Methods xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

# Practical guide for dynamic monitoring of protein oxidation using genetically encoded ratiometric fluorescent biosensors of methionine sulfoxide

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ARTICLE INFO

*Article history:*
Received 3 May 2016
Received in revised form 22 June 2016
Accepted 22 June 2016
Available online xxxx

*Keywords:*
Methionine sulfoxide
Sensors
Methionine

ABSTRACT

In cells, physiological and pathophysiological conditions may lead to the formation of methionine sulfoxide (MetO). This oxidative modification of methionine exists in the form of two diastereomers, R and S, and may occur in both free amino acid and proteins. MetO is reduced back to methionine by methionine sulfoxide reductases (MSRs). Methionine oxidation was thought to be a nonspecific modification affecting protein functions and methionine availability. However, recent findings suggest that cyclic methionine oxidation and reduction is a posttranslational modification that actively regulates protein function akin to redox regulation by cysteine oxidation and phosphorylation. Methionine oxidation is thus an important mechanism that could play out in various physiological contexts. However, detecting MetO generation and MSR functions remains challenging because of the lack of tools and reagents to detect and quantify this protein modification. We recently developed two genetically encoded diasterospecific fluorescent sensors, MetSOx and MetROx, to dynamically monitor MetO in living cells. Here, we provide a detailed procedure for their use in bacterial and mammalian cells using fluorimetric and fluorescent imaging approaches. This method can be adapted to dynamically monitor methionine oxidation in various cell types and under various conditions.



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http://dx.doi.org/10.1016/j.ymeth.2016.06.022
1046-2023/

## 1. Introduction

Living cells are constantly exposed to oxidants such as reactive oxygen species (ROS), chloramines or peroxynitrites [1,2]. Under physiological conditions, ROS concentrations are controlled and can regulate processes such as proliferation, differentiation and apoptosis [3,4]. However, several conditions can lead to a loss of redox homeostasis upon dysregulation of ROS production or elimination, leading to the accumulation of ROS to detrimental levels [1,4]. ROS can oxidize proteins and virtually all amino acids may be affected. The sulfur-containing amino acids, cysteine and methionine, are prone to oxidation at their sulfur atom, and they are the only identified amino acids for which oxidation is reversible [5]. In the case of signal transduction, ROS action generally occurs through oxidation of critical cysteine residues of metabolic enzymes or signaling proteins, such as kinases and transcription factors [1,3]. Numerous enzymatic and non-enzymatic systems exist that reduce oxidized cysteines [4]. Methionine (Met) can also undergo oxidation by the addition of one oxygen atom on the lateral chain leading to the formation of methionine sulfoxide (MetO), which exists as two diastereomers, *R* (Met-*R*-O) and *S* (Met-*S*-O). Oxidation can occur in free amino acid and in protein-based methionine residues, and organisms possess several enzymes of the methionine sulfoxide reductase (MSR) group of proteins that reduce the oxidized form [6]. The most widespread of these enzymes are methionine sulfoxide reductases A (MSRA) and B (MSRB), which are found in almost all organisms and are specific for the reduction of the *S* and *R* diastereomers, respectively [7,8]. MSRA can reduce both free and protein-based Met-*S*-O, whereas generally MSRB only reduces Met-*R*-O in proteins [6,9]. It should also be noted that plants possess numerous MSRBs and, among them, some display the capacity to reduce free Met-*R*-O [10,11]. Typical MSRA and MSRB use the reducing power provided by the NADPH-dependent thioredoxin reductase/thioredoxin (Trx) system in a mechanism involving disulfide exchange (see Section 2) [12]. Glutaredoxin and glutathione are also used by some MSRs as the regenerating system [13–17]. Prokaryotes and single-cell eukaryotes possess another enzyme, f*R*MSR, which reduces the free form of the *R*-diastereomer of MetO [18,19]. In bacteria, several enzymes of the dimethyl sulfoxide reductase family containing a molybdenum cofactor can have a MSR activity, particularly, the biotin sulfoxide reductase BisC, which reduces only the free form of the *S*-diastereomer of MetO and was shown to have relevant activity for MetO reduction *in vivo* [20]. Very recently, another molybdoenzyme was identified in the bacterial periplasm and shown to reduce free and protein-based MetO without any stereospecificity, making it a 'lethal weapon' for the protection of the bacterial envelop against deleterious protein oxidation [21,22].

Little is known regarding the effect of free Met oxidation in cells, but it was shown that, in the absence of an appropriate reducing enzyme, MetO cannot be used as a source of Met in auxotroph organisms [20,22,23]. In proteins, methionine residues are not equally sensitive to oxidation, but it appears that the surface-exposed ones are more sensitive to oxidation than the buried residues. Moreover, the amino acid environment determines the sensitivity to oxidation and the propensity to form one or the other diastereomer, although no sequence 'signature' of Met oxidation could be clearly defined [9,24,25]. Oxidation of Met in proteins can have several consequences (see [26,27] for reviews), and depending on these consequences, oxidized proteins can be classified into four groups [9]: (i) proteins not impaired by Met oxidation, which could fulfill, together with MSRs, an antioxidant function through cyclic oxidation and reduction of Met [28], (ii) proteins damaged by Met oxidation, such as those involved in neurodegenerative diseases [29–31], (iii) unfolded proteins and nascent polypeptides whose protein core Met are susceptible to oxidation thereby affecting their proper folding which has been shown to greatly accelerate their degradation [9], and (iv) proteins whose functions are actively regulated by cyclic Met oxidation/reduction. This class, where the oxidation is targeted and purposeful, is a novel emerging field in biochemistry and cell physiology. Studies in recent years yielded several new pathways involving redox modification of Met. For example, the oxidation can be a necessary biochemical reaction step in crosslinking collagen IV by peroxidasin in the extracellular matrix [32–34]. In other cases, the protein function is regulated by cyclic Met oxidation/reduction, such as the calcium/calmodulin-dependent protein kinase II [35], the bacterial HypT transcription factor [36], and the actin for which Met oxidation was shown to be catalyzed by MICAL monooxygenases [37–39].

Genetic studies and quantification of MSR activity showed that MSRs are implicated in the protection against oxidative stress in numerous organisms and suggest that MetO accumulation is linked with neurodegenerative diseases and aging [29,40–42]. However, the actual quantification of the MetO content was rarely made, and the data supporting these hypotheses are based on the idea that modification of MSR activity is correlated with the MetO content in cells. However, this does not offer a possibility to quantify the effect and help determine if the observed phenotype results from global protein oxidation or from the oxidation of few specific Met residues in key proteins. Related to this, it is particularly important to note that not all oxidized Met in proteins can be reduced by the MSR system depending on accessibility of MetO [9]. This was shown for the oxidized α-synuclein, which possesses four oxidizable Met, of which only two can be reduced *in vivo* by the MSR system [29]. To study oxidation and reduction of α-synuclein in cells, the authors used an interesting method which introduced an oxidized $^{15}$N-labeled protein into cells and followed its oxidation state by NMR.

Prior to the genetically encoded fluorescent sensors of MetO, for which a practical guide is given in this paper, two methods were published for quantification of MetO. The first is an HPLC-based method which consists of a total amino acid quantification in protein extracts [28,43]. This method allowed to estimate the levels of MetO in several tissue types and cells, which were 2–10% of total Met content, and to show their increase up to 60% in response to oxidative stress [28,44–46]. The second method is a proteomic approach that identified oxidized Met in protein extracts by mass spectrometry [24,47]. This protocol has the advantage of identifying the affected Met in protein contexts and reporting relative abundance of MetO for each protein. Neither HPLC nor proteomic methods require genetic manipulation, but