Molecular Mapping of the Chloride-binding Site in von Willebrand Factor (VWF)

ENERGETICS AND CONFORMATIONAL EFFECTS ON THE VWF/ADAMTS-13 INTERACTION

Physiological concentrations of NaCl inhibit the hydrolysis of von Willebrand factor (VWF) by ADAMTS-13. This effect is because of the specific binding of chloride ions to VWF. Urea-induced unfolding was measured in the presence of NaCl, CH₃COONa, and NaClO₄ at pH 8.0, 25 °C, for multimeric VWF, the recombinant A1-A2-A3 VWF domains, and the A1 domain. Chloride stabilizes the folded conformation of the A1-A2-A3 and A1 domains more efficiently than acetate but less strongly than perchlorate. Spectroscopic evidence showed that chloride binds to both the A1 and A1-A2 domain but not to the isolated A2 domain. Binding of Cl⁻ to both wild type (WT) and the natural mutant p.R1306W A1-A2-A3 domains of VWF has a large heat capacity change equal to −1 and −0.4 kcal mol⁻¹ K⁻¹ for WT and p.R1306W A1-A2-A3 domains, respectively. This result implies that a burial of a vast apolar surface area is caused by conformational transitions linked to chloride binding. At any temperature, chloride affinity was higher for WT than for the mutant p.R1306W form. Chloride ions inhibit hydrolysis by ADAMTS-13 of the A1-A2-A3 and A1-A2 domains in the presence of either urea or high shear stress, whereas this effect was either absent or negligible in experiments using A2 and A2-A3 domains. These findings show that the A1 domain contains the binding site of chloride ions that control allosterically the proteolysis by ADAMTS-13 of the Tyr¹₆₀₅–Met¹₆₀₆ bond in the A2 domain and that the R1306W mutation of type 2B VWD quenches the binding of chloride ion to the A1 domain.

The degradation of von Willebrand factor (VWF) depends on the activity of the zinc protease ADAMTS-13, which cleaves this multimeric adhesive protein at the peptide bond between residues Tyr¹₆₀₅ and Met¹₆₀₆ of the A2 domain (1–5). In contrast with other zinc proteases, such as the metalloproteases, ADAMTS-13 does not need activation by other hydrolases to become proteolytically competent toward VWF (1). Hence this protease must be regulated by other factors to avoid the continuous cleavage of high molecular weight VWF multimers, which play a key role in primary hemostasis. The regulatory mechanisms of the VWF/ADAMTS-13 interaction are linked to the high conformational mobility of VWF. The adhesion of platelets to the vessel wall, under the high flow/shear conditions of blood in the microcirculation, is mediated by VWF and in particular by sequences within the A1 and A3 domains. The A1 domain (residues 1260–1479) binds to the platelet glycoprotein Ib-IX-V complex (GpIb), to subendothelial glycosaminoglycans, to sulfatides of the cell surface (6), and to nonfibrillar collagen type VI (7). Native VWF in blood has no appreciable binding to platelets. This interaction can take place by means of such exogenous nonphysiologic modulators as ristocetin and botrocetin (8, 9) and by fluid shear stress (5, 10) or can be the consequence of gain-of-function mutations in the A1 domain causing type 2B von Willebrand disease (VWD) (11, 12). It is generally assumed that the modulation of GpIbα binding activity involves conformational changes in VWF that lead to the exposure of functional sites normally cryptic when the molecule is in solution. In vivo, this may occur under high shear stress (5, 10) or when VWF interacts with such subendothelial components as collagen after the integrity of the vessel wall is disrupted (13). The formation of VWF-GpIbα complex was recently found to accelerate the specific cleavage of VWF by ADAMTS-13 (14). This finding implies that the conformation of the A1 domain regulates through allosteric mechanisms the accessibility of the ADAMTS-13 cleavage site in the A2 domain of VWF. On the other hand, the VWF A-like domain is conserved in several plasma and matrix proteins involved in different biological functions and is now considered the prototype for a protein superfamily containing at least 75 proteins that are similar in sequence (15). For many of these proteins the VWA domain is characterized by a strong ability to transmit allosteric signals in virtue of its high conformational mobility (16, 17). The hydrolysis of VWF can be obtained in vitro under static conditions, using a mild denaturing milieu, which generates partially unfolded VWF conformers that are susceptible to pro-
teolytic attack by ADAMTS-13. Moreover, early studies by Furlan et al. (2), recently extended by our group (17) on the specificity of the VWF/ADAMTS-13 interaction showed that physiological concentrations of NaCl inhibit allosterically the hydrolysis of VWF in the presence of mild denaturing urea concentrations. We largely ascribed this effect to the specific binding of chloride ions to VWF (17), but the molecular mapping of the anion-binding site is still elusive. The goal of this study was to assess within the A1-A2-A3 domains of VWF the effect of chloride binding on the unfolding of the protein and on its conformational stability. A further goal of the study was to map the binding site of chloride ions within the A1-A3 domains and to analyze the allosteric linkage with the hydrolysis of the Tyr1605–Met1606 peptide bond in the A2 domain. The natural type 2B VWD mutant, p.R1306W VWF, was also used to investigate how this mutation is allosterically linked to both chloride binding and to the efficiency of VWF hydrolysis by ADAMTS-13.

MATERIALS AND METHODS

Production of Vectors Containing A1-A2-A3 and A1-A2 VWF cDNA

Step 1 — The sequence encoding domains A1, A2, and A3 was amplified with designed primers (set A) available on request, using as a template the pSV-VWFH vector, a kind gift of Lysiane Hilbert and Claudine Mazurier (Lille, France), which contains the full-length human VWF cDNA (18). The PCR product was purified and digested with AarII and EcoRV (New England Biolabs, Beverly, MA) and then ligated into pSV-VWFH vector, which had been digested with the same enzymes, in order to obtain the pSV-A1A2A3V5 vector.

Step 2 — The construction of the pSV-SPHA1A2A3V5 cloning vector, containing also the VWF signal peptide and the His6 tag sequence, required a second PCR (PCR condition and primers, set B, are available on request). The pSV-VWFH was used as a template, and the PCR product was purified, digested with AvrII and Tth111I, and ligated into the pSV-A1A2A3V5 vector, which had been digested with the same enzymes, in order to obtain the pSV-A1A2A3V5 vector.

Step 3 — The pSV-SPHA1A2A3V5 vector was further amplified with designed primers available on request (set C). The PCR product was purified and digested with HindIII and EcoRV and then ligated into the pcdNA3.1 mammalian expression vector (Invitrogen), which had been digested with the same enzymes, in order to obtain the pcdNA3.1SPHA1A2A3V5 expression vector. The pcdNA3.1SPHA1A2A3V5 vector, which had been digested with the same enzymes, was amplified with designed primers available on request (set C). The PCR product was purified and digested with HindIII and EcoRV and then ligated into the pcdNA3.1 mammalian expression vector (Invitrogen), which had been digested with HindIII and Pmel, in order to obtain the pcdNA3.1SPHA1A2A3V5 expression vector. The ligase was feasible because both EcoRV and Pmel cause blunt ends.

Step 4 — The p.R1306W mutation was introduced into pcdNA3.1SPHA1A2A3V5 using a QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). A forward primer (5'-GGACATGATGGAGTGGCTGCGCATCTCC-3') and a reverse primer (5'-GGAGATGGCCAGCCACTCCATCATGCCATC-3') were used to introduce c.3916C>T (designated as T), to obtain pcdNA3.1SPHA1A2A3V5-R1306W.

Step 5 — A small portion of pcdNA3.1SPHA1A2A3V5 vector, containing a short sequence of the A2 domain and the V5 tag sequence, was amplified (primer set D available on request). The product of this PCR was purified, digested with BspEl and DraII, and then ligated into pcdNA3.1SPHA1A2A3V5 vector, which had been digested with the same enzymes, in order to obtain pcdNA3.1SPHA1A2A3V5. Cloned inserts were sequenced to confirm the correct sequence (19).

Transient Transfection of Wild Type (WT) and Mutant VWF Fragment Expression Vectors

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium enriched with nutrient mixture F-12 supplemented with 10% fetal bovine serum, 2 mmol/liter L-glutamine, and 100 mg/ml penicillin/streptomycin (Invitrogen) in a 5% CO2 atmosphere at 37 °C. Approximately 5 × 106 HEK293 cells were independently transfected with pcdNA3.1SPHA1A2A3V5, pcdNA3.1SPHA1A2A3V5-R1306W, and pcdNA3.1SPHA1A2A3V5 expression vectors by using a method described previously (20). Serum-free conditioned medium was applied to confluent cells 14 h after transfection, collected after additional 72 h, and added with phenylmethylsulfonyl fluoride (Sigma) to a final concentration of 1 mM.

Production and Expression of Recombinant A2 and A2-A3 VWF Fragments

The sequence encoding domains A2 and A2-A3 were amplified with primer sets E and F, respectively (available on request), using as a template the pcdNA3.1SPHA1A2A3V5 vector. The PCR product obtained with primer set E was purified, digested with XhoI and HindIII, and then ligated into prSET-A (Invitrogen) vector, which had been digested with the same enzymes, in order to obtain the prSET-A2V5 vector. The PCR product obtained with primer set F was purified and digested with the KpnI and HindIII and then ligated into the prSET-A2V5 vector, which had been digested with the same enzymes in order to obtain the prSET-A2A3V5 vector. Both prSET-A2V5 and prSET-A2A3V5 vectors were expressed in BL21 Escherichia coli cells (Invitrogen). Transformed bacteria were grown in LB broth at 37 °C and shaken. Recombinant protein production was induced with 1 mM isopropyl β-d-thiogalactoside overnight. Bacteria were harvested and lysed using CellLytic™ Express (Sigma) reagent, and the soluble fraction was collected.

Purification of VWF Fragments

Conditioned media of transfected HEK293 and the soluble fraction from bacteria were dialyzed into phosphate-buffered saline overnight at 4 °C, and recombinant proteins were purified on a nickel-chelated agarose column ( Pierce) according to manufacturer’s instructions. Purified proteins were dialyzed into 10 mM Heps, pH 7.0, overnight at 4 °C. Recombinant proteins were analyzed by SDS-PAGE and Coomassie staining, and the concentration was quantified using a protein assay (Bio-Rad) and spectrophotometrically at 280 nm, using extinction coefficients (g/liter) of 0.574, 0.682, 0.999, 0.612, and 0.548 for WT A1-A2-A3, p.R1306W A1-A2-A3 mutant, A2, A1-A2, and A2-A3 domains, respectively. The A2-A3 domains obtained from the above procedure under nonreducing conditions showed a series of bands with a molecular mass of greater than 48 kDa (not shown). Western blot analysis showed that all these bands reacted with rabbit horseradish peroxidase-labeled polyclonal anti-VWF antibody from Bethyl Laboratories, Inc. (Prodotti Gianni, Milano, Italy). These bands disappeared
under reducing conditions, where a single band of ~48 kDa showed up. This finding suggested that several constructs randomly stem in bacteria from the folding process, using different arrangements of the free cysteine residues present in the construct. Thus, a further purification was performed by RP-HPLC using a C4 column (5–60% CH3CN in 0.1% trifluoroacetic acid in 40 min). The peaks corresponding to the 48-kDa species were dried under vacuum and resuspended in 10 mM Hepes, pH 7.50. Finally, to check the correct folding of this species, a collagen binding assay (Life Therapeutics, Cabru, Peregalo di Lesmo, Italy) was performed on this material and compared with the behavior of purified A1-A3 and isolated A2 domains, knowing that only the A1 and A3 domains interact with equine tendon collagen (7). The assay confirmed that the A2 domain did not interact with immobilized collagen, whereas 0.05–0.15 mg/ml of both A1-A2-A3 and A2-A3 bound to collagen with progressively higher avidity (in both cases an absorbance value of >5-fold with respect to blank at 0.05 mg/ml). This finding showed that the A2-A3 domains support significant binding to collagen, strongly suggesting that the HPLC-purified A2-A3 domains folded correctly.

**Purification of Blood-derived Full-length VWF and the 39/34-kDa A1 Domain**

**Multimeric VWF**—Pharmaceutical preparations of VWF concentrates (Fahndi, Grifols, Ghezzano, Italy) were used as source material to purify VWF. Briefly, 20,000 units of preparation were suspended in 20 ml of distilled water and filtered using 2-μm pore-sized membranes. This solution was gel-filtered at a flow rate of 2.0 ml/min onto a 70×30 cm Sephacryl S500 column equilibrated with 10 mM Tris-HCl, 0.15 M NaCl, pH 8.0, at 25 °C to eliminate albumin present in the preparation. The peak containing VWF was pooled and passed through a heparin-Sepharose column (25×1.5 cm; Amersham Biosciences) equilibrated in the same buffer at a flow rate of 0.8 ml/min. A gradient of 0.1–0.5 M NaCl was applied for 60 min. The last peak to be eluted (0.29 M NaCl) was pooled, and aliquots were stored at −80 °C. SDS-PAGE of this material showed under reducing conditions the existence of a single band with an apparent molecular mass of about 230 kDa. The concentration of this purified material was measured by a standardized method using a commercial kit for VWF antigen (Instrumentation Laboratory, Milan, Italy) and an automatic instrument (Instrumentation Laboratory). By using the same instrument and a commercially available assay (VWF activity; Instrumentation Laboratory), the ratio between VWF antigen and ristocetin cofactor activity (expressed as percentage) was equal to 1.05, demonstrating that the purified protein had an intact functional activity. Accordingly, purified VWF showed a 110% aggregating behavior of purified A1-A3 and isolated A2 domains, knowing that only the A1 and A3 domains interact with equine tendon collagen (7). The assay confirmed that the A2 domain did not interact with immobilized collagen, whereas 0.05–0.15 mg/ml of both A1-A2-A3 and A2-A3 bound to collagen with progressively higher avidity (in both cases an absorbance value of >5-fold with respect to blank at 0.05 mg/ml). This finding showed that the A2-A3 domains support significant binding to collagen, strongly suggesting that the HPLC-purified A2-A3 domains folded correctly.

**Expression and Purification of Recombinant ADAMTS-13**

Full-length human ADAMTS-13 was expressed and purified as reported previously (17). SDS-PAGE on 4–12% gradient gels showed that under reducing conditions the purified material consisted of a single band of ~170 kDa.

**Urea-induced Unfolding Experiments Monitored by Intrinsic Protein Fluorescence**

Urea-induced unfolding curves were fitted to a two-state equation, as described previously (22), to calculate the thermodynamic parameters ΔG, Cm, and m. The observed free energy of unfolding, ΔG, is assumed to be a linear function of urea molarity with ΔG = ΔG0 − m[U], where m represents the sensitivity of the transition to the urea concentration, and ΔG0 is the free energy of unfolding in the absence of denaturants at a standard state of 1 M. Another derived parameter, U1⁄2, defined as the ratio (ΔG0/m), is the concentration of urea where the fraction of unfolded protein equals that in the native state. The values of these parameters and their standard deviations are the average of at least two unfolding experiments. For each determination of the unfolding free energy under a particular set of salt conditions, increasing urea concentrations were analyzed by adding stepwise to VWF and its A domains a stock urea solution (9 M) buffered with 5 mM Tris-HCl, pH 8.0, at 25 °C. The recorded fluorescence spectra were corrected for the contribution of urea alone and the dilution factor of the protein solution. The extent of denaturation was monitored at 25 °C by tryptophan and tyrosine fluorescence at 337 nm on a thermostated spectrophotometer (Varian Cary Eclipse) with an excitation wavelength of 295/280 nm (5 nm bandwidth at both excitation and emission wavelength).

Specifically, fluorescence intensity (F) as a function of urea concentration ([U]) was fitted to Equation 1,

\[
F = \frac{(F_{\text{ON}} + s_0[U]) + (F_{\text{OD}} + s_0[U]) \exp^{-\frac{(\Delta G - m[U])/kT}{1 + \exp^{-\frac{(\Delta G - m[U])/kT}}}}}{1 + \exp^{-\frac{(\Delta G - m[U])/kT}}}
\]

(Eq. 1)
where $\Delta G$ and $m$ are the intercept and slope, respectively, of the linear extrapolation of the unfolding free energy to zero urea concentration; $F_{\text{ON}}$ and $s_N$ are the intercept and slope, respectively, of the native state base line; $F_{\text{OD}}$ and $s_D$ are their counterparts of the denatured state base line, and $K_p$ and $T$ are the Boltzmann’s constant and the absolute temperature. Each sample, thermostatted at 25 °C, was allowed to equilibrate for at least 15 min, and the final VWF and the concentration of its domains were always ~0.1 mg/ml, to avoid inner filter effects.

Fluorescence experiments of guanidine chloride-dependent unfolding were also performed to demonstrate the applicability of the two-state model of unfolding for the A1-A2-A3 domain.

Data were analyzed by the linear extrapolation method, using GRAFIT (Erithacus Software, Horley Surrey, UK). To study the effect of salts on $\Delta G$, samples with the same concentration of salts were prepared in duplicate, and fluorescence data sets in the different repeats were fitted to Equation 1 with $\Delta G$, $m$, $F_{\text{ON}}$, $s_N$, $F_{\text{OD}}$, and $s_D$ as specific to a particular data set. Standard errors of fitting parameters were calculated along with the best fit values.

**Temperature Dependence of Cl$^-$ Interaction with the A1-A2-A3 Domains of VWF**

The temperature dependence of Cl$^-$ binding was analyzed by change of the intrinsic fluorescence of the various VWF domains, as reported previously (17). The experiments were performed over a temperature range from 7 to 40 °C, in a 5 mM Tris-HCl buffer, pH 8.0, at all temperatures. The pH of the buffer solution was always set at 25 °C, using a $\Delta pH/\Delta T$ value of Tris buffer of -0.027. In some experiments, intrinsic fluorescence of the A1-A2-A3 domains was studied as a function of unfraccionated heparin (from porcine intestinal mucosa; Calbiochem) up to a concentration of 10 μM. The thermodynamic parameters of binding were calculated using the van’t Hoff analysis and fitting for $\Delta C_p$, $T_p$, and $T_S$, contained in Equation 2, which assumes a large negative heat capacity change (23),

$$-\log K_d = \Delta C_p/R \cdot 2.303 (T_p/T - \ln(T_p/T) - 1) \tag{Eq. 2}$$

where $\Delta C_p$ is the heat capacity change, and $T_p$ and $T_S$ define the characteristic temperatures where the enthalpic ($\Delta H$) and entropic ($\Delta S$) contributions to the free energy change are 0, respectively.

**Hydrolysis of Recombinant VWF Constructs by rADAMTS-13**

Recombinant ADAMTS-13 (final reaction concentration 5–10 nM) was incubated in 5 mM Tris-HCl, 1.5 mM urea, 3 mM BaCl_2, NaCl from 0 to 0.15 M, pH 8.0, at 25 °C with 0.05 mg/ml of purified A1-A2-A3 (WT and p.R1306W), A1-A2, A2-A3, and A2 domains of VWF. In the latter case urea was omitted from the buffer solution, because the denaturant partially inhibits the protease activity of ADAMTS-13. At different time points, 50 µl of the samples were removed; the reaction was stopped with 10 mM EDTA, and the reaction products were analyzed by RP-HPLC. Samples were analyzed by means of a 250 × 4.6-mm RP-304 column (Bio-Rad). The chromatographic run was performed by applying the following conditions: 5% acetonitrile for 5 min, from 5 to 60% acetonitrile in 0.1% trifluoroacetic acid for 60 min for A1-A2-A3 domains and 40 min for the A2 domain, whereas the acetonitrile gradient was 20% for 5 min and from 20 to 65% in 45 min for the A1-A2 and A2-A3 fragments. The peaks were detected routinely at 214 or 254 nm.

For the shear stress experiments, a cone-plate rheometer (model CSL 500) from Carried (now TA Instruments, Waters S.p.A., Vinmodrone, Italy) was used. The used cone had a diameter of 4 cm and the cone-plate gap was of 10 μm. The experimental conditions used were 5 mM Tris-HCl, 3 mM BaCl_2, salt either 0.01 or 0.15 M NaCl, pH 8.0, at 37 °C. The purified A1-A2-A3 domains of VWF were used at 0.05 mg/ml, whereas recombinant ADAMTS-13 was employed at 10 nM. To ensure achievement at the desired temperature, samples were applied on the cone-plate apparatus for about 1 min under static conditions. The shear stress applied was either of 0 or 40 dynes/cm², corresponding from the geometry of the system (0.2°) to a shear rate ranging from 818 to 850 s⁻¹. The total volume of the applied sample was of 344 μl. The shear was applied for 1 or 5 min. At the end, the cone-plate was stopped; the entire volume of the sample was recovered, and 10 mM EDTA (final concentration) was added to the solution to stop the reaction. The fragments produced by ADAMTS-13 were identified and quantified by RP-HPLC as described above. To check the specificity of the shear-induced VWF cleavage by ADAMTS-13, a control experiment was performed under the same experimental conditions except for the presence of 10 mM EDTA, and no significant VWF hydrolysis was observed. In the kinetic study with 1.5 mM urea, in virtue of the pseudo-first order conditions (concentration $< K_o$, of the reaction), the percent of the peptide cleaved at time $t$, $P(t)$, was fitted to Equation 3,

$$ P(t) = 100 \cdot (1 - \exp(-k_{\text{obs}} t)) \tag{Eq. 3}$$

$k_{\text{obs}}$ is the pseudo-first order rate of its hydrolysis, equal to $e_0 / K_{\text{cat}}$ ($e_0$ is the ADAMTS-13 concentration). The calculated $k_{\text{obs}}$ values were subsequently analyzed as a function of chloride concentration. The experimental data were analyzed on the basis of Scheme 1,

$$ E + S \underset{k_1}{\overset{k_{\text{cat}}}{\rightleftharpoons}} ES \underset{k_{-1}}{\longrightarrow} E + P_1 + P_2 $$

$$ E + \text{SCI}^- \underset{k_{1}}{\overset{k_{\text{cat}}}{\rightleftharpoons}} E \cdot \text{SCI}^- \underset{k_{-1}}{\longrightarrow} E + P_1 + P $$

**Scheme 1**

where $E$ is ADAMTS-13; $S$ is the VWF substrate; Cl$^-$ is chloride, and $P_1/P_2$ are the N- and C-terminal peptides produced by hydrolysis of the peptide bond between Tyr$^{1606}$ and Met$^{1606}$. Chloride acts as effector, whose binding and dissociation from VWF was considered much faster than that of VWF to the enzyme. Under these conditions, the pseudo-first order of the ADAMTS-13/VWF interaction, $k_{\text{obs}}$, was analyzed by the following linkage Equation 4 (17),

$$ k_{\text{obs}} = k_0 + k_{\text{Cl}}(\text{Cl}^-/K_d)/Z \tag{Eq. 4}$$
Chloride Binding to VWF

where \( Z \) is equal to \( (1 + (\text{Cl}^- / K_{d})) \), \( k^0 \), and \( k^{1}\) are pseudo-first order rate constants of hydrolysis pertaining to the Cl\(^-\)-free and Cl\(^-\)-bound VWF domain, and \( K_{d} \) is the equilibrium dissociation constant of chloride binding to VWF domain.

RESULTS

Production of VWF A Domains—In all cases expression and purification of the various VWF A domains allowed pure preparations to be obtained. The purity and molecular weight of these constructs were checked by SDS-PAGE on 4–12% gradient gels and were found equal to 72.5 kDa for WT and p.R1306W A1-A2-A3 domains, 53.4 kDa for the A1-A2 domain, 27.8 kDa for the A2 domain, and 48 kDa for the A2-A3 domain. Fig. 1 shows the SDS-PAGE of the A1-A2-A3, A1-A2, A2-A3, A1, and A2 domain used in this study.

Dependence on Salt Concentration of Free Energy for Urea-induced Unfolding of VWF Constructs—Urea-induced unfolding was measured versus NaCl, CH\(_3\)COONa, and NaClO\(_4\) concentration at pH 8.0, 25 °C, for full-length VWF, recombinant A1-A2-A3 domains, and purified A1 domain. First, the unfolding free energy \( \Delta G \) was measured versus urea molarity at a fixed salt concentration and extrapolated back to 0 M urea to give the free energy change associated with unfolding of VWF constructs under nondenaturing conditions. This procedure is repeated at a series of salt concentrations, and \( \Delta G^0 \) value is plotted versus salt. Control experiments with guanidine chloride were also carried out to validate the correctness of the two-state unfolding model. As shown in Fig. 2 in the absence of NaCl the pre- and post-unfolding fluorescence base lines extrapolated to zero denaturant give the same values regardless of which denaturant is used. This finding demonstrates that the two-state approach is consistent with the experimental data. This means that the difference in fluorescence values between native and unfolded proteins is really a property of the protein, independent of the degree of unfolding cooperativity \( (m \text{ values}) \) calculated from application of Equation 1. However the effect of chloride on the thermodynamics of unfolding of the A1-A2-A3 domain could be studied using urea alone, in consideration of the equimolar concentration of chloride present in the guanidine preparation. In Fig. 3, the spectroscopic data pertaining to unfolding by urea of full-length VWF, the A1-A2-A3 and A1 domains, are shown under the two NaCl concentrations (0–150 mM). It is known that the exposure to solvent water of aromatic amino acids leads to a decrease of their fluorescence intensity because of the quenching effect of water molecules (24). However, this principle can be apparently violated in some proteins, where the interactions between tryptophan and tyrosine residues in the buried region of the folded molecule lead to their mutual quenching, determining an unchanged or increased fluorescence intensity after partial or full unfolding (24). In this study, both constant and increased fluorescence intensity were recorded as a function of salt concentrations in the pre-unfolding segment of the curve (Fig. 3). In the latter case the increase in intensity could be explained only if the mutual quenching of aromatic residues in a folded conformation of the molecule is stronger than the quenching effect of the solvent. This effect, observed for both full-length VWF and the A1-A2-A3 domains, was always observed in the absence of NaCl, whereas physiologic concentrations of the salt eliminated this effect (Fig. 3, A and B). In contrast, the increase of fluorescence change in the absence of NaCl was not observed in the A1 domain (Fig. 3C). The fluorescence changes observed as a function of urea represent a real denaturation process, because a progressive increase of the emission wavelength (>350 nm) at all NaCl concentrations was always observed using urea concentrations >1 M (data not shown). Furthermore, similar results were obtained by exciting the protein solutions at 280 nm instead of 295 nm and fitting the experimental data to Equation 1 (data not shown). NaCl increases the stability to urea denaturation, expressed by the \( U_{1/2} \) parameter, of both A1-A2-A3 and A1 domains, although this effect was not observed for full-length VWF, which is probably affected by interchain apolar bonds not sensitive to salt effects (see Table 1 and Fig. 4). In all the VWF forms studied, the value of \( \Delta G^0 \) was progressively enhanced by NaCl, as reported in Table 1 and shown in Fig. 5. This parameter describes the free energy change linked to the stability of the protein under non-denaturing conditions, so that this finding implies that chloride ions stabilize a “super-folded” conformation of VWF forms in solution. More-
over, NaCl induces in both the A1-A2-A3 and A1 domains a moderate but significant increase of the m value (see Table 1). Because the m value reflects, at least in part (25), the protein surface area newly exposed on denaturation, the above finding might suggest that in the presence of chloride ions urea induces an exposition to bulk solvent of a higher surface area upon denaturation of the VWF A domains. This finding is in agreement with the hypothesis that chloride ions stabilize a super-folded conformation of the A1 and A1-A2-A3 domains, different from that of the chloride-free protein forms. Altogether these findings demonstrate that chloride ions affect the \( \Delta G^o \) values of all the VWF forms studied, indicating a conformational effect of the anion on VWF species under native conditions. The linkage between the \( \Delta G^o \) values and NaCl is substantially driven by the structural and functional properties of the smallest VWF fragment studied, i.e. the A1 fragment encompassing the amino acid residues Leu\(^{1243}\) to Gly\(^{1481}\). This conclusion was also corroborated by the finding that the isolated A2 domain does not change its intrinsic fluorescence in the presence of NaCl, whereas both A1-A2 and A1-A2-A3 domains decrease their fluorescence as a function of chloride ions (see below). The potential misfolding of the A2 domain as cause of the lack of chloride binding and subsequent spectroscopic signals could be reasonably ruled out by the finding that the \( k_{cat}/K_m \) value of its hydrolysis by ADAMTS-13 was similar to that calculated for the A1-A2-A3 domains (see below).

These results support the conclusion that the A1 domain contains the binding site of chloride ions, which inhibit the interaction with ADAMTS-13 of the entire A1-A2-A3 domains. The significant increase of fluorescence in the pre-unfolding segment of the denaturation curves of both full-length and A1-A2-A3 domains as a function of urea concentration implies that along the unfolding process these proteins pass through intermediate species or molten globules, which generally are relatively close to the native state (26). In these globules considerable native-like character can exist in terms of secondary structure and the overall fold, although there is generally extensive disorder in the side chains, and the global structural fluctuations are much greater than those of the native state. This scenario can be applied also to VWF A1-A2-A3 domains in the presence of 1.5M urea, which is at a concentration well below that needed to unfold half of the protein, as experimentally found (see Table 1). At this urea concentration, however, a molten globule of the A1-A2-A3 domains should be significantly populated, as this species is sensitive to proteolysis by ADAMTS-13. Many theoretical considerations and experimental findings indicated that a protease can bind and act on a segment in a protein only if this segment is sufficiently flexible to adapt its conformation to the specific stereochemistry of the protease active site. It has been demonstrated that the sites of limited proteolysis in a globular protein are characterized by enhanced backbone flexibility. Therefore, the mobility of the chain segment appears to be the key feature of the site(s) of limited proteolysis (27). Of course, the notions of accessibility and sequence specificity are also required properties of the sites of cleavage but are not sufficient to explain the selective proteolysis of one single peptide bond among the hundred(s) of bonds, as often observed in limited proteolysis.

![FIGURE 3. Urea-induced unfolding of different VWF forms. A, full-length VWF; B, A1-A2-A3 construct; C, 39/34-kDa VWF fragment. The excitation wavelength was 295 nm, whereas the emission wavelength was 337 nm. The open symbols refer to the experiments performed in the absence of NaCl, whereas the full symbols are obtained in the presence of 150 mM NaCl. The concentration of the various species were as follows: A, 0.1 mg/ml in the absence of NaCl and 0.089 mg/ml in the presence of 150 mM NaCl; B, 0.11 mg/ml in the absence of NaCl and 0.088 mg/ml in the presence of 150 mM NaCl; C, 0.118 mg/ml in the absence of NaCl and 0.117 mg/ml in the presence of 150 mM NaCl. The spectral bandwidth was equal to 5 nm in both excitation and emission wavelength. The continuous lines were drawn using the best fit parameter values contained in Equation 1 and listed in Table 1.](image-url)
domains, analyzing the dependence of anion binding on the conformational stability of the A1-A2-A3 unfolding transition.

The increase of tryptophan fluorescence in the pre-unfolding segment of the denaturation curves was observed only for full-length VWF and A1-A2-A3 domains, but not for the isolated A1 domain (see Fig. 3, for full-length VWF and A1-A2-A3 domains, but not for the iso-

These considerations can be applied to the interaction of ADAMTS-13 with the region of the VWF A2 domain in the presence of sub-denaturing concentrations of urea. Because the A1 domain is demonstrated to bear the chloride-binding site, the effect on the hydrolysis by ADAMTS-13 of the peptide bond present in the A2 domain implies the existence of an allosteric linkage between the anion binding to the A1 domain and the conformational state of the A2 domain around the Tyr$^{1605}$–Met$^{1606}$ peptide bond. The increase of tryptophan fluorescence in the pre-

A1-A2-A3 domains as a function of both the chemical nature and concentration of the anion (sodium salts): acetate (E), chloride (C), and perchlorate (F). Conditions are as follows: pH 8.0, 25 °C, 5 mM Tris. The data are fitted by linear regression according to the best fit parameters values: acetate, $\Delta G^0 = 5.506 \pm 0.08$ kcal mol$^{-1}$, slope $= 10.8 \pm 1.1$ kcal mol$^{-1}$ M$^{-1}$ ($r = 0.98$); chloride, $\Delta G^0 = 5.74 \pm 0.14$ kcal mol$^{-1}$, slope $= 28.2 \pm 1.9$ kcal mol$^{-1}$ M$^{-1}$ ($r = 0.99$); perchlorate, $\Delta G^0 = 5.8 \pm 0.2$ kcal mol$^{-1}$, slope $= 63.5 \pm 3.4$ kcal mol$^{-1}$ M$^{-1}$ ($r = 0.99$).

Table 1

| VWF form | NaCl m/o | $\Delta G^0$ kcal/mol | m | $U_{1/2}$ kcal/mol | $\Delta G/\Delta U$ kcal/mol |
|----------|----------|-----------------------|---|-------------------|-----------------------------|
| Multimer | 0        | 6.7 ± 0.5             | 1.2 ± 0.2 | 5.66             | 6.5 ± 0.9                   |
|          | 5        | 8.3 ± 1.7             | 1.6 ± 0.3 | 5.19             | 4.2 ± 0.7                   |
|          | 25       | 9.45 ± 1.8            | 1.7 ± 0.4 | 5.55             | 5.1 ± 0.6                   |
|          | 50       | 11.1 ± 1.8            | 1.9 ± 0.4 | 5.83             | 2.50 ± 0.3                  |
|          | 100      | 13.3 ± 2              | 2.3 ± 0.4 | 5.78             | 2.0 ± 0.3                   |
|          | 150      | 16.5 ± 2.2            | 2.9 ± 0.5 | 5.61             | 1.8 ± 0.4                   |
| A1-A2-A3 | 0        | 5.4 ± 0.2             | 1.3 ± 0.05| 4.18             | 7.4 ± 0.4                   |
|          | 5        | 6.0 ± 0.3             | 1.29 ± 0.06| 4.65             | 5.6 ± 0.5                   |
|          | 25       | 6.5 ± 0.6             | 1.31 ± 0.07| 4.96             | 5.1 ± 0.6                   |
|          | 50       | 7.4 ± 0.4             | 1.32 ± 0.04| 5.69             | 4.1 ± 0.5                   |
|          | 100      | 8.7 ± 0.5             | 1.33 ± 0.1 | 6.59             | 1.9 ± 0.4                   |
|          | 150      | 9.8 ± 0.4             | 1.36 ± 0.06| 7.20             | 1 ± 0.03                    |
| A1 fragment | 0        | 3.62 ± 0.4     | 0.88 ± 0.07| 4.11             | 0.9 ± 1                     |
|          | 5        | 4.3 ± 0.3             | 0.95 ± 0.05| 4.53             | 0.1 ± 0.1                   |
|          | 25       | 5.12 ± 0.3            | 0.91 ± 0.05| 5.63             | 0.4 ± 0.3                   |
|          | 50       | 5.88 ± 0.5            | 0.93 ± 0.04| 5.79             | 0.08 ± 0.06                 |
|          | 100      | 6.78 ± 0.4            | 1.02 ± 0.07| 6.65             | 0.07 ± 0.05                 |
|          | 150      | 7.7 ± 0.4             | 1.11 ± 0.06| 6.94             | 0.06 ± 0.05                 |
the salt concentrations used in this study (<0.15 M), bulk water structure is not greatly influenced by the nature of the salt present in solution outside the first hydration shell. Thus the Hofmeister effects of the anions used with the A1-A2-A3 domains need to be explained by interactions of the ions with the macromolecule and its first hydration shell. Ionic species that are strongly hydrated do not easily shed their innermost hydration shell and hence have the weakest binding constants for the protein-binding site. This may explain the difference of the stabilization efficacy of about 6 kcal mol\(^{-1}\) between NaClO\(_4\) and CH\(_3\)COONa. This sensitivity strongly suggests that binding of anions is important for the stability and conformation of the A1-A2-A3 and, as a consequence, of the entire VWF monomer. This finding led to further investigations of the thermodynamic parameters of chloride binding to VWF and its A domains.

**Temperature Dependence of Chloride Binding to A1-A2-A3 Domains of VWF**—No inner filter effect was observed in the titration experiments even at the highest (initial) concentration of the VWF constructs employed. Control experiments were performed by progressive dilution of the various VWF species starting from 0.1 mg/ml. The fluorescence signal was perfectly linear \((r \geq 0.98)\) in all cases, whereas significant deviation from linearity was observed for concentrations >0.15 mg/ml. NaCl caused at pH 8.00 a 6–20% decrease of the intrinsic fluorescence of both A1-A2-A3, and A1-A2 domains, as shown in Fig. 6A, in analogy with previous results obtained with full-length VWF (17). No significant change was observed for isolated A2 domain (data not shown). The maximum emission wavelength was unchanged (338 nm) as a function of NaCl concentration up to 0.1 M, whereas at higher concentrations (>0.15 M) a blue shift of about 5 nm was observed. The fluorescence data of both A1-A2 and A1-A2-A3 domains were fitted to the following single site binding isotherm shown in Equation 5,

\[
F_{\text{obs}}(\%) = 100 - \Delta F\%(NaCl)/[(NaCl) + K_d] \tag{Eq. 5}
\]

where \(F_{\text{obs}}(\%)\) is the experimental value of fluorescence measured as a function of NaCl concentration ranging from 0 to 0.225 M depending on the temperature varying from 7 to 40 °C; \(\Delta F\%\) is the % decrease of the fluorescence at any NaCl concentration, and \(K_d\) is the phenomenological equilibrium dissociation constant of chloride binding to any domain of VWF. In all instances a single site binding isotherm was adequately fitted to the experimental data, as shown by Fig. 6A. Although a single site binding isotherm was sufficient to analyze the data, the experimental strategy, in the absence of more detailed structural data, does not allow us to rule out completely that multiple binding sites with a similar affinity for chloride ions are present in the A1-A2-A3 and A1-A2 domains. At all temperatures the apparent affinity of chloride for the WT A1-A2-A3 was higher than that for the mutant p.R1306W A1-A2-A3 domains. Once the \(K_d\) value was calculated at each temperature, a van’t Hoff plot allowed us to calculate the relevant thermodynamic parameters contained in Equation 2. Fig. 6B shows this plot and reports the best fit parameter values of A1-A2-A3 domains. The experimental points are not linearly correlated with the temperatures values but show a downward curvature, implying that the enthalpy of interaction is not constant over the temperature range studied (7–40 °C) or, in other words, that a large negative heat capacity change characterizes chloride binding to A1-A2-A3 domains. Such a negative heat capacity change is not expected for a purely electrostatic interaction and indicates that hydrophobic and other interactions are also involved in the binding equilibrium. A large heat capacity change was measured for both the WT and the p.R1306W mutant A1-A2-A3 domains, although the value of the WT was double that of the mutant construct (\(-1.05 \pm 0.18 \text{ kcal mol}^{-1} \text{ K}^{-1}\) for p.R1306W, \(-0.4 \pm 0.02 \text{ kcal mol}^{-1} \text{ K}^{-1}\) for WT). The change in heat capacity associated with any protein/ligand interaction is a fundamental energetic parameter because it provides insight into the hydration changes involved and also affords a key to the correct interpretation of the rest of the energetic parameters in structural terms. The change in heat capacity caused by protein/ligand interaction reflects mainly the contribution from hydration or interaction with the solvent. By analyzing a large set of data with model compounds and structural analysis of the energetics of protein denaturation, a quantitative relation between the \(\Delta C_p\) (expressed in cal K\(^{-1}\) mol\(^{-1}\)) and the change in water-accessible surface area of buried apolar residues was formulated as shown in Equation 6 (29),

\[
\Delta C_p = -0.33(\pm 0.09)\Delta AS_{\text{ap}} \tag{Eq. 6}
\]
of van der Waals and hydrogen bonds (29). In a previous study, Spolar et al. (30), examining the energetics of binding of ligands and proteins and their relationship to the structural alterations brought about by the binding, found that in all cases where the binding involves a significant conformational change, the absolute values of $\Delta C_p$ may be hundreds of cal K$^{-1}$ mol$^{-1}$ or even more than 1 kcal K$^{-1}$ mol$^{-1}$. The values of $\Delta C_p$ for chloride binding to WT and the p.R1306W mutant imply that in both cases a vast apolar surface area ($\approx 3000$ and $\approx 1200$ A$^2$ for WT and p.R1306W mutant, respectively) is buried upon chloride binding, as a consequence of a folding conformational transition, and that this phenomenon is much more evident in WT than in the p.R1306W mutant. These conclusions are in agreement with the results of urea denaturation experiments performed as a function of NaCl. In that case, the $m$ parameter of Equation 1 was indeed found to increase as a function of NaCl for all the VWF constructs used (see Table 1). Because it is known that the $m$ parameter reflects, at least in part, the amount of newly accessible surface area exposed upon denaturation (25), this means that upon urea denaturation, the newly accessible surface area exposed is higher for the Cl$^{-}$-bound VWF conformer than for the Cl$^{-}$-free form. Cl$^{-}$ binding, vice versa, induces a folding conformational transition in the A1-A2-A3 domains, favors a burial of apolar surface area, as emerged from the thermodynamic analysis of the fluorescence data set. Globally taken, these experiments show that chloride binds specifically to the A1 domain of VWF and induces a folding conformational transition with burial of a vast apolar surface area and change of the hydration shell bonded to the surface of the protein.

Hydrolysis of Recombinant VWF Constructs by rADAMTS-13—Several VWF constructs were used to study the effects of chloride on cleavage by ADAMTS-13 as follows: 1) WT A1-A2-A3 domains; 2) p.R1306W A1-A2-A3 domains (2B VWD mutant); 3) WT A1-A2 domain; 4) isolated WT A2 domain; and 5) A2-A3 domain. In all the cases a substrate concentration lower than the $K_m$ value ($\approx 0.5 \mu M$) was used to meet pseudo-first order conditions. The value of the apparent pseudo-first order rate constant, equal to $k_{cat}/K_m$, was calculated using Equation 3 and then analyzed as a function of chloride concentration. The separation of the hydrolysis products of the various VWF constructs was accomplished by RP-HPLC, using a C4 column. This method allowed the measurement of the pseudo-first order rate constant. Under the low concentration of the various A domains used, which are all below the corresponding $K_m$ values of the interaction with ADAMTS-13, the kinetic rate constant is equal to ($k_{cat}/K_m$) times the concentration of ADAMTS-13. By a simple calculation, the $k_{cat}/K_m$ value was found equal to $7.26 \pm 0.2 \times 10^5$ M$^{-1}$ s$^{-1}$, $1.36 \pm 0.1 \times 10^5$ M$^{-1}$ s$^{-1}$, and $7.47 \pm 0.3 \times 10^4$ M$^{-1}$ s$^{-1}$ for WT A1-A2-A3, p.R1306W A1-A2-A3, and A1-A2 domains, respectively (see Fig. 7, A and B). The value of $k_{cat}/K_m$ of A2 and A2-A3 hydrolysis was equal to $7.24 \pm 0.3 \times 10^4$ and $6.56 \pm 0.3 \times 10^4$ M$^{-1}$ s$^{-1}$, in good agreement with those recently found by others with similar methods for the hydrolysis of similar A2 and A2-A3 domains (31). The 2B VWD construct p.R1306W is hydrolyzed by ADAMTS-13 faster than the corresponding WT form. This finding shows that when the A1-A2-A3 domains are stabilized by a mutation in the GpIb-bound like conformation, the catalytic interaction with ADAMTS-13 is facilitated, in agreement with recent findings (14).

The dependence of the $k_{cat}/K_m$ values was then studied as a function of NaCl, and the results were analyzed with the linkage of Equation 4. As reported in the legend to Fig. 8, the apparent $K_n$ value of chloride binding to WT A1-A2-A3 domains is significantly lower than that of the p.R1306W A1-A2-A3 domains (35 versus 158 mM), with values close to those measured in fluorescence experiments (48 versus 190 mM). No significant effect was observed with both A2 and A2-A3 domains, as the small decrease of the $k_{cat}/K_m$ value observed at NaCl $> 150$ mM was likely due to ionic strength effects. These experiments showed that chloride is able to induce a significant decrease of the specificity constant of hydrolysis by ADAMTS-13 of both the WT and p.R1306W A1-A2-A3 domains, that the apparent affinity of chloride ion for the WT A1-A2-A3 domains is 4–5 times higher than that for p.R1306W A1-A2-A3 domains, and that no effect was observed for the isolated A2 domain, which most likely does not contain a specific binding site for the anion. Changes in $k_{cat}/K_m$ values show that chloride acts as an alloste-
ric effector, whose binding to the A1 domain induces conformational transitions that propagate from the A1-A2 interface to the scissile peptide bond in the A2 domain, which becomes unavailable to the proteolysis by ADAMTS-13.

Application of a shear stress of 40 dynes/cm$^2$ to the A1-A2-A3 domains also induced a conformational change in the molecule, so that it recognized and reacted with ADAMTS-13 in the absence of any denaturant. When this reaction was carried out in the presence of different concentrations of NaCl (10 and 150 mM), it was clearly inhibited by chloride. At 150 mM NaCl, in the presence of 10 nM recombinant ADAMTS-13 the proteolytic reaction produced after 1 min $\sim$10% of the total product, whereas at 10 mM the reaction proceeded to $\sim$78%, as shown in Fig. 9. These findings showed that binding of chloride can oppose the conformational transitions leading to ADAMTS-13 interaction with VWF induced by both chemical (denaturants) and physical (shear stress) potentials.

**DISCUSSION**

The thermodynamic and kinetic data presented in this study provide strong evidence that the previously discovered conformational effects of chloride ions on VWF multimers (17) occur via specific binding to the A1 domain. The latter is characterized by a $\alpha/\beta$ fold, with a $\beta$-sheet composed of six $\beta$-strands flanked on each side by three $\alpha$-helices. The $\beta_2/\beta_3$ and the $\beta_3/\alpha_2$ loop have a high $B$-factor in crystal structures and thus have a flexible conformation (32). In particular, that the A1 domain has a very high conformational flexibility is also confirmed by natural mutations such as I1309V, where the involvement in a hydrogen bond network of an additional water molecule in the inner part of the A1 domain induces at a distance of more than 25 Å a conformational rearrangement of a cluster of basic residues from Lys$^{1332}$ to Arg$^{1336}$, leading to type 2B VWD with enhanced affinity for GpIb (33, 34). In this study, another typical 2B VWD mutant, p.R1306W, shows how a mutation in the A1 domain causes at the same time an enhanced affinity for GpIb, a reduction of chloride affinity, and an enhanced rate of hydrolysis by ADAMTS-13. This finding implies the existence of a conformational linkage between the A1 and A2 domains in the VWF molecule, affecting both chloride and GpIb binding to the A1 domain and ADAMTS-13 interaction at the A2 domain. The p.R1306W natural mutant has a higher affinity for GpIb and the mutation, stabilizing a conformation of the A1 domain in a GpIb-bound like state, reduces at the same time the A1 domain affinity for chloride, as demonstrated previously for the ristocetin-bound conformer of VWF (17). These findings corroborate the hypothesis that a negative thermodynamic linkage does exist between chloride and GpIb binding to VWF (17). Both fluorescence and enzymatic data indicate that the A1 domain bears a chloride-binding site; however, the detailed location of this site remains unclear. Inspection of the crystal structure of the A1 domain in the free and GpIb-ligated conformations (32, 35) reveals that all the positively charged side chains are exposed to solvent. Although the crystal structure of the entire A1-A2-A3 is yet unknown, the structure of the A1 domain (30, 35) and the MD simulation of the A2 domain (36) support the prediction that some of these positively charged residues mediate the interaction between the A1 and A2 domains, presumably forming hydrogen bonds/salt bridges at the interface between the A1 and A2 domains.

The thermodynamic data obtained from denaturation studies showed that NaCl enhances the $\Delta G^0$ value, i.e. the stability of the A1-A2-A3 domains under nondenaturing conditions. The mechanism by which chloride stabilizes the A1-A2-A3 domains could be in part the formation/stabilization of hydrogen bonds/salt bridges between charged residues at the A1-A2 interface upon its binding to a specific site in the A1 domain. Furthermore, because from molecular simulation studies it was shown that the peptide bond Tyr$^{1605}$-Met$^{1606}$ in the A2 domain cleaved by ADAMTS-13 is buried in the molecule (36), it may be hypothesized that the “folding” effect of chloride would render more compact the whole A1-A2-A3 domain. This effect would cause the Tyr$^{1605}$-Met$^{1606}$ peptide bond to be less available to the catalytic attack by ADAMTS-13, as also experimentally shown.
Chloride Binding to VWF

Based on these findings, possible candidates for the anion-binding sites in the A1 domain are likely to be positively charged “clusters” on the surface of the molecule. X-ray diffraction studies on the A1 domain-GpIb complex showed that the $\alpha_1/\beta_2$ loop of the A1 domain, containing the clusters formed by Lys$^{1342}$ to Arg$^{1346}$ and from Arg$^{1346}$ to Arg$^{1350}$, undergoes allosteric conformational transitions upon GpIb interaction or by mutations causing type 2B VWD (32, 37). Extensive site-directed mutagenesis will be an important tool in addressing the question of whether or not these positively charged residues are involved in the formation of the anion-binding sites. Fluorescence experiments showed that high heparin caused no intrinsic fluorescence of the VWF A1-A2-A3 domains (data not shown) nor did it change at a concentration of 10 $\mu$M the apparent affinity of chloride for the A1-A2-A3 domains at 25 °C. This finding would imply that the two clusters of charged residues involved in the formation of the interaction sites for heparin, consisting of one region from Arg$^{1341}$ to Lys$^{1348}$ and from Lys$^{1405}$ to Lys$^{1408}$ (38, 39), are not involved in the formation of the chloride-binding site. On the other hand, previous studies showed that heparin accelerates the hydrolysis of VWF by ADAMTS-13 (14), thus having an effect opposite to that exerted by chloride ions. Therefore, functional evidence and spectroscopic evidence are in agreement to show that heparin and chloride ions do not share the same binding site in the A1 domain.

Physiological Relevance of Chloride Binding to VWF for Its Interaction with ADAMTS-13—In a solution under normal physiological conditions, the hydrolysis of VWF by ADAMTS-13 is controlled by its conformation to prevent the unwanted cleavage of the most efficient hemostatic forms. In the presence of physiological temperatures, pH, and Cl$^-$ concentrations, hydrolysis is slow, based on the functional effects of Cl$^-$ binding to the A1 domain, which is approximately half-saturated by the anion at 37 °C ($K_d \approx 150 \text{ mM}$), as shown in this study. This binding determines the unavailability of the Tyr$^{1605}$-Met$^{1606}$ peptide bond to cleavage by ADAMTS-13. However, when VWF exposed to high shear binds to a regulatory protein, such as GpIb, Cl$^-$ is released from the A1 domain and a new VWF conformer is stabilized by this interaction. The allosteric displacement of chloride ions by GpIb binding causes a much faster rate of hydrolysis of the susceptible bond by ADAMTS-13. This allosteric mechanism could play a relevant role in such body compartments as the renal glomerulus, where chloride concentration changes during the process of plasma filtration. Any process that alters on the endothelium the delicate balance between the interactions favoring (GpIb, shear forces) or opposing (chloride) the interaction with ADAMTS-13 may contribute to the occurrence of thrombotic microangiopathies, especially those forms without severe deficiency of ADAMTS-13 (40). The fact that the kidney is often a target organ of thrombotic microangiopathies (41) may be the consequence of the active involvement of chloride in the pathogenesis of these disorders. The concentration of chloride ions changes along the nephron (especially in the distal tubule), with potential effects on VWF/ADAMTS-13 interactions, especially if additional factors cooperate to inhibit the activity of the metalloprotease. On the contrary, in some hemorrhagic disorders such as type 2B VWD, chloride ions bind to VWF with lower affinity as demonstrated in this study, and the rate of hydrolysis by ADAMTS-13 increases. This can contribute, along with the enhanced binding of high molecular weight VWF multimers to platelets, to the depletion of these VWF forms usually observed in these patients (42), in analogy with recent findings concerning type 2A VWD (43).

In conclusion, these data show how a physiological inorganic anion, such as chloride, may be involved in the allosteric regulation of a complex macromolecular interaction, such as that between VWF and ADAMTS-13.

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