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Review

Structural and functional consequences of methionine oxidation in thrombomodulin

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Abstract

Thrombomodulin (TM) is an endothelial cell surface glycoprotein that is responsible for switching the catalytic activity of thrombin away from fibrinogen cleavage (pro-coagulant) and towards protein C cleavage (anticoagulant). Although TM is a large protein, only the fourth and fifth epidermal growth factor-like (EGF-like) domains are required for anticoagulant function. These two domains must work together, and the linker between the two domains contains a single methionine residue, Met 388. Oxidation of Met 388 is deleterious for TM activity. Structural studies, both X-ray and NMR, of wild type and variants at position 388 show that Met 388 provides a key linkage between the two domains. Oxidation of the methionine has consequences for the structure of the fifth domain, which binds to thrombin. Oxidation also appears to disrupt the interdomain contacts resulting in structural and dynamic changes. The functional consequences of oxidation of Met 388 include decreased anticoagulant activity. Oxidative stress from several causes is reflected in lower serum levels of activated protein C and a higher thrombotic tendency, and this is thought to be linked to the oxidation of Met 388 in TM. Thus, TM structure and function are altered in a subtle but functionally critical way upon oxidation of Met 388.

Keywords: Anticoagulant; Protein C; Thrombin; Coagulation; NMR; EGF domain

1. Introduction

The biological activity of many proteins can be significantly affected by oxidation of methionine to methionine sulfoxide. Proteins that show decreased activity upon oxidation include calmodulin [1], α_1-protease inhibitor [2,3], high mobility group protein HMG-D [4], chymotrypsin [5], and thrombomodulin [6].

Thrombomodulin (TM) binds to thrombin and inhibits the fibrinogen cleavage activity, which results in fibrin clot formation. When TM binds to thrombin, it does not block the thrombin active site, instead it imparts new substrate specificity such that the TM–thrombin complex cleaves and activates protein C. In turn, activated protein C cleaves and inactivates the essential cofactors required for the coagulation cascade, thus shutting down further production of thrombin [7].

TM has six epidermal growth factor (EGF)-like domains. The 81-amino-acid TM fragment composed of the fourth and fifth EGF-like domains (TMEGF45) is the smallest active TM fragment (Fig. 1) [8]. The fifth domain contains most of the thrombin binding residues and has an uncrossed disulfide bonding pattern [9]. When the individual EGF-like domains were tested for thrombin binding, only the fifth domain was able to bind thrombin on its own [10]. TMEGF45, containing both the fourth and fifth EGF-like domains, binds more tightly than the fifth domain alone and is the smallest fragment with anticoagulant cofactor activity. Addition of the sixth domain results in TMEGF456, which has no additional cofactor activity but a 10-fold smaller K_d

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(low nanomolar) than TMEGF45 [8]. Cofactor activity assays and binding kinetic studies have shown that TMEGF45 has full cofactor activity (as measured by k_{cat} for protein C activation by the thrombin-TMEGF45 complex). The fourth and fifth EGF-like domains work together; addition of the fourth and fifth domains separately does not result in anticoagulant cofactor activity [8]. It is really quite amazing that such a small fragment of TM can alter the activity of thrombin. It is thought that TM undergoes a conformational change upon binding to thrombin, but exactly how it modulates thrombin activity is not well understood [11]. The crystal structure of thrombin bound to TMEGF456 also shows a large interface of extensive contacts between thrombin and the TM fragment. It also shows a single methionine, Met 388, buried between the fourth and fifth EGF-like domains, suggesting its critical role in bringing these two essential parts of TM together (Fig. 2) [12].

Oxidation of Met 388 in thrombomodulin (TM) with chloramine T or H_2O_2 results in a 75–90% loss of TM-mediated anticoagulant cofactor activity [6]. Met 388 is located in the short three-residue linker between the fourth and fifth EGF-like domains. It is conserved in mouse, bovine and human TM, and if Met 388 is mutated to any other residue other than Leu, the anticoagulant cofactor activity of TM decreases [13]. In protein C activation kinetic assays, TMEGF45ox had a 3.5-fold lower k_{cat}, and a 3.3-fold higher K_{mTM} compared to wild-type TMEGF45 [14]. These results agree with previous reports that the K_d of the TM–thrombin interaction increased from 4.4 to 10.9 nM upon oxidation of full-length TM [13]. Although Met or Leu is required at position 388 for efficient activation of protein C, the Met 388 to Ala mutant of TM is still capable of efficient activation of the thrombin-activatable fibrinolysis inhibitor (TAFI) [15]. The activation of TAFI requires residues in the third as well as fourth EGF-like domains of

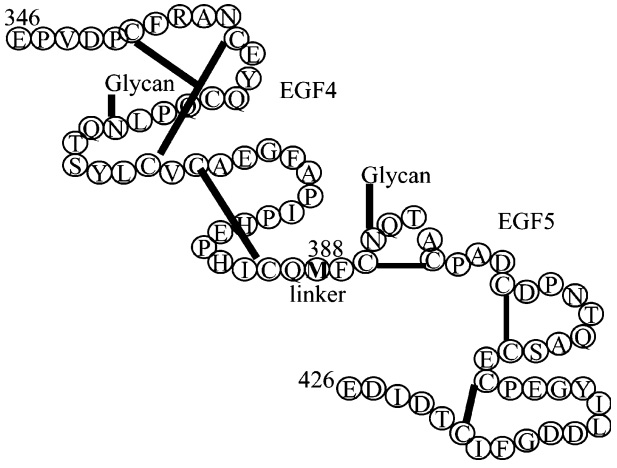

Fig. 1. Schematic diagram of the smallest active fragment of thrombomodulin. The amino acids are indicated in single letter code and Met 388 is in bold. The disulfide bonding pattern determined previously [9] is indicated in solid lines between the cysteine residues. The two N-linked glycosylation sites are indicated with flags.

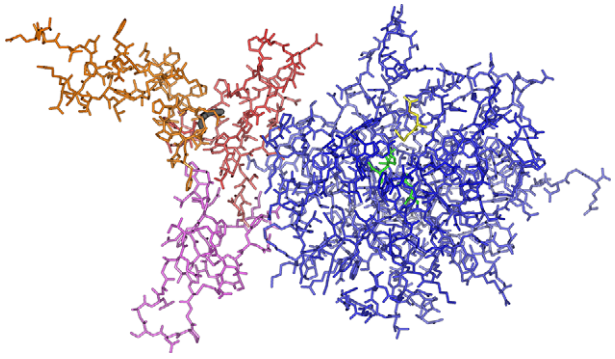

Fig. 2. Structure of the thrombin–TMEGF456 complex determined by X-ray crystallography [12]. Thrombin is colored blue with the active site residues in green and the inhibitor bound at the active site in yellow. The TM EGF-like domains are colored orange (fourth domain), red (fifth domain) and pink (sixth domain). Met 388 is colored black.

TM, and thus a different TM surface may be utilized for both thrombin-binding vs. TAFI activation. It is not yet known whether oxidation of Met 388 will have any affect on TAFI activation.

2. Structural consequences of methionine oxidation in TM

Structural studies of TMEGF45 [11] and of the TMEGF456–thrombin complex [12] suggest that Met 388 may play a critical function in the interaction between the fourth and fifth EGF-like domains of TM. In both structures, Met 388 is the central player in side-chain interactions that connect the fourth and fifth domains. As seen in Fig. 2, Met 388 is buried between the fourth and fifth EGF-like domains, and these are the two domains essential for TM-mediated anticoagulant cofactor activity against thrombin. Alanine mutagenesis revealed that Phe 376, located in the fourth domain, is critical for cofactor activity, and this residue is seen to participate in many hydrophobic contacts with Met 388 [11,12,16]. In addition, Met 388 interacts with the Cys 390–Cys 395 and Cys 409–Cys 421 disulfide bonds and is buried within the core of the fifth domain [11,12]. In the structure of the fifth domain alone, Met 388 does not interact with these disulfide bonds and has no apparent structural role [17]. In comparing the structure of the fifth domain alone with that of the fifth domain in TMEGF45, we hypothesized that insertion of Met 388 into the hydrophobic core of the fifth domain may function to expose the thrombin-binding residues within the fifth domain [11]. These residues pack against the disulfide bonds in the structure of the fifth domain alone, but are solvent-exposed in the structure of TMEGF45. It is also possible that the interaction between Phe 376 and Met 388 may connect the fourth and fifth domains in a way that perhaps is important for anticoagulant cofactor activity.

The structure of TMEGF45 in which Met 388 has been oxidized (TMEGF45ox) and the structure of TMEGF45 in

which Met 388 has been mutated to Leu (TMEGF45ML) have been solved by NMR [14]. A comparison of both structures with the wild-type protein reveals the flexible interdomain connection mediated by Met 388.

Fig. 3 shows the final structural ensembles of the NMR solution structures of each TMEGF45 variant that were solved overlaid on the fourth domains [14]. The structure of the fourth domain in TMEGF45ox is very similar to structures of the fourth domain solved previously [11,18]. Similarly, the fourth domain in TMEGF45ML was not significantly different from the fourth domain in the wild-type protein. For both TMEGF45ox and TMEGF45ML, the fourth domain forms a β-sheet between residues Tyr 358 and Cys 372 and subtle differences in the juxtaposition of the sheet with the other loops in the domain are observed. In particular, the structure of TMEGF45M388L notably diverges at the C-loop of the fourth domain containing Phe 376, a region very well determined for all structures. Long-range NOEs were observed between Phe 376 and Tyr 358, which were not observed in the spectra for TMEGF45 or TMEGF45ox, and more strong NOEs were observed from Leu 388 to Phe 376. These additional restraints structure the C-loop differently than in TMEGF45 and TMEGF45ox, with a bend in the backbone toward the fifth domain.

Changes at position 388 notably affected the juxtaposition of the three separately disulfide bonded loops in the fifth domain. Few long-range NOE restraints were observed in any of the fifth domains, and they differed widely between the different forms of the protein [14]. The C-loop in TMEGF45ML does not pack back against the rest of the domain as it does in the wild type and TMEGF45ox structures, possibly because the side chain of Leu 388 is not long enough to interact with this region of the fifth domain as it did in the wild type.

Oxidation of Met 388 apparently decreases the hydrophobicity of the side chain enough that it no longer interacts with the core of the fifth domain. As a result, the fifth

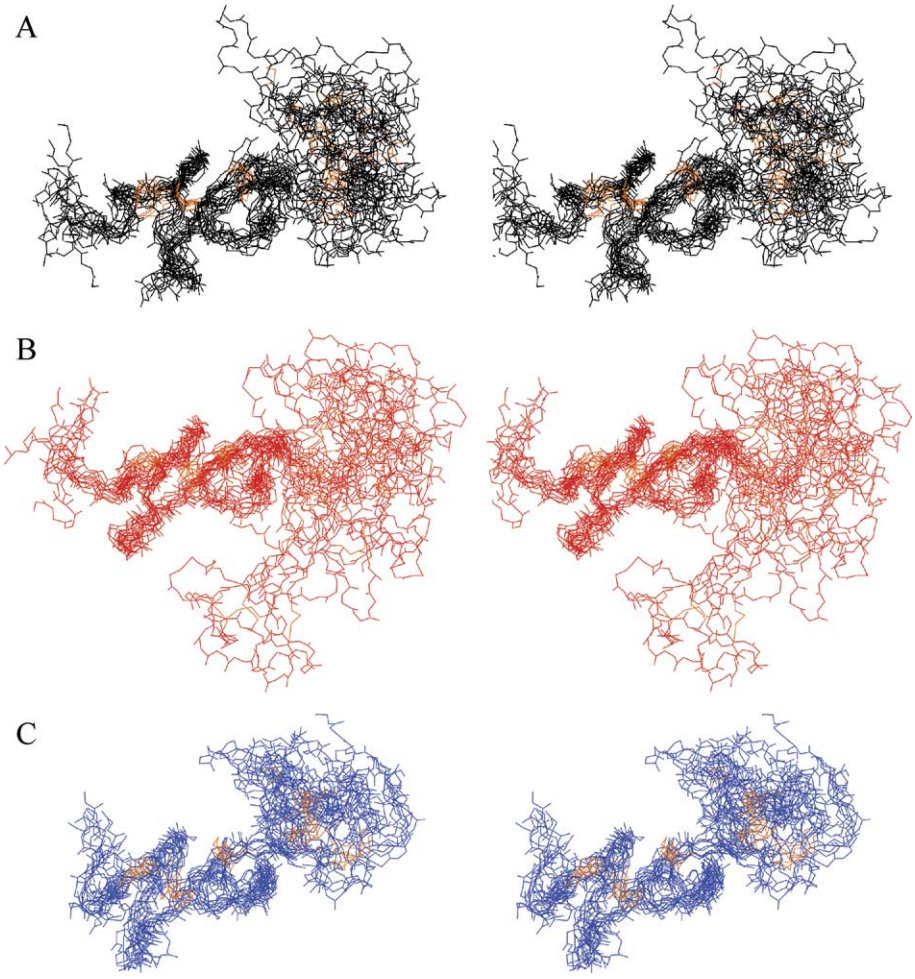

Fig. 3. Stereoviews of the overlay of the backbone atoms of TMEGF45 (A), TMEGF45ox (B) and TMEGF45ML (C). The molecules are superimposed on the fourth domain residues. The backbone atoms are all displayed in black and the disulfide bonds are displayed in orange. The structures were all superimposed and then displayed separately to ensure a consistent orientation. The N-terminus is on the left and the C-terminus is on the right in each structure. The fifth domain of TMEGF45 is defined to a specific quadrant of space by the long-range NOEs from Met 388 to the fifth domain. The fifth domain of TMEGF45ML is also defined to a narrow quadrant, but it is rotationally distinct from the wild-type structure. The TMEGF45ox structure lacks interdomain NOEs and therefore the orientation of the fifth domain is not defined with respect to the fourth domain.

domain is now structured differently than it was in TMEGF45. For example, a new interaction is observed between Phe 419 and Ala 405 located in the B-loop of the fifth domain, which causes a different orientation of the B-loop and fixes the B and C-loops with respect to each other. Another difference is that the majority of the interactions between the A-loop and the C-loop is not observed in TMEGF45ox. The lack of contacts between Met 388 and the Cys 390–Cys 395 and Cys 409–Cys 421 disulfide bonds in the fifth domain of TMEGF45ox apparently causes these loops to be further apart. New contacts between Leu 415 and Cys 421 draw the thrombin-binding residues Tyr 413–Asp 417 back into the domain so that they are not as free to interact with thrombin. The fifth domain of TMEGF45ox differs from wild type and TMEGF45ML in that the thrombin-binding residues Tyr 413, Ile 414, and Leu 415 have NOEs back to the hydrophobic core of the fifth domain. This was also seen in the structure of the fifth domain alone [17]. The packing of the thrombin binding residues back into the domain may explain the weaker thrombin-binding affinity observed for the fifth domain alone (20-fold) and for TMEGF45ox (fourfold).

The linker region of TMEGF45ML had similar numbers of long-range restraints to both the fourth and fifth domains, as were observed for TMEGF45. However, the identity of the interacting residues differed; presumably the branched side chain of Leu 388 cannot reach as far into the core of the fifth domain. The different geometry of leucine as compared to methionine and the different side chain interactions that it made resulted in a difference in the twist angle between the two domains in TMEGF45ML.

The interdomain linkage in TM is hydrophobic and the residue that mediates the domain–domain interaction is Met 388. The hydrophobicity of Met 388 is apparently critical for the interdomain interactions because in TMEGF45ox, no long-range NOEs are observed between Met 388 and residues in the fifth domain, and this effectively disrupts the fourth and fifth domain–domain interaction. Once oxidized, Met 388 no longer showed long-range NOEs to residues in the fifth domain. The two domains do not interact with each other and the linker residues are now exclusively associated with the fourth domain. This causes the domain–domain orientation in TMEGF45ox to be undefined (Fig. 3), in contrast to the structure of TMEGF45 [11].

3. Dynamic consequences of methionine oxidation in TM

The structure of TMEGF45ML and the structure of the wild-type protein both showed that the position of the fourth domain relative to the fifth domain was relatively fixed, with many long-range restraints spanning from the fifth domain to the fourth mediated through the residue at position 388. In contrast, the fifth domain in TMEGF45ox is not fixed relative to the fourth (Fig. 3). This is consistent with the lack of NOE constraints between the two domains due to the "pulling-out" of the oxidized methionine from the core of the fifth domain.

Consistent with the lack of NOE constraints between the linker and the fifth domain, heteronuclear NOE values (Fig. 4) for residues in the fifth domain of TMEGF45ox were slightly lower than in the wild-type molecule [14]. In particular, the hNOEs for the A-loop and part of the C-loop of the fifth domain were lower for the TMEGF45ox as compared to the wild-type protein.

Analysis of the overall tumbling of the fourth domain relative to the fifth domain showed that in wild type and TMEGF45ML, the two domains tumble as a single unit whereas in TMEGF45ox, the two domains tumble with different time constants. The R_2'/R_1' ratios, experimentally determined from ^{15}N relaxation rates of individual amides for TMEGF45 and TM45ox, are shown in Fig. 5. Fushman et al. [19] have shown that the R_2'/R_1' ratios for a protein comprised of two domains reflect the interdomain reorientation. From the R_2'/R_1' ratio, an apparent correlation time, τ_{app}, can be calculated from relaxation data for proteins that do not tumble isotropically [19]. If the proteins were tumbling isotropically the τ_{app} would coincide with the overall correlation time τ_c. The τ_c for TMEGF45 was measured with time-resolved fluorescence anisotropy decay to be 7 ns [20]. In TMEGF45, the averages for τ_{app} for the

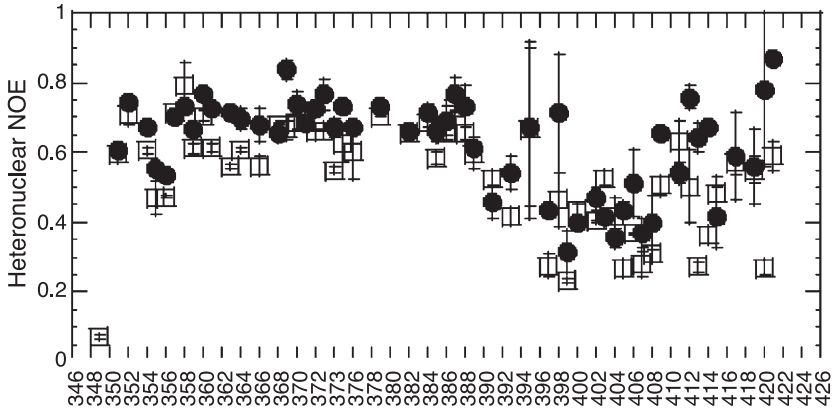

Fig. 4. Heteronuclear NOEs measured at 600 MHz for TMEGF45 (closed circles) and TMEGF45ox (open squares).

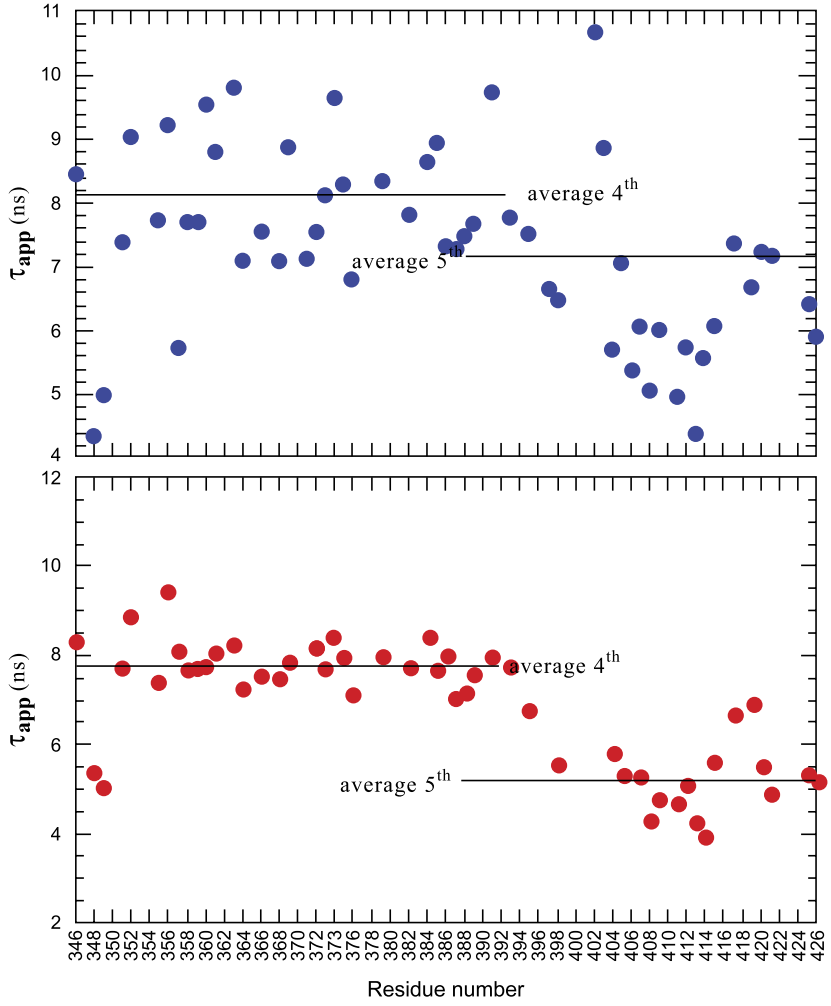

Fig. 5. The apparent tumbling time of each residue in (A) TMEGF45 and (B) TMEGF45ox calculated from the R_1, R_2 and heteronuclear NOE data for each protein. The averages across the fourth domain (residues 326–387) and across the fifth domain (residues 389–426) were calculated and are also shown on each plot.

fourth and fifth domains are 7.90 and 6.50 ns, which are very close to the τ_c of 7 ns calculated using fluorescence measurements (Fig. 5). This again suggests that the entire TMEGF45 molecules tumble in solution as one unit. The case is dramatically different for TMEGF45ox, which has average τ_{app} values of 7.67 and 5.07 ns for the fourth and fifth domains, respectively. These average values are less than those for TMEGF45 and the average difference between the two domains is much greater [21]. This suggests that the rotational diffusion properties of each domain are significantly different in TMEGF45ox. Also, the lower average τ_{app} values for TMEGF45ox compared to TMEGF45 indicate some degree of interdomain flexibility between the fourth and fifth domains in TMEGF45ox. Similar patterns were seen in the Abl SH(32), where τ_{app} decreased when the two domains were not ligated, suggesting that the domains became more flexible with respect to each other [19]. The changes in relaxation rates between TMEGF45 and TMEGF45ox complement the observation that the fourth and fifth domains are specifically oriented with respect to one another in the wild-type protein, but not when Met 388 is oxidized.

4. Functional consequences of methionine oxidation in TM

One of the functional consequences of Met 388 oxidation is that the TM–thrombin protein–protein interaction is weakened. In the structure of the TMEGF456–thrombin complex, only the fifth and sixth domains contact thrombin, and Met 388 inserts into the fifth domain. It might therefore be concluded that the insertion of Met 388 is essential for maintaining the thrombin-binding capability of the fifth domain [12]. We previously postulated that the expulsion of the thrombin binding residues Tyr 413, Ile 414, and Leu 415 from the hydrophobic core by the insertion of Met 388 may lead to an improved binding affinity for thrombin as compared to the fifth domain alone. TMEGF45ox, which has the key thrombin-binding residues packed into the

hydrophobic core of the fifth domain, has a weakened K_{mTM} relative to that of TMEGF45. On the other hand, TMEGF45ML, which has an adequately large and hydrophobic residue located in the linker region, and the key thrombin-binding residues exposed for binding, has a K_{mTM} comparable to that of wild type.

The domain–domain orientation in TMEGF45ox is disrupted, leading to a wider distribution in the structural ensemble and disruption of the dynamic connection between the two domains. This breaking of the TMEGF45ox interdomain linkage most likely accounts for the fivefold decrease in the k_{cat} of protein C activation by the TMEGF45ox–thrombin complex. This hypothesis is well founded because TMEGF45ML, which has a slightly better than wild-type k_{cat}, also shows a tight interdomain linkage. Many residues in the fourth domain of TM are essential for protein C activation, but not required for thrombin binding, and the role that interdomain communication plays in TM cofactor activity is still an open question [16].

Oxidation of Met 388 in TM is known to reduce its anticoagulant cofactor activity by some 90% [6]. So far, it has not been possible to isolate enough TM from patients to quantitatively analyze the amount of oxidized protein in vivo in order to determine whether methionine oxidation is relevant in vivo. Despite this, evidence is building that oxidation of TM may have pathophysiological consequences. A very recent study of coagulation markers in patients with type 2 diabetes mellitus has been performed. This disease is associated with higher levels of lipid oxidation, and levels of thrombin–TM activity as well as activated protein C were markedly decreased ($P<0.05$) in these patients [22].

Why then has evolution not substituted leucine for methionine at position 388 in TM? In mouse, bovine and human TM, Met 388 is conserved, but if it is mutated to leucine, the protein is oxidatively resistant and the cofactor activity increases [13]. One reason that a methionine is conserved in this position and not the more kinetically optimal leucine may be that the methionine could play a physiological role in rapidly down-regulating the anticoagulant potential of TM. At the site of injury, the exposed endothelium attracts and activates neutrophils, releasing oxidants in a localized environment and creating a more pro-clotting environment, which is needed to stop the bleeding. Indeed, Met 388 can be efficiently oxidized by activated neutrophils, and neutrophils are known to synthesize inactive TM [23]. The functional consequences of the need to maintain oxidative sensitivity of Met 388 may lie, therefore, in the need to maintain a pro-coagulant environment at the site of a wound. During some inflammatory states, however, a more generalized TM oxidation and inactivation in the microcirculation may result in the pathophysiological condition of disseminated intravascular coagulation (DIC). Administration of soluble TM ameliorates DIC in rats and clinical trials in which activated protein C was administered to persons suffering from DIC have shown great promise [24].

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