Chapter 14
Glutathione Peroxidase-4

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Abstract Within the family of glutathione peroxidases (GPxs), GPx-4 is the sole monomeric enzyme that contains Sec at the active site. Phylogenetically, it is closer to the Cys-containing homologues (CysGPx) of invertebrata and vertebrata than to the tetrameric GPxs of vertebrata containing Sec. Nonetheless, the catalytic site is fully conserved in the whole family, suggesting a similar reactivity. As the tetrameric homologues, GPx-4 accepts GSH in the reductive steps of the catalytic cycle, while a redoxin is the preferred reducing substrate of the invertebrata CysGPxs. GPx-4 is also competent for oxidizing a quite heterogeneous series of thiol substrates. Reduction of complex membrane phospholipid and cholesterol hydroperoxides in cooperation with vitamin E accounts for the inhibition of lipid peroxidation by GPx-4. By no means, however, GPx-4 can be seen as just an antioxidant enzyme. Indeed reduction of lipid hydroperoxides accounts for the anti-apoptotic and anti-inflammatory effect of GPx-4 activity, and oxidation of specific protein thiols is its peculiar function in the late phase of spermatogenesis. Whether this reaction is relevant in other biochemical pathways, where a redox switch drives a functional shift in specific proteins, remains as an open and challenging option. In this chapter, the enzymology of GPx-4 will be reviewed focusing on the two best-characterized aspects: (1) inhibition of lipid peroxidation, and (2) oxidation of specific protein motifs. We refer to other chapters in this book for insights contributed by inverse genetic studies and for the general aspects of selenium catalysis in peroxidases.

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14.1 Introduction: The Discovery and Purification of a New Enzyme

Discovery, purification, and characterization of enzymes were the landmarks of the evolution of biochemistry in the pre-genomic era of the twentieth century. Usually, enzyme discovery was the successful outcome of having identified an enzymatic activity and purified the protein that catalyzes it. GPx-4 was among the last enzymes identified by following its activity – inhibition of lipid peroxidation – and purified through chromatographic steps following an increase of the specific activity [1].

The purified protein inhibited microsomal lipid peroxidation in the presence of GSH, accounting for the effect of a "cytosolic factor" previously identified by Paul McCay and coworkers [2], whose enzymatic activity was unknown. Just on a theoretical basis, and primed by ongoing studies on the mechanism of lipid peroxidation in our laboratory [3, 4], we resorted to search for the peroxidase activity on hydroperoxide derivatives of phospholipids of the "peroxidation inhibiting protein" (PIP) that we had purified. The identification of such an activity [5] prompted us to rename PIP as a new enzyme, i.e., "phospholipid hydroperoxide glutathione peroxidase" (PHGPx) [6]. Although the peroxidatic reaction was similar to that of the already known tetrameric Glutathione Peroxidase (now GPx-1) [7], the remarkable difference was the failure of the latter to reduce hydroperoxide derivatives of complex lipid substrates [8], which was in agreement with its failure to inhibit microsomal lipid peroxidation induced by NADPH or ascorbate and an iron complex [2].

The analogy of PHGPx with the tetrameric glutathione peroxidase prompted a search for a selenium moiety, which was indeed successful, and led to the final evidence for the second mammalian selenoperoxidase (SecGPx) [6]. The possibility that PHGPx could be just a monomer of the tetrameric glutathione peroxidase was definitely ruled out when, in the laboratory of Leopold Flohé, the sequence analysis on a sample of purified PHGPx disclosed a protein poorly related to the tetrameric glutathione peroxidase, which was indeed a product of a new gene [9, 10]. Eventually, although PHGPx was the second glutathione peroxidase purified, it was systematically named as GPx-4, following the discovery of the tetrameric GPx-3 (also called plasma GPx) [11, 12] and GPx-2 (also called gastrointestinal GPx, GIGPx) [13].

14.2 Enzymology

14.2.1 Activity, Substrate Specificity, and Kinetics

The usual substrate for measuring GPx-4 activity is phosphatidylcholine hydroperoxide (PC-OOH) dispersed in Triton X-100 micelles. Soybean phosphatidylcholine, which is a mixture of phosphatidylcholines containing different fatty acids, or a specific molecular species, is routinely used to prepare the hydroperoxy substrate either by auto-oxidation or enzymatic hydroperoxidation in the presence of Soybean

Lipoxygenase IV [14]. Apparently, the enzyme lacks specificity toward the oxidizing substrate. GPx-4 indeed reduces, besides H_2O_2, all the species of lipid hydroperoxide (R-OOH) so far tested – i.e., the hydroperoxides of different free fatty acids (FFA-OOH), phospholipids, and triglycerides as well as cholesterol and cholesterol ester hydroperoxides [6, 15]. Specificity appears restricted only to the hydroperoxy group whatever it is bound to an H atom or to a large and complex lipid substrate. Notably, among the physiological oxidizing substrates, only H_2O_2 and FFA-OOH are shared with tetrameric GPx-1.

The interfacial character of the GPx-4 reaction has never been analyzed in detail, and relevant questions about interactions with specific membranes, or specific lipid rafts, are still unaddressed, although it is expected physiologically quite relevant. We currently know that the possibility of accommodating large hydroperoxide substrates results from the absence of the tetrameric interface-containing loop (see below) [16]. While considering that the acquisition of this loop is a late achievement in evolution of the family [17], a challenging question emerges about the actual relevance of the quaternary structure of GPxs that, paradoxically, does not seem to have resulted in anything else so far, but a loss of function.

GPx-1 and -4 also diverge in respect of the specificity for the reducing substrate, only GPx-1 being highly specific for GSH, while GPx-4 accepts several structurally unrelated small molecular weight thiols [18, 19]. Furthermore, GPx-4 oxidizes also specific protein thiols under the permissive condition of a low concentration of GSH, the competing substrate for the reductive step of the peroxidatic reaction (see also below) [20, 21].

In spite of the above distinct substrate specificity, the kinetic mechanism of GPx-1 and GPx-4 is apparently identical, as indeed expected, since the active site has been strictly conserved [22, 23]. The steady-state kinetic analysis fits a model of a ping-pong mechanism where the interaction of the enzyme with the substrate and the release of the products are much slower than the redox transitions. This prevents the accumulation of enzyme–substrate complexes, and thus the V_{max} and K_m are infinite. For more details on GPxs kinetics see Chap. 13.

14.2.2 Structure

The *GPx-4* gene produces three distinct mRNAs differing in their 5′ ends, encoding for the mitochondrial, the cytosolic, and the nuclear proteins [24]. While the first two mRNAs result from a longer or shorter transcript of the first exon, respectively, either including or lacking an upstream ATG, the nuclear protein is built up by an alternate promoter on a distinct transcriptional initiation site in an alternate exon located within the first intron of *GPx-4*. The N-terminal extension is cleaved off completely in the mitochondrial and partially in the nuclear product. Thus, in the rat, while the cytosolic and the mitochondrial products are identical and have an MW of 19,146 Da, the nuclear GPx-4 has a variable N-terminal extension and has a higher MW by 3,403 or 3,272 Da. All these forms coexist in nuclei [25].

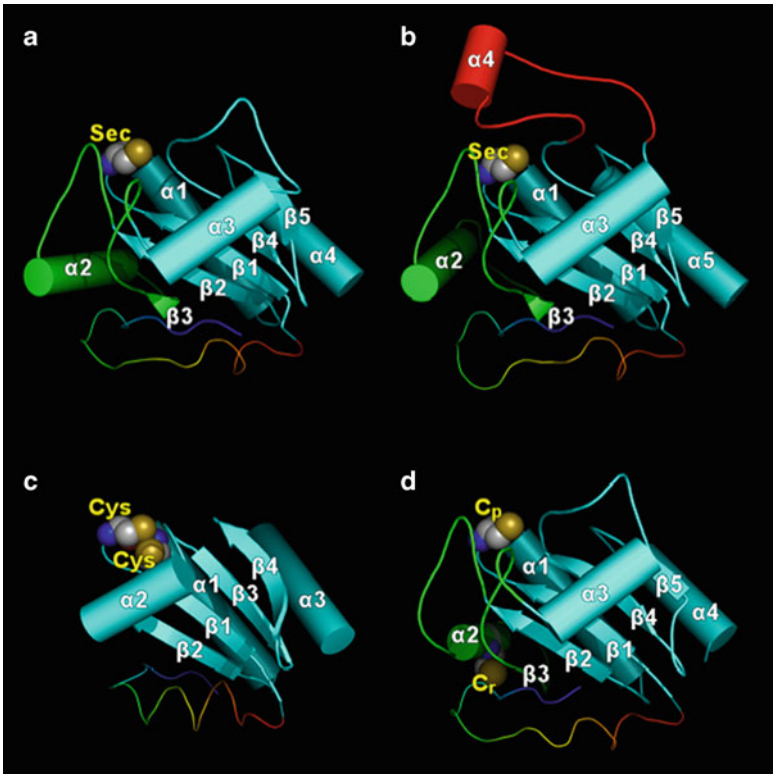

Fig. 14.1 Canonical topology and least common secondary structural elements, shown in cyan, shared by proteins belonging to the thioredoxin fold (see text). All GPxs possess an additional alpha helix (*green* α2 shown in (**a**), (**b**), and (**d**)) compared to the thioredoxin reference structure (**c**). Tetrameric GPxs shown in (**b**) have an extra alpha helix (α4 in *red*) required for oligomerization. Redox sensitive/catalytic Cys or Sec are reported as spheres. In (**a**), human monomeric GPx-4 (pdb id: 2OBI); in (**b**), single subunit of human tetrameric GPx-1 (pdb id: 2F8A); in (**c**), human thioredoxin 2 (pdb id: 1UVZ); and in (**d**), model of monomeric *Drosophila melanogaster* GPx with the peroxidatic Cys indicated as Cp and the resolving Cys as Cr within the α2-helix

Since the definition of the primary structure of cytosolic GPx-4, several hundreds of homologous sequences have been deposited in databanks. This information, integrated by crystallographic data of the U46C GPx-4 mutant [26] and homologous proteins, permitted the definition of the structural features of the whole family of GPxs [17]. GPxs are folded according to the pattern first described for thioredoxin (Trx) (Trx fold) [27, 28] and shared with several families of oxidoreductases. In the Trx fold, the typical secondary structure pattern gives rise to a conformation where the four β sheets are flanked by three α helices yielding two layers α/β/α sandwich. The minimal common motif of the Trx fold is shown in Fig. 14.1. The core pattern starting from the N-terminus is the following: β1-α1-β2-α2-β3-β4-α3. In the GPx

fold, an additional α-helix and a small β-sheet are inserted between β2 and α2 of the Trx fold consensus, whereas exclusively tetrameric GPxs, such as GPx-1, encompass another α-helix between α2 and β3.

Multiple sequence alignments and fold recognition analysis of a large number of homologous proteins revealed that the monomeric pattern is much more diffused in nature than the tetrameric pattern, the latter being apparently restricted to vertebrata and descending from the insertion of the additional α-helix generating the inter-subunit interface [17]. Notably, the large majority of non-mammalian monomeric GPxs contain a Cys substituting for Sec at the active site [17] that is associated, with few exceptions, with the presence of a second, functionally relevant, Cys residue in a variable position in the α2-helix (Fig. 14.1). This serves as resolving Cys in the peroxidatic cycle when, upon oxidation, it forms a disulfide with the peroxidatic Cys [16, 22]. This disulfide is eventually reduced by a redoxin. The formation of a disulfide within the catalytic cycle, which is eventually reduced by a Trx redox center, mirrors the catalytic mechanism of "atypical" peroxiredoxins [22].

14.2.3 Phylogeny and Homology Considerations

The GPx superfamily encompasses eight members, whose phylogeny could be reconstructed thanks to the vast amount of available sequence data in public databases (Fig. 14.2) [17]. Though a putative common ancestor may be recognized for vertebrate GPxs, the same cannot be unequivocally detected if the whole set of GPxs coming from all living organisms are taken into account. This means that complex relationships may have arisen and putative convergent evolution or lateral gene transfer, especially in bacteria, may have occurred. What unequivocally comes to light is the uneven distribution of selenium usage in GPxs during evolution. Selenium, indeed, seems a recent acquisition of the family, maybe dating back to the metazoan radiation when organisms substituted Sec for Cys as the redox-active moiety. Almost contemporary to the capacity to insert Sec, the acquisition of an α-helix favored the aggregation in tetramers (Fig. 14.1). As in the classical view of paralogy, the eight members of GPxs found in most vertebrata, and definitely in mammalia, have diversified their tissue/substrate specificity and function. The phylogenetic relationship of the vertebrata monomeric forms of GPx-4, which contain a catalytic Sec, with the majority of GPxs from invertebrata, containing a Cys residue at identical positions, is surprising: GPx-4 is indeed far closer to these Cys homologues than to the tetrameric paralogs, which carry the Sec residue at the active site. This might suggest that vertebrate GPx-4 forms have conserved the fold and scaffold features of the hypothetical monomeric Cys-based common ancestor and at the same time have undergone minimal but drastic change of acquiring the capability of inserting Sec. To some extent, the evolutionary recent vertebrate GPx-4 forms may be considered "fossil" enzymes, given that they preserve at best the features of distantly related sequences that the other members do not, and for this reason GPx-4 can be seen as a landmark peroxidase, representative of the GPx superfamily.

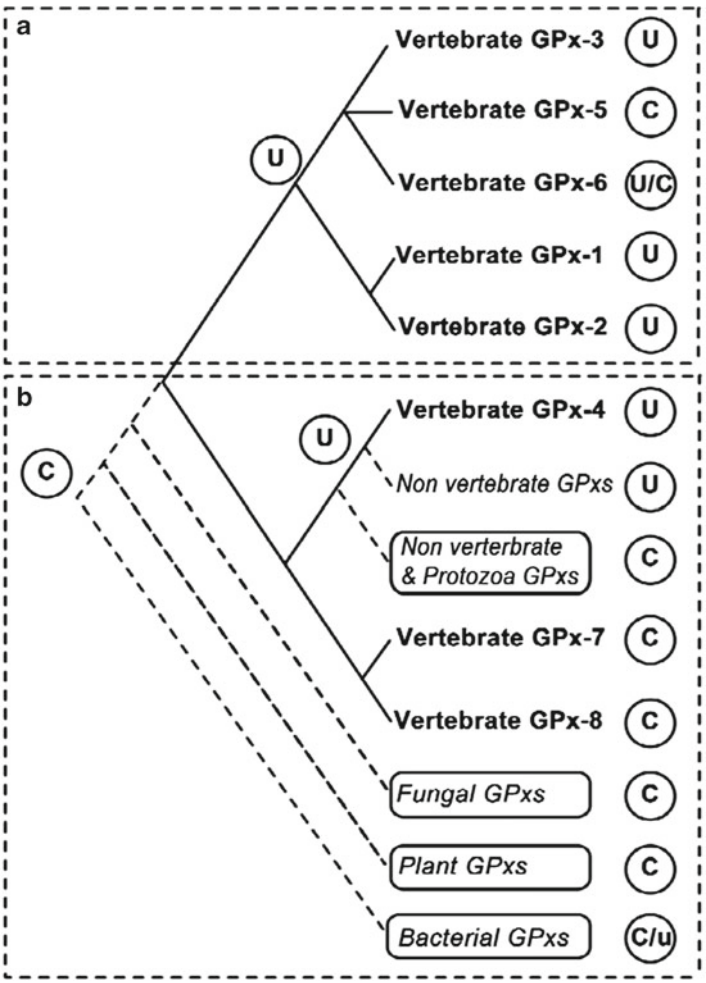

Fig. 14.2 Phylogeny of the GPx superfamily. Confirmed evolutionary reconstructions are reported in *solid lines* whereas *dashed lines* are uncertain or nonvalidated relationships (see text). Tetrameric GPxs are grouped in box a and monomeric GPxs in box b, including some reported dimeric forms in plants. Present GPxs are shown as leaves of the tree, while circles indicate either Cys (C) or Sec (U) in the catalytic center; u indicates an extremely rare, so far unique event. Internal nodes report the putative reconstruction of the original ancestor species carrying either Cys or Sec. Boxed GPxs contain the resolving cysteine (Cr) within the α2-helix (see text)

14.2.4 Active Site

The structure of the catalytic site, originally proposed from the crystal structure of bovine GPx-1, was found strictly conserved in vertebrate GPx-4, encompassing, besides the catalytic Sec, a Trp and a Gln residue, located in distant regions in the

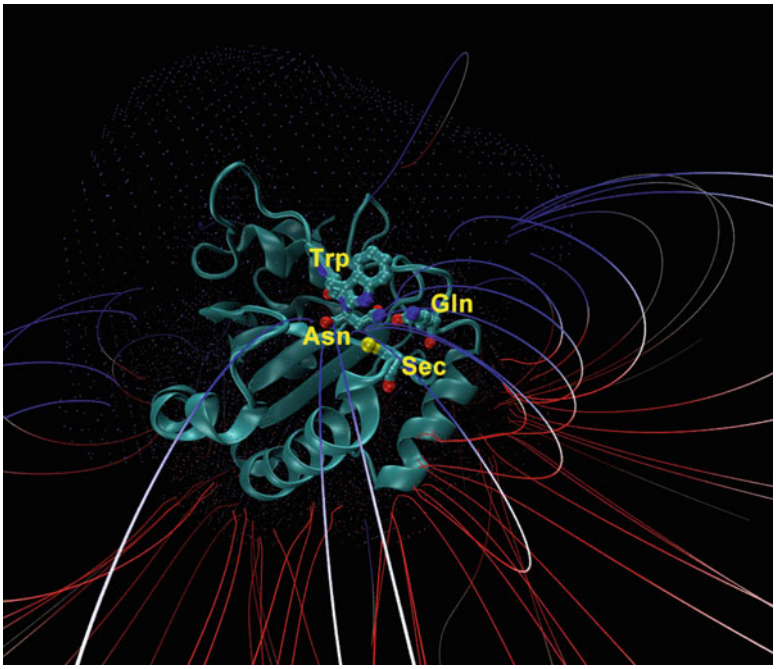

Fig. 14.3 The catalytic tetrad of GPx-4 and electric field lines in GPx-4 (PDB id: 2OBI, human enzyme). The amino acids indicated represent the experimentally validated catalytic tetrad. Electrostatic potential has been calculated on human GPx-4 by Adaptive Poisson–Boltzmann Equation and positive (*blue dots*) and negative (*red dots*) isosurfaces are shown at ±2kT/e. Electric field lines show the catalytic site surrounded by a strong cationic potential. In addition, a negative surface (*red lines* and *red dots*) is present, opposite to the positively charged catalytic pocket, creating a dipole moment as shown by the arcs connecting "*blue*" and "*red*" zones

primary structure. This catalytic triad, which was functionally validated by site-directed mutagenesis [29], has been recently revisited as a tetrad when an Asn residue was seen fully conserved in multiple sequence alignments and homology modeling and functionally validated by mutagenesis [30] (Fig. 14.3).

In GPx-4, the redox-active Se (or S in the mutant used for crystallization) lies in a flat surface that Adaptive Poisson Boltzman Solver Equation tool indicates as cationic [23] (Fig. 14.3). On the edge of this area, the oligomerization loop, found in GPx-1 but not in GPx-4, fulfills the evidence that large hydroperoxidic substrates cannot be accommodated in the tetrameric GPxs (Fig. 14.1a, b). The calculated electrostatic field also reveals that GPx-4 is highly polarized (Fig. 14.3). The cationic side overlapping the active site could be relevant for addressing the GPx-4 to specific locations in membranes where the enzyme might interact by specific electrostatic interactions instead of unspecific lipophilicity, as usually suspected for an enzyme whose action is on membranes.

14.2.5 Catalytic Mechanism

In a minimalist view, the groups surrounding the redox-active residue of GPxs (Sec or Cys), which are involved in catalysis, are the indole of Trp, the two amides of Asn and Gln, and possibly the amide in the backbone of the Gly one residue downstream the catalytic Sec/Cys residue. These are conserved at identical positions in nearly all members of the GPx family, the most remote derivatives included. For an efficient reduction of an R-OOH, the active site must fulfill the following minimal requirements: (1) ionization of the redox-active chalcogen; (2) "activation" of the O–O bond, reasonably through polarization or stretching of the bond; and (3) protonation of the leaving group $R\text{-}O^-$.

Dissociation is essential for the nucleophilic displacement reaction. This notion supported the widely diffused concept that the lower pK_a of selenol than thiol must account for the actual advantage of having Sec rather than Cys at the active site. Moreover, in *Dm*GPx, a CysGPx, the mutation of the amino acids surrounding the peroxidatic Cys affects the rate constant of the reaction much more than the pK_a of the chalcogen, clearly showing that the role of the active-site residues is broader than just lowering the pK_a of the redox-active moiety [30]. In the nucleophilic displacement reaction, where the enzyme reacts with the hydroperoxide, selenolate is expected to be a better nucleophile, but this accounts for an advantage of Se in the range of just one order of magnitude, as deduced from the comparison of kinetic analysis of a SecGPx (namely GPx-4) with a CysGPx (namely *Dm*GPx) on the same peroxidatic substrate [23].

An accurate quantum chemistry computational approach has been applied to study the steps of the catalytic mechanism in the active site of human GPx-4 or its Cys mutant. The computational protocol, rooted in advanced Density Functional Theory methodologies (DFT), has been optimized to minimize energetically the chosen set of amino acids surrounding the pocket [31]. Intriguingly, the proton of the selenol or the thiol has been observed dislocated in the catalytic cage and optimized in most of the tested locations, leading to the conclusion that it is displaced in the positively charged catalytic pocket rather than exclusively bound to the selenol or thiol [31]. In any case, the protonation of the amide of Trp seems to be somehow favored compared to the other sites, but good energies have been reported for the amides of Asn and Gln in terms of a low energy minimum and a conserved geometry of the catalytic site. In other words, the system is energetically relaxed and this "moving" proton is eventually bound to $R\text{-}O^-$ formed by R-OOH reduction. This mechanism fits two constraints of the catalysis of the reaction: ionization of the chalcogen and protonation of the leaving group. For the R-OOH substrate in the active site, the quanto-mechanical approach also indicated stretching and distortion of the O–O bond that is expected to further contribute to the catalysis. From this computational analysis, the outcome of the reaction mechanism is the instantaneous oxidation of selenium or sulfur by the hydroperoxide, without the formation of any enzyme–substrate complex. This nicely fits the non-saturation kinetics observed under steady-state conditions [19].

In GPx-4 and in the other SecGPx, the chemical form of the oxidized Se moiety has not been clarified. While the formation of a sulfenic acid derivative of the

enzyme has been experimentally observed for *Dm*GPx [16], and computationally confirmed by a well-defined energy minimum for the Cys mutant of human GPx-4 (unpublished), no analytical evidence has been so far obtained for the formation of a selenenic acid derivative of GPx-4. Furthermore, the transition state from the reduced enzyme to the oxidized form containing a selenenic acid derivative was not computationally supported by DFT calculations. This suggests the existence of a different oxidized intermediate in Cys- and in SecGPxs. In addition, a high sensitivity mass spectrometry analysis of oxidized GPx-4 and GPx-1 clearly showed a 2 a.m.u. decrease in respect to the reduced form (unpublished), thus suggesting the loss of two hydrogen atoms instead of the expected addition of one oxygen atom. This could indicate that either selenenic acid immediately extracts a hydrogen atom from suitable neighboring residue and releases water, or the oxidized form is initially different from selenenic acid.

In the reductive phase of the catalytic cycle, the oxidized intermediate is reduced by two thiol groups (e.g., 2GSH) in two steps. The formation of a mixed selenodisulfide as the first catalytic intermediate has been demonstrated for GPx-4 by MS/MS. In second step of the reductive phase, which is the last step of the peroxidatic cycle when the reduced enzyme is regenerated, the mixed disulfide is reduced by the second thiol. Very little is known about the mechanism of the catalysis of this exchange reaction. However, a faster reaction is expected for Sec than CysGPx, since selenolate is a much better leaving group than thiolate. This conclusion is supported by the NMR evidence that the rate of selenium diselenide exchange is several orders of magnitude faster than that of thiol disulfide [32]. Notably, in the last step of the peroxidatic cycle, the advantage of selenol vs. thiol is a more electrophilic character while just the opposite – more nucleophilic – is true for the first reaction of the cycle when the chalcogen is oxidized by the hydroperoxide [33].

14.3 Functions

GPx-4 is a vital enzyme. When its expression is abrogated in knockout models, the embryo dies at the gastrula stage [34]. This provides nonambiguous evidence that the maintenance of cellular homeostasis and survival strictly depends on GPx-4 activity and that the functions of this enzyme are not rescued by alternative biochemical pathways. The phenotypes obtained by deleting the different forms of GPx-4 are reviewed in Chap. 43. Here we summarize the biochemical evidence for the impact of the redox transitions catalyzed by GPx-4 on biological events.

14.3.1 Reduction of Lipid Hydroperoxides: Inhibition of Lipid Peroxidation

The notion that inhibition of lipid peroxidation by GPx-4 and GSH is due to the enzymatic reduction of R-OOH in membranes has twofold relevance. Besides leading

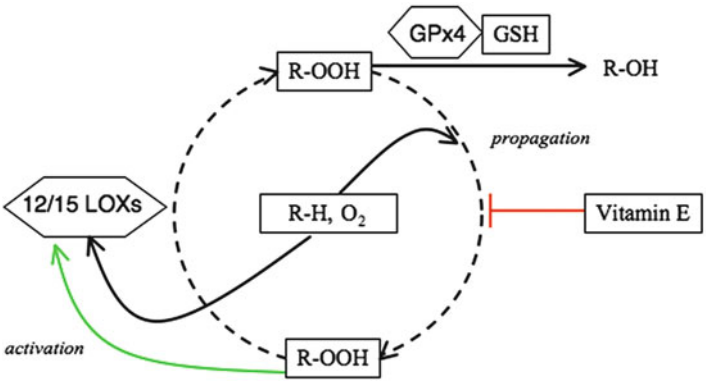

Fig. 14.4 Scheme of the synergism between GPx-4 and vitamin E in inhibiting lipid peroxidation and 12/15 lipoxygenases (LOXs). For sake of simplicity, individual specific reactions are not reported and lipid peroxidation is initiated from decomposition of preexisting phospholipid hydroperoxides (R-OOH)

to the discovery of a new enzyme, it contributed to focus the dual role of hydroperoxides that, besides being the major products of peroxidation, are also crucial initiators. Lipid peroxidation, first described more than a century ago by De Saussure, who observed the weight increase of polyunsaturated lipids exposed to air [35], is a process of oxidative degradation of lipids [36] producing, besides hydroperoxides, an array of secondary products including reactive and toxic electrophiles.

Consistently with a mechanism where, following a formation of an initiating species, lipid peroxyl radicals (R-OO$^{\bullet}$) drive the oxidative chain reaction and new chain reactions branch from radicals deriving from hydroperoxides, the protective mechanisms operating at different levels are integrated with each other [37]. While chain breaking antioxidants, such as vitamin E, slow down the R-OO$^{\bullet}$-driven propagation, the reduction of R-OOH to the corresponding alcohols (R-OH) prevents secondary initiations starting from hydroperoxide breakdown [38] (Fig. 14.4).

Rapoport and Schewe et al. first brought into light the role of enzymatically produced R-OOH in vivo. They showed that the maturational breakdown of reticulocyte mitochondria depends on a lipid peroxidation process driven by a 12/15 lipoxygenase (LOX) activity [39], thus demonstrating a physiological outcome of enzymatically produced R-OOH. The 12/15 LOX isoforms use as substrate intact membrane phospholipids and require at the same time R-OOH to become fully active [14, 40, 41] (Fig. 14.4). Consistent with its ability to reduce R-OOH in microsomal lipid peroxidation, GPx-4, in the presence of GSH, modulates the activity of reticulocyte 12/15-LOX and preserves the specificity of the oxidative process by preventing the nonenzymatic extension of lipid peroxidation [41].

The recent observation obtained by inverse genetic studies, that deletion of *GPx-4* in cells leads to apoptosis by a caspase-independent mechanism requiring a functional 12/15 LOX, and that vitamin E complements *GPx-4* deficiency [42], suggests

a molecular mechanism that is remarkably in agreement with the model of interplay between GPx-4 and a chain braking antioxidant, as summarized previously [38]. The synergistic interplay between different redox transitions working in a concerted mechanism discloses a scenario where the presence of a minute, steady-state concentration of R-OOH in membranes is physiological, and cell death descends from the unbalance of this steady state. Although further studies are required to elucidate this possibility, the control of apoptosis initiated by the activating effect of an R-OOH on LOXs seems at present sufficient to account for GPx-4 being a vital enzyme.

14.3.2 Reduction of Lipid Hydroperoxides: Regulation of Lipoxygenases and Inflammation

While the formation of oxidized phospholipids by LOXs is a relatively new emerging concept [43–45], evidence has been accumulated in the last decades about the physiological functions of enzymatically produced specific oxidation products of polyunsaturated fatty acids [46–48]. Nonconflicting evidence indicates that GPx-1 and GPx-4 are both competent for inactivating 5-LOX [49, 50], implying that the physiological activator is a common substrate for both peroxidases. In agreement with the concept of self-activation of LOXs by their reaction product, we could reasonably argue for a role of an FFA-OOH. Finally, overexpression studies show that GPx-4 prevents the activation of NF-κB much more efficiently than GPx-1 [51], thus suggesting its role in the control of inflammatory pathways where complex oxidized lipid mediators are apparently involved in regulatory redox transitions.

14.3.3 Oxidation of Protein Thiols: Spermatogenesis

In spermatids, GPx-4 is highly expressed as an active peroxidase that is transformed into an enzymatically inactive cross-linked structural protein during final sperm maturation. As such, it makes up at least 50% of the keratin-like material surrounding the helix of mitochondria in the mid-piece of spermatozoa [21]. This "moonlighting" of GPx-4 is primed by a critical GSH depletion [52], increasing its redox potential, a condition usually associated to cellular differentiation [21, 53]. Deprived of its most abundant reducing substrate, GPx-4 can react with protein thiols as alternate substrates [20, 21]. Mass spectrometric analysis revealed that during this process a selective, intermolecular reaction takes place between Sec-46 of GPx-4 and Cys-148 (porcine numbering of GPx-4 used), resulting in linear polymers representing dead-end intermediates of the peroxidatic cycle [54]. The formation of mitochondrial capsules also requires the participation of the "Sperm Mitochondrion-associated Cysteine-rich Protein" (SMCP). The involvement of SMCP has been verified by reproducing the oxidative polymerization of the capsule components

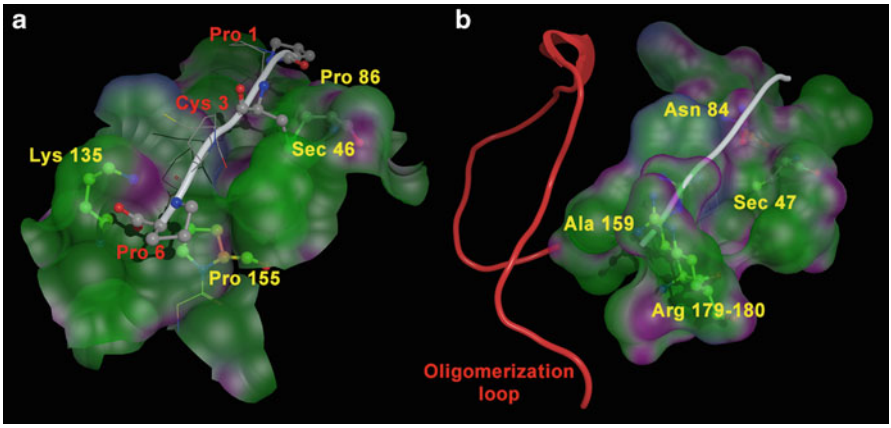

Fig. 14.5 (**a**) Molecular docking and dynamic of the PPCCPP peptide in the surface of the catalytic pocket of GPx-4 (*green* apolar, *violet*/*blue* polar residues). The Pro residues at position 155 and 86 contribute to keep flat and open the catalytic pocket of GPx-4 favoring the pose of bulky substrates. (**b**) Speculated pose of PPCCPP peptide on GPx-1. It is impossible to dock the peptide in silico due to steric clash given by Arg 179, Arg 180 (human GPx-1 numbering), and the oligomerization loop (*red ribbon*)

in vitro that is, seemingly, initiated by the oxidation of peculiar adjacent Cys motifs in SMCP [20]. Once formed, this unusual disulfide is prone to reshuffling, eventually driving cross-linking of different Cys-containing proteins [20, 55, 56]. Remarkably, similar adjacent Cys motifs, which are quite rare in the genome, are also present in protamines [57], which are the basic proteins that, by keeping compact spermatozoa chromatin, stabilize the structure and prevent transcription. The interaction of protamines with DNA is implemented by the formation of disulfides catalyzed by GPx-4 [58]. Consistently, selenium deficiency gives rise to spermatozoa with a chromatin prone to unwinding in the presence of denaturing agents [59, 60]. This role of GPx-4 in contributing to sperm chromatin stability has been validated in mice, by targeted deletion of the nuclear form of GPx-4 [61].

The ability of GPx-4 to oxidize specific peptide motifs containing adjacent Cys residues is not shared with GPx-1. This aspect has been investigated in silico using a computational approach based on molecular docking. From docking simulations, the access of large substrates to the active site in tetrameric GPx-1 is hampered by bulky amino acids, Arg and His, at position 179 and 180 (with reference to the human sequence) (Fig. 14.5). These residues, instead, are constantly replaced by Pro and light side-chain amino acids in all known GPx-4 homologues. This eases the access of bulky peptides to the catalytic pocket thanks to the flat surface of the Pro residues and the external orientation of the amino acids at position 180. Furthermore, in tetrameric GPx-1, the oligomerization loop contributes to restrict the accessibility to the catalytic site (Fig. 14.5). Intriguingly, Arg 179 has been proposed to contribute to the stabilization of GSH into the catalytic pocket of GPx-1, whereas its lack accounts for its low docking specificity in GPx-4 [22].

The small and unstructured peptide, PPCCPP, a peculiar motif in SMCP experimentally validated as substrate of GPx-4 [20], has been chosen for monitoring the docking simulation. This peptide, which does not react with GPx-1, shows instead a high binding affinity with human GPx-4. The simulation suggests that the binding zone is placed in a confined surface area located close to the catalytic Sec 46. The peptide interacts through a couple of Pro–Pro interactions (GPx-4 Pro 86 and peptide Pro 1, GPx-4 Pro 155, and peptide Pro 6) which are further stabilized by the electrostatic interaction between the peptide C-terminal and Lys 135, on one side of GPx4, and the polar interaction between the peptide N-terminal and, on the other side, the backbone carbonyl group of Gln 45. In this arrangement, the Cys residue at position 3 of the peptide is located at about 2 Å from GPx-4 catalytic Sec 46. The PPCCPP peptide, instead, cannot interact with the catalytic area of GPx-1, because Pro 86, Pro 155, and Lys 135 of GPx-4 are replaced in GPx-1 by Asn 84, Arg 179–180, and Ala 159, respectively (human enzymes numbering system used) (unpublished). This suggests that: (1) the interaction of GPx-4 with peptides and proteins involves a small surface area responsible for the correct orientation of the substrate toward the catalytic site; and (2) only the GPx-4 homologues are endowed with the correct combination of water-exposed amino acids, allowing the interaction of Sec with the substrate. In general terms, this observation is evocative of a role for GPx-4 as the catalyst of redox switches, reasonably primed by the increased redox potential of GSSG/GSH couple.

14.4 Concluding Remarks and Perspectives for Future Research

In conclusion, almost 30 years after the discovery of GPx-4 and a huge number of published studies, this enzyme is still largely uncharacterized regarding the relevance of the biological issues connected to its activity, which must be judged as still largely unresolved. Since deletion of *GPx-4* is lethal, its capability of reducing membrane hydroperoxides is not compensated by any other enzyme, including Peroxiredoxin 6, for which an activity on phospholipids hydroperoxides in vitro has been described [62]. Nevertheless, this fact also pinpoints the relevance of membrane hydroperoxides generated by specific 12/15 LOX isoforms and competent for priming a caspase independent cell death pathway [42]. This could be relevant in chronic degenerative diseases, when an imbalance between hydroperoxide production and elimination is in favor of the first. However, through the same mechanism, GPx-4 comes forth as a key player in cancer, when its activity could be relevant in controlling a physiologically useful apoptosis.

The specific capability of GPx-4 to oxidize specific protein thiols could be relevant in specific physiological functions, besides the first discovered stabilization of spermatozoa mitochondrial capsules and chromatin. This would expand the role and the functions of GPx-4 toward the area of functional redox switches [63], an emerging field in regulation of biological functions.

The final, unsolved question is about the substantial advantage of having selenium rather than sulfur at the active site. From basic chemistry, we know that selenium can be both more nucleophilic and more electrophilic than sulfur [33], and reasonably this speeds up the peroxidatic cycle. But, can this be enough? As a matter of fact, the introduction of selenium substituting for sulfur is rather limited in nature and in the same mammalian cells GPx-4 coexists with some Cys homologues. Definitely, it is hard to believe that the only difference could be limited to a not dramatic difference in the reaction rate.

References

1. Ursini F, Maiorino M, Valente M et al (1982) Biochim Biophys Acta 710:197
2. Gibson DD, Hornbrook KR, McCay PB (1980) Biochim Biophys Acta 620:572
3. Maiorino M, Ursini F, Valente M et al (1980) Atti dell'Istituto Veneto di Scienze, Lettere ed Arti Tomo CXXXVIII:131
4. Ursini F, Maiorino M, Ferri L et al (1981) J Inorg Biochem 15:163
5. Daolio S, Traldi P, Ursini F et al (1983) Biomed Mass Spectrom 10:499
6. Ursini F, Maiorino M, Gregolin C (1985) Biochim Biophys Acta 839:62
7. Flohé L (1971) Klin Wochenschr 49:669
8. Tappel WA (1980) Measurement of and protection from in vivo lipid peroxidation. In: Free radicals in biology Pryor W (ed) Academic Press, New York, pp 2
9. Schuckelt R, Brigelius-Flohé R, Maiorino M et al (1991) Free Radic Res Commun 14:343
10. Brigelius-Flohé R, Aumann KD, Blocker H et al (1994) J Biol Chem 269:7342
11. Maddipati KR, Gasparski C, Marnett LJ (1987) Arch Biochem Biophys 254:9
12. Takahashi K, Akasaka M, Yamamoto Y et al (1990) J Biochem 108:145
13. Chu FF, Doroshow JH, Esworthy RS (1993) J Biol Chem 268:2571
14. Maiorino M, Gregolin C, Ursini F (1990) Methods Enzymol 186:448
15. Thomas JP, Geiger PG, Maiorino M et al (1990) Biochim Biophys Acta 1045:252
16. Maiorino M, Ursini F, Bosello V et al (2007) J Mol Biol 365:1033
17. Toppo S, Vanin S, Bosello V et al (2008) Antioxid Redox Signal 10:1501
18. Roveri A, Maiorino M, Nisii C et al (1994) Biochim Biophys Acta 1208:211
19. Ursini F, Maiorino M, Brigelius-Flohé R et al (1995) Methods Enzymol 252:38
20. Maiorino M, Roveri A, Benazzi L et al (2005) J Biol Chem 280:38395
21. Ursini F, Heim S, Kiess M et al (1999) Science 285:1393
22. Flohé L, Toppo S, Cozza G et al (2010) Antioxid Redox Signal. doi:10.1089/ars.2010.3397
23. Toppo S, Flohé L, Ursini F et al (2009) Biochim Biophys Acta 1790:1486
24. Maiorino M, Scapin M, Ursini F et al (2003) J Biol Chem 278:34286
25. Maiorino M, Mauri P, Roveri A et al (2005) FEBS Lett 579:667
26. Scheerer P, Borchert A, Krauss N et al (2007) Biochemistry 46:9041
27. Martin JL (1995) Structure 3:245
28. Atkinson HJ, Babbitt PC (2009) PLoS Comput Biol 5:e1000541
29. Maiorino M, Aumann KD, Brigelius-Flohé R et al (1995) Biol Chem Hoppe Seyler 376:651
30. Tosatto SC, Bosello V, Fogolari F et al (2008) Antioxid Redox Signal 10:1515
31. Orian L, Polimeno A, Maiorino M et al (2010) Selenium 2010 Kyoto (Japan) P-022
32. Pleasants JC, Guo W, Rabenstein DL (1989) J Am Chem Soc 111:6553
33. Steinmann D, Nauser T, Koppenol WH (2010) J Org Chem 75:6696
34. Imai H, Hirao F, Sakamoto T et al (2003) Biochem Biophys Res Commun 305:278
35. De Saussure NT (1804) Recherches chimiques sur la végétation Chez la veuve Nyon librairie Paris

36. Hochstein P, Ernster L (1963) Biochem Biophys Res Commun 12:388
37. Sevanian A, Ursini F (2000) Free Radic Biol Med 29:306
38. Maiorino M, Coassin M, Roveri A et al (1989) Lipids 24:721
39. Rapoport SM, Schewe T (1986) Biochim Biophys Acta 864:471
40. Jones GD, Russell L, Darley-Usmar VM et al (1996) Biochemistry 35:7197
41. Schnurr K, Belkner J, Ursini F et al (1996) J Biol Chem 271:4653
42. Seiler A, Schneider M, Forster H et al (2008) Cell Metab 8:237
43. Kühn H, Borchert A (2002) Free Radic Biol Med 33:154
44. Thomas CP, Morgan LT, Maskrey BH et al (2010) J Biol Chem 285:6891
45. Bochkov VN, Oskolkova OV, Birukov KG et al (2010) Antioxid Redox Signal 12:1009
46. Brash AR (1999) J Biol Chem 274:23679
47. Funk CD (2001) Science 294:1871
48. Smith WL, DeWitt DL, Garavito RM (2000) Annu Rev Biochem 69:145
49. Haurand M, Flohé L (1988) Biol Chem Hoppe Seyler 369:133
50. Imai H, Narashima K, Arai M et al (1998) J Biol Chem 273:1990
51. Brigelius-Flohé R, Friedrichs B, Maurer S et al (1997) Biochem J 328(Pt 1):199
52. Bauché F, Fouchard MH, Jégou B (1994) FEBS Lett 349:392
53. Kirlin WG, Cai J, Thompson SA et al (1999) Free Radic Biol Med 27:1208
54. Mauri P, Benazzi L, Flohé L et al (2003) Biol Chem 384:575
55. Carugo O, Cemazar M, Zahariev S et al (2003) Protein Eng 16:637
56. Hudàky I, Gàspàri Z, Carugo O et al (2004) Proteins 55:152
57. McKay DJ, Renaux BS and Dixon GH (1985) Biosci. Rep. 5: 383-391
58. Godeas C, Tramer F, Micali F et al (1997) Biol Reprod 57:1502
59. Behne D, Weiler H, Kyriakopoulos A (1996) J Reprod Fertil 106:291
60. Watanabe T, Endo A (1991) Mutat Res 262:93
61. Conrad M, Moreno SG, Sinowatz F et al (2005) Mol Cell Biol 25:7637
62. Fisher AB (2010) Antioxid Redox Signal. doi:10.1089/ars.2010.3412
63. Wouters MA, Fan SW, Haworth NL (2010) Antioxid Redox Signal 12:53