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ORIGINAL ARTICLE

The reaction of methionine with hydroxyl radical: reactive intermediates and methanethiol production

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Abstract The mechanisms of reaction of methionine with hydroxyl radical are not fully understood. Here, we unequivocally show using electron paramagnetic resonance spin-trapping spectroscopy and GC–FID and GC–MS, the presence of specific carbon-, nitrogen- and sulfur-centered radicals as intermediates of this reaction, as well as the liberation of methanethiol as a gaseous end product. Taking into account the many roles that methionine has in eco- and biosystems, our results may elucidate redox chemistry of this amino acid and processes that methionine is involved in.

Keywords Methionine · Hydroxyl radical · EPR · GC · Free radical · Methanethiol

Introduction

The understanding of the mechanism of the reaction between methionine (Met) and hydroxyl radical ($^{\cdot}$OH) may have a great impact on environmental and biochemical studies. Met is present in wastewaters, sediments and freshwaters, as it is a component of decomposing proteins and involved in the sulfur cycle (Kiene et al. 1990; Caron and Kramer 1994; Higgins et al. 2008). In such systems, $^{\cdot}$OH is produced via iron- or copper-involving Fenton mechanism ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$), photo-Fenton reaction ($Fe^{3+} + \text{light} \rightarrow Fe^{2+}$ followed by Fenton reaction) or ionizing radiation-provoked radiolysis of water (Sedlak et al. 1997; White et al. 2003; Nakatani et al. 2007; Vermilyea and Voelker 2009). On the other hand, Met represents an essential amino acid for humans. Met is incorporated in proteins and it is important for methylation reactions and other important processes (Korendyaseva et al. 2010; Hasegawa et al. 2011). In living systems, $^{\cdot}$OH is prevalently produced by the incompletely chelated iron- or copper-involving Fenton reaction, which is implicated in a large number of human pathophysiologies (Valko et al. 2005; Kell 2010). Another very important source of $^{\cdot}$OH in living systems is from the radiolysis of water provoked by ionizing radiation, which may come from radiological events, environmental sources, occupational exposure and therapeutic irradiation (Spotheim-Maurizot and Davídková 2011).

The mechanism of Met + $^{\cdot}$OH reaction has undergone periodical revisions during the past, to take into account various reactive intermediates and end products (Taniguchi et al. 1972; Hiller et al. 1981; Hiller and Asmus 1983; Miller et al. 1998; Pogocki et al. 2001; Nukuna et al. 2001; Abedinzadeh 2001; Schöneich et al. 2003; Mishra et al. 2009). The identification of free radicals is a formidable task, due to their exceptionally short lifetime and versatility of reactive species produced in various systems (Halliwell and Whiteman 2004; Valko et al. 2007). Electron paramagnetic resonance (EPR) spin-trapping spectroscopy could overcome these limitations and represents probably

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the most efficient technique for qualitative detection of radicals. Sophisticated EPR spin-trap reagents such as BMPO (Zhao et al. 2001), are capable of simultaneously detecting different reactive species, which makes them appropriate for the detection of free radicals in systems whose redox chemistry has not been fully explored (Bačić et al. 2008; Spasojević 2010). The spin-trapping technique is based on the reaction in which the transient radical species reacts with 'EPR silent' spin-traps to yield a more persistent nitroxide spin-adducts. These are readily detected by EPR spectroscopy, whereby different species can be distinguished with high sensitivity. In addition, the end products of $^{\cdot}OH$-provoked Met decomposition still represent subjects of investigation. For example, the production of homocysteine in Met + $^{\cdot}OH$ reaction has not been recognized until recently (Bern et al. 2010), although the level of Met has been strongly correlated with the homocysteine level in biological systems (Mori and Hirayama 2000; Campolo et al. 2006; Bonaventura et al. 2009). Moreover, $^{\cdot}OH$-mediated degradation of Met in proteins has been proposed to be responsible for the emission of methanethiol (CH_3SH) and some other volatile sulfur compounds in different ecosystems (Kiene et al. 1990; Caron and Kramer 1994; Higgins et al. 2008), and it has been implicated in CH_3SH production in the biochemical milieu (Tzeng et al. 1990; Toborek and Hennig 1996; Lei and Boatright 2006, 2007). However, to the best of our knowledge, gaseous products of this reaction have not been shown clearly using a simple biochemical system.

Pertinent to these facts, the aim of the present study was to examine free radicals and gaseous end products of Met + $^{\cdot}OH$ reaction using EPR spin-trapping and GC–FID and GC–MS methods.

Materials and methods

Reagents

High purity methionine (TraceCERT) was purchased from Fluka (Buchs, Switzerland). Fenton reaction was performed by combining 1 mM H_2O_2 (Renal, Budapest, Hungary) and 0.2 mM $FeSO_4$ (Merck, Darmstadt, Germany). Methionine was added at the final concentration of 6.7 mM. Spin-trap BMPO (5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide) was purchased from Enzo Life Sciences International (Plymouth Meeting, PA, USA). The trap was added prior to the reaction initiation at a final concentration of 15 mM. All experiments were performed using bidistilled deionized ultrapure (18 MΩ) water. The pH value of the system composed of Fenton reaction reagents and methionine was 4.71.

EPR spectroscopy

EPR spectra were recorded using a Varian E104-A EPR spectrometer operating at X-band (9.572 GHz) with the following settings: modulation amplitude, 0.2 mT; modulation frequency, 100 kHz; microwave power, 20 mW; time constant, 32 ms; scanning time, 4 min. The temperature in the cavity was controlled at 293 K. Recordings were performed using EW software (Scientific Software, Bloomington, IL, US). Samples were drawn into 10 cm-long gas-permeable Teflon tubes to maintain constant O_2 level in the sample (wall thickness, 0.025 mm; internal diameter, 0.6 mm; Zeus industries, Raritan, NJ, USA). Measurements were performed using quartz capillaries in which Teflon tubes were placed. Recordings were conducted 2 min after the reaction had started. Spectral simulation of each spectrum was performed using WINEPR SimFonia computer program (Bruker Analytische Messtechnik GmbH, Darmstadt, Germany).

Gas chromatography

Samples (final volume 1 mL) were prepared in gas-tight vials with 1 mL of headspace and incubated for 15 min with steering. The GC–MS analyses were performed on an Agilent 7890A GC system equipped with 5975C inert XL EI/CI MSD and an FID detector connected by capillary flow technology two-way splitter with make-up gas. An HP-5 MS capillary column (Agilent Technologies, Santa Clara, CA, USA; 25-mm i.d., 30-m length, 0.25-μm film thickness) was used. Samples were injected manually in split mode at 10:1. The injection volume was 150 μl and the injector temperature was 250°C. The carrier gas (He) flow rate was 1.5 ml/min at 30°C (constant pressure mode). The column temperature was 30°C and run time was 5 min. The transfer line was heated to 280°C. The FID detector temperature was 300°C. EI mass spectra (70 eV) were acquired in *m/z* range of 10–550 and the ion source temperature was 230°C. A library search and mass spectral deconvolution and extraction were performed by using NIST AMDIS (Automated Mass Spectral Deconvolution and Identification System) software, ver. 2.64. The search was performed against our own library containing 4,951 spectra, and the commercially available NIST05 Willey07 library containing approximately 500,000 spectra.

Results and discussion

The mayor signal observed in the Fenton system originates from the adduct of $^{\cdot}OH$ radical—BMPO/OH, showing characteristic hyperfine coupling constants (HCC): a_N = 1.356 mT, a_H^{β} = 1.230 mT, a_H^{γ} = 0.066 mT (diastereomer

I; 81.6%) and $a_N = 1.347$ mT, $a_H^{\beta} = 1.531$ mT, $a_H^{\gamma} = 0.062$ mT (diastereomer II; 18.4%) (Zhao et al. 2001), as confirmed by spectral simulation (Fig. 1a). In the presence of Met, EPR spectrum was changed drastically (Fig. 1b), being composed of signals of BMPO/OH adduct (12%) and of the adducts of carbon-centered radical(s) BMPO/CR (67%; HCC: $a_N = 2.20$ mT, $a_H = 1.45$ mT), nitrogen-centered radical BMPO/NR (12%; HCC: $a_N = 1.45$ mT, $a_H = 1.65$ mT, $a_N = 0.19$ mT) and sulfur-centered radical BMPO/SR [9%; HCC: $a_N = 1.43$ mT, $a_H^{\beta} = 1.47$ mT, $a_H^{\gamma} = 0.13$ mT (diastereomer I; 57%) and $a_N = 1.436$ mT, $a_H^{\beta} = 1.66$ mT, $a_H^{\gamma} = 0.18$ mT (diastereomer II; 43%)]. These results are in line with previous assertion that carbon-, nitrogen- and sulfur-centered reactive intermediates are formed in the reaction of Met with ˙OH radical (Fig. 2).

Three distinct carbon-centered reactive intermediates have been proposed previously in ˙OH-provoked degradation of Met [Fig. 2, structures (1a–c)]. HCC that have been used here for BMPO/C adduct simulation ($a_N = 2.20$ mT, $a_H = 1.45$ mT) are similar to the previously reported parameters for BMPO/CH_3 adduct ($a_N = 2.17$ mT, $a_H = 1.53$ mT) (Xu and Kalyanaraman 2007). A slightly

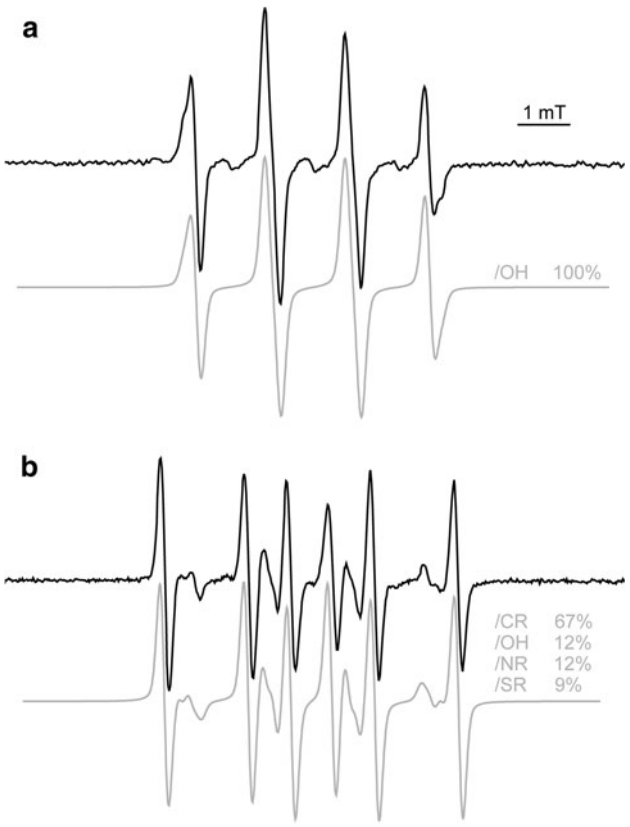

Fig. 1 EPR signals of BMPO adducts. **a** Fenton system; **b** Met + Fenton system. *Gray* spectral simulations with presented percentages of adducts contributing the spectrum

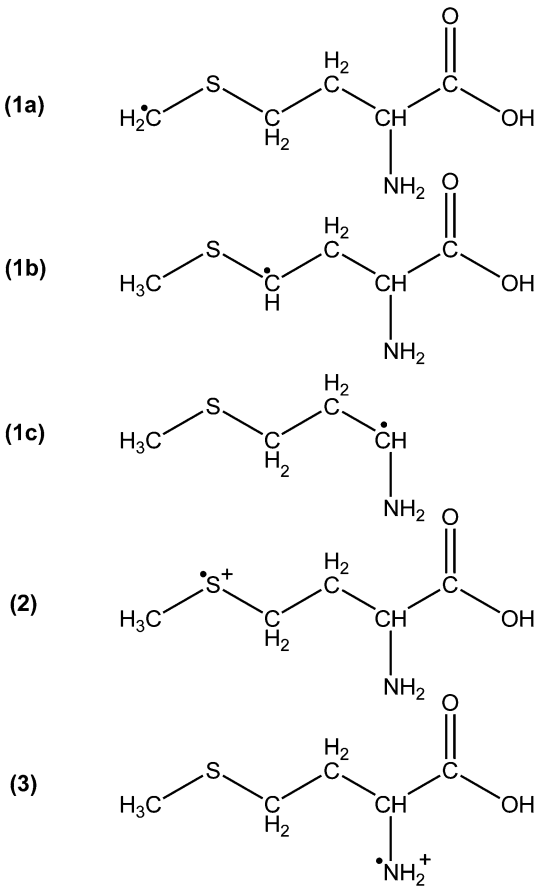

Fig. 2 Proposed structures of radical intermediates in the Met + ˙OH reaction. *1* Carbon-centered radicals; *2* sulfur-centered radical; *3* nitrogen-centered radical

smaller value for a_H may be explained by the presence of bulk structure in the Met-derived adduct pushing ^1H in BMPO further away from the unpaired electron, implying that the structure (1b) is the most probable. Although our results unequivocally show the presence of carbon-centered radical(s) in the system, we are not able to tell apart between three structures. HCC used here to simulate the signal of BMPO/SR adduct were previously reported for BMPO adduct of sulfur-centered radical with large bulk group (glutathionyl radical) (Zhao et al. 2001), which is similar to structure (2) presented in Fig. 2. Finally, to simulate the signal of BMPO/NR, we applied HCC previously reported for BMPO adduct formed with unpaired electron-bearing N-terminal NH_2 group of Gly–Gly–Gly tripeptide (Hawkins and Davies 2002). This verifies the production of nitrogen-centered radical on NH_2 group of Met in the presence of ˙OH [Fig. 2, structure (3)]. The presence of previously proposed $RSCH_2OO^{\cdot}$ radical intermediate (Francisco-Marquez and Galano 2009) was not observed.

Figure 3 presents the results obtained in the GC–FID and GC–MS analysis of headspace over the Met + ˙OH

system. The obtained data show that the degradation of Met initiated by ˙OH has methanethiol (CH_3SH) as an end gaseous product. An additional peak to the peak originating from air gases can be observed in Fig. 3a. The composition of this peak showing 1.3 min retention time was further analyzed using GC–MS (Fig. 3b). The *m/z* values obtained are from the following products: 48, M (methanethiol); 49, M +1 (+1 is from ^{13}C isotope); 50, M +2 (+2 is from ^{34}S isotope); 47, M −1 (−1 is from the loss of one ^{1}H via fragmentation); 46, M −2H; 45, M −3H; 33, SH. Interestingly, by comparing the arbitrary units for peaks 48 and 50, we obtained 23:1 ratio which is close to the ratio of $^{32}S/^{34}S$ isotope abundances in the nature verifying that the detected compound contains one sulfur atom. It should be stressed that EI-MS spectrum in Fig. 3b is almost identical to the spectrum of methanethiol available in the Wiley 07 NIST 05 database. Importantly, Met + ˙OH may also end in decarboxylation (Bobrowski and Schöneich 1996; Mishra et al. 2009). In our experimental setup, CO_2 signal in GC was overlapped by strong signal from air. However, the production of CO_2 in this reaction has been already shown (Bobrowski and Schöneich 1996), so we gave priority to performing experiments under settings resembling eco- and biosystems (air atmosphere).

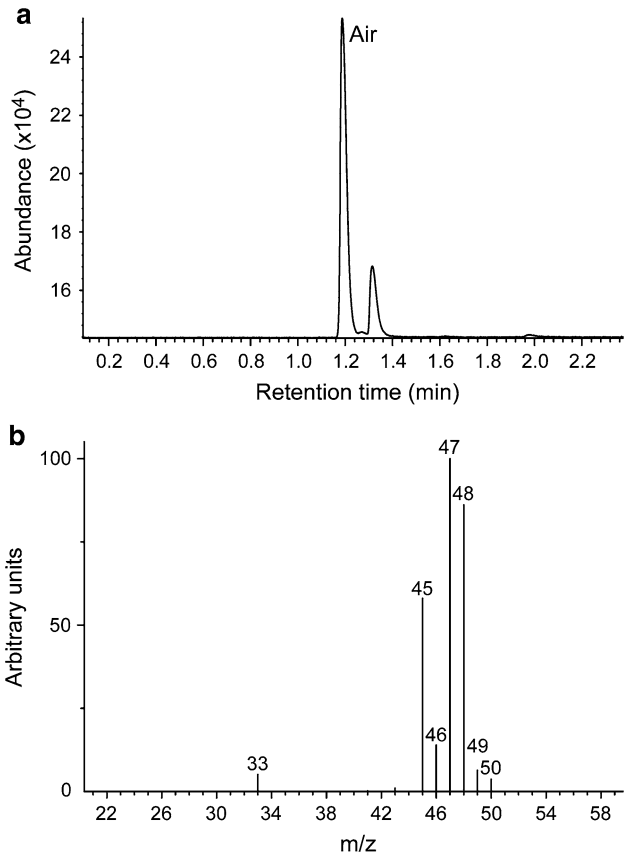

Fig. 3 GC–MS data of headspace over the Met + ˙OH water system. **a** Total ion current chromatogram; **b** EI-MS spectrum at 1.3 min retention time

At pH 4.71, Met behaves similarly in a rather wide pH range between 2.22 (pKa of –COOH group) and 9.27 (pKa of $–NH_3$) (Hiller and Asmus 1983), covering most of the pH values characteristic for ecological systems as well as the physiological pH values. It is important to note that Met reacts with ˙OH very rapidly, showing the reaction rate between 7.4 and 8.9 × 10^9 dm^3 mol^{-1} s^{-1} at pH ca. 7 in water (http://kinetics.nist.gov/solution/index.php). The knowledge of Met + ˙OH reaction mechanism is interesting by itself, but the presence of specific reactive intermediates is particularly important for living systems, since differently 'centered' radicals show different biological targets, which is in contrast to the generally non-selective ˙OH radical (Halliwell and Gutteridge 2007; Jacob and Winyard 2009). Showing higher one-electron reducing potential (Buettner 1993), carbon-centered radicals may target thiol groups (e.g., in cysteine) to produce thiyl radicals (Hioe and Zipse 2010). In addition, carbon-centered radicals may perform demethylation combined with the production of another carbon-centered radical (Hioe and Zipse 2010). The biochemistry of sulfur-centered radicals is not fully understood, but it is known that they react with unsaturated fatty acids, such as arachidonic, linoleic and oleic, to form thioesters and carbon-centered radicals (Jacob and Winyard 2009). Met-based sulfur-centered radicals may also attack cysteine residues in proteins leading to the formation of protein-bound thiyl radicals or disulfide bonds (Hioe and Zipse 2010). Nitrogen-centered radicals are known to target thiol groups and double bonds in organic molecules (Nicolau and Dertinger 1970). The significance of knowing the reactive intermediates in ˙OH-provoked Met oxidation goes beyond potential secondary reactions. The oxidation of specific Met residues in proteins seems to be implicated in the regulation and maintenance of redox state of cells, but also in different human pathological conditions and aging (Butterfield and Boyd-Kimball 2005; Bigelow and Squier 2005; Choi et al. 2006). It is interesting that Met dietary restriction leads to increase in the glutathione pool (Richie et al. 1994). It seems plausible that reactive intermediates could play some role in these effects.

The oxidation of Met has been implicated previously in the production of CH_3SH in various water ecosystems (Kiene et al. 1990; Caron and Kramer 1994; Higgins et al. 2008). Our data unequivocally prove such assertions. A similar mechanism has been proposed for biological systems (Finkelstein and Benevenga 1986; Scislowski and Pickard 1994). Generally, CH_3SH is not present free in living systems, as it is rapidly metabolized or it binds to cysteine residues in proteins from which it may be liberated by reducing agents (Tangerman 2009). Methanethiol

inhibits catalase (Finkelstein and Benevenga 1986), one of the enzymes in the first line of antioxidative defense responsible for H_2O_2 removal, as well as various other enzymes (Valentine et al. 1987). It has been reported to affect some other aspects of metabolism, such as amino acid transport, protein and DNA synthesis, intracellular pH, cell migration, membrane permeability and electrical properties (De Santis et al. 1990; Johnson et al. 1992; Lancero et al. 1996), as well as mitochondrial functions (Scislowski and Pickard 1994).

Dietary restriction of Met and this amino acid only prolongs mammalian lifespan (Orentreich et al. 1993; Zimmerman et al. 2003; Miller et al. 2005). It is important to note that Western human populations generally consume levels of Met that are several-fold higher in comparison to dietary requirements (Sanz et al. 2006; Gomez et al. 2009). The negative effects of Met on lifespan have been strongly related with the unpleasant ability of this amino acid to promote oxidative stress, which is implicated in the mechanism of aging. Met restriction strongly decreases the production of reactive oxygen species in mitochondria, as well as oxidative damage in proteins, membranes and mitochondrial DNA, while increased supplementation of Met shows just the opposite effects (Orentreich et al. 1993; Sanz et al. 2006; Gomez et al. 2009). However, the mechanisms via which Met acts in such a manner in mammalian cells are not understood. Taking into account that (i) presented intermediates target biological molecules by attacking thiol groups, double bonds and performing demethylation, and that (ii) methanethiol acts as a mild toxin by inhibiting antioxidative enzymes, components of mitochondrial electron transfer chain and other aspects of metabolism, our results may help understand the mechanisms by which methionine promotes oxidative stress and aging in mammalian cells.

It seems that the oxidation of Met may play quite a different role in the mechanisms initiated by ionizing radiation. Thiol-containing compounds, including cysteamine and cysteine are known to possess radioprotective properties, but their therapeutic utility is limited by their side effects at therapeutic doses. To avoid this, prodrugs with 'shielded' thiol groups, such as thiazolidine compounds and alpha-methyl-homocysteine thiolactone, have been examined showing positive beneficial effects (Roberts et al. 1995; Koch et al. 1997). There is a very interesting analogy of Met with these prodrugs, since Met also comprises 'shielded' –SH group, which becomes free following the decomposition, in the form of homocysteine (Bern et al. 2010) or CH_3SH (as shown here). While the radioprotective properties of homocysteine have been documented (Koch et al. 1997), the effects of methanethiol in systems exposed to high-dose radiation are to be further investigated. However, $^{\cdot}OH$ radical-initiated production of CH_3SH from Met, and methanethiol-provoked inhibition of catalase (Valentine et al. 1987) may at least partially explain how low-dose irradiation activates signaling cascades leading to beneficial effects known as hormesis (Rattan 2008). According to this sequence of events, radiation-provoked oxidation of Met in living systems results in increased level of H_2O_2, which represents a signaling species that may initiate a multifaced adaptation response (Kim et al. 2001). For example, low-dose radiation has been reported to stimulate cell proliferation via MAPK/ERK pathway (Liang et al. 2011), which is known to be activated by H_2O_2 (Bhat and Zhang 1999).

Interestingly, Valentine et al. (1987) have proposed that CH_3SH may represent a gaseous signaling species almost 30 years ago. The hypothesis has been overlooked thereafter, but the emerging results require that the potential signaling function of CH_3SH need to be reconsidered. Nevertheless, in comparison with NO, CO and H_2S, CH_3SH may represent an excellent tool for examination of mechanisms underlying gaseous signaling function.

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Conflict of interest The authors declare that they have no conflict of interest.

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