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Review

Unique metabolic roles of methionine both free and in proteins

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ABSTRACT

Our laboratory has a long interest in methionine (Met) biosynthesis, metabolism and its role in oxidative damage. Although the primary goal of this review was to summarize more recent studies on the role of Met oxidation in proteins on cellular function, we have used this opportunity to describe some of the early studies that elucidated the unique and important functions that Met has in one carbon metabolism, and as an initiator of protein synthesis. Oxidative damage, our main focus, is believed to be a major factor in age related diseases and the aging process. The oxidation of Met residues in proteins to methionine sulfoxide has turned out to be an important biomarker of oxidative damage since there are specific enzyme systems, methionine sulfoxide reductases, that can repair this damage and which play an important role in protecting cells against oxidative damage.

KEYWORDS: methionine metabolism, oxidative damage

INTRODUCTION

Met is a sulfur-containing amino acid that has distinctive properties that set it apart from other amino acids present in proteins. In all cells, Met is known to play a major role in 1) one carbon metabolism and 2) the initiation of protein synthesis. In recent years it has also become apparent that the oxidation of Met in proteins is a major indicator of oxidative stress and the enzymatic systems that repair this protein damage are important in protecting cells against oxidative damage and may play a role in age related diseases and the aging process. In this review, we will briefly summarize some of the reactions involved in Met biosynthesis and metabolism and review how cells protect against oxidation of Met residues in proteins, and the importance of this cellular protective mechanism. Because of the huge volume of studies on Met metabolism and its role in oxidative stress, and our focus on studies carried out in our laboratory, the authors apologize to the many researchers whose studies have contributed to our knowledge, but are not mentioned.

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Met biosynthesis and the role of Met in one carbon metabolism

Only plants and microbes can synthesize Met *de novo*. Animals must obtain some Met, along with the other essential amino acids, from their diets. As a nutrient for animals, Met has some unique properties compared to other amino acids. For example, Met restriction, similar to total caloric restriction, has been shown to promote longevity of flies and rodents [1, 2] and Met alone increases fecundity in the fruitfly, *Drosophila melanogaster*, on a calorie restricted diet [3]. These may reflect the unique role of Met in protein synthesis, cellular oxidation and possibly signaling pathways in some animals.

The Met biosynthetic pathway in enteric bacteria and plants has largely been elucidated. Met gets

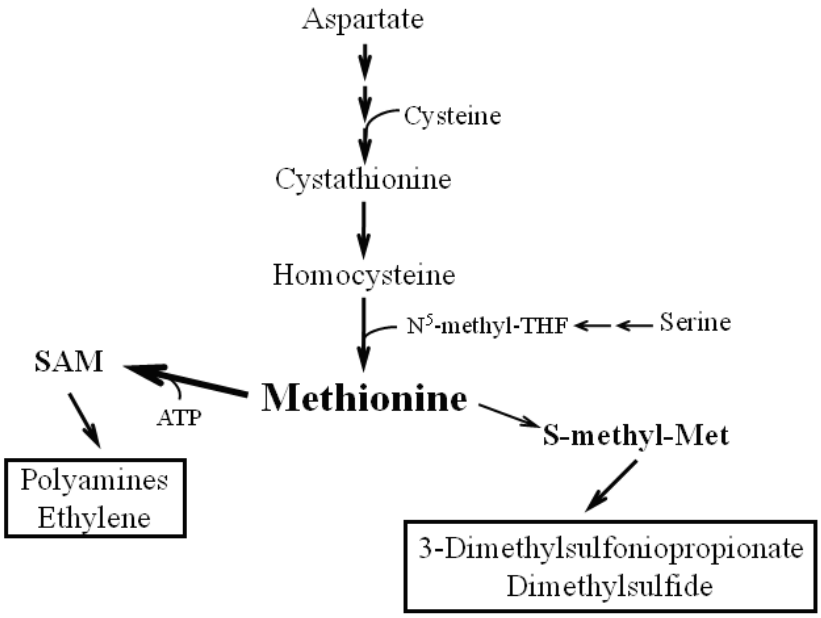

Figure 1. Biosynthesis and metabolism of methionine (Met) in plants. SAM, S-adenosylmethionine. THF, tetrahydrofolate. See text for further details.

its sulfur atom from cysteine, the carbon backbone is derived from aspartate and the methyl group from serine as shown in Figure 1 [4-6]. As also shown in Figure 1, Met and its major metabolite S-adenosylmethionine (SAM) are precursors to other important plant metabolites such as ethylene and polyamines. Furthermore, a Met derivative, S-methylmethionine, is essential for transporting reduced sulfur, an immediate precursor of the osmoprotectant, 3-dimethylsulfoniopropionate and the volatile sulfur derivative, dimethylsulfide [7, 8]. In fact, it is estimated that in the aquatic plant *Lemna paucicostata*, ~80% of Met metabolism is for SAM synthesis, while only 20% is for protein synthesis [9]. In plants, Met synthesis and catabolism are regulated coordinately at both post-transcriptional and post-translational levels [10].

Animal cells do not synthesize homocysteine *de novo* but can carry out the terminal methyl transfer from N^5-methyl-tetrahydrofolate (N^5-methyl-THF) to homocysteine to form Met. This terminal reaction has been studied in great detail in bacterial and animal cells since the 1960s and a coenzyme form of vitamin B_{12}, methyl-B_{12}, is involved in the methyl transfer reaction [11-15]. This reaction in animal tissues has special importance for it explains the well documented metabolic inter-relationship among folic acid, vitamin B_{12} and one carbon metabolism [16]. This is summarized in Figure 2. The Met formed is not only the precursor of SAM, a major methylating agent, but the terminal reaction is essential to regenerate THF for use as a one carbon carrier for purine and pyrimidine synthesis as well as other metabolites. In addition, in eukaryotic mitochondria and chloroplasts, N^{10}-formyl-THF supplies the formyl group for the synthesis of the formyl-Met-tRNA (fMet-tRNAi), the initiator tRNA in eukaryotic organelle protein synthesis. Another interesting aspect of the terminal B_{12} methyl transferase (Figure 2) is that it also requires SAM to activate the isolated enzyme, transferring the first methyl group via a methyl-B_{12} on the enzyme to homocysteine, before the methyl groups are transferred from N^5-methyl-THF [17]. In recent years the crystal structure of the vitamin B_{12} dependent terminal methyl transferase has made it possible to further elucidate the molecular mechanism and what regions of the protein are important in SAM binding and the methyl transfer involving the methyl-B_{12} on the enzyme [18]. As also shown in Figure 2, Met residues in proteins can be oxidized by reactive oxygen species (ROS) and the importance of this post-translational modification will be described below.

In plants and many bacteria the terminal methyl transfer from N^5-methyl-THF to homocysteine to form Met (Figure 1) is catalyzed by an enzyme that does not require vitamin B_{12}. This non-B_{12} transmethylase, first purified from *Escherichia coli* [19, 20], catalyzes a much simpler mechanistic methyl transfer, but is critical in supplying Met for both protein synthesis and one carbon metabolism in plants.

Metabolic role and metabolism of SAM

The major metabolic pathways of Met in both animals and plants are incorporation into proteins and conversion to SAM, which as mentioned, is the major methylating agent in eukaryotic cells. Over 90% of the SAM in cells is used for methylation of nucleic acids, proteins and other macromolecules, vitally important reactions for cellular function. The presence of a salvage pathway for Met synthesis in animal cells explains why it is not considered an absolutely essential amino acid. As also shown in Figure 2, SAM, after methylating a suitable substrate, is converted to S-adenosylhomocysteine (SAH). Animal and plant cells can hydrolyze SAH to adenosine and homocysteine [21]. In animals, the homocysteine can then be converted to Met by the B_{12} dependent transmethylase, which reduces the need for Met in the diet.

Role of Met in the initiation of protein synthesis

Soon after the genetic code was elucidated in the early 1960s, it was noted that bacterial cells contained a Met derivative, identified as N-formylmethionine [22]. Subsequent studies showed that there was a specific initiator tRNA that could be charged with Met and then formylated (fMet-tRNAi) [22-24]. The enzyme that carried out this important formylation of Met-tRNAi was initially purified from *E. coli* [25]. It was soon evident that essentially all bacterial nascent proteins had N-formyl-Met at their N-terminus. In the initiation reaction the fMet-tRNAi, specifically recognized by the bacterial initiation factors, is carried to the ribosomes containing an mRNA having an AUG codon for Met in the first

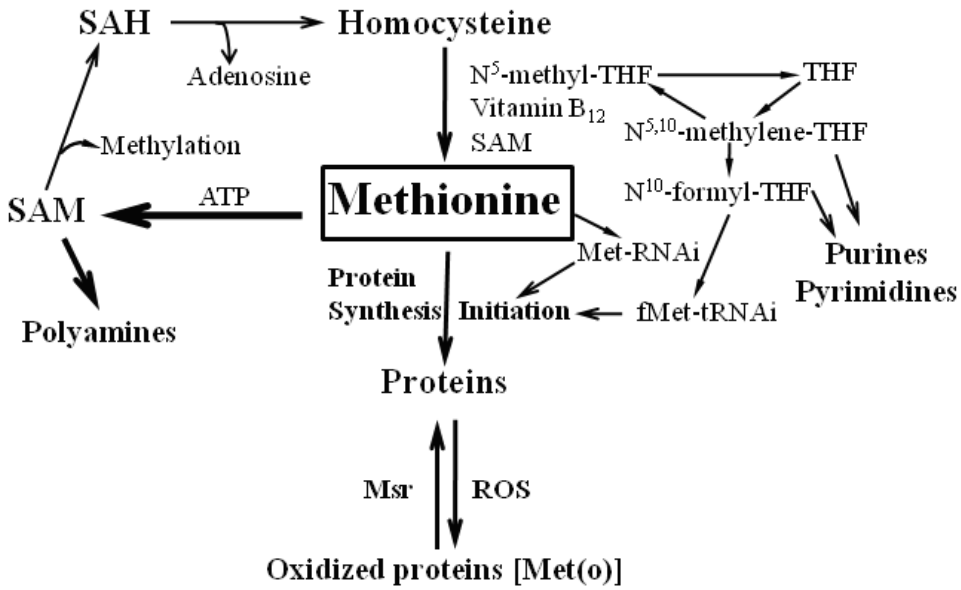

Figure 2. Metabolic relationship involving folate acid, vitamin B_{12} and one carbon metabolism. The terminal reaction in methionine synthesis in animal cells is linked to folate and one carbon metabolism since this methyl transferase reaction regenerates THF which is required for other biosynthetic pathways involving one carbon metabolism. In addition, methionine is the precursor of SAM, a major methyl donor in most cells. A salvage pathway involving the hydrolysis of S-adenosylhomocysteine (SAH) to adenosine and homocysteine helps to regenerate methionine. See text for more details.

position of the open reading frame. However, the mature proteins in the bacterial cells do not contain a formyl group and many do not contain Met at the N-terminus. This is due to a deformylase that first removes virtually all of the formyl groups from the nascent protein chains [26] and a Met aminopeptidase which then removes the N-terminal Met from many of the nascent proteins [27]. The initiation of protein synthesis in eukaryotic cells is slightly different in that these cells lack a Met-tRNAi transformylase in their cytoplasm, although Met is still the initiator amino acid [28]. As in bacteria, there is a specific initiator tRNA that is charged with Met and recognizes an AUG codeword on the mRNA in the first position of the coding sequence. Likewise, there is an enzyme in eukaryotic cells that can remove the N-terminal Met from the nascent protein chains [29]. Eukaryotic organelles that contain DNA that direct the synthesis of proteins, such as mitochondria and chloroplasts, are bacterial-like with regard to their protein synthesis mechanism and initiate their DNA coded proteins with fMet. It is likely that these organelles originated from bacteria that were incorporated into the eukaryotic cells by endosymbiosis [30].

To our knowledge there is no good explanation as to why Met, with few exceptions, initiates the synthesis of all proteins in all organisms. It is quite amazing that the evolutionary process has selected Met to be the initiator of protein synthesis. In other respects there is nothing unusual about the number of Met residues in proteins or their location. In most mature proteins Met is, in fact, under represented (around 2-3%). A vast majority of cellular proteins contain at least one Met residue, in addition to the N-terminal initiator Met, which is often removed. Our analyses of the *Arabidopsis thaliana* proteomes (Table 1) show that among 33,248 annotated proteins (including hypothetical peptides and proteins) that are encoded by nuclear genes, only 1,174 proteins do not have an internal Met residue, accounting for 3.5% of the cytosol proteome. In addition, our analyses show that these Met-free peptides and proteins vary in size from 15 to 894 amino acids, most of which are less than 250 amino acids. By contrast, 21% and 8.5% of the chloroplast- and mitochondrial-encoded proteins of *Arabidopsis* have no internal Met residues, respectively. All thirteen human mitochondrial-encoded proteins have Met, but analyses of four human nuclear chromosomes show that between 2.3 and 3.1% of the proteins are lacking an internal Met residue (Table 2). As indicated, the analysis of internal Met residues in proteins has provided no clues as to why Met is the universal initiator amino acid in proteins, and this remains an interesting question in evolution.

Importance of Met oxidation in proteins

In proteins there are six amino acids that can be readily oxidized by reactive oxygen species (ROS). These biological oxidants include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion ($O^{\cdot}_2$) and hydroxyl radical ($HO^{\bullet}$). The amino acids and their oxidation products are shown in Table 3. Met is one of the most easily oxidized being converted to methionine sulfoxide [Met(o)] as shown in Figure 3. ROS can oxidize both free Met and Met in proteins forming Met(o). Since there is a chiral sulfur atom in Met(o), this chemical oxidation

Table 1. Number of methionine-free proteins in the *Arabidopsis thaliana* proteomes.

Genomes	Protein No.	Met-free protein No.	Met-free protein%
Chromosome 1	8,703	258	3.0
Chromosome 2	5,277	222	4.2
Chromosome 3	6,534	257	3.9
Chromosome 4	5,079	179	3.5
Chromosome 5	7,655	257	3.4
Total	33,248	1,173	3.5
Chloroplast	85	18	21.2
Mitochondrion	117	10	8.5

Table 2. Presence of methionine-free proteins in representative human proteomes.

Genomes	Protein No.	Met-free protein No.	Met-free protein%
Chromosome 1	3163	99	3.1
Chromosome 11	1951	52	2.7
Chromosome 20	854	25	2.9
Chromosome 22	704	16	2.3
Mitochondrion	13	0	0

Table 3. Amino acids in proteins sensitive to oxidation by ROS.

Amino acids	Oxidation products
Cysteine	Cystine
Histidine	Imidazole oxidation
Lysine	Carbonyl derivatives
Methionine	Met sulfoxide
Tyrosine	Ring oxidation
Tryptophan	Oxyindole

yields equal amounts of the two epimers of Met(o), Met-S-(o) and Met-R-(o). Further oxidation to the sulfone does occur *in vivo*, but the mechanism and relevance of this reaction remain unknown. As also noted in Figure 3, Met(o) in proteins can be reduced back to Met by the methionine sulfoxide reductase enzymes, MsrA and MsrB, in the presence of a reducing system. This important protective mechanism will be discussed in more detail below.

In plants, chloroplasts are known to be in a constant high oxidative state and a major site of ROS production. Since chloroplasts are also the major, if not sole, organelle for Met biosynthesis in plants [5], it is probable that the newly-synthesized Met, as well as Met in chloroplast proteins, are initial and major targets for oxidation. The consequences of oxidation of free Met are not clear since free Met is rapidly metabolized in most tissues. Also, since Met(o) cannot be incorporated into protein, free Met(o) in cells should not affect protein structure or function.

The situation with Met residues in proteins is quite different since proteins have long half lives and protein oxidation is known to occur. The Met residues located on the protein surface would be expected to be most easily oxidized by ROS to Met(o). The conversion of Met to Met(o) makes the compound more hydrophilic which is known to affect protein structure, dimer formation and antibody recognition as initially shown for *E. coli* ribosomal protein L12 [31, 32]. In addition, these changes could lead to alterations in protein function or more susceptibility to proteolytic degradation. However, the relationship between the extent of Met oxidation and changes in protein function is not necessarily straightforward since in most cases oxidation of Met residues may not significantly alter the function of a protein. Bigelow and Squier [33] postulate that because Met(o) is known to weaken protein helices, the use of proteomic and bioinformatic tools will aid in identifying Met sites falling within anticipated helical structures and thus identify proteins that are altered during oxidative stress.

As shown below, despite the low abundance of Met residues in proteins, they appear to play an important role in protecting cells against oxidative stress. Oxidative metabolism produces small amounts of partially reduced ROS that have been implicated in aging and age related diseases. Thus, oxidation is an unavoidable process during normal growth and development and in response to environmental conditions. Fortunately, cells have evolved enzymatic systems to deal with the oxidative stress, such as the Msr system to protect against Met oxidation.

Discovery of the MsrA and MsrB enzymes

Like many serendipitous discoveries in science, the identification and initial purification of MsrA resulted from studies unrelated to oxidative damage. One of us (HW) had been involved in studies on the mechanism of protein synthesis from the mid-1960s, in collaboration with the Nirenberg laboratory at the National Institutes of Health (USA), shortly after the genetic code was worked out.

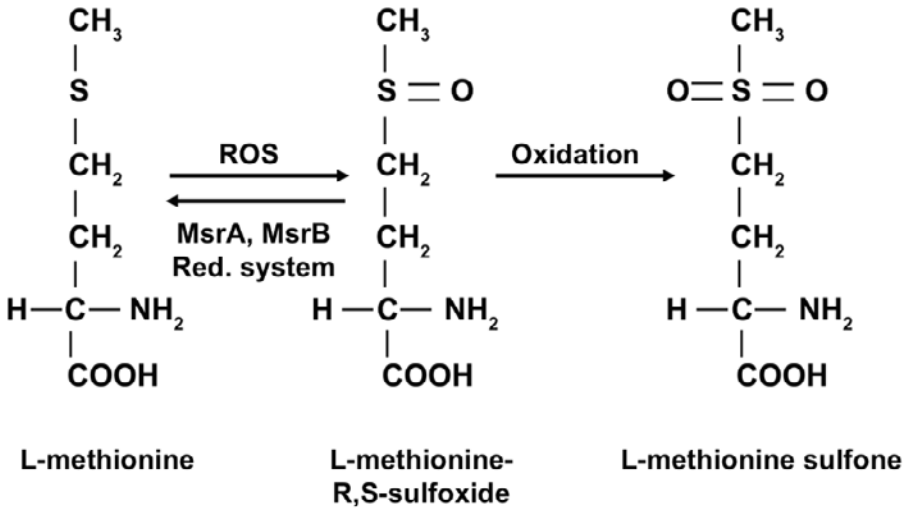

Figure 3. Methionine (Met) oxidation by reactive oxygen species (ROS). Chemical oxidation of methionine by ROS forms methionine sulfoxide [Met(o)], which is a mixture of the R and S epimers. Further oxidation yields methionine sulfone. Met(o) in proteins can be enzymatically reduced back to Met by the Msr enzymes, MsrA and MsrB. See text for further details. Modified from Weissbach *et al.* [53].

These early studies had focused on the initiation and elongation steps of protein synthesis in *E. coli*, especially the role of GTP in the translation process, and it soon became apparent that GTP was involved in all three phases (initiation, elongation and termination) of the translation process [34]. Studies with elongation factor Tu showed that Tu-GTP was required to bring the aminoacyl-tRNA to the ribosome, resulting in the hydrolysis of GTP and the release of Tu-GDP from the ribosome [35]. An exchange reaction, catalyzed by elongation factor Ts, regenerated Tu-GTP from Tu-GDP [36-38]. It also appeared that ribosomal protein L12 (and its deacetylated form, L7) might be the recognition sites on the 50S large ribosomal subunit for Tu-GTP, and other translation factors that bind GTP [39, 40]. An important finding was that ribosomal protein L12 could be readily removed from the ribosomes by high salt extraction which inactivated the ribosome. However, these ribosomes could be reactivated by incubating them with the soluble L12 in low salt [40]. This made it possible to perform structure/function studies on L12, free of ribosomes, in order to determine how it might function to recognize Tu-GTP, and other translation factors, on the ribosome. It became apparent that L12 could be inactivated due to oxidation of one or more of the three Met residues in the protein to Met(o) [32]. Since such an oxidation could be occurring on the ribosome in *E. coli* grown aerobically, soluble extracts of the organism were prepared to see whether there was a repair enzyme that could reverse the oxidative damage to L12. Two types of Met(o) reductases were initially separated. The first could reduce free Met(o) [41], but not protein bound Met(o), and based on more recent studies this enzyme is very likely reducing the R epimer of free Met(o) [42, 43]. The second enzyme, which could reduce both Met(o) in L12 and free Met(o), was initially referred to as peptide methionine sulfoxide reductase [44], but is now known as MsrA. This enzyme was later shown to specifically reduce the S epimer of Met(o) [45, 46] and has been identified in almost all eukaryotic and prokaryotic organisms [47].

After the discovery of MsrA, and its specificity for the S epimer of Met(o), it was apparent that there must be another enzyme to reduce the R epimer of Met(o) in proteins. However, it took twenty years after the discovery of MsrA to identify MsrB, which selectively reduces the R form of Met(o) in proteins. Initial attempts to detect such an activity in *E. coli* had been unsuccessful, and a major breakthrough came when the sequence of MsrA was compared from a variety of organisms. Except for the bacterium *Neisseria gonorrhoeae*, all of the MsrA

genes initially examined coded for a protein having a molecular weight of about 25 kDa [48]. However, the *N. gonorrhoeae* PilB protein was about 57 kDa, and was shown to have an MsrA sequence located in the N-terminal portion of the molecule and exhibited MsrA activity [48]. Also Huang *et al.* [49] had identified a human gene with homology to the carboxyl portion of the PilB protein called CBS-1 that was later shown to be a member of the MsrB family. The first clear demonstration of Met-R-(o) activity came from the studies of Grimaud *et al.* [50] in *E. coli*. These investigators cloned and expressed a gene with homology to the carboxyl end of the PilB protein and showed that this *E. coli* protein could reduce the R epimer of Met(o). The final piece to the puzzle was solved when the carboxyl end of PilB was also shown to possess Met-R-(o) reductase activity [51]. This activity was called MsrB. Thus, PilB has both MsrA and MsrB activities in the same protein. A few other microbial organisms have both activities combined in a single protein (discussed in Weissbach *et al.* [52, 53]) but in most organisms, including all eukaryotes, the *msr*A and *msr*B genes are distinct [47].

The studies of plant Msr enzymes have lagged behind those in microbial and animal systems. However, a very early study, in which kidney bean and turnip leaf discs were incubated with [^{14}C]-methionine sulfoxide, reported that ~10-26% of the Met(o) was reduced to Met [54]. These authors proposed the possible presence of methionine sulfoxide reductases in leaves that may be involved in a redox system. They also found no evidence for a Met oxidase and correctly suggested that Met could be readily oxidized non-enzymatically. Despite their prediction, it took 30 years before the first plant *msr*A gene was isolated from *Brassica napus* [55]. The plant *msr*B genes were first described in *Arabidopsis thaliana* [56] soon after MsrB was identified in *E. coli* [50].

Role of the Msr enzymes in protein repair and as scavengers of ROS

Unlike the other amino acids whose oxidations are irreversible, cysteine and Met oxidation in proteins can be reversed. As described above, Met(o) can be specifically reduced to Met by the Msr enzymes. The two major members of this class of enzymes are MsrA, which reduces Met-S-(o), and MsrB, which reduces Met-R-(o) (Figure 3). In animal cells there is one *msr*A gene and three *msr*B genes [57] and their intracellular locations are shown in Table 4. MsrA has a broad specificity and can reduce both free Met(o) and protein-bound Met(o). In contrast, MsrB2 and B3 are relatively specific for protein bound Met(o) with only trace activity toward free Met(o), although there is recent evidence suggesting that MsrB1 may have a broader specificity [58]. Msr-mediated reduction of protein-bound Met(o) can serve three main functions, 1) as a repair system for protein oxidation, 2) as part of an ROS scavenger system, and 3) possibly as a cellular regulatory mechanism.

With regard to the repair function, if a Met residue located at the active site of a protein is oxidized, the Msr system can repair this oxidative damage and restore the original activity of the protein, without the need to synthesize new proteins. This was first shown with *E. coli* ribosomal protein L12 where oxidation of selected Met residues in *E. coli* ribosomal protein L12 inhibits its function [32]. As noted above, the study of this oxidation and its reversal led to the discovery of MsrA [44]. Another well studied early example is the oxidation of Met358 to Met(o) in α1-proteinase inhibitor. This oxidation abolishes its proteinase inhibiting activity [59], and reducing Met(o) to Met by MsrA can partially restore the activity of the oxidized α1-proteinase inhibitor *in vitro* [60, 61]. Since the cellular balance of proteinase/ antiproteinase may be involved in the disease emphysema, regulation of proteinase inhibitor activity through Met oxidation/reduction may be physiologically and clinically important. Another example is when Met oxidation occurs at position

Table 4. Msr enzymes in mammalian cells and their location*

Enzyme	Substrate	Location
MsrA	Met-S-(o)	cytoplasm, mitochondria
MsrB1	Met-R-(o)	cytoplasm
MsrB2	Met-R-(o)	mitochondria
MsrB3	Met-R-(o)	mitochondria, ER

*See text and Kim and Gladyshev [57].

three in the *Drosophila* shaker voltage-dependent K^+ channel [62]. The presence of the polar oxygen alters the properties of the protein's inactivating ball domain and reduction of the Met(o) allows the channel to recover very rapidly from the inactivated state. This system implicates Met-oxidation in the regulation of cellular signal transduction cascades, with ramifications in learning and memory processes [62]. Finally, when one of two C-terminal Met residues is oxidized in calmodulin, there is a 30-fold decrease in the calcium affinity and a reduced ability of calmodulin to bind and activate the plasma membrane ATPase, allowing calmodulin to adjust metabolism under oxidizing conditions [63].

In plants, the conserved Met residues of the chloroplast-localized heat shock protein Hsp21 require MsrA to reduce the oxidized residues so that it can function as a chaperone to bind and protect other proteins from aggregation [64, 65]. Likewise, Met oxidation in the bacterial enzyme glutamine synthetase leads to a decrease in Mg^{+2}-dependent, but not Mn^{+2}-dependent, γ-glutamyl transferase activity [66]. There are now many other examples, very few in plants though, of proteins that lose their activity upon Met oxidation, *in vitro*, which can be reversed by the Msr enzymes [67, 68]. It is of interest that there are two examples where Met oxidation may stimulate protein function. Oxidation of a Met residue to Met(o) in ubiquitin results in a 50% increase in activity [69] and Met oxidation can increase the activity of the calcium/calmodulin-dependent protein kinase II [70].

The second function of the Msr system is related to its participation in an ROS scavenger system in which the Met residues in proteins can function as catalytic antioxidants. The ease by which Met residues in proteins can be oxidized raises the possibility that Met residues in proteins could function as a cellular antioxidant, especially if the Met(o) formed could be reduced back to Met by cellular enzyme systems. This was first proposed by Levine *et al.* [66]. In this process, all of the exposed Met residues in proteins, in theory, can be oxidized to Met(o) by ROS with the concurrent destruction of one equivalent of the ROS for each Met molecule oxidized. Since MsrA and MsrB can reduce the protein bound Met(o) back to Met, this permits the Met residues to function catalytically

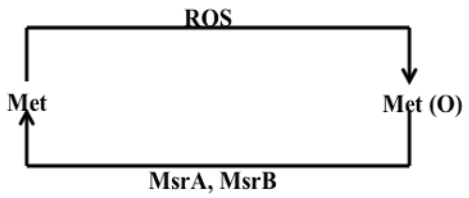

Figure 4. Dual role of the Msr system in protecting cells against oxidative damage. See text for further details.

as scavengers of ROS. The protein repair and scavenger functions of the Msr system are summarized in Figure 4. Support for this mechanism comes from animal cell culture studies that have shown that over-expression of MsrA decreases the levels of ROS in pheochromocytoma (PC12) and lens cells [71, 72] and knocking out MsrA in human lens cells causes the level of ROS to increase [73].

Similarly, in plants over-expression of the plastidial MsrA lowers the sulfoxide content and under-expression leads to an increase in sulfoxide content in *Arabidopsis thaliana* [74], suggesting a correlation between MsrA activity and ROS levels in plant cells. In one study of the endoplasmic reticulum-localized *Arabidopsis* AtMsrB3, it was shown that the MsrB3 lacking plants had a larger increase in Met(o) and H_2O_2 content and electrolyte leakage (membrane damage), as compared to the wild type and AtMsrB3-over-expressing plants [75]. Similarly, MsrB2-silenced pepper plants exhibited increased production and accumulation of ROS, resulting in the acceleration of cell death [76]. All of these studies in both animal and plant systems support the notion that the Msr system is involved in regulating ROS levels in cells.

Another potential function of the Msr system is to participate in regulating the activity of proteins. One could easily imagine that Met oxidation/reduction would be a simple way to modulate the activity of proteins, similar to protein phosphorylation/dephosphorylation. Examples that

suggest this possibility are studies with a voltage gated K^+ ion channels [62], calcineurin [77], thrombomodulin [78], calmodulin and protein kinases [70, 79]. The difficulty in proving a regulatory function for the Msr system is that the production of ROS, which would be required to regulate these proteins at specific times, is not under strict control in cells. Therefore, it is hard to imagine how protein oxidation can be fine-tuned to modulate the activity of a protein exactly when needed. The identification of an enzyme that stereospecifically oxidizes Met residues, and could work in conjunction with the Msr enzymes, would be a major step forward in elucidating a regulatory role for Met oxidation/reduction. It is of interest that Lim *et al.* [80] have recently shown that MsrA can catalyze the reverse reaction, namely, the oxidation of Met to Met-S-(o). The oxidative reaction is much slower than the reductase activity and there is, as yet, no evidence that the oxidative activity of MsrA has physiological significance.

There are some data in plants to indicate that the oxidation/reduction of Met residues may directly or indirectly influence other regulatory processes. A study of the plant mitochondrial pyruvate dehydrogenase (PDH) α subunit (E1α) demonstrated that *in vitro* oxidation of a Met residue inhibits the phosphorylation of an adjacent serine [81]. Because phosphorylation of the E1α subunit switches off the mitochondrial PDH complex activity and dephosphorylation reactivates the complex [82], Met oxidation/reduction of PDH could modulate the mitochondrial respiratory activity. More significantly, many proteins may contain phosphorylating serine residues that are immediately adjacent or close to Met residues. Oxidation of these critically positioned Met residues could potentially disturb or disrupt the serine phosphorylation/dephosphorylation reactions and the binding ability and specificity of these proteins (e.g. 14-3-3 proteins) that mediate signal transduction (83). Therefore, even though protein oxidation by ROS may be primarily a non-enzymatic chemical process, Met residue oxidation/reduction may regulate the function and/or activity of a wide range of proteins.

In summary, Msr activity in cells not only repairs damage to proteins whose function has been affected by Met oxidation, but Met-containing proteins also contribute to the regulation of overall levels of cellular ROS because the Met residues in proteins can function as catalytic anti-oxidants in the presence of the Msr enzymes. In addition, Met-derived protein oxidation, along with other protein modification processes, may constitute an intricate regulatory network for animals and plants to modulate physiological and biochemical processes in response to ever changing environmental conditions such as oxidative stress [84].

Physiological role of the Msr system in protecting cells against oxidative damage and aging

The first studies that clearly showed a protective effect of MsrA were done using *E. coli* mutants lacking MsrA, where it was shown that they were more sensitive to oxidation from H_2O_2 [85, 86]. Also, other transgenic studies in microbes and animals have shown phenotypes by just modulating MsrA activity. An earlier study using an MsrA knockout in mice showed that these animals were sensitive to high oxygen tension, had a neurological defect and a shorter life span [87]. A recent study, however, has questioned the life span result, but does verify that the animals are more sensitive to oxidative stress [88]. Over-expression of MsrA in nerve cells of *Drosophila* markedly extends the life span (Figure 5) and these animals are more resistant to oxidative stress [89]. Studies of yeast under aerobic conditions also showed a significant effect on life span by over-expression of MsrA or by knocking out *msr*A, whereas little or no effect of altering MsrB (SelR) levels was found [90].

In animals it was shown that the number of Met(o) residues in proteins increases while Msr activity decreases in various aging models [91], and it is generally accepted that oxidative stress plays a role in the pathogenesis of many age associated diseases. Alzheimer's disease (AD) is a neurodegenerative disease caused or aggravated by pathways that stimulate ROS or weaken antioxidants in the brain [92, 93]. The beta amyloid peptide (Aβ) is the primary constituent of plaques found in brains from AD patients, and the Met residue in Aβ may play an important role in the toxicity of the peptide. This 42 amino acid peptide contains one Met at position 35 (Met-35) and the oxidized-Aβ [Aβ-Met(o)] has been found in large

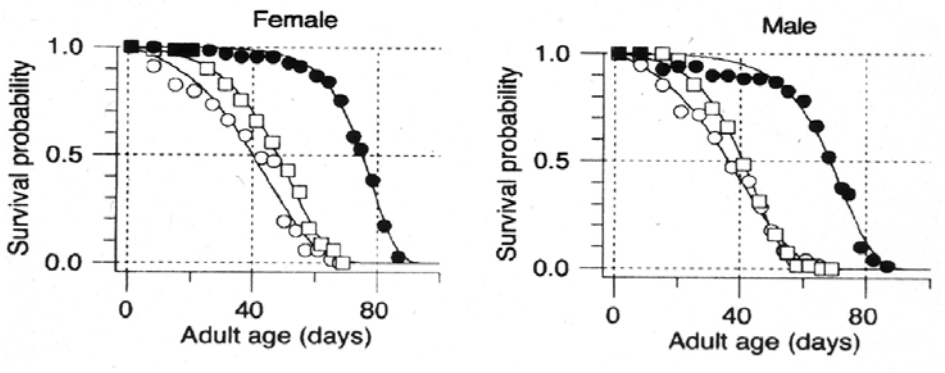

Figure 5. Over-expression of MsrA in neuronal tissue in flies extends their life span. Open symbols are control flies and filled symbols are MsrA transgenic flies. From Ruan *et al.* [89]. The United States National Academy of Sciences holds copyright to this work.

amounts in post-mortem AD plaques [94, 95]. Furthermore, there is a decrease in MsrA activity in AD brains [96]. Met-35 in Aβ is necessary for Aβ's lipid and protein oxidative properties, as well as its free radical generation and neurotoxicity properties *in vitro* [97]. Butterfield *et al.* [98] have also shown that the Met-35 of the Aβ peptide is necessary for protein carbonyl generation and lipid peroxidation (two oxidative stress parameters) *in vivo* in the mammalian brain. These studies suggest a potential therapeutic pathway involving prevention of Met oxidation in Aβ and/or stimulating Msr activity as a means towards delaying the progression of AD. Of great interest is a report that transducing PC12 cells with a TAT-MsrA fusion protein protected these cells against Aβ toxicity [99].

One of the factors believed to cause age-related macular degeneration (AMD) is also oxidative stress. Retinal pigment epithelial cells (RPE), due to their location between the retina and choroidal blood, are particularly susceptible to oxidative damage and it is believed that oxidative damage to RPE cells plays a role in AMD. Liang and Godley [100] showed that mitochondrial DNA in RPE cells was damaged by H_2O_2-induced oxidation, whereas nuclear DNA was not and they suggest that this mitochondrial damage causes RPE cell apoptosis. Over-expression of MsrB2 in stably-transfected RPE cells was shown to provide protection to three oxidative stressors: tert-butyl-hydroperoxide, all-trans retinoic acid and a highly toxic oxysterol [101]. However, silencing of MsrB2 in these cells did not increase susceptibility to oxidative damage, suggesting compensation by other Msr or protective enzymes [101].

Other age-related eye diseases include cataract and glaucoma. Pathology of the eye is directly related to the normal aging process, which includes decline of antioxidant systems, increased levels of ROS, and buildup of oxidized proteins resulting in cell death. ROS increase in these cells is due to both environmental and intracellular factors [102].

Physiological role of the Msr system in plants

Plants possess multiple Msr-encoding genes. For example, *Arabidopsis thaliana* has five *msr*A and nine *msr*B genes [103]. Corresponding to the wide distribution of ROS in the cell, three of the *Arabidopsis* MsrA enzymes are localized in the cytosol and one MsrA each in plastids (chloroplasts) and endoplasmic reticulum (ER) or an associated secretory pathway. Likewise, there are six MsrB enzymes predicted to be present in the cytosol, two in plastids and one in the ER. The presence of the Msr enzymes in these organelles could explain the need to repair protein damage caused by Met oxidation and to control the level of ROS. Studies have demonstrated that over-expressing the plastidic MsrA lowers the Met(o) content and confers plants tolerance to photooxidative damage to photosynthetic membranes, whereas plants in

which MsrA is suppressed are more susceptible to oxidative stress [74]. Since protein oxidation is often a consequence of a general stress process, reduction of Met(o) in proteins by Msr enzymes can also be considered as a cellular defense response at the molecular level. In plants, Msr gene expression and enzyme activities have been linked to a wide range of stress conditions such as long night, high salt, high light intensity, low and high temperatures, pathogen infections and pathogen virulence [64, 74-76, 104-107]. Although ROS are found throughout the cell, the major ROS production sites in plants are chloroplasts, mitochondria and microbodies where active oxidizing reactions or electron transfer flows occur [108]. In contrast to cytosol or chloroplasts, the Msr enzymes, unexpectedly, appear to be absent in plant mitochondria even though mitochondria are thought to be a main target for oxidative damage. This conclusion was reached initially by our analysis of the fully sequenced nuclear genomes of four plants (*Arabidopsis thaliana, Oryza sativa, Populus trichocarpa* and *Vitis vinifera*) which failed to predict any mitochondrion-targeting MsrA or MsrB proteins. In addition, analysis of the *Arabidopsis* mitochondrial proteome with over 400 proteins by 2-D gel electrophoresis or mass spectrometry did not identify any Msr proteins [109]. However, it should be noted that the green algae and the moss *Physcomitrella patens*, as well as animals, all apparently possess mitochondrial MsrA and MsrB. Obviously, studies are needed to specifically address whether the plant mitochondria truly lack Msr proteins and Met(o) reductase activities and, if so, how this organelle deals with Met oxidation of proteins that must occur.

There is limited information on Msr activity in nuclei. In mammalian cells MsrB1 has been reported to be present in both the cytoplasm and nucleus, even though it does not have a typical nuclear localization signal [57].

Evolutionary implications of the R and S epimers of Met(O)

One intriguing question is whether the S- and R-epimers of Met(o) have different biological effects on protein structure and function. As mentioned, chemical oxidation of Met, such as with ROS, produces a statistically equivalent amount of Met-S-(o) and Met-R-(o). This means that in cells one oxidized Met residue will be either the S-epimer or R-epimer (cannot be both). In the population of Met-oxidized protein molecules there should be the same molar number of S- and R-epimers. Since MsrA can only catalyze the reduction of Met-S-(o) and MsrB only works on Met-R-(o), the complete reduction of Met(o) would require both enzymes. In all extant organisms surveyed, an organism can have MsrA only, MsrA/B (a protein containing both activities), or MsrA and MsrB. However, no organism has been found that contains only MsrB [47]. As for plants, the relative importance of the MsrA/MsrB ratio is not clear. Over- or under-expression of either enzyme did not produce a phenotype but did cause changes in plants' responses to stress [110]. All of these observations raise the question of whether the S- and R-epimers of Met(o) have different effects on protein activity. Accordingly, MsrA may have predated MsrB in evolution and has secured a more prominent role in life [47].

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