Zinc and Calcium Ions Cooperatively Modulate ADAMTS13 Activity*

Received for publication, April 26, 2005, and in revised form, October 7, 2005 Published, JBC Papers in Press, November 11, 2005, DOI 10.1074/jbc.M504540200

Patricia J. Anderson‡, Koichi Kokame§, and J. Evan Sadler†,

From the †Howard Hughes Medical Institute, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110 and the §National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

ADAMTS13 is a metalloproteinase that cleaves von Willebrand factor (VWF) multimers. The metal ion dependence of ADAMTS13 activity was examined with multimeric VWF and a fluorescent peptide substrate based on Asp1596–Arg1668 of the VWF A2 domain, FRETS-VWF73. ADAMTS13 activity in citrate-anticoagulated plasma was enhanced ~2-fold by zinc ions, ~3-fold by calcium ions, and ~6-fold by both ions, suggesting cooperative activation. Cleavage of VWF by recombinant ADAMTS13 was activated up to ~200-fold by zinc ions (K_D app ~0.5 mM), calcium ions (K_D app ~4.8 mM), and barium ions (K_D app ~1.7 mM). Barium ions stimulated ADAMTS13 activity in citrated plasma but not in citrate-free plasma. Therefore, the stimulation by barium ions of ADAMTS13 in citrated plasma appears to reflect the release of chelated calcium and zinc ions from complexes with citrate. At optimal zinc and calcium concentrations, ADAMTS13 cleaved VWF with a K_m app of 3.7 ± 1.4 µM (~15 nM for VWF subunits), which is comparable with the plasma VWF concentration of 5–10 µg/ml. ADAMTS13 could cleave ~14% of VWF pretreated with guanidine HCl, suggesting that this substrate is heterogeneous in susceptibility to proteolysis. ADAMTS13 cleaved FRETS-VWF73 with a K_m app of 3.2 ± 1.1 µM, consistent with an ~200-fold decrease in affinity compared with VWF. ADAMTS13 cleaved VWF and FRETS-VWF73 with strongly comparable catalytic efficiency of 55 µM{sup}-1 min{sup}-1 and 18 µM{sup}-1 min{sup}-1, respectively. The striking preference of ADAMTS13 for VWF suggests that substrate recognition depends on structural features or exozymes on multimeric VWF that are missing from FRETS-VWF73.

The von Willebrand factor (VWF) cleaving proteinase ADAMTS13 is a member of the ADAMTS family (a disintegrin and metalloprotease with thrombospondin repeats) (1–3). Since the identification of ADAMTS13, evidence has increased concerning the association of severe ADAMTS13 deficiency with the disease thrombotic thrombocytopenic purpura (TTP) (4–6). TTP is characterized by disseminated microvascular thrombi containing platelets and multimers of VWF, which is a plasma protein that mediates platelet adhesion by tethering platelets to the extracellular matrix (7, 8). In the absence of ADAMTS13 activity, ultra-large multimers of VWF accumulate, causing persistent intravascular platelet aggregation and TTP. Congenital TTP, or Upshaw-Schulman syndrome, is caused by compound heterozygous or homozygous mutations in the ADAMTS13 gene (2, 9, 10). Acquired idiopathic TTP usually affects adults and is caused predominantly by autoimmune responses to ADAMTS13 (6). The mortality rate is ~90% if untreated; however, plasma exchange therapy has reduced this rate to ~20% (11–13).

Many proteinases of the ADAMTS family are involved in extracellular matrix remodeling, angiogenesis, and development, where they typically cleave large multimeric proteins (14, 15). ADAMTS13 regulates the size of plasma VWF multimers by proteolytic cleavage at Tyr1596–Met1600 within the A2 domain of VWF subunits (4, 16). ADAMTS proteinases contain a reprolysin-like zinc metalloproteinase domain, a disintegrin domain, a cysteine-rich and spacer region, several thrombospondin type 1 repeats, and variable C termini, which in ADAMTS13 includes two CUB domains (named for the first identified proteins containing this motif, complement C1r/C1s, Uegf, and Bmp1) (17). The metalloproteinase domain of ADAMTS13 has a putative zinc ion catalytic site (H224EXYHXGXGK235), one predicted calcium ion-binding site coordinated by residues Glu81, Asp173, Cys281, and Asp284, and a conserved Met349 that supports the active site zinc ion in a “Met turn”; these features identify ADAMTS13 as a member of the “metzincin” family (1, 18, 19). The metzincins, which include the homologous matrix metalloproteinases and ADAMs (a disintegrin and metalloprotease), achieve optimal activity with both zinc and calcium ions (18–22).

The role of divalent metal ions in ADAMTS13 activity is not fully understood. Previous studies of ADAMTS13 activity reported that a combination of barium and calcium ions was optimal for cleavage of VWF (4, 5, 23). The addition of zinc ions has yielded inconsistent results; some studies found no effect, whereas others found that zinc ions restored the activity of EDTA-treated ADAMTS13 (4, 23). In addition, interactions of ADAMTS13 with VWF depend upon ionic strength and pH, but optimal conditions vary considerably among several reports. When assayed at pH 8.0, ADAMTS13 activity was greatest under conditions of low ionic strength (4). Other studies have demonstrated proteolysis of VWF by ADAMTS13 at low ionic strength (I = 75 mM) in the absence of added metal ions (6).

The previous studies of ADAMTS13 proteolysis of VWF have established that interactions between the enzyme and substrate are dependent upon metal ions and electrostatic interactions. However, these studies have generally employed reaction conditions unlike those prevailing in vivo. Therefore, the properties of ADAMTS13 were investigated under physiological conditions of pH and ionic strength. The enzyme was found to be activated by calcium and zinc ions at concentrations typical of plasma. Additionally, the K_m app for VWF was within the range of plasma VWF concentrations, but the K_m app for a synthetic peptide based on the sequence of cleavage site within the VWF A2-domain was ~210-fold higher. This difference indicates that structural features or
exosites within the native multimeric VWF molecule are required for efficient substrate recognition by ADAMTS13.

**EXPERIMENTAL PROCEDURES**

**Materials**—Aliquots of normal human plasma (American Red Cross, St. Louis, MO) were stored at −20 °C until use. One unit of ADAMTS13 activity was defined as the activity in 1 ml of pooled normal human plasma. The concentration of purified human plasma VWF (Laboratoire Français du Fractionnement et des Biotechnologies, Lille, France, generously provided by Claudine Mazurier) in phosphate-buffered saline was determined by absorbance at 280 nm with an absorption coefficient of 1.0 mg ml⁻¹ cm⁻¹ and correction for light scattering at 340 nm as described (24).

**Expression of Recombinant ADAMTS13**—A cDNA encoding ADAMTS13 with a C-terminal V5-His tag (1, 25) was cloned into the tetracycline-inducible vector pcDNA4/TO (Invitrogen) at the EcoRI site to yield plasmid pcTO-ADAMTS13. Trекс 293 cells (Invitrogen) were transfected with pcTO-ADAMTS13 (1 μg) using Lipofectamine 2000 (Invitrogen). Stable cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% tetracycline-approved fetal bovine serum (Clontech or Invitrogen). 300 μg/ml zeocin, 5 μg/ml blasticidin, 2 mM glutamine, 5 units/ml penicillin, and 5 μg/ml streptomycin. Protein expression was initiated in 70–80% confluent roller bottles with 1 mM tetracycline. Prior to addition, VWF was preincubated for 30 min at 4 °C. The concentration of each chaotropic agent in 50 mM Hepes, pH 7.4, 150 mM NaCl, and 1 mg/ml bovine serum albumin. Metal ion stock solutions were prepared in distilled water. Some metal ions required the addition of small amounts of HCl for solubility, specifically ZnCl₂. All reported metal ion concentrations represent the total concentration added to reactions. In complete reactions containing buffer, substrate, and plasma, Zn²⁺ was <2 μM and Ca²⁺ was <250 μM as determined by inductively coupled plasma spectroscopy (Mayo Clinic, Rochester, MN).

**Effects of sodium ions on plasma ADAMTS13** were determined in reaction buffer containing 50 mM Hepes, pH 7.4, 0.25 mM ZnCl₂, 5 mM CaCl₂, containing either 150 mM NaCl or 150 mM choline chloride ([2-hydroxyethyl]trimethylammonium chloride), and the effects of ionic strength on plasma ADAMTS13 activity were determined by varying the concentration of NaCl in reaction buffer. The effects of guanidine HCl and urea on the cleavage of VWF were studied by varying the concentration of each chaotropic agent in 50 mM Hepes, pH 7.4, 150 mM NaCl. The reactions were preincubated at 37 °C for 10 min prior to the addition of VWF to a final concentration of 20 μg/ml or 2 μg/ml, followed by incubation at 37 °C for 1 h.

The reactions were quenched by the addition of sample loading buffer (62.5 mM Tris, pH 6.8, 1% SDS, 0.01% bromphenol blue, 5% glycercol (final concentrations)) and analyzed by SDS-PAGE on 4% or 5% gels (Invitrogen or Bio-Rad, respectively). The proteins were transferred to polyvinylidene difluoride membranes by electrophoretic blotting, and the 350-kDa product was detected by Western blotting with a 1:2500 dilution of horseradish peroxidase-conjugated rabbit anti-human VWF (Dako, Carpinteria, CA) (26).

The observed product band densities were quantitated from scanned films using NIH Image 1.61 (rsb.info.nih.gov/nih-image/) or by chemiluminescence or fluorescence detected by a STORM Imager and integration of the peaks using ImageQuant TL (Amersham Biosciences). The rate constants were calculated from the change in band density (ΔD₅₀₅nm/h). The activity with added metal ions was expressed as a ratio to the activity without added metal ions and analyzed by nonlinear least squares fitting of the quadratic binding equation, with the maximal change in fold activation (ΔF₄₄₀nm) and apparent dissociation constant (Kᵥₒᵤ) as the fitted parameters (27). The stoichiometric factor (n) and concentration of ADAMTS13 were fixed at 1.0 and 1 nm, respectively.

**Barium Ion Effects on Citrated and Noncitrated Plasma**—Plasma samples were collected from healthy volunteer donors according to a protocol approved by the institutional review board of Washington University School of Medicine. Noncitrated plasma was obtained by collection into 75 μM FPR-Ck, 75 μM FFR-Ck, 75 μM hirudin (Sigma), and 32 μg/ml corn trypsin inhibitor (Hematologic Technologies, Inc.) (final concentrations) (28). The whole blood was centrifuged at 2000 rpm in a Sorvall 6000 for 15 min, and the recovered plasma was incubated further by adding an additional 75 μM FPR-Ck, 75 μM FFR-Ck, and 32 μg/ml corn trypsin inhibitor. VWF was preincubated in 5 mM Hepes, pH 8.0, 1.23 mM guanidine HCl, for 30 min at 37 °C. ADAMTS13 (0.6 mM) from citrated or noncitrated plasma was incubated with predenatured VWF (2 μg/ml) in the absence or presence of varying concentrations of BaCl₂ in 5 mM Hepes, pH 8.0, at 37 °C for 1 h. Effects of barium ions on 30 mM recombinant ADAMTS13 were assessed in 50 mM Hepes, pH 7.4, 150 mM NaCl, and 1 mg/ml bovine serum albumin. The reactions were quenched by the addition of 50 mM EDTA, pH 8.0, and sample loading buffer and analyzed by gel electrophoresis and Western blotting as described above. The activation of recombinant ADAMTS13 by barium ions was calculated and expressed as a ratio to the activity in the absence of added metal ions, and the data were fitted to the quadratic binding equation as described above for calcium and zinc ions. The stoichiometric factor (n) and concentration of recombinant ADAMTS13 were fixed at 1 and 30 nm, respectively.

**Kinetics of VWF Cleavage**—The reactions were performed with varying concentrations of VWF with plasma ADAMTS13 (0.6 mM) in assay buffer at 37 °C. The densities of the 350-kDa VWF product band were quantitated as described for metal ion-dependent reactions, and the cleavage rates were calculated as the change in product band density per hour (ΔD₅₀₅nm/h). The rates of product generation as a function of VWF concentration were fit to the Michaelis-Menten equation to determine...
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the apparent Michaelis constant ($K_m$) and maximal observed density ($D_{obs\, max}$).

Progress curve analysis of VWF proteolysis was determined under first order reaction conditions where the $[VWF]$ was $~0.5 \times K_m$. The reactions contained 2 µg/ml VWF and 0.6 mM plasma ADAMTS13, in assay buffer at 37 °C. The 350-kDa VWF product band was detected by chemifluorescence. The area of the product band as a percentage of the total area from each lane as a function of time was calculated by integration of peaks using ImageQuantTL software (Amersham Biosciences). The percentage of area was multiplied by the concentration of substrate in the reaction to determine the concentration of the 350-kDa band. The progress curve of the changes in the 350-kDa VWF product band as a function of time was fit to Equation 1 to obtain the apparent first order rate constant ($k_{obs}$) and the maximum product generated ($[VWF]_{max}$), where $k_{obs} = E_kcat/K_m$.

$$[VWF]_t = [VWF]_{max}(1 - e^{-k_{obs}t}) \quad (1)$$

Kinetics of FRETS-VWF73 Cleavage—A peptide containing the cleavage site of ADAMTS13, consisting of Asp$^{1596}$–Arg$^{1668}$ of VWF, was synthesized by the Peptide Institute (Osaka, Japan). Two amino acids flanking the cleavage site (Gln$^{1599}$ and Asn$^{1610}$) were substituted with 2,3-diamino-propionic acid modified with 2-(methylamino)benzoyl and 2,4-dinitrophenol, respectively, as fluorescence resonance energy transfer donor and quencher pairs (30). Fluorescence intensities were measured with a PerkinElmer LS55 using a plate reader accessory in white 96-well plates. Increases in fluorescence intensities ($F_{obs} - F_o = \Delta F$) recorded with time using an excitation wavelength of 340 nm (5-nm band pass) and an emission wavelength of 450 nm (5-nm band pass). Various concentrations of substrate were incubated with 0.17 nM (5-nm band pass) and an emission wavelength of 450 nm (5-nm band pass). Various concentrations of substrate were incubated with 0.17 nM plasma ADAMTS13 in assay buffer. FRETS-VWF73 (2 µM) was cleaved to completion (17.5 h) to establish the relationship between product concentration and fluorescence intensity ($\Delta F$). The observed velocities ($\Delta F$/min) as a function of substrate concentration were fit to the Michaelis-Menten equation to obtain the maximum velocity ($V_{max}$) and the $K_m$. Nonlinear least squares analyses were performed with Sci-entist software (Micromath).

Cleavage of 2 µM FRETS-VWF73 by 0.3 mM recombinant ADAMTS13 as a function of calcium and zinc ion concentration was assayed similarly in 50 mM Hepes, pH 7.4, 150 mM NaCl, and 1 mg/ml bovine serum albumin, except the instrument used was a PerkinElmer Victor$^2$ V plate reader accessory with 340-nm 25-nm bandwidth excitation filter, and 450-nm 10-nm bandwidth emission filter. The errors in the fitted parameters are reported as ±2 S.D.

RESULTS

Metal Ion Dependence of ADAMTS13 Activity—Previous studies of ADAMTS13 demonstrated that barium and calcium ions enhanced the rate of proteolysis of VWF (4), but these studies employed conditions of pH 8.0 and barium ion concentration (10 mM) that do not occur in vivo. Therefore, the metal ion activation of ADAMTS13 from both plasma and recombinant sources was re-evaluated under more physiological conditions. The interactions of metal ions with plasma ADAMTS13 were characterized by analyzing the changes in the density of the 350-kDa VWF cleavage product by Western blotting. In contrast to previous results (4), increasing concentrations of BaCl$_2$ (up to 5 mM) had little effect on the activity of ADAMTS13 at pH 7.4 (Fig. 1). Similarly, Mg(SO$_4$) and Cu(SO$_4$) had little effect on the activity of ADAMTS13 at pH 7.4. Increasing concentrations of CoCl$_2$, Mn(SO$_4$)$_2$, and Ni(SO$_4$)$_2$ inhibited ADAMTS13 activity. Calcium ions enhanced the activity by at least ~3-fold (up to 5 mM). Zinc ions enhanced the activity by at least ~2-fold (at 1 mM) (Fig. 1). ADAMTS13 activity was undetectable at concentrations of zinc ions above 3 mM, possibly because of inhibition of the enzyme by Zn(OH)$^+$ (31). Approximately 50% of the susceptible VWF was cleaved in this experiment at the optimal concentrations of CaCl$_2$ or ZnCl$_2$, and the extent of activation by these ions is underestimated slightly because of decreases in substrate concentration during the reaction. Nevertheless, the results indicate that low concentrations of zinc or calcium ions enhance the cleavage of VWF by ADAMTS13.

To determine the optimal concentration of zinc ions for plasma ADAMTS13 activity, zinc ion concentrations were varied below 250 µM (Fig. 2A). Increasing concentrations of ZnCl$_2$ enhanced the activity of ADAMTS13 by ~2-fold (Fig. 2, A and C). The addition of 5 mM CaCl$_2$ in the presence of increasing concentrations of ZnCl$_2$ further enhanced the activity of ADAMTS13 by ~6-fold (Fig. 2, B and C). The ~6-fold increased activation demonstrated ADAMTS13 proteolysis of VWF to be dependent on both zinc and calcium ions and suggested a cooperative role of the two divalent cations.

Partial unfolding of VWF by chaotropic agents such as urea or guanidine HCl is required for rapid cleavage by ADAMTS13 in the absence of fluid shear stress (4–6, 23). To determine the effects of zinc and calcium ions on the proteolysis of VWF under these conditions, VWF was pretreated in varying concentrations of guanidine HCl or urea and then diluted 10-fold into reactions containing ADAMTS13. In the presence of zinc and calcium ions, the optimal concentration of guanidine HCl was between 1.0 and 1.25 M guanidine HCl (initial concentrations) for maximal substrate proteolysis (data not shown), which is similar to previous results (5). In contrast, preincubation of VWF in up to 2 M urea did not accelerate cleavage by ADAMTS13 (data not shown). These results suggest that distinct VWF structures are produced upon incu-
calcium ions (100 μM) further increased ADAMTS13 activity (Fig. 3C). Similarity, increasing concentrations of zinc ions enhanced the activity of recombinant ADAMTS13 by ~200-fold, with a $K_{D_{app}}$ of 0.5 ± 0.3 μM. Similarly, increasing concentrations of calcium ions enhanced the activity of recombinant ADAMTS13 by ~160-fold, with a $K_{D_{app}}$ of 4.8 ± 3.0 μM. The addition of zinc ions to reactions containing near saturating calcium ions (100 μM) further increased ADAMTS13 activity (Fig. 3E).

These results indicate a cooperative role for calcium and zinc ions in supporting ADAMTS13 activity.

**Effects of Barium Ions on ADAMTS13 Activity**—Plasma ADAMTS13 activity is enhanced by the addition of barium ions, when reactions are performed using citrate anticoagulated plasma in low ionic strength buffer at pH 8.0 (4). However, little or no rate enhancement by barium ions was observed at $I = 75$ mM and pH 7.4 (Fig. 1), and the ability of citrate to buffer divergent metal ions suggested that the reported barium ion effects might not accurately reflect the properties of ADAMTS13. Therefore, the effect of barium ions was determined using plasma samples that were anticoagulated with sodium citrate or with nonchelating inhibitors (FPR-CK, FFR-CK, hirudin, and corn trypsin inhibitor), and reactions were performed in at low ionic strength ($I = 2.5–22.5$ mM) such that the citrate concentration was either 1 or 0 mM (Fig. 4A). In the presence of citrate, ADAMTS13 activity was enhanced ~2-fold by increasing concentrations of barium ions. In the absence of sodium citrate, however, ADAMTS13 activity was not affected by barium ions. Chelation of zinc and calcium ions by citrate also inhibited recombinant ADAMTS13 (Fig. 4B), supporting the conclusion that anticoagulation of plasma by citrate inhibits ADAMTS13.

To avoid the confounding effects of zinc and calcium ions in plasma, activation by barium ions was investigated using recombinant ADAMTS13. Barium ions supported ADAMTS13 activity comparable with that achieved by calcium ions, but with a $K_{D_{app}}$ value of $1.7 ± 0.8$ mM (Figs. 3D and 4C) that is ~350-fold higher than observed for calcium ions (Fig. 3D).

**Effects of Ionic Strength and Sodium Ions on ADAMTS13 Activity**—In reactions containing plasma VWF and urea denaturant, ADAMTS13 in citrated plasma is markedly inhibited by increasing ionic strength and is almost inactive in 150 mM NaCl (4). In vivo, however, ADAMTS13 must
operate efficiently at physiological ionic strength. Therefore, the dependence of ADAMTS13 activity on ionic strength was investigated with optimal concentrations of zinc and calcium ions (Fig. 5). Under these conditions ADAMTS13 was fully active at ionic strengths up to \( I = 285 \text{ mM} \) (including the contribution of ionized guanidine HCl). Several zinc metalloproteinases and serine proteinases have demonstrated a dependence on sodium ions for activity (33, 34). However, increasing concentrations of sodium chloride, maintaining constant ionic strength with choline chloride, did not alter ADAMTS13 activity (data not shown), indicating that sodium ions do not have a specific effect on ADAMTS13. The results indicate that high ionic strength does not necessarily inhibit ADAMTS13.

**Kinetics of VWF Cleavage**—The kinetic properties of ADAMTS13 were assessed under conditions approximating physiological pH, ionic strength, and concentrations of zinc and calcium ions. The purified VWF substrate was pretreated with 1.2 \( \text{mM} \) guanidine HCl to induce a conformation susceptible to cleavage (5) and diluted 10-fold into the reaction. Proteolysis of VWF was dependent on both enzyme (Fig. 6A) and substrate concentrations (Fig. 6B). The rate of proteolysis displayed a hyperbolic dependence on VWF concentration with a \( K_{\text{m,app}} \) of 3.7 ± 1.4 \( \mu\text{g/ml} \) or 15 \( \text{nm} \) in VWF subunits (Fig. 6C). ADAMTS13 binds directly to surface-immobilized VWF with a \( K_{\text{d,app}} \) of \( \sim4 \mu\text{g/ml} \) (35), and the concentration of VWF in plasma is \( \sim5–10 \mu\text{g/ml} \) (36), suggesting that ADAMTS13 functions within a physiological substrate concentration range. The enzyme displayed exponential progress curves at a relatively low VWF concentration of 2 \( \mu\text{g/ml} \) (\( \sim0.5 \times K_{\text{m,app}} \)), consistent with first order reaction kinetics and an apparent first order rate constant \( k_{\text{obs}} \) of 0.033 ± 0.021 \( \text{min}^{-1} \) and \( [\text{VWF}]_{\text{max}} \) of 0.34 ± 0.07 \( \mu\text{g/ml} \) VWF cleaved (Fig. 7). These results indicate a value for \( k_{\text{cat}} \) of \( \sim0.83 \text{ min}^{-1} \). This value must be considered a rough estimate because the generation of observed 350-kDa VWF cleavage product requires the cleavage of two adjacent subunits. Determining the true kinetic constants for VWF will require the use of an assay that measures the cleavage of single bonds. Control experiments showed that ADAMTS13 activity was stable for at least 3 h under reaction conditions. Thus, ADAMTS13 cleaves VWF efficiently under physiological conditions of pH, ionic strength, and metal ion concentration. However, a maximum of 14 ± 5% of the total VWF substrate was susceptible to ADAMTS13.

The incomplete cleavage of VWF (Fig. 7) suggested that the substrate was heterogeneous in sensitivity to ADAMTS13 or became resistant during the course of the reaction. To distinguish these possibilities, VWF pretreated with guanidine HCl was diluted 10-fold into reaction buffer and incubated for 100 min prior to the addition of ADAMTS13. Similar reaction kinetics were observed, and a maximum of 6 ± 2% of the substrate was cleaved (data not shown). Thus, the partial cleavage of VWF was reduced \( \sim50% \) but not eliminated by preincubation without enzyme. The state of the uncleavable VWF is unclear. Denaturation with low concentrations of guanidine may never allow VWF to adopt a cleavable conformation, or upon dilution, some VWF may immediately refold and become resistant to ADAMTS13. These results are consistent with the effects of guanidine HCl on the cleavage of plasma VWF (5) and recombinant VWF (23) by highly purified plasma ADAMTS13.

**FIGURE 4. Effects of citrate and barium ions on ADAMTS13 activity at low ionic strength.** A, plasma ADAMTS13 (0.6 \( \mu\text{M} \)) was assayed in 5 \( \text{mM} \) Hepes, pH 8.0, with VWF substrate (2 \( \mu\text{g/ml} \)) and increasing concentrations of barium ion with or without 1 \( \text{mM} \) sodium citrate. The 350-kDa VWF cleavage product was detected by gel electrophoresis and Western blotting. B, effects of sodium citrate on recombinant ADAMTS13 (30 \( \mu\text{M} \)) in the presence and absence of zinc and calcium ions. C, recombinant ADAMTS13 (30 \( \mu\text{M} \)) was assayed as in A with increasing concentrations of barium ions and without sodium citrate. D, the activation (fold) of ADAMTS13 as a function of barium ion concentration was determined by chemiluminescence analysis of Western blots from several experiments including those in panel B. The maximum extent of VWF cleavage product formed was \( <15\% \) of total cleavable VWF all conditions. The line represents the least squares fit of the quadratic binding equation to the data using the parameters described in the text.

**FIGURE 5. Dependence of ADAMTS13 activity on ionic strength and sodium ion concentration.** Plasma ADAMTS13 was assayed in 50 \( \text{mM} \) Hepes, pH 7.4, 250 \( \mu\text{M} \) \( \text{ZnCl}_2 \), and 5 \( \text{mM} \) \( \text{CaCl}_2 \) with increasing concentrations of sodium chloride (mM) without (−) or with (+) 10 \( \mu\text{M} \) EDTA. The 350-kDa VWF cleavage product was detected by gel electrophoresis and Western blotting.

**FIGURE 6. Activity of ADAMTS13 at physiological pH and ionic strength.** A, the concentration of plasma ADAMTS13 was varied in reactions (1 h) containing 250 \( \mu\text{M} \) \( \text{ZnCl}_2 \), 5 \( \text{mM} \) \( \text{CaCl}_2 \), and a constant VWF concentration (20 \( \mu\text{g/ml} \)). B, the concentration of VWF was varied in reactions containing 250 \( \mu\text{M} \) \( \text{ZnCl}_2 \), 5 \( \text{mM} \) \( \text{CaCl}_2 \), and a constant ADAMTS13 concentration (0.6 \( \mu\text{M} \)). C, the densities of the 350-kDa product band as a function of VWF concentration were calculated from scanned films for the experiments performed as in B. The line represents the least squares fit of the Michaelis-Menten equation with the parameters described in the text. Under these conditions, VWF cleavage product generation was linear with time and \( <15\% \) of susceptible substrate was cleaved during the assay.
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FRET-VWF73 Cleavage—The activity of ADAMTS13 toward a structurally homogeneous substrate was investigated using a fluorescent synthetic peptide, FRET-VWF73, corresponding to residues Asp1596–Arg1668 of VWF domain A2 and containing the Tyr1605–Met1606 bond cleaved by ADAMTS13 (30). The reactions were performed at approximately physiological pH, ionic strength, and concentrations of zinc and calcium ions. Proteolysis of FRET-VWF73 was linear with time up to 2 h (data not shown), confirming the stability of ADAMTS13 activity. The reaction demonstrated typical Michaelis-Menten kinetics, with a \( k_{\text{cat}} \) of 3.2 ± 1.1 \( \mu \text{M} \) and a \( V_{\text{max}} \) of 1.04 ± 0.10 \( \Delta F/\text{min} \), or a \( k_{\text{cat}}/K_{\text{m}} \) of 58 min \(^{-1} \) (Fig. 8), which is 70-fold greater than the \( k_{\text{cat}}/K_{\text{m}} \) for VWF cleavage of 0.83 min \(^{-1} \). The 210-fold increase in \( k_{\text{cat}}/K_{\text{m}} \) compared with multimeric VWF treated with guanidine HCl (Fig. 6) suggests that FRET-VWF73 lacks tertiary structure or possibly exosites within full-length VWF that may be required for efficient substrate recognition.

The metal ion dependence of FRET-VWF73 cleavage was qualitatively similar to that of VWF cleavage, although the \( K_{\text{d}} \) for calcium ion was higher. Activity was undetectable without added metal ions and was increased by calcium ions with a \( K_{\text{d}} \) of 60 ± 25 \( \mu \text{M} \). In the presence of 10 \( \mu \text{M} \) zinc ions, calcium supported a similar level of activity with a \( K_{\text{d}} \) of 74 ± 35 \( \mu \text{M} \) (Fig. 9A). Zinc ions alone stimulated ADAMTS13 activity at concentrations <20 \( \mu \text{M} \), but higher concentrations were markedly inhibitory. In the presence of optimal calcium ions, concentrations of zinc ion <10 \( \mu \text{M} \) supported full activity, and higher concentrations were inhibitory (Fig. 9B).

Barium ions also stimulated the cleavage of FRET-VWF73 (2 \( \mu \text{M} \)). Normalized to the maximal activity with 10 \( \mu \text{M} \) zinc and 1.5 \( \text{mM} \) calcium ions (100%), the activity observed with 10 \( \text{mM} \) barium ions was 66%, and that with 10 \( \text{mM} \) barium plus 10 \( \mu \text{M} \) zinc ions was 89%.

**DISCUSSION**

ADAMTS13 shares several metal ion binding properties with other metalloproteinases. The active site zinc ion of ADAMTS13 binds to the sequence HEXXHXGXXHHD \(^{285} \) (1), which is common among other metalloproteinases including members of the matrix metalloproteinase and the ADAM families (18, 19, 37). For some metalloproteinases, divalent cations other than zinc can support catalytic activity. For example, reconstitution of apo-stromelysin (MMP-3) with cobalt ions restored the activity by 80% (38), and reconstitution of apo-matriplisin (MMP-7) with manganese, nickel, or cobalt ions also restored activity (39, 40). In addition, calcium, magnesium, and manganese ions have been demonstrated to support procollagen N-proteinase (ADAMTS2) activity, whereas copper and high concentrations of zinc ions inhibit the enzyme (41, 42). Cobalt or copper ions supported the catalytic activity of astacin, and the crystal structure showed that these ions had pentagonal bipyramidal coordination states similar to that of the catalytic zinc ion in native astacin. In contrast, mercury or nickel ions displayed different coordination geometries and inhibited the enzyme (43). In the present study, copper ions slightly enhanced the activity of ADAMTS13, whereas nickel ions inhibited the enzyme and cobalt ions had no effect (Fig. 1). These results are broadly similar to the divalent metal ion effects reported previously for plasma ADAMTS13 under somewhat different assay conditions including pH 8.0, 1 \( \text{mM} \) urea and low ionic strength (4). Additional experiments will be necessary to determine whether divalent metal ions other than zinc can support ADAMTS13 enzyme activity.

Like several other metalloproteinases, ADAMTS13 is inhibited by excessively high concentrations of zinc ions, perhaps because of the formation of Zn(OH)\(^{+} \) that binds to the catalytic Glu or Asp residue within the active site. For example, carboxypeptidase A is inhibited by zinc ions with a \( K_{\text{f}} \) of 24 \( \mu \text{M} \) (31). The amount of added zinc ions required to activate or inactivate ADAMTS13 depends on the concentration of metal ion chelators. In reactions containing 1 \( \text{mM} \) EDTA,
plasma ADAMTS13 was reported to be fully active with 2 mM total zinc ions but inactive with 3 mM zinc ions (23). In the present study, ADAMTS13 in citrated plasma was activated at least ~2-fold by 1 mM zinc ions but was inactive at concentrations >3 mM (Fig. 1), whereas recombinant ADAMTS13 with no added chelators was activated ~200-fold by 5 μM zinc ions (Figs. 3 and 9) and inactivated by >50 μM zinc ions (Fig. 9). This exquisite sensitivity to inhibition by excess zinc ions suggests that ADAMTS13 assays should precisely control the free concentrations of divalent metal ions, particularly in assays developed for clinical use.

Calcium ions play a structural role in many metalloproteinases and stimulate ADAMTS13 activity (4, 5, 23), suggesting a functional interaction between zinc and calcium ion binding. Calcium ions dramatically activate recombinant ADAMTS13 cleavage of VWF and bind with a $K_{D,app}$ of ~4.8 μM (Fig. 3), well below the plasma free calcium ion concentration of ~1.2 mM. The $K_{D,app}$ was significantly higher, ~60 μM, when assayed with the FRETS-VWF73 peptide substrate (Fig. 9A). The cause of this difference is not known. Although not studied in detail, the ability of calcium ions alone to stimulate ADAMTS13, a putative zinc-dependent metalloprotease, implies that a very low concentration of zinc ions is present in the dialyzed recombinant enzyme preparations used in these studies (Figs. 3D and 9A) and suggests that zinc and calcium ions bind cooperatively to ADAMTS13. Molecular modeling based on the crystal structures of adamalysin II (44) and ADAM33 (45), which are homologous to ADAMTS13, indicates that calcium ion- and zinc ion-binding sites are located on opposite sides of the ADAMTS13 metalloprotease domain, separated by ~24 Å (1) (data not shown). Therefore, cooperative interactions between the zinc and calcium ions must be mediated indirectly through changes in protein structure. Studies using apo-ADAMTS13 will need to be performed to further establish the role of zinc and calcium ions in the activity of ADAMTS13.

Barium and strontium ions reportedly are more potent than calcium ions for activating ADAMTS13, and clinical ADAMTS13 assays frequently employ supplementation with barium ions (4, 5). The stimulation of ADAMTS13 activity might be due to the occupancy of calcium-binding sites by barium ions, but this explanation probably is incomplete. At low ionic strength (I = 2.5 to 22.5 mM), ADAMTS13 activity in citrated plasma was enhanced ~2-fold by 5–10 mM barium ions (Fig. 4). In contrast, under conditions of physiological ionic strength and pH, the addition of increasing concentrations of barium ions had little or no effect on the activity of ADAMTS13 in citrated plasma (Fig. 1). Barium ions did not enhance ADAMTS13 activity in plasma that was free of citrate (Fig. 4A) and did not stimulate recombinant ADAMTS13 when present at concentrations comparable with the maximally effective concentrations of calcium ions (Fig. 4C). However, much higher levels of barium ions (~5 mM) did stimulate recombinant ADAMTS13 activity (Fig. 4D), suggesting that barium ions can occupy the calcium ion site on ADAMTS13 but with substantially lower affinity. Citrate also inhibits the calcium- and zinc-dependent activity of ADAMTS13 (Fig. 4B). Consequently, the ability of barium ions to activate ADAMTS13 in citrated plasma samples probably is due to the displacement of more potent calcium or zinc ions from complexes with citrate.

These results suggest that sodium citrate is not the optimal anticoagulant for assays of ADAMTS13 activity in blood samples. ADAMTS13 activity is normal in heparinized plasma (5), suggesting that heparin might be an acceptable nonchelating substitute for citrate. Alternatively, anticoagulation may be dispensable because ADAMTS13 appears to be recovered quantitatively in serum (4).

ADAMTS13 is inhibited markedly by increasing ionic strength when assayed in the presence of 1–1.5 mM urea (4, 46). However, little or no effect of ionic strength is observed in the absence of urea. Guanidine HCl induces a VWF conformation that is susceptible to ADAMTS13 but also relatively stable after the guanidine HCl concentration is reduced by dilution. The cleavage of guanidine-treated VWF is insensitive to sodium chloride (Fig. 5) or choline chloride (data not shown), indicating that the binding of ADAMTS13 to this form of the substrate does not depend strongly on ionic strength. Although the reaction conditions in these studies vary in other ways that could be significant, a primary role for urea in causing the differences is consistent with the well characterized ability of ionic strength to stabilize proteins against denaturation by urea (47, 48). Consequently, neutral salts like sodium chloride may simply prevent urea-induced changes in the conformation of the VWF substrate that would make it susceptible to ADAMTS13.

This conclusion is supported by changes in VWF conformation induced by physiological concentrations of sodium chloride and by other sodium salts, as monitored by intrinsic protein fluorescence (46).

The cleavage of the small fragment of VWF contained in FRETS-VWF73 is relatively insensitive to ionic strength (49) and does not require denaturants (30, 49). This isolated fragment also lacks significant secondary structure by NMR spectroscopy (50). These findings are consistent with the following model in which urea and ionic strength interact and modulate the cleavage of native VWF by ADAMTS13: the transition from a resistant to a susceptible conformation of VWF is facilitated by urea and inhibited by neutral salts, but the recognition and cleavage of the susceptible VWF conformation is largely independent of ionic strength.

Although FRETS-VWF73 does not require denaturation to be cleaved by ADAMTS13, guanidine-treated VWF has a ~210-fold lower $K_{m,app}$ (Fig. 8). This discrepancy implies that ADAMTS13 interacts with specific, extended structural features of multimeric VWF. Molecular modeling of the A2 domain suggests that it has a characteristic α/β-fold with a six-stranded β-sheet surrounded by three α-helices on each side. The Tyr1605–Met1606 peptide bond is predicted to be buried within the β-sheet, supporting the need for large conformational changes of the A2 domain prior to proteolysis (51, 52). Efficient cleavage also requires the segment Glu1660–Arg1666 in the C-terminal α-helix of the A2 domain (49). The fragment of VWF represented in FRETS-VWF73 corresponds to approximately three β-strands and three α-helices of the A2 domain, but when removed from the context of the complete domain this isolated peptide has no distinct secondary structure (50). Therefore, the relatively poor $K_{m,app}$ value for FRETS-VWF73 may reflect the large entropic cost of adopting a conformation that can bind ADAMTS13. In addition, such a small fragment of the A2 domain may lack additional sites on VWF that interact with ADAMTS13. For example, the adjacent A1 domain may bind cofactors that affect cleavage (53), and the A3 domain may provide a docking site for ADAMTS13 (54). Such interactions may explain why VWF has both a ~210-fold lower $K_{m,app}$ and a ~70-fold lower $k_{cat}$ than FRETS-VWF73. These changes have the effect of minimizing the difference in catalytic efficiency ($k_{cat}/K_{m,app}$) between VWF (55 μM⁻¹ min⁻¹) and FRETS-VWF73 (18 μM⁻¹ min⁻¹). These kinetic constants must be compared cautiously because the values for VWF are distorted by heterogeneity of the substrate and the complexity of the assay. Nevertheless, independent measurements of ADAMTS13 binding to immobilized VWF (35) yielded an equilibrium constant ($K_D = 14$ nM, per subunit of VWF) similar to the $K_{m,app}$ for VWF cleavage of 15 nM.

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*E. A. Tuley and P. J. Anderson, unpublished results.*
These studies demonstrate that both plasma and recombinant ADAMTS13 function efficiently under physiological conditions. Once VWF adopts a suitable conformation, perhaps under the influence of fluid shear stress, ADAMTS13 can cleave it rapidly at the pH levels, ionic strengths, and divalent metal ion concentrations that prevail in vivo. The ionic strength and denaturant concentrations that are optimal for ADAMTS13 assays in vitro, in the absence of fluid shear stress, promote conformational changes in VWF necessary to make it susceptible to cleavage. However, these nonphysiological conditions may impair the recognition of susceptible VWF by ADAMTS13.

The effect of calcium or barium ions on ADAMTS13 activity probably reflects binding to a structural metal ion site in the metalloprotease domain, as predicted by molecular modeling (1) The VWF substrate also might bind calcium ions, but this has not been reported. The VWF A1, A2, and A3 domains do not have MIDAS metal ion sites found in the homologous A domains of certain integrin subunits, and the crystal structures of the VWF A1 and A3 domains do not show metal ions (55, 56). Whether other VWF domains bind metal ions is unknown. Calcium ions are required for optimal cleavage of the FRETS-VWF73 peptide substrate (Fig. 9), which is disordered in solution (50) and presumably unable to bind calcium ions with high affinity. Therefore, calcium ions probably bind directly to ADAMTS13 to stimulate substrate cleavage. Although one calcium site is likely to be in the metalloprotease domain, additional sites in other domains cannot be excluded. For example, the CUB domains in complement component C1s have well defined calcium binding sites (57), suggesting that the two C-terminal CUB domains of ADAMTS13 might have similar sites. Detailed understanding of how ADAMTS13 regulates platelet adhesion should be facilitated by further characterization of the requirements for substrate exposure and recognition by ADAMTS13.

Acknowledgments—We thank Claudine Mazurier (Laboratoire Francais du Fractionnement et des Biotechnologies, Lille, France) for the generous gift of purified VWF, Mary Burritt and John Buts (Mayo Clinic, Rochester, MN) for performing inductively coupled plasma spectroscopy analyses, and Paul E. Bock (Vanderbilt University School of Medicine) for helpful discussions of data analysis.

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