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Med Hypotheses. Author manuscript; available in PMC 2007 April 10.

Published in final edited form as:
Med Hypotheses. 2007 ; 68(4): 811–821. doi:10.1016/j.mehy.2006.09.009.

Does the oxidation of methionine in thrombomodulin contribute to the hypercoaguable state of smokers and diabetics?

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Abstract

The leading cause of premature death in smokers is cardiovascular disease. Diabetics also suffer from increased cardiovascular disease. This results, in part, from the hypercoagulable state associated with these conditions. However, the molecular cause(s) of the elevated risk of cardiovascular disease and the prothrombotic state of smokers and diabetics remain unknown. It is well known that oxidative stress is increased in both conditions. In smokers, it is established that oxidation of methionine residues takes place in α_1-antitrypsin in lungs and that this leads to emphysema. Thrombomodulin is a key regulator of blood clotting and is found on the endothelium. Oxidation of methionine 388 in thrombomodulin is known to slow the rate at which the thrombomodulin-thrombin complex activates protein C, a protein which, in turn, degrades the factors which activate thrombin and lead to clot formation. In analogy to the cause of emphysema, it is hypothesized that oxidation of this methionine is elevated in smokers relative to non-smokers and, perhaps, in conditions such as diabetes that impose oxidative stress on the body. Evidence for the hypothesis that such an oxidation and concomitant reduction in activated protein C levels would lead to elevated cardiovascular risk is presented.

Introduction

Cardiovascular disease is the most common cause of premature death in smokers [1]. Smoking related cardiovascular diseases are the cause of 140,000 premature deaths annually in the United States [2]. The most common cardiovascular diseases in smokers are the thrombotic arterial occlusive diseases, in particular myocardial infarction and stroke. While narrowing of arteries from atherosclerosis is an important component of these diseases, equally important is the fact that the blood of smokers is much more prone to clot than that of non-smokers. While these facts are well established, the molecular origin of this prothrombotic state has remained unclear, despite intensive research. We present here a hypothesis for the molecular root of this hypercoagulability. Further, we believe that this hypothesis is equally plausible for explaining the molecular origin of a similar prothrombotic state [3–5] and increased cardiovascular risk in diabetics and may also explain, in part, why elevated levels of homocysteine are a risk factor for heart disease.

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Thrombomodulin is a key regulatory protein in hemostasis

Central to our hypothesis is thrombomodulin. Detailed reviews of hemostasis and blood coagulation in general [6–11] and thrombomodulin in particular [12–15] have been recently published and will not be repeated at length here. Briefly however, thrombomodulin is critically important in regulation of clotting. Thrombomodulin was isolated initially by Esmon's group in 1981 [16]. Thrombomodulin serves, as the name implies, to regulate the activity of thrombin. In complex with thrombin it activates protein C, which degrades key factors in the clotting cascade [17]. Deficiency in protein C or activated protein C is well established as increasing the risk of thrombosis [17,18]. Low levels of thrombomodulin are a well established risk factor for heart disease [19]. Without a doubt, thrombomodulin plays a key role in slowing or stopping clotting.

While superficially it may seem contradictory, more recent discoveries have shown that thrombomodulin [20–22], again in complex with thrombin, activates thrombin activatable fibrinolysis inhibitor (TAFI) [23], which stabilizes clots [24], Reflection on this point emphasizes the critical role thrombomodulin plays in clotting regulation, since it controls both the rate at which clots form and the rate at which clots breakdown. As we shall see, a critical question is the molecular mechanism by which thrombomodulin strikes a balance between promoting clot formation or degradation.

Thrombomodulin is found anchored on the luminal surface of the endothelium. In rats, it is found predominantly in the lungs, at much higher levels than even other highly vascularized organs such as liver or kidney [25,26]. Thrombomodulin does undergo endocytosis with subsequent degradation, but significant amounts of thrombomodulin are cleaved from the surface to circulate in the blood before being cleared through the urine [27]. High levels of thrombomodulin in plasma have been reported in a variety of conditions including diabetes, lupus, pre-eclampsia, and disseminated intravascular coagulation [12] and are believed to serve as a good marker of endothelial damage [28,29]. Several small studies have found that levels of plasma thrombomodulin are not correlated with the incidence of cardiovascular disease [30–32] but work on a larger study population has found that high levels, taken to be indicative of higher levels of thrombomodulin expression, appear protective [19].

Oxidation of methionine 388 is critical in the regulation of thrombomodulin activity

In 1992 a paper was published showing that the oxidation of a single methionine, residue 388, destroyed most of the activity of the thrombomodulin-thrombin complex in proteolytically activating protein C [33]. Oxidation of other methionines in the protein did not appear to alter activity [34]. Substitution of the methionine with a leucine result in a mutant capable of activating protein C, without sensitivity to oxidation.

Numerous studies [35–38] have shown that binding and activation of thrombin only requires the 81 amino acid fragment of thrombomodulin corresponding to EGF domains 4 and 5, although domain 6 increases the K_m of thrombomodulin for thrombin by a factor of 10 without altering the k_{cat} of the thrombomodulin-thrombin complex for protein C [39]. A thrombomodulin fragment consisting of domains 5 and 6 does bind to thrombin, but the complex fails to activate protein C [40]. Met 388 is one of three residues linking domains 4 and 5 [34,35].

This study was undertaken because an increased tendency toward coagulation is a common complication of inflammation, and thrombosis is an important contributor to death in inflammatory processes such as sepsis. The examination of methionine oxidation was a natural

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extension of various observations of regulation of activity by methionine oxidation wherein methionine is oxidized to the sulfoxide form by the reactive oxygen species generated by leukocytes and neutrophils during inflammation [41–45]. To cite just one example, it has been shown that the C5 component of the complement system, normally activated by proteolysis, can be activated by the oxidation of a specific methionine residue [46–48]. Active C5 triggers the complement cascade. These workers argue that leukocytes, which generate various oxygen radicals when active, may thus also activate the complement system to aid in the immune response.

In 2000, it was further shown that oxidation of Met388 in thrombomodulin has no effect on the clot stabilizing activation of TAFI by the thrombomodulin-thrombin complex [49]. Thus, oxidation of this methionine removes the downregulation of clotting by thrombomodulin with no effect on the coagulation enhancing regulatory capacity. While both of these effects have only been demonstrated *in vitro*, it seems likely that the combination of effects acts *in vivo* to increase the propensity to form clots whenever significant amounts of methionine oxidation have occurred. It is plausible that this methionine forms a crucial molecular switch which controls the balance between clot formation and breakdown. In the course of this work, this group reconfirmed the original observation that Met388 oxidation that oxidation of Met388 dramatically decreases the activation of protein C by the thrombomodulin-thrombin complex.

Even more recently the structural basis of the inactivation of thrombomodulin has been determined. Wood *et al.* [34,50] solved the structure of thrombomodulin fragments by both NMR and x-ray crystallography. Structures of the unoxidized and oxidized forms showed clear structural differences in the fifth domain of thrombomodulin. Phenylalanine 376 packs against the hydrophobic methionine but occupies a substantially different position when the hydrophilic sulfoxide form is present, making it a key part of the conformational switch. These structural changes bury several residues which interact with thrombin in the structure of the thrombomodulin-thrombin complex [35].

Although the primary focus of this work by the Komives group at UCSD was structural, they also performed binding and activity assays [34]. Oxidation of Met388 increased the K_m for thrombin from 140 ± 5 nM to 460 ± 70 nM. Oxidation left K_m for the binding of the thrombomodulin-thrombin complex with protein C unchanged within experimental error. However, k_{cat} for the activation of protein C by the thrombomodulin-thrombin complex dropped from 5.0 ± 0.1 s^{-1} to 1.4 ± 0.1 s^{-1}. Specific activity fell by almost an order of magnitude, the oxidized form showing just 15% of the activity of the unoxidized form.

We note again that this effect is not likely a fluke unique to humans since this methionine is conserved in all thrombomodulin genes that have been sequenced, as shown in Figure 1. Phenylalanine 376, the other half of the conformational switch, is also conserved.

This work has contributed to the acceptance of thrombomodulin as a key link between inflammation and coagulation [22,51–53]. We argue here that a strong case can be made that oxidation of methionine 388 in thrombomodulin is important in a variety of other human diseases.

Is a decrease in thrombomodulin activity biologically relevant?

The question of biological relevance has been addressed by mutation of thrombomodulin in mice [54]. In the same numbering system as elsewhere in this paper, the glutamate at position 387, normally a glutamine side chain in humans, was substituted with a proline. This is, of course, immediately adjacent to the methionine of concern in the hypothesis put forward here. As might be expected, the ability of this mutant protein to activate protein C suffered, an

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estimated factor of about a thousand fold reduced efficiency in protein C activation with physiological concentrations of the various proteins.

Despite this virtual elimination of the activation of protein C, the mutant animals are viable, but are impaired in ways reminiscent of cardiovascular disease. The initial paper focused more on the creation of the strain and the reproductive effects, but the most notable observation from the perspective of cardiovascular disease is that mutant mice suffer from increased fibrin deposition in the heart and lungs [54], by as much as ten fold over wild-type in 3 to 6 month old mice [55]. Enhanced fibrin deposition has been linked to myocardial infarction [56]. That these mice are hypercoaguable was shown in subsequent work by an accelerated rate of platelet thrombus growth after $FeCl_3$ injury to the carotid artery [57]. The time at which flow was reduced to 50% of the initial flow (t_{50}) was reduced by approximately 22% [57]. Mice with the same mutation in a slightly different genetic background, when subjected to the same $FeCl_3$ insult, showed complete thrombotic occlusion in 80% of the mutant animals, versus only 27% of the wild-type mice [58]. Similarly, surgical occlusion of the carotid artery resulted in extensive stasis-induced thrombosis, extending the entire length of the artery in many of the mutant mice. In wild-type mice occlusion was restricted to within less than 1 mm of the ligation [57]. These mice with reduced capability of thrombomodulin to activate protein C also exhibited increased sensitivity to lipopolysaccharide-induced septicemia. Injection of the wild-type LD_{50} dose of LPS resulted in 100% mortality in mutant mice [57]. Further, the mutant mice succumbed much earlier than the wild-type mice.

Methionine oxidation in proteins

This is an appropriate point at which to quickly review the literature on methionine oxidation more generally. Proteins are well known to be sensitive to oxidative damage, often with important biological effects. Protein oxidation has been suggested as a causative or contributory factor in many diseases [59]. Oxidized proteins have been found to increase in aged organisms, leading to the proposal that protein oxidation contributes to the aging process [60,61].

Methionine, cysteine, tryptophan, tyrosine, and histidine residues are susceptible to oxidation. Cysteine and methionine are the most easily oxidized. The oxidation of the cysteine thiol to the disulfide form is a normal, beneficial, and familiar reaction. Oxidation of methionine may be less familiar, but still readily occurs. The oxidation to a sulfone can be accomplished by fairly strong oxidants, but it is the oxidation of methionine to the sulfoxide form (Figure 2) that concerns us here, as it has been shown to occur in a wide variety of proteins with both mild and strong oxidizing species, such as H_2O_2, hypochlorous acid, and superoxide [62–66].

Oxidants found naturally in biological systems [33,44,65–67], cigarette smoke [68–72], and ozone [73,74] or other environmental oxidants [75,76] have all been demonstrated to cause methionine sulfoxide formation in proteins and peptides. Methionine oxidation often reduces or eliminates biological activity [77,78]. Methionine oxidation is therefore of serious concern when proteins are used as pharmaceuticals because oxidation, which may occur readily during processing or storage, often alters activity [79–82]. The alteration in activity is undoubtedly due to the considerable alteration in the character of the methionine. The side chain alters in size and geometry, but more importantly the sulfoxide is very polar and hydrophilic, with significant partial positive charge on the sulfur and negative charge on the oxygen. The oxygen is an excellent hydrogen bond acceptor. In contrast the reduced form is very non-polar and hydrophobic. This can readily lead to changes in the stability of different protein conformations or in the ability of a binding site to recognize another protein or substrate.

Reactive oxygen species have lately become more widely recognized as biologically important messengers [44,83–87] and methionine is one likely target for oxidation by such species [44,

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45,88,89]. Indeed, recent and somewhat surprising work has shown that the rate of reaction of methionine with hypochlorous acid is faster than the reaction with cysteine [90]. Methionine as a target of redox signaling is particularly interesting since sulfoxide formation is reversible. Indeed, all organisms have a variety of enzymes whose specific role to reduce methionine sulfoxide in proteins and peptides back to the thioether [91].

Thus, there are many well documented, biologically relevant examples of methionine oxidation in other proteins. Evidence that methionine oxidation is an important reversible regulator of biological activity is accumulating at a rapid pace. It is plausible that oxidation of methionine 388 in thrombomodulin plays an important role in the regulation of hemostasis. Whether or not oxidation of Met388 is an inappropriate activation of a normal signaling system or just coincidently deleterious is not strictly relevant to the hypothesis advanced here. It is clear however that Met388 oxidation causes a profound biological effect and that such effect is a reasonable result of methionine oxidation is well supported by analogy in other proteins.

Link to smoking

Tobacco smoke is a complex mixture, but includes many oxidizing species that impose significant oxidative stress on the body [92–100]. These oxidizing species include organic radicals and hydrogen peroxide, which can oxidize methionine. Further, smokers are known to have increased levels of immune system cells such as activated neutrophils in their lungs [101,102], cells which in turn release still more oxidizing agents. In addition, it has been shown that smokers have elevated levels of iron in their lungs [103]. Iron catalyzes the Fenton reaction of ascorbate, simultaneously consuming this key antioxidant and producing oxidizing radicals [104].

Most importantly, there is a well established linkage between the disease of emphysema in smokers and methionine oxidation of another protein, α_1-antitrypsin [43,68,105–108], Oxidation of either methionine 351 or 358 in the binding site of α_1-antitrypsin destroys the protein's ability to bind to and inhibit elastase [109], The degradation of elastin by elastase is an important step in enabling immune cells to infiltrate the site of an infection. The generation of reactive oxygen species by leukocytes and neutrophils during inflammation thus facilitates the immune response to infection. However, it is now indisputable that components of cigarette smoke can carry out this oxidation [71,72,110–112], leading to the inappropriate and chronic activation of elastase and, hence, causing emphysema.

Another common cause of emphysema is chronic exposure to mineral dust, such as in coal and hard rock miners. It has more recently been shown that mineral dusts can cause oxidation of methionine in α_1-antitrypsin *in vitro* [113]. Even more convincingly, the ability of different dusts to cause breakdown of elastin *in vivo* in rats was correlated with the ability of the dust to oxidize methionine in the inhibitor *in vitro*. Thus, two apparently different causes of emphysema seem to have the same molecular origin: methionine oxidation.

Curiously, despite a much greater toll in human lives, the molecular cause of the hyperthrombotic state in smokers has remained unclear. There is growing evidence that free radical, oxidative damage to the endothelium is very important in the development of cardiovascular disease in smokers, although most attention seems focused on oxidative impairment of nitric oxide signaling [114,115]. It is our belief that the precedent established for the cause of emphysema and the known effect of methionine oxidation upon thrombomodulin activity make it extremely likely that a similar oxidation is taking place in smokers, making it an important molecular root of their cardiovascular ills. In addition to their pronounced tendency to clot, there is growing evidence that a prothrombotic state contributes to atherogenesis [116–120], thus thrombomodulin oxidation may increase the risk of smokers for atherosclerosis as well.

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We remind the reader that the predominant location for thrombomodulin is the lung [25,26, 121], a location which obviously renders it even more vulnerable to oxidation by the witch's brew of reactive oxidizing species in cigarette smoke and the high levels of activated immune cells caused by smoking. The linkage of low levels of thrombomodulin with increased risk of heart disease further strengthens our hypothesis [19]. We note again that while the levels of thrombomodulin in smokers and non-smokers have been examined, no group has ever examined thrombomodulin methionine oxidation *in vivo* in smokers or non-smokers.

The Fernández group published a paper strengthening the case for a linkage between smoking and thrombomodulin oxidation as an important molecular cause of their cardiovascular disease. Apparently reaching the same hypothesis that we present here, they tested the levels of activated protein C in non-smokers and smokers [122]. (Low levels of activated protein C have been found by others to be a strong, independent risk factor for venous thromboembolism [123, 124] and may be a risk factor for ischemic stroke [125].) Circulating levels of activated protein C were a very statistically significant 23.3% lower in smokers than nonsmokers. While other causes, such as reduced expression of protein C and increased degradation of protein C or of activated protein C can not be ruled out, one possible cause of low protein C levels is that smokers have reduced thrombomodulin activity, which could be due to methionine oxidation.

The circumstantial case for our hypothesis is strong. This hypothesis postulates a plausible molecular mechanism linking the oxidative stress imposed by smoking to the disruption of the key endothelial function of hemostasis, leading to thrombosis. Further, it is not a terribly speculative stretch to imagine that this oxidative modification of thrombomodulin may be a very useful biomarker for cigarette smoke exposure and for cardiovascular risk [56].

Oxidative stress is present in other conditions which are prone to thrombosis

Oxidative stress in general is linked to increased tendency to coagulate, but the molecular mechanism is clearly complex and significant factors remain unknown [115,126–129]. We have already mentioned sepsis, which motivated the original study of methionine 388 oxidation. Three other conditions where oxidative stress and hypercoagulability are present bear mention in particular.

Diabetes is well known to cause oxidative stress, thought to be due to oxidation of glucose in the presence of transition metal ions with concomitant product of hydrogen peroxide and because increased metabolic flux through unusual pathways increases mitochondrial superoxide production [130–132]. Diabetics are equally well known to have an elevated risk of thrombosis [3–5,133,134] and cardiovascular disease [135]. The molecular causes are by no means fully understood. However, we found very interesting a recent paper reporting that activated protein C levels in type-2 diabetics are significantly depressed relative to normal controls [136].

Elevated homocysteine levels are a very strongly established marker for increased risk of heart disease and thrombosis [137,138]. The molecular cause of thrombosis in individuals with hyperhomocysteinemia is unclear [139–143]. Oxidative stress is known to be present in individuals with elevated levels of homocysteine [144,145]. There is some controversy over whether homocysteine is the cause of the oxidative stress (from disulfide formation in the presence of metals and oxygen and concomitant peroxide and thio radical formation [146]) or if it is just a marker of oxidative stress caused by an underlying folate deficiency [147]. However, all agree hyperhomocysteinemia is associated with oxidative stress, whether or not homocysteine directly causes it. It is less clear if our hypothesis fits this condition, since one study has reported that activated protein C levels are no different in individuals with and without elevated homocysteine [148] and another study failed to find increased activation of the coagulation system in healthy volunteers under methionine load to induce mild

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hyperhomocysteinemia [149]. Still, the appearance again of an increased risk of thrombosis in a condition associated with oxidative stress is interesting and suggestive.

Similarly, it is also known the patients with hyperthyroidism are subject to elevated levels of oxidative stress [150–152]. Such patients are also known to be at increased risk for thrombosis [153,154]. Indeed, as many as 18% of patients with thryotoxicosis actually die from embolism [155]. We draw attention to a hypothesis that the cause of elevated levels of death from cardiovascular disease in end-stage renal failure, another condition closely linked with diabetes, is due to the elevated levels of oxidative stress known to exist in this condition [129,156,157]. Oxidative stress and an elevated tendency to coagulate [157,158] are clearly present in this patient population. Lastly, oxidative stress and inflammation are clearly associated with one another in many diseases, including the classic inflammatory disease, arthritis [159]. It is intriguing that arthritics were recently shown to have an elevated risk of cardiovascular disease [160], although superficially there is no reason to link inflammation in the joints to cardiovascular problems. Arthritic patients appear to be prothrombotic as well [161,162]. In short, oxidative stress or inflammation in general seems to increase cardiovascular risk and blood coagulability. Oxidation of methionine 388 in thrombomodulin may be a key molecular linkage between these disparate conditions and cardiovascular risk.

Thrombosis is the result of a multitude of factors and oxidative stress can have a multitude of effects

Lastly, we recognize that oxidative stress in general and smoking, diabetes, or hyperhomocysteinemia, in particular, have other significant health effects beyond hypercoagulability. We have discussed briefly above the development of emphysema through methionine oxidation and, to name just one other effect of oxidative stress, the role of lipid oxidation in LDL in atherothrombosis is becoming clearer. Again, although other important effects of oxidative stress and methionine oxidation have been identified, it seems certain that additional effects are waiting for identification.

Similarly, we wish to make clear that we recognize that many contributing factors, both environmental and genetic, are responsible for the medical conditions discussed in this paper. Many of these other causative agents are very well supported by huge amounts of evidence and we have touched lightly, if at all, on those factors. Even though important contributors to thrombotic disorders have been identified, it seems virtually certain our understanding of identified causes is incomplete and that other contributing factors remain to be identified. We have emphasized in the discussion above just one such possible factor disrupting hemostasis, and while the overall causes of cardiovascular disease are undoubtedly more complicated, we feel the case is strong that thrombomodulin Met388 oxidation is important in the health effects of smoking, diabetes, and other conditions that impose oxidative stress.

Acknowledgments

Our gratitude to Samuel Gellman for the conversations that sparked interest in methionine oxidation. We thank Martin Hauer-Jensen, Enrico Di Cera, and Elizabeth Komives for helpful discussion and criticism. This work was supported by the Arkansas Biosciences Institute and NIH grant 1R15HL078994-01A1.

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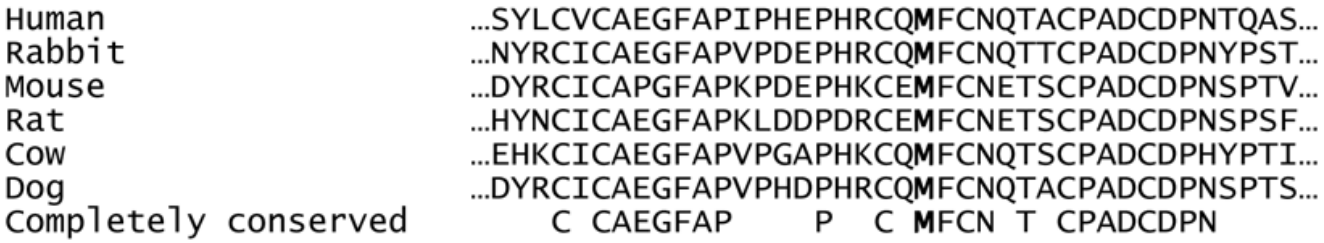

Figure 1.
Alignment of thrombomodulin genes sequenced to date in the region of interest. Squirrel monkey, rhesus monkey, and chimpanzee thrombomodulin genes are virtually identical to human, including at Met388, and are not shown. Methionine 388 is indicated in bold and is conserved. Note the conservation of phenylalanine 376 as described in the text as well as the cysteines, involved in disulfide bonds, and of asparagine 391, which has been shown to be glycosylated in humans.

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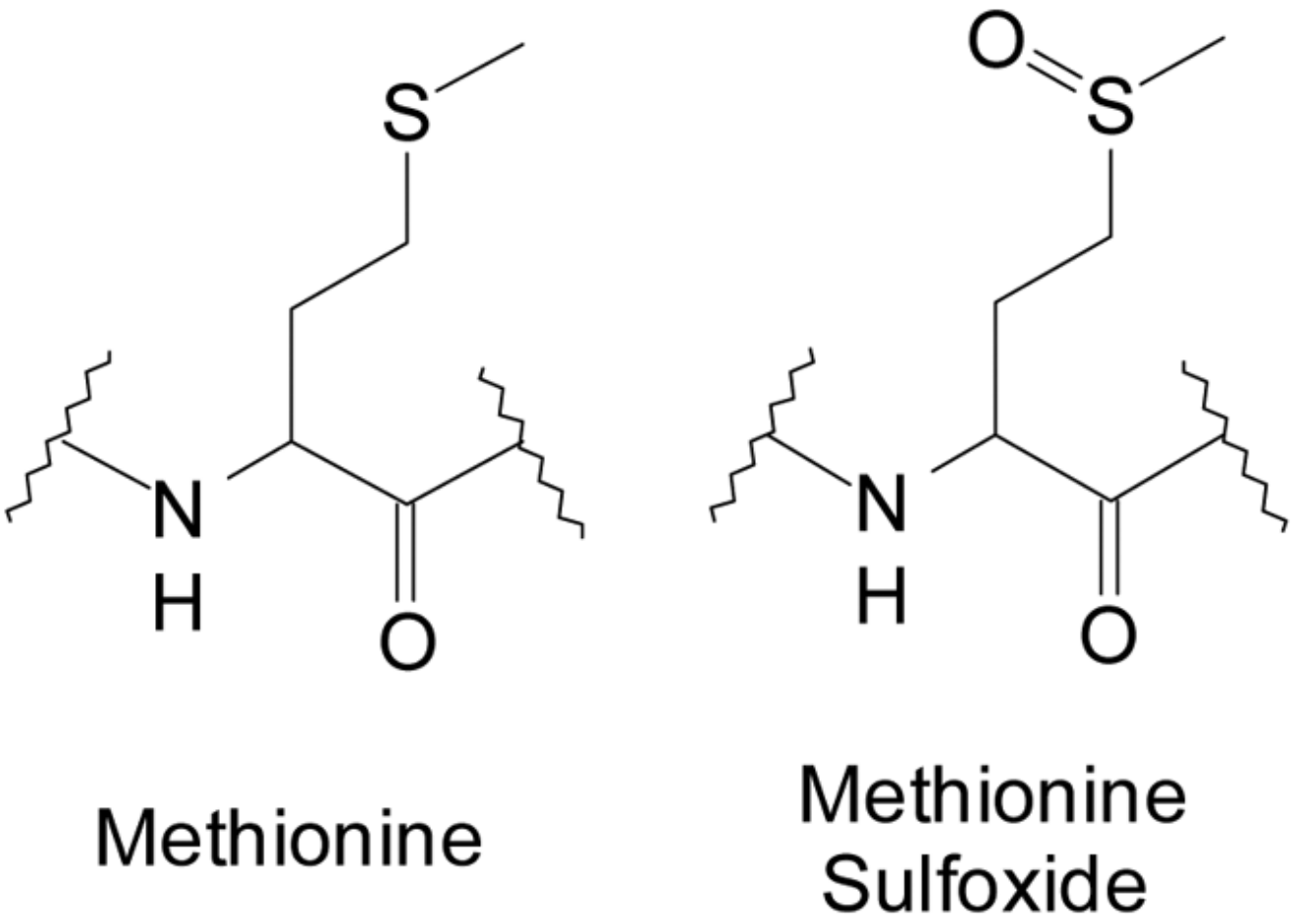

Figure 2.
Structures of methionine and methionine sulfoxide residues.