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REVIEW ARTICLE

Functions and evolution of selenoprotein methionine sulfoxide reductases

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Abstract
Methionine sulfoxide reductases (Msrs) are thiol-dependent enzymes which catalyze conversion of methionine sulfoxide to methionine. Three Msr families, MsrA, MsrB, and fRMsr, are known. MsrA and MsrB are responsible for the reduction of methionine-*S*-sulfoxide and methionine-*R*-sulfoxide residues in proteins, respectively, whereas fRMsr reduces free methionine-*R*-sulfoxide. Besides acting on proteins, MsrA can additionally reduce free methionine-*S*-sulfoxide. Some MsrAs and MsrBs evolved to utilize catalytic selenocysteine. This includes MsrB1, which is a major MsrB in cytosol and nucleus in mammalian cells. Specialized machinery is used for insertion of selenocysteine into MsrB1 and other selenoproteins at in-frame UGA codons. Selenocysteine offers catalytic advantage to the protein repair function of Msrs, but also makes these proteins dependent on the supply of selenium and requires adjustments in their strategies for regeneration of active enzymes. Msrs have roles in protecting cellular proteins from oxidative stress and through this function they may regulate lifespan in several model organisms.

Keywords: methionine sulfoxide, MsrA, fRMsr, MsrB1, MsrB2, MsrB3, selenoprotein, selenocysteine, aging, antioxidants, ROS

1. Introduction

Cellular components in aerobic organisms are often confronted with reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). Molecular oxygen is indispensible for survival of aerobic organisms, which use it for respiration and numerous other processes, but the use of oxygen is also associated with the generation of ROS [1]. ROS may damage various biomolecules, such as DNA, proteins, and lipids, and may contribute to the development of cancer, neurodegenerative diseases and other maladies. Oxidative damage to DNA may lead to mutations, which in turn may result in cancer [2, 3]. Lipid oxidation by ROS may change membrane rigidity and permeability. In addition, it may disrupt membrane networks and generate toxic products, such as malondialdehyde, through lipid peroxidation [4, 5]. Oxidized proteins may become nonfunctional due to structural changes and catalytic malfunction. Sulfur-containing amino acids, methionine and cysteine, are the major targets of ROS and are the amino acids most susceptible to oxidation. Oxidized methionine exists in the form of two diastereomers, methionine-*S*-sulfoxide and methionine-*R*-sulfoxide [6, 7]. To counteract ROS damage, organisms evolved defense systems, including low molecular weight compounds and antioxidant enzymes that protect against oxidative stress. The latter include glutathione peroxidase (GPx) [8, 9], superoxide dismutase (SOD) [10, 11], catalase [12], thioredoxin reductase (TR) [13], methionine sulfoxide reductase (Msr) [14–17], and several other proteins. Among these antioxidant enzymes, Msrs are prominent because of their roles as repair enzymes and indirect scavengers of ROS. These proteins utilize catalytic redox-active cysteine residues to reverse oxidized methionine back to methionine. Many Msrs use the thioredoxin system *in vivo* to regenerate the active catalytic cysteine, but other reductants may also be used [16–20]. Because of their protein repair functions, the studies involving Msrs are broadly linked to various research areas such as aging, neurodegenerative disease, cancer, cell signaling, and protein function.

2. Three types of methionine sulfoxide reductases

The first Msr enzyme, MsrA, was discovered some 30 years ago. It was found that this enzyme can restore the function of oxidized ribosomal protein L12 in *Escherichia coli* [21]. After this initial finding, Msrs have been investigated from a variety of organisms, new Msr forms were discovered, and currently these enzymes can be divided into three large groups according to their substrate specificity and family clustering. MsrA is specific for the reduction of free and protein-based methionine-*S*-sulfoxide. This

protein is the only known enzyme in mammals capable of reducing methionine-*S*-sulfoxide [22–25], but it can also reduce other compounds such as *N*-acetyl-methionine-*S*-sulfoxide, dimethyl sulfoxide, ethionine-*S*-sulfoxide, *S*-sulindac, *S*-sulforaphane, etc. [26–28]. It was found that MsrA exists in a selenoprotein form in some lower organisms, such as green algae and bacteria, wherein it utilizes catalytic selenocysteine in place of cysteine [29, 30].

A second Msr, known as MsrB (described previously as SelR or SelX), is specific for the reduction of protein-based methionine-*R*-sulfoxide, but also, with low efficiency, of free methionine-*R*-sulfoxide. For example, catalytic efficiency of *E. coli* MsrB for the reduction of free methionine-*R*-sulfoxide is 1000-fold lower than that of *E. coli* MsrA for free methionine-*S*-sulfoxide. In addition, when the *MsrB* gene was disrupted in *E. coli*, specific activity of cell extract for the reduction of free methionine-*R*-sulfoxide was essentially unchanged [31, 32], whereas mutation of another Msr gene (see below) in yeast resulted in the reduced activity with this substrate [33]. In human SK-Hep1 cells, MsrBs were also found to be inefficient reductants of free methionine-*R*-sulfoxide. Thus, this study revealed that mammals have important differences in the reduction of free methionine-*S*-sulfoxide and free methionine-*R*-sulfoxide [34].

In mammals, there are three MsrB proteins, including MsrB1, MsrB2, and MsrB3. All three contain Zn, coordinated by two CxxC motifs (i.e., two Cys separated by two residues), which stabilizes MsrB structure. Of these three enzymes, MsrB1 is a selenoprotein that contains selenocysteine in place of the catalytic cysteine residue normally present in other MsrBs [35, 36].

A third Msr type, fRMsr, was only recently discovered [37] and it catalyzes the reduction of free methionine-*R*-sulfoxide [33, 34, 37]. This enzyme is a GAF-domain-containing protein and is highly specific for its substrate (i.e., it does not act on protein-based sulfoxides) [33, 37, 38]. Interestingly, comparative genomic analysis of fRMsr revealed that its occurrence is limited to unicellular organisms, whereas multicellular organisms, including mammals, lack this protein [33, 37]. This observation suggested that fRMsr was lost during evolution in mammals, i.e., they are deficient in the reduction of free methionine-*R*-sulfoxide (Figure 1).

The three Msrs support protein repair and methionine metabolism functions by reducing free and protein-based methionine sulfoxides. The key function of Msr is to repair oxidatively damaged proteins, in which ROS oxidized methionine residues. This oxidation may interfere with protein function, because it leads to an increased negative charge and size, and consequently may result in structural changes and loss of function [6, 7, 39]. In addition, Msrs may work indirectly as ROS scavengers through cyclic oxidation and reduction of methionine residues. The methionine/Msr system may assist other redox systems, such as thioredoxin and glutathione systems, in maintaining cellular redox homeostasis. This idea is consistent with the data showing that deficiency in various Msrs results in oxidative stress [14, 16, 40, 41]. Methionine sulfoxides and Msrs may also play a role in sensing altered redox status and regulating certain proteins. Recently, it was shown that CaMKII (calcium/calmodulin (Ca^{2+}/CaM)-dependent protein kinase II) was activated by oxidation of its methionine residue in the regulatory domain in the absence of Ca^{2+}/CaM and that this activation could be reversed by MsrA [42]. In addition, human large conductance Ca^{2+}-activated K channels (BKCa or Slo), *E. coli* Ffh, human potassium channel HERG1, and calmodulin are known to be regulated by oxidation of methionine residues and the repair activity of Msrs [43–45]. Along with these Msr functions, several reports showed that MsrA knockout mice exhibit a tip-toe walking behavior consistent with cerebellar dysfunction [46], enhanced neurodegeneration in hippocampus [47], and impaired dopamine regulation [48]. However, the specific link between Msrs and neurodegenerative diseases remains poorly understood.

3. Localization of mammalian MsrA and MsrBs

Mammals have a single *MsrA* gene containing a typical mitochondrial signal peptide [49]. However, mouse and rat MsrAs occur not only in mitochondria, but also in cytosol and nucleus. It was suggested that the rate of folding is a crucial factor in the subcellular distribution of MsrA, even though the molecular mechanism and regulation remain unclear [50]. It was also reported that alternative first exon splicing generates an additional MsrA form lacking a mitochondrial signal, which resides in cytosol and nucleus [51–53]. It should be noted, however, that the alternatively spliced MsrA form was not detected in examined mouse tissues, including brain, kidney, and liver, by western blot assays, and only a weak signal was detected at the mRNA level. Studies showed that other alternative forms of MsrA mRNA, such as an additional exon 2, could be detected in rat aortic vascular smooth muscle cells and aortic tissue preparations [53] and that this form was localized exclusively to mitochondria [53, 54].

Among three mammalian MsrBs, MsrB1 is present in the cytosol and nucleus and exhibits the highest catalytic activity due to selenocysteine in its active site. Recently, it was found that MsrB1 occurs in two forms, 14 kDa and 5 kDa, in mouse tissues and human HEK 293 cells and that both forms are selenoproteins. The 5 kDa form corresponded to a C-terminal sequence of the 14 kDa MsrB1. However, neither function nor regulation of the 5 kDa MsrB1 is known [55, 56]. MsrB2, also known as CBS1, is targeted to mitochondria with the guidance of its N-terminal signal peptide. MsrB2 has cysteine as the catalytic residue. It shows high activity with methionine-*R*-sulfoxide, but is inhibited

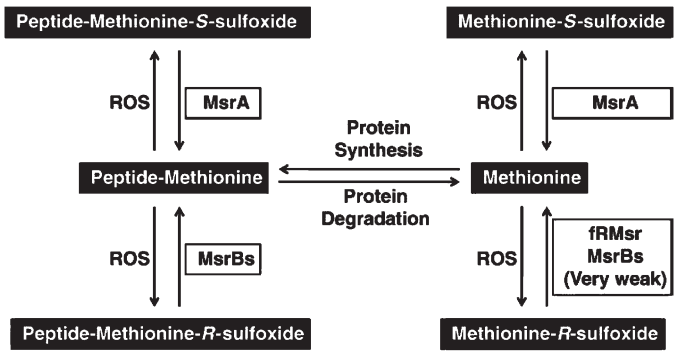

Figure 1. Reversible generation and reduction of methionine sulfoxide. MsrA can reduce both free and protein-based methionine-*S*-sulfoxide. MsrB mainly reduces protein-based methionine-*R*-sulfoxide and its activity towards free methionine-*R*-sulfoxide is very weak. fRMsr can only reduce free methionine-*R*-sulfoxide.

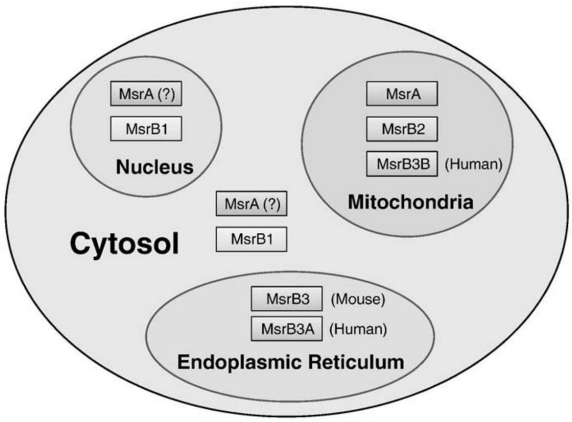

Figure 2. Localization of Msrs in mammals. MsrA and MsrB1 are located in the nucleus and cytosol. MsrA, MsrB2, and MsrB3B are targeted to mitochondria by their mitochondrial targeting signals. Human MsrB3A is located in the ER and has N-terminal ER targeting and C-terminal retention signals, whereas human MsrB3B is a mitochondrial protein. Mouse MsrB3 is located in the ER. There are alternatively spliced variants of MsrA, which are not shown in the figure and localize to cytosol and nucleus, or to mitochondria.

by elevated concentrations of substrate [35, 57, 58]. MsrB3 consists of two forms, MsrB3A and MsrB3B, generated by alternative first exon splicing in humans. MsrB3A is targeted to the endoplasmic reticulum (ER) by an N-terminal ER signal peptide and an ER retention signal at the C-terminus, whereas MsrB3B is targeted to mitochondria by its N-terminal mitochondrial signal peptide. Interestingly, mouse MsrB3 also has the ER and mitochondrial signal peptides located consecutively at the N-terminus, but it is targeted only to the ER due to masking of the mitochondrial signal by an upstream ER signal. Even though a possibility cannot be excluded that small amounts of mouse MsrB3 targeted to mitochondria exist, studies found no evidence of alternative splicing in this gene based on EST, RT-PCR, and western blot analyses [35, 59]. The localization pattern of mammalian MsrA and MsrBs is shown in Figure 2.

4. Catalysis by the different Msr types

Mammalian MsrA has three conserved cysteines, which participate in the reaction as catalytic and resolving cysteines. A sulfenic acid intermediate at the catalytic cysteine is generated when this residue attacks the sulfur of methionine-*S*-sulfoxide and then thiol–disulfide exchange involving two resolving cysteines leads to a disulfide bond on the protein surface, which is finally reduced by thioredoxin or DTT [23, 60]. Recently, it was proposed that yeast fRMsr also has three conserved cysteines that follow a similar sulfenic acid/thiol–disulfide exchange mechanism. In addition, the thioredoxin system can function in recycling fRMsr as it does in the case of MsrA. A difference between MsrA and fRMsr involves accessibility of the substrate. Yeast fRMsr has a narrow binding pocket, which limits access to bulky substrates. This may explain differences in substrate specificity between MsrA and fRMsr, even though the overall catalytic process involving catalytic and resolving cysteines is similar in these enzymes (Figure 3A and B) [33, 37].

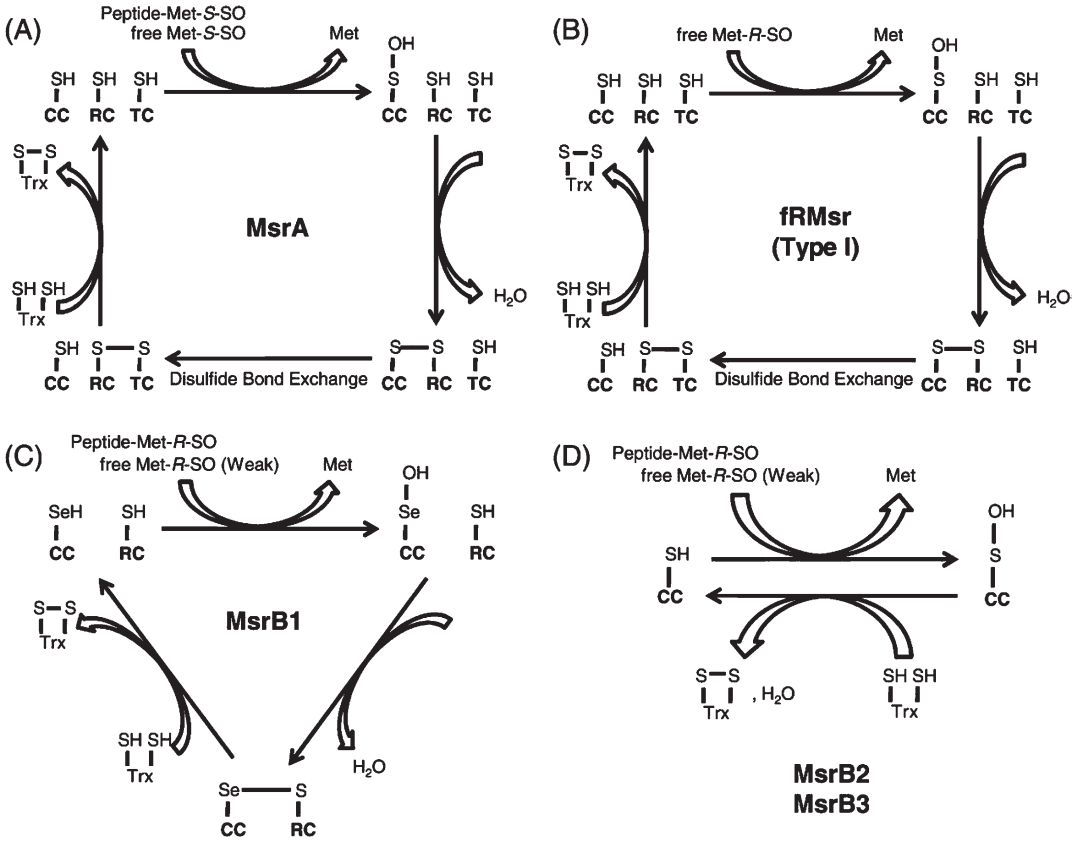

Figure 3. Catalytic mechanisms of Msrs. Catalytic cysteine (CC), resolving cysteine (RC), and/or third cysteine (TC) take part in the catalysis by (A) MsrA, (B) fRMsr, (C) MsrB1, and (D) MsrB2 and MsrB3. MsrA and fRMsr have three conserved cysteines. MsrB1 has one conserved cysteine and one conserved selenocysteine. MsrB2 and MsrB3 have only one redox cysteine and its intermediate sulfenic acid may be directly reduced by thioredoxin or certain compounds.

MsrB1 has one conserved cysteine in the N-terminal portion and the catalytic selenocysteine in the C-terminal region. During catalysis, selenocysteine forms selenenic acid intermediate by attacking the sulfur of methionine-*R*-sulfoxide and then this intermediate rearranges into selenenylsulfide with the help of the resolving cysteine and is finally reduced by thioredoxin (Figure 3C) [61]. In contrast, MsrB2 and MsrB3 have only one conserved cysteine and appear to have evolved a different catalytic mechanism for the reduction of their sulfenic acid intermediate (i.e., without disulfide formation between catalytic and resolving cysteines), which is reduced directly by thioredoxin (Figure 3D) [61–63]. Although thioredoxin is the most important reductant for Msrs, it was shown that thioredoxin-independent Msr reduction is also possible [19, 20]. For example, thionein, selenocystamine, and CDP32 could be used for the reduction of human MsrB2 and MsrB3, and such thioredoxin-independent reaction may even be more efficient than that involving thioredoxin.

5. Selenoproteins and catalytic benefits of selenocysteine utilization

Selenoproteins exist in organisms in all three domains of life. Selenium is inserted into these proteins co-translationally in the form of selenocysteine, in response to UGA codon. Selenocysteine insertion is dependent on the presence of a stem-loop structure, known as the SECIS (selenocysteine insertion sequence) element, in the 3′-UTRs of selenoprotein genes. Selenium is essential in mammals because of its occurrence in selenoproteins (Figure 4). These proteins have various functions and show widely different knockout phenotypes. For example, GPx4 reduces lipid peroxides and its knockout in mice results in embryonic lethality [64]. Another selenoprotein, Selenoprotein P (SelP), transports selenium from liver to brain, thyroid gland, kidney, testis and likely other organs. Without SelP, a much higher selenium intake is needed to support selenoprotein synthesis [65–67]. Selenoprotein N (SelN) is a protein of unknown function and mutations in this gene are linked to various muscular diseases, such as rigid spine muscular dystrophy [68]. Many of the 25 human selenoproteins contribute to the regulation of redox homeostasis, but the functions of several proteins are not known. Perhaps, the best studied selenoprotein families are GPxs and TRs. There are 5 selenoprotein GPxs in humans and 4 in mice, which function as thiol-dependent peroxidases. TR 1, 2, and 3 regulate cellular redox homeostasis by controlling the redox state of thioredoxins [69]. SelW and SelK might also play a role in redox regulation. For example, SelW binds glutathione and its overexpression increases resistance to oxidative stress in CHO cells and H1299 cancer cells in a glutathione-dependent manner [70, 71]. Overexpression of SelK reduces intracellular ROS levels and protects cardiomyocytes from oxidative stress-induced toxicity [72].

Like other functionally characterized selenoproteins, MsrB1 is an oxidoreductase. Selenocysteine in this enzyme supports high catalytic efficiency of MsrB1 (> 100-fold higher than that of the corresponding cysteine-containing mutant) [35, 61, 73, 74]. This enhancement of activity was also demonstrated for a selenocysteine-containing MsrA from *Chlamydomonas* [30]. Thus, selenocysteine, strategically positioned in the active site, is a key catalytic residue, whereas its replacement with cysteine leads to a significant drop or loss of catalytic activity. Similar observations were also made with iodothyronine deiodinase in oocytes [75, 76] and clostridial glycine reductase selenoprotein A [75, 77].

However, selenocysteine is not always beneficial under physiological conditions. When the catalytic cysteine in MsrB2 and MsrB3 was replaced with selenocysteine, activities of these enzymes were increased up to 100-fold in the presence of DTT as a reducing agent, but these proteins were not active with thioredoxin. One possibility is that a selenenic acid intermediate could not be efficiently reduced by thioredoxin and therefore the enzymes could not be regenerated for the next catalytic cycle, whereas a selenenylsulfide bond, formed between catalytic selenocysteine and resolving cysteine residues, was reducible by thioredoxin. This hypothesis was recently supported by the studies on a natural selenocysteine-containing MsrA and an artificial selenoprotein MsrB from *Clostridium* sp., an anaerobic bacterium [29, 62]. It was shown that these proteins take advantage of incorporating selenocysteine as the catalytic residue, but they could not be reduced by thioredoxin, suggesting that the selenenic acid intermediate requires other reductants under physiological conditions.

On the other hand, the use of selenocysteine in MsrB and MsrA is rare when viewed from the evolutionary perspective. The absolute majority of these enzymes are cysteine-containing proteins, and many of these are highly efficient in standard *in vitro* assays. Interestingly, Msrs are absent in many hyperthermophylic organisms because at higher temperatures, methionine sulfoxide reduction may not require catalysis. By the same token, selenoprotein Msrs might be needed under conditions when their cysteine-containing counterparts are ineffective.

6. Aging and Msrs

Since the oxidative stress theory of aging was first introduced half a century ago, free radicals and other ROS emerged as important factors that regulate the aging process [1, 78, 79]. Along with the idea of ROS-induced damage, defense mechanisms received much attention, especially in the area of aging research, because efficient protection from oxidative stress and elimination of damage might delay the aging process. It has been shown that various antioxidant enzymes, such as catalase and SOD, could protect from oxidative stress and increase lifespan in some model organisms [80–84]. As an antioxidant en-

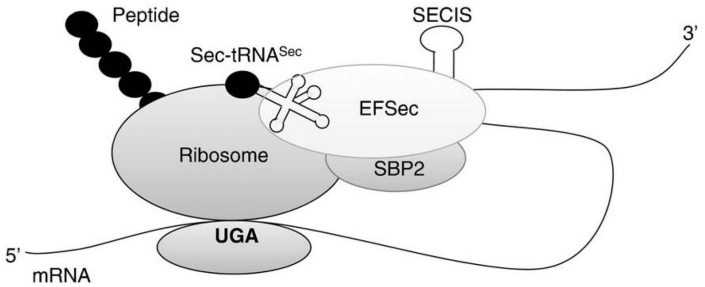

Figure 4. Selenoprotein biosynthesis. Selenocysteine is inserted into protein at UGA codons with the help of various factors that form the selenocysteine insertion machinery as shown in the figure. Sec-tRNA^{Sec}, a tRNA specific for selenocysteine; SBP2, SECIS binding protein 2; EFSec, elongation factor specific for Sec-tRNA.

zyme, MsrA has also been examined, and it was found that its overexpression could extend lifespan by 70% in *Drosophila* [84]. Furthermore, knockout of MsrA in mice or yeast reduced lifespan of these organisms [46, 85]. In contrast to MsrA, the effect of MsrB is less obvious. It was reported that overexpression of MsrB extends lifespan of yeast cells grown under caloric restriction conditions, but not in cells grown in regular medium, i.e., conditions where MsrA could increase lifespan [86].

As mentioned above, MsrA and MsrB have clear differences in their ability for reduction of free methionine sulfoxide as well as in their cellular locations, and these differences might account for the observations in the aging studies. Much of the amino acid metabolism occurs in mitochondria, which is also the organelle that generates significant amounts of ROS [87–90]. Since methionine is one of the amino acids most susceptible to oxidation by ROS, it is possible that it can serve as a defense system through cyclic oxidation and reduction by mitochondria-targeted Msrs. In addition, methionine is a precursor for other antioxidants and redox compounds, such as cysteine, taurine, and glutathione. Furthermore, S-adenosylmethionine, the principal methyl donor in the cell, is also an intermediate in the methionine metabolism, which may link Msr function to epigenetic regulation [34, 78, 91–93]. Possible negative effects of excess methionine as a factor that regulates lifespan should be also considered. For example, overflow of methionine may generate excessive amounts of homocysteine [94] (Figure 5). Even though efficient reduction of free methionine sulfoxide might be a factor in protecting cells from ROS and extending lifespan, it should be

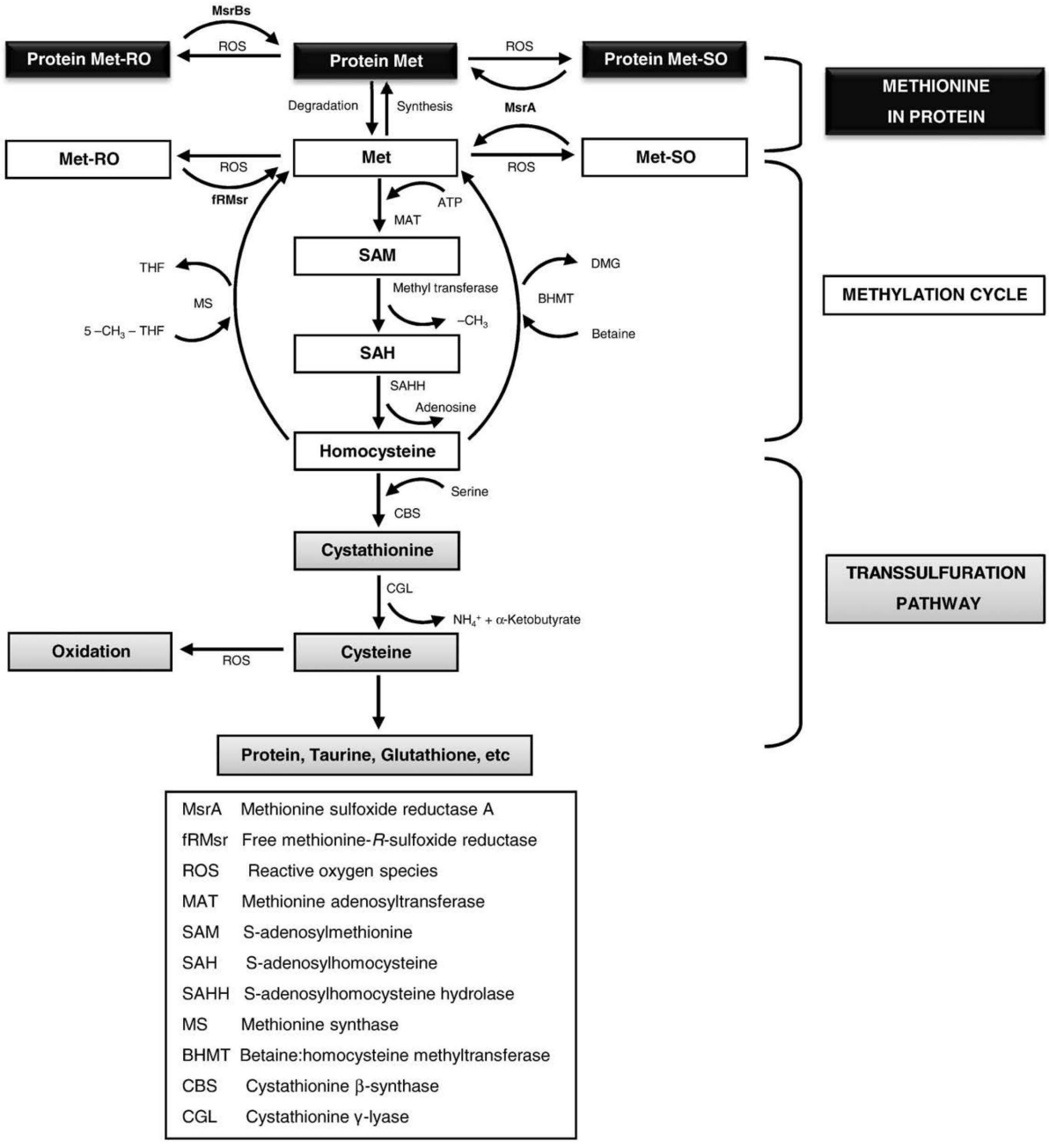

Figure 5. Methionine and methionine sulfoxide metabolism in mammals. Protein synthesis, methionine cycle and transsulfuration pathways represent the major uses of free methionine.

viewed in the context of overall methionine metabolism under both stress and normal conditions. Finally, since MsrB1 is a selenoprotein, it would be important to examine its effect as well as the effect of dietary selenium on lifespan in mammals.

Acknowledgments

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