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FEYS et al

STRUCTURE-FUNCTION OF ADAMTS13 S119F

Mutation of the H-bond acceptor S119 in the ADAMTS13 metalloprotease domain reduces secretion and substrate turnover in a patient with congenital thrombotic thrombocytopenic purpura

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Abstract

Hereditary thrombotic thrombocytopenic purpura is caused by mutations in ADAMTS13 resulting in defective processing of von Willebrand factor (VWF) which causes intravascular platelet aggregation culminating in thrombocytopenia with shistocytic anemia. In this study the functional and structural role of a recently identified ADAMTS13 metalloprotease domain mutation S119F was investigated. Secretion from heterologous cells was hampered but not completely eliminated. Secreted S119F was active towards multimeric VWF and FRETs-VWF73 but with abnormal kinetics having a significantly reduced k_{cat} ($0.88 \pm 0.04 \text{ s}^{-1}$ vs $2.78 \pm 0.11 \text{ s}^{-1}$) and slightly smaller K_{M} ($1.4 \pm 0.2 \mu\text{M}$ vs $2.3 \pm 0.3 \mu\text{M}$). A computational model of the metalloprotease domain demonstrates both steric and polar interaction effects caused by S119F. Interestingly, mutant S119A has properties similar to S119F ($k_{\text{cat}} = 0.82 \pm 0.03 \text{ s}^{-1}$ and $K_{\text{M}} = 1.1 \pm 0.1 \mu\text{M}$) allowing to assign distorted kinetics to the loss of the H-bond with conserved residue W262. We conclude that the S119-W262 H-bond is crucial for maximal turnover.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is an acute thrombotic disorder characterized by consumptive thrombocytopenia and microangiopathic hemolytic anemia.¹ In most cases TTP is acquired, but a small number of cases bears mutations in the *ADAMTS13* (A Disintegrin And Metalloprotease with ThromboSpondin Motifs) gene resulting in the same phenotype.^{2,3}

ADAMTS13 is a metalloprotease specifically hydrolyzing the Y1605-M1606 peptide bond in multimeric von Willebrand factor (VWF). VWF multimer size reduction is pivotal in preserving normal hemostasis because uncleaved multimers spontaneously bind platelets causing occlusion of microvasculature; the TTP hallmark.

Over 50 *ADAMTS13* gene mutations have been reported since its identification in 2001.⁴ Most of these were not investigated by *in vitro* mutagenesis and expression analysis, but those that were mainly cause secretion defects.³ This is in line with the observation that *ADAMTS13* antigen is absent in plasma samples from patients with hereditary TTP.⁵ Nonetheless, some *ADAMTS13* mutants are secreted^{3,6,7} but are less or not functional.

Recently, high resolution structures of the metalloprotease domains of *ADAMTS*-1, -4 and -5 were published providing insight in active-site and small molecule inhibitor orientation.^{8,9} The structures of the metalloprotease domains of *ADAM/ADAMTS* proteins generally superimpose with little variation except in the variable loops making up domain periphery.¹⁰

In this study, we characterize a recently identified *ADAMTS13* metalloprotease domain missense mutation which causes a partial secretion defect and a three-fold reduction in the turnover number k_{cat} . We correlate kinetic data with rational assumptions from a homology model of the *ADAMTS13* metalloprotease domain.

Materials and methods

Patient characterization

This study was approved by the KULeuven Ethical Committee of the Katholieke Universiteit Leuven and its hospitals. Informed consent was obtained in accordance with the Declaration of Helsinki. The propositus is a 14-year old Caucasian pediatric patient with typical congenital TTP successfully treated with prophylactic plasma infusion therapy. *ADAMTS13* exons with exon-intron boundaries were amplified from genomic DNA using polymerase chain reaction (PCR) with primers described elsewhere.¹¹ Sequencing reactions were outsourced (GATC Biotech AG, Konstanz, Germany).

Citrated plasma was used for analysis of *ADAMTS13* activity and antigen. Normal human pooled plasma (NHP, $n=20$) was used as a control. Antigen was measured by ELISA as described⁵ and by immunoblotting with monoclonal antibody (mAb) 17B10 binding to the third *ADAMTS13* thrombospondin-1 repeat. Bound mAb was detected by peroxidase labeled goat-anti-mouse antiserum (GAM-HRP, Jackson ImmunoResearch, West Grove, PA).

Mutagenesis and expression of recombinant proteins

Mutagenesis was performed with the QuickChange[®] XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) using primers bearing the desired mutations. All mutants were constructed using the inducible expression plasmid pGene/V5-His (Invitrogen) containing the *ADAMTS13* cDNA.⁵ Vectors were lipofected in Chinese Hamster Ovary GeneSwitch[™] cells and Human Embryonic Kidney 293 (HEK293) GeneSwitch[™] cells for stable and transient expression, respectively. Serum-free conditioned medium was purified by ion exchange (Q-sepharose, GE Healthcare, Waukesha, WI).¹²

ADAMTS13 activity and kinetic measurements

Activity towards multimeric VWF was measured using mild denaturants as described previously.¹³ Reactions contained 10 $\mu\text{g/mL}$ purified VWF (CSL Behring, King of Prussia, PA) and 0.6 nM recombinant *ADAMTS13* (r*ADAMTS13*) and were incubated for 16h. Proteolysis was analyzed by

residual collagen binding.¹⁴ Proteolytic fragments were visualized by western blotting with anti-VWF monoclonal antibodies RG46, 52K-2 and 52K-8 (Dr. Ruggeri, La Jolla, CA)¹⁵ and GAM-HRP.

Determination of kinetic parameters k_{cat} and K_M using the FRET-VWF73¹⁶ substrate and 0.1nM of enzyme was as described previously.¹⁷

ADAMTS13 metalloprotease domain homology model

MODELLER 9v4¹⁸ was used to generate a homology model based on the structures of ADAMTS1, ADAMTS4 and ADAMTS5 (respective PDB-codes: 2V4B, 3B2Z and 3B8Z). Sequence identity between ADAMTS13 and templates varied between 25% and 30%. Modeling preserved both the active-site zinc ion and the zinc-coordinating water molecule. The dead-end elimination method¹⁹ was used to optimize orientation of all side-chains and polar hydrogens. Idealization of bond geometry and removal of unfavorable non-bonded contacts was performed by a conjugate gradient energy minimization with the GROMACS v3.3.1 software package²⁰ in the presence of explicit water and counter ions. We applied the GROMOS96 43a1 force field. Mutant structure S119F was generated using the BRUGEL package²¹ and least squares fitting was done using PyMOL™ (DeLano Scientific, Palo Alto, CA).

Results

Homozygous mutation 356 C>T in the ADAMTS13 metalloprotease coding sequence

ADAMTS13 antigen was severely reduced to 5% in remission phase and undetectable (<5%) in acute phase plasma as determined by western blot (Figure 1A, *left*) and ELISA. Both samples contained no measurable VWF73 cleaving activity (not shown). A homozygous missense mutation 356C>T and a known single nucleotide polymorphism (SNP) 1342C>G were identified, encoding a serine to phenylalanine substitution (S119F) and a glutamine to glutamic acid exchange (Q448E), respectively. Meyer *et al* recently reported S119F as the first congenital TTP case on the African continent.²² Importantly, the serine residue is conserved among ADAMTSs except in ADAMTS16 and -18²³ where a threonine resides at the corresponding position, alluding to a role of the side-chain hydroxyl group.

S119F kinetic parameters k_{cat} and K_M are reduced

Transient expression of both the single (S116F) and the double mutant (S119F/Q448E) revealed normal expression but markedly impaired secretion (Figure 1A, *right*). VWF multimers were completely cleaved by 0.6 nM ADAMTS13 in NHP while an identical concentration of either single or double mutant only partially cleaved the substrate (Figure 1B). Western blotting of the reduced VWF N-terminal reaction products revealed no obvious size difference between VWF fragments generated by S119F and WT indicating site-specificity was unaltered (Figure 1C).

Importantly, k_{cat} of S119F ($0.88 \pm 0.04 \text{ s}^{-1}$, mean $\pm$ SD) was significantly smaller than WT ($2.78 \pm 0.11 \text{ s}^{-1}$) while K_M was only slightly smaller ($1.4 \pm 0.2 \mu\text{M}$ vs $2.3 \pm 0.3 \mu\text{M}$) as determined by FRETs-VWF73 kinetic measurements (Figure 2A). A reduced k_{cat} indicates that substrate turnover and/or product release is affected by the mutation. Furthermore, K_M is only slightly different indicating ground-state binding is comparable with WT or somewhat better. As a consequence, the specificity constant (k_{cat}/K_M) reduces by half from $1.2 \mu\text{M}^{-1} \text{ s}^{-1}$ (WT) to $0.6 \mu\text{M}^{-1} \text{ s}^{-1}$ (S119F). This parameter is inversely related to the free energy of transition state complex formation.²⁴ Therefore, more energy is required to attain transition state in S119F following the enzyme substrate binding step. Hence, higher concentrations of S119F

are needed for optimal VWF processing and TTP may precipitate at enzyme levels which would be safe if no mutation were present.

Importance of the S119-W262 H-bond

In our metalloprotease domain model (Figure 2B), S119 resides on a short α -helix juxtaposed to the C-terminus of the S1' loop (residues 247-261) (Figure 2C).^{9,25} This loop lacks secondary structure and is important for substrate recognition.¹⁰ The side-chain hydroxyl group from S119 accepts a H-bond from the backbone amine of conserved residue W262. Furthermore, the W262 indole side-chain is orientated in a hydrophobic pocket which is conserved among ADAMTSs and compiled of aliphatic residues from nearby strands including the short α -helix harboring S119 (Figure 2C).^{8,9} Both the H-bond and the optimal orientation of the short α -helix are hence affected in S119F. To distinguish between these mechanisms underlying the abnormal kinetics, a mutant S119A was constructed. This mutant prevents H-bond formation but exerts no steric strain on the helix bearing S119. Interestingly, kinetic parameters were not different between S119A and S119F ($k_{cat}=0.82\pm0.03s^{-1}$ and $K_M=1.1\pm0.1\mu M$). This indicates that steric interference in S119F is not the leading cause of decreased catalytic efficiency. The data show that the S119-W262 H-bond is crucial for optimal turnover (reduced k_{cat}) although not required for substrate recognition or binding (equal K_M). Alternatively, disruption of the H-bond induces structural changes in the active-site shape which would concomitantly explain the poor secretion. In conclusion, as the C- and N-terminus of the S1' loop meet, it adopts a Ω -shape which is in part sustained by the hydrophobic interactions including C-terminal W262 keeping the ends of the flexible strand together in the pocket. Loss of the W262 H-bond with S119 might render the backbone of the S1' loop more flexible, influencing overall structure and/or optimal alignment for transition state complex formation.

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Authorship

Contribution: H.B.F. designed and performed research, analyzed and interpreted data, and wrote the paper. I.P. performed research and interpreted data. R.V. performed research and critically reviewed the manuscript. H.D., M.D.M. and C.V.G. provided essential reagents and/or patient material and critically reviewed the manuscript. K.V. designed research, analyzed and interpreted data, and wrote the paper.

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Reference List

1. Moake JL. Thrombotic thrombocytopenic purpura: the systemic clumping "plague". *Annu Rev Med*. 2002;53:75-88.
2. Levy GG, Nichols WC, Lian EC et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature*. 2001;413(6855):488-494.
3. Kokame K, Matsumoto M, Soejima K et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci U S A*. 2002;99(18):11902-11907.
4. Zheng XL, Sadler JE. Pathogenesis of thrombotic microangiopathies. *Annu Rev Pathol*. 2008;3:249-277.
5. Feys HB, Liu F, Dong N et al. ADAMTS-13 plasma level determination uncovers antigen absence in acquired thrombotic thrombocytopenic purpura and ethnic differences. *J Thromb Haemost*. 2006;4(5):955-962.
6. Uchida T, Wada H, Mizutani M et al. Identification of novel mutations in ADAMTS13 in an adult patient with congenital thrombotic thrombocytopenic purpura. *Blood*. 2004;104(7):2081-2083.
7. Peyvandi F, Lavoretano S, Palla R et al. Mechanisms of the interaction between two ADAMTS13 gene mutations leading to severe deficiency of enzymatic activity. *Hum Mutat*. 2006;27(4):330-336.
8. Gerhardt S, Hassall G, Hawtin P et al. Crystal structures of human ADAMTS-1 reveal a conserved catalytic domain and a disintegrin-like domain with a fold homologous to cysteine-rich domains. *J Mol Biol*. 2007;373(4):891-902.
9. Mosyak L, Georgiadis K, Shane T et al. Crystal structures of the two major aggrecan degrading enzymes, ADAMTS4 and ADAMTS5. *Protein Sci*. 2008;17(1):16-21.
10. Takeda S. Three-dimensional domain architecture of the ADAM family proteinases. *Seminars in Cell & Developmental Biology*. 2009;20(2):146-152.
11. Kokame K, Miyata T. Genetic defects leading to hereditary thrombotic thrombocytopenic purpura. *Semin Hematol*. 2004;41(1):34-40.
12. Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood*. 2001;98(6):1662-1666.
13. Furlan M, Robles R, Lammle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*. 1996;87(10):4223-4234.
14. Gerritsen HE, Turecek PL, Schwarz HP, Lammle B, Furlan M. Assay of von Willebrand factor (vWF)-cleaving protease based on decreased collagen binding affinity of degraded vWF - A tool for the diagnosis of thrombotic thrombocytopenic purpura (TTP). *Thromb Haemost*. 1999;82(5):1386-1389.
15. Mohri H, Fujimura Y, Shima M et al. Structure of the von Willebrand factor domain interacting with glycoprotein Ib. *J Biol Chem*. 1988;263(34):17901-17904.

16. Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRET-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol*. 2005;129(1):93-100.
17. Anderson PJ, Kokame K, Sadler JE. Zinc and calcium ions cooperatively modulate ADAMTS13 activity. *J Biol Chem*. 2006;281(2):850-857.
18. Fiser A, Sali A. Modeller: generation and refinement of homology-based protein structure models. *Methods Enzymol*. 2003;374:461-491.
19. De Maeyer M, Desmet J, Lasters I. The dead-end elimination theorem: mathematical aspects, implementation, optimizations, evaluation, and performance. *Methods Mol Biol*. 2000;143:265-304.
20. Van Der SD, Lindahl E, Hess B et al. GROMACS: fast, flexible, and free. *J Comput Chem*. 2005;26(16):1701-1718.
21. Delhaise P, Bardiaux M, De Maeyer M et al. The Brugel Package - Toward Computer-Aided-Design of Macromolecules. *J Mol Graph*. 1988;6:219.
22. Meyer SC, Jeddi R, Meddeb B et al. A first case of congenital TTP on the African continent due to a new homozygous mutation in the catalytic domain of ADAMTS13. *Ann Hematol*. 2008;87(8):663-666.
23. Nicholson AC, Malik SB, Logsdon JM, Van Meir EG. Functional evolution of ADAMTS genes: Evidence from analyses of phylogeny and gene organization. *BMC Evol Biol*. 2005;5(1):11.
24. Copeland RA. Enzymes, A Practical Introduction to Structure, Mechanism, and Data Analysis. New York, NY: Wiley-VCH; 2000.
25. Gomis-Ruth FX. Structural aspects of the metzincin clan of metalloendopeptidases. *Mol Biotechnol*. 2003;24(2):157-202.

Figure Legends

Figure 1. Expression levels, activity and site-specificity of S119F. (A, *left*) Representative western blot of endogenous ADAMTS13 in acute (AC) and remission (RE) phase of the propositus and in NHP. Blotted protein was detected with anti-ADAMTS13 mAb17B10 and GAM-HRP. Secondary antibody alone was negative (not shown). (A, *middle and right*) Transient expression of wild-type rADAMTS13 (WT), mutants S119F (SF) and S119F/Q448E (SFQE) Protein in medium (*middle*) and cell lysate (*right*) was detected with peroxidase labeled anti-V5 antibodies. (B) VWF collagen binding ELISA to assess residual VWF activity. 10 ug/mL purified multimeric VWF was incubated with 0.6 nM ADAMTS13 in NHP (●), 0.6 nM rADAMTS13 S119F (◆) or S119F/Q448E (▼, dotted line) for 16h in 1M urea. (●) All reactions were inhibited by 10 mM EDTA (dashed lines) as shown for NHP (○) and S119F (◇). (C) Analogous samples as in B, in the presence (+) or absence (-) of EDTA, were analyzed in Western blot under reducing conditions where VWF product bands were detected by anti-A1 domain mAbs and GAM-HRP.

Figure 2. Model of the ADAMTS13 metalloprotease domain and kinetics of S119 mutants. (A) Michaelis-Menten kinetics of WT rADAMTS13 (○), mutants S119F (▽) and S119A (◆). Initial velocity in function of FRET-S-VWF73 concentration ([S]) at fixed enzyme concentration (0.1 nM) is shown. (B) Surface representation of the ADAMTS13 metalloprotease model. Depicted in red and green are the S1' and S2' loop, respectively. The loops constitute part of the active site cleft. Mutated residue serine 119 (magenta) is located to the bottom-left facing the C-terminus of the S1' loop. (C) Cartoon representation zoomed into the S1' loop (red) and inner molecule. Depicted in sticks are S119, M249 (Met-turn) and residues making up the hydrophobic patch to which the W262 indole side-chain is oriented (V248, L120 and L114). Hydrogen bonds are shown in dashes. Mutation of residue S119 to phenylalanine (overlayed in orange) disrupts the hydrogen bond and causes a sterical clash between the S1' loop for all possible rotamers at fixed backbone positions (one rotamer shown).

Figure 1

Figure 1

Figure 1

C

Figure 2

Figure 2

B

Figure 2