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Identification of cysteine thiol-based linkages in ADAMTS13 in support of a non-proteolytic regulation of von Willebrand factor

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Abstract

Background: ADAMTS13, a plasma metalloprotease, cleaves von Willebrand factor (VWF) to regulate its function. Additionally, ADAMTS13 is thought to regulate lateral association of VWF multimers to form fibrillar structures through its free thiols.

Objective: The purpose of the present study is to obtain direct evidence for ADAMTS13 to engage in thiol/disulfide exchange reactions.

Methods: Covalent complexes between ADAMTS13 and VWF were determined by agarose gel electrophoresis under nonreducing conditions. Free thiols in ADAMST13 were identified by a reversed phase high-performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry system.

Results: We demonstrate formation of covalent linkage between ADAMTS13 and VWF, which is time, concentration, temperature, and shear dependent. This interaction is independent of proteolytic activity of ADAMTS13 but depends on the C-terminal domains comprising the fifth through eighth thrombospondin type 1 repeats and C1r/C1s, Uegf, Bmp1 (CUB) domains. The interaction can be blocked by thiol-reactive agents, indicating that association is accomplished through disulfide bridge formation. Several partially reduced free thiols are identified in ADAMTS13, with cysteines 1254 and 1275 being the most prominent, although a point mutation (C1275S) in ADAMTS13 does not alter its ability to form covalent linkages with VWF. This suggests functionally relevant disulfide plasticity in ADAMTS13. Interestingly, ADAMTS13 also forms homo-oligomers under the same conditions as required for the generation of hetero-oligomeric complexes of ADAMTS13 and VWF.

Conclusions: Our results suggest that a dynamic network of free thiols in ADAMTS13 undergoing intra- and inter-molecular redox reactions may add another layer of regulation to VWF function under various conditions.

1 | INTRODUCTION

Hemostasis is the platelet-dependent clotting process that is critical for minimizing blood loss at sites of vascular lesion. In a healthy subject, this highly complex pathway consists of multiple layers of control involving a variety of soluble and cellular components. A number of severe diseases are caused by failure to control hemostasis, either resulting in an increased propensity to bleed or unwanted thrombus formation within blood vessels. One illustrative example is von Willebrand factor (VWF), whose blood clotting function is impaired in patients with von Willebrand disease,[1] whereas thrombotic thrombocytopenia purpura is caused by failure to regulate VWF activity through proteolytic cleavage by ADAMTS13.[2,3] In addition, ADAMTS13 has been reported to act on VWF in a novel manner involving a thiol-dependent activity,[4,5] but the evidence of direct interaction between ADAMTS13 and VWF is lacking.

VWF is a plasma glycoprotein composed of identical subunits that are linked to multimers ranging in size from 500 to 20 000 kDa.[6,7] The protein is synthesized in megakaryocytes and endothelial cells, and is released into plasma as ultralarge VWF multimers.[8] VWF multimers circulate in blood in a globular conformation that changes to an elongated shape upon fluid shear stress.[9,10] Under flow, VWF multimers can undergo lateral self-association, leading to the formation of VWF fibers with augmented platelet adhesion properties.[11,12] Such fibrillation is generated through thiol/disulfide exchange reactions of unpaired cysteine thiols between VWF molecules.[11] VWF is very rich in cysteines, with as many as 234 of the 2813 amino acids of pre-pro VWF being cysteine residues.[7,13]

ADAMTS13 is a glycosylated plasma protein that consists of a metalloproteinase domain, a disintegrin-like domain, a thrombospondin type 1 (TSP1) repeat, a cysteine-rich domain, a spacer domain, seven additional TSP1 repeats and two CUB domains.[14,15] The main function of ADAMTS13 is to regulate the hemostatic activity of VWF by cleaving it between tyrosine 1605 and methionine 1606 (Y1605–M1606), resulting in smaller, less adhesive multimers.[16-20]

Further experimental data have shown that recombinant ADAMTS13 (rADAMTS13; BAX 930, TAK-755, Baxalta Innovations GmbH, a member of the Takeda group of companies, Vienna, Austria) is able to reduce the disulfide bonds between VWF multimers, thereby regulating their lateral association into fibrillar structures. This activity is localized to the C-terminal domain of ADAMTS13 and seems to be thiol specific because it can be blocked by pretreating rADAMTS13 with the thiol-blocking agent iodoacetamide (IAA).[4] Using a mouse model for oxidative endothelial injury, evidence for the pathophysiological relevance of this disulfide bond–reduction activity was recently provided by demonstrating an antithrombotic effect of the C-terminal domain of ADAMTS13.[5]

Essentials
- ADAMTS13 is thought to regulate von Willebrand factor (VWF) also through a redox mechanism.
- ADAMTS13's capability for redox-sensitive thiol/disulfide exchange reactions is assessed.
- ADAMTS13 forms covalent linkages with VWF and itself, independent of its proteolytic activity.
- Dynamic, redox-active thiols in ADAMTS13 could additionally control hemostatic activity of VWF.

The mature ADAMTS13 protease has 77 cysteine residues, implying that at least one of the cysteine residues remains in its thiol form.[21] Using mass spectrometry, two independent studies found six[4] and eight[5] free thiols in the C-terminal TSP1 and CUB1 domains of rADAMTS13. Among the cysteines identified by both groups, residue C1275 of the CUB1 domain appeared to be a good candidate for catalyzing dissociation of lateral VWF multimers, as the remaining four cysteines of the CUB1 domain were predicted to form consensus structural disulfide bonds.[22] Nonetheless, the situation is probably more complex because under denaturing conditions, the number of free cysteine thiols increased to between 24 and 28. This indicates that in addition to the surface-exposed thiols, several others are buried inside the structure of the enzyme; these may also become accessible under shear stress.[4]

The present study investigated the relevance of free cysteine thiols in ADAMTS13 to the molecular mechanism of its proposed non proteolytic activity of defibrillating VWF. We characterized the enzyme's cysteine thiol activity using a novel method for demonstrating disulfide bond formation between ADAMTS13 and VWF, reasoning that in the absence of a redox regeneration system, the postulated thiol-dependent interaction of ADAMTS13 with VWF should lead to the formation of covalent heteromeric adducts that would be readily discernible by multimer gels. We were also interested in identifying any free cysteine thiols under these conditions and determining whether an irreversible blocking of the free cysteine thiols would prevent adduct formation. Results are discussed in reference to the existence of redox-active cysteines in ADAMTS13 and their role in regulation of VWF function.

2 | MATERIALS AND METHODS

2.1 | Recombinant and plasma-derived proteins

Production of purified recombinant ADAMTS13, VWF, and factor IX (FIX) from Chinese hamster ovary cell lines has previously been

described.[23-25] Truncated versions of ADAMTS13 used in this study included MDTCS, an N-terminal fragment spanning from the metalloprotease to the spacer domain and containing a C-terminal V5-His-tag, and the C-terminal fragments referred to as T2C and T5C,[5] which consist of TSP1 repeats 2–8 plus CUB domains and TSP1 repeats 5–8 plus CUB domains, respectively. All C-terminal fragments contain an N-terminal flag-tag and a C-terminal V5-His tag. The point-mutated variant ADAMTS13 C1275S has the cysteine at position 1275 replaced by serine.[22] The point-mutated full-length and all truncated ADAMTS13 variants were expressed in Chinese hamster ovary cell lines and were purified by affinity chromatography using a commercially available Ni-column (GE Healthcare). Plasma-derived ADAMTS13 was purified from a human plasma pool using a combination of affinity and conventional chromatography steps (not shown).

2.2 | Incubation of ADAMTS13 and variants with VWF

Unless stated otherwise, the reaction mixtures (total volume 200 μL) contained 40 μg/mL ADAMTS13 and 100 μg/mL VWF in a buffer composed of 20 mmol/L histidine, 2 mmol/L $CaCl_2$, 30 mmol/L NaCl, and 0.05% Tween 80; pH 7.2. The mixtures were incubated for the indicated times under static conditions at room temperature (RT) and 37°C, and under shear stress at RT. Shear stress conditions were applied by subjecting the reaction mixtures in 1.5 mL Eppendorf tubes to vigorous agitation at the rotation rate of 2500 rpm on a thermomixer (MixMate; Eppendorf), according to Han et al.[26] As a control, the same molar concentration of 12 μg/mL FIX was used instead of ADAMTS13. To identify the optimal ratio between ADAMTS13 and VWF in the reaction, 40 μg/mL ADAMTS13 was mixed with increasing concentrations of VWF (30–475 μg/mL). The effect of an inhibitor of the proteolytic activity of ADAMTS13 on VWF adduct formation was tested by adding 19.2 mmol/L ethylenediaminetetraacetic acid (EDTA) to the reaction mixture. ADAMTS13 activity was analyzed using the synthetic fluorogenic FRETS-VWF73 minimal peptide substrate (Peptanova GmbH) in a reaction buffer composed of 5 mmol/L Bis-Tris, 25 mmol/L $CaCl_2$, 0.005% Tween-20, pH 6, essentially as described.[27] To narrow down the region within ADAMTS13 that was responsible for VWF complex formation, the ADAMTS13 fragments MDTCS (18.3 μg/mL), T2C (21.7 μg/mL), and T5C (15.8 μg/mL) were incubated with 100 μg/mL VWF under static and shear stress conditions at RT; complex formation of 40 μg/mL ADAMTS13 C1275S harboring a mutation in the most prominent free thiol of ADAMTS13 with VWF was similarly tested.

2.3 | Multimer analysis of VWF and ADAMTS13

Analysis of multimeric VWF was carried out as described[28] using a polyclonal rabbit anti-human VWF antibody (A0082; Dako) in combination with an alkaline phosphatase–conjugated polyclonal anti-rabbit antibody (Promega) for detection of VWF. Multimeric ADAMTS13

was similarly analyzed by vertical agarose gel electrophoresis under nonreducing conditions. Appropriately diluted ADAMTS13 samples (22.5 μL) were mixed with 9 μL of a 750 mmol/L IAA solution and incubated for 10 minutes in darkness at RT. Following the addition of 31.5 μL sample buffer (70 mmol/L Tris, 4 mmol/L EDTA, 24 g/L sodium dodecyl sulfate [SDS], 9 mol/L urea, 0.01 g/L bromophenol blue; pH 6.7), samples were incubated for 1 hour at 37°C before loading onto the agarose gel. After blotting, ADAMTS13 was detected with a polyclonal rabbit anti-ADAMTS13 antibody (K1-4; Baxter) in combination with an alkaline phosphatase–conjugated polyclonal anti-rabbit antibody (Promega).

2.4 | Experiments with thiol-reactive N-ethylmaleimide and 2-IAA

To test the effect of blocking agents on the formation of ADAMTS13-VWF complexes, 40 μg/mL ADAMTS13 was incubated with 100 μg/mL VWF in the presence of 3.3 mmol/L N-ethylmaleimide (NEM) or 10 mmol/L IAA for 24 hours under shear stress conditions and at 37°C.

2.5 | Glutathione-triggered oligomerization of ADAMTS13

The effect of thiol-sensitive agents on ADAMTS13 oligomerization was tested by incubating 230 μg of ADAMTS13 in the presence of reduced (GSH) or oxidized (GSSG) glutathione, L-cysteine, and N-acetyl cysteine at concentrations of 2 mmol/L and 8 mmol/L for 3 hours at RT in the presence or absence of shear stress.

2.6 | Free thiol analysis

For fluorescence labeling of free thiol groups in ADAMTS13, 200 μg ADAMTS13 in phosphate-buffered saline (PBS; 8 g/L NaCl, 1.45 g/L $Na_2HPO_4.2H_2O$, 0.2 g/L KCl, 0.5 mL/L Tween 20; pH 7.2) was spiked with 1 mmol/L 5-IAA fluorescein (Thermo Fisher Scientific) and incubated at 37°C or RT for 1 hour. Removal of free 5-IAA fluorescein was carried out by ultrafiltration using 6 mol/L guanidinium chloride or PBS buffer. ADAMTS13 was then reduced using 10 mmol/L dithiothreitol at 37°C for 1 hour and thereafter immediately alkylated with 20 mmol/L IAA (Sigma) at 37°C for 1 hour. Following buffer exchange to 100 mmol/L NH_4HCO_3 by ultrafiltration, tryptic digestion was performed through the addition of 3 μg trypsin and overnight incubation at 37°C. Peptides with fluorescence-labeled free thiols were analyzed using reverse-phase high-performance liquid chromatography (HPLC) with fluorescence detection. For peptide identification, HPLC was coupled to an electrospray ionization quadrupole time-of-flight mass spectrometry (Synapt G2 HDMS; Waters Corporation) instrument operated in positive ion mode. Data analysis was performed using BiopharmaLynx 1.3.4 software (Waters Corporation).

3 | RESULTS

3.1 | ADAMTS13 can form covalent complexes with VWF independently from its proteolytic cleavage activity

To determine whether ADAMTS13 could interact with VWF not only through established exosites associated with proteolysis,[19] but also by forming covalent bonds during the postulated thiol/disulfide exchange reactions,[4,5] a method based on nonreducing gel electrophoresis was developed. These studies revealed that ADAMTS13 could indeed form high molecular weight (HMW) adducts with VWF (Figure S1). Formation of HMW complexes was enhanced by elevated temperature and shear stress (Figure 1A), whereas ADAMTS13-catalyzed proteolytic cleavage of VWF was only increased under shear stress (Figure 1B). That the extent of VWF cleavage was only modest is due to the selection of an incubation buffer optimized for ADAMTS13 stability rather than proteolytic activity.

Prolonged incubation of ADAMTS13 with VWF under conditions that favor covalent complex formation did not lead to a drop in ADAMTS13's proteolytic activity (Figure S2). HMW ADAMTS13-VWF complexes also formed when plasma-derived ADAMTS13 was used (Figure S3), indicating that the observed adduct formation was not due to a fortuitous property of rADAMTS13. Formation of HMW ADAMTS13-VWF complexes was also specific, as no such complexes were seen when VWF or ADAMTS13 were incubated with FIX, an unrelated plasma protein (Figures S4 and S5).

Inhibition of the proteolytic action of ADAMTS13 by EDTA, which deprives the metalloprotease domain of essential Zn^{2+} and Ca^{2+} ions,[29] did not prevent formation of these HMW complexes (Figure S6). The time course of HMW complex accumulation was comparable irrespective of enzyme inhibition, but the size of the bands was larger and did not change over time in the presence of EDTA, indicating adduct formation between ADAMTS13 and uncleaved (and thus larger) VWF multimers (Figure 2). Furthermore, in all of the experiments described, a modest increase in ADAMTS13 homo-oligomerization was noted with elevated temperature and shear stress.

3.2 | The C-terminus of ADAMTS13 is required to form covalent complexes with VWF

To explore the extent of involvement of non proteolytic regions of ADAMTS13 in complexing with VWF, reaction mixtures were set up to include VWF and three staggered ADAMTS13 fragments: T2C, T5C, and MDTCS (Figure 3A). The first two fragments, T2C and T5C, represented N-terminally deleted variants, and were designed to be sufficient for disulfide bond formation but to lack proteolytic activity. By contrast, the C-terminally deleted variant MDTCS was engineered to retain full proteolytic activity but to lack the ability for disulfide bond formation with VWF.[5] Mixtures were incubated for 24 hours at 37°C and under shear stress, and resolved by electrophoresis.
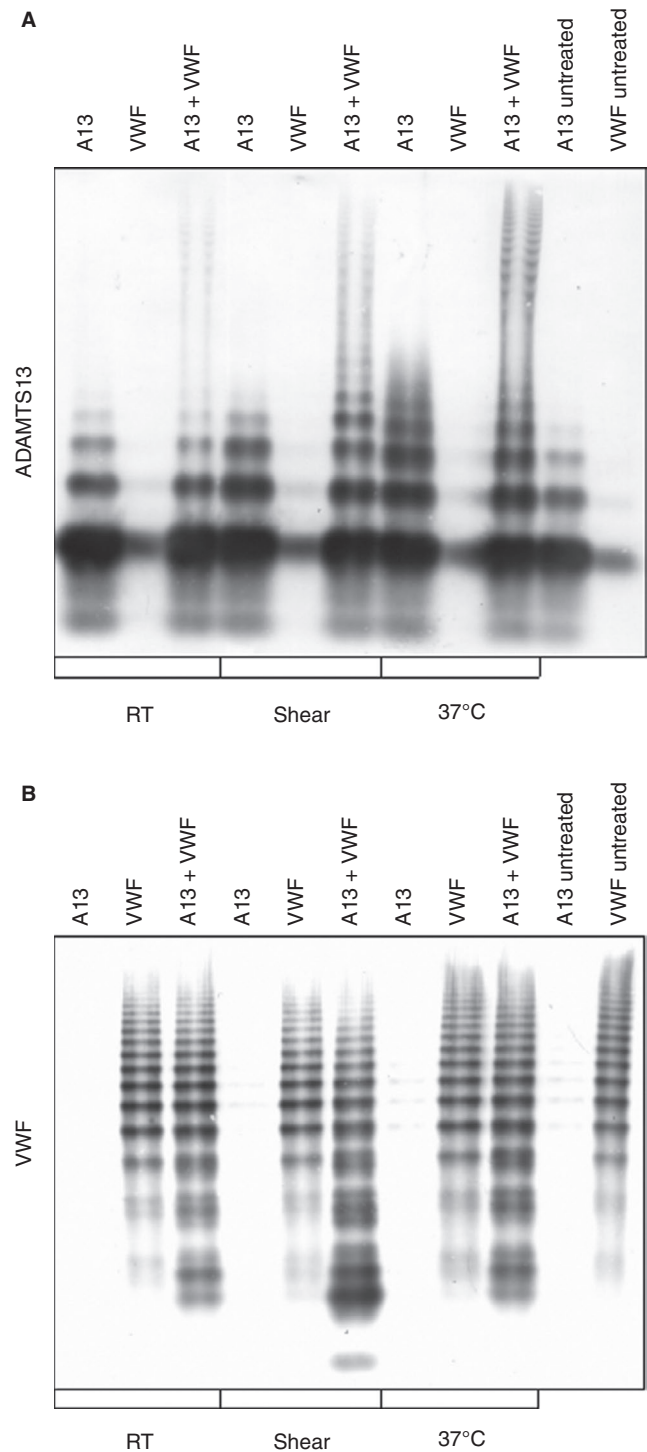

FIGURE 1 Covalent complex formation of ADAMTS13 with von Willebrand factor (VWF). ADAMTS13 (40 μg/mL) was mixed with VWF (100 μg/mL) and incubated for 24 hours at room temperature (RT) under static conditions and shear stress, as well as under static conditions at +37°C. As controls, the single components were incubated under otherwise identical conditions (A13, VWF). In addition, freshly thawed aliquots of the single components were loaded on the gel (A13 untreated, VWF untreated). Samples were separated by 2.5% agarose gel electrophoresis and subjected to immunoblot analysis using (A) anti-ADAMTS13 and (B) anti-VWF antibodies. Note the appearance of oligomeric high molecular weight bands for the samples containing ADAMTS13 and VWF
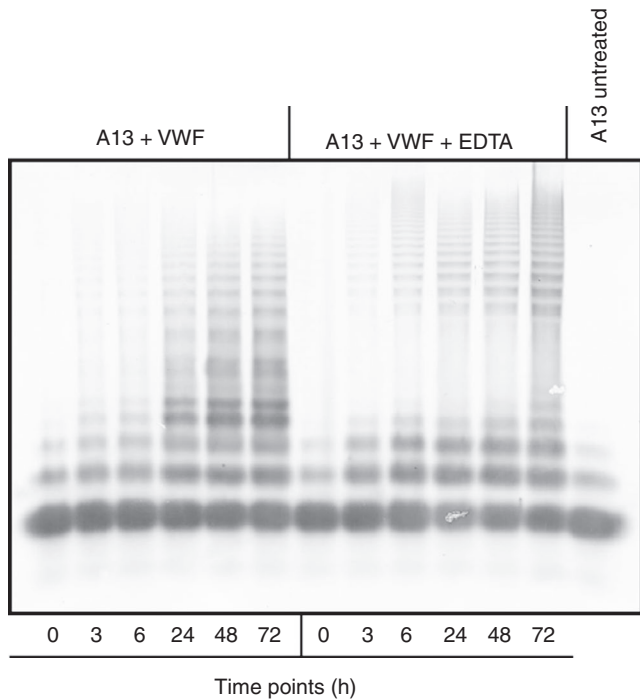
FIGURE 2 Covalent complex formation of ADAMTS13 and von Willebrand factor (VWF) does not require the proteolytic activity of ADAMTS13. Influence of ethylenediaminetetraacetic acid (EDTA) on complex formation. VWF (100 µg/mL) was mixed with ADAMTS13 (40 µg/mL) and incubated in the presence or absence of 20 mmol/L EDTA (A13 + VWF + EDTA and A13 + VWF, respectively) under shear stress conditions at room temperature for up to 72 hours. Samples were separated by 2.5% agarose gel electrophoresis and immunoblotted with anti-ADAMTS13 antibodies and compared with a nonincubated preparation (A13 untreated). High molecular weight bands were clearly discernible under both conditions

The ADAMTS13 positive control for HMW complex formation gave rise to a pattern of bands that was consistent with a specific association with VWF. Additionally, the T2C and T5C fragment variants could be seen to form complexes with VWF at 37°C (Figure 3B) and under shear stress (Figure 3C), albeit the resolution of the complexes was not as good as with full-length ADAMTS13, likely because of the lower purity of this material. By contrast, incubation of the MDTCS variant with VWF did not result in covalent complex formation with VWF even when blots were exposed longer to adjust for the lower sensitivity of the polyclonal anti-ADAMTS13 antibody toward MDTCS (Figure 3B,C). Because complexes with VWF were discernible with the two C-terminal fragments but not the MDTCS fragments, we narrowed the minimal region in ADAMTS13 responsible for association of the protein with VWF.

3.3 | Covalent complex formation is prevented by thiol-reactive reagents

To test whether the HMW complexes formed between ADAMTS13 and VWF emerged through thiol/disulfide exchange reactions, a series of reaction mixtures were set up to include IAA and NEM, two reagents that irreversibly modify free thiols. The reagents were added to reaction mixtures that contained ADAMTS13 and VWF, and were incubated under shear stress for 24 hours. A control test for specificity of the reagents to leave unaltered other ADAMTS13-VWF interactions was preservation of the proteolytic activity of ADAMTS13 toward VWF. Following incubation, the reaction mixtures were separated using electrophoresis.

Build-up of HMW ADAMTS13-VWF complexes was decreased by IAA and was completely prevented by NEM (Figure 4A), whereas these reagents did not affect proteolytic cleavage of VWF by ADAMTS13 (Figure 4B). This reaffirmed not only the covalent nature of the VWF-ADAMTS13 complexes but also that the reagents used did not cause any detectable levels of protein degradation or other structural alterations. Likewise, ADAMTS13 homo-oligomerization was prevented by NEM and was diminished by IAA (Figure 4A).

3.4 | Homo-oligomerization of ADAMTS13 can be stimulated by glutathione

To further characterize the free thiol–sensitive reactions of ADAMTS13, we addressed whether physiologic agents with a reactive cysteine such as glutathione would resolve ADAMTS13 homo-oligomers (Figure 5). Surprisingly, incubation with GSH triggered multimerization of ADAMTS13, particularly in the presence of shear stress. GSSG did not have such an effect and even prevented the slight increase in the degree of multimerization upon exposure of ADAMTS13 to shear stress (Figure 5A).

The apparent GSH concentration-dependent increase in oligomers was quantified by using size exclusion chromatography. Following incubation for 3 hours under static conditions at RT, more than 99% of ADAMTS13 was monomeric in absence of GSH, but monomeric content gradually decreased with increasing GSH concentrations in favor of oligomeric ADAMTS13 (Figure 5B). At the same time, the proteolytic activity of ADAMTS13 gradually declined, indicating that the nonphysiologic high concentrations of GSH also disrupted disulfide bonds important for the catalytic activity of ADAMTS13 (Figure 5B). A detection of free thiols by biotin-conjugated maleimide (MBP) confirmed the GSH-triggered concentration increase of free cysteines (Figure 5C). The data further support our hypothesis that ADAMTS13 possesses several reactive cysteines with an avid propensity to form disulfide bonds, either with itself or its substrate VWF.

3.5 | Determination of free thiols in ADAMTS13 reveals a redundancy of reactive cysteines

In a first step toward understanding the contribution of particular cysteines to disulfide bond formation with VWF, we labeled free thiols in ADAMTS13 with 5-IAA fluorescein and, following trypsin digestion, the resulting peptides were separated by HPLC and identified by mass spectrometry.
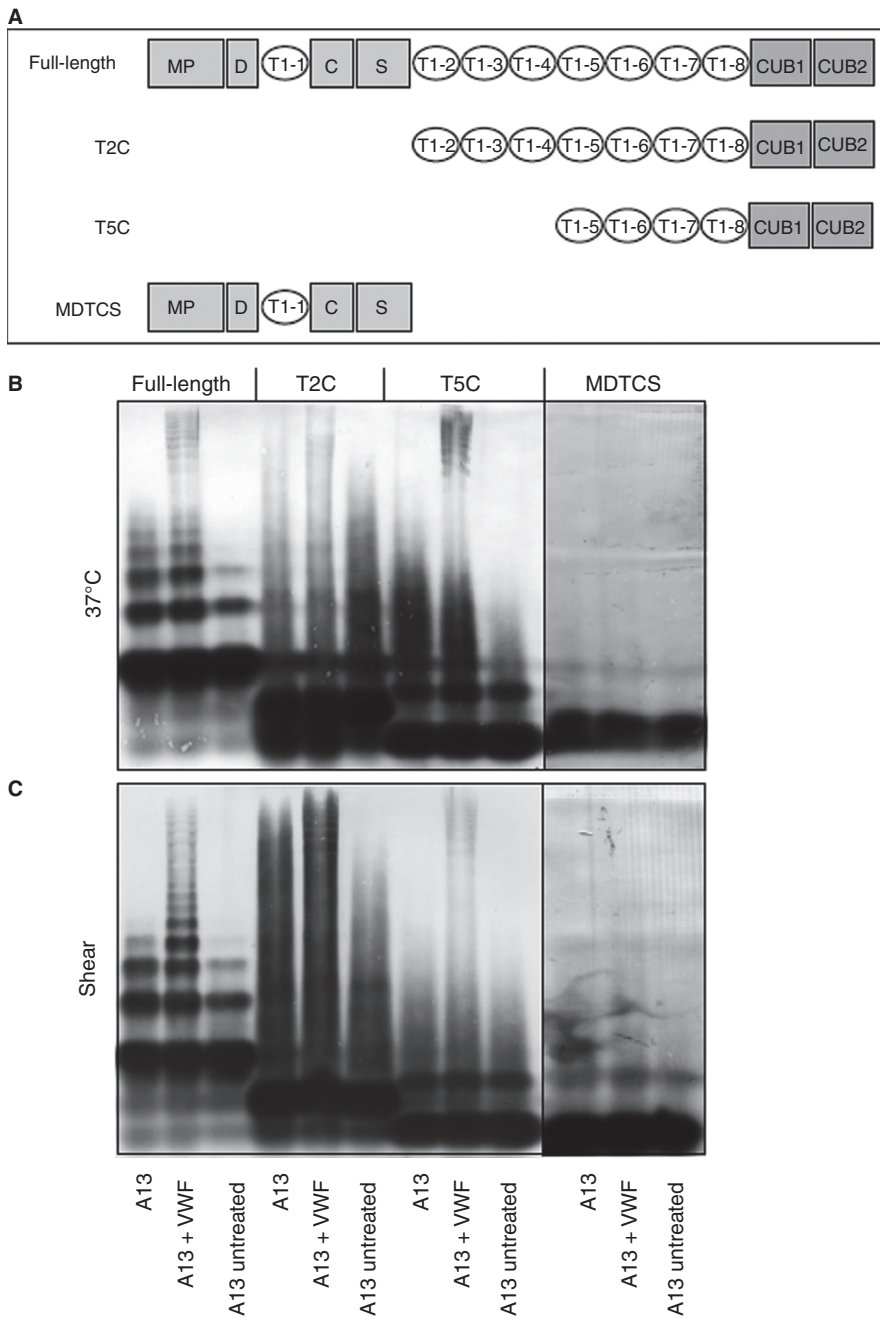
A

B

C

FIGURE 3 The C-terminal region of ADAMTS13 is responsible for covalent complex formation with von Willebrand factor (VWF). (A) Schematic drawing of the ADAMTS13 fragments used. Full-length ADAMTS13 (40 µg/mL) or equimolar quantities of the ADAMTS13 fragments MDTCS (18.3 µg/mL), T2C (21.7 µg/mL), and T5C (15.8 µg/mL) were mixed with VWF (100 µg/mL) and incubated for 24 hours (B) under static conditions at +37°C and (C) at room temperature under shear stress. As controls, ADAMTS13 and the respective fragments were incubated as single components under otherwise identical conditions (A13, MDTCS, T2C, T5C). Freshly thawed aliquots of these components were also loaded on the gel (untreated). Samples were separated by 2.5% agarose gel electrophoresis and immunoblotted with anti-ADAMTS13 antibodies. High molecular weight bands were discernible for full-length ADAMTS13, T2C, and T5C, but not for MDTCS, even after extended imaging of the blot. CUB, C1r/C1s, Uegf, Bmp1; D, disintegrin; MP, metalloprotease; T1, thrombospondin type 1

The results showed that cysteine-bearing peptides could be assigned to all the prominent peaks (Figure S7A). Among those peptides, cysteines 1254 and 1275 were most prominent and coincidentally were identified twice owing to the presence of peptides with no and one missed trypsin cleavage. Moreover, each of the identified labeled peptides could be matched with the corresponding nonlabeled peptide in the sample, indicating that the respective cysteines were only partially reduced.

Furthermore, to increase our confidence in the results obtained above, the labeling procedure was repeated at different temperatures (37°C and RT) and the washing procedure was altered from using guanidinium chloride to PBS buffer so as to exclude labeling artifacts, owing to the denaturation of ADAMTS13 during the washing

step. Both HPLC profiles resembled that of the previous run, albeit peak intensities were reduced (Figure S7B). Comparison of the identified cysteines with previously published reports revealed a strong divergence in that although we too identified C1275, we additionally detected two cysteines each in the disintegrin domain and the CUB1 and CUB2 domains (Table 1).

Our detection of a multitude of partially reduced cysteines led us to consider the potential existence of a dynamic network of redundant free thiols undergoing intra- and inter-molecular redox reactions, commensurate with disulfide bond plasticity, in ADAMTS13.[30] To obtain experimental evidence for this hypothesis, we chose to mutate C1275 in ADAMTS13 to a serine because the former residue was identified as being partially reduced both here as well as in the
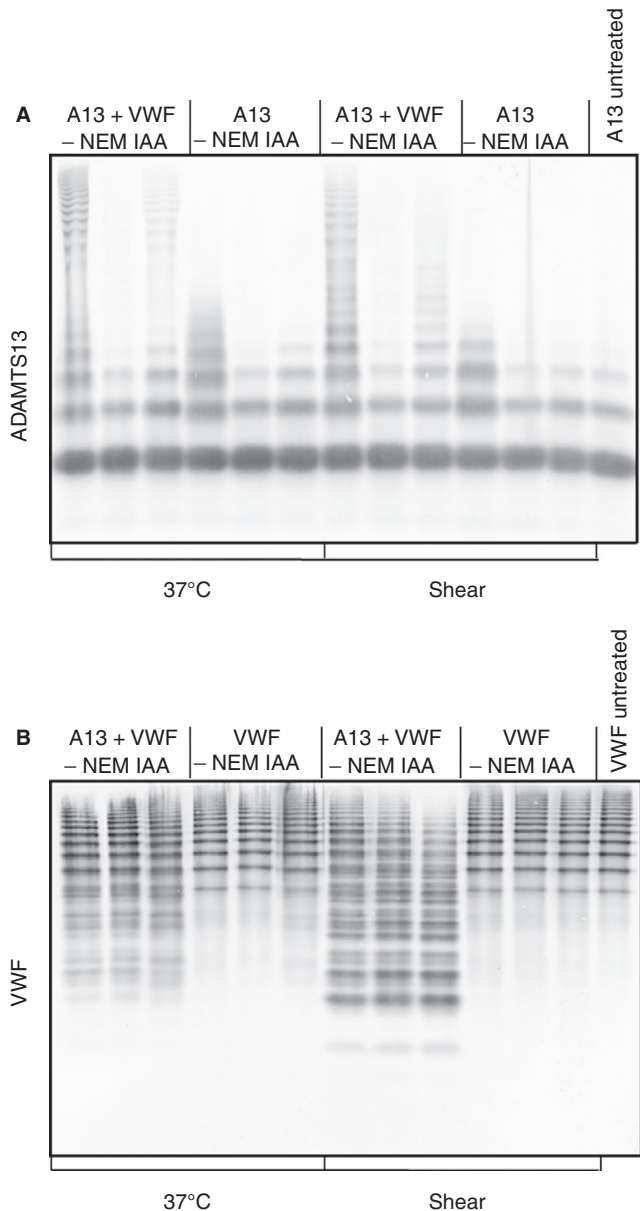
FIGURE 4 Impact of thiol-sensitive reagents on complex formation of ADAMTS13 with von Willebrand factor (VWF). ADAMTS13 (40 μg/mL) was mixed with VWF (100 μg/mL) and incubated for 24 hours with 3.3 mmol/L N-ethylmaleimide (NEM), 10 mmol/L iodoacetamide (IAA), or without any agent (—) under static conditions at +37°C or under shear stress conditions at room temperature. As controls, the single components were incubated under otherwise identical conditions (A13, VWF). Samples were separated by 2.5% agarose gel electrophoresis and immunoblotted with (A) anti-ADAMTS13 antibodies and (B) anti-VWF antibodies in comparison with nonincubated preparations. A13 untreated and VWF untreated in (A) and (B), respectively. NEM and IAA interfered with the complex formation of ADAMTS13 and VWF, but did not affect the oligomeric profile of VWF and its proteolytic degradation by ADAMTS13

aforementioned published studies. We tested this C1275S mutant version of ADAMTS13 for its ability to homo-oligomerize as well as to form complexes with VWF. Following incubation at 37°C or shear stress for 24 hours, reaction mixtures containing the mutated enzyme and VWF were resolved by electrophoresis. Duplicate mixtures were treated with thiol-modifying NEM to better monitor the extent of oligomerization of the ADAMTS13 variant.
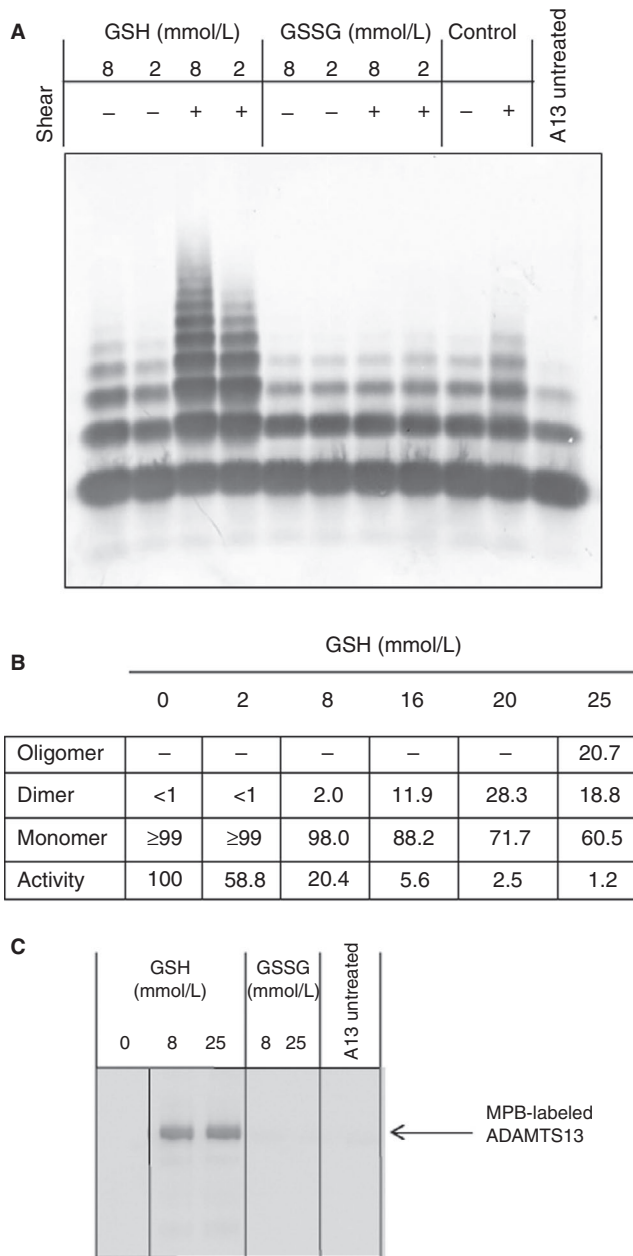
The C1275S mutant was indistinguishable from wild-type ADAMTS13 with respect to HMW ADAMTS13-VWF complex formation and homo-oligomerization (Figure 6A), and with respect to proteolytic cleavage of VWF (Figure 6B). However, when NEM was added, formation of both HMW complexes was prevented, as was homo-oligomerization. This indicated that in the absence of C1275, ADAMTS13 was capable of forming alternative bonds that were nevertheless still sensitive to NEM (Figure 6A). Hence, we reasoned that the bonds between ADAMTS13 and VWF were due to cysteines other than C1275, perhaps C1254, and that those bonds responsible for the multimerization of ADAMTS13 were probably related to them.

4 | DISCUSSION

Here, we showed that the VWF-cleaving protease ADAMTS13 can also form disulfide bridges with VWF based on a novel mechanism involving a panel of redundant and only partially reduced cysteine thiols. We narrowed down the region of ADAMTS13 responsible for these thiol interactions to the C-terminus and showed that a single mutation of the most prominent of these cysteine residues (C1275) did not prevent formation of VWF-ADAMTS13 complexes. The described process would be in line with ADAMTS13 curtailing VWF fibrillation,[4,5] as it masks the ability of VWF multimers to oligomerize further.

The observed thiol/disulfide exchange between ADAMTS13 and VWF may also be relevant for the recently described redox switch in VWF. The redox status of two vicinal cysteines (C1669 and C1670) affects the conformation of the A2 domain, which in turn affects VWF binding to platelet GpIbα and accessibility to the ADAMTS13 cleavage site.[31] Regulation of this switch by a plasma reductase has been postulated.[32] Although not the scope of the current study, it is tempting to speculate that this reductase could be ADAMTS13, which would then control VWF activity by yet another mechanism; clearly, future work with a recombinant VWF A2 domain and ADAMTS13 is required to substantiate this thought.

By incubating ADAMTS13 with VWF, covalent enzyme-substrate complexes could be discerned as additional HMW bands in SDS multimer gels. The possibility that ADAMTS13 and VWF had associated through electrostatic interactions could be excluded because the ionic detergent SDS contained in the sample buffer would have disrupted all noncovalent protein–protein interactions. Furthermore, the thiol-modifying reagents NEM and IAA prevented the formation of these HMW adducts, which strongly indicated that these complexes emerged through disulfide bond formation. Non proteolytic interactions between ADAMTS13 and VWF have been explored previously. ADAMTS13 was shown to release VWF from maleimide-polyethylene glycol 2-biotin beads in a thiol-dependent

A

B

	GSH (mmol/L)					
	0	2	8	16	20	25
Oligomer	–	–	–	–	–	20.7
Dimer	<1	<1	2.0	11.9	28.3	18.8
Monomer	≥99	≥99	98.0	88.2	71.7	60.5
Activity	100	58.8	20.4	5.6	2.5	1.2

C

FIGURE 5 Impact of the thiol-sensitive agent glutathione on ADAMTS13 structure and function. ADAMTS13 (230 µg/mL) was treated with 2 or 8 mmol/L of reduced (GSH) or oxidized (GSSG) glutathione and incubated for 3 hours at room temperature (RT) under static (–) or shear stress (+) conditions. As control, ADAMTS13 was incubated in bulk drug substance buffer. Samples were separated by 2.5% agarose gel electrophoresis under nonreducing conditions and ADAMTS13 was detected by immunoblot analysis with anti-ADAMTS13 antibodies (A). For comparison, nonincubated ADAMTS13 was also loaded (A13 untreated). An increase in homo-oligomers was specifically detected under shear stress in the presence of GSH but not GSSG. ADAMTS13 homo-oligomerization was quantified by size exclusion chromatography, for which ADAMTS13 was similarly incubated for 3 hours under static conditions with increasing concentrations of GSH (2–25 mmol/L) (B). The resulting chromatograms were evaluated for the relative content (%) of ADAMTS13 monomers, dimers, and oligomers. The same samples were assayed for ADAMTS13 proteolytic activity using the FRETS-VWF73 assay, with activities being presented in percentage of the control incubation without GSH (defined as 100%) (B). To assess the concentration of free cysteine thiols in glutathione-treated ADAMTS13, the protein was incubated with 8 or 25 mmol/L GSH or GSSG for 3 hours at RT and, following dialysis, incubated with maleimide-polyethylene glycol 2-biotin (MBP) for 15 minutes to label free cysteine thiols (C). Unbound MBP was removed by dialysis and bound MBP visualized by immunoblot analysis using horseradish peroxide–coupled streptavidin. A freshly thawed aliquot of ADAMTS13 was also loaded on the gel (A13 untreated). The amount of free cysteine thiols was substantially higher in the GSH-treated samples

be removed via existing scavenging mechanisms. A depletion of ADAMTS13 has indeed been reported during acute systemic inflammation where release of VWF is induced.[34] However, this possibility would defy the rationale behind the existence of an enzyme capable of dealing with a large flux of substrate. In a second scenario, we evoked the possibility that the in vivo reaction was reversible, which would have necessitated a hitherto unidentified regeneration system to release ADAMTS13 from VWF. As a consequence, formed complexes would be transient, therefore becoming difficult to detect under normal conditions. Based on the existence of various partially reduced cysteines, regeneration of ADAMTS13 may be accomplished by intramolecular thiol/disulfide exchange reactions; however, this would require redox systems to reduce the cysteines involved in releasing ADAMTS13.

The recently described interaction of the N- and C-termini of quiescent ADAMTS13 is disrupted upon substrate-dependent activation of the enzyme.[35] This conformational change is thought to be a key physiologic measure for the proteolytic regulation of VWF. It will be important to determine whether formation of disulfide bonds between the C-terminal domains of ADAMTS13 and VWF will likewise disrupt the autoinhibitory conformation of ADAMTS13.

Identification of the cysteines involved in these exchange reactions was challenging. We could narrow the activity to the C-terminal portion of ADAMTS13, as the T2C and T5C fragments (but not

manner, but the study did not report on HMW adducts.[4] In another study, by applying coimmunoprecipitation, some ADAMTS13 was found to bind constitutively to VWF, leading to the presumption that this represented a priming step ahead of proteolytic cleavage upon exposure to shear stress, but it was not addressed whether the observed interaction was covalent or not.[33]

In reference to the observed covalent interaction of ADAMTS13 with VWF, this is in favor of the postulated mechanism for the nonproteolytic activity of ADAMTS13, which would require an intermittent covalent association between both proteins. In our hands, the thiol/disulfide exchange between ADAMTS13 and VWF occurred simply by mixing the two components in the absence of any redox regeneration system, allowing the complexes to form irreversibly.

This left open the question of the fate of these HMW structures in vivo. One possibility could be that these complexes might

TABLE 1 Identification of free thiols by LC-MS

Peptide	Cys residue	A13[a] domain	This study	Yeh et al[2]	Bao et al[17]
AGLAWSPCSR	256	MP	–	X	–
AVACTFAR	322	DIS	X	–	–
EHLDMCQALSCHTDPLDQSSCSR	332, 337, or 347	DIS	X	–	–
WVNYSCLDQAR	710	TSP1-2	–	–	X
CVEAQGSLLK	769	TSP1-3	–	X	X
AGAQQPAVALETCNPQPCPAR	799	TSP1-3	–	–	X
FLCMDSALR	919	TSP1-5	–	–	X
VPVQEELCGLASKPGSR	933	TSP1-5	–	X	X
RILYCAR	977	TSP1-6	–	–	X
LLPGPQENSVQSSACGR	1192	CUB1	–	X	–
GPGQADCAVAIGRPLGEVVTLR	1213	CUB1	–	X	X
MCR	1254	CUB1	X	–	–
RCGRPGGGVLLR	1275	CUB1	X	X	X
ECDMQLFGPWGEIVSPSLSPLSPATSNAGGCR	1299, 1325	CUB2	X	–	–

Cys, cysteine; DIS, disintegrin; LC-MS, liquid chromatography mass spectrometry; MP, metalloprotease; TSP1, thrombospondin type 1.
Main peaks are marked in bold-face type.
[a]ADAMTS13 domains: MP, DIS, TSP1, CUB, C1r/C1s, Uegf, Bmp1.

the MDTCS fragment) were proficient in forming complexes with VWF, in line with the antithrombogenic effect of these fragments in vivo.[5] Labeling of reduced cysteines led to the identification of six partially reduced residues, four of which were located in the C-terminal CUB domains. Comparison with results from two previous studies[4,5] yielded only a single match with cysteine 1275, but the respective point-mutated variant was not different from the wild-type protein in any of the assays performed. We cautiously reasoned that the disparate results might point toward a set of cysteines in ADAMTS13 that are used in a network of redox-based reactions that regulate activity of VWF. This hypothesis would explain why all labeled cysteines were only partially reduced and why separate laboratories identified different cysteines, because alternating subsets of cysteines could be reduced depending on the experimental conditions.

Other cysteine residues identified (C1213 and C1254) were previously postulated to form consensus structural disulfide bonds, thereby excluding them from involvement in the reactive thiol network.[22] However, partially reduced cysteines of a disulfide bond are reminiscent of the disulfide bond of VWF, C2431-C2453, which was found to be reduced in ~75% of the molecules and key for the lateral association of VWF.[36] Reactive cysteines relevant for VWF regulation might actually be located in the C-terminal portion of ADAMTS13 and possibly limited to the CUB domains, the possibility of which is supported by the observation that expression of the isolated CUB1 domain leads to thiol-dependent aggregate formation.[22] Nonetheless, because even a fragment comprising CUB1 and CUB2 had a low propensity to aggregate, in the context of the full-length protein, it would appear that this reaction might be tightly regulated. In addition, the C1275S mutation prevented aggregation

of the CUB1 domain,[22] but did not alter the activity of full-length ADAMTS13 to form oligomers.

The ADAMTS13 material used was largely monomeric, but contained a portion of dimers, trimers, and tetramers. A quantification by size exclusion chromatography assigned 99% of the material to the monomer and only ~1% to the cumulative oligomeric forms of ADAMTS13. Interestingly, homo-oligomerization of ADAMTS13 increased under the same reducing conditions that also led to VWF interaction. Incubation of ADAMTS13 with GSH (but not GSSG) in the low mmol/L range led to a further, nonphysiological increase in multimeric ADAMTS13. At first glance, the observation of increased homo-dimerization of ADAMTS13 owing to the addition of a reducing compound was counterintuitive, as one would predict a decrease in the degree of multimerization, expecting that saturation of the cysteines would make for a less sticky protein. However, for a mechanism using thiol/disulfide exchange reactions, cysteine thiol-enhanced generation of reactive thiolate anions would also promote ADAMTS13 self-assembly. Although we cannot entirely exclude that oligomerization was an experimental artifact caused by much higher ADAMTS13 concentrations than those measured in the circulation (0.5–1.0 µg/mL), it is tempting to suggest that this event is functionally linked to the regulation of VWF multimerization by ADAMTS13. Future experimental work is required to substantiate this idea. For instance, our TTP mouse model, where a therapeutic dose of recombinant human ADAMTS13 resolves the TTP symptoms triggered by recombinant human VWF at 2000 U/kg body weight,[37] could be used to identify such complexes in vivo through coimmunoprecipitation of ADAMTS13 and VWF from mouse plasma.

We conclude that our results demonstrate that ADAMTS13 forms disulfide bridges with VWF, on the basis of a novel mechanism
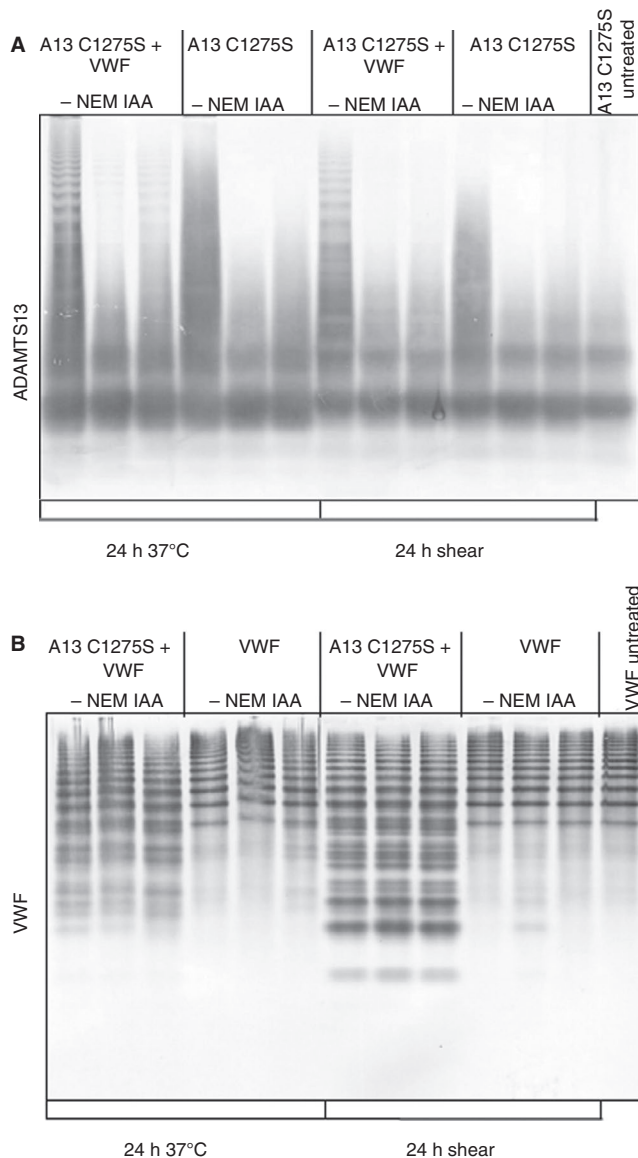
FIGURE 6 Impact of mutating cysteine 1275 on complex formation with von Willebrand factor (VWF) in ADAMTS13. The point-mutated variant ADAMTS13 C1275S (40 μg/mL) was mixed with VWF (100 μg/mL) and incubated for 24 h with 3.3 mmol/L N-ethylmaleimide (NEM), 10 mmol/L iodoacetamide (IAA), or without any agent (—) under static conditions at +37°C or under shear stress conditions at room temperature. As controls, the single components were incubated under otherwise identical conditions (A13 C1275S, VWF). Samples were separated by 2.5% agarose gel electrophoresis and immunoblotted with (A) anti-ADAMTS13 antibodies and (B) anti-VWF antibodies in comparison with nonincubated preparations (A13 C1275S untreated and VWF untreated in (A) and (B), respectively). The NEM-sensitive appearance of oligomeric high molecular weight bands indicated that ADAMTS13 C1275S retained the ability to form covalent complexes with VWF

involving a panel of redundant and only partially reduced cysteine thiols, providing the first direct evidence for thiol/disulfide exchange reactions modulating multimerization of an enzyme and its substrate, i.e. ADAMTS13 and VWF.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.