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BRIEF REPORT

Temperature-dependent irreversible conformational change of recombinant ADAMTS13 upon metal ion chelation

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Abstract

Background: The catalytic domain of ADAMTS13 possesses one Zn^{2+} and up to three putative Ca^{2+} binding sites and can be inactivated by chelating agents. Although replenishment with an appropriate metallic cation is thought to restore the enzyme's proteolytic activity fully, ADAMTS13 stability in a metal ion-depleting environment has not been explored.

Objectives: To address the stability of ADAMTS13 in citrated human plasma.

Methods: ADAMTS13 activity was measured using the FRETS-VWF73 fluorogenic assay. The molar ratio of metals bound to ADAMTS13 was determined by size exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICP-MS). Higher-order structural changes were analyzed using Fourier-transformed infrared spectroscopy and dynamic light scattering.

Results: ADAMTS13 was stable at room temperature for up to 24 hours irrespective of the presence of citrate (0.38%). However, at 37°C, citrate caused a time-dependent activity decrease. No ADAMTS13 activity decrease was seen in heparinized plasma, but the addition of citrate again caused ADAMTS13 instability at 37°C. Scavenging of citrate by the addition of Ca^{2+} or Zn^{2+} prior to but not postincubation prevented the activity decrease of the enzyme. The SEC-ICP-MS analyses showed that ADAMTS13 only bound Zn^{2+} and that its reduced activity correlated with a gradual loss of bound Zn^{2+}. Concomitant higher-order structural analyses demonstrated structural changes in ADAMTS13 that are typical of less-ordered protein structures.

Conclusions: Zn^{2+} is required to stabilize ADAMTS13 structure at physiologic temperature, thereby preventing irreversible loss of enzyme activity. This finding is particularly important to consider when using citrated human plasma as a source of ADAMTS13 in clinical settings.

KEYWORDS

ADAMTS13 protein, blood plasma, protein conformation, protein stability, zinc

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1 | INTRODUCTION

The plasma metalloprotease ADAMTS13 is the key physiologic regulator of multimeric von Willebrand factor (VWF).[1–3] Enzyme deficiencies due to mutations in the *ADAMTS13* gene or to inhibition by autoantibodies result in an excess of platelet aggregation and disseminated VWF/platelet-rich thrombus formation, which are cardinal features of thrombotic thrombocytopenic purpura (TTP).[2,4–6]

The catalytic metalloprotease domain of ADAMTS13 possesses a number of predicted metallic cation binding sites. These include an essential Zn^{2+} coordination site in the active site, with the consensus sequence HEXXHXXGXXHD harboring three critical histidine residues,[7] and three putative Ca^{2+} binding sites. Based on mutational

Essentials

- Thrombotic thrombocytopenic purpura therapy involves ADAMTS13-containing citrated human plasma.
- ADAMTS13 stability and structure in the presence of citrate or heparin were assessed.
- ADAMTS13 activity decreased in citrated but not heparinized plasma.
- Zn^{2+} stabilizes ADAMTS13 at 37°C and prevents irreversible activity loss.

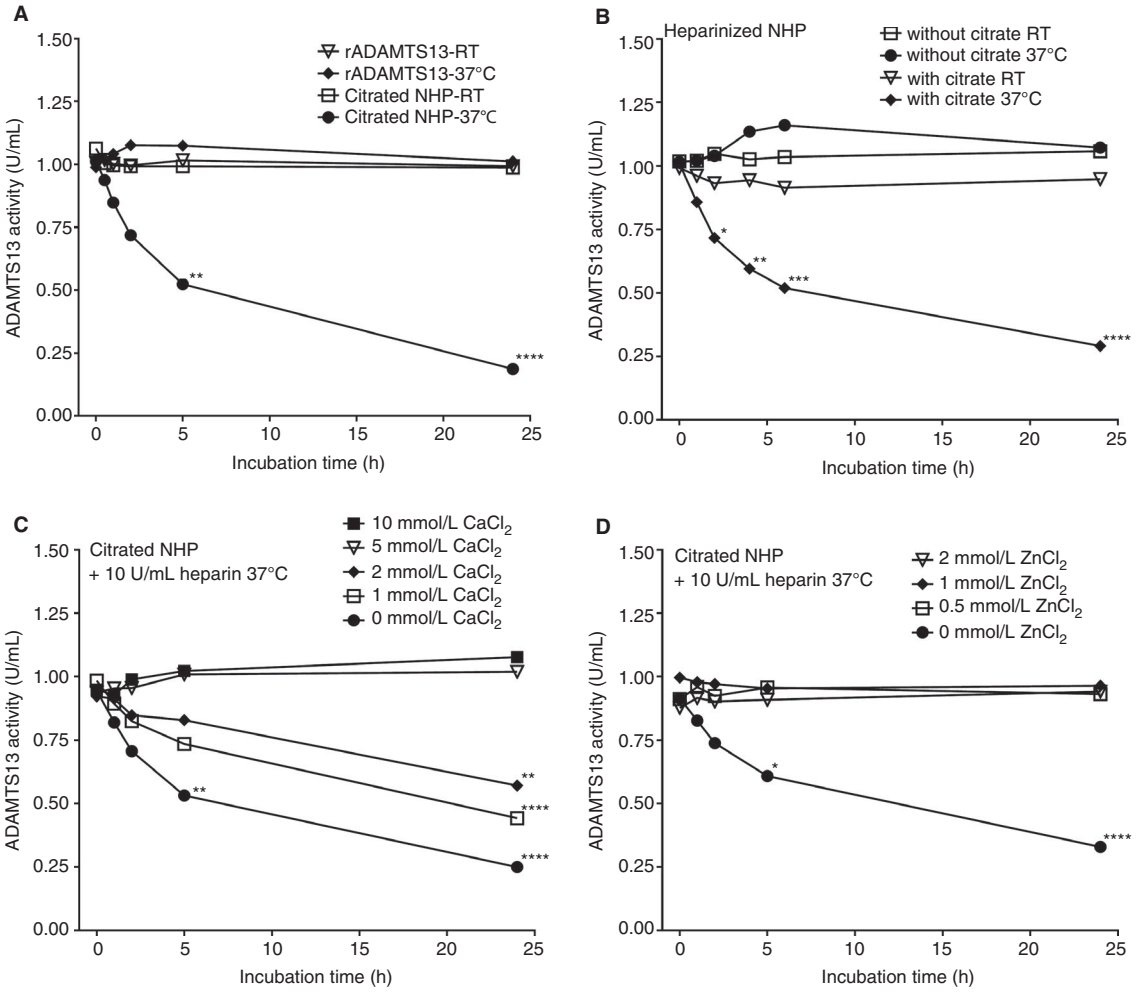


FIGURE 1 Instability of ADAMTS13 normal human plasma (NHP) at physiologic temperature (37°C) is caused by citrate. A, ADAMTS13 activity was measured in citrated pooled NHP and in a purified system (recombinant human ADAMTS13) at room temperature (RT) and at 37°C at 0, 0.5, 1, 2, 5, and 24 h of incubation. ADAMTS13 activity in NHP decreased at 37°C but not at room temperature in a time-dependent manner. B, ADAMTS13 activity was measured in heparinized pooled NHP with or without citrate (0.38% w/v) at room temperature and at 37°C at 0, 1, 2, 4, 6, and 24 h of incubation. ADAMTS13 activity in heparinized NHP was maintained at both temperatures, whereas the addition of citrate caused a gradual loss of activity at 37°C. C, Citrated pooled NHP plus 10 U/mL heparin as anticoagulant was incubated in the presence of increasing concentrations of Ca^{2+} at 37°C for 24 h, and ADAMTS13 activity was measured at 0, 1, 2, 5, and 24 h of incubation. ADAMTS13 activity was fully maintained at a concentration of 5 mmol/L Ca^{2+} or higher. D, Citrated pooled NHP plus 10 U/mL heparin was incubated in the presence of increasing concentrations of Zn^{2+} at 37°C for 24 h, and ADAMTS13 activity was measured at 0, 1, 2, 5, and 24 h. ADAMTS13 activity was fully maintained at a concentration of 0.5 mmol/L Zn^{2+} or higher. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ as compared with control (A: citrated NHP, RT; B: heparinized NHP, RT; C: citrated NHP + 10 mmol/L $CaCl_2$, 37°C; D: citrated NHP + 2 mmol/L $ZnCl_2$, 37°C)

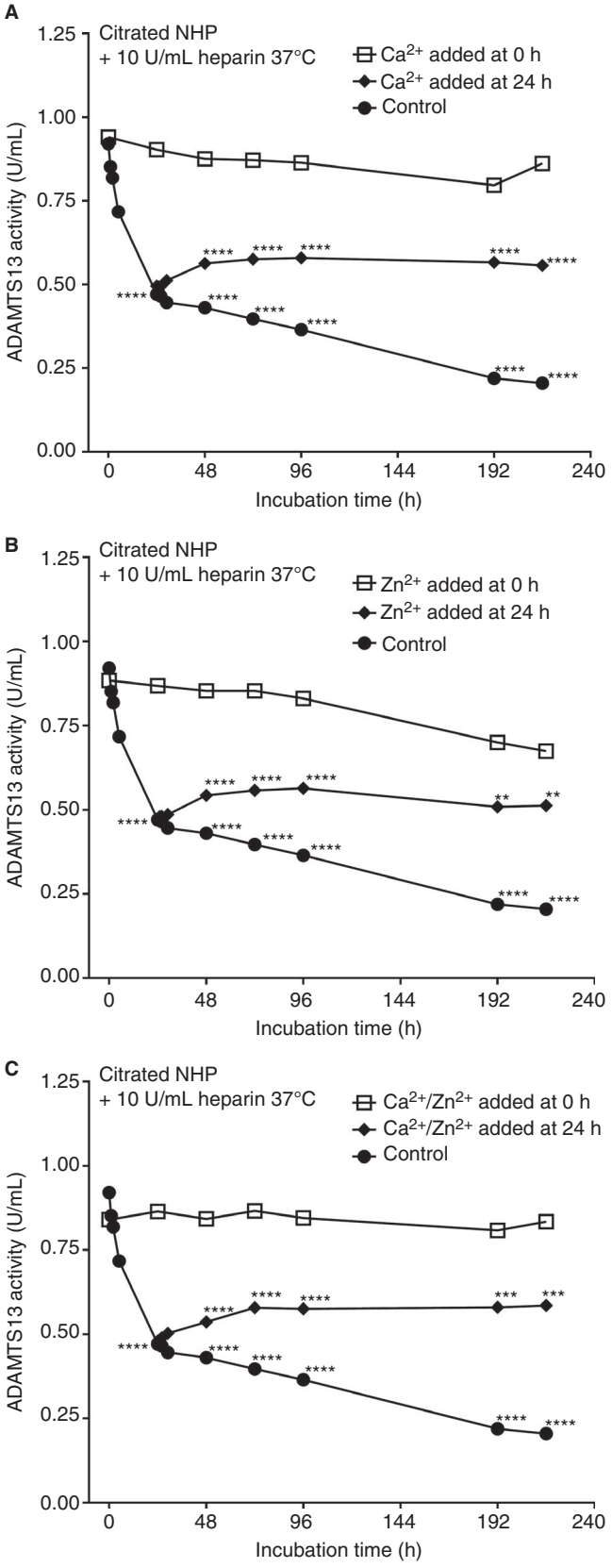


FIGURE 2 Ca^{2+} and Zn^{2+} stabilize but cannot restore ADAMTS13 activity in citrated normal human plasma (NHP). Citrated pooled NHP plus 10 U/mL heparin was incubated at 37°C for 9 days in the absence (control) or presence of A, 5 mmol/L Ca^{2+}; B, 1 mmol/L Zn^{2+}; or C, both ions added after 24 h. ADAMTS13 activity was measured at 0, 1, 2, 5, 24, 25, 26, 29, 48, 72, 96, 192, and 216 h. As control for maintenance of ADAMTS13 activity at 37°C, the respective metal ions were added already at the start of the experiment. For those samples, ADAMTS13 activity was measured at time 0, 24, 48, 72, 96, 192, and 216 h. The addition of metal ions after 24 h was not able to restore full ADAMTS13 activity. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as compared with control (A: citrated NHP, Ca^{2+} added at 0 h; B: citrated NHP, Zn^{2+} added at 0 h; C: citrated NHP, Ca^{2+} + Zn^{2+} added at 0 h)

analyses, only a single site seems to be important for enzyme activity.[8] The dependence of ADAMTS13 activity on Zn^{2+} and Ca^{2+} was demonstrated by the observation that the cation-chelating molecules ethylenediaminetetraacetic acid[9,10] and doxycycline[11] render ADAMTS13 inactive and that the addition of Zn^{2+} and/or Ca^{2+} can restore ADAMTS13 activity.[8,10,12] The 3D structure of the protease domain of ADAMTS13 has been modeled using the available 3D structures of adamalysin II[8] or the more closely related ADAMTS family members ADAMTS1, 4, and 5.[13,14]

In clinical practice, normal human plasma (NHP) anticoagulated with citrate is the typical enzyme source for standard clinical assays such as the FRETS-VWF73 ADAMTS13 activity assay and the anti-ADAMTS13 inhibitor assay.[15,16] It has long been recognized that the chelating properties of citrate influence the Ca^{2+} concentration required for full ADAMTS13 activity and can be managed by adjusting the Ca^{2+} concentration accordingly.[8,17]

The impact of citrate on enzyme stability is less clear. ADAMTS13 activity was reported to be stable in citrated NHP at room temperature for up to 48 hours,[18] and to be only very slowly inactivated at 37°C, with a half-life longer than 1 week.[19] On the other hand, cleavage of denatured multimeric VWF by citrated NHP was shown to be complete at 22°C and 4°C and only partial at physiologic temperature.[20] Similarly, Kraisin et al found a negative influence of physiologic temperature on the stability of ADAMTS13 activity in citrated normal plasma, with a half-life ranging between 24 and 48 hours.[21]

Here we explored the temperature-dependent stability of ADAMTS13 using various sources of NHP and recombinant human ADAMTS13 (rADAMTS13) in different experimental setups. We further aimed to identify the cause of the observed instability in the presence of citrate at physiological temperature by determining whether enzyme inactivation correlates with reduced metal ion binding and structural disintegration.

2 | METHODS

2.1 | Sources of ADAMTS13

Purified rADAMTS13 (BAX 930/TAK755, Baxalta Innovations GmbH, a member of the Takeda group of companies, Vienna, Austria) was formulated in a low-salt buffer, physiologic pH, containing 0.1% Tween 80 and used at 1 U/mL for all experiments

except the inductively coupled plasma mass spectrometry (ICP-MS) measurements, which required a highly concentrated solution (404 μg/mL). Pooled NHP anticoagulated with citrate was from George King Biomedical (Overland Park, KS). Pooled NHP anticoagulated with heparin was from Fitzgerald Industries International (Acton, MA).

2.2 | Stability testing of ADAMTS13 activity

All samples of a test series were measured for ADAMTS13 activity using the synthetic fluorogenic FRETS-VWF73 peptide substrate (Peptanova GmbH, Sandhausen, Germany), as previously described.[22] The effect of citrate on ADAMTS13 stability was tested by adding one part of sodium citrate 3.8% (w/v; Sigma, St. Louis, MO) to nine parts of pooled NHP anticoagulated with heparin. Alternatively, citrate was scavenged by Zn^{2+} and Ca^{2+} using increasing concentrations of $CaCl_2$ or $ZnCl_2$ spiked into citrated NHP. To prevent clotting after the addition of metal ions, citrated NHP was pretreated with 10 U/mL lithium heparin (Sigma). Restoration of ADAMTS13 activity following a 24-hour incubation step at 37°C was assessed by spiking citrated NHP (plus 10 U/mL lithium heparin) with 5 mmol/L $CaCl_2$, 1 mmol/L $ZnCl_2$, a mixture of 5 mmol/L $CaCl_2$ and 1 mmol/L $ZnCl_2$, or water as control.

2.3 | Analysis of ADAMTS13 metal ion binding by size exclusion chromatography inductively coupled plasma mass spectrometry

The SEC-ICP-MS analysis was carried out essentially as described previously.[23] An Ultimate 3000 ×2 Dual Titanium HPLC system (Thermo Fisher Scientific, Karlsruhe, Germany) controlled by Chromeleon 6.8 software was used for the size exclusion separation. For elemental detection, the system was combined with an Elan 6100 DRCII quadrupole ICP-MS from Perkin-Elmer-Sciex (Toronto, Canada). The reaction cell was filled with oxygen to induce the formation of $^{32}S^{16}O$ and circumvent the $^{16}O_2$ interference on the most abundant sulfur isotope; ^{32}S. Zinc was detected as ^{66}Zn. The intensity ratio between the ^{66}Zn and the $^{32}S^{16}O$ signal was determined using bovine Cu/Zn-SOD (Sigma-Aldrich Chemie GmbH, Vienna, Austria). The stoichiometric zinc ratio was calculated via the number of sulfur atoms per protein unit.

2.4 | Analysis of ADAMTS13 conformation by dynamic light scattering and Fourier-transformed infrared spectroscopy

Dynamic light scattering (DLS) was performed using a Malvern NanoZetasizer ZS (Malvern Instruments Ltd, Malvern, UK) and a Haake Rheostress 1 (Thermo Fisher Scientific) equipped with a cone with a 60-mm diameter/0.5° angle essentially as described previously.[24] The protein's angle was set to 173° backscatter to measure its size.

Fourier-transformed infrared (FTIR) spectroscopy was performed using the FTIR spectroscope TENSOR 27 (Bruker Optik GmbH, Ettlingen, Germany) equipped with a BioATR II cell working in attenuated total reflection mode essentially as described previously.[25] The secondary structure (percent α-helix and β-sheet) of the protein was calculated by the evaluation software OPUS 6.0 (Bruker Optik GmbH), which contains a database of approximately 40 proteins of known secondary structure.

2.5 | Statistical evaluation

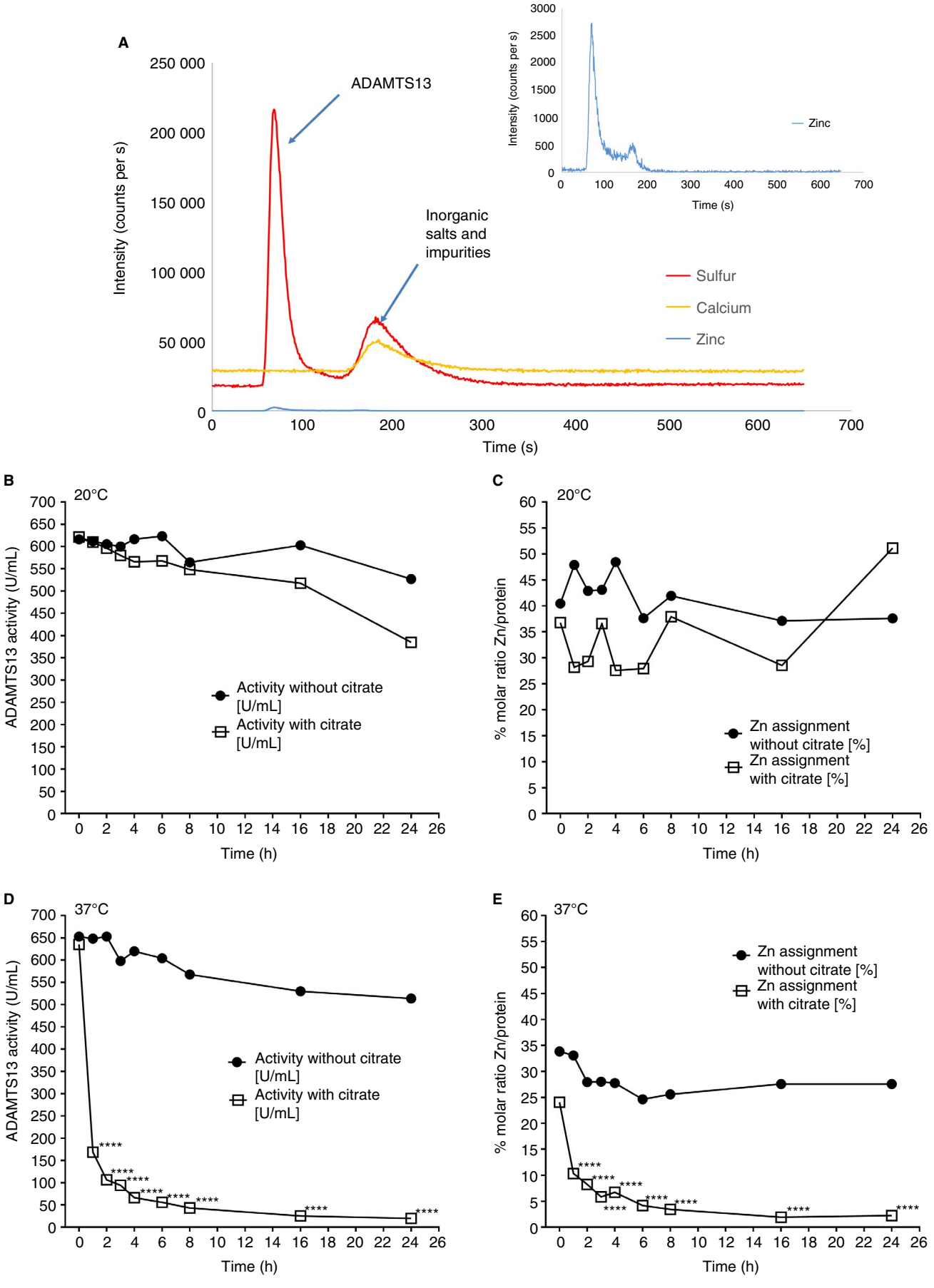
All calculations were performed using GraphPad (7.03) software. Graphs were subjected to spline or point-to-point regression and analyzed by two-way ANOVA.

3 | RESULTS AND DISCUSSION

3.1 | Stability of ADAMTS13 is compromised by citrate at physiologic temperature (37°C)

The temperature-dependent stability of ADAMTS13 in plasma was initially assessed by measuring FRETS-VWF73 fluorogenic activity in pooled citrated NHP incubated at room temperature and at 37°C. While ADAMTS13 activity was maintained over the entire incubation period of 24 hours at room temperature, a time-dependent activity reduction was observed at 37°C. In a purified system using recombinant ADAMTS13 however, the enzyme's activity was maintained regardless of the temperature (Figure 1A). In NHP anticoagulated with heparin, ADAMTS13 activity was also stable over 24 hours at 37°C (Figure 1B); however, addition of citrate to the heparinized NHP caused a time-dependent drop in ADAMTS13 activity,

FIGURE 3 The citrate-dependent activity drop correlates with a loss of ADAMTS13's bound Zn^{2+}. A, Demonstration of (substoichiometric quantities of) Zn^{2+} bound to ADAMTS13. Shown is a representative chromatogram of purified recombinant human ADAMTS13 obtained via size exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICP-MS). Dynamic reaction cell technology was applied utilizing oxygen as reaction gas, enabling the interference-free measurement of sulfur as SO^+ (m/z = 48). Zn^{2+} and Ca^{2+} were measured at m/z = 66 and m/z = 43, respectively. The inset shows a 100-fold magnification of the Zn^{2+} trace. A Ca^{2+} peak was not detected at the retention time of the protein. Some remaining unbound Ca^{2+} impurities could be detected in the lower cutoff of the size exclusion chromatogram. B-E, ADAMTS13 selectively loses Zn^{2+} at physiologic temperature in the presence of citrate. Recombinant human ADAMTS13 was incubated in the presence or absence of citrate (0.38% w/v) at 20°C, B, C, and 37°C, D, E. At 0, 1, 2, 3, 4, 6, 8, 16, and 24 h, the retained activity of ADAMTS13, B, D, was compared with Zn^{2+} assignment as determined by SEC-ICP-MS and expressed as the molar ratio of Zn^{2+} to ADAMTS13, C, E. The graphs indicate a clear relationship between enzyme activity and Zn^{2+} assignment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 as compared with control (A: activity without citrate; B: Zn assignment without citrate; C: activity without citrate; D: Zn assignment without citrate)


A
ADAMTS13
Inorganic salts and impurities
Intensity (counts per s)
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B
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Activity without citrate [U/mL]
Activity with citrate [U/mL]
Time (h)
C
20°C
% molar ratio Zn/protein
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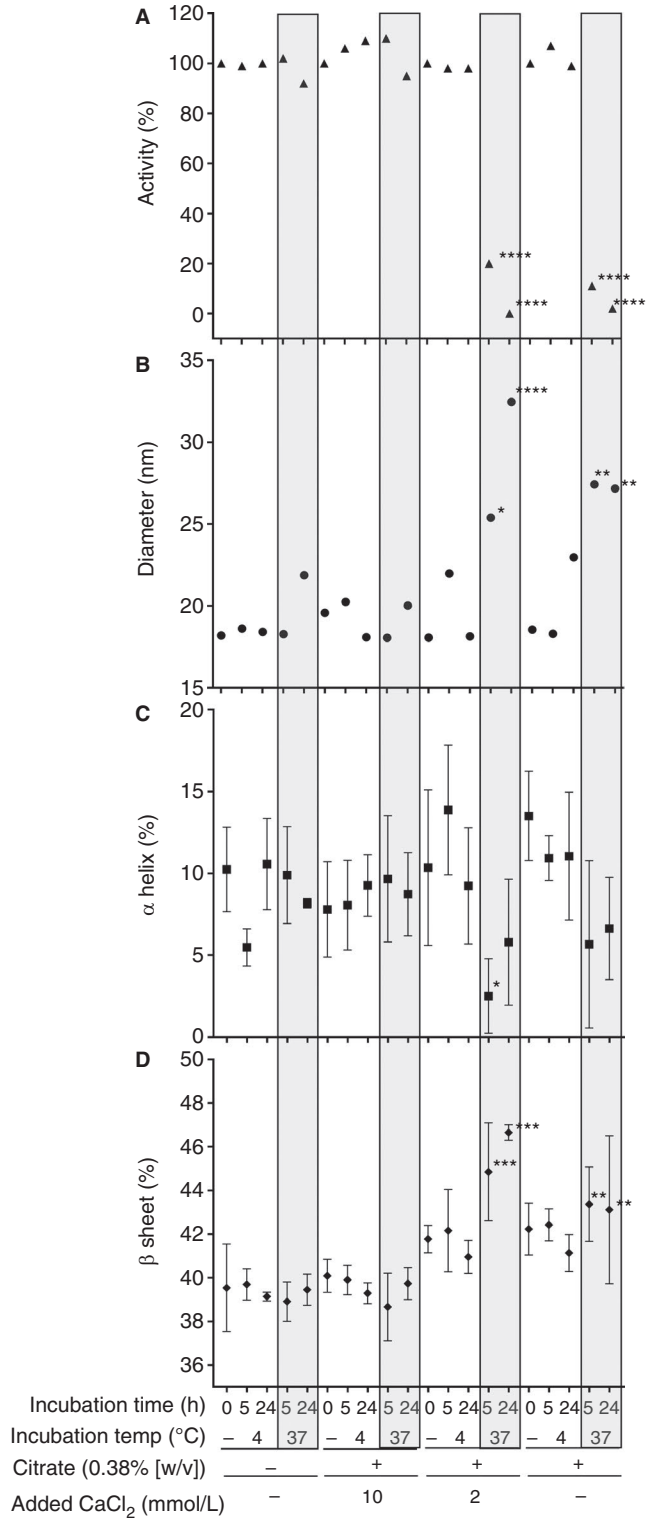

FIGURE 4 The citrate-dependent activity drop correlates with changes in the higher-order structure of ADAMTS13. Recombinant human ADAMTS13 was incubated in the presence or absence of citrate (0.38% w/v) and increasing concentrations of Ca^{2+} at 4°C and 37°C for up to 24 h. At 0, 5, and 24 h, samples were analyzed for the activity of the enzyme, A, as well as its structural integrity, using dynamic light scattering (hydrodynamic diameter in nanometers) and Fourier-transformed infrared spectroscopy (α-helix and β-sheet content). In the presence of citrate at physiologic temperature, the drop in ADAMTS13 activity was accompanied by an increase of the enzyme's hydrodynamic diameter, B, a drop in its α-helical content, C, and a slight increase in its β-sheet content, D. Calculated is the average of three runs per sample. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ as compared to control (A-D: rADAMTS13 without citrate, 4°C)

but only at 37°C. Addition of increasing concentrations of Ca^{2+} or Zn^{2+} to citrated NHP and monitoring of ADAMTS13 activities over 24 hours at 37°C showed that supplementation of $CaCl_2$ at 5 mmol/L or higher (Figure 1C) and $ZnCl_2$ at 0.5 mmol/L or higher (Figure 1D) completely restored enzyme stability. Taken together, chelation of divalent cations by citrate negatively affected ADAMTS13 stability at physiologic temperature.

3.2 | Destabilization of ADAMTS13 by citrate at physiologic temperature is irreversible

We next tested whether the addition of Ca^{2+} and/or Zn^{2+} to citrated NHP that had been incubated at 37°C for 24 hours would restore ADAMTS13 activity (Figure 2). ADAMTS13 activity was monitored for 8 days, but neither Ca^{2+} (Figure 2A) nor Zn^{2+} (Figure 2B) alone nor the mixture of both metal ions (Figure 2C) fully restored enzyme activity. Nonetheless, stabilization of the residual activity was achieved when compared with the untreated citrated NHP sample. Activity remained stable in control samples where the addition of metal ions occurred at the zero-hour time point. These data suggested that, at physiologic temperature, citrate slowly depleted ADAMTS13 of divalent cations, most likely Ca^{2+} and Zn^{2+}, which resulted in inactivation of the enzyme.

3.3 | Citrate promotes ADAMTS13 destabilization by the removal of Zn^{2+}

To monitor potential changes in metal ion binding with the addition of citrate directly, we determined the amount of bound Ca^{2+} and Zn^{2+} in purified rADAMTS13 using SEC-ICP-MS (Figure 3A). The resulting chromatogram revealed congruent sulfur and Zn^{2+} peaks consistent with ADAMTS13's essential Zn^{2+} binding site in its active center. No Ca^{2+} peak was discernible at the retention time of the sulfur and Zn^{2+} peaks, even at 100× magnification. This is likely because all the loosely associated Ca^{2+} was removed during the purification process, as observed previously.[8] Calculation of the zinc-to-sulfur stoichiometric ratio, using Cu/Zn-SOD as reference for determining the molar ^{66}Zn to $^{32}S^{16}O$ intensity ratio of the instrument,[23] revealed a zinc loading density for rADAMTS13 that fluctuated between the measurements from 25% to 40%. These data suggest that the active site-associated Zn^{2+} is also not as tightly bound as the metal ions in the dismutase, which maintains its activity even under denaturing conditions.[26]

A stability study focusing on changes in rADAMTS13's molar ratio of Zn^{2+} to protein in dependence of citrate and temperature showed a quick drop at 37°C in the presence of citrate, but not at 20°C or in the absence of citrate (Figure 3C,E). The kinetics of

the decrease coincided with the reduction of ADAMTS13 activity (Figure 3B,D), demonstrating a close relationship between these two parameters.

3.4 | Citrate-triggered Zn^{2+} depletion at physiologic temperature results in a destabilization of ADAMTS13's higher-order structure

Since the observed ADAMTS13 activity decline was not due to proteolysis (evidenced by SDS PAGE of rADAMTS13 and addition of protease inhibitors to citrated NHP; not shown), we studied the protein's structural integrity by measuring its hydrodynamic diameter using DLS. Diameters remained in a narrow range for all conditions and time points where ADAMTS13 activity was not reduced (Figure 4A,B). In contrast, a marked increase in the diameter was noted for all four samples with a low residual activity (<20%). Since such a size increase is typical for proteins that transition into a less-ordered structure,[27] a correlation between the loss of activity and structural integrity of ADAMTS13 could be hypothesized.

To substantiate this correlation, the change in percentage of secondary structural elements in ADAMTS13 was followed by FTIR spectroscopy. The citrate-dependent decrease in ADAMTS13 activity at 37°C was indeed associated with a marked reduction of α-helical content (Figure 4C) and a slight increase in β-sheet content (Figure 4D), typical for transitions into less-ordered protein structures.[28]

3.5 | Novel insights on ADAMTS13 structure-function relationship

This study shows that zinc is not only required for maintaining the catalytic activity of ADAMTS13 but also for stabilizing its conformation. Our observation of ADAMTS13 being slowly inactivated by citrate selectively at 37°C further indicates that chelation of bound Zn^{2+} requires a free energy threshold above which conformational fluctuations in ADAMTS13's active site would enable temporary exposure of the coordinated Zn^{2+} atom to the citrate-containing solvent. Demetalation would in turn lead to local unfolding of the metalloprotease domain followed by a more global unfolding process causing the enzyme to disintegrate structurally and functionally, as measured by two orthogonal higher-order structure-determining methods. These collapsed molecules were also refractory to renaturation upon supplementation with Ca^{2+} or Zn^{2+}, demonstrating that ADAMTS13 stability was irreversibly compromised, perhaps because dimers or aggregates had already formed. To understand these rearrangements better, elucidation of ADAMTS13's metalloprotease domain structure and its dynamics upon citrate-dependent demetalation at 37°C will be required.

3.6 | Clinical relevance

Citrate is a commonly used anticoagulant to arrest the clotting cascade through chelation of Ca^{2+}.[29] The effect on ADAMTS13 stability reported here should be considered when measuring inhibitory anti-ADAMTS13 antibodies in plasma samples from patients with TTP, as the enzyme activity for added citrated NHP will decrease by approximately 25% during preincubation at 37°C for 2 hours. Treatment of TTP involves plasma infusion and exchange to replace the missing metalloenzyme.[2] Frozen citrate plasma is thawed at 37°C prior to infusion, but will usually be infused within 30 minutes. Plasma separation takes 2-4 hours, but the plasma to be substituted is normally kept at room temperature, and the dwell time in low-calcium milieu is around 1-2 minutes for a typically applied flow rate of 100 mL/min. Furthermore, a check of the calcium concentration in postfilter plasma indicated presence of residual free Ca^{2+} (0.2 mmol/L). A similar Ca^{2+} concentration (0.3 mmol/L) was measured for a patient undergoing continuous hemofiltration with citrate anticoagulation. These considerations suggest that destabilization of ADAMTS13 in the presence of citrate at physiological temperature has relatively little impact with regard to the therapeutic use of citrated NHP.

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DISCLOSURE OF CONFLICTS OF INTEREST

Hanspeter Rottensteiner, Stefan Kaufmann, Barbara Kink, Barbara Plaimauer, and Friedrich Scheiflinger are employees of Baxalta Innovations GmbH, a member of the Takeda group of companies, Vienna, Austria, and may own stock and/or stock options in Takeda Pharmaceutical Company Limited. Peter Matthiessen was an employee of Baxalta Innovations GmbH, a member of the Takeda group of companies, Vienna, Austria, at the time of the study. Barbara Kink and Peter Matthiessen have a patent (EP2480198) issued for stabilized liquid and lyophilized ADAMTS13 formulations. Anna Rathgeb and Stephan Hann have nothing to disclose.

AUTHOR CONTRIBUTIONS

Hanspeter Rottensteiner, Stefan Kaufmann, Anna Rathgeb, Barbara Kink, Barbara Plaimauer, Peter Matthiessen, Stephan Hann, and Friedrich Scheiflinger were the key investigators for the study. Hanspeter Rottensteiner, Barbara Plaimauer, and Friedrich Scheiflinger conceptualized the study; Stefan Kaufmann, Anna Rathgeb, and Barbara Kink performed the data analyses; Hanspeter

Rottensteiner, Barbara Plaimauer, Peter Matthiessen, and Stephan Hann supervised the research; and Hanspeter Rottensteiner drafted the manuscript. All authors met the ICMJE criteria for authorship and read and approved the final manuscript for submission.

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