Protection against Peroxynitrite by Selenoproteins*
Helmut Sies, Lars-Oliver Klotz, Victor S. Sharov, Annika Assmann and Karlis Briviba
Institut für Physiologische Chemie I and Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-Universität Düsseldorf, Postfach 101007, D-40001 Düsseldorf, Germany
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Cellular defense against excessive peroxynitrite generation is required to protect against DNA strand-breaks and mutations and against interference with protein tyrosine-based signaling and other protein functions due to formation of 3-nitrotyrosine. We recently demonstrated a role of selenium-containing enzymes catalyzing peroxynitrite reduction. Glutathione peroxidase (GPx) protected against the oxidation of dihydrorhodamine 123 (DHR) by peroxynitrite more effectively than ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a selenoorpoxidase, 2 x 10^6 M^-1 s^-1. The maintenance of protection by GPx against peroxynitrite requires GSH as reductant. Similarly, selenomethionine but not selenomethionine oxide exhibited inhibition of rhodamine 123 formation from DHR caused by peroxynitrite.

In steady-state experiments, in which peroxynitrite was infused to maintain a 0.2 µM concentration, GPx in the presence of GSH, but neither GPx nor GSH alone, effectively inhibited the hydroxylation of benzoate by peroxynitrite. Under these steady-state conditions peroxynitrite did not cause loss of 'classical' GPx activity. GPx, like selenomethionine, protected against protein 3-nitrotyrosine formation in human fibroblast lysates, shown in Western blots. The formation of nitrite rather than nitrate from peroxynitrite was enhanced by GPx, ebselen or selenomethionine.

The selenoxides can be effectively reduced by glutathione, establishing a biological line of defense against peroxynitrite.

The novel function of GPx as a peroxynitrite reductase may extend to other selenoproteins containing selenocysteine or selenomethionine.

Recent work on organo tellurium compounds revealed peroxynitrite reductase activity as well. Inhibition of dihydrorhodamine 123 oxidative correlated well with the GPx-like activity of a variety of diaryl tellurides.

Introduction
Peroxynitrite, a biological oxidant, is generated, e.g., by endothelial cells, Kupffer cells, neutrophils and macrophages (see (Beckman, 1997) for review). Peroxynitrite is a mediator of toxicity in inflammatory processes with strong oxidizing properties towards biological molecules, including sulphydryls, ascorbate, lipids, aminoaclids and nucleotides, and it can cause strand-breaks in DNA. Free or protein-bound tyrosine residues and other phenolics can be nitrated by peroxynitrite. Protein tyrosine nitration may interfere with phosphorylation/dephosphorylation signaling, and the in vivo occurrence of protein nitration in the human has been demonstrated (Beckman et al., 1994; MacMillan-Crow et al., 1996).

Peroxynitrite (ONOO^-) is a relatively stable species as compared with free radicals, but peroxynitrous acid (ONOOH) decays with a rate constant of 1.3 s^-1.

The selenium-containing compound, ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) (Masumoto and Sies, 1996a) and its main metabolite in vivo, 2-(methylseleno) benzalilide (Masumoto and Sies, 1996b), react with peroxynitrite very efficiently. Ebselen, selenocystein and selenomethionine protected DNA from single-strand break formation caused by peroxynitrite more effectively than their sulfur-containing analogs (Roussyn et al., 1996). Furthermore, these selenocompounds...
were protective in model oxidation and nitration reactions mediated by peroxynitrite (Briviba et al., 1996). Ebselen is known as a mimic of the GSH peroxidase (GPx) reaction. We hypothesized that its newly found reactivity with peroxynitrite mimics a so far undescribed peroxynitrite reductase activity of selenoproteins (Sies and Masumoto, 1997). Recent evidence established a protective function for GPx, ebselen and selenomethionine against peroxynitrite (Assmann et al., 1998; Sies et al., 1997b).

**Glutathione Peroxidase (GPx) Protects Against Peroxynitrite**

**Dihydrorhodamine 123 oxidation by peroxynitrite**

The peroxynitrite-mediated oxidation of dihydrorhodamine 123 to fluorescent rhodamine 123 is an efficient and selective probe of peroxynitrite production in model systems (Kooy et al., 1994). When peroxynitrite (100 nM) was added to 500 nM dihydrorhodamine 123, about 10 nM rhodamine 123 was formed, and addition of a GPx preparation from bovine erythrocytes up to 200 nM had no effect on rhodamine 123 formation. However, in the presence of GSH at the low concentration of 1 µM, GPx exhibited a pronounced inhibition of rhodamine 123 formation. The addition of 1 µM GSH alone, without GPx, led to a 15% loss of rhodamine 123 production. The half-maximal inhibitory concentration of GPx was 150 nM (for details, see Sies et al, 1997b).

**Hydroxylation of benzoate under steady-state infusion of peroxynitrite**

A suitable detector system for examining steady-state conditions is given by the hydroxylation of benzoate (Szabo et al., 1997). GPx in the presence of GSH completely suppressed benzoate hydroxylation, and the GSH/peroxynitrite ratio necessary for the inactivation of peroxynitrite in the presence of GPx was 2/1. These data (Sies et al., 1997b) established that GPx inactivates peroxynitrite in a catalytic reaction at the stoichiometry known for that of hydroperoxide reduction, i.e. the ‘classical’ GPx reaction.

**Nitrite formation from peroxynitrite**

As the spontaneous decay of peroxynitrite generates nitrate, the increase in the yield of nitrite rather than nitrate in the presence of selenocompounds is a measure of peroxynitrite reduction. There was an increase in the formation of nitrite from peroxynitrite by GPx and GSH in the steady-state. Correspondingly, the levels of nitrate were lowered.

**Ebselen**

Ebselen, an organoselenium compound with GPx-like activity, inhibits peroxynitrite-mediated oxidation of dihydrorhodamine 123 with a half-maximal inhibitory concentration of 0.2 µM whereas the oxidation product, ebselen selenoxide is practically ineffective.

**Selenomethionine**

Similarly, selenomethionine exhibits efficient protection against peroxynitrite-mediated oxidation of dihydrorhodamine, whereas methionine is less effective. The oxidation of selenomethionine by peroxynitrite leads to the formation of methionine selenoxide which does not protect against dihydrorhodamine 123 oxidation. Selenomethionine oxide can be reduced back to selenomethionine by thiols, i.e. GSH (Assmann et al., 1998).

Selenomethionine generates a pronounced increase (up to 70% at 0.5 mM) in nitrite formation when 100 µM peroxynitrite was employed (Sies et al., 1997b). This indicates successful competition with the spontaneous decay to nitrate.

**Organotellurium compounds**

The activities of selenoorganic compounds in inhibiting dihydrorhodamine 123 oxidation, benzoate hydroxylation and 4-hydroxyphenylacetate nitration are also found with a variety of organotelluric compounds (Briviba et al., 1998). Regarding the compounds tested, bis(4-aminophenyl) telluride offered the most pronounced protection against dihydrorhodamine 123 oxidation, being 11 times more effective than selenomethionine.

When peroxynitrite was infused to maintain a steady-state concentration, bis(4-aminophenyl) telluride in the presence of GSH, but neither bis(4-aminophenyl) telluride nor GSH alone, effectively inhibited peroxynitrite-mediated hydroxylation of benzoate. The capabilities of protecting against peroxynitrite-induced oxidation and nitrat-
tion reactions of a series of organotellurium compounds correlates with their glutathione peroxidase activity (Briviba et al., 1998).

Regarding nitration reactions, bis(4-hydroxyphenyl) telluride was most effective in inhibiting 4-hydroxyphenylacetate nitration with a half-maximal inhibitory concentration about 3–4 times lower than that of selenomethionine or ebselen.

Protein nitration in cells

Western blots from human fibroblast lysates exposed to peroxynitrite using a monoclonal anti-3-nitrotyrosine antibody showed several bands of nitrated protein, e.g. 25 kDa and 41 kDa, assigned to Mn-superoxide dismutase and actin, respectively (Sies et al., 1997b). Reduced GPx, but not oxidized (untreated) GPx, and selenomethionine as well as bis(4-aminophenyl) telluride were protective against tyrosine nitration by peroxynitrite. Ebselen also protected, yet less efficiently. From such data, the relative efficiencies in blocking protein nitration caused by peroxynitrite are found to be bis(4-aminophenyl) telluride > selenomethionine > ebselen. Thirty μM of reduced GPx completely abolished protein nitration. This is more effective than any of the low-molecular-weight compounds tested. As GPx is a tetramer, however, 30 μM of GPx corresponds to 120 μM of selenol, which still makes GPx more effective than ebselen and approximately as effective as selenomethionine.

Discussion

Peroxynitrite reductase

Selenoproteins, and selenocysteines in particular, carry out a variety of catalytic functions, many of which are redox reactions. We recently reported a novel function for selenoproteins, the reduction of peroxynitrite (Sies et al., 1997a, b). Our studies were prompted by the observation of a very efficient reduction of peroxynitrite by ebselen (Masumoto and Sies, 1996a), exhibiting the highest second-order rate constant for a low-molecular-weight compound with peroxynitrite known so far, 2.0 x 10^6 M⁻¹ s⁻¹ (Masumoto et al., 1996). In analogy to the reaction cycle for ebselen, Fig. 1A presents the proposed sequence: in the first step, the selenocysteine, probably as the selenolate, reacts with peroxynitrite to be oxidized to the corre-
sponding selenenic acid, yielding nitrite. However, peroxynitrous acid may also react to yield nitrous acid. The subsequent two steps in the reaction cycle are facile regeneration reactions at the expense of reducing equivalents provided by GSH in cells, known from the extensive work on GPx (see Flohé, 1989). Regarding the chemical mechanism, it might be concluded that the selenolate form of the selenocysteine residue is required. However, a selenol moiety is not strictly necessary for peroxynitrite reductase activity, in contrast to the GSH peroxidase action, since the carboxymethylated selenium derivative maintained activity. This is in accord with the high rate constant obtained for 2-(methylseleno) benzanilide (Masumoto and Sies, 1996b) and for selenomethionine (Padmaja et al., 1996).

Physiological significance

There is protection by selenoorganic compounds against peroxynitrite-induced single-strand breaks in plasmid DNA or base modifications sensitive to Fpg protein in bacteriophage DNA (Roussyn et al., 1996, Epe et al., 1996). It is possible that selenomethionine and selenocysteine residues in proteins in general may carry out similar functions, i.e. that selenoproteins or selenopeptides might have a biological function as a defense line against peroxynitrite (Briviba et al., 1996; Sies and Masumoto, 1997; Sies et al., 1997b). A number of different selenopeptides and selenoproteins, many of them with still unknown function, have been described in vivo.

While the 100- to 1000-fold higher second-order reaction rate constants of the selenium-containing compounds as compared to sulfur analogs make for a kinetic advantage, it should be considered that there are multiple other defense mechanisms against peroxynitrite in the organism. For example, there is prevention of the formation of peroxynitrite by control of nitric oxide synthase and by control of the level of nitric oxide by oxyhemoglobin and other binding sites, as well as control of superoxide levels by superoxide dismutase. Secondly, there are reactions of peroxynitrite, once formed, with other compounds such as ascorbate (Whiteman and Halliwell, 1996), GSH (Quijano et al., 1997) or CO₂ (Zhu et al., 1992; Gow et al., 1996), all of which will share in the modulation of potentially deleterious reactions caused by peroxynitrite.

Organoselenium compounds

A special feature of the peroxynitrite reductase activity of selenoproteins resides in the catalytic nature and in the high efficiency of the reaction. The capacity of protecting against peroxynitrite-dependent reactions can be maintained in the presence of thiol equivalents at micromolar concentrations. While the reduction of methionine sulfoxide requires the enzymatic activity of methionine sulfoxide reductases (Levine et al., 1996), glutathione is effective in reducing selenomethionine oxide (Assmann et al., 1998), suggesting that non-enzymatic regeneration of organoselenium compounds is sufficient. Thus, there is a low-molecular-weight defense system against peroxynitrite maintained by selenosubstituted methionine and cysteine residues in proteins, using glutathione (Assmann et al., 1998).

Organotellurium compounds

Not only organosulfur or organoselenium compounds but, as one proceeds down in group 16 of the periodic table, also organotellurium compounds with glutathione peroxidase-like activity protect against oxidation and nitration reactions caused by peroxynitrite (Briviba et al., 1998). The previously observed GPx-like activity of diaryl tellurides (Andersson et al., 1993) has been ascribed to the ready oxidation of the heteroatom from the divalent to the tetravalent telluroxide state by hydrogen peroxide or organic hydroperoxides. As with GPx, regeneration of the active species then occurs via thiol reduction with disulfide formation. We suggest that diaryl tellurides act as scavengers of peroxynitrite by an oxygen transfer mechanism similar to that observed with hydroperoxides.

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