). Biolayer interferometry (BLI) used Octet RED384 instrument and software (ForteBio). Streptavidin-functionalized sensors were dipped in biotinylated GPIIb α (1 μ M) for 60 s and quenched with 10 μ g/mL biotinyl-lysine for 40 s. Binding and dissociation responses were recorded at varying A1 concentrations in 20 mM HEPES pH 7.5, 0.02 % Tween-20 and either 10, 30 or 150 mM NaCl as indicated. Response in a buffer-only reference well was subtracted (internal reference). The experiment was also carried out using the same concentrations of A1 and the internal reference but with sensors that had no absorbed GPIIb α (parallel reference). Parallel reference responses were subtracted from the experimental responses with Octet software. Data at different A1 concentrations were fit globally to a single k_{on} and k_{off} value for each experimental condition and plotted using Originlab (Origin, Northampton, MA). The ionic strength of 20 mM HEPES, pH 7.5 was calculated using <https://www.liverpool.ac.uk/pfg/Research/Tools/BufferCalc/Buffer.html>.

Isothermal calorimetry (ITC). Proteins were dialyzed overnight against 150 mM NaCl, 20 mM Tris-HCl pH 7.5, degassed, and centrifuged at 20,000 $\times$ g for 10 min. GPIIb α (250 μ M except 550 μ M with A1+OGly+N+C) was titrated into an A1 protein solution (22 μ M except 50 μ M with A1+OGly+N+C) in a MicroCal iTC200 (GE Healthcare Life Sciences). A priming injection of 0.4 μ L (not included in data analysis) was followed by 2 μ L injections every 180 s. Data averaged over 2-s windows were analyzed using Originlab 7.

Stability to denaturant and heat. For urea denaturation, A1 (0.25 to 0.5 mg/mL) in 10 μ L of 20 mM HEPES pH 7.5, 150 mM NaCl was mixed with 10 μ L of 200 mM HEPES pH 7.5, 1.5M NaCl, x μ L of 7.5 M urea, and (80 - x) μ L of water, where x gave final urea concentrations of 0.135 - 6 M in 24 concentration steps in 100 μ L, and equilibrated for 24 hours at 20°C. Intrinsic Trp fluorescence measured at 330 nm and 350 nm upon excitation at 275 nm with a Prometheus NT48 (NanoTemper) with automatic adjustment of excitation power.

Fluorescence intensity ratio (*FIR*), emission intensities at 350 nm over 330 nm, was fit as a function of urea concentration to a 3-state transition model using the Linear Extrapolation Method (Santoro & Bolen, 1988). *FIR* at each urea concentration (C_{urea}) is modeled as the population weighted contribution from the native state (N), intermediate state (I), and the denatured state (D): $FIR(C_{\text{urea}}) = FIR_N \cdot P_N + FIR_I \cdot P_I + FIR_D \cdot P_D$ where FIR_N , FIR_I and FIR_D represent the linear baseline for the N, I and D states, respectively, which can be expressed as $FIR_j = b_j + s_j \cdot C_{\text{urea}}$, with b_j and s_j as intercept and slope of the j state. P_N , P_I and P_D represent the population of N, I and D states at each urea concentration, respectively. The population of state j (P_j) in the system can be related to the free energy of each state (N, I, D) based on the Boltzmann distribution, $P_j = \frac{\text{Exp}(-\frac{\Delta G_j}{R \cdot T})}{\text{Exp}(-\frac{\Delta G_N}{R \cdot T}) + \text{Exp}(-\frac{\Delta G_I}{R \cdot T}) + \text{Exp}(-\frac{\Delta G_D}{R \cdot T})}$, where R is the gas constant, and T is absolute temperature of the system. With the N state as reference state ($\Delta G_N = 0$), the free energy of the I and D states at each urea concentration can be expressed as $\Delta G_I(C_{\text{urea}}) = \Delta G_I^0 + m_I \cdot C_{\text{urea}}$ and $\Delta G_D(C_{\text{urea}}) = \Delta G_D^0 + m_D \cdot C_{\text{urea}}$, respectively, where ΔG_I^0 and ΔG_D^0 are the free energy of the I and D state in absence of urea, and m_I and m_D are the cooperativity of transition from the N state to I state, and from N state to D state, respectively. For a cooperative two-state transition induced by a particular denaturant, the m -value is proportional to the surface area exposed upon protein denaturation and is generally proportional to the protein size. The linear relationship between m -values and globular protein chain lengths have been reported for denaturant-induced protein unfolding (Ghosh & Dill, 2009). Thus, the final fitting function for the 3-state transition is as follows:

$$FIR(C_{\text{urea}}) = \frac{(b_N + s_N \cdot C_{\text{urea}}) + (b_I + s_I \cdot C_{\text{urea}}) \cdot \text{Exp}(-\frac{\Delta G_I^0 + m_I \cdot C_{\text{urea}}}{R \cdot T}) + (b_D + s_D \cdot C_{\text{urea}}) \cdot \text{Exp}(-\frac{\Delta G_D^0 + m_D \cdot C_{\text{urea}}}{R \cdot T})}{1 + \text{Exp}(-\frac{\Delta G_I^0 + m_I \cdot C_{\text{urea}}}{R \cdot T}) + \text{Exp}(-\frac{\Delta G_D^0 + m_D \cdot C_{\text{urea}}}{R \cdot T})} \quad (\text{Equation 1})$$

Nonlinear least square fit to Equation 1 was employed to fit the urea denaturation data for four A1 constructs. The data were globally fit with shared s_N , s_I , s_D , m_I and m_D , and individual b_N , b_I , b_D , ΔG_I^0 and ΔG_D^0 for each construct. The rationale for fitting the baseline slope for each state to a shared value between the four constructs is that the N- and C- terminal linker does not contain any tryptophan. The rationale for the four

constructs to share the same m-values for the I state and D states is that the four constructs only differ in the O-glycosylated linkers which are not folded.

For heat denaturation, A1 (0.25 to 0.5 mg/mL) was in 20 mM HEPES pH 7.5, 150 mM NaCl. FIR was measured in the Prometheus NT48 while temperature was ramped from 30 to 85°C at a rate of 1°C/min using an excitation power of 50%. Additionally, the intensity of backscattered light was recorded as a measure of aggregation.

Hydrogen deuterium exchange mass spectrometry. Hydrogen deuterium exchange experiments were essentially as reported previously (Iacob, 2013; Le et al., 2018). Comprehensive experimental details and parameters are provided in Table 1, in the recommended (Masson et al., 2019) tabular format. All HDXMS data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository and the dataset identifier is pending.

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TABLE 1. HDX MS Data Summary and list of experimental parameters

Data Set	A1-short	A1N	A1C	A1NC
HDX reaction details^a	15-fold dilution with labeling buffer at 20 °C, final D ₂ O level=93.3%, pH _{read} =7.10; equal volume quench buffer at 0 °C, pH _{read} =2.5			
HDX time course	10s, 1m, 10m, 1hr, 4hr			
HDX controls	3 undeuterated for each condition			
Back-exchange	30-35%			
Number of peptides	102	103	101	108
Sequence coverage	93.2%	92.5%	91.8%	90.9%
Avg. peptide length (a.a.)	12.3	12.2	12.3	12.3
Redundancy	5.4	5.14	4.89	5.77
Replicates	2 technical for each condition			
HDX Repeatability^b	± 0.15 relative Da			

^a labeling buffer: 20 mM HEPES, 150 mM NaCl, 99% D₂O, pD 7.5; quench buffer: 300 mM sodium phosphate, 0.25 M tris (2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), H₂O pH 2.5

^b All reported values are the average relative deuterium level as given by the DynamX software, which in some cases is an average of more than one charge state, across all peptides in both technical replicates. No statistical tests were applied to the HDX MS measurements

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428 Briefly, A1 short and each O-glycosylated protein construct (28 µM in 20 mM HEPES, 150 mM NaCl)

429 was diluted 15-fold into 20 mM HEPES, 150 mM NaCl, 99% D₂O (pD 7.5) at room temperature. At deuterium

430 exchange time points from 10 s to 240 min, an aliquot was quenched by adjusting the pH to 2.5 with an equal

431 volume of 300 mM sodium phosphate, 0.25 M tris (2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), H₂O.

432 Samples were analyzed as previously described (Iacob, 2013; Wales, 2008). They were digested offline with 10
 433 mg/mL pepsin in water for 5 min on ice, then injected into a custom Waters nanoACQUITY UPLC HDX
 434 ManagerTM. All mass spectra were acquired using a Waters Synapt G2-Si HDMS^E mass spectrometer.
 435 Comparison experiments were done under identical experimental conditions such that deuterium levels were not
 436 corrected for back-exchange and are therefore reported as relative (Wales & Engen, 2006). All experiments were
 437 performed in duplicate. The error of measuring the mass of each peptide was ± 0.15 Da in this experimental setup.
 438 The peptides were identified using PLGS 3.0.1 software and the HDX MS data were processed using DynamX
 439 3.0 (Waters Corp., USA). Peptides common to A1+OGly+N+C and the shorter constructs were followed with
 440 HDX, with an overall sequence coverage of 90.9%. In total, 108 peptic peptides were followed with hydrogen
 441 deuterium exchange uptake plots (Supplemental Figures 1-5).

Supplementary Materials.

Figure 5 - Figure Supplement 1. Complete HDX peptide coverage at each indicated time point. (A-

B) Peptides from the indicated A1 fragments are colored according to the key with deuterium exchange at all timepoints as % of the available amide backbone H atoms in each peptide. Residue number based on the immature sequence indicated above and secondary structures indicated below.

Figure 5 - Figure Supplement 2. Complete HDX peptide coverage at each indicated time point. (A-

B) Peptides from the indicated A1 fragments are colored according to the key with deuterium exchange at all timepoints as % of the available amide backbone H atoms in each peptide. Residue number based on the immature sequence indicated above and secondary structures indicated below.

Figure 5 - Figure Supplement 3. Full HDX kinetics for peptides, one of three figures.

Figure 5 - Figure Supplement 4. Full HDX kinetics for peptides, two of three figures.

Figure 5 - Figure Supplement 5. Full HDX kinetics for peptides, three of three figures.

Source Data Files.

Figure 4D – Source Data 1. A1 Short.

Figure 4D – Source Data 2. A1 Short+ C.

Figure 4D –Source Data 3. A1 Short+N.

Figure 4D – Source Data 4. A1 Short+NC.

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Acknowledgements. We would like to thank Prof. Thomas Wales for helpful discussions and a research collaboration with the Waters Corporation (JRE). We thank Yang Su for writing and using code to display HDXMS data for figure preparation. We acknowledge support from the National Hemophilia Foundation and from the Austrian Science Fund (FWF), Erwin Schrödinger Fellowship number J4081-B21, NIH grant F32HL126386, and NIH grant R01-HL148755.

Author Contributions. Investigation: KB, REI, and NEH. Formal analysis: KB, REI, JRE, and JL. Conceptualization: TAS. Methodology: TAS, JRE, and CL. Writing- Original Draft: KB and TAS. Writing- Review and Editing: TAS, JRE, CL, KB, and JL. Supervision: JRE and TAS. Funding acquisition: TAS, KB, NEH, and JRE.

Data and materials availability statement. All data are contained within the manuscript. All HDXMS datafile have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository and the dataset identifier is pending.

Conflict of Interest Disclosures. The authors declare that they have no conflict of interest with the contents of this article.

A. A1short

B. A1+Ogly+N

Figure 5 - Figure Supplement 1. Complete HDX peptide coverage at each indicated time point. (A-B) Peptides from the indicated A1 fragments are colored according to the key with deuterium exchange at all timepoints as % of the available amide backbone H atoms in each peptide. Residue number based on the immature sequence indicated above and secondary structures indicated below.

A. A1+Ogly+C

B. A1+Ogly+N+C

Figure 5 - Figure Supplement 2. Complete HDX peptide coverage at each indicated time point. (A-B) Peptides from the indicated A1 fragments are colored according to the key with deuterium exchange at all timepoints as % of the available amide backbone H atoms in each peptide. Residue number based on the immature sequence indicated above and secondary structures indicated below.

✕ A1+OGly+N+C ◆ A1+OGly+N ● A1 short ■ A1+OGly+C

Figure 5 - Figure Supplement 3. Full HDX kinetics for peptides, one of three figures.

✕ A1+OGly+N+C ◆ A1+OGly+N ● A1 short ■ A1+OGly+C

Figure 5 - Figure Supplement 4. Full HDX kinetics for peptides, two of three figures.