THE ROLE OF DISULFIDE BOND FORMATION IN GPIB-FC AGGREGATION

Tyler Carter[1]; Prachi Bhoskar[1]; Jing Wen[1]; Hanwei Zhao[1]; Stephen Raso[2]; Thomas Porter[2]; Jin Xu[1]

[1]University of Massachusetts Lowell, Lowell, MA; [2]Pfizer Inc., Andover, MA

Abstract

Glycoprotein Ibα (GPIbα), the major component of the platelet membrane bound GPIb-IX-V receptor complex, plays a key role in platelet aggregation and thrombus formation by binding to von Willebrand factor (VWF) on exposed subendothelial collagen following vascular injury. There are 7 cysteines in each GPIbα(1-290 amino acid) molecule with a free thiol group at Cys^{65}. It has been speculated that the unpaired Cys^{65} residue could cause *in vitro* GPIbα aggregation. The mechanism of GPIbα aggregation was studied using a recombinant GPIbα-Fc fusion protein (GPIb-Fc) and GPIbα. GPIb-Fc aggregates were SDS-stable and reducible on SDS-PAGE, and purified aggregates were stable in MALDI mass spectrometry, indicating that aggregation was caused by covalent disulfide bond formation. Based on analytical ultracentrifugation and on-line size exclusion HPLC-light scattering data, aggregates ranged in size from dimer to 20mer. Far-UV circular dichroism and intrinsic Trp fluorescence spectroscopy revealed an altered GPIbα structure in the aggregates. Additionally, these aggregates were inactive in *in vitro* VWF binding by surface plasmon resonance (SPR, Biacore). Heat-stress stability studies demonstrated increased covalent aggregation at higher pH and partially denaturing conditions. Experiments using GPIb-Fc containing a single amino acid mutation (Cys^{65}Ser) confirmed the role of the unpaired Cys residue in initiating GPIb-Fc aggregation. The thiol labeling ratio was similar for both monomeric and aggregated GPIb-Fc, indicating that aggregation was caused by thiol-mediated disulfide shuffling instead of disulfide bond formation between free thiol groups. To further elucidate the aggregation pathway, peptide mapping was performed on tetramethylrhodamine (TMR)-maleimide labeled monomeric and aggregated GPIbα. The results demonstrated that all three GPIbα disulfide bonds could potentially be scrambled by the unpaired Cys and form intermolecular disulfide bonds. These results serve as a case study for fusion protein aggregation. This unique aggregation pathway may possibly have physiological implications.

Methodology – Initial Heat Stress Study

Size Analyses

Size Exclusion Chromatography (SEC): GPIb-Fc at physiological conditions (PBS pH 7.2) was incubated for 15 hours at 37°C. Heat-stressed GPIb-Fc was injected at 2.5 hour intervals onto a Waters HPLC system equipped with a Tosoh TSKgel G3000SWxl column. The mobile phase was 10 mM tris pH 7.2, 150 mM sodium chloride and was delivered at a flow rate of 1 mL/min. UV absorbance was monitored at 280 nm.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): SEC-purified GPIb-Fc and aggregates were mixed with 4X LDS Sample Buffer (Invitrogen) with or without 5 mM dithiothreitol (for reducing and nonreducing conditions, respectively), incubated at 70°C for 10 minutes and subsequently loaded onto a NuPAGE Novex 4-20% Tris-Glycine gel (Invitrogen) with flanking molecular weight markers. The program was 200 V for 35 minutes. After thrice rinsing with deionized water, the gel was stained with SimplyBlue SafeStain (Invitrogen) for one hour before being destained overnight with deionized water.

Analytical Ultracentrifugation (AUC): SEC-purified GPIb-Fc aggregates were analyzed by AUC against a GPIb-Fc control. The instrument was a Beckman Proteomelab XL-1.

Multiangle Laser Light Scattering (MALLS): Aggregated GPIb-Fc was analyzed by SEC coupled with MALLS. The instrument configuration included a Dawn EOS and Optilab DSP Interferometric Refractometer.

Structural Analyses

Intrinsic Trytophan Fluorescence: Aggregated GPIb-Fc was analyzed by intrinsic tryptophan fluorescence against a non-stressed GPIb-Fc control. The instrument was a Perkin Elmer LS55 fluorescence spectrophotometer. The spectra ranged from 300 to 450 nm with excitation and emission bandwidths of 10 and 5 nm, respectively. The PMT voltage and scanning speed settings were 700 V and 5 nm/min, respectively.

Far-UV Circular Dichroism (CD): Aggregated GPIb-Fc was analyzed by far-UV CD against a non-stressed GPIb-Fc control. The instrument was a Jasco J-810 Spectropolarimeter. The data were collected from 190 nm to 250 nm with a bandwidth of 1 nm, data pitch at 0.1 nm and scanning speed of 5 nm/min.

Functional Analysis

Surface Plasmon Resonance (SPR): Aggregated GPIb-Fc was analyzed by surface plasmon resonance against a monomeric GPIb-Fc control. The instrument used was a Biacore 3000. Aggregated and control GPIb-Fc were immobilized on a sensor chip coated with Protein A, after which VWF was passed through the flow cell.

Sizing Analyses – SEC & SDS-PAGE

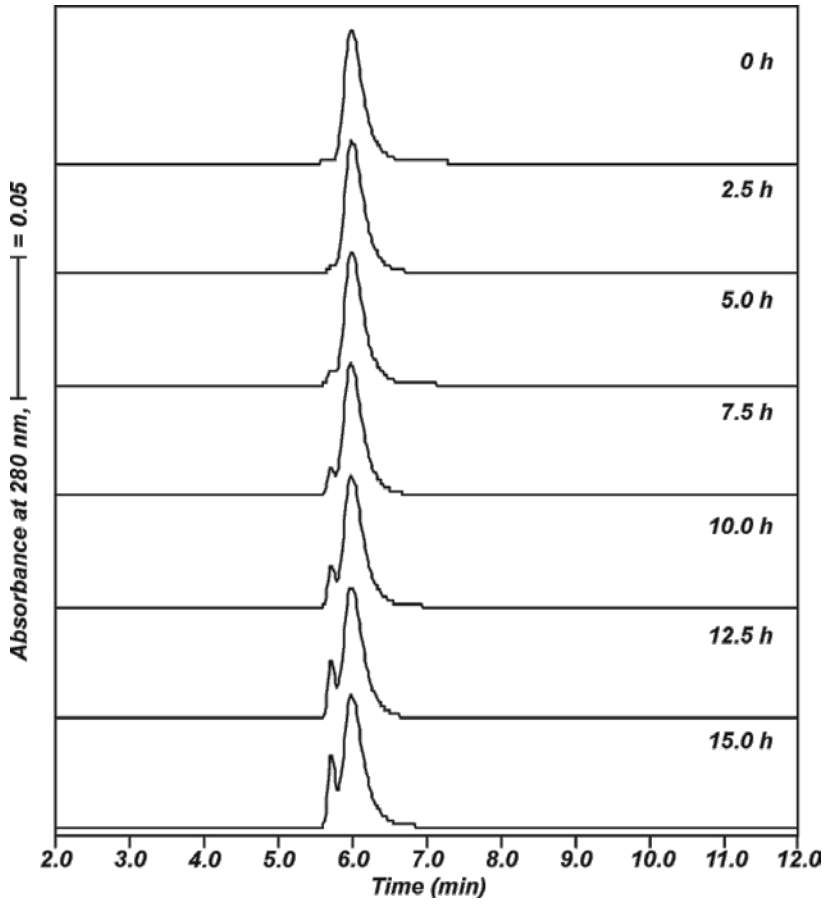


Figure 1. SEC Overlays of Heat-Stressed GPIb-Fc.

Significant aggregation was observed after incubating at 37°C for 15 hours. GPIb-Fc monomer and high molecular weight (HMW) fractions were collected for further analysis.

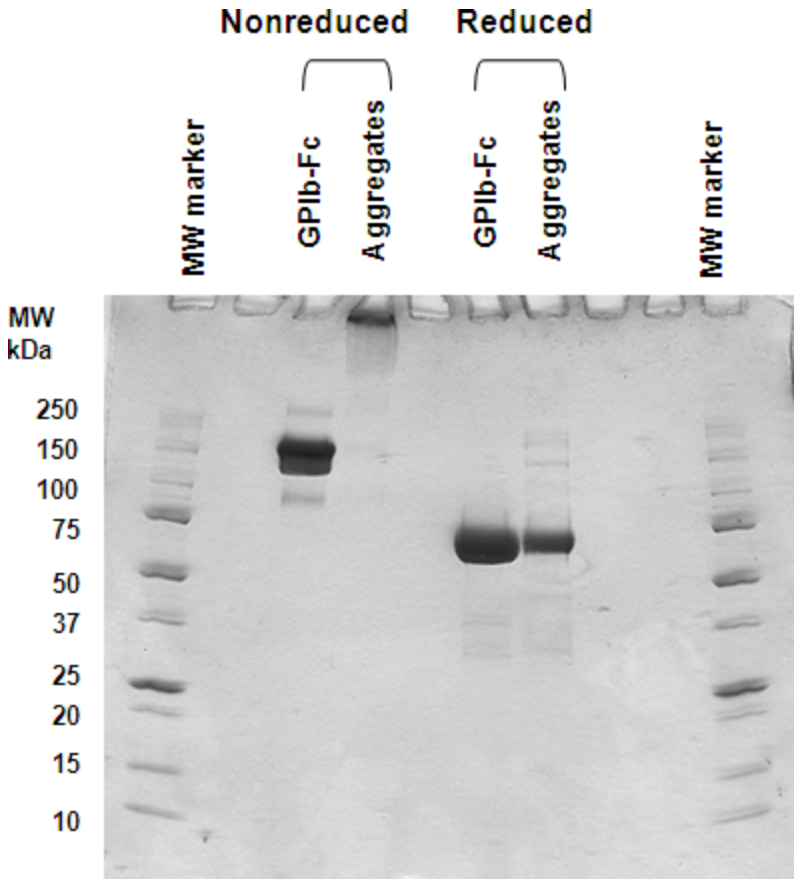


Figure 2. SDS-PAGE Analysis of GPIb-Fc Aggregates.

The SEC-purified GPIb-Fc aggregates were SDS-stable, but reducible to monomeric GPIb-Fc.

Sizing Analyses – SEC-MALLS & AUC

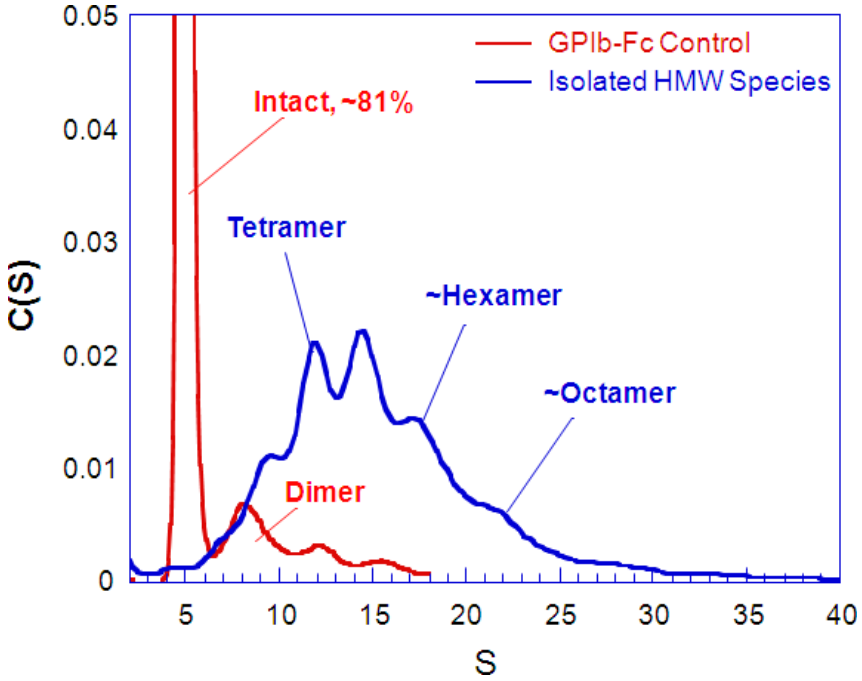


Figure 3. Native AUC of SEC-Purified GPIb-Fc Aggregates.

GPIb-Fc aggregates ranged in size from dimer to larger oligomers.

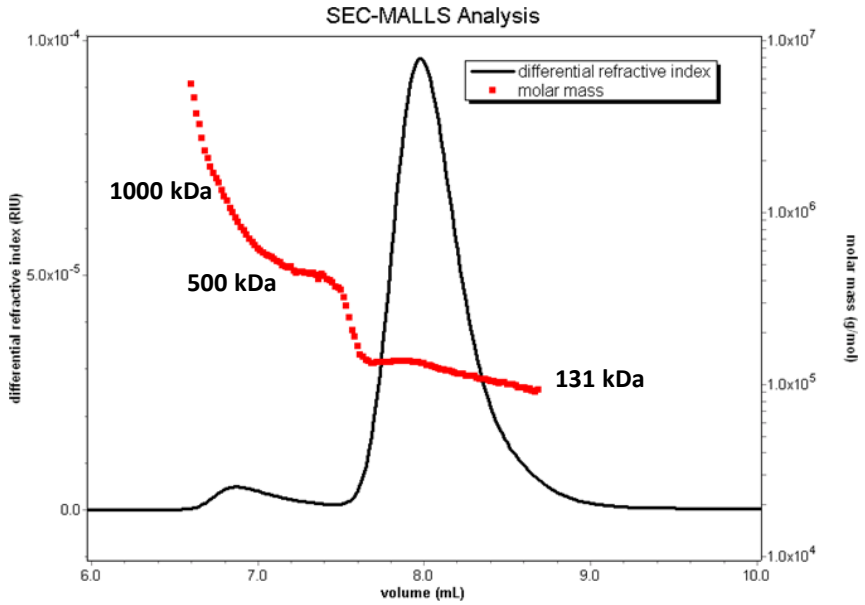


Figure 4. SEC-MALLS Analysis of Heat-Stressed GPIb-Fc.

The most dilineated GPIb-Fc aggregate was tetramer (~500 kDa), above which the exclusion limit of the column resulted in a broad distribution of oligomers.

Structural Analyses

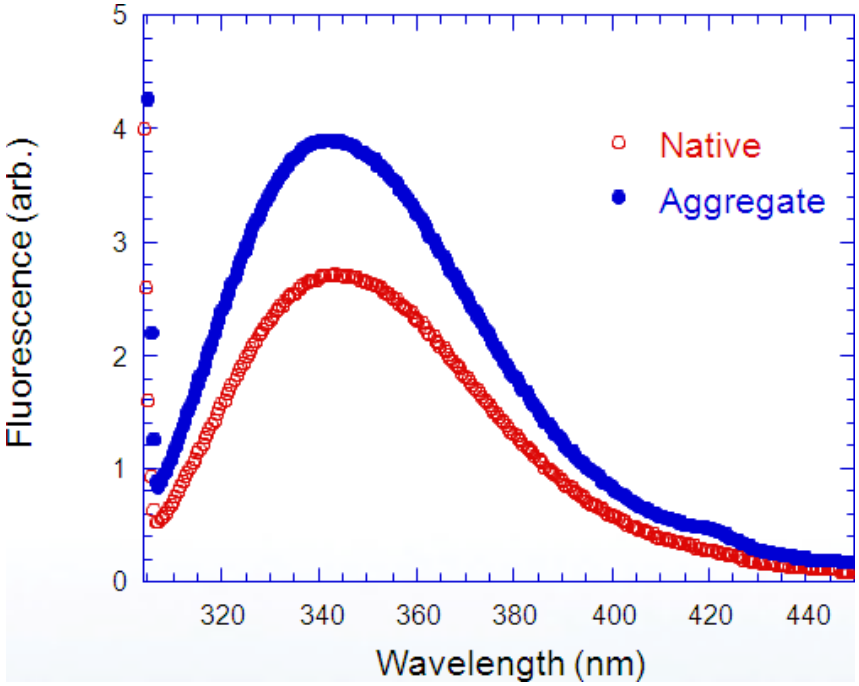


Figure 5. Intrinsic Tryptophan Fluorescence of GPIb-Fc Aggregates.

A shift in fluorescence intensity ~345nm was indicative of significant structural change.

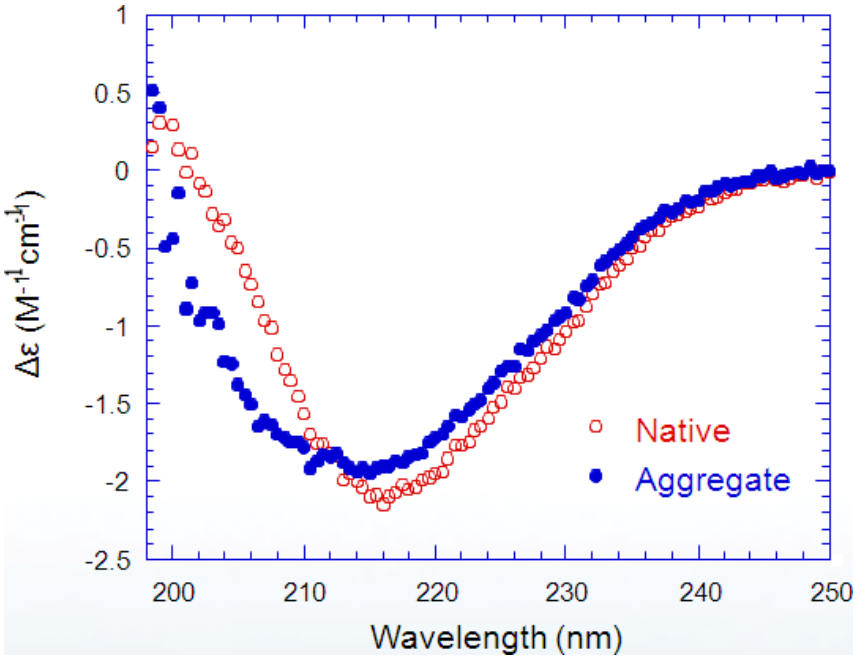


Figure 6. Far-UV CD of GPIb-Fc Aggregates.

The GPIb-Fc spectrum features a minima ~216 nm and maxima ~198 nm, indicative of strong β-sheet character. For the GPIb-Fc aggregates, a shift was observed for this minima and maxima, as well as for the overall far-UV spectrum, which was indicative of significant structural change.

Functional Analyses

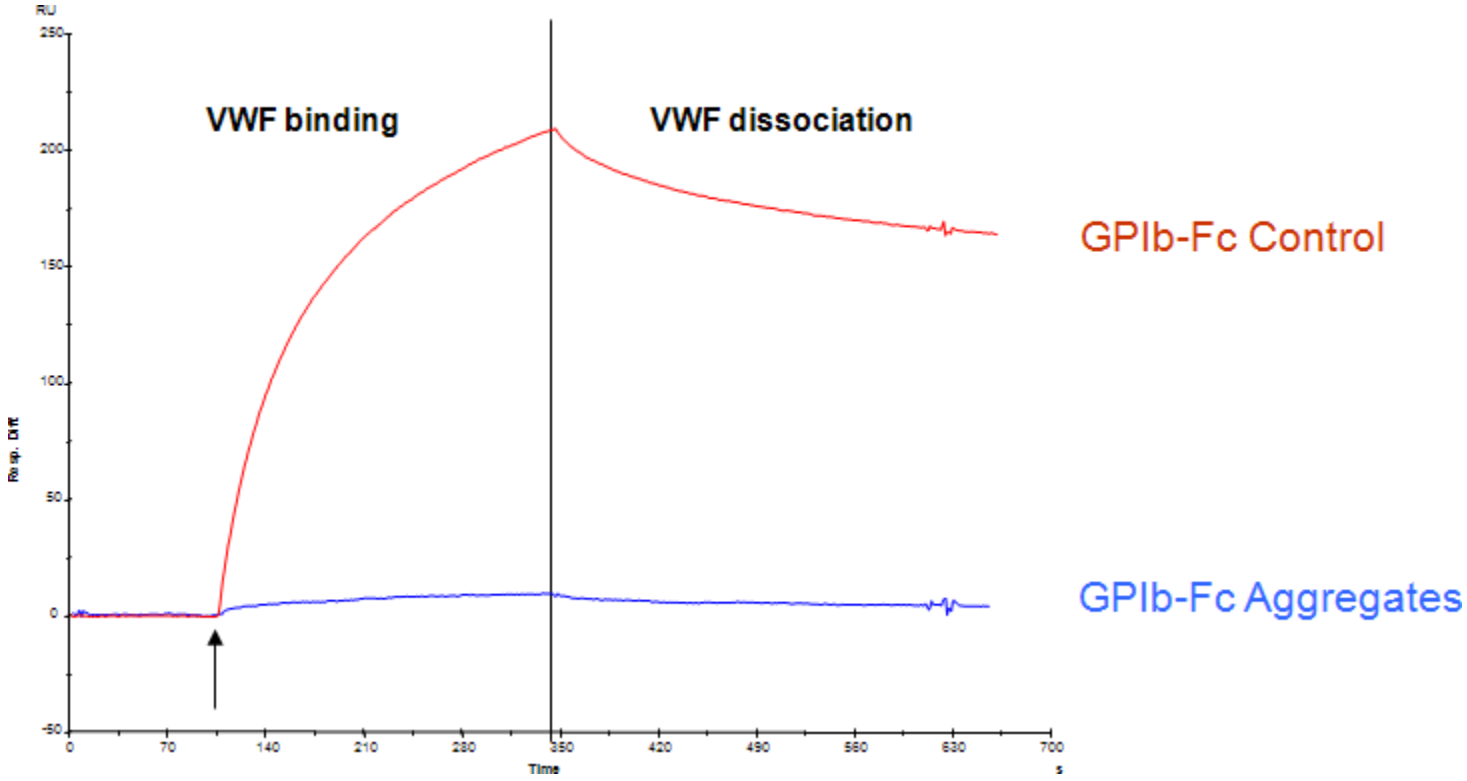


Figure 7. VWF Binding by SPR Analysis.

GPIb-Fc aggregates are essentially unable to bind VWF.

Methodology – Determining GPIb-Fc Aggregation Mechanism

Heat-Stressing of Alkylated/Denatured GPIb-Fc: To determine whether thiol groups were involved in GPIb-Fc aggregation, GPIb-Fc was separately alkylated and denatured with 10 mM iodoacetamide and 2 M guanidine hydrochloride conditions and incubated at 40°C for 20 hours. Controls included heat-stressed and non-stressed GPIb-Fc. All samples and controls were subsequently analyzed by SEC and reducing/nonreducing SDS-PAGE.

Heat-Stressing of Cys^{65}-Ser Mutant: To ascertain the involvement of the free thiol in GPIb-Fc aggregation, GPIb-Fc engineered with a Cys^{65}-Ser mutation was heat-stressed at 40°C for 20 hours along with an original GPIb-Fc control. Other controls included non-stressed original and mutant GPIb-Fc. The sample and controls were subsequently analyzed by SEC and reducing/nonreducing SDS-PAGE.

GPIbα Aggregation Properties: To delineate the GPIb-Fc thiol aggregation pathway, GPIb-Fc was digested 10:1 (w:w) with mocarhagin for 30 minutes at 37°C, after which GPIbα was purified by protein A chromatography. GPIbα was heat-stressed at 40°C for 20 hours, with non-stressed GPIbα serving as a control. The sample and control were subsequently analyzed by SEC and reducing/nonreducing SDS-PAGE.

Thiol Labeling by TMR-Maleimide/LC-MS Peptide Mapping: GPIb-Fc and GPIb-Fc aggregates were labeled with TMR-maleimide at molar ratios of 1:10 and 1:100 under native and denaturing conditions, and their labeling ratios quantified. To determine the disulfide bond shuffling mechanism initiated by Cys^{65}, TMR-labeled GPIbα aggregates and control GPIbα were subsequently digested with Lys-C and analyzed on a Waters Symmetry300 C18 (3.9 x 150 mm, 5 µm) and Waters ESI-TOF MS system.

Location of Cys^{65}

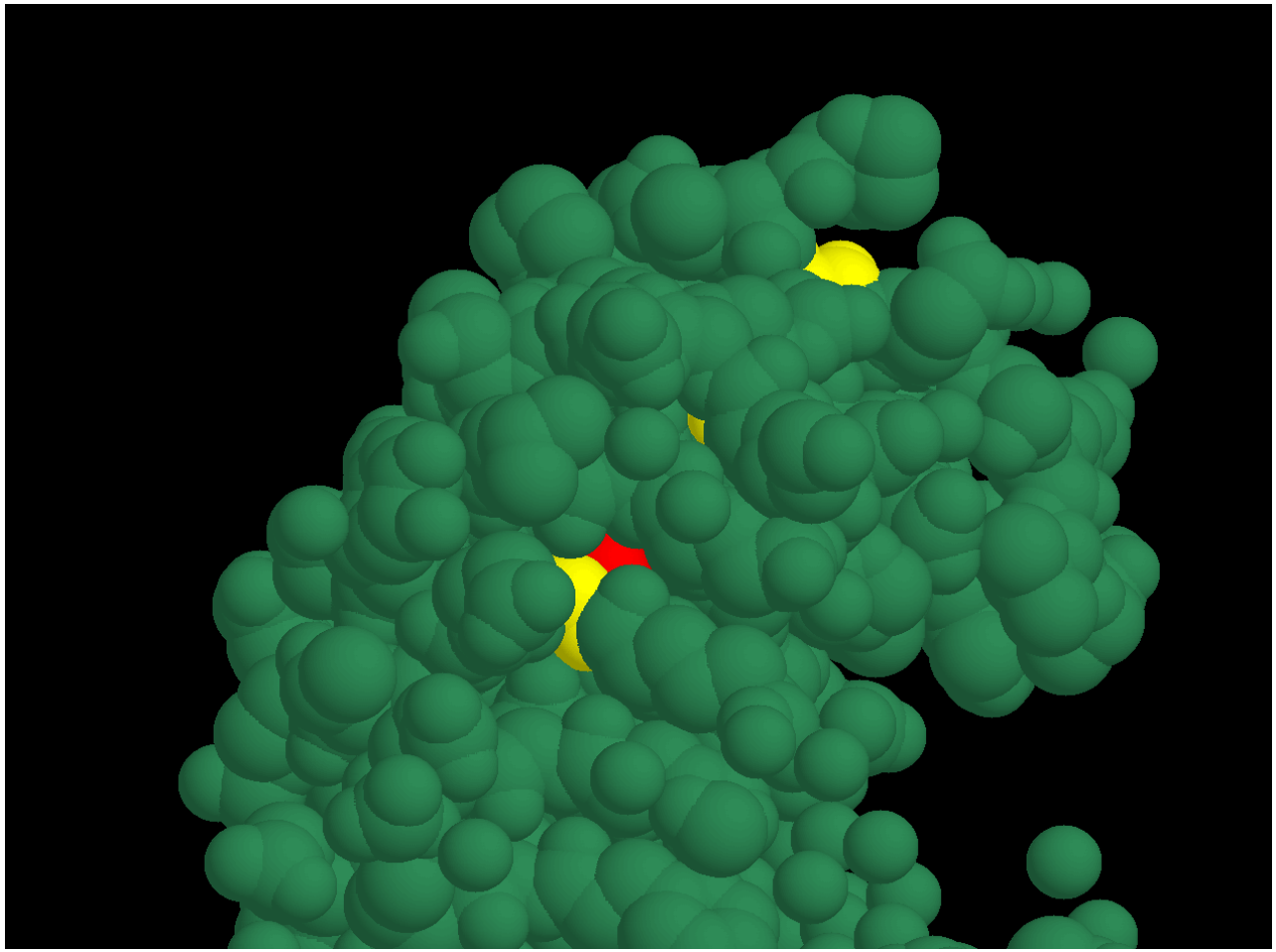
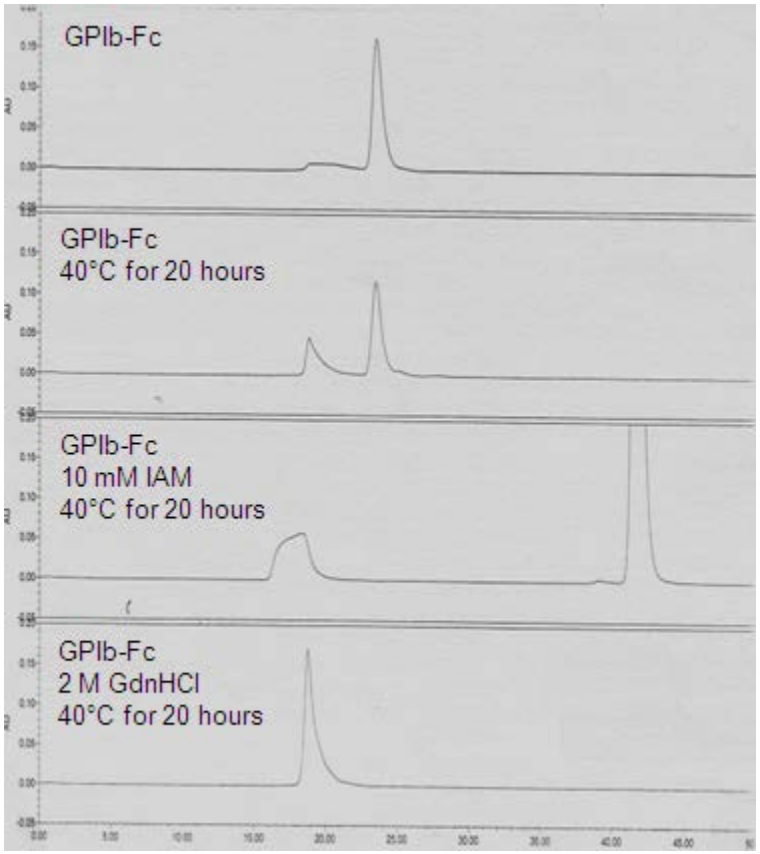


Figure 8. Location of Cys^{65} in GPIbα Crystal Structure.

Aggregation of Alkylated/Denatured GPIb-Fc



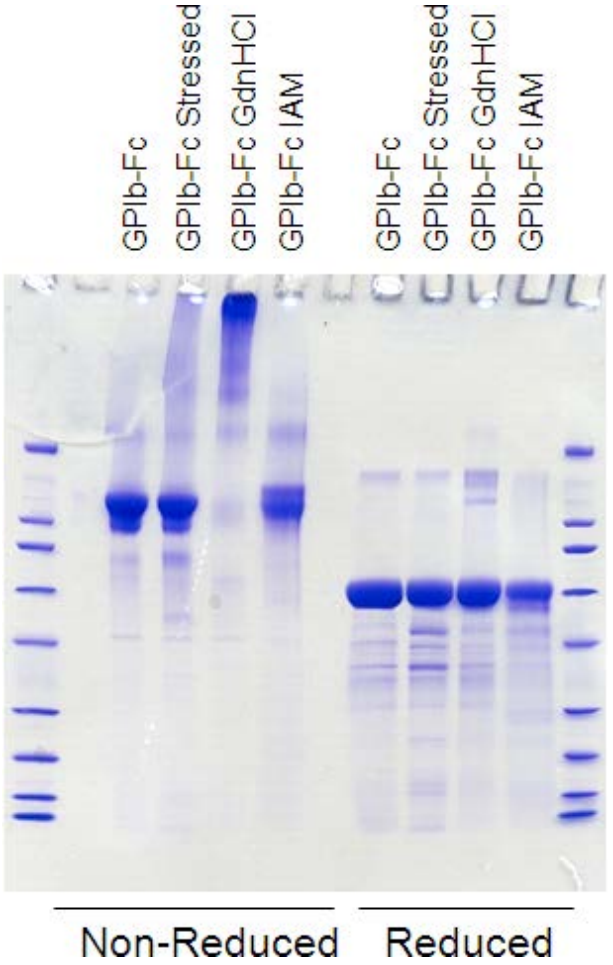
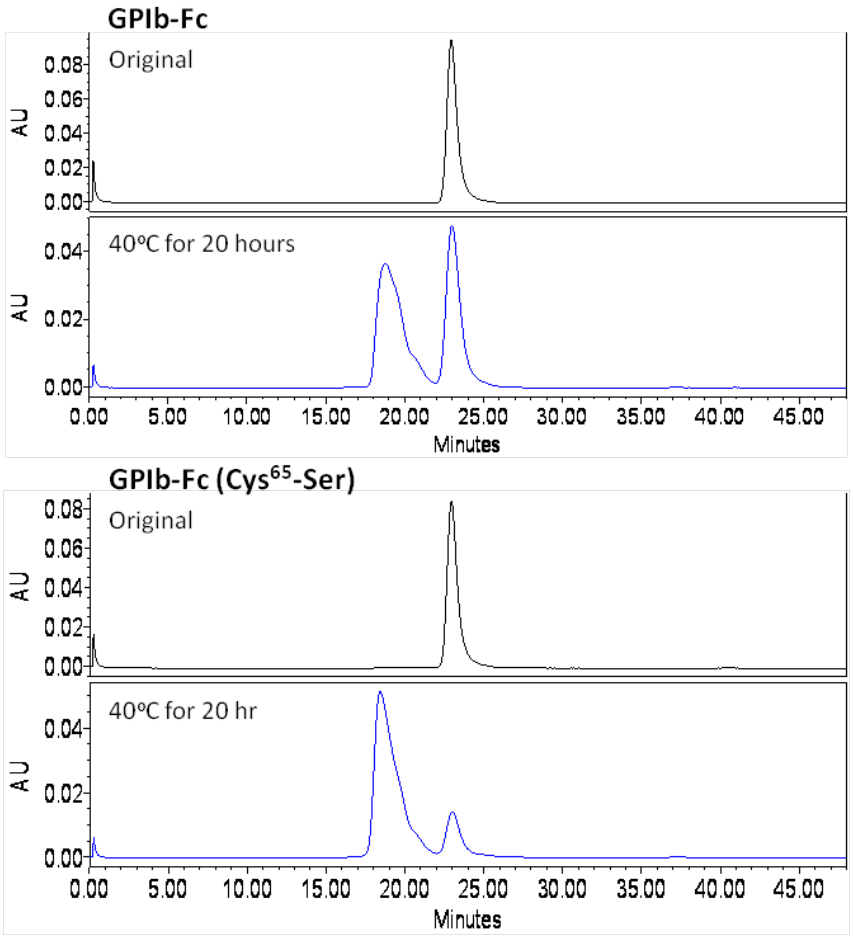


Figure 9. SEC/SDS-PAGE Analyses of Heat-Stressed Alkylated/Denatured GPIb-Fc.

Thiol groups were involved in covalent aggregation; however, significant noncovalent aggregation was also evident in the presence of IAM.

Aggregation of GPIb-Fc ($Cys^{65}Ser$) Mutant



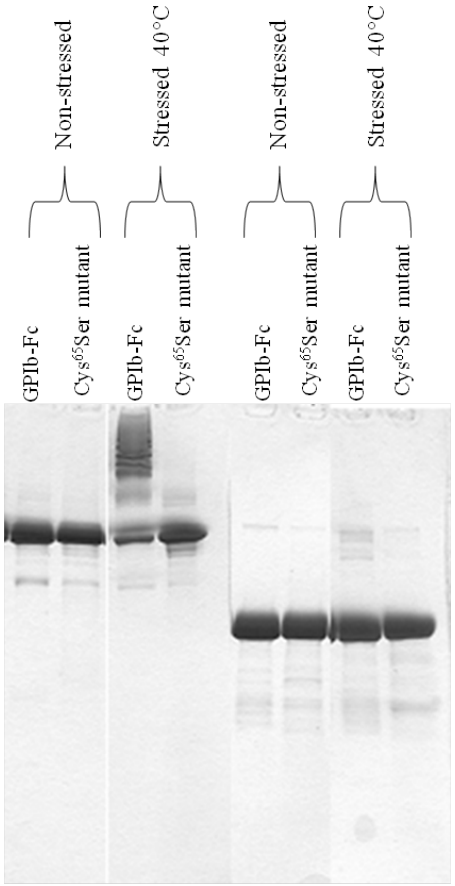


Figure 10. SEC/SDS-PAGE Analyses of Heat-Stressed GPIb-Fc (Cys^{65}-Ser) Mutant.

While aggregates were observed for the heat-stressed Cys^{65}-Ser mutant on SEC, the absence of aggregates on SDS-PAGE suggests these aggregates are noncovalent/SDS-dissociable. This indicates that Cys^{65} initiates covalent aggregation in GPIb-Fc.

Potential Aggregation Pathways

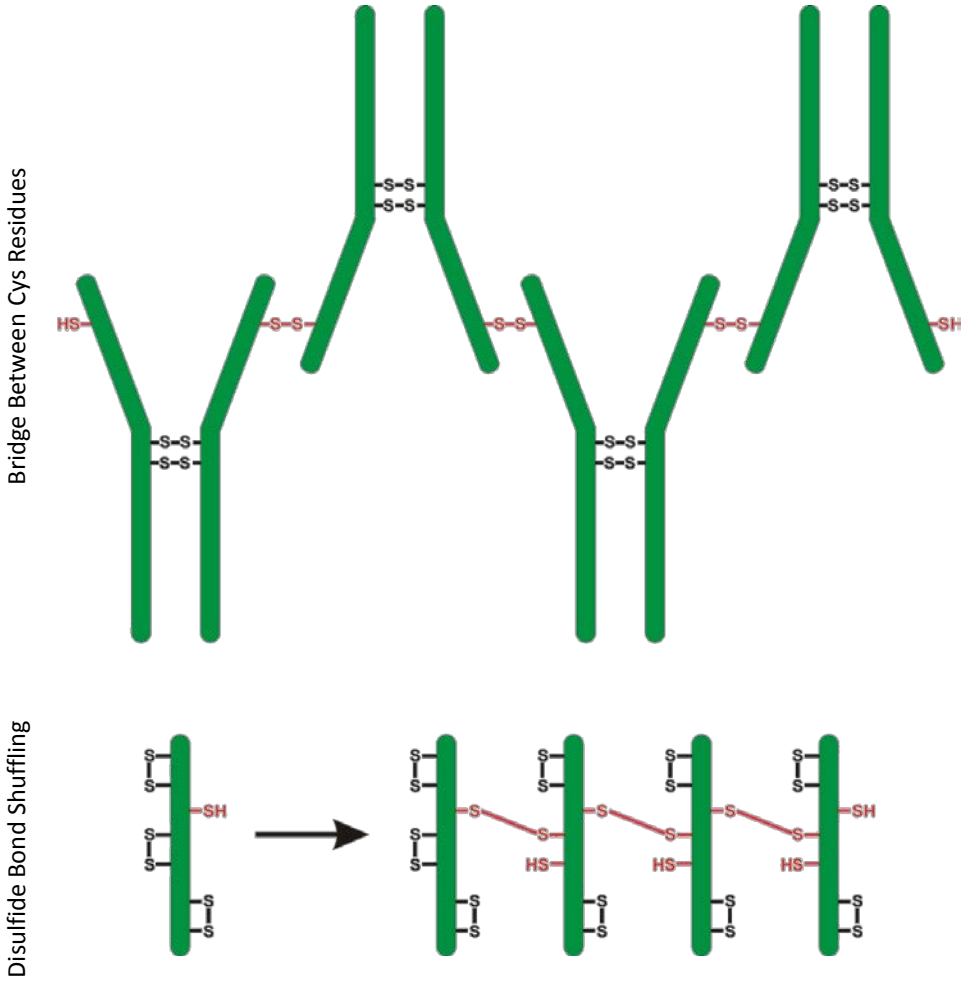
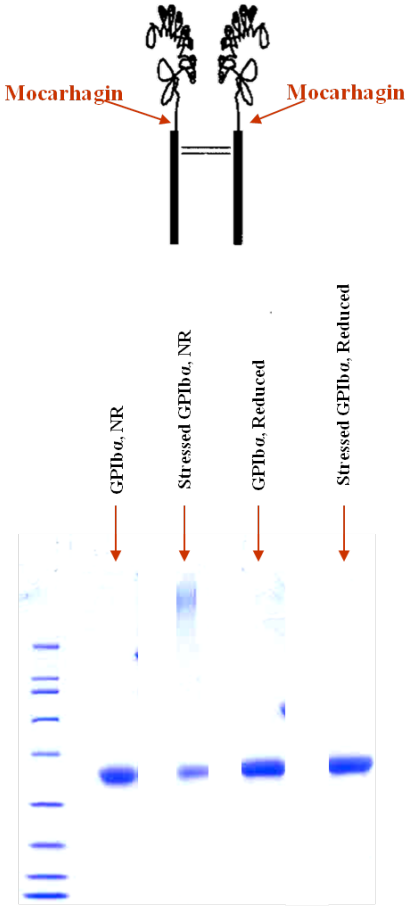


Figure 11. Potential GPIb-Fc aggregation pathways initiated by Cys[65].

GPIbα Aggregation Properties



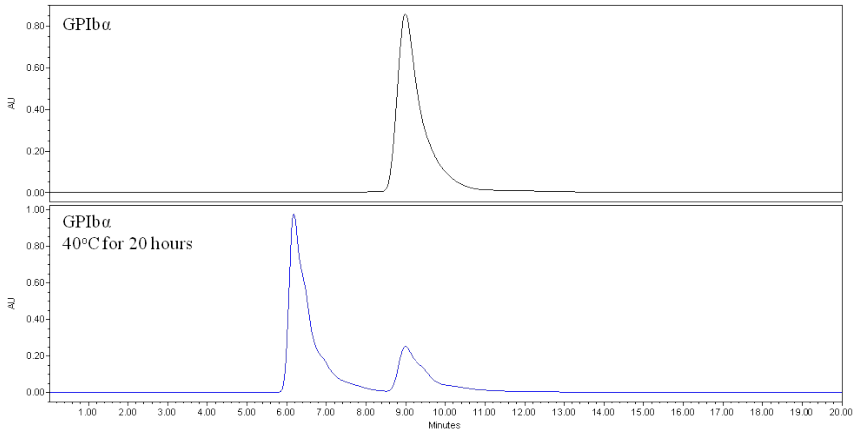


Figure 12. SEC/SDS-PAGE Analyses of Heat-Stressed GPIbα Monomer

SEC/SDS-PAGE analysis of heat-stressed GPIbα was consistent with the disulfide bond shuffling pathway.

Thiol Labeling by TMR-Maleimide

	Sample	TMR-Maleimide:Protein, mol/mol	
		10:1	100:1
Labeling Ratio	GPIbα	0.47	0.81
	HMW	0.44	0.78

Table 1. Thiol-Labeling Analysis of GPIbα Monomer and Aggregates.

The labeling ratios did not differ significantly between GPIbα and GPIbα aggregates, indicating that the total thiol content remained constant upon aggregation.

Aggregation of GPIb-Fc ($Cys^{65}Ser$) Mutant

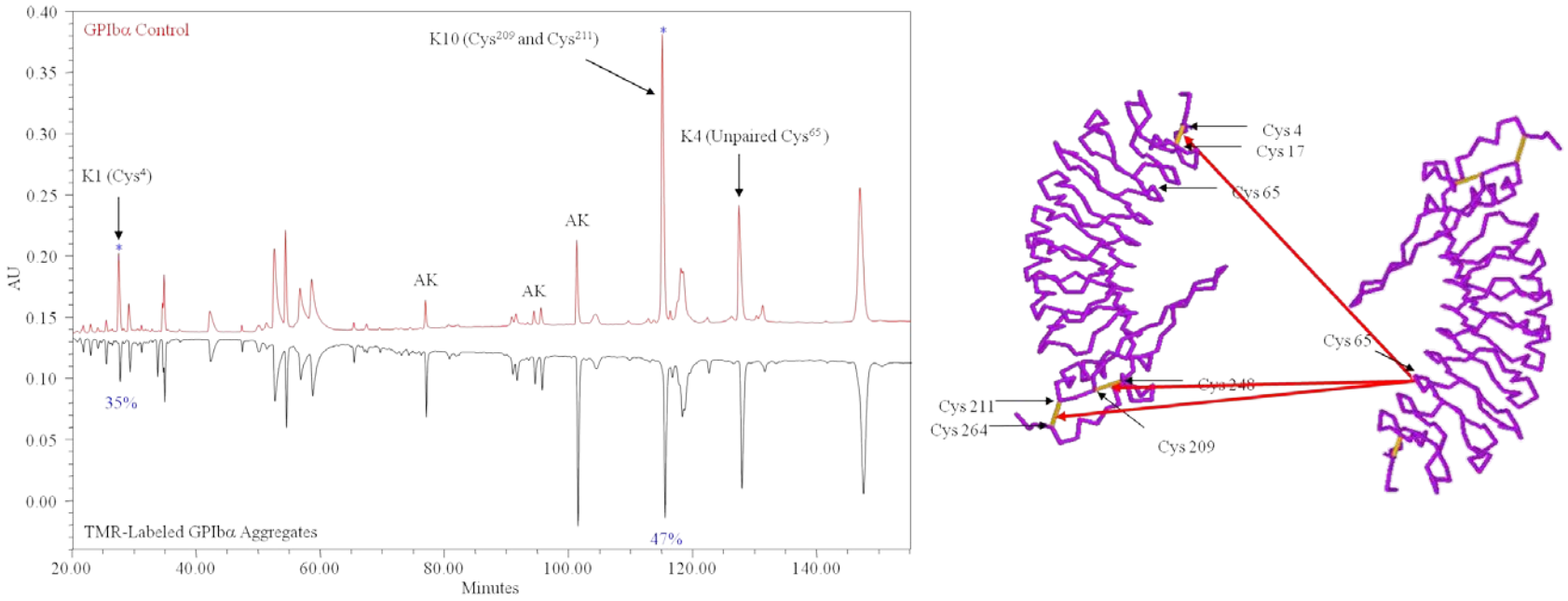


Figure 13. C18 ESI-TOFF MS Peptide Mapping of TMR-Labeled GPIb-Fc Aggregates.

The reduced prevalence of the K1 and K10 peptides for the GPIbα aggregates indicated that their respective cysteines (Cys^{4} for K1, Cys^{209} and Cys^{211} for K10) underwent disulfide bond shuffling initiated by Cys^{65}. The structure to the right depicts the disulfide bonds susceptible to Cys^{65} attack.

Conclusions

- GPIb-Fc aggregates are covalent and range in size from dimer to octomer.
- The aggregates have non-native higher order protein structure and are not active.
- Unpaired Cys^{65} attacks disulfide bonds, leading to intermolecular disulfide bond shuffling and covalent aggregation.