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Original Contribution

Catalytic activity of selenomethionine in removing amino acid, peptide, and protein hydroperoxides

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ABSTRACT

Selenium is a critical trace element, with deficiency associated with numerous diseases including cardiovascular disease, diabetes, and cancer. Selenomethionine (SeMet; a selenium analogue of the amino acid methionine, Met) is a major form of organic selenium and an important dietary source of selenium for selenoprotein synthesis in vivo. As selenium compounds can be readily oxidized and reduced, and selenocysteine residues play a critical role in the catalytic activity of the key protective enzymes glutathione peroxidase and thioredoxin reductase, we investigated the ability of SeMet (and its sulfur analogue, Met) to scavenge hydroperoxides present on amino acids, peptides, and proteins, which are key intermediates in protein oxidation. We show that SeMet, but not Met, can remove these species both stoichiometrically and catalytically in the presence of glutathione (GSH) or a thioredoxin reductase (TrxR)/thioredoxin (Trx)/NADPH system. Reaction of the hydroperoxide with SeMet results in selenoxide formation as detected by HPLC. Recycling of the selenoxide back to SeMet occurs rapidly with GSH, TrxR/NADPH, or a complete TrxR/Trx/NADPH reducing system, with this resulting in an enhanced rate of peroxide removal. In the complete TrxR/Trx/NADPH system loss of peroxide is essentially stoichiometric with NADPH consumption, indicative of a highly efficient system. Similar reactions do not occur with Met under these conditions. Studies using murine macrophage-like J774A.1 cells demonstrate a greater peroxide-removing capacity in cells supplemented with SeMet, compared to nonsupplemented controls. Overall, these findings demonstrate that SeMet may play an important role in the catalytic removal of damaging peptide and protein oxidation products.

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Hydroperoxides are a major product of the reactions of radicals and excited-state species with biological molecules in the presence of molecular oxygen. These species can be generated on most biological targets and are formed in particularly high yields on lipids and proteins. Proteins are likely to be a major target for such oxidation, as a result of their abundance in cells (proteins compose ca. 70% of the dry weight of cells), plasma, and most tissues and their rapid rates of reaction with many oxidants [1]. Hydroperoxides have been reported to be formed on most amino acids, peptides, and proteins on exposure to $HO^{\bullet}/O_2$ with up to 65% efficiency [2,3]. Hydroperoxides are also major products of singlet oxygen ($^1O_2$) attack on Tyr, Trp, and His residues of proteins (reviewed in [4]). Recent studies have demonstrated that hydroperoxides are also formed in plasma, cell lysates, and intact cells on exposure to systems that generate $^1O_2$ or reactive radicals [5–9].

Direct evidence for the formation of protein hydroperoxides in intact biological systems is lacking, as these materials readily decompose at elevated temperatures and in the presence of one- and two-electron reductants (reviewed in [1,4]). These reactions can induce damage to other biomolecules via both radical and molecular (two-electron) oxidation reactions. Thus damage can be induced on DNA (with resultant formation of oxidized DNA bases, strand cleavage, and formation of protein–DNA crosslinks) and other proteins (reviewed in [1,4]).

Oxidation of Cys residues by amino acid and protein hydroperoxides is a facile molecular reaction, with this resulting in inactivation of thiol-dependent enzymes (reviewed in [1,4]), including those critical to the maintenance of cellular reducing equivalents (glyceraldehyde-3-phosphate dehydrogenase), apoptosis (caspases), cell signaling (protein tyrosine phosphatases), protein turnover (cathepsins), Ca^{2+} levels (sarco-endoplasmic reticulum Ca^{2+}-ATPases), and reduced enzymatic cofactors (glutathione reductase). Thus initial oxidation of proteins to yield hydroperoxides can result in chain reactions and amplification of the initial damage (reviewed in [1]).

Previous studies have shown that exposure of macrophages to extracellular amino acid, peptide, and protein hydroperoxides [5,10] results in the loss of the hydroperoxide, formation of alcohols from the

Abbreviations: BSA, bovine serum albumin; FOX, Fe(II)–xylenol orange complex; GPx, glutathione peroxidase; GSSG, glutathione disulfide; GSH, L-glutathione; Sec, selenocysteine; SeMet, seleno-L-methionine; SeMetO, seleno-L-methionine selenoxide; Trx, thioredoxin; TrxR, thioredoxin reductase.

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hydroperoxide as a result of two-electron or possibly radical reactions [10,11], depletion of intracellular L-glutathione (GSH) and protein thiols, and loss of activity of the specific thiol-dependent enzymes cysteine proteases, including caspases 3/7 and cathepsins B and L [12].

Cytosolic antioxidant enzymes such as catalase, peroxiredoxin, glutathione peroxidase (GPx), thioredoxin (Trx), and thioredoxin reductase (TrxR) are responsible for the direct or indirect detoxification of biological peroxides. In mammalian cells, several isoforms of GPx and TrxR contain the selenium-containing amino acid selenocysteine (Sec) and utilize the redox properties of this amino acid in their catalytic activity. The selenium required for the synthesis of these selenoproteins comes from the diet and, particularly, seleno-L-methionine (SeMet) [13,14]. The corresponding sulfur-containing amino acid, methionine (Met), has been proposed to have beneficial dietary effects as a precursor of cysteine residues for GSH synthesis [15] and as a direct scavenger of reactive oxidants (both as a free amino acid and when incorporated into proteins) [1,16,17]. Previous studies have shown that SeMet can be randomly incorporated into proteins in place of Met [18], and there are limited data that this species can act as an oxidant scavenger. Thus previous studies have shown that SeMet can be oxidized by peroxynitrite, hypothiocyanous acid (with rate constants, k, in each case ~$2–3\times10^3\ M^{-1}\ s^{-1}$ [19,20]), and hydrogen peroxide [21]. Furthermore, it has been shown that the initial oxidation product, selenomethionine selenoxide (SeMetO), arising from oxidation of SeMet, can be recycled by low-molecular-mass thiols such as GSH and, to a lesser extent, ascorbate [22,23].

In the light of the above data we hypothesized that SeMet might react with amino acid-, peptide-, and protein-bound hydroperoxides and hydroperoxides present in human plasma. In addition we have examined whether the GSH and TrxR–Trx–NADPH systems can reduce the initial oxidation products, making reduction catalytic in nature. We have also investigated whether supplementation of cells with SeMet increased their capacity to detoxify these hydroperoxides.

Materials and methods

Materials

SeMet, Met, GSH, L-ascorbic acid, bovine lactalbumin, and soybean trypsin inhibitor were obtained from Sigma–Aldrich (Australia). *N*-Ac-Trp-OMe, Gly-His-Gly, and Gly-Tyr-Gly were from Bachem (Bubendorf, Switzerland). H_2O_2, bovine serum albumin (BSA; Cohn faction V, essentially fatty acid free), and NADPH were obtained from Roche (Castle Hill, Australia). *Escherichia coli* Trx and TrxR, and rat recombinant TrxR, were obtained from IMCO Corp. (Stockholm, Sweden). Blood was obtained from multiple healthy donors after informed consent by venipuncture, with plasma isolated by centrifugation at 1800*g*, for 10 min, at 4 °C. Plasma protein concentrations were determined using bicinchoninic acid reagent and diluted to 10 mg protein ml^{-1}. All other chemicals were commercial samples of high purity. Aqueous solutions and buffers were prepared in MilliQ Nanopure water.

Preparation and quantification of amino acid, peptide, and protein hydroperoxides

Hydroperoxides were generated on BSA (50 mg ml^{-1}), bovine lactalbumin (50 mg ml^{-1}), soybean trypsin inhibitor (10 mg ml^{-1}), *N*-Ac-Trp-OMe (1 mM), Gly-His-Gly (2.5 mM), Gly-Tyr-Gly (2.5 mM), and human plasma (10 mg ml^{-1}) by photolysis with visible light (from a Kodak S-AV 2050 slide projector) as described previously [12]. Samples were kept at 4 °C and continuously aerated during the photolysis period (BSA, bovine lactalbumin, and soybean trypsin inhibitor, ca. 45 min; *N*-Ac-Trp-OMe, Gly-His-Gly, Gly-Tyr-Gly, and plasma, ca. 60 min). On cessation of photolysis, samples were treated with catalase (Sigma; bovine liver, 5 mg ml^{-1}, 62.5 U ml^{-1}) for 10 min at 4 °C to remove any photogenerated H_2O_2 and subsequently frozen in aliquots at −80 °C. Hydroperoxide concentrations were determined by reaction with a Fe(II)-xylenol orange complex (FOX assay) in the absence of sorbitol [24]. The assay was standardized with commercial H_2O_2, and the hydroperoxide concentrations are reported as H_2O_2 equivalents due to the unknown stoichiometry of the FOX reagent with the hydroperoxides under study.

Reaction of hydroperoxides with SeMet

For time-dependent experiments, reaction mixtures (100 µl) containing hydroperoxides alone or mixtures of SeMet and hydroperoxides (1:1 molar ratio) were incubated for 0–30 min, for peptide-bound hydroperoxides, or 0–60 min, for H_2O_2 and BSA-bound hydroperoxides, at 22 °C. At the indicated time points, samples were diluted to 1 ml with MilliQ water and residual hydroperoxides quantified using the FOX assay. For dose-dependent experiments, 50 µl of hydroperoxide-containing samples (250–300 µM) were reacted with 50 µl of SeMet (0 to 20-fold molar excess, prepared in MilliQ water) at 22 °C. The reaction mixture was briefly vortexed, diluted to 1 ml with MilliQ water, and reacted with 50 µl of FOX reagents within 3 min.

Quantification of SeMet, oxidation products, and redox cycling by GSH and Trx–TrxR

The fate of SeMet and the products formed after reaction with hydroperoxides were studied by HPLC [23]. Twenty microliters of sample (filtered using a 0.2-µm Nanosep filter device; Pall Scientific, Australia) was injected onto a reverse-phase C-18 column (Beckman Ultrasphere ODS, 5 µm, 4.6×250 mm) and the materials were eluted at a flow rate of 1 ml min^{-1}, using a gradient solvent system. Solvent A (100%; 7.5 mM H_3PO_4, pH 2.3) was used for 6.5 min before initiation of a gradient to 5% solvent B (7.5 mM H_3PO_4, pH 2.3 in 50% (v/v) acetonitrile) over 1 min. This was maintained for 1 min before being further increased to 10% solvent B over 7 min. After a total of 15 min, the gradient reverted to 100% solvent A, with this maintained for another 9 min. Under these conditions SeMetO eluted at ca. 2.9 min, GSH at ca. 6.3 min, SeMet at ca. 8.2 min, and glutathione disulfide (GSSG) at ca. 14.3 min. Materials were detected by UV absorbance at 220 nm and identified and quantified using authenticated standards (GSH, SeMet, and GSSG from Sigma–Aldrich) except in the case of SeMetO, which was prepared by incubating SeMet with hypochlorous acid (HOCl) at a 1:1 molar ratio for 30 min at 22 °C, with the yield assumed to be 100%.

The ability of GSH to reduce SeMetO was investigated using a 10-fold molar excess of GSH (3 mM) added either before or after reaction of SeMet with hydroperoxides (1:1 molar ratio, 300 µM). The reactions were performed in MilliQ water and allowed to progress for 15 min at 22 °C before filtering and analysis by HPLC (as above).

The effect of GSH-mediated recycling of SeMet on removal of hydroperoxides was assessed by incubating hydroperoxides with a 0.1-fold molar concentration of SeMet (relative to the hydroperoxide concentration), a 10-fold molar excess of GSH, or both components for 0–30 min at 22 °C. Identical experiments were also carried out with Met in place of SeMet. Residual hydroperoxides were quantified by FOX assay at the indicated times. Analogous experiments assessing the effect of ascorbic acid-mediated recycling of SeMet were carried out using an equimolar concentration of ascorbic acid relative to the hydroperoxide concentration.

The capacity of a NADPH–Trx–TrxR system (and components of this) to reduce SeMetO was investigated in experiments using 250 µM SeMetO ± 500 µM NADPH ± 20 µM *E. coli* Trx ± 61.25 nM *E. coli* TrxR in 50 mM phosphate-buffered solution, pH 7.4, with samples incubated for 30 min at 22 °C. Rat recombinant TrxR (62.5 nM) was substituted for the *E. coli* enzyme in some experiments. Samples were filtered (as above) after incubation and subsequently analyzed for SeMetO and

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