SHEAR-INDUCED STRUCTURAL CHANGES IN BLOOD PROTEINS

Efrosyni Themistou[1], Indrajeet Singh[1], Sathy V. Balu-Iyer[2], Paschalis Alexandridis[1], and Sriram Neelamegham[1]

[1]Department of Chemical and Biological Engineering
[2]Department of Pharmaceutical Sciences
University at Buffalo - The State University of New York (SUNY)
Buffalo, NY 14260-4200, USA

Introduction

The physiological and pathological functions of blood proteins are imparted by changes in their structure. Von Willebrand factor (VWF) is a large, multimeric, multidomain glycoprotein in human blood [1]. VWF exists both as a dimer with molecular weight (MW) of ~500 kDa and as a multimer (polymer) with MW in the range of $10\text{-}20\times10^6$ Da [2]. It is commonly believed that conformation changes in VWF are important regulators of both the protein's physiological and pathological role [3-6]. While there is some biochemical evidence of VWF structural changes with shear [4,7-8], specific probes to rapidly detect conformational changes are yet unidentified. Such structural changes play a key role in regulating arterial thrombosis, by aiding platelet deposition at sites of vascular injury [3-4,6,9].

We are examining the biophysical and biological features regulating the size and structure of VWF in solution, both under static and fluid shear conditions, by using small angle neutron scattering (SANS) [10] and fluorescence methods. Here, we discuss the use of 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt (bis-ANS), a fluorescence probe that partitions into protein hydrophobic domains [11-13], to investigate conformational changes in VWF solution structure by probing non-polar/hydrophobic domains of this protein that are exposed upon shear application.

In this study, we demonstrated that shear-dependent VWF changes in solution structure are accompanied by exposure of hydrophobic domains within the protein. More specifically, the fluorescence intensity due to probe-VWF interaction increased, suggesting that new hydrophobic domains were exposed upon fluid shear application at shear rates (G) greater than 2300-6000/s and at shear times (t_{sh}) greater than 1min. Such fluid shear-induced conformational changes are specific to VWF on the basis of control experiments on bovine serum albumin (BSA).

The importance of this work is two-folded: (1) Develop a new technique, bis-ANS fluorescence probing, to assess and quantify shear-induced structural changes of blood proteins. This new, easy to use, technique is able to quantify the effect of G and t_{sh} on protein conformation, by using small sample volumes. (2) To examine shear-mediated features in VWF that may regulate the cascade of events that contribute to arterial thrombosis and other blood coagulation processes. The findings emerging from this work may be applicable to other blood proteins.

Materials and Methods

Materials

Multimeric human VWF was purified from blood plasma cryoprecipitate obtained from Community Blood Bank (Erie, PA) [10,14]. Bis-ANS was from Invitrogen - Molecular Probes (Eugene, OR). BSA (96%) was purchased from Sigma-Aldrich (St. Louis, MO). The VWF concentration was measured using the Coomassie/Bradford protein assay kit (Pierce Biotechnology, Rockford, IL). Quantitative amino acid analysis was performed on non-sheared and sheared VWF samples at the Protein Chemistry Laboratory (Texas A&M University) to calibrate the Bradford assay.

Fluorescence Experiments

The fluorescent probe bis-ANS was dissolved in Na-K phosphate buffer and the probe solution was stored at 4°C for 3 days prior use. The optimum probe concentration for each experiment was found by measuring the fluorescence signal of the probe incubated with ~60 µg/mL protein. Thus, for studies with VWF and BSA (control protein), protein was incubated with 9.7 and 1.2 µg/mL of bis-ANS, respectively. Protein solutions were sheared using a cone-and-plate viscometer (Haake VT550, 0.5° cone angle, 5cm diameter) by varying (i) G=0-9600 /s and (ii) t_{sh}=1-5 min. 140µl of protein sample were incubated with 15 µl of bis-ANS stock solution prior to each measurement. The absolute fluorescence was measured using a QuantaMaster Fluorometer (PTI, Birmingham, NJ) at ambient temperature to detect probe-protein binding. The signal of the maximum peak of the emission spectrum was used to evaluate the extent of bis-ANS binding to VWF or BSA. The excitation wavelength used was 375 nm and an emission scan was collected between 420 and 620 nm. Experiments were performed using VWF (~60µg/ml) and BSA (~50µg/ml), both dissolved in Hepes buffer.

For sheared protein samples, the following parameters were measured or calculated: (i) $F^{(G)}$ (counts per second, cps): absolute fluorescence (raw data). (ii) $F^{(G)}_{max}$ (cps): peak emission fluorescence at fixed wavelength. (iii) $F_c^{(G)}$ (cps.mL/µg): concentration-normalized emission spectra after accounting for fluorescence due to free bis-ANS in Hepes buffer ($F^{(G)}_{Hepes+bis\text{-}ANS}$), intrinsic protein fluorescence in buffer ($F^{(G)}_{protein+Hepes}$), and fluorescence of Hepes buffer alone ($F^{(G)}_{Hepes}$): $F_c^{(G)} = (F^{(G)}_{protein+Hepes+bis\text{-}ANS} - F^{(G)}_{Hepes+bis\text{-}ANS} - F^{(G)}_{protein+Hepes} + F^{(G)}_{Hepes})$/concentration. (iv) $F_c^{(G)}{}_{max}$ (cps.mL/µg): Peak concentration-normalized emission fluorescence at fixed wavelength. All data are presented as mean $\pm$ standard deviation. The Students t-test was performed in some cases and a p value <0.05 was considered significant.

Results and Discussion

Shear-induced exposure of VWF hydrophobic domains

We used bis-ANS binding assay to detect shear-induced conformational changes in VWF by investigating the exposure of hydrophobic domains on its surface that is often correlated with alterations in protein function [11-12,15]. The effect of shear on the fluorescence intensity of the probe-protein solution was examined by comparing the

fluorescence signal when either VWF or the control protein BSA was sheared at G=9600/s with that of the non-sheared protein.

Figures 1A and 1B are representative spectra of experiments performed with VWF and BSA, respectively. $F^{(G)}$ was measured for protein sheared at G=9600/s for 5 min (black patterned line) and this was compared to non-sheared sample (G=0/s, grey patterned line). In all cases, bis-ANS was added exactly 1 min after shear stoppage and data were recorded 1 min after that. The fluorescence intensity was low for Hepes buffer containing bis-ANS alone, Hepes buffer alone and protein alone samples. In Figure 1A, a shift towards lower wavelengths is observed upon addition of protein since $F^{(G)}_{max}$ appears at 490 nm in the presence of VWF and at 508 nm for dye alone in Hepes buffer. This suggests that the probe binds protein hydrophobic domains. $F^{(G)}_{max}$ increased by ~335% upon applying a G of 9600/s to VWF. This enhancement in fluorescence signal and probe binding is indicative of exposure of new hydrophobic domains in VWF subjected to shear. These new sites were likely inaccessible in the native protein. For the control protein BSA (Figure 1B), there was no significant change in $F^{(G)}_{max}$ intensity after applying high G (9600/s). Thus, BSA does not undergo conformation change and the signal change in Figure 1A is protein specific.

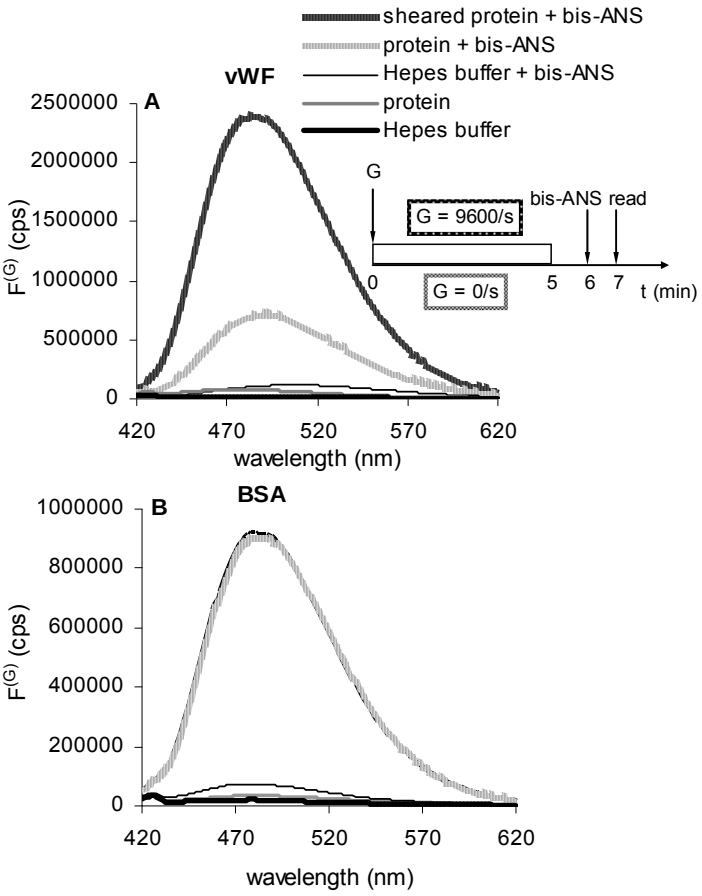

Figure 1. Shear-induced protein conformational changes detected by fluorescence of bis-ANS for (A) VWF and (B) BSA. The schematic presents the experimental protocol indicating the shear rate (G), the shear time, time of the probe addition and time of measurement.

Time and shear – dependent enhancement in bis-ANS binding

The physiological functions of VWF, including its binding interaction with blood platelets during atherothrombosis, are regulated by the magnitude of applied fluid shear. By using Annexin-V as a marker of platelet activation in a previous study of our group [14] significant platelet activation was mediated via GpIb-VWF interactions upon high shear (*G*=9600/s) even at the very low t_{sh} of 10 s. Higher G and t_{sh} were found to increase the extent of platelet activation [16]. By comparing the data from those studies, we conclude that VWF solution structure may undergo shear-induced conformational changes that occur at small time scales and are amplified at higher t_{sh}, relevant with the platelet activation.

Here, we apply the bis-ANS binding assay to find the G and t_{sh} at which conformational changes in VWF solution structure begin and also to quantify those changes.

Shear time effect

We examined the effect of t_{sh} on probe-binding and VWF conformation change. To this end, studies were performed at 9600/s with t_{sh} between 1 and 5 min (Figure 2A and 2B). $F^{(G)}$ was measured and normalized with respect to the protein concentration. The signal increases due to bis-ANS binding to VWF, but not to the control protein BSA, in a time-dependent manner. This change is observed in Figure 2A where there is an increase in the fluorescence intensity in the emission spectra with increasing the t_{sh}. Thus, the exposure of hydrophobic domains in VWF is enhanced with time. This increase in the fluorescence signal is also shown in Figure 2B that presents $F_c^{(G)}{}_{max}$ at different t_{sh}. No conformational change was observed for BSA since in Figure 2B the $F_c^{(G)}{}_{max}$ value is similar for t_{sh} of 1 and 5 min.

Shear rate effect

G values over the range of physiological and pathological conditions were applied to VWF to examine its solution structural changes. Upon step-increasing G, the bis-ANS fluorescence signal is increasing with shear, indicating a progressive increase in the extent of protein conformation change. Figures 2C and 2D describe the effect of varying G (0-9600 /s) over a 5 min time interval demonstrating that the level of bis-ANS binding to VWF increases with increasing G. Probe binding was not altered upon shearing the control protein BSA. Emission spectra of VWF samples are shown in Figure 2C. These same data are presented in Figure 2D that presents $F_c^{(G)}{}_{max}$ at different G. Thus, these studies measure the extent of hydrophobic domain exposure as a function of G. We observed that a minimum G of 6000/s is required for augmented probe binding, giving a 3-fold increase in the fluorescence signal. A higher G of 9600/s causes further enhancement of fluorescence intensity; a 4-fold increase of the probe signal is observed. These findings suggest that VWF specific conformational changes in solution that accompany hydrophobic domain exposure increase with shear above 6000/s in a shear-dependent manner.

Those results are in agreement with our SANS studies where the amount of neutron scattering also increases with G due to extended structural changes at the highest G that were used in these experiments. Both studies are indicative of the fact that VWF conformation changes are dependent on the magnitude of the applied shear. However, in our studies with bis-ANS as well as with SANS low levels of structural changes were observed at G below 2300/s, whereas at higher G high levels of VWF hydrophobic domain exposure were observed, giving an enhanced bis-ANS fluorescence signal.

It is important to compare our results with the literature, which shows that above a threshold G between 2000/s and 5000/s, fluorescently-tagged VWF structure changes from a compact to an elongated one [7]. From those results, it is suggested that at low, physiological G (<3000/s) small length scale changes occur due to rearrangement of domain level features, while at higher G (>2300/s) large length scale changes expose VWF hydrophobic domains, showing that the former ones may be a prerequisite for the second ones. Also, a study shows that the different domains of VWF show different stability since it was found that the VWF A2 domain may be most susceptible to unfolding than other VWF A domains [17].

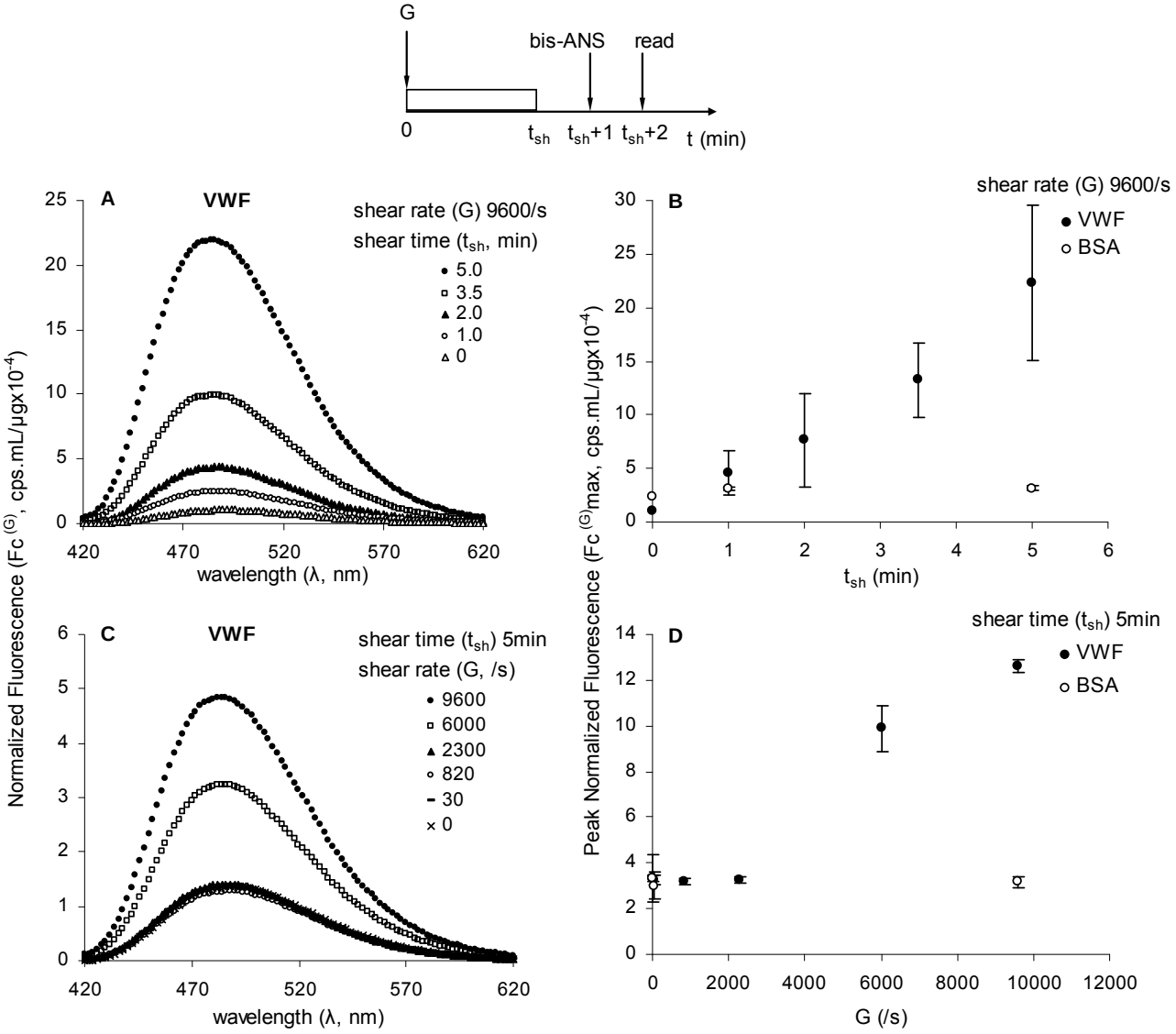

Figure 2. Shear and time dependence. A. Fluid shear was applied to VWF at 9600/s for various shear times (t_{sh}). Bis-ANS was added 1 min after shear stoppage and fluorescence spectra were measured 1 min thereafter as illustrated in the schematic. Normalized fluorescence due to bis-ANS VWF interaction $F_c^{(G)}$ was calculated as described in Experimental Part. **B.** Peak $F_c^{(G)}$ value at a fixed wavelength ($F_c^{(G)}{}_{max}$) is presented for 3 experiments. **C.** Studies similar to panel A were performed at a fixed t_{sh} (5 min) and the magnitude of fluid shear (G) was varied. **D.** Peak $F_c^{(G)}$ value at a fixed wavelength ($F_c^{(G)}{}_{max}$) is presented for 3 experiments.

Conclusions

A new, easy to use, technique is introduced here to investigate shear-induced conformational changes in large length scale features of VWF and measure/quantify their strength at different G and t_{sh}. Shear-induced exposure of VWF hydrophobic domains was observed at high G up to 9600/s and t_{sh} up to 5 min, indicated by the increase of the binding of the fluorescence probe bis-ANS to the sheared VWF compared to the non-sheared protein. The current work suggests that structural changes at large length scales in VWF are more obvious from physiological to high G and at t_{sh} higher than 1 min, whereas they are more dominant at higher t_{sh} and G. Such conformational changes may regulate alterations in VWF function upon shear [4,6,8], and enhance VWF binding to hydrophobic substrates and collagen [7,8], or VWF self-association [14]. The implications of these structural changes in VWF to arterial thrombosis are currently under investigation in our laboratory.

Acknowledgement

This work was supported by NIH grant HL77258.

References

1. Sadler, J. E. “Biochemistry and genetics of von Willebrand factor” *Annu. Rev. Biochem.* **1998**, *67*, 395-424.
2. Fowler, W. E.; Fretto, L. J.; Hamilton, K. K.; Erickson, H. P.; McKee, P. A. “Substructure of human von Willebrand factor” *J. Clin. Invest.***1986**, *76*, 1491-1500.
3. Levy, G. G.; Nichols, W. C.; Lian, E. C.; Foroud, T.; McClintick, J. N.; McGee, B. M.; Yang, A. Y.; Siemieniak, D. R.; Stark, K. R.; Gruppo, R.; Sarode, R.; Shurin, S. B.; Chandrasekaran, V.; Stabler, S. P.; Sabio, H.; Bouhassira, E. E.; Upshaw, J. D.; Jr.; Ginsburg, D.; Tsai, H. M. “Mutations in a member of the *ADAMTS* gene family cause thrombotic thrombocytopenic purpura” *Nature* **2001**, *413*, 488-494.
4. Siedlecki, C. A.; Lestini, B. J.; Kottke-Marchant, K. K.; Eppell, S. J.; Wilson, D. L.; Marchant, R. E. “Shear-dependent changes in the three-dimensional structure of human von Willebrand factor” *Blood* **1996**, *88*, 2939-2950.
5. Tsai, H. M. “Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion” *Blood* **1996**, *87*, 4235-4244.
6. Tsai, H. M.; Sussman I. I.; Nagel, R. L. “Shear stress enhances the proteolysis of von Willebrand factor in normal plasma” *Blood* **1994**, *83*, 2171-2179.
7. Schneider, S. W.; Nuschele, S.; Wixforth, A.; Gorzelanny, C.; Alexander-Katz, A.; Netz, R. R.; Schneider, M. F. “Shear-induced unfolding triggers adhesion of von Willebrand factor fibers” *Proc. Nat. Acad. Sci. USA* **2007**, *104*, 7899-7903.
8. Novak, L.; Deckmyn, H.; Damjanovich, S.; Harsfalvi, J. “Shear-dependent morphology of von Willebrand factor bound to immobilized collagen” *Blood* **2002**, *99*, 2070-2076.
9. Singh, I.; Shankaran, H.; Beauharnois, M. E.; Xiao, Z.; Alexandridis, P.; Neelamegham, S. “Solution structure of human von Willebrand factor studied using small angle neutron scattering” *J. Biol. Chem.* **2006**, *281*, 38266-38275.

10. Kasirer-Friede, A.; Cozzi, M. R.; Mazzucato, M.; De Marco, L.; Ruggeri, Z. M.; Shattil, S. J. "Signaling through GP Ib-IX-V activates αIIbβ3 independently of other receptors" *Blood* **2004**, *103*, 3403-3411.
11. Kosinski-Collins, M. S.; King, J. "In vitro unfolding, refolding, and polymerization of human gammaD crystallin, a protein involved in cataract formation" *Protein Sci.* **2003**, *12*, 480-490.
12. James, E. L.; Bottomley, S. P. "The mechanism of alpha 1-antitrypsin polymerization probed by fluorescence spectroscopy" *Arch. Biochem. Biophys.* **1998,** *356*, 296-300.
13. Balasubramanian, S. V.; Bruenn, J.; Straubinger, R. M. "Liposomes as Formulation Excipients for Protein Pharmaceuticals: A Model Protein Study" *Pharm. Res.* **2000***, 17,* 344-350.
14. Shankaran, H.; Alexandridis, P.; Neelamegham, S. "Aspects of hydrodynamic shear regulating shear-induced platelet activation and self-association of von Willebrand factor in suspension" Blood **2003**, *101*, 2637-2645.
15. Pierce, A.; deWaal, E.; Van Remmen, H.; Richardson, A. Chaudhuri, A. "A novel approach for screening the proteome for changes in protein conformation" *Biochemistry* **2006**, *45*, 3077-3085.
16. Zhang, J. N.; Bergeron, A. L.; Yu, Q.; Sun, C.; McIntire, L. V.; Lopez, J. A.; Dong, J. F. "Platelet aggregation and activation under complex patterns of shear stress" *Thromb. Haemost.* **2002**, *88*, 817-821.
17. Auton, M.; Cruz, M. A.; Moake J. "Conformational stability and domain unfolding of the Von Willebrand factor A domains" *J. Mol. Biol.* **2007**, *366*, 986-1000.