Radical production from free and peptide-bound methionine sulfoxide oxidation by peroxynitrite and hydrogen peroxide/iron(II)

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Abstract Methionine sulfoxide is a post-translational protein modification that has been receiving increasing attention in the literature. Here we used electron paramagnetic resonance spin trapping techniques to show that free and peptide-bound methionine sulfoxide is oxidized by hydrogen peroxide/iron(II)-EDTA and peroxynitrite through the intermediacy of the hydroxyl radical to produce both $^{\bullet}CH_3$ and $^{\bullet}CH_2CH_2CH$ radicals. The results indicate that methionine sulfoxide residues are important targets of reactive oxygen- and nitrogen-derived species in proteins. Since the produced protein-derived radicals can propagate oxidative damage, the results add a new antioxidant route for the action of the enzyme peptide methionine sulfoxide reductase.

Key words: Methionine sulfoxide; Methionine sulfoxide reductase; Protein-derived radical; Electron paramagnetic resonance; Oxidative stress; Aging

1. Introduction

Together with cysteine, tyrosine and tryptophan, methionine belongs to the most easily oxidized amino acids [1]. The first stable product of methionine oxidation is methionine sulfoxide, a post-translational protein modification whose cellular role has been increasingly investigated [1–10]. Thus, oxidation of methionine to the sulfoxide can result in significant conformational and/or functional changes in proteins and, indeed, specific protein activities have been shown to be both up- and down-regulated following methionine oxidation [1]. Also, in specially designed model peptides, methionine oxidation has been shown to serve as a conformational switch between α-helical and β-sheet structures [2], a process that has similarities to those occurring in neurodegenerative diseases [3,4]. Relevantly, other than glutathione oxidation, methionine sulfoxide is the only amino acid modification that counts on an enzymatic repair system, the ubiquitous peptide methionine sulfoxide reductase (Msr) (Fig. 1) [1,5–10]. Based on the potential reversibility of methionine sulfoxide formation through enzymatic repair, it has been suggested that methionine oxidation may have regulatory functions [5–9]. Moreover, the reversible oxidation of methionine to methionine sulfoxide may serve an endogenous antioxidant function within some proteins, where methionine reacts with oxidants that would otherwise attack functionally more important residues [10]. A pivotal role for the peptide Msr in the antioxidant defense and lifespan of an organism has been inferred from recent studies with mutants and transgenic animals [5–9].

Methionine oxidation other than sulfoxide formation has received less attention in the literature. However, if methionine acts as an antioxidant by reacting with reactive oxygen- and nitrogen-derived species [9,10], it should be oxidized by both two- and one-electron mechanisms [11,12]. Indeed, the oxidation of free and peptide-bound methionine to radical intermediates has been demonstrated before [11–15]. Likewise, methionine oxidation beyond the sulfoxide level may occur, particularly through one-electron mechanisms because methionine sulfone has rarely been detected under physiological conditions [1]. Here we show that, indeed, oxidation of free and peptide-bound methionine sulfoxide by hydrogen peroxide/iron(II)-EDTA and peroxynitrite produces free radical intermediates (Fig. 1).

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma, Merck or Fisher and were analytical grade or better. 3,5-Dibromo-4-nitrosobenzenesulfonic acid (DBNBS) and peroxynitrite were synthesized as previously described [16]. The concentration of peroxynitrite stock solutions was determined spectrophotometrically at 302 nm using an extinction coefficient of 1.67×10^3 M^{-1} cm^{-1} [16]. The dipeptide (GM) was from a commercial source and the tripeptide (GM(O)G) was synthesized manually. The nonapeptides (IRDRGPMYD and IRDRGPM(O)YD) were synthesized with a Shimadzu PSSM8 (Kyoto, Japan) automated benchtop simultaneous multiple solid-phase synthesizer. The C-terminal of the synthesized peptides was amidated. 9-Fluorenylmethoxycarbonyl-protected amino acids and TGR™ resin were purchased from Novabiochem (San Diego, CA, USA). All peptides were cleaved from the resin by treatment with King's reagent (80% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% ethanedithiol, 5.0% anisol, 5.0% water, and 5.0% phenol) [17]. The peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column using an acetonitrile (5–95%) gradient in 0.1% trifluoroacetic acid. The HPLC instrument was from Shimadzu (Kyoto, Japan). The quality and characterization of the peptides was assessed by mass spectrometry (triple quadrupole Quattro II and MALDI-

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Abbreviations: EPR, electron paramagnetic resonance; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; MNP, 2-methyl-2-nitrosopropane; Msr, methionine sulfoxide reductase; peroxynitrite, the sum of peroxynitrite anion ($ONOO^-$, oxoperoxonitrate (−1)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified

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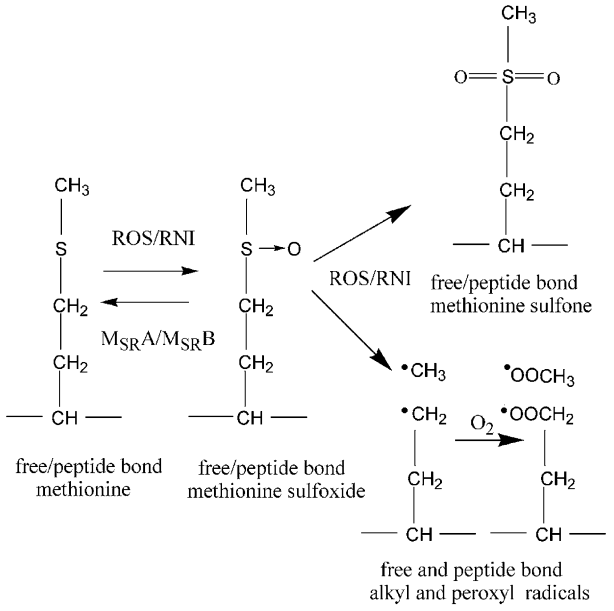

Fig. 1. Schematic representation of free and peptide-bound methionine oxidation by reactive oxygen (ROS) and nitrogen (RNI) species. Peptide-bound methionine sulfoxide can be repaired by the peptide Msr [5–10] or, as shown here, be further oxidized to radical intermediates.

TOF TofSpec-E Micromass spectrometers). Buffers were pre-treated with Chelex-100 to remove contaminant metal ions. All solutions were prepared with distilled water purified with a Millipore Milli-Q system.

2.2. Electron paramagnetic resonance (EPR) experiments

The incubation mixtures were transferred to a flat cell and the spectra recorded at room temperature 1 min after the addition of the oxidants on a Bruker ER 200 D-SRC upgraded to an EMX instrument. Computer simulation of spectra was performed using a program written by Duling [18].

3. Results and discussion

3.1. Radicals from free methionine sulfoxide

Incubations of methionine sulfoxide (10–50 mM) with hydrogen peroxide (1–3 mM), iron(II)-EDTA (0.2–0.5 mM) and DBNBS (20 mM) in phosphate buffer, pH 7.3, led to an EPR composite spectrum of two radical adducts (see, for instance, Fig. 2A). One of the adducts was easily identified as the DBNBS/$^{\bullet}CH_3$ radical adduct by its characteristic spectrum showing the quartets of three equivalent hydrogens (a_N = 1.43 mT; $a_{H(3)}$ = 1.36 mT; $a_{H(2m)}$ = 0.09 mT) (Fig. 2A) [16]. The spectrum of the second radical adduct showed three sets of triplets that indicated one nitrogen and two sets of equivalent hydrogens, one of them due to the meta hydrogens of DBNBS itself (a_N = 1.40 mT; $a_{H(2)}$ = 1.18 mT; $a_{H(2m)}$ = 0.05 mT). This suggests a DBNBS/$^{\bullet}CH_2$-R structure. To further characterize this radical, parallel experiments with a spin trap that lacks EPR-visible hydrogens, 2-methyl-2-nitrosopropane (MNP), were performed. In this case, the EPR composite spectrum contained mainly the MNP/$^{\bullet}CH_3$ (a_N = 1.72 mT; $a_{H(3)}$ = 1.43 mT) and the MNP/$^{\bullet}CH_2CH_2CH(NH_3^+)(COO^-)$ (a_N = 1.66 mT; $a_{H\beta(1)}$ = 1.21 mT; $a_{H\beta(1)}$ = 1.12 mT, $a_{H\gamma(2)}$ = 0.06 mT) radical adducts (Fig. 3) [14,19]. This result characterizes the DBNBS/$^{\bullet}CH_2$-R radical adduct (Fig. 2A) as the DBNBS/$^{\bullet}CH_2CH_2CH(NH_3^+)(COO^-)$ radical adduct.

Relevantly, no EPR signal was detected in the absence of iron(II)-EDTA (Fig. 2B) or hydrogen peroxide (data not shown), indicating that methionine sulfoxide oxidation depended on the hydroxyl radical produced from hydrogen peroxide/Fe(II)-EDTA [16,20]. In agreement, peroxynitrite, whose proton-catalyzed decomposition produces a 30% yield of nitrogen dioxide and hydroxyl radical [16,21–23], also oxidized methionine sulfoxide to produce the DBNBS/$^{\bullet}CH_3$ and the DBNBS/$^{\bullet}CH_2CH_2CH(NH_3^+)(COO^-)$ radical adducts (Fig. 2C). As expected, addition of methionine sulfoxide to previously decomposed peroxynitrite produced no detectable radical adducts (Fig. 2D). In this case, MNP was not used because its adducts are not stable in the presence of peroxynitrite [24].

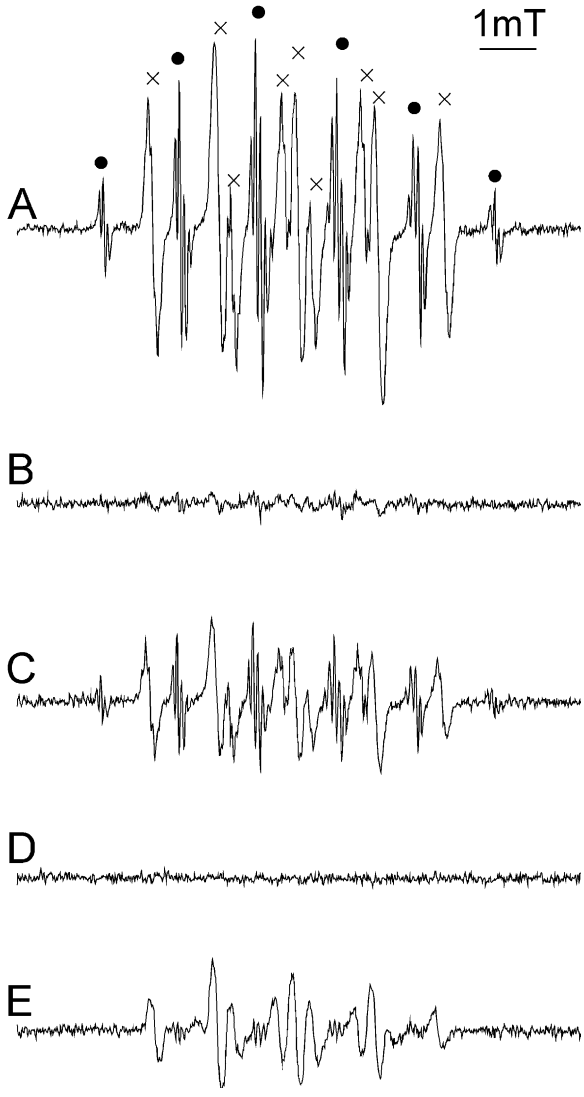

Fig. 2. EPR spectra of DBNBS radical adducts obtained from incubations of DBNBS (20 mM) in phosphate buffer (200 mM), pH 7.3, with: (A) methionine sulfoxide (50 mM), hydrogen peroxide (3 mM) and iron(II)-EDTA (0.5 mM); (B) the same as A except for the absence of iron(II)-EDTA; (C) methionine sulfoxide (50 mM) and peroxynitrite (1 mM); (D) decomposed peroxynitrite (1 mM) and methionine sulfoxide (50 mM); and (E) methionine (50 mM) and peroxynitrite (5 mM). Instrumental conditions: microwave power, 20 mW; time constant, 163.84 ms; sweep time, 167.77 s; modulation amplitude, 0.5 G; gain, 1×10^6. Detected radical adducts are labeled as (●) DBNBS/$^{\bullet}CH_3$ and (×) DBNBS/$^{\bullet}CH_2$R radical adducts.

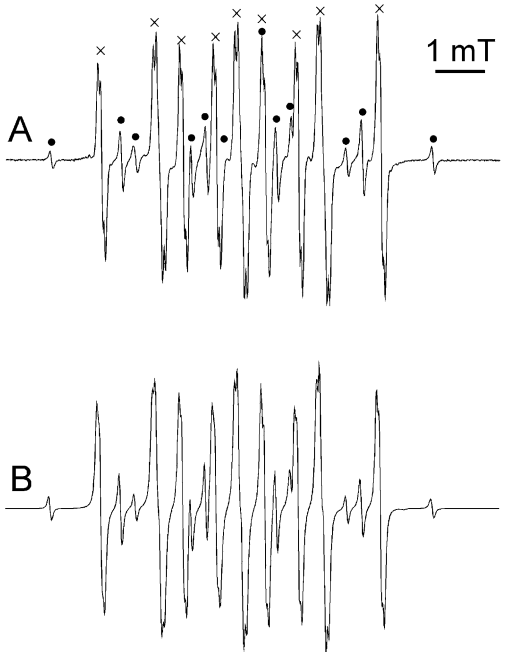

Fig. 3. EPR spectra of MNP radical adducts obtained from incubation of MNP (50 mM) in phosphate buffer (200 mM), pH 7.3, with methionine sulfoxide (50 mM), hydrogen peroxide (3 mM) and iron(II)-EDTA (0.5 mM) (A). B is the computer simulation of A considering two radical adducts (●) MNP/•CH_3 (8% yield) and (×) MNP/•$CH_2CH_2CH(NH_3^+)(COO^-)$ (92% yield) which were labeled in the composite spectrum of A. Instrumental conditions: microwave power, 20 mW; time constant, 327.680 ms; sweep time, 335.544 s; modulation amplitude, 0.5 G; gain, 2.0×10^4.

In the absence of methionine sulfoxide, none of the oxidants produced detectable adducts with MNP. With DBNBS, however, they produced the expected DBNBS/•OH radical adduct ($a_N = 1.26$ mT) as previously reported [25,26]. Taken together, these results show that methionine sulfoxide is oxidized by hydrogen peroxide/iron(II)-EDTA and peroxynitrite through the intermediacy of hydroxyl radicals to produce •CH_3 and •$CH_2CH_2CH(NH_3^+)(COO^-)$ radicals resulting from the cleavage of both thioether bonds (Fig. 1).

Peroxynitrite [27,28] and hydrogen peroxide/iron(II)-EDTA [11,12] are known to oxidize methionine to methionine sulfoxide among other products. The main radical adducts detected from methionine oxidation by these oxidants, however, were different from those produced from methionine sulfoxide oxidation (Fig. 2). Indeed, only traces of the DBNBS/•CH_3 radical adduct were detected from methionine and in the presence of high peroxynitrite concentrations (≥ 5 mM) (Fig. 2E). Under these and other tested conditions, the EPR spectrum was dominated by that of a radical adduct with one set of two equivalent hydrogens ($a_N = 1.40$ mT; $a_{H(2)} = 1.10$ mT). This indicates a DBNBS/•CH_2-R structure different from the one produced from methionine sulfoxide oxidation. The only possibility consistent with the methionine structure (Fig. 1) is the DBNBS/•$CH_2SCH_2CH_2CH(NH_3^+)(COO^-)$ radical adduct.

These results confirm and extend previous studies by demonstrating that methionine oxidation by peroxynitrite [27,28] and hydrogen peroxide/iron(II)-EDTA [11,12] produces methionine sulfoxide and radical intermediates. As we show here, methionine sulfoxide is oxidized to •CH_3 and •$CH_2CH_2CH(NH_3^+)(COO^-)$ radicals, although the latter was barely detectable in incubations starting with methionine itself (Figs. 2 and 3). These radicals, however, have been previously detected during treatment of methionine with stronger oxidants such as γ-irradiation and hydrogen peroxide photolysis [14,15]. Likewise, the •CH_3 and the •$CH_2CH_2CH(NH_3^+)$-(COO^-) radicals are likely to be produced in vivo because methionine sulfoxide has been found in various tissues [1].

3.2. Radicals from peptide-bound methionine sulfoxide

To investigate whether radical intermediates were produced from the oxidation of peptide-bound methionine sulfoxide, the tripeptide GM(O)G was synthesized and incubated with 1 mM peroxynitrite and DBNBS at pH 7.3. The EPR spectrum obtained (Fig. 4A) was similar to that obtained with free methionine sulfoxide (Fig. 2C) but less resolved, as expected from some degree of immobilization of a peptide-bound DBNBS/•CH_2CH_2CH-peptide radical adduct [24]. The DBNBS/•CH_3 radical adduct produced in parallel showed the expected mobile spectrum (Fig. 4A) confirming the cleavage of both thioether bonds of the methionine sulfoxide side chain (Fig. 1). Replacement of 1 mM peroxynitrite with 3 mM hydrogen peroxide/0.5 mM iron(II)-EDTA led to the same EPR spectrum except for the intensity that was higher (about twice) than that observed before in the case of free methionine sulfoxide oxidation (Fig. 2). When the GM(O)G tripeptide was replaced with the GM dipeptide, no signal was detected (Fig. 2B), even at higher peroxynitrite concentrations (up to 5 mM) (data not shown). This result suggests that the fate of

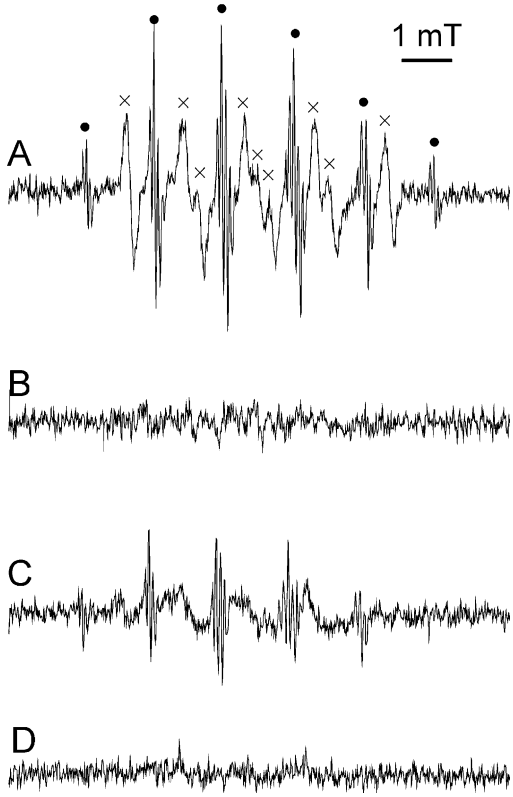

Fig. 4. EPR spectra of DBNBS radical adducts obtained from incubations of DBNBS (20 mM), peroxynitrite (1 mM) in phosphate buffer (200 mM) with: (A) GM(O)G (50 mM), pH 7.3; (B) GM (50 mM), pH 7.3; (C) IRDRGPM(O)YD (10 mM), pH 5.0, under a N_2 flow; and (D) IRDRGPMYD (10 mM), pH 5.0, under a N_2 flow. Instrumental conditions as described in Fig. 2.

the initially produced methionine sulfide radical cation in the dipeptide is different from that of the free methionine that yielded EPR-detectable radical adducts (Fig. 2E). In agreement, stabilization of peptide-bound methionine sulfide cation by interaction with nucleophiles such as the amino group of other residues has been described to occur during the one-electron oxidation of peptide-bound methionine [11,12].

Longer peptides containing either methionine sulfoxide (IRDRGPM(O)YD) or methionine (IRDRGPMYD) were also synthesized and treated with hydrogen peroxide/iron(-II)-EDTA and peroxynitrite. Again, no radical adduct was obtained from the oxidation of the nonapeptide containing methionine (Fig. 4D) whereas both DBNBS/$^{\bullet}CH_3$ and DBNBS/$^{\bullet}CH_2CH_2CH$-peptide radical adducts were obtained with the nonapeptide containing methionine sulfoxide (Fig. 4C). As expected, as the length of the peptide increased, the spectrum of the DBNBS/$^{\bullet}CH_2CH_2CH$-peptide radical adduct became more immobilized and then less resolved (compare Fig. 4A and C). The spectrum shown in Fig. 4C was obtained with 1 mM peroxynitrite at pH 5.0 but the same spectrum was obtained with peroxynitrite at pH 7.3 and with hydrogen peroxide/iron(II) at pH 7.3 (data not shown). What varied was the intensity of the signal, which was about the same for hydrogen peroxide/iron(II)-EDTA at pH 7.3 and peroxynitrite at pH 5.0 but lower for peroxynitrite at pH 7.3. This behavior is consistent with the fact that proton-catalyzed peroxynitrite decomposition produces a higher yield of hydroxyl radical able to oxidize exogenous targets at acid than at neutral pH [16,21].

It is important to note that the presence of an easily oxidizable amino acid such as tyrosine in the nonapeptide did not prevent oxidation of methionine sulfoxide or produce detectable radical adducts other than those derived from it (Fig. 4). This is in line with the properties of the hydroxyl radical that preferentially adds to tyrosine rather than oxidizing it to tyrosyl radicals [29]. Although the addition product can decay to the tyrosyl radical, the latter did not attain concentrations high enough to be trapped under the tested conditions (Fig. 4). Overall, these results indicate that methionine sulfoxide residues are important targets of the hydroxyl radical in proteins and that they are oxidized to $^{\bullet}CH_3$ and protein-derived alkyl radicals. These are reactive species that can propagate damage either directly [30,31] or through reaction with molecular oxygen to produce peroxyl radicals (Fig. 1) [32]. The damaging properties of protein-derived peroxides, in particular, have been extensively demonstrated [32].

4. Concluding remarks

The oxidation of methionine sulfoxide by the hydroxyl radical was not an unexpected result [11–15]. The major point here was to demonstrate that this oxidation occurs in peptides by the action of biologically important species such as peroxynitrite and hydrogen peroxide/iron(II). The peroxynitrite action was particularly relevant to demonstrate because its biological half-life (estimated to be less than 100 ms) is long enough to permit diffusion within biomolecules and even through cells [22]. In most biological environments peroxynitrite is expected to act through the radicals produced from its reaction with ubiquitous carbon dioxide, that is, nitrogen dioxide and carbonate radical anion. In acid pH, however, its proton-catalyzed decay to nitrogen dioxide and hydroxyl radical becomes relevant [21–23]. Thus, the oxidation of methionine sulfoxide residues to protein-derived radicals is likely to occur in acid environments such as those found at cell surfaces, in phagolysosomes of phagocytic cells and in ischemic tissues. Since the produced protein-derived radicals can propagate oxidative reactions, repair of methionine sulfoxide to methionine would reduce oxidative damage. In this scenario, our results add a new antioxidant route for the action of the enzyme peptide Msr.

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