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Review

The effect of shear stress on protein conformation
Physical forces operating on biochemical systems: The case of von Willebrand factor

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ABSTRACT

Macromolecules and cells exposed to blood flow in the circulatory tree experience hydrodynamic forces that affect their structure and function. After introducing the general theory of the effects of shear forces on protein conformation, selected examples are presented in this review for biological macromolecules sensitive to shear stress. In particular, the biochemical effects of shear stress in controlling the von Willebrand Factor (VWF) conformation are extensively described. This protein, together with blood platelets, is the main actor of the early steps of primary haemostasis. Under the effect of shear forces >30 dyn/cm^2, VWF unfolding occurs and the protein exhibits an extended chain conformation oriented in the general direction of the shear stress field. The stretched VWF conformation favors also a process of self aggregation, responsible for the formation of a spider web network, particularly efficient in the trapping process of flowing platelets. Thus, the effect of shear stress on conformational changes in VWF shows a close structure–function relationship in VWF for platelet adhesion and thrombus formation in arterial circulation, where high shear stress is present. The investigation of biophysical effects of shear forces on VWF conformation contributes to unraveling the molecular interaction mechanisms involved in arterial thrombosis.

Contents

1. Introduction

The physico-chemical properties of a system are thermodynamically defined by a set of macroscopic quantities accessible to experimental measurements and related to the laws of statistical mechanics governing its microscopic parts [1]. Moreover, biochemical systems can also sense mechanical forces although the mechanisms at the base of consequent biomolecular conformation and biochemical signaling pathway have long remained elusive [2].

A unique aspect of biological systems is the dimension of particles involved in processes so that, apart from the existence of huge polymers, also simpler proteins are constituted of different interacting domains. Overall macromolecular functional properties arise from the

Abbreviations: ADAMTS-13, *A D*isintegrin-like *A*nd *M*etalloprotease with *T*hrombo*S*pondin type I repeats; Gplb, glycoprotein Ib; GpIIb–IIIa, glycoprotein IIb–IIIa; GpVI, glycoprotein VI; rhDNase, recombinant human deoxyribonuclease; rhGH, recombinant human growth hormone; TTP, thrombotic Thrombocytopenic Purpura; ULVWF, ultra large von Willebrand factor; VWA, von Willebrand factor A-like domain; VWD, von Willebrand disease; VWF, von Willebrand factor.

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contribution of these structural domains and can be defined as the sum of their specific role and mutual interactions [2]. The analysis of binding phenomena, macromolecular aggregation, protein stability and conformational transitions are generally treated as a function of chemical potential of particles in solution and a specific interaction of molecules at a well-defined site can be transmitted to other domains, thus modifying the system properties. Moreover, chemical ligand-binding processes are associated to changes in heat (enthalpy) and volume of the system. Therefore, general concepts of linkages between ligand activity, and thermal transition temperatures or enthalpy change accompanying isothermal titration of a macromolecule, have been used to establish the state function properties of proteins [3]. Consequently, changes in enthalpy, as a function of ligand activity, will provide further information regarding the linkage relation between enthalpic changes and chemical ligand binding processes for the macromolecular system. Hypothetical, but not easily achievable, experiments based on the exploration of volume changes as a function of pressure or ligand activity would provide a specific characterization of the meaning of thermodynamic states and reactions of biological macromolecules [1].

The conformational changes in different domains of a macromolecule induced by a site specific binding are due to a rearrangement of hydrophobic, electrostatic and van der Waals interactions, thus modifying the molecule structure [4]. However, theoretically, energy involved in a protein transition, normally induced by ligand binding or thermodynamic factors, could also derive from mechanical forces causing a similar structural change [5]. Unfortunately, because of the microscopic dimension of a macromolecular domain, it could be experimentally difficult to apply mechanical forces to a specific site (without involving the adjacent domains), thus inducing only the desired functional transition, without affecting the molecule general properties. Moreover, primary structure, hydrophobic and electrostatic interactions among residues, and presence of vicinal disulphide bonds confer flexibility or rigidity to different domains and consequently the capacity to transmit mechanical tensions and perturbations [4].

However, the development of ultrasensitive instruments for nanomanipulation, such as atomic force microscopy and optical and magnetic tweezers, has allowed the effect of forces on protein conformation and function to be probed at the single-molecule level and to reach a specific target domain thus inducing localized perturbations [5].

In an increasing number of proteins (see next sections), an induced mechanical transition “in vivo” has been identified and the mechanism, generally linked to pathophysiological processes in vascular districts, is determined by the effect of blood flow on the molecules (shear stress). Experimental results indicate that similar transitions and functional states can be partially reproduced “in vitro” by inducing specific or non-specific chemical bindings [6–10].

An example of protein that undergoes shear stress or chemical allosteric transition is the VWF molecules. Their aggregation is triggered by the exposure of specific binding sites uncovered after the protein shear stress activation [11–13]. The process can be also experimentally reproduced by the interaction of the protein with ristocetin or batrocetin or partially unfolding the molecule [10].

2. What is shear stress? Effect of shear on protein conformation: general aspects

A shear stress is defined as a stress which is applied in parallel or tangentially to a face of a material, as opposed to a normal stress which is applied perpendicularly [14]. Real fluids moving along a solid boundary will incur a shear stress on that boundary. The no-slip condition [15] dictates that the speed of the fluid at the boundary is zero, but at some height from the boundary it must equal that of the fluid. For all Newtonian fluids in laminar flow, the shear stress is proportional to the strain rate in the fluid where the viscosity is the constant of proportionality. However, for non-Newtonian fluids, this is no longer the case, as for these fluids the viscosity is not constant. The shear stress is imparted onto the boundary as a result of this loss of velocity [14,16]. The shear stress, for a Newtonian fluid, at a surface element parallel to a flat plate, at the point y, is given by:

$$\tau = \mu \frac{\partial u}{\partial y} \tag{1}$$

where μ is the dynamic viscosity of the fluid, u is the velocity of the fluid along the boundary, and y is the height of the boundary.

Blood in all but the smallest vessels (<500 μm), at all but the lowest shear rates (<200 s^{-1}), can be treated as a homogeneous Newtonian fluid [14,16].

At shear rates higher than 200 s^{-1}, the blood viscosity asymptotically approaches a constant value of about 3 cp. For fully developed Poiseuille laminar flow, the shear stress at the wall of the tube is related to the flow Q (volume/time) and the radius R by

$$\tau_{wall} = \mu \frac{4Q^3}{\pi R} \ . \tag{2}$$

In the case of arterial stenosis, the flow geometry is irregular and cannot be accurately described by the Poiseuille equation. A more adequate model for calculating shear stress in a stenosed vessel is given by the Navier–Stokes equations, a set of partial differential equations derived independently by G.G. Stokes, in England, and M. Navier, in France, in the early 1800s. These equations describe how the velocity, pressure, temperature, and density of a moving fluid are related. The Navier–Stokes equations consists of a time-dependent continuity equation for conservation of mass, three time-dependent conservation of momentum equations and a time-dependent conservation of energy equation [14,17]. Biomolecules exposed to blood circulation experience hydrodynamic forces that, particularly in microvessels, affect their function and induce mechanical strains on their structures. Well-defined solutions exist for the estimation of forces applied on particles localized near a substrate under fluid flow [18], for aggregates composed of two equal-sized particles [19] and doublets composed of unequal-sized particles separated by a finite distance [16]. Many biological particles, including neutrophils, platelets and metastatic cells aggregates, cell-surface receptors and soluble molecules like VWF, can be represented as a pair of (un)equal spheres linked by a rigid tether.

The effects of many kinds of solvent conditions and physiochemical parameters on the thermodynamic stability of protein molecules have been thoroughly investigated in many biochemical studies. Chaotropic agents (urea and guanidinium ion), pH, and temperature have been shown to alter or denature proteins [8,20,21]. Likewise, an effect of shear stress on changes in protein conformation may be expected, and experimentally explored for a large number of biologically and bio-technologically relevant proteins [22]. It is also widely believed that shear stresses arising from fluid flow can affect protein stability. Due to the polymer nature of proteins, large velocity gradients are theoretically able to induce conformational transitions or even unfolding in these molecules and these effects may result in the loss of enzyme activity, molecular aggregation or even disruption of its covalent backbone. However, contrasting results concerning the effect of high shear on protein stability and function have been reported in literature. For instance, IgG molecules even under prolonged exposure to shear undergo only <0.3% reversible aggregation in solution [23]. In this study the authors suggested that air entrapment, surface effects, and particulate contamination have a cooperative effect in causing protein aggregation/loss under shear [23,24]. In contrast, using more sophisticated methods, such as particle image velocimetry and Raman spectroscopy, to measure

lysozyme structure *in situ*, other researchers have recently observed a rapid and reversible unfolding of this protein when it was subjected to high shear [25]. Moreover, recombinant human growth hormone (rhGH) and recombinant human deoxyribonuclease (rhDNase) were used to investigate the role of shear in causing conformational transitions or even denaturation in these molecules [26]. Notably, it was found that high shear rate did not have a significant effect on protein aggregation. However, scanning microcalorimetry allowed to deduce the occurrence of shear-induced conformational transitions, in agreement with a lower melting temperature and enthalpy, detected for highly sheared rhGH [26]. SDS-PAGE indicated also the presence of low molecular-weight fragments, suggesting that high shear caused peptide bond breakage [26]. rhDNase was relatively more stable than rhGH under high shear stress. No conformational changes and protein fragments were observed in this case. In another study Jaspe and Hagen [27] detected no changes in protein conformation of a small globular protein, cytochrome c, under shear up to a shear rate of 2×10^5/s. The theoretical calculations of these investigators suggested that only very high levels of shear ($\sim10^7$/s) are able to alter the structure of small globular proteins. Thus, beside the absolute value of shear stress or rate, also the size and conformational state of the protein under static conditions play a role in the phenomena of protein conformational changes induced by high fluid shear stress.

Moreover, many experimental evidences have shown that the influence of mechanical load on conformational changes of buried binding sites in proteins may be a general mechanism for biological mechano-sensors. In the field of cytoskeleton proteins, for instance, it was demonstrated that activation of the strain sensing kinase of the giant muscle occurs by exploiting a force-induced activation mechanism [28,29]. Notably, in this case the applied mechanical force is able to unfold the C-terminal domain of the protein, canceling its autoinhibitory function. After this process, ATP can bind to the enzyme, phosphorylation takes place and force adaptation processes in muscle can be effected [28]. Another protein involved in the organization of actin cytoskeleton, talin, containing several buried binding sites for vinculin, can expose these interaction sites only upon application of a mechanical force. Binding of vinculin to these sites activate signaling. Thus, talin acts as a mechanosensor that only upon its partial unfolding by mechanical forces is bound by vinculin and generates signaling.

In the field of haemostasis and thrombosis research, a large multimeric protein called von Willebrand Factor (VWF) has been shown to undergo structural changes upon application of hydrodynamic shear. One of the methodological problems concerning the study of shear-induced conformational effects on protein is the reversibility of these phenomena and the possibility to monitor in real time their occurrence. The first evidence of an extensive 3D conformational change induced by high shear stress on VWF was obtained in an elegant study using force atomic microscopy [30]. The results presented in this study demonstrated that VWF undergoes a shear stress-induced structural transition from a globular state to an extended or stretched chain conformation exposing individual globular domains [30]. On a hydrophobic surface, the conformational transition occurs under the critical shear stress regime of >31 dyn/cm^2. VWF exhibits strong adhesion with hydrophobic surfaces, which suggest a possible structurally dependent adhesive mechanism that may aid specific VWF binding to biological macromolecules and receptors. More recently, other authors confirmed these original data by using different and sophisticated microfluidic devices [12,31,32]. Together, these data suggest that protein conformational changes may take place under specific conditions. Further, the peculiarity of the very high molecular weight of VWF further confirmed that the size of protein may be a critical factor in regulating shear-induced conformation changes in these macromolecules. In the case of VWF, the conformational changes induced by high shear stress are of particular relevance, since the different VWF conformers have a very different efficiency both in performing the haemostatic functions of the protein and in favoring its proteolytic processing by the metalloprotease ADAMTS-13, as extensively explained below. Hence, the functional strategies used to monitor the effects of shear on VWF are able to disclose with high sensitivity and under different standpoints the generation of shear-induced conformers in this haemostatic protein.

3. VWF conformation is highly sensitive to shear stress

Von Willebrand factor (VWF) plays a key-role in primary haemostasis [33,34]. This protein derives from a very large gene (180 kb, 52 exons) and is synthesized by endothelial cells and megakaryocytes as pre-pro-VWF. The latter includes a 22-residue signal peptide and a 741-residue propeptide [35] and undergoes extensive posttranslational processing, glycosylation, and assembly in the endoplasmic reticulum, Golgi and post-Golgi [35,36]. The mature protein consists of ~250 kDa monomeric subunits, which form disulfide-linked multimers of 500 to 20,000 kDa [37,38]. The latter initiate platelet adhesion at sites of vascular injury [35]. Much of the functional activity of VWF multimers resides within the three tandem A domains, A1, A2, and A3. VWF multimers interact with platelet glycoprotein Ib and collagen through binding sites localized at the A1 and A3 domains, respectively [39–41]. Vascular endothelial cells upon stimulation by agonists as thrombin, histamine or prostaglandins also secrete long strings of ultra large (UL), haemostatically hyperactive VWF multimers that are anchored to the endothelial cell surface [6]. The A2 domain, situated between domains A1 and A3, does not form a disulfide-bonded loop as the latter and contains a peptide bond between Tyr1605 and Met1606 specifically cleaved by a metalloprotease, *A D*isintegrin *A*nd *M*etalloprotease with *T*hrombo*S*pondin motifs, and a Zn^{2+}/Ca^{2+}-dependent protease, member n. 13 of the ADAMTS enzyme family (ADAMTS-13), that proteolyzes ULVWF-platelet strings [42].

Inherited absence or acquired immune-mediated reduction in ADAMTS-13 activity is associated with the systemic microvascular thrombosis characteristic of thrombotic thrombocytopenic purpura (TTP) [43]. ULVWF multimeric strings are subject to the shear stress of flowing blood and the tensile stress of attached platelets [44,45]. These stresses assist ADAMTS-13 proteolysis of ULVWF multimers, likely by altering sites in the A1 and A3 domains that allow ADAMTS-13 docking and associated cleavage of an exposed peptide bond in the A2 domain.

The above described structural and functional properties of VWF are fundamental for its functioning in repairing vascular damage, during the phenomenon globally referred to as primary haemostasis. In virtue of this process, the platelet receptor GpIb binds to the extracellular matrix components through the intermediation of VWF multimers [46]. The evident pro-thrombotic risk associated with this protein is mainly avoided as this process does not occur under static conditions and requires a “pre-activation” of soluble VWF multimers involving a conformational change that exposes the binding site for GpIb in the A1 domain of VWF. Interaction of the platelet GpIb receptor with the A1-domain of immobilized VWF [47] results in initial adhesion, characterized by a continuous surface translocation of the platelets [48]. The process ultimately leads to stable platelet adhesion by means of interaction with the platelet collagen receptors GpVI and GpIa/IIa [49], activation of the platelet GpIIb/IIIa receptor complex [50], and finally platelet aggregation. The “pre-activation” mechanism is triggered either by mechanical forces, such as the high shear stress (>5000 s^{-1}) found in arterial circulation, or by interaction with external surfaces and chemicals/snake venoms [11,13,33,51–53]. Notably, type 2B von Willebrand disease (2B-VWD) also results in stabilizing the stretched conformation of VWF

multimers, that become, even under lower shear rates, more prone both to GpIb interaction [33,54].

High shear stress causes micro- and macro-conformational changes in VWF [7]. Moreover, the same shear-induced conformational changes expose also in the VWF molecule a peptide bond in the A2 domain of VWF that is cleaved by the metalloprotease ADAMTS-13 that proteolyzes the ultra large VWF multimers, thus limiting their high pro-haemostatic properties [55].

Another relevant effect stemming from conformational changes induced by shear forces is the ability of inducing a self-association of VWF multimer in solution [12]. Although self-association and aggregation under high shear stress of VWF was an already recognized phenomenon [52], the molecular mechanism driving the process has only recently been clarified [13,56]. VWF multimers undergo a dramatic shape change as a function of the shear rate [30] that is responsible for the formation of VWF fibers, which are organized in spider web-like structures [12]. The latter represents an ideal anchor that serves to capture flowing platelets as the primary haemostasis process takes place. These past studies showed that in all cases the aggregation and web-formation processes are essentially linked to the mechanical force generated by shear. On the other hand, ristocetin, or other molecules, such as the venom botrocetin, also promotes the interaction of VWF with GpIb. The antibiotic ristocetin is a polyphenol molecule, which interacts with the A1 domain of VWF, thus providing a model for changes in VWF conformation that may occur in vivo, as we demonstrated in a recently published paper [10]. It could also be considered of special interest as a model for the conformational changes that occur in natural VWF molecules affected by the mutations that cause the different forms of type 2B VWD. It is noteworthy that the transition from a globular conformational state of VWF protein to a stretched active one is a very fast process. Thus, both physical (shear) and chemical (ligand binding) potentials can generate the free energy change required to promote the conformational transitions in VWF. This property is fundamental for the primary haemostasis to properly occur. In the next section, the mechanistic aspects by which the shear forces promote the conformational transition in VWF molecule will be analytically described.

4. Mechanisms of shear-induced conformational transitions in VWF multimers

As anticipated above, the VWF multimers with ultra large size (ULVWF) are secreted in response to thrombogenic stimuli. The secreted ULVWF is in part bound locally to endothelial cells and partly to collagen at sites of tissue injury, through its A3 domain. These hydrodynamic forces cause conformational changes in VWF that exposes a binding site in the A1 domain for the platelet glycoprotein Ib (GpIb) molecule [57]. It has to be noted that, once secreted by endothelial cells, ULVWF is trimmed by ADAMTS-13, with production of smaller VWF fragments that have a lower pro-haemostatic potential [57]. Notably, VWF multimers extracted from platelet α-granules, which do not undergo during their storage any proteolytic activity by ADAMTS-13, have multimers with higher molecular weight than plasma-derived VWF [58]. This is additional evidence concerning the relevance of ADAMTS-13 activity in VWF fragmentation, which limits its thrombogenic potential. In the absence of ADAMTS-13 activity, due to genetic mutations or formation of anti-ADAMTS-13 auto antibodies, a life-threatening disease, referred to as thrombotic thrombocytopenic purpura, does occur, causing an uncontrolled micro vascular thrombosis [43]. At variance with this clinical setting, some mutations in the A2 domain that likely destabilize it cause both excessive cleavage by ADAMTS-13 and a shift in the size distribution to smaller VWF multimers with less haemostatic potential [59]. This effect causes a severe bleeding disorder defined as type 2A von Willebrand disease [59]. VWF is cleaved by ADAMTS-13 within the A2 domain at its Tyr1605–Met1606 bond [37,60,61]. The latter is buried at the middle of VWF A2 domain [62,63], but is exposed and can be attacked by ADAMTS-13 by shear when A2 is present in full length VWF multimers, whereas it is proteolyzed, although with lower specificity, as isolated domain [55,61,64]. This phenomenon is likely due to the tensile forces that act on proteins in shear flow increase as a function of protein length [16,63]. From the seminal work of Shankaran [16] we know that, considering a protein multimer composed of N monomers, the tensile force on a monomer increases as a function of the distance from the nearest end of the multimer and that at the mid part of the multimer chain the force is proportional to N^2. In particular, following the Shankaran theory, tensile force increases with the square of length of the multimer chain, as both its size and the difference in velocity between the shear lamina of the fluid in which the two ends of the multimer are present, increase with length. Accordingly, the tensile force $F(j)$ to the inside of any sphere pair j in a multimer chain with N dimers (considering this scheme as the reference structure of a multimer VWF chain) is the sum of the force on all the outer dimer pairs: $F(j) \approx [(N+j)\ (N+1-j)/2]\ f\ (d+2a)$, where $(d+2a)$ is the length of a VWF dimer in electron microscopy of 120 nm. From the above relation it can easily seen that the tensile force at the middle of the multimer is proportional to the square of the number of the monomers. Notably, the quadratic proportionality between tensile force and length of the multimer shows the relevance of VWF multimer size for the unfolding of the protein needed to allow both an efficient interaction with ADAMTS-13 and the exposition of numerous interaction sites for platelet GpIb. Recently, the study of Schneider and colleagues has shown that shear flow is able to stretch VWF (2), and tensile force exerted on the multimer could cause conformational change in the A2 domains that enables the cleavage of the scissile bond that is buried in the unsheared state of VWF [37,57,62].

Based on the above findings and considerations the relevant question arises as to whether under the shear force found in the microcirculation, that is 50–100 dyn/cm^2, the tensile force generated at the middle of a long VWF multimer (>100 monomer) may be sufficient to induce unfolding of the A2 domain, responsible for its availability to the proteolytic activity of ADAMTS-13 and exposition of A1 binding sites for GpIb. Considering the length of a VWF dimer of 120 nm and applying the relation reported above, it can be calculated that under a shear stress of 100 dyn/cm^2 the tensile force generated at the middle of a 200mer VWF is about 10 pN. The latter value was experimentally found to characterize the unfolding of isolated A2 domain [65]. Notably, the VWF multimers with the highest molecular weight, found in the normal circulation, was characterized by about 200 monomers [55]. Multimers of this length are indeed generated by ADAMTS-13 within 2 h after the secretion of ULVWF by endothelial cells. Thus, based on theoretical and experimental findings, unfolding and folding of the A2 domain may occur at forces that might be experienced by VWF in its transit through the circulation or at sites of haemostasis and thrombosis, and that tensile force acts as a cofactor to unfold A2 for cleavage by ADAMTS-13 [65]. From these findings another important feature of the central part of the VWF monomer emerges and concerns the clear conformational linkage existing between the A2 and A1 domains. Thus, conformational transitions occurring in the A1 and A2 domains are mutually and positively linked, whereby the increased availability of the Tyr1605–Met1606 peptide to the proteolytic attack by ADAMTS-13 is associated to an increased availability of GpIb binding site in the A1 domain. This is another example of balance in haemostasis, whereby a pro-haemostatic function is counter-balanced by an anti-haemostatic effect.

5. How does the energy from tensile force induce conformational transitions in the A2 domain of VWF required for ADAMTS-13 interaction?

The recently solved crystal structure of the VWF A2 domain shows evolutionary adaptations to shear sensor function. The A2 fold with α-

helices and β-strands that alternate in sequence (Fig. 1). Notably, at variances with other VWA domains, such as A1 and A3 domains in the same VWF monomer, in the A2 domain one α-helix is missing. In place of the α4-helix, a long loop runs indeed from the C terminus of the β4-strand to the N terminus of the β5-strand. Because of this structural peculiarity, Zhang and coworkers defined as "α4-less loop." the long β4–β5 loop that in A2 occupies the same topological position as the α4-helix in A1 and A3 [63] (see Fig. 1).

The ADAMTS-13 cleavage site at residues Tyr1605 and Met1606 is present near the middle of the central β4-strand (Fig. 1). However, while α-helices have extensive H-bond networks able to stabilize their secondary structure, the α4-less lacks this connecting network, having a lesser number of residues that stabilize its association with neighboring structural elements. This structural and conformational arrangement allows the α4-less loop to lower the force required for unfolding the A2 domain. Thus, this effect facilitates the exposure of the Tyr-Met peptide to be cleaved by ADAMTS-13. Moreover, if we compare the folding/unfolding mechanisms and kinetics of other VWA domains, it may be hypothesized that the α4-less loop serves also to slow refolding of the A2 domain. This functional effect is particularly useful to allow ADAMTS-13 to proteolyze the A2 domain in VWF under physiological shear conditions. In addition, another unique property of the β4-strand that may further facilitate the proteolytic attack by ADAMTS-13 is its poor packing. In the A2 β4-strand, Leu1603, Met1606, and Thr1608 have indeed alternative side-chain rotamers. Of interest, the alternative side-chain rotamers of Leu1603 and Met1606 differ substantially in position from one another [63]. Met1606 in A2 is neighbored by Val1502 in the β1-strand, instead of the larger Leu residue found at the same position in A1 and A3 domains [63]. An additional factor that contributes to impede packing of other residues around Leu1603 is constituted by the large side chain of Tyr1605, which extends over Leu1603 toward the α6-helix. Finally, if compared to other VWA domains, the A2 domain contains a buried water molecule, H-bonded to hydroxyl group of Ser1517, in an environment that is largely hydrophobic. In other von Willebrand factor A-like domains (VWA), Ser1517 is substituted with hydrophobic residues. The presence of this polar interaction in a highly hydrophobic environment may destabilize the region where the cleavage site between Tyr1605 and Met1606 is localized. Globally, all these features contribute to reduce the force needed to unfold the A2 region and also to slow the refolding process.

In proteins, disulfide bonds between vicinal cysteines are very rare and, when present, often are functionally important [66]. When they are found in particular proteins, the two cysteines form a typical ring constituted by 8 members (the main chain and side chains of vicinal disulfide-bonded cysteines), as shown in Fig. 2. Noteworthy, vicinal disulfides are not found in any other known VWA domains and, due to their relative rigidity, they are thought to play a relevant role in setting an energy barrier for mechanically-induced unfolding of proteins [66]. In the particular case of VWF A2 domain, the C-terminal residue Ser1671 is H-bonded to Cys1670 only. Thus, all elongational forces that act on the C terminus will be transmitted to Cys1670. Since Cys1669 and Cys1670 are the last 2 residues of the α6-helix, any applied force will be sensed by these residues. It has to be remarked that the amides of Cys1669 and 1670 are H-bonded with the Val1665 and Leu1666 carbonyls. Likewise, hydrophobic interactions occur between the side chains of the vicinal disulphide and the hydrophobic pocket formed by the side chains of Leu1497, Met1528, Ile1535, Leu1603, Tyr1605, and Leu1666 [63]. Thus, any elongation force that overwhelms the mechanical resistance of the vicinal disulphide is transmitted to the middle of the β4-strand, where the Tyr1605–Met1606 is localized. This "domino effect" is an efficient mechanism by which the shear forces acting on VWF are traduced into conformational transitions that render the peptide bond Tyr-Met in the A2 domain available to ADAMTS-13's attack. Finally, another structural element, which is involved in the mechanisms that transform mechanical energy of shear into conformational effects of the A2 domain, is the presence of cis-proline1645 at position 1645. Proline is an amino acid generally considered to be an α-helix- and β-sheet-breaking residue, although proline residues are sometimes found in the middle of α-helices where they cause some geometry deformation (kink formation) [67]. The torsion angle of a peptide

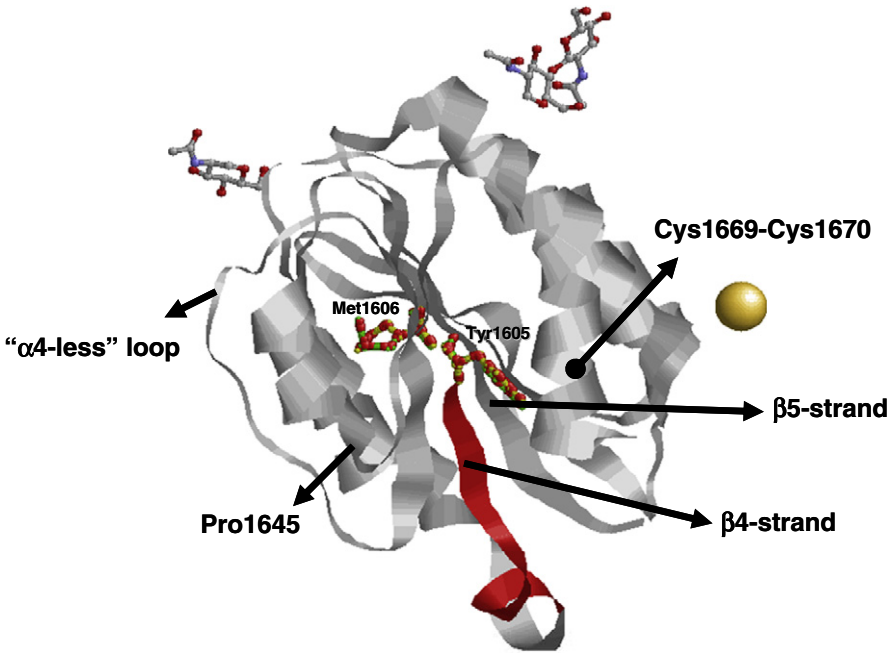

Fig. 1. Ribbon plot of the crystal structure of the VWF A2 domain (pdb file 3GXB, [63]). The main domains involved in shear-induced conformational transitions are indicated by arrows. The side chain of Tyr1605 and Met1606 are shown as ball and sticks. The detailed description of the mechanisms driving the shear-induced conformational transitions of the VWF A2 domain is reported in the text. The sphere on the right is s sulphate ion, while the separate molecules depicted as sticks are N-acetyl-glucosamine used to crystallize the A2 domain. The software RasMol version 2.7.4.2 was used to represent the molecule.

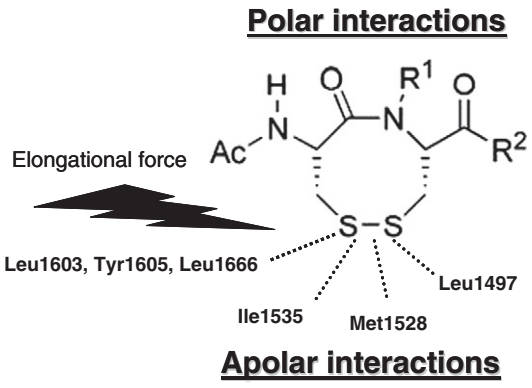

Fig. 2. Scheme of the interaction of the vicinal disulphide region of 1669–1670 in the VWF A2 domain (see Fig. 1). The main polar and apolar interaction of the disulphide with polar and apolar interactions are also reported. The breakage/alteration of these interactions induced by the sensitivity to shear forces of the vicinal disulphide cause a domino-like effect involved in the unfolding of the central β4-strand with the exposure of the Tyr1606–Met1606 peptide bond trimmed by ADAMTS-13.

bond (referred to as ω) may be either *cis* or *trans* and the latter form is energetically favorable due to steric hindrance in *cis* [68]. Unfavorable conformational energy of the *cis* peptide bond is the main cause of its relatively rare appearance in the native structure of proteins. The only exception applies to proline, which has a far higher probability (0.1–3.3) to form a *cis* peptide bond with the preceding amino acid residue than any of the remaining 19 common amino acids which have a probability of 10^{-3} to form the *cis* peptide bond [68]. The higher probability of finding proline in *cis* peptide bonds can be due to lower activation energy barrier (13 kcal/mol) for an X-Pro bond vs 20 kcal/mol for other peptide bonds [69]. Thus, in the case of VWF A2 domain, conversion to a *trans* Trp1644–Pro1645 peptide bond in the vasculature should be preferred only under shear forces. This phenomenon bond would greatly delay A2 domain refolding, once it had been unfolded for unusually long periods of time or been exposed to unusually high shear, which can accelerate *cis* to *trans* peptide conversion [69]. However, it can predicted that VWF multimers in the bloodstream is exposed to moderate shear forces for a short time, and, under these condition, refolding is rapid compared with the estimated time for cleavage by ADAMTS-13 [65]. However, when VWF is bound to its receptors on endothelial cells (GpIb, P-selectin) or to subendothelial proteins (collagen) at a site of hemorrhage, it interacts with numerous platelets, which favor the generation of much higher shear forces over longer periods of time. These phenomena promote the stable conversion to a *trans* Trp1644-Pro1645 peptide bond and thus delay refolding and expedite trimming by ADAMTS-13.

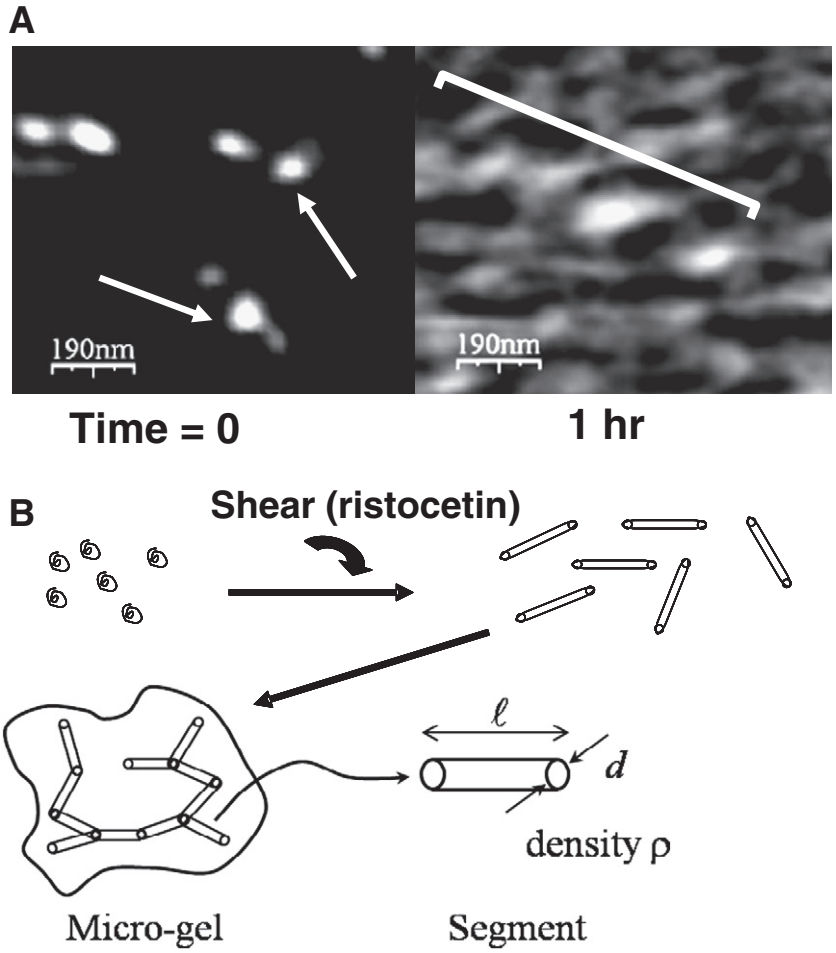

Fig. 3. A) AFM images of the supramolecular aggregates of VWF protein in the absence of ristocetin and 1 h after its addition. Supramolecular structures evolve with time from small globular particles with a mean hydrodynamic radius of about 50 nm (arrows) to elongated and branched fibrils (square bracket). (modified from [72]). B) Cartoon schematizing the formation of a micro-gel structure of VWF multimers, upon their conformational changes induced by high shear stress or ristocetin binding in the absence of shear. The micro-gel is a fractal assembly of segments of length l, diameter d, and density ρ, joined together end-to-end with only a few branching points [72]. The segment length, should not be confused with the average fiber length between the branching points, but is rather likely related to the persistence length of the fiber.

6. Functional consequences of VWF unfolding on its aggregation propensity

Recently, it has been demonstrated that upon application of appropriate shear forces (>30 dyn/cm^2) VWF multimers assume a stretched conformation in solution and form spider web-like structures [12]. As anticipated above, this network represents an ideal anchor to capture flowing platelets during the process of primary haemostasis [12,13]. This behavior was already shown in previous studies. For instance, a shear-dependent self-association of VWF in suspension was demonstrated in experiments using cone-plate viscometry [31,52]. These studies showed that in all cases the aggregation and web-formation process is essentially linked to the mechanical force generated by shear. Moreover, it was recently demonstrated that formation of VWF macro-aggregates may be also induced by binding of chemical effectors, such as the antibiotic ristocetin. The latter is a polyphenol glycopeptides synthesized by the actinomycete Nocardia lurida, although its potent antibiotic activity was largely unfruitful in clinical practice, because it caused frequent drug-induced thrombocytopenia, which was demonstrated to be associated with extensive intravascular platelet agglutination [70,71]. Later on, in vitro studies discovered that this molecule is able to specifically bind to the A1-domain of VWF, triggering a large conformational change of VWF molecule, responsible for its binding to platelet GpIbα [56]. This property is currently used in the clinical diagnostics of VWD, to detect both defective (type 2A and 2M) and even hyper-reactive (type 2B) VWF mutants that can be identified due to their enhanced sensitivity to ristocetin. The use of ristocetin and of the light scattering technique have been recently exploited to monitor the kinetic process of VWF self-association in solution, in a process that phenomenologically resembles that one induced by high shear stress [10,72].

The extensive conformational changes induced by shear stress in vivo or ristocetin binding to VWF in vitro disrupt the multiple domain interactions that occur in globular VWF structure, as demonstrated by atomic force microscopy (AFM) experiments (see [12] and Fig. 3). Upon stabilization of the extended conformation through ristocetin binding or mechanical stretching by shear stress, homotypic domain interactions are substituted by heterotypic A-domain associations, which contribute to the formation of the network as shown in the presence of high shear stress or under unsheared conditions in the presence of the allosteric effector ristocetin [12,13] (Fig. 3A–B). The mechanism is a physical example of the model known as "3D swapping" [73], whereby conformational transitions in a multidomain protein alter internal homotypic interactions, thereby allowing heterotypic domain interactions, which promote the formation of aggregates. This mechanism optimizes the generation of a spider web-like network, which anchors circulating platelets through binding of the GpIb-IX-V membrane complex [12]. The reversibility and the kinetics of the above shear-stress-induced conformational transitions in VWF multimers finely regulate the mechanism of the primary haemostasis in physiological and pathological settings [10,72].

In conclusion, VWF multimers undergo extensive conformational transitions under high shear stress. Some structural elements, especially the presence of the vicinal Cys1669–Cys1670 disulfide bond in the A2 domain of VWF, act as transducers of physical tensile forces and stabilize a stretched conformation of VWF multimer. Upon these conformational changes, unfolded VWF multimers can self-associate. It is likely that the same interactions occurring between the multiple domains present in each VWF multimer are responsible for maintaining the globular structure of VWF under resting conditions. Once these interactions are disrupted by shear, interactions between homotypic and/or heterotypic regions might result in self-association. This mechanism allows the formation of a VWF fibers network that easily entraps flowing platelets at the site of vascular lesions.

The processes described above show another mechanism that confirms the holistic feature of energy in nature: that is how the energy arising from a push can turn into a blood clot.

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