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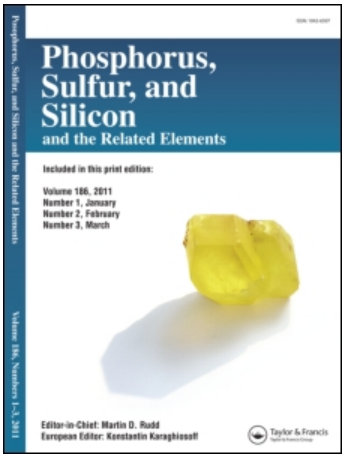

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Role of Selenium in the Enzymatic Reduction of Hydroperoxides

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Selenium-mediated catalysis in the enzymatic reduction of hydroperoxides is reviewed. Out of the growing number of mammalian selenoproteins four are peroxidases. Their common catalytic mechanism involves redox shuttling of a selenocysteine residue in the active site, where it forms a characteristic catalytic triad with hydrogen-bonded tryptophan and glutamine residues. These peroxidases differ in tissue distribution, substrate specificity, regulation, responsiveness to selenium restriction, and likely in their biological role. Cytosolic glutathione peroxidase, which predominates in balancing hydroperoxide toxicity, rapidly declines in selenium deficiency. Its activity can therefore be taken as a sensitive indicator of the selenium status. Indirectly, selenium can be involved in peroxide reduction by proteins formerly known as antioxidant proteins and now named peroxiredoxins. They reduce hydroperoxides by means of a particularly reactive cysteine residue. Some members of this protein family proved to be thioredoxin-dependent peroxidases. In mammals the regeneration of reduced thioredoxin depends on the selenoprotein thioredoxin reductase. Selenium supplementation proved mandatory in areas severely deficient in this trace element and may be considered reasonable also in asymptomatic moderate selenium deficiency whenever clinical conditions known to cause oxidative stress have to be anticipated.

Keywords: glutathione peroxidases, peroxiredoxins, reaction mechanism, substrate specificity, selenium

In Retrospect: Four Decades of Selenium Biochemistry

The seventh ICCST could look back to 40 years of research on selenium biochemistry and related deficiency syndroms in mammalian organisms. It was in 1957 when Klaus Schwarz searching for essential micronutrients discovered "factor 3", a low molecular weight selenium-containing compound of till now unidentified structure[1]. Factor 3, like a variety of bioavailable selenium-containing compounds, was able to prevent an acute liver necrosis in rats fed a diet deficient in selenium and vitamin E. Since, selenium has been discussed as an antioxidant trace element, but the molecular mechanism of this antioxidant action remained an enigma for another 15 years. In 1972 finally[2], another symptom of selenium deficiency, the tendency of red blood cells to undergo hemolysis upon oxidative stress, drew the attention to a metabolic pathway well known to balance the risks of aerobic life, the glutathione-mediated reduction of H_2O_2 at the expense of NADPH. Out of the enzymes involved, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase, and glutathione peroxidase (GPx) only the latter proved to be selenium-dependent[2] and could be shown to contain stoichiometric amounts of selenium[3].

The role of GPx as an integral component of the biological antioxidant system appeared well established and it became common to

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explain selenium deficiency symptoms in terms of inadequate GPx activities (for review of older literature see [4]). In the meantime, however, it has become evident that at least four distinct selenium-containing glutathione peroxidases coexist in mammals. They are phylogenetically related and their basic catalytic mechanism is identical, but they differ markedly in specificity, tissue-specific expression, regulation, and response to selenium supply (reviewed in [5]). Not only for this reason the monistic interpretation of the selenium deficiency syndrom as GPx deficiency needs to be revised. More recently the deiodinases responsible for the thyroid hormone metabolism proved to be selenoproteins[6, 7]. Further, a selenocysteine residue was discovered in human thioredoxin reductase[8], and the activity of this enzyme was demonstrated to respond to selenium depriviation / supplementation in tissue culture[9]. This implies that also the redox status of the pleiotropic redox mediator thioredoxin[10] may crucially depend on selenium[11, 12]. The biological role of further selenoproteins known by sequence such as the muscle selenoprotein W[13] and the extracellular selenoprotein P with up to twelve selenocysteine residues[14] is still largely unknown, and the abundance of putative selenoproteins evidenced by pulse labelling experiments with ^{75}Se[15] promises future surprises. For sure, mammalian organisms vitally depend on one or the other selenoprotein, since abrogation of the biosynthesis of all selenoproteins by genetic disruption of the selenocysteyl-t-RNA gene proved to be lethal in the homozygous state. This does not imply that selenoproteins are indispensible for life in general. Despite the abundance of selenoproteins in various phyla[16], exceptions obviously

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exist. The best documented example of a selenium-independent organism is yeast. Specific incorporation of selenium into a particular yeast protein could never be unambiguously demonstrated and the absence of any selenocysteyl-t-RNA gene in the yeast genome, which has now been sequenced completely, renders the synthesis of distinct selenoproteins extremely unlikely for this organism.

The Catalytic Mechanism of the Selenoperoxidases

The classical glutathione peroxidase (cGPx), like its congeners, reduces H_2O_2 as well as a large variety of alkylhydroperoxides with concomittant oxidation of glutathione (GSH) to the corresponding disulfide GSSG[4,5]. The catalytic mechanism involves oxidation followed by stepwise reduction of the enzyme itself. This type of catalysis is reflected in a kinetic pattern which is known as ping-pong kinetics and is characteristic of enzyme-substitution mechanisms. In essence, the selenocysteine moiety of the enzyme is oxidized by a hydroperoxide thereby adopting the oxidation state of a selenenic acid (eq. 1).

$$E\text{-}Se^- + ROOH + H^+ \xrightarrow{k_{+1}} E\text{-}SeOH + ROH \qquad (1)$$

The selenenic acid form of the enzyme readily reacts with a thiol, e.g. GSH, to form a intermediate in which the reducing substrate is covalently bound to the enzyme by a selenadisulfide bridge (eq. 2).

$$E\text{-}SeOH + GSH \xrightarrow{k_{+2}} E\text{-}Se\text{-}SG + H_2O \qquad (2)$$

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The ground state enzyme is then regenerated by means of a second GSH according to eq. 3:

$$\text{E-Se-SG} + \text{GSH} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \text{E-Se}^- + \text{GSSG} + \text{H}^+ \qquad (3)$$

The bimolecular reactions of eqs. 1-3, of course, may involve the formation of specific enzyme substrate complexes, which, however, are not evident from the kinetic analysis, since substrate saturation typical of Michaelis-Menten-type kinetics is not observed with the selenoperoxidases. This does not imply that such complexes are not formed. It rather means that their formation is slower than the consecutive formation of enzyme intermediates. The infinitely high Michaelis constants, which are observed with all these enzymes, do therefore not necessarily indicate a low affinity between enzymes and substrates but may instead reflect the high reactivity within the complexes. In the first step of the catalytic cycle the formation of a typical enzyme substrate complex is indeed unlikely, since the enzymes apparently react promiscuously with any hydroperoxo group which is sterically accessible. In contrast, cGPx at least is highly specific for GSH which can only be explained by the interim formation of specific complexes. Plausible models of such complexes of the oxidized enzymes forms and GSH have been constructed[17] based on the established three-dimensional structure[18]. They involve primarily electrostatic interactions with arginine and lysine residues surrounding the catalytic centre with the negatively charged carboxy groups of GSH, whereby the SH group of the substrate is forced into close

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proximity to the oxidized selenium in the active site of the enzyme. Pertinent molecular dynamics calculations also reveal that the optimum orientation of the highly flexible peptide substrate may take considerably longer than the reaction of its thiol group with the active site selenium once a reasonable strategic orientation has been achieved.

The rate constants for the reaction of the reduced selenoperoxidases with H_2O_2 (see eq. 1, k_{+1}) range between 10^7 and 10^8 M^{-1} s^{-1} and, thus, come close to diffusion-limited rates if futile collisions are taken into account. This extremely high reactivity in the active site selenium is obviously achieved by a particular catalytic triad of amino acid residues wherein the selenol of selenocysteine is hydrogen-bonded to the imino group of a tryptophan and the amido group of a glutamine residue (see Fig. 1A). Thereby the selenol is forced into full dissociation and freely exposed at the enzyme surface. The catalytic relevance of this configuration had already been inferred from the x-ray analysis of cGPx[18], was strongly supported by modelling the ground state of cGPx and homologous selenoperoxidases[5], and could be unambiguously demonstrated by kinetic analysis of molecular mutants of phospholipid hydroperoxide glutathione peroxidase (PHGPx)[19]. These investigations first of all underlined the pecularity of selenium versus sulfur in reacting with peroxides. Substituting cysteine for selenocysteine in PHGPx caused a decrease in k_{+1} by three orders of magnitude[19]. A similar decline of reactivity had previously been shown when the analogous substitution was performed in cGPx[20]. Double mutants in which the cysteine-coordinated tryptophan and glutamine residues were replaced by

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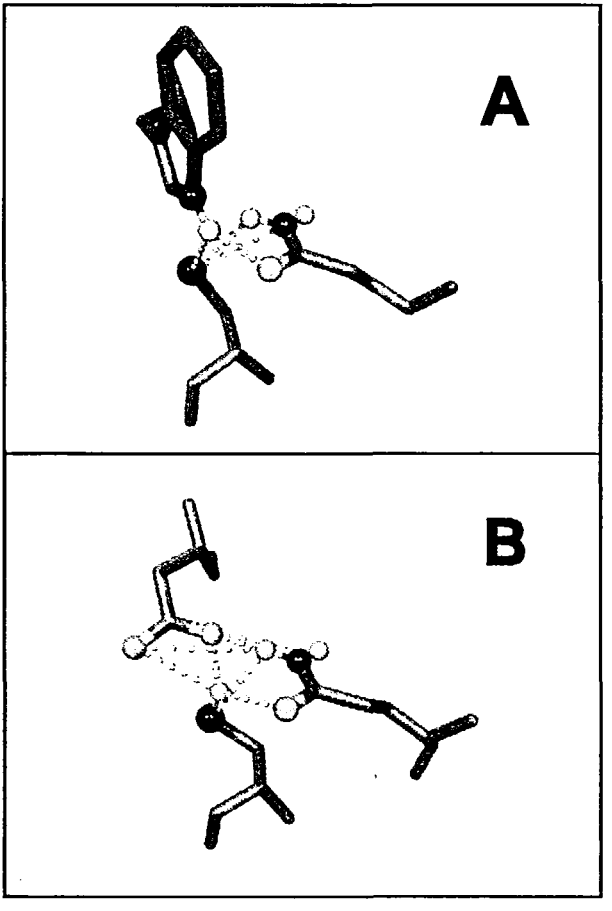

FIGURE 1 Molecular models of the catalytic triad of PHGPx muteins. The lines of small balls represent potential hydrogen bridges as evidenced by energy minimization and molecular dynamics simulation[19]. A: Cysteine homolog of bovine PHGPx showing the SH function of cysteine 46 being forced into dissociation by hydrogen bonds arising from the amido group of glutamine 81 and the imino group of tryptophan 136. B: Double mutant having tryptophan 136 exchanged against an acid aspartic residue favouring association of cysteine 46 and thereby rendering this mutant almost unreactive towards peroxides.

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histidine remained reasonably active suggesting that the proton donating potential of histidine can functionally substitute for the activation by the tryptophan and glutamine residues. In contrast, the k_{+1} values shrinked to hardly measurable levels, when tryptophan or glutamine was replaced by acidic residues forcing the cysteine SH into association[19] (Fig. 1B). The particular arrangement of selenocysteine, tryptophan, and glutamine, which is strictly conserved in all selenoperoxidases and with cysteine in further members of the GPx family of proteins, has thus to be considered a novel catalytic triad optimizing selenium or sulfur, respectively, for the reaction with hydroperoxides.

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Diversifications within the Glutathione Peroxidase Family of Proteins

With the advance of fast sequencing techniques more and more genes and protein sequences are being discovered revealing that the GPx family of proteins is an ancient one and has spread over all phyla of life (Fig. 2). Interestingly, however, in most of the examples cysteine replaces selenocysteine in the catalytic centre. The biological role of those GPx homologs is unclear. Based on the kinetic analysis of artificially produced cysteine homologs of mammalian GPx species[19,20], they cannot be considered as efficient peroxidases. In *Plasmodium falciparum*, which is known to be highly susceptible to peroxides, such cysteine homolog of GPx might represent the only device substituting for the more efficient heme- or selenium-containing peroxidases[22]. Other observations like induction by salt stress in *Citrus*

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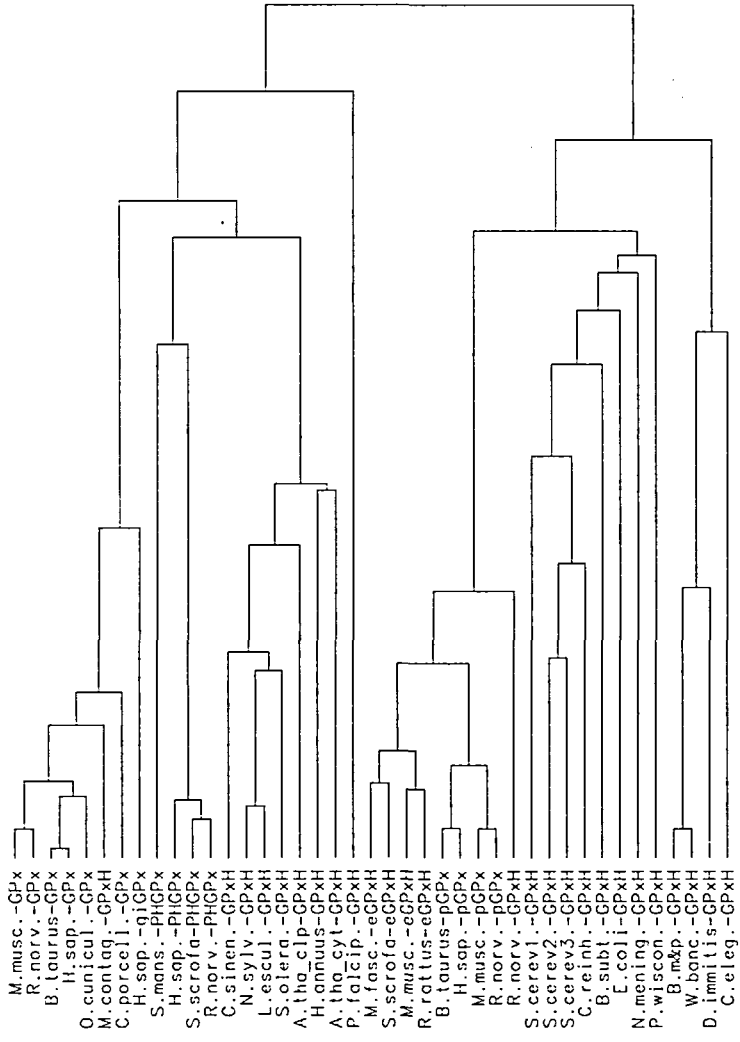

FIGURE 2 Dendrogram of the GPx superfamily of proteins. Molecular entities abbreviated as GPx (GPx, cellular glutathione peroxidase; pGPx, plasma glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; giGPx, gastrointestinal glutathione peroxidase) are selenoproteins. The

cysteine-containing homologs are abbreviated as GPxH (GPxH, cellular glutathione peroxidase homolog; eGPxH, epididymal glutathione peroxidase homolog). The molecular evolution within the protein family was calculated by the GENMON program, version 5.0 (GBF, Braunschweig, Germany) based on conserved amino acid residues corresponding to exon 3 of porcine PHGPx[21]. The real selenoperoxidases are clearly split into four molecular clades; a viral cGPx homolog (*Molluscum contagiosum*) closely related to mammalian cGPx is suggestive of being derived from natural gene transfer; the mammalian extracellular GPx homologs (eGPxH) are obvious late offsprings of the pGPx types, while the GPxH groups of plants, bacteria and lower eukaryots debranched independently earlier. Sequences were obtained from either SWISSPROT or GENEBANK. Species abbreviations and accession numbers (in brackets) are as follows: **GPx**: M.musc., *Mus musculus* (P11325); R.norv., *Rattus norvegicus* (P04041); B.taurus, *Bos taurus* (P00435); H.sap., *Homo sapiens* (P07203); O.cunicul., *Oryctolagus cuniculus* (P11909); C.porcell., *Cavia porcellus* (U39842); **pGPx**: *Bos taurus* (P37141), *Homo sapiens* (P22325), *Mus musculus* (P46412), *Rattus norvegicus* (P23764); **giGPx**: *Homo sapiens* (P18283); **PHGPx**: S.mans., *Schistosoma mansoni* (Q00277), S.scrofa, *Sus scrofa* (P36968), *Homo sapiens* (P36969), *Rattus norvegicus* (P36970); **GPxH**: M.contag., *Molluscum contagiosum* (U60315), C.sinen., *Citrus sinensis* (Q06652), N.sylv., *Nicotiana sylvestris* (P30708), L.escul., *Lycopersicon esculentum* (Y14762), S.olera., *Spinacia oleracea* (D63425), A.tha_clp, *Arabidopsis thaliana* chloroplast (P52032), A.tha_cyt, *Arabidopsis thaliana* cytosol (AC002335), H.annuus, *Helianthus annuus* (Y14707), P.falcip., *Plasmodium falciparum* (Z68200), S.cerev., *Saccharomyces cerevisiae* (1 - P36014, 2 - P38143, 3 - P40581); C.reinh., *Chlamydomonas reinhardtii* (AF014297); B.subt., *Bacillus subtilis* (P52035); E.coli, *Escherichia coli* (P06610); N.mening., *Neisseria meningitidis* P52036); B.m&p., *Brugia malayi & Brugia pahangi* (P35665); W.banc., *Wuchereria bancrofti* (P35666); D.immitis, *Dirofilaria immitis* (P52033); C.eleg., *Caenorhabditis elegans* (Z81015); P.wiscon., *Pseudomonas wisconsinensis* (U88907), *Rattus norvegicus* (M76733); **eGPxH**: M.fasc., Macaca fascicularis (P28714), *Sus scrofa* (D37916), *Mus musculus* (P21765), R.rattus, *Rattus rattus* (P30710).

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plants[23] or by androgens in mammalian epididymis[24] suggest that the mutation causing an exchange of selenocysteine to cysteine in the GPx family was associated with a switch from a detoxifying device to a regulatory protein.

A marked diversification has also taken place within the real selenoperoxidases. We can clearly distinguish four molecular clades, the classical cytosolic glutathione peroxidases (cGPx), the extracellular forms (pGPx), gastrointestinal variants (giGPx), and the phospholipid hydroperoxide glutathione peroxidases (PHGPx). While they all share the catalytic triad, the surroundings of the active centre are diverse suggesting differences in donor substrate specificities. In fact, only cGPx has been shown to be strictly specific for GSH[25]. The positively charged residues of the substrate binding area of cGPx[5,17] are partially conserved in giGPx, less well conserved in pGPx and completely absent in PHGPx. Correspondingly, pGPx reportedly accepts also thioredoxin and glutaredoxin as reducing substrates[26]. PHGPx is reduced by a variety of thiols including dihydrolipoic acid but surprisingly shows some specificity for GSH despite the absence of any residues suggestive of selective GSH binding[27]. Its reaction rates with GSH are, however, substantially lower than those of cGPx which is in line with poor specificity[5], and it could well be that the physiological donor substrate of PHGPx has not yet been identified.

Differences between the GPx types are also observed in respect to their specificities for hydroperoxides. cGPx does not attack hydroperoxo groups of complex lipids probably because the active sites are located in valleys near the subunit interfaces of the tetrameric

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enzyme. In contrast, the active site in the monomeric PHGPx is freely exposed at the surface and can readily accept hydroperoxo groups of phospholipids and cholesterol integrated into biomembranes[5,28].

Further differences between the clades concern selenium responsiveness of expression and other regulatory phenomena. Under limited availability of selenium the biosynthesis of selenoproteins follows a hierachic order considered to reflect their biological importance[15,29]. cGPx ranks low in this hierachy responding fast to selenium restriction, whereas PHGPx can hardly be depleted. This differential response to selenium supply is not only due to different utilization of the selenocysteyl-tRNA by the pertinent mRNAs but also to different mRNA stabilities. By still unknown mechanisms the mRNA encoding cGPx and pGPx is downregulated in selenium deficiency[29], PHGPx mRNA is more stable[29] and the mRNA encoding giGPx is obviously not at all affected by selenium restriction (Wingler and Brigelius-Flohé, unpublished data).

In accordance with the antioxidant role of cGPx, oxygen-responsive elements have been identified in the cGPx gene[29,30]. Corresponding data for the other GPx types are not available. Instead, PHGPx exhibits an expression pattern suggestive of a role in endocrine tissue. In vertebrates it is highly expressed in testis[31] and there preferentially in late round spermatids[32]. Interestingly, the only example of a selenoperoxidase in a non-vertebrate organism, the PHGPx of *Schistosoma mansoni*, appears almost restricted to the genital tract, in this case, however, to the vitelline glands essential for egg maturation[33].

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The alternate Use of Sulfur and Selenium in Hydroperoxide Metabolism

Over the last ten years a new family of proteins was discovered which now are called peroxiredoxins. Some of them proved to be reasonably fast peroxidases using a variety of donor substrates which have in common reactive, usually vicinal thiol groups. Typical examples of well defined peroxidases of the peroxiredoxin family are thioredoxin peroxidase of yeast and vertebrates[34], the AhpC component of the alkyl hydroperoxide reductase system in bacteria[35], and the tryparedoxin peroxidase of trypanosomatids[36]. The functional group of the peroxiredoxins appears to be a cysteine residue embedded in a highly conserved VCP motif which occurs once or twice in these proteins[37]. In analogy to the selenoperoxidases the first step of the catalysis is presumed to involve oxidation of this cysteine residue by the peroxide to a sulfenic acid derivative followed by formation of a disulfide bridge, probably between the subunits of the homooligomeric protein[37]. The rate constant for this first step has been determined for tryparedoxin peroxidase and, interestingly is very similar to the corresponding k_{+1} values of the sulfur analogs of the selenoperoxidases (Tab. 1). This implies that the sulfur in the active site should be optimally activated as is the sulfur or selenium in the GPx family. How this is achieved in the peroxiredoxins is unknown, since not a single 3-D-structure of this protein family has so far been worked out. A consensus sequence of the peroxiredoxins with experimentally established peroxidase function, however reveals only very few amino acid residues which could potentially activate the cysteine residue like

TABLE I Rate constants for Se/S-Peroxidases with H_2O_2

Se/S-Peroxidase	Rate constant	Reference
Se-cGPx	1×10^7 mM^{-1} min^{-1}	Flohé *et al.* 1972[38]
S-cGPx	1×10^4 mM^{-1} min^{-1}	Rocher *et al.* 1992[20]
Se-PHGPx	8×10^5 mM^{-1} min^{-1}	Ursini *et al.* 1985[39]
S-PHGPx	3×10^3 mM^{-1} min^{-1}	Maiorino *et al.* 1995[19]
(S)TXNPx	6×10^3 mM^{-1} min^{-1}	Nogoceke *et al.* 1997[36]

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in GPx, two tryptophans, one glutamine and one arginine[40]. In tryparedoxin peroxidase, the arginine residue could be exchanged without affecting activity (Montemartini, unpublished data). It thus could well be that in the peroxiredoxins, which are phylogenetically unrelated to the glutathione peroxidases, a GPx-like catalytic triad comprising cysteine, glutamine and tryptophan could have developed as an example of convergent molecular evolution.

In yeast and trypanosomatids the peroxiredoxin-catalysed peroxide removal is independent of selenium, and there may substitute for the more efficient selenoperoxidases. The mammalian thioredoxin peroxidase, however, is ultimately fueled with reduction equivalents by thioredoxin reductase, which as mentioned, is a selenoprotein and in its activity depends on adequate selenium supply. Thus, mammalian hydroperoxide metabolism will be comprised by selenium deficiency irrespective of the pathway preferentially utilized. The biological implications of this statement, however, are far from being clear. Multiple forms of peroxiredoxins have been discovered in mammals, most of them expressed in particular cells or under specific conditions

only as evident from their original designations like "proliferation associated gene product", "macrophage stress protein", natural killer cell enhancing factor", etc. It therefore is unlikely that the mammalian peroxiredoxins play a major role in the defence against unspecific oxidative stress as does cGPx. Rather the discovery of this new pathway may shed light on the intriguing, still poorly understood question of how selenium affects specific immune functions.

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Implications

From the data and considerations outlined in this minireview it is evident that selenium plays a crucial role in mammalian hydroperoxide metabolism. The selenoprotein in charge of defense against unspecific oxidative stress appears to be cGPx. Out of all known selenoproteins it declines fastest in selenium deficiency. Such decline of cGPx activity, even if complete, is obviously compatible with life under normal conditions, but certainly represents a risk factor. With regard to the toxic and mutagenic potential of H_2O_2 and other hydroperoxides, prophylactic optimization of the GPx activity by adequate selenium supplementation should be considered whenever situation of massive oxidative stress like septicemia, reperfusion injury or other types of acute and chronic inflammation must be anticipated.

Whether the pathology associated with severe selenium deficiency as in Keshan and Kashin-Beck disease are due to impaired peroxide metabolism remains to be demonstrated. Certainly, the fast mutation of Coxsackie virus implicated in the development of Keshan disease[41] might be favoured by high peroxide tone. Similarly, impaired

metabolism or increased formation of hydroperoxy intermediates of lipid mediators might contribute to inflammatory episodes in Kashin-Beck disease. But deficiencies in other selenium-dependent pathway may be equally relevant. Irrespective of the pathogenic mechanisms involved, selenium-supplementation in endemic areas is mandatory to balance serious deficiency.

A problem of continuing topicality is the need of selenium supplementation in chronic marginal to moderate selenium deficiency as it may occur in Finland, New Zealand, some parts of Germany and the United States and many other countries. Epidemiological studies suggest an inverse relationship of selenium status and certain forms of cancer and cardiovascular diseases[42]. Prospective studies designed to substantiate a beneficial effect of selenium supplementation in populations considered a risk, however, did not yet allow conclusions which are generally accepted by the scientific community.

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