Protection against Peroxynitrite by Selenoproteins*

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Z. Naturforsch. **53c,** 228–232 (1998); received January 15/February 2, 1998

Peroxynitrite, Glutathione Peroxidase, Phospholipid Hydroperoxide Glutathione Peroxidase, Selenium, Ebselen

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 selenoorganic compound exhibiting a high second-order rate constant for the reaction with peroxynitrite, 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 organotellurium compounds revealed peroxynitrite reductase activity as well. Inhibition of dihydrorhodamine 123 oxidation 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 sulfhydryls, ascorbate, lipids, aminoacids 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) benzanilide (Masumoto and Sies, 1996b), react with peroxynitrite very efficiently. Ebselen, selenocystine 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

* This contribution was presented at a Workshop on the occasion of the 20th anniversary of the "Oxygen Club Munich", organized by M. Saran and E. F. Elstner.

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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 spontanous 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 nitra-

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 selenocysteine 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, $2.0 \times 10^6\ M^{-1}s^{-1}$ (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-

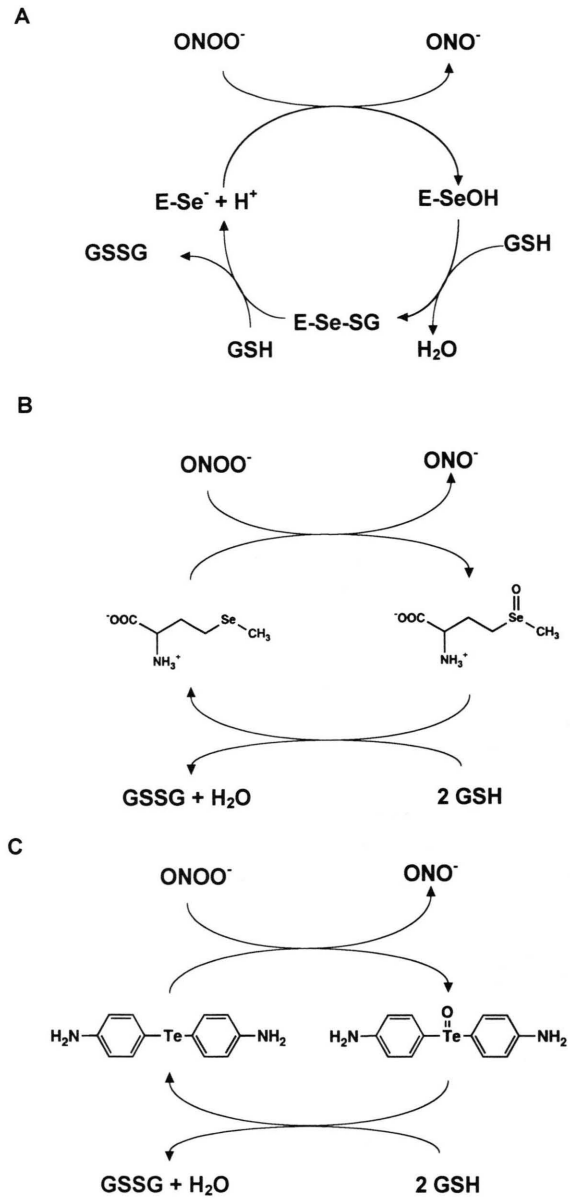

Fig. 1. Proposed catalytic mechanism of selenoperoxidases (A), selenomethionine (B), and diaryl tellurides, as exemplified by bis(4-aminophenyl) telluride (C), in the reduction of peroxynitrite to nitrite (or peroxynitrous acid to nitrous acid).
The mechanism for (A) is based on that established for GSH peroxidases and the mimic, ebselen (Flohé (1989); Sies (1993); Maiorino *et al.* (1995)) which use ROOH and ROH as substrate and product, respectively. See Sies *et al.* (1997b); Briviba *et al.* (1998).
GSH, GSSG = reduced, oxidized glutathione, respectively.

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 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 CO_2 (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.

Acknowledgements

We are grateful to Dr. Hiroshi Masumoto for helpful comments.

This study was supported by the Deutsche Forschungsgemeinschaft, SFB 503, Project B1, and by the National Foundation for Cancer Research, Bethesda. V. S.Sharov was a Research Fellow of the Alexander von Humboldt Foundation, Bonn, Germany.

Andersson C. M., Hallberg A., Brattsand R., Cotgreave I. A., Engman L. and Persson J. (1993), Glutathione peroxidase-like activity of diaryl tellurides. Bioorg. Med. Chem. Lett. **3,** 2553–2558.

Assmann A., Briviba K. and Sies H. (1998), Reduction of methionine selenoxide to selenomethionine by glutathione. Arch. Biochem. Biophys. **349**, 201–203

Beckman J. S., Ye Y. Z., Anderson P. G., Chen J., Accavitti M. A., Tarpey M. M. and White C. R. (1994), Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. Biol. Chem. Hoppe Seyler **375**, 81–88.

Beckman J. S. (1996), The physiological and pathological chemistry of nitric oxide. In: Nitric Oxide. Principles and Actions (J. Lancaster, ed.) Academic Press San Diego, pp. 1-82.

Briviba K., Roussyn I., Sharov V. S. and Sies H. (1996), Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. Biochem. J. **319**, 13–15.

Briviba K., Tamler R., Klotz L. O., Engman L., Cotgreave I. A. and Sies H. (1998), Protection by organotellurium compounds against peroxynitrite-mediated oxidation and nitration reactions. Biochem. Pharmacol. **55**, 817–823.

Epe B., Ballmaier D., Roussyn I., Briviba K. and Sies H. (1996), DNA damage by peroxynitrite characterized with DNA repair enzymes. Nucleic Acid Res. *24*, 4105–4110.

Flohé L. (1989), The selenoprotein glutathione peroxidase. In: Glutathione. Chemical, Biochemical and Medical Aspects. Part A (Dolphin, D., Poulson, R., and Avramovic, O., eds.). Wiley Interscience, New York, pp. 643–731.

Gow A. J., Duran D., Malcolm S. and Ischiropoulos H. (1996), Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS Lett. **385**, 63–66.

Kooy N. W., Royall J. A., Ischiropoulos H. and Beckman J. S. (1994), Peroxynitrite-mediated oxidation of dihydrorhodamine 123. Free Radic. Biol. Med. *16*, 149–156.

Koppenol W. H., Kissner R. and Beckman J. S. (1996), Syntheses of peroxynitrite: to go with the flow or on solid grounds? Methods Enzymol. **269**, 296–302.

Levine R. L., Mosoni L., Berlett B. S. and Stadtman E. R. (1996), Methionine residues as endogenous antioxidants in proteins. Proc. Natl. Acad. Sci. USA **93**, 15036–15040.

MacMillan-Crow L. A., Crow J. P., Kerby J. D., Beckman J. S. and Thompson J. A. (1996), Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc. Natl. Acad. Sci. U. S. A. **93**, 11853–11858.

Maiorino M., Aumann R., Brigelius-Flohé R., Doria D., Heuvel v.d. J., McCarthy J., Roveri A., Ursini F. and Flohé L. (1995), Probing the Presumed Catalytic Triad of Selenium-Containing Peroxidases by mutational analysis of phospholipid hydroperoxide glutathione peroxidase (PHGPx). Biol. Chem. Hoppe-Seyler **376**, 651–660.

Masumoto H., Kissner R., Koppenol W. H. and Sies H. (1996), Kinetic study of the reaction of ebselen with peroxynitrite. FEBS Lett. **398**, 179–182.

Masumoto H. and Sies H. (1996a), The reaction of ebselen with peroxynitrite. Chem. Res. Toxicol. **9**, 262–267.

Masumoto H. and Sies H. (1996b), The reaction of 2-(methylseleno)benzanilide with peroxynitrite. Chem. Res. Toxicol. **9**, 1057–1062.

Padmaja S., Squadrito G. L., Lemercier J. N., Cueto R. and Pryor W. A. (1996), Rapid oxidation of DL-selenomethionine by peroxynitrite. Free Radic. Biol. Med. **21**, 317–322.

Quijano C., Alvarez B., Gatti R. M., Augusto O. and Radi R. (1997), Pathways of peroxynitrite oxidation of thiol groups. Biochem. J. **322**, 167–173.

Roussyn I., Briviba K., Masumoto H. and Sies H. (1996), Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. Arch. Biochem. Biophys. **330**, 216–218.

Sies H. (1993), Ebselen, a selenoorganic compound as glutathione peroxidase mimic. Free Radic. Biol. Med. **14**, 313–323.

Sies H., Masumoto H., Sharov V. S. and Briviba K. (1997a), Defenses against peroxynitrite. In: Oxygen Homeostasis and Its Dynamics (Y. Ishimura, ed.) pp. 505–509. Tokyo: Springer Verlag.

Sies H., Sharov V. S., Klotz L. O. and Briviba K. (1997b), Glutathione peroxidase protects against peroxynitrite-mediated oxidations: a new function for selenoproteins as peroxynitrite reductase. J. Biol. Chem. **272**, 27812–27817

Sies H. and Masumoto H. (1997), Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. Adv. Pharmacol. **38**, 229–246.

Szabo C., Ferrer Sueta G., Zingarelli B., Southan G. J., Salzman A. L. and Radi R. (1997), Mercaptoethylguanidine and guanidine inhibitors of nitric-oxide synthase react with peroxynitrite and protect against peroxynitrite-induced oxidative damage. J. Biol. Chem. **272**, 9030–9036.

Whiteman M. and Halliwell B. (1996), Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants. Free Radic. Res. **25**, 275–283.

Zhu L., Gunn C. and Beckman J. S. (1992), Bactericidal activity of peroxynitrite. Arch. Biochem. Biophys. **298**, 452–457.