Role of Chloride Ions in Modulation of the Interaction between von Willebrand Factor and ADAMTS-13*

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The degradation of von Willebrand factor (VWF) depends on the activity of a zinc protease (referred to as ADAMTS-13), which cleaves VWF at the Tyr1605-Met1606 peptide bond. Little information is available on the physiological mechanisms involved in regulation of ADAMTS-13 activity. In this study, the role of ions on the ADAMTS-13/VWF interaction was investigated. In the presence of 1.5 M urea, the protease cleaved multimeric VWF in the absence of NaCl at pH 8.00 and 37 °C, with an apparent $k_{cat}/K_m = 3.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, but this value decreased by ~10-fold in the presence of 0.15 M NaCl. Using several monovalent salts, the inhibitory effect was attributed mostly to anions, whose potency was inversely related to the corresponding Jones-Dole viscosity $B$ coefficients ($\text{ClO}_4^- > \text{Cl}^- > \text{F}^-$). The specific inhibitory effect of anions was due to their binding to VWF, which caused a conformational change responsible for quenching the intrinsic fluorescence of the protein and reducing tyrosine exposition to bulk solvent. Ristocetin binding to VWF could reduce the apparent affinity and reverse the inhibitory effect of chloride. We hypothesize that, after secretion into the extracellular compartment, VWF is bound by chloride ions abundantly present in this milieu, becoming unavailable to proteolysis by ADAMTS-13. Shear forces, which facilitate GpIbα binding (this effect being artificially obtained by ristocetin), can reverse the inhibitory effect of chloride, whose concentration gradient across the cell membrane may represent a simple but efficient strategy to regulate the enzymatic activity of ADAMTS-13.

The degradation of von Willebrand factor (VWF) plays a key role in hemostasis by mediating platelet adhesion to the sites of vascular damage and acting as a carrier protein for coagulation factor VIII. VWF is synthesized in vascular endothelial cells and megakaryocytes and is present in these cells, plasma, and the subendothelial matrices. VWF is initially released into circulating blood as ultra-large multimeric forms, which are more hemostatically active than the smaller multimeric forms but are rapidly degraded into smaller multimeric forms ranging in size from 500 to 20,000 kDa in normal human plasma (1). Degradation depends on the presence of the von Willebrand factor-cleaving protease ADAMTS-13, which cleaves VWF at the peptide bond between residues Tyr1605 and Met1606 (2). In the past few years, several studies have clearly shown the involvement of ultra-large VWF and ADAMTS-13 in the occurrence of the syndrome referred to as thrombotic thrombocytopenic purpura, which is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and neurologic signs and other symptoms related to widespread formation of platelet thrombi in the microcirculation. Ultra-large VWFs are often present in the plasma of patients with thrombotic thrombocytopenic purpura, due to the deficiency of ADAMTS-13 (1–3). The abnormal presence of ultra-large VWF in plasma is thought to cause widespread platelet aggregation in the microcirculation and thrombus formation, which underlies the pathogenesis of thrombotic thrombocytopenic purpura.

In contrast with other zinc proteases, such as the matrix metalloproteases, ADAMTS-13 does not need activation by other hydrolases to become proteolytically competent toward VWF (2). VWF hydrolysis can be obtained in vitro under mild denaturing conditions, which generate partially unfolded VWF conformers that are susceptible to proteolytic attack by ADAMTS-13. Moreover, early studies by Furlan et al. (7) on the specificity of the ADAMTS-13/VWF interaction showed that physiological concentrations of NaCl inhibit hydrolysis of VWF. Based on this observation, current functional assays that measure ADAMTS-13 activity are carried out either in the absence of NaCl or at very low NaCl concentrations (7–9). Taken together, these observations prompted us to further investigate the effect of different ions on ADAMTS-13 interactions with VWF, in order to elucidate the mechanisms of control of ADAMTS-13 enzymatic activity under physiological conditions.

MATERIALS AND METHODS

Cell Culture and Stable Transfection—A pCDNA3.1 expression vector (kindly provided by Dr. F. Scheiffinger; Baxter Bioscience, Vienna, Austria) containing the entire coding region and a small part of the 3’-untranslated region of the ADAMTS-13 gene (pCDNA3.1-ADAMTS-13 WT) was used for stable transfection experiments. Human embryonic kidney HEK293 cells were maintained in Dulbecco’s modified
Eagle’s medium enriched with nutrient mixture F-12 (DMEM-F-12) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 µg/ml penicillin/streptomycin (Invitrogen) in a 5% CO2 atmosphere at 37 °C. 30% to 50% confluent cells were infected with 50 µg of pCDNA 3.1-ADAMTS-13 WT by electroporation with an EasyJet Plus (Equibio-Celbio, Milano, Italy) according to the manufacturer’s instructions. For selection, the medium was supplemented with 450 µg/ml Genetin G418 sulfate (Invitrogen). Serum-free conditioned medium was applied to equivalent numbers of confluent cells and collected at 96 h. The functional activity of recombinant ADAMTS-13 was measured using an ELISA based on the media of the transfected cells, using the previously described collagen binding activity (CBA) assay (8) for identifying collagen-bound VWF with a commercial kit (Gradipore Ltd., French Forest, New South Wales, Australia) (10). The medium of untransfected cells was used as a negative control.

Purification of Recombinant ADAMTS-13—Recombinant ADAMTS-13 (rADAMTS-13) was purified from cell supernatants according to a previously described method (11), with the following modifications. The ammonium sulfate precipitate (35%) was applied in room temperature to a butyl-Sepharose column (1.2 × 6 cm; Amersham Biosciences) at a flow rate of 0.6 ml/min. The eluted fractions were pooled, and ammonium sulfate was added to 40% saturation. The precipitate was collected by centrifugation, dissolved in −3 ml of 50 mM Tris-HCl (pH 8.5), gently stirred for 1 h with 0.5 mM phenylmethylsulfonyl fluoride and 5 mM iodoacetamide, and applied to a D10 Bio-Rad column previously equilibrated with 50 mM Mops-NaOH buffer (pH 6.6/0.1 M NaCl) or one buffer change. The sample was further purified by heparin-Sepharose affinity chromatography and DEAE ion exchange chromatography (1.5 × 10 cm), as previously described (11). Purity was tested by SDS-PAGE using a 4–15% acrylamide gradient under reducing and nonreducing conditions. The band corresponding in the SDS-PAGE to the species with a molecular mass of 190 kDa was blotted, and N-terminal PAGE using a 4–15% acrylamide gradient under reducing and nonreducing conditions. The activity of the purified enzyme was determined using an E(280 nm) (mg/ml) −1 cm −1 = 1.45, based on the protein composition.

Purification of VWF—Purified VWF was obtained from either a therapeutical factor VIII/VWF plasma concentrate (Fahndi, Grifol’s, Pisa, Italy) or as a recombinant preparation (a generous gift of Dr. Peter Turecek; Baxter Bioscience, Vienna). VWF purification was obtained using a two-step procedure, in which 5 ml of the reconstituted plasma concentrate at a concentration of 3000 units/ml in 5 ml Tris-HCl, 0.1 mM NaCl (pH 8.0) at 25 °C was percolated through a 2 × 70-cm column containing 50 g of agarose (Amersham Biosciences) to remove nonfunctional human albumin and other proteins. The peak containing VWF was further purified on a heparin-Sepharose column, using a NaCl concentration gradient (100–290 mM in 60 min) at a flow rate of 0.8 ml/min. The last peak to be eluted (200 mM NaCl) was pooled and further analyzed. SDS-PAGE under reducing conditions in 4–15% gradient gels showed the presence of a single band with an approximate molecular mass of 250 kDa.

Assay of VWF-cleaving Protease Activity by CBA Assay for Kinetic Studies in the Presence of Urea—The VWF substrate was prepared by resuspending the purified preparation in 1.5 mM urea, 5 mM Tris (pH 8.0).

Digestion of VWF by rADAMTS-13 was investigated as a function of the enzyme concentration and different salts. rADAMTS-13 (2.5 µM in 50 mM Tris-HCl (pH 8), 280 mM NaCl) was diluted with 1.5 mM urea, 5 mM Tris (pH 8.0). Aliquots of 50 µl were then incubated with 5 µl of 90 mM BaCl2 or 50 mM NaCl, NaF, NaClO4 or 3.34, and 1 mM) of different salts to change both cations and anions (NaCl, NaF, NaClO4, tetramethylammonium chloride, and CsCl). The final concentrations of Na+ and Cl− were also checked potentiometrically by a Hitachi 717 automated analyzer (Roche Applied Science). In some experiments, BaCl2 was replaced by Ba(COO)2 at the same final concentration of 3 mM to investigate the potential effect of chloride present in the barium salt.

Assay of VWF-cleaving Protease Activity by CBA Assay for Kinetic Studies in the Presence of Ristocetin—The CBA assay was also applied to evaluate the conformational effects induced in VWF molecules by ristocetin instead of urea. Accordingly, different concentrations of ristocetin (0.6, 0.8, 1, 1.2, and 1.5 mg/ml) were used in absence and presence of different concentrations of NaCl, NaF, and NaClO4 (ranging from ~1 to 300 mM). To avoid ristocetin (sulfate salt) precipitation in the presence of BaCl2 used in the CBA assay, pretreatment of the ristocetin sulfate preparation (Helena Laboratories Corp., Beaumont, TX) with stoichiometric amounts of BaCl2 or BaClO4 was always carried out to eliminate sulfate ions. Briefly, ristocetin was suspended in buffer at a final concentration of 15–20 mg/ml. The molarity of the ristocetin solution was calculated using a molecular mass value of 2168 Da. A stoichiometric amount of BaCl2 and BaClO4 was added, and then the suflated BaCl2 was eliminated by centrifugation of the sample at 4000 × g for 15 min. The concentration of de-sulfated ristocetin in the supernatant was then calculated spectrophotometrically at 280 nm, using 4.41 as the extinction coefficient (0.1%, w/v). An aliquot of this solution was also used to check the presence of potentially contaminating sulfate ions by adding an excess of BaCl2; in no instance was further sulfate precipitation observed. The de-sulfated ristocetin was then used for the functional experiments that evaluated the activity of ADAMTS-13 toward VWF.

Assay of VWF-cleaving Protease Activity by Electrophoretic Analysis of the Distribution of VWF—The VWF-cleaving protease activity of ADAMTS-13 was also evaluated using a modification of the immunoblotting assay described by Furlan et al. (7). The recombinant form of VWF and plasma-derived ADAMTS-13 were used in these experiments. Citrated plasma samples were re-centrifuged at 2000 × g for 15 min and diluted 1:20 with a solution of 0.15 mM NaCl, 10 mM Tris (pH 7.4) containing 1 mM Pefabloc. The protease was activated by preincubation with 10 mM BaCl2 for 5 min at 37 °C. 100 µl of the activated protease solution was immediately added to 50 µl of recombinant VWF (5 units/ml) and applied on the surface of a hydrophilic VWF. The samples containing 50 µl of dialysis buffer (1.5 mM) were washed with 50 µl of 1.5M sodium sulfate was eliminated by centrifugation of the sample at 4000 × g for 15 min. The concentration of de-sulfated ristocetin in the supernatant was then calculated spectrophotometrically at 280 nm, using 4.41 as the extinction coefficient (0.1%, w/v). An aliquot of this solution was also used to check the presence of potentially contaminating sulfate ions by adding an excess of BaCl2; in no instance was further sulfate precipitation observed. The de-sulfated ristocetin was then used for the functional experiments that evaluated the activity of ADAMTS-13 toward VWF.

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Fluorescence Studies—Steady-state fluorescence titration measurements were carried out as described previously (14, 15) on purified VWF. Fluorescence emission spectra (λex = 280 nm) were recorded at 25 °C in a 1-cm quartz cell, using a Spex (Edison, NJ) FluoroMax spectrophotometer, in 5 mM Tris (pH 8.0) and increasing amounts of salts (NaCl, NaF, and NaClO4) or unfraccionated porcine heparin (100
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units/mg; Calbiochem) with a mean molecular mass of 14.5 kDa. Any background signal (fluorescence or scattered light) from the buffer was subtracted, and fluorescence intensity was corrected for the progressive protein dilution (14, 15). The decrease in fluorescence signal, usually defined as “bleaching effect,” due to the iterative exposure of the sample to the high intensity light beam, was restricted to <5% of the initial intensity.

Second Derivative UV Spectroscopy—All measurements were conducted at least in duplicate at 25 ± 0.2 °C in 5 mM Tris-HCl buffer (pH 8.0). Protein concentration was determined by UV absorbance at 280 nm on a double beam Varian model Cary 2200 spectrophotometer (Palo Alto, CA), using extinction coefficients (ε) calculated on the basis of the amino acid composition. The ε values for VWF (as monomer) and rADAMTS-13 were taken as 0.44 and 1.45 ml/mg cm, respectively. The proteins used in these experiments had an absorbance value of ~0.2 at 280 nm. The average exposure (α) of tyrosine residues to solvent in the VWF and ADAMTS-13 structures was calculated according to the method of Ragone et al. (16), under 0 and 150 mM NaCl, NaClO₄, and NaF. The unfolded state of the proteins was obtained in the presence of 6 M urea in 5 mM Tris buffer (pH 8.0).

RESULTS

Purification of Recombinant ADAMTS-13

The purification procedure described yielded a single component of ~190 kDa (Fig. 1). In some preparations, a band at ~90 kDa was also observed, perhaps an auto-hydrolytic product of ADAMTS-13 at the C terminus, because the same N-terminal sequence AAGGILHLGLLVAVG was observed in the two preparations containing >10% of the 90-kDa form were not used in the kinetic experiments. In the most abundant preparations, a minor band slightly above the major band of 190 kDa was also observed. The N-terminal sequence of this component was MHQHRHPRA, suggesting that human embryonic kidney cells secrete an aliquot of ADAMTS-13 containing the signal peptide (11). The major 190-kDa component had a specific activity toward VWF of 100 ± 10%/μg enzyme, as demonstrated by CBA assay, using pooled normal plasma from 50 healthy individuals as a reference.

Kinetics of VWF Hydrolysis by rADAMTS-13: CBA Assay

ADAMTS-13 at concentrations ranging from 1.6 to 13.3 nM yielded a pseudo-first order kinetics of VWF hydrolysis. Fig. 2 shows that kinetic curves were fitted to a single exponential decay equation, allowing us to obtain pseudo-first order rate constants. If this rate constant is actually under pseudo-first-order conditions, i.e. VWF ≪ K_m, then doubling VWF concentrations should not change the value of the rate constant, and these values should be linearly related to enzyme concentration. These pseudo-first order conditions were met because 6.6 or 13.2 μg/ml VWF (taken as monomeric species) was hydrolyzed by ADAMTS-13 with the same k value, i.e. ~3 × 10⁻⁴ s⁻¹ (data not shown). Moreover, the observed pseudo-first order rate constants were linearly correlated to ADAMTS-13 concentrations allowing calculation of the k_cat/K_m value on the basis of the relation: k_cat = k_o * K_cat/K_m (17). Fig. 2 shows that under the experimental conditions of 1 mM NaCl, the k_cat/K_m value was equal to 3.4 ± 0.13 × 10⁻⁴ M⁻¹ s⁻¹. Fig. 2 also shows that increasing the concentrations of NaCl from 1 to 150 mM caused a severe reduction of the hydrolysis rate, which decreased ~10-fold at the highest salt concentration. Because this finding suggests a strong dependence of VWF/ADAMTS-13 interaction on the ionic strength of the solution, we chose to investigate the dependence of this interaction on different salts, changing both the cation and the anion. NaCl, NaF, NaClO₄, CaCl₂, and tetramethylammonium chloride at different concentrations were used to study the kinetics of VWF cleavage by ADAMTS-13. Fig. 3 shows that a Debye-Hückel plot of the experimental data yielded a slope of ~3.3, which is among the highest values reported in the literature for protein-protein interactions (18). However, the data reported in Fig. 3 showed that the salts used in these experiments had effects linked to the presence of specific anions. Whereas different cationic chloride salts caused similar effects on the kinetics of VWF/ADAMTS-13 interactions (see Fig. 4A), the change of the anionic component in sodium salts gave different magnitudes of effect (Fig. 4B). This finding raised the question of whether or not specific anion binding to either ADAMTS-13 or VWF (or both) might generate this negative effect. The pseudo-first order rate constants (kobs) of VWF cleavage were analyzed as a function of the individual anion concentration (Λ), using a simple scheme whereby one anion binding to one of the interacting proteins is able to completely suppress the ADAMTS-13/VWF interactions; accordingly,

\[ k_{obs} = k^* [1 + (\Lambda/K_d)] \]

where k* is the rate constant in the absence of salt, and K_d is the apparent equilibrium dissociation constant of anion binding. The calculated K_d values followed the order K_d(ClO₄) < K_d(Cl) < K_d(F), (Fig. 4B), indicating that the apparent affinity of fluoride is lower than that of chloride, which in turn has a lower affinity than perchlorate ion. In the inset of Fig. 4B, the values of the apparent equilibrium dissociation constants calculated at 37 °C for F⁻, Cl⁻, and ClO₄⁻ are plotted as a function of the respective Jones-Dole viscosity B coefficients (19). The plot shows that there is a definite inverse relationship between these parameters, corroborating the concept that the inhibitory effect of anions results from specific binding regulated by the physico-chemical nature of the anion involved.

Control experiments were also carried out to check whether or not the presence of chloride present as BaCl₂ could affect the measurement of the apparent K_d of chloride binding. This was not the case, because the kinetics of VWF hydrolysis in the presence of added NaCl at concentrations of either 1 or 150 mM was almost the same when BaCl₂ was replaced by Ba(COO)₂ (the latter salt was chosen because its solubility is much higher than that of BaF₂), as shown in Fig. 5. This implies that the small amount (3 mM) of chloride present as BaCl₂ was much lower than the corresponding K_d of its binding; the experimental assay was not able to detect the effect of this small amount of additional chloride, which was, however, taken into consideration for the fitting procedures of experimental data involving chloride ion. On the basis of both the differential effect and the magnitude of the apparent K_d values, we presume that the contribution of ionic strength to the observed inhibition of ADAMTS-13/VWF interaction is actually negligible. The phenomenological effect of anions was specific and linked to the chemical nature of the anion involved. The potency series indicated above is compatible with the Hofmeister series (20). In the majority of cases, the positions of ions in the Hofmeister
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**Fig. 2.** Kinetics of VWF cleavage by ADAMTS-13. The VWF and ADAMTS-13 concentration was equal to 6.6 μg/ml (as monomer form) and 6.7 nm, respectively. The continuous lines were drawn using the single exponential decay equation: VWF = VWF0 exp(−kt), where VWF, is the VWF concentration at time t, and k is the pseudo-first rate constant of VWF cleavage. At NaCl = 1 mM (○), the k value was equal to 3.2 ± 0.2 × 10−4 s−1; at 3.3 mM NaCl (●), k = 2.9 ± 0.1 × 10−4 s−1; at 5.68 mM NaCl (□), k = 2.1 ± 0.2 × 10−4 s−1; at 10.375 mM NaCl (■), k = 2.03 ± 0.2 × 10−4 s−1; at 19.75 mM NaCl (△), k = 2.05 ± 0.1 × 10−4 s−1; at 38 mM NaCl (▲), k = 2 ± 0.2 × 10−4 s−1; at 75 mM NaCl (▼), k = 1 ± 0.1 × 10−4 s−1; and at 151 mM NaCl (▼), k = 3.8 ± 0.9 × 10−5 s−1. Inset, values of kobs of VWF hydrolysis (0.6 μg/ml) measured at 1 mM NaCl as a function of rADAMTS-13 concentration. The linear fit (r = 0.93) has a slope = 3.4 ± 0.13 × 106 M−1 s−1.

**Fig. 3.** Debye–Hückel plot of the pseudo-first order rate constant of VWF hydrolysis by ADAMTS-13 as a function of the square root of the ionic strength of the solution (I), changed by means of NaCl (○), CsCl (●), tetramethylammonium chloride (□), NaClO4 (△), and NaF (▲). The slope of the straight line is equal to −3.34 ± 0.2 (r = −0.934).

series correspond to the degree of their hydration (21). Accordingly, small ions of high charge density bind water tightly, whereas large monovalent ions of low charge density bind water weakly, relative to water–water interaction strength: this is expressed by the Jones-Dole viscosity B coefficients (19). However, the relative position in the series should be considered as indicative only because there will be considerable variation depending on type of protein, pH, temperature, and ion pair effects (22).

**Effect of Ristocetin on VWF/ADAMTS-13 Interactions**

Recent findings showed that binding to platelet GpIbα accelerates the hydrolysis of VWF by ADAMTS-13 (23). With this as background, we chose to investigate whether or not the negative linkage between anion binding and kinetics of VWF cleavage by ADAMTS-13 was influenced by another allosteric effector of VWF such as ristocetin. This molecule is known to trigger, upon specific binding to the A1 domain (24), a conformational change in the VWF molecule that favors the interaction with GpIbα. Thus, under saturating concentrations of ristocetin, VWF is stabilized in a “GpIb-bound-like” conformation (24). Ristocetin accelerates the hydrolysis rate of VWF severalfold. The same result was obtained by evaluating VWF cleavage using the immunoblotting assay (data not shown). However, the accelerating capacity of ristocetin depended on the concentration of salt present in the solution.

These results indicate that a negative linkage does exist between anion and ristocetin binding to VWF. In the presence of 1.5 mg/ml ristocetin, the rate constant of VWF hydrolysis was even higher than that in the presence of 1.5 M urea (see Fig. 6). This result suggests that, although urea is able to induce in VWF a conformation suitable for ADAMTS-13 interaction, this denaturing agent might also cause a partial denaturation of the ADAMTS-13 molecule, inhibiting in part the catalytic activity of the protease. Moreover, the apparent Kd of anions in the presence of a saturating concentration of ristocetin was higher than that in the presence of 1.5 M urea (Fig. 6) This finding suggests that the conformational change induced by urea in VWF is different from that induced by ristocetin and that the latter stabilizes a VWF conformation with lower affinity for anions than that induced by urea. Additional experiments were also performed to investigate the linkage between the apparent Kd of different anions, such as chloride and perchlorate, and ristocetin over a concentration ranging from 0.6 to 1.5 mg/ml. A plot of the log of the apparent Kd values of anion binding as a function of the log of ristocetin concentration showed a linear relationship (Fig. 7). This finding implies that the higher the concentration of ristocetin, the lower the apparent anion affinity. The slope of this linkage graph is 1 for both NaCl and NaClO4, suggesting that when 1 mol of ristocetin is bound to monomeric VWF, approximately 1 mol of anion is released into solution (25). Furthermore, the fact that under the same concentration of ristocetin but in the presence of a fixed concentration of different anions (Cl−, F−, or ClO4−), we observed different rate constants of VWF hydrolysis, as shown in Fig. 6, allowed us to reasonably exclude pure dielectric effects of these anions on ristocetin binding to VWF because under these conditions, the ionic strengths of the solutions were the same.

**Kinetics of VWF Hydrolysis by rADAMTS-13:**

**Electrophoretic Analysis of Distribution of VWF Multimers**

The findings reported above prompted us to investigate the effect of salt on VWF/ADAMTS-13 interactions using a different protease assay method, based upon the electrophoretic analysis of VWF multimers after proteolysis by ADAMTS-13. Using the same concentrations of different anions (sodium salts), the efficiency of VWF cleavage changed as a function of
the anion used (Fig. 8). In particular, at a salt concentration of 75 mM, the inhibition potency decreased according to the series NaF > NaCl > NaClO₄, confirming the results obtained with the CBA assay.

Spectroscopic Studies

Fluorescence Spectroscopy—The observed strong inhibitory effects of anions on the cleavage of VWF by ADAMTS-13 triggered the need to establish which of the two proteins (or both) was involved in salt binding. The first approach was based on evaluation of the intrinsic fluorescence of both proteins in the presence of different salts (NaCl, NaF, and NaClO₄). Fig. 9A shows VWF fluorescence intensity at the maximum peak (337 nm) as a function of the concentration of different anions. No peak shift was observed during titration. However, Fig. 9A shows that a 10–20% decrease of intrinsic fluorescence was observed in the presence of chloride, perchlorate, and fluoride. Thus, in the presence of anions, intrinsic fluorescence is quenched by conformational transitions linked to anion binding. The apparent Kₐ values of anions, obtained fitting the experimental points according to a single-site binding model (25), were in the order of ≈30 mM, which, although measured at a different temperature, is of the same order of magnitude as those derived from kinetic experiments. The magnitude of fluorescence quenching was significantly smaller in the presence of chloride anion (12%) compared with fluoride (20%), suggesting that non-identical conformational rearrangements occurred in the two experimental conditions.

In all cases, progressive dilution (up to 1:50) of VWF samples with plain buffer or buffer containing 0.15 M NaCl, NaF, or NaClO₄ gave fluorescence signals linearly correlated (r = 0.95 in all cases) to dilutions at four different temperatures (10 °C, 15 °C, 25 °C, and 35 °C). This demonstrated the absence of any significant fluorescence signal change driven by noncovalent polymerization of the protein under these experimental conditions.

A high molecular weight anionic polymer such as unfractionated heparin (average molecular weight = 15,000), recently reported to accelerate VWF cleavage by ADAMTS-13 (23), caused no change of fluorescence in the VWF molecule up to a concentration of 50 μM, confirming the specificity of the effects.
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Fig. 5. Kinetics of VWF cleavage by ADAMTS-13. The VWF and ADAMTS-13 concentration was equal to 6.7 μg/ml (as monomer form) and 10 nM, respectively. Activation of ADAMTS-13 was accomplished by either 3 mM (final concentration) BaCl₂ (open symbols) or Ba(OO)₂ (filled symbols). The lines (dashed line, in the presence of BaCl₂; solid line, in the presence of Ba(OO)₂) were drawn using the single exponential decay equation as described in the Fig. 2 legend. At NaCl = 1 mM, the k value was equal to 3.96 ± 0.09 × 10⁻⁴ s⁻¹ in the presence of BaCl₂ (○) and 3.85 ± 0.08 × 10⁻⁴ s⁻¹ in the presence of Ba(OO)₂ (●). At NaCl = 151 mM, the k value was equal to 4.3 ± 0.9 × 10⁻⁵ s⁻¹ in the presence of BaCl₂ (□) and 4.05 ± 1 × 10⁻⁵ s⁻¹ in the presence of Ba(OO)₂ (■).

Fig. 6. Kinetics of VWF cleavage by ADAMTS-13 in the presence of 1.5 mM urea (filled symbols) and 1.5 mg/ml ristocetin (open symbols) and in the presence of NaCl (circles), NaF (triangles), and NaClO₄ (squares). The concentration of VWF and ADAMTS-13 was equal to 6.7 μg/ml and 12 nM, respectively. The lines were drawn using the linkage Eq. 1 described under “Materials and Methods.” The vertical bars are the S.E. In the presence of urea, the Kd values were 18.3 ± 0.8, 33 ± 3, and 50 ± 6 nM for ClO₄⁻, Cl⁻, and F⁻, respectively. In the presence of ristocetin, the apparent Kd values were 32 ± 2, 62 ± 7, and 125 ± 6 nM for ClO₄⁻, Cl⁻, and F⁻, respectively.

Fig. 7. Values of the apparent Kd of NaClO₄ (○) and NaCl (●) binding (log values) as a function of the molar ristocetin concentration (log values). The straight line was obtained by linear regression with the best fit slope factor equal to 0.75 ± 0.06 and 0.77 ± 0.04 for Cl⁻ and ClO₄⁻, respectively, with r > 0.98 in both cases.

of chloride on the intrinsic fluorescence of VWF. Taken together, the above experiments, although not excluding a potential role of cations as well as an involvement of ADAMTS-13 in ion binding, strongly suggested that the negative role played by salts in the VWF/ADAMTS-13 interaction arose from conformational changes occurring in the VWF molecule upon binding of specific anions.

Average Exposure of Tyrosine Residues Measured by Second Derivative UV Spectroscopy—The second derivative UV spectra obtained at 25 °C (pH 8.00) in the presence of 0.15 mM NaCl, NaClO₄, and NaF showed that sodium chloride and perchlorate induced a ∼25% decrease of tyrosine exposure to solvent because the fractional tyrosine exposure decreased from 0.95 ± 0.05 in the absence of NaCl to 0.72 ± 0.04 in the presence of 0.15 mM NaCl and to 0.70 ± 0.06 in the presence of perchlorate. On the other hand, fluoride did not significantly change the average tyrosine exposure to solvent (0.88 ± 0.08) (Fig. 9B).

Spectroscopic Experiments using rADAMTS-13

No change in intrinsic fluorescence was observed when rADAMTS-13 was used in titration experiments with different salts (data not shown). Likewise, second derivative spectra of r-ADAMTS-13 under different salt concentrations (NaCl, NaF, and NaClO₄) did not show any significant difference (data not shown).

DISCUSSION

The effects of salts on the structure and association of proteins are complex and may be specific or nonspecific, i.e., salts may bind directly to the side chains of the proteins or interact with solvent water. In addition to the electrostatic effects, anions interact with solvent water and can affect both the water-water and protein-water interactions, leading to an increase or decrease in the hydrophobic interactions of proteins. Generally, the effects of low salt concentrations are mostly due to neutralization of the protein net charge, whereas the effects of high salt concentrations are due to the net effect of the three competing preferential interactions (with water, protein, and salt ions) (20).

The association of VWF to ADAMTS-13 may in principle involve both hydrophobic and electrostatic interactions between side chains, as well as changes in hydration. If the Debye-Hückel screening had the main contribution, then the effect of various anions would be determined by the ionic strength of the solution. In our study, similar concentrations were used for all the monovalent salts, and therefore the solutions had similar ionic strengths. However, different salts showed significantly different degrees of inhibition on the kinetics of VWF/ADAMTS-13 interactions. Thus, we surmise that the major effect of anions in modulating the rate of the interaction is not due to Debye-Hückel screening phenomena. The potency of the inhibitory effects of anions followed the Hofmeister series, that is ClO₄⁻ > Cl⁻ > F⁻ (26) and was inversely related to the relative Jones-Dole viscosity B coeffi-
Chloride Ions in Modulation of VWF/ADAMTS-13 Interaction

The inhibitory effect of anions was attributed to their influence on VWF structure, on the basis of the changes of its spectroscopic properties. These effects could be reasonably attributed to neutralization of VWF charges, a phenomenon that may lead to conformational changes of a protein known to undergo allosteric transitions upon binding of ligands and water molecules to specific sites (28, 29). In particular, these conformational changes cause a quenching of intrinsic protein fluorescence without causing a shift of the peak. This finding ruled out any denaturing effect of salts on VWF proteins (30).

Although the side chain of tyrosine can contribute to intrinsic fluorescence of proteins, the main fluorophore is represented by the indole moiety of tryptophan. This group is highly sensitive to environment, making it an ideal choice for monitoring protein conformational changes and interactions with other molecules. Two common quenchers of tryptophan fluorescence are water molecules and peptide bonds, although the side chains of amino acids, tyrosine, cysteine, and positively charged histidine, and cysteine should be the best quenchers in proteins (31). Although the assignment of identity of tryptophan quencher(s) will need more sophisticated techniques for structural studies, our UV spectroscopic data suggest that burial of tyrosine residues in VWF, linked to chloride binding, can be responsible for the quenching of intrinsic fluorescence.

The molecular mapping of the potential anion binding site in the VWF molecule is not in the realm of this study. However, it is known that one of the VWF A1 faces is strongly electropositive, involving the areas surrounding the N terminus of the α3 helix and the C termini of strand C and of helix 4 as well as of the α5, α6, and β19 helices (32). This vast area contains the binding site for heparin, i.e. a high molecular weight anionic polymer (32). Moreover, the A1 domain of VWF (containing three tryptophan residues) acts as an allosteric modulator of VWF function because it also contains the binding site for GpIbα and ristocetin (24). Recent x-ray diffraction studies showed that the basic residues Lys542-Lys545 in the A1 region of VWF undergo a conformational rearrangement upon GpIbα binding (33, 34) and also that Trp550 is involved in this conformational change (33, 34). Additional studies are needed to assess whether or not this region is also involved in chloride binding, contributing to the decrease of intrinsic fluorescence linked to the anion interaction. Site-directed mutagenesis of different residues in critical regions of the A1-A3 domains of VWF will be an important tool in addressing the question of whether or not the above-mentioned charged residues are involved in the formation of the anion binding site. Thus, we are further investigating this issue, keeping in mind that anion binding sites do not have to be formed only by charged side chain in proteins. As previously shown by x-ray analysis of a number of proteins, the anion binding sites are formed predominantly by polar groups, and the role of charged groups in the formation of the anion binding site may be unimportant (35). Therefore, only extensive site-directed mutagenesis and spectroscopic studies will help solve this relevant issue.

Based on our findings, the conformational transitions induced by anions on VWF may represent the other side of the coin with respect to the changes of VWF triggered by shear forces, GpIbα, or ristocetin/urea binding. The Tyr-Met peptide bond, predicted by molecular dynamics to be buried between two helix domains in the VWF A2 domain (36), may enhance its own exposure to bulk solvent by shear stress and GpIbα/ristocetin binding, thereby allowing proteolytic attack by ADAMTS-13. The observed effect of ristocetin, which reverses the inhibition of VWF cleavage by anions, is in agreement with the hypothesis that anion binding stabilizes a “closed” conformation of the high molecular mass forms of VWF, making them unable to interact with the metalloprotease. Previous data obtained by force atomic microscopy showed that high shear stress induces a stretching of the VWF multimer, enabling the adhesive protein to gain full hemostatic efficiency (37). Based on the present findings, we propose that an opposite mechanism may be triggered by anion binding to VWF, which would be stabilized in a more folded conformation, with burial of the Tyr1605-Met1606 peptide bond cleaved by ADAMTS-13.

It remains to be established whether or not the inhibitory effect of anions (particularly chloride) may have physiological relevance. In human plasma, in which both VWF multimers and ADAMTS-13 circulate in solution, the effects of ionic strength may be considered of very limited relevance because only minor changes may take place. Specific binding of different anions may play a key role in the modulation of VWF functions if one considers that a gradient of ion concentration

![Figure 9](image-url)
exists between the intra- and extracellular compartments. In particular, it is known that intracellular anions are represented mainly by phosphates, sulfates, and protein carboxylates, whereas the chloride ion concentration is low, amounting to 1–3 mM (38). On the other hand, the extracellular chloride concentration is much higher (98–106 mM) and may easily form inner sphere ion pairs with the chaotropic cation groups on proteins (protonated imidazolium, ε-amino group of lysine). The net effect on VWF structure of anions present in the intracellular compartment is not known. However, once secreted into the extracellular compartment, VWF is readily bound by chloride ions, which are abundantly present in that compartment and have a high affinity for the protein. This binding would in turn change the VWF conformational state, making VWF unavailable to proteolytic activity by ADAMTS-13. This might be a simple and elegant strategy to regulate the enzymatic activity of ADAMTS-13 through conformational changes of the protease substrate. These findings may pave the way to investigate whether and how this regulatory mechanism could be also involved in the pathogenesis of some thrombotic microangiopathies.

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