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REVIEW

# Hydroperoxide-Reducing Enzymes in the Regulation of Free-Radical Processes

**Mars G. Sharapov[1,a]*, Sergey V. Gudkov[2,3,4], and Vadim Z. Lankin[5]**

[1]*Institute of Cell Biophysics, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia*
[2]*Prokhorov Institute of General Physics, Russian Academy of Sciences, 119991 Moscow, Russia*
[3]*Lobachevsky State University of Nizhny Novgorod, 603022 Nizhny Novgorod, Russia*
[4]*All-Russian Research Institute of Phytopathology, 143050 Bolshiye Vyazemy, Russia*
[5]*National Medical Research Center of Cardiology, Ministry of Health of the Russian Federation, 121552 Moscow, Russia*
[a]*e-mail: sharapov.mars@gmail.com*

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**Abstract**—The review presents current concepts of the molecular mechanisms of oxidative stress development and describes main stages of the free-radical reactions in oxidative stress. Endogenous and exogenous factors of the oxidative stress development, including dysfunction of cell oxidoreductase systems, as well as the effects of various external physicochemical factors, are discussed. The review also describes the main components of the antioxidant defense system and stages of its evolution, with a special focus on peroxiredoxins, glutathione peroxidases, and glutathione S-transferases, which share some phylogenetic, structural, and catalytic properties. The substrate specificity, as well as the similarities and differences in the catalytic mechanisms of these enzymes, are discussed in detail. The role of peroxiredoxins, glutathione peroxidases, and glutathione S-transferases in the regulation of hydroperoxide-mediated intracellular and intercellular signaling and interactions of these enzymes with receptors and non-receptor proteins are described. An important contribution of hydroperoxide-reducing enzymes to the antioxidant protection and regulation of such cell processes as growth, differentiation, and apoptosis is demonstrated.

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## ROS, FREE RADICALS, AND OXIDATIVE STRESS

Oxygen consumption is an inherent feature of aerobic organisms. Transition to the oxygen-mediated oxidation of organic substrates has made energy generation more efficient and promoted explosive evolutionary progress of living organisms [1]. However, this type of metabolic progress has its downside, which is formation of aerobic respiration side products such as reactive oxygen species (ROS).

**Sources of ROS.** Approximately 90% ROS in a cell are formed in the mitochondria due to the leakage of electrons to molecular oxygen in the respiratory chain [2, 3]. According to calculations, the leakage of electrons in the mitochondrial electron transport chain (ETC) amounts to 1-2% of the total electron flux through the mitochondria [4]. About 0.2% of the consumed oxygen is transformed into superoxide anion radical $O_2^{\bullet-}$, and 0.4% is transformed into $H_2O_2$. Interestingly, this ratio between $O_2^{\bullet-}$ and $H_2O_2$ (1 : 2) remains the same under normal conditions as well and does not depend on the atmospheric oxygen concentration and mitochondrial respiratory rate. Furthermore, the higher is the cell metabolic activity, the more ROS are generated [5]. In particular, the myocardium, the content of mitochondria in

*Abbreviations*: $C_P$, peroxidatic cysteine; $C_R$, resolving cysteine; Grx, glutaredoxin; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione, reduced; HO$^\bullet$, hydroxyl radical; In$^\bullet$, antioxidant free radical; L$^\bullet$, lipid alkyl radical; LH, polyunsaturated fatty acid; LO$^\bullet$, lipoxal radical; LOO$^\bullet$, lipoperoxy radical; LOOH, lipid hydroperoxide; RHS, reactive halogen species; ROS, reactive oxygen species; RNS, reactive nitrogen species; LPO, lipid peroxidation; $O_2^{\bullet-}$, superoxide anion radical; GST, glutathione S-transferase; PDI, protein disulfide isomerase; Prx, peroxiredoxin; RO$^\bullet$, alkoxy radical; SOD, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase.

* To whom correspondence should be addressed.

which reaches 25-30% of the tissue mass, consumes approximately 8 mM of oxygen per minute and produces ~0.1 mM of superoxide anion radical in the process [4]. Excessive oxygen consumption by the cells is extremely dangerous because the rates of electron leakage in the mitochondria and ROS formation under hyperoxic conditions are directly proportional to the increase in the oxygen partial pressure. Thus, healthy adult rats die within 72 h after exposure to 100% oxygen (i.e., concentration that is five times higher than the normal oxygen content in the air) [6].

The remaining 10% ROS that form in a cell under normal conditions are produced due to the activity of induced oxidoreductases, such as NADPH oxidase (NOX), xanthine oxidase (XO), cyclooxygenase (COX), NO synthase (NOS), and others [7]. The contribution of induced oxidoreductases to the formation of endogenous ROS could be much higher. For example, in neutrophils, macrophages, and microglial cells, NADPH oxidases could convert up to 90% of the consumed oxygen into $O_2^{\bullet-}$ and $H_2O_2$ [7].

**Classification of ROS.** ROS can be divided into two large groups according to their physicochemical properties: radical and non-radical. The radical forms of ROS contain one or several unpaired electrons, which makes them highly unstable and reactive. Radical ROS readily recombine with each other or react with various substrates. A radical can either donate its unpaired electron or, vice versa, accept an electron from another molecule to stabilize its electron shell. The molecule attacked by a radical becomes, in turn, a free radical itself, which initiates a chain reaction resulting in the damage of biomolecules located in a close vicinity [8].

Superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydroperoxide radical ($HO_2^{\bullet}$), alkoxy radical ($RO^{\bullet}$), peroxy radical ($RO_2^{\bullet}$), etc. are examples of radical ROS, while non-radical ROS include hydrogen peroxide ($H_2O_2$), organic hydroperoxides (ROOHs), singlet oxygen ($^1O_2$), and ozone ($O_3$) [1].

The term ROS describes a broad array of inorganic and organic compounds with different properties. This is related to the fact that ROS (especially, free-radical ones) are capable of reacting with virtually any molecule. In particular, ROS react with nitrogen compounds, thus facilitating formation of reactive nitrogen species (RNS), such as nitrogen monoxide ($NO^{\bullet}$), nitrogen dioxide ($NO_2^{\bullet}$), peroxynitrite ($ONOO^-$), peroxynitric acid (ONOOH), and others. Interaction of ROS with halogens results in the formation of hypohalogenites (HOCl, HOBr, HOI) that belong to reactive halogen species (RHS). The products of the chain reaction of lipid oxidation are lipid radicals (alkyl $L^{\bullet}$, alkoxyl $LO^{\bullet}$, lipid peroxyl $LOO^{\bullet}$) and lipid hydroperoxides (LOOHs), which are sometimes named reactive lipid species [9]. Free radicals of protein nature ($P^{\bullet}$, $RS^{\bullet}$) are often called long-lived protein radicals or long-lived reactive protein species [10]. ROS, RNS, and RHS react with each other in the course of biochemical and free-radical processes, thus transitioning from one form to another and producing complex effects on biological systems [11]. The characteristic features of typical ROS, RNS, and RHS found in living organisms are presented in Table 1.

As can be seen in Table 1, free-radical ROS, such as hydroxyl ($HO^{\bullet}$) or alkoxyl ($RO^{\bullet}$) radicals, are the most reactive species (based on the standard electrode potential) that can modify any molecule. At the same time, less reactive ROS have longer lifetimes and can migrate to greater distances from their source, which could be eventually more damaging for the cell.

Based on the presented data, reduction of hydrogen peroxide and organic (mostly lipid) hydroperoxides by peroxidase enzymes (peroxiredoxins, glutathione peroxidases, and glutathione-S-transferases) should play a key role in the prevention of oxidative stress (see below), because $H_2O_2$ and natural organic hydroperoxides (ROOHs, LOOHs) are the main sources of active free radicals ($HO^{\bullet}$, $RO^{\bullet}$, and $LO^{\bullet}$, respectively), which are produced upon degradation (homolysis) of these compounds and can initiate or continue the free-radical chain oxidation of biomolecules.

## OXIDATIVE STRESS

Oxidative stress is a state of biological systems, when the level of generated ROS exceeds the capacity of the antioxidant systems to utilize them, resulting in the disruption of redox regulation and accumulation of damaged biomolecules. Currently, oxidative stress is subdivided into eustress and distress [20].

Oxidative eustress is induced by a short-term increase in the ROS content within the physiological range (for $H_2O_2$, below 100 nM), which facilitates the processes of cellular signaling, cell defense against pathogens, etc. Oxidative changes of macromolecules observed in oxidative eustress are reversible. ROS generated during oxidative eustress are mostly endogenous and are associated with the activation of cellular oxidases (see above) [20]. Endogenous ROS regulate the activity of many proteins by interacting with the redox-sensitive elements (RSEs), such as methionine, cysteine, and selenocysteine (Sec) residues, as well as with prosthetic groups containing transition metal ions. Important regulatory proteins with RSEs includes protein kinases (ERK, JNK, STAT, p38), phosphatases (PTEN, SHP-2), metalloproteinases (MMPs), growth factors (TGF, TNFα, EGF), transcription factors (Nrf2, NF-κB, HIF, AP-1), and many more [21, 22].

Oxidative distress is induced by the short- or long-term increase in the ROS content up to the concentrations significantly higher than the physiological ones (for $H_2O_2$, above 100 nM), which causes irreversible oxidative

**Table 1.** Main types of ROS, RNS, RHS, and free radicals in biological systems

| | Free radical/ oxide/peroxide | Formula | $t_{1/2}$, s | $R_d$,μm | E°, V (pH 7.0)* | Reaction of ROS formation | Catalysis** |
|---|---|---|---|---|---|---|---|
| ROS | Singlet oxygen | $^1O_2$ | $10^{-6}$ | 0.3 | – | $2H_2O_2 \rightarrow H_2O + {}^1O_2$,<br>$H_2O_2 + Cl^- \rightarrow H_2O + OCl^-$,<br>$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + {}^1O_2$,<br>$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + HO^- + {}^1O_2$,<br>$O_2^{\cdot-} + OH^{\cdot} + H^+ \rightarrow H_2O + {}^1O_2$ | Cat<br>–<br>MPO<br>MPO<br>– |
| | Superoxide anion radical | $O_2^{\cdot-}$ | $10^{-6}$ | 0.3 | $O_2/O_2^{\cdot-} = -0.33$<br>$O_2^{\cdot-}/H_2O_2 = 0.93$ | $O_2 + \bar{e} \rightarrow O_2^{\cdot-}$ | NOX, XO, CytC |
| | Ozon | $O_3$ | seconds | – | $O_3/O_3^{\cdot-} = 1.03$<br>$O_3/O_2 = 1.66$ | $3O_2 + h\nu \rightarrow 2O_3$<br>285 kJ/mol | UV |
| | Hydroxyl radical | $HO^{\cdot}$ | $10^{-9}$ | <0.01 | $HO^{\cdot}/H_2O = 2.34$ | $O_2^{\cdot-} + H_2O_2 \rightarrow HO^{\cdot} + HO^- + {}^1O_2$,<br>$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + Fe^{3+} + HO^-$,<br>$ClO^- + Fe^{2+} + H^+ \rightarrow HO^{\cdot} +$<br>$+ Fe^{3+} + Cl^-$ | –<br>–<br>– |
| | Perhydroxyl radical | $HOO^{\cdot}$ | $10^{-6}$-$10^{-3}$ | 10 | $HO_2^{\cdot}/H_2O_2 = 1.48$ | $O_2^{\cdot-} + H_2O \rightarrow HOO^{\cdot} + HO^-$ | – |
| | Hydrogen peroxide | HOOH | stable | 1-10 | $H_2O_2/H_2O = 1.35$ | $O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$,<br>$2HO_2^{\cdot} \rightarrow H_2O_2 + {}^1O_2$ | SOD<br>– |
| | Alkoxyl radical | $RO^{\cdot}$ | $10^{-6}$ | # | $RO^{\cdot}/ROH = 1.60$ | $Fe^{2+} + ROOH \rightarrow Fe^{3+} + HO^- + RO^{\cdot}$ | – |
| | Peroxyl radical | $ROO^{\cdot}$ | $10^{-5}$ | # | $RO_2^{\cdot}/RO_2H = 1.00$ | $R^{\cdot} + O \rightarrow ROO^{\cdot}$ | – |
| | Alkyl hydroperoxide | ROOH | stable | # | – | $ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$,<br>$ROO^{\cdot} + Fe^{2+} + H^+ \rightarrow ROOH$ | – |
| | Lipoxyl radical | $LO^{\cdot}$ | $10^{-6}$ | # | – | $Fe^{2+} + LOOH \rightarrow Fe^{3+} + HO^- + LO^{\cdot}$ | – |
| | Lipoperxyl radical | $LOO^{\cdot}$ | $10^{-3}$ | # | – | $L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$ | – |
| | Lipid hydroperoxide | LOOH | stable | # | – | $LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$,<br>$LOO^{\cdot} + Fe^{2+} + H^+ \rightarrow LOOH$ | – |
| RNS | Hypochlorous acid | HOCl | stable | – | $ClO^-/Cl^- = 1.28$ | $H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$ | MPO, EPX |
| | Hypobromous acid | HOBr | stable | – | $BrO^-/Br^- = 1.59$ | $H_2O_2 + Br^- + H^+ \rightarrow HOBr + H_2O$ | |
| | Hypoiodous acid | HOI | stable | – | $HIO_3/I_2 = 1.19$ | $H_2O_2 + I^- + H^+ \rightarrow HOI + H_2O$ | |
| RNS | Nitrogen monoxide [nitrogen (II) oxide, nitrosyl radical] | $NO^{\cdot}$ | 1-10 | 50-1000 | $NO/N_2O = 1.59$ | L-Arg + $NADPH_2$ + $O_2 \rightarrow$<br>$\rightarrow NO^{\cdot}$ + L-citrulline | NOS |
| | Nitrogen dioxide [nitrogen (IV) oxide] | $NO_2^{\cdot}$ | $10^{-1}$-1 | 100 | $NO_2^{\cdot}/NO_2^- = 1.04$ | $ONOOCO_2^{\cdot} \rightarrow CO_3^{\cdot-} + NO_2^{\cdot}$ | – |
| | Peroxynitrite | $ONOO^-$ | 0.5-1 | 1-4 | $ONOO^-/NO_2^- = 1.20$<br>$ONOO^-/NO_2^{\cdot} = 1.40$ | $O_2^{\cdot} + NO^{\cdot} \rightarrow ONOO^-$<br>$ONOO^- + H^+ \rightarrow OH^{\cdot} + NO_2^{\cdot}$ | – |

**Table 1** (Contd.)

| | Free radical/ oxide/peroxide | Formula | $t_{1/2}$, s | $R_d$, μm | E°, V (pH 7.0)* | Reaction of ROS formation | Catalysis** |
|---|---|---|---|---|---|---|---|
| RNS | Peroxynitric acid | ONOOH | 1.3 | – | $NO_3^-/NO = 0.95$<br>$NO_3^-/HNO_2 = 0.93$ | $ONOO^- + H^+ \rightarrow ONOOH$ | – |
| | Nitrosyl cation | $NO^+$ | stable | – | – | $NO^{\cdot} - \bar{e} \rightarrow NO^+$ | – |
| | Nitroxyl anion | $NO^-$ | stable | – | – | $NO^{\cdot} + \bar{e} \rightarrow NO^-$ | – |
| | Dinitrogen trioxide [nitrogen (III) oxide, nitrous anhydride] | $N_2O_3$ | seconds | – | $N_2O_3/N_2O_3^- = 0.9$ | $NO + NO_2 \rightarrow N_2O_3$ | – |
| | Dinitrogen tetroxide | $N_2O_4$ | seconds | – | $N_2O_4/HNO_2 = 1.06$ | $2NO_3^- + 2H_2O + 2\bar{e} \rightarrow N_2O_4 + 4HO^-$ | – |
| | Nitrous acid | $HNO_2$ | seconds | – | $HNO_2/N_2O = 1.29$<br>$HNO_2/NO = 0.98$<br>$HNO_2/H_2N_2O = 0.86$ | $N_2O_4 + 2H_3O^+ + 2\bar{e} \rightarrow$<br>$\rightarrow 2HNO_2 + 2H_2O$ | – |
| | Nitryl chloride | $ClNO_2$ | seconds | – | – | $H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$,<br>$NO_2^- + HOCl + H^+ \rightarrow$<br>$\rightarrow ClNO_2 + H_2O$ | MPO |
| Other free radicals | Alkyl radical | $R^{\cdot}$ | $>10^{-6}$ | # | – | $HO^{\cdot} + RH \rightarrow H_2O + R^{\cdot}$,<br>$RO^{\cdot} + LH \rightarrow ROH + L^{\cdot}$ | – |
| | Lipoalkyl radical | $L^{\cdot}$ | $10^{-8}$ | # | – | $HO^{\cdot} + LH \rightarrow H_2O + L^{\cdot}$,<br>$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot}$ | – |
| | Thiyl radical | $RS^{\cdot}$ | $>10^{-6}$ | # | – | $R^{\cdot} + R'\text{-}SH \rightarrow R'\text{-}S^{\cdot} + RH$ | – |
| | Protein radicals | $P^{\cdot}$ | $1\text{-}10^5$ | # | – | $P + \gamma \rightarrow P^{\cdot} + h\nu$ | – |
| | Antioxidant radicals | $In^{\cdot}$ | $10^{-3}$ | # | – | $LOO^{\cdot} + InH \rightarrow In^{\cdot}$ | – |

Note. Table is constructed based on information published previously in [10, 12-19].

* Values of standard electrode potential (E°) taken from publications [15, 16]. # Radius of diffusion ($R_d$) depends on the size of molecule. Average rate of lipid diffusion in a membrane is 2 μm/s [17]. Based on the lifetime of radicals and hydroperoxides, radius of diffusion of such molecules is $10^{-6}$-$10^{-3}$ μm.

** Cat, catalase; MPO, myeloperoxidase; EPX, eosinophil peroxidase; NOX, NADPH oxidase; XO, xanthine oxidase; CytC, cytochrome *c* oxidase; NOS, NO synthases; hν, photons; γ, high-energy photons (gamma-quant, X-ray radiation).

modification of macromolecules, initiation of pathological processes, cell cycle arrest, and cell death [20]. Elevation of the ROS concentration during oxidative distress is often caused by various damaging external factors (ionizing radiation, UV radiation, xenobiotics, etc.).

Hence, ROS play a dual role in the life of aerobic organisms. On one hand, ROS participate in the defense against pathogens (oxidative burst mediated by NADPH oxidase of immune cells), neutralization of xenobiotics, and signal transduction within or between the cells (during eustress). On the other hand, when ROS production exceeds the antioxidant capacity of the cell (oxidative distress), this leads to the irreversible damage at all levels of organism organization (molecular, cellular, tissue, and organ levels) and might provoke development of multiple pathologies [23].

## ROS-INDUCED DAMAGE

ROS are extremely reactive molecules that readily interact with various classes of compounds, resulting in the oxidative modification of the latter. ROS can induce oxidative damage of the most important biological molecules (nucleic acids, proteins, lipids), thus disrupting their structure and functions, followed by the damage of

cells and tissues. Excessive production of ROS, especially in combination with the insufficient compensatory activity of the antioxidant systems, results in the redox imbalance and development or aggravation of pathological changes at all levels of organism organization [8]. An increase in the ROS content is often directly proportional to the severity of pathological states. ROS generation in the human organism intensifies with aging. The majority of these processes occur relatively slowly, so that an organism has enough time to adapt to the changes, which offsets the ROS-mediated damage in the long term. Therefore, the rate of changes in the pro- or anti-oxidative balance (rapid increase in the ROS levels or decrease in the activity of antioxidant systems) is extremely important in the etiology and development of any pathology. However, evaluation of the balance between the pro- and antioxidant systems is challenging in practice, therefore, the extent of cell, tissue, and organism damage by the oxidative stress is determined based on its consequences, e.g., the presence of modified biomolecules. A wide range of methods and tests have been developed in recent years for quantification of biological molecules modified as a result of oxidative stress [11].

**Oxidative DNA damage** is the most critical consequence of oxidative stress in the cell. It is associated with mutagenesis, carcinogenesis, aging, and a number of age-related pathologies. By now, more than a hundred products of oxidative DNA damage have been identified [24], including pyrimidine dimers, DNA-protein crosslinks, single- and double-strand DNA breaks, oxidatively damaged deoxyribose, formamidopyrimidine derivatives of purines, hydroxylated derivatives, and other oxidized derivatives of nitrogenous bases. One of the major markers of oxidative DNA damage is 8-oxo-7,8-dihydroguanidine (8-oxoguanine) [25].

8-Oxoguanine is formed by DNA exposure to singlet oxygen and hydroxyl radicals and comprises at least 5% of the total number of oxidized bases in DNA in the oxidative stress of any etiology. Based on this fact, 8-oxoguanine is currently considered as one of the main markers of oxidative stress and free-radical DNA damage [26]. 8-Oxoguanine has ambiguous coding properties, which results in the point mutations in DNA and disruption of DNA replication and transcription. In human cells, 8-oxoguanine is recognized and reduced by the specific DNA repair enzyme hOGG1 (human oxoguanine glycosylase 1). The loss of one *hOGG1* gene allele promotes the mutagenic effect of 8-oxoguanine. An increase in the level of 8-oxoguanine in DNA increases the number of transversions, mostly often, G–C → T–A, which is one of the most common somatic mutations in oncological diseases [27].

**Oxidative damage of proteins** causes significant disruptions in the normal functioning of multiple cellular systems and can be manifested as changes in the functions of receptors, enzymes, and transport proteins, as well as formation of antigens capable of initiating the autoimmune response. Protein oxidation is accompanied by a number of structural changes, such as oxidative damage of amino acids (formation of disulfide bonds, interprotein crosslinks, polypeptide chain breaks) and denaturation and aggregation of protein molecules [28]. Moreover, oxidative damage of proteins can initiate the secondary damage of other biological molecules (the classic example is the damage and inactivation of DNA repair enzymes that eventually alters the coding properties of DNA).

It is commonly accepted that the free-radical oxidation of proteins in mammalian organisms is closely associated with aging and various physiological disorders. The free-radical oxidation of proteins is considered as an important factor in the development of arteriosclerosis, arthritis, diabetes, cataract, muscle dystrophy, pulmonary heart disease, and neurodegenerative disorders [29]. Analysis and systematization of protein damage are challenging tasks. Protein oxidation by ROS could result in the formation of amino acid radicals that produce peroxyl radicals in the reactions with oxygen, which, in turn, can generate numerous oxidation products. Most often, protein oxidation is initiated by the hydroxyl radical:

$$RH + HO^{\bullet} \rightarrow R^{\bullet} + H_2O. \quad (1)$$

The generated alkyl radical forms peroxyl radical in the reaction with oxygen:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}. \quad (2)$$

Furthermore, various carbonyl compounds (>C=O) are formed in the course of protein oxidation according to the following mechanisms:

$$R^{\bullet} + HO^{\bullet} \rightarrow R(=O)H \text{ (aldehyde)}, \quad (3)$$

$$ROO^{\bullet} + HO_2^{\bullet} \rightarrow R(=O)OH + O_2 \text{ (carboxylic acid)}, \quad (4)$$

$$ROO^{\bullet} + H^{\bullet} \rightarrow R(=O)OH \text{ (carboxylic acid)}, \quad (5)$$

$$R\text{-}(CH_2)_4\text{-}NH_2 + HO^{\bullet} \rightarrow NH_3 + R\text{-}(CH_2)_3\text{-}C(=O)H \text{ (aminoadipic semialdehyde)}, \quad (6)$$

$$...–(R1)CH\text{-}C(=O)\text{-}NH\text{-}CH(R2)–... + HO^{\bullet} + O_2 + Fe^{2+} + H^{+} \rightarrow ...–(R1)C=O + O=C=N\text{-}CH(R2)–... + 2H_2O + Fe^{3+} \text{ (breakage of peptide bond)}. \quad (7)$$

Carbonyl groups are the most commonly used markers of protein oxidative damage. They are produced by direct oxidation of amino acid residues by ROS and interaction of amino acid residues with aldehydes (including those formed as secondary products of lipid peroxidation) [30]. The main products of oxidative damage of amino acids are dityrosine, formylkynurenine, hydroxy-

lated valine and leucine, dihydroxyphenylalanine, aminoadipic semialdehyde, and others. Dityrosine is not catabolized in the organism and is excreted in urine, which makes it a suitable biomarker of the amino acid damage during oxidative stress.

**Oxidative damage of lipids.** Polyene lipids are extremely susceptible to the free-radical oxidation due to the presence of double bonds. Free-radical lipid peroxidation (LPO) occurs via the chain mechanism. Hydroxyl radical plays an important role in the LPO initiation. As a small and uncharged molecule, it can penetrate into the hydrophobic lipid layer and react with polyunsaturated fatty acids (LHs) in the composition of the supramolecular lipid–protein complexes – biological membranes and blood plasma lipoproteins. Lipid radicals are formed in the reaction:

$$HO^{\cdot} + LH \rightarrow H_2O + L^{\cdot}. \quad (8)$$

The formed lipid radical ($L^{\cdot}$) reacts with molecular oxygen dissolved in the medium, leading to formation of lipoperoxyl radical ($LOO^{\cdot}$):

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}. \quad (9)$$

Lipoperoxyl radical attacks a phospholipid molecule in its vicinity with the generation of lipoperoxide (LOOH) and a new $L^{\cdot}$ radical.

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}. \quad (10)$$

Alternating reactions 9 and 10 comprise the chain LPO reaction. The presence of divalent iron ions significantly accelerates LPO through branching of the lipid oxidation chain reaction:

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + HO^{-} + LO^{\cdot}. \quad (11)$$

The formed $LO^{\cdot}$ radical initiates a new chain of lipid oxidation:

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot}. \quad (12)$$

Under experimental conditions, the chain reactions of free-radical oxidation of biological membranes can include ten or more steps, while in living organism, the number of branchings is usually limited to 2-3 due to the presence of a large number of quenchers that terminate the chain reaction. The free-radical chain oxidation of lipids can be terminated as a result of interaction of free radicals with antioxidants (inhibitors, InHs), transition metal ions, or with each other:

$$LOO^{\cdot} + Fe^{2+} + H^{+} \rightarrow LOOH + Fe^{3+}, \quad (13)$$

$$LOO^{\cdot} + InH \rightarrow In^{\cdot} + LOOH, \quad (14)$$

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow L{=}O + LOH + {}^1O_2 + h\upsilon. \quad (15)$$

Reaction 13 is accompanied by chemiluminescence, the intensity of which reflects the rate of LPO [9].

In conclusion, free-radical LPO results in the formation of primary molecular products, hydroperoxides of unsaturated fatty acids, with subsequent accumulation of unsaturated aldehydes as the secondary products. 4-Hydoxynonenal and malondialdehyde (MDA), the most recognized LPO secondary products, are commonly considered as biomarkers of oxidative stress. Other potential oxidative stress biomarkers are isoprostanes, which are specific oxidation products of some highly unsaturated fatty acids, such as arachidonic, eicosapentaenoic, and docosahexaenoic acids. MDA and other low-molecular-weight dicarbonyls can form covalent bonds with proteins and nucleic acids [30]. In biological systems, potentially hazardous lipid peroxides (precursors of carbonyl compounds) are neutralized by the hydroperoxide-reducing enzymes, such as peroxiredoxins (Prxs), glutathione peroxidases (GPxs), and glutathione S-transferases (GSTs), which will be discussed below.

## ANTIOXIDANTS

Compounds that neutralize ROS or prevent their formation are called antioxidants. Under physiological conditions, natural low-molecular-weight antioxidants (mostly phenolic compounds) neutralize ROS via non-enzymatic reactions due to their ability to donate or accept electron(s), thus eliminating uncoupled/unstable states of the radicals. Interaction of inhibitors of free-radical reactions (InHs) with free radicals results in the formation of free-radical forms of antioxidants ($In^{\cdot}$), which are less reactive and less damaging than the original free radicals [1]. Hence, the function of natural antioxidants is not a complete termination of free-radical oxidation, but rather its delay due to the capture of uncoupled electrons from the active radicals:

$$RO^{\cdot}\ (LO^{\cdot}) + InH \rightarrow ROOH\ (LOOH) + In^{\cdot}. \quad (16)$$

Therefore, active free-radical oxidation may be accompanied by the decrease in the concentration of reduced forms of phenolic inhibitors and accumulation of semiquinones capable of re-initiation of oxidation of organic substrates. Living organisms contain “buffering” reducing systems (ascorbate, glutathione) that participate in the non-enzymatic regeneration of antioxidants. In the activity of these systems is insufficient, inhibition of free-radical processes by antioxidants (especially by their high concentrations) could result in the prooxidative effect, i.e., conversion of the antioxidant effect into the prooxidant one, due to the initiation of oxidation of biological substrates by the accumulated free-radical forms of the

antioxidants [31], which impedes the use of low-molecular-weight antioxidants for medical purposes.

The toxic effect of ROS in living organism is prevented by various low-molecular-weight antioxidants, such as tocopherols, flavonoids, ascorbic acid, reduced glutathione (GSH), melatonin, ergothioneine, and others, as well as by specialized enzymes (which can be considered as high-molecular-weight antioxidants) [1]. Enzymatic catalysis is undoubtedly the most efficient way to eliminate ROS. Various antioxidant enzymes have emerged in the course of evolution, including superoxide dismutases (SODs) that catalyze reaction of superoxide anion radical dismutation, and a wide spectrum of peroxidases, including catalases (CATs), GPxs, GSTs, glutamyl-cysteine synthases (GCSs), glutaredoxins (Grxs), thioredoxins (Trxs), Prxs, etc. In this study, we have focused out attention on the hydroperoxide-reducing enzymes (Prxs, GPxs, and GSTs).

**Evolution of antioxidant systems.** Specialized enzymatic antioxidant defense systems have been found in all studied aerobic organisms. The systems of ROS detoxication have became a necessity after the Great Oxidation Event, or Oxygen Catastrophe, which was the emergence and accumulation of free oxygen in the Earth atmosphere ~2.4-2.5 billion years ago and has caused a global change of the reducing atmosphere into the oxidizing one. There is an opinion that during the time of oxygen accumulation in the Earth atmosphere, the respiratory chains of bacteria and eukaryotic mitochondria had initially served for the $O_2$ detoxication, i.e., the functions of energy supply and antioxidant defense had been performed by the same biocatalysts (enzymes or their analogues). These systems with the dual activity could include components of the electron transport chain, with hemoproteins and iron-sulfur proteins playing the major role. Later, in the course of specialization, these proteins have likely diverged into the energy-transforming and defensive (antioxidant) ones [32]. It cannot be ruled out that the divergence of the low-molecular-weight compounds into cofactors and protective molecules (antioxidants) have occurred already at the stage of pre-biological evolution. However, some quinones (ubiquinone, plastoquinone, menadione, etc.) function in modern organisms both as electron transporters and antioxidants. The presence of oxygen in water and in the atmosphere should have resulted in spontaneous formation of its semi-reduced form – superoxide anion radical ($O_2^{\bullet-}$). Spontaneous $O_2^{\bullet-}$ dismutation (reaction rate, $7\times10^5$ $M^{-1}\cdot s^{-1}$) generates hydrogen peroxide; this process is efficiently catalyzed by SOD (reaction rate, $2\times10^9$ $M^{-1}\cdot s^{-1}$), which is present in all Kingdoms of life, as well as by the iron-sulfur protein rubredoxin (superoxide reductase) found in archaea and bacteria [33]. It should be mentioned that in many prokaryotes, iron-sulfur proteins regulate expression of the antioxidant enzyme genes [34]. It is possible that SODs have originated at the dawn of the Great Oxidation Event and is among the first antioxidant enzymes. SOD-encoding genes are found in all known aerobic organisms (from bacteria to humans). It is believed that SODs have originated from two common predecessors: one gene gave rise to the Cu/Zn-containing SODs, and another – to the enzymes containing Mn, Fe, and Ni ions. SODs are highly conserved enzymes; their active sites are highly homologous between the enzymes from different Kingdoms of life. Mammals have three SOD isoforms found in the mitochondria (Mn-containing SOD2), cytoplasm (Cu/Zn-containing SOD1), and extracellular space (Cu/Zn-containing SOD3) [35]. In both prokaryotes and eukaryotes, SOD expression is activated with the increase in the atmospheric $O_2$ concentration, which results in the upregulation of SOD activity in the cells and ameliorates the toxic effects of $O_2$ [36, 37]. Hydrogen peroxide ($H_2O_2$) formed by the SOD-catalyzed $O_2^{\bullet-}$ radical dismutation (reaction 17) is very hazardous, as its degradation in the presence of iron ions leads to the formation of the highly reactive hydroxyl radical ($HO^{\bullet}$) (reactions 18 and 19), whose oxidative and damaging potential is much higher than that of $O_2^{\bullet-}$ (Table 1):

$$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2 \text{ (catalyzed by SOD)}, \quad (17)$$

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^- + Fe^{3+} \text{ (Fenton reaction)}, \quad (18)$$

$$H_2O_2 + O_2^{\bullet-} \rightarrow HO^{\bullet} + OH^- + O_2 \text{ (Haber–Weiss reaction)}. \quad (19)$$

Reduction of $H_2O_2$ formed by the superoxide anion radical dismutation is catalyzed by peroxidases. Peroxidases belong to multiple enzyme families, whose active sites contain heme, Mn ions, and SH- or SeH-groups. First, we should mention catalase (CAT) as a heme-containing enzyme reducing $H_2O_2$ to water and molecular oxygen ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$). CAT displays the maximum activity at very high $H_2O_2$ concentrations (hundreds mM), which are not normally observed in live cells. This is likely the reason why up to 80% intracellular CAT is located in the specialized cellular organelles, peroxisomes, where the content of hydrogen peroxide can increase to the millimolar concentrations [38]. Cys- or Sec-containing peroxidases, such as Prxs, Se-containing GPxs, and non-selenium containing GSTs, are more efficient $H_2O_2$ reducers. It is important to mention that some of these enzymes are capable of reducing not only $H_2O_2$, but also hydroperoxides. It is likely that the hazard of oxidation of organic substrates (first of all, polyene lipids) by the $HO^{\bullet}$ radical has broadened the substrate specificity of peroxidases in the course of evolution. The availability of GPxs and Prxs in higher organisms has eliminated the need for CAT [4]. Indeed, the hereditary absence of CAT in the tissues (acatalasemia, or Takahara disease) is not fatal, while the knockout of GPx genes results in embryonic lethality. The knockout of Prxs leads to severe oxidative tissue damage (see below). These enzymes efficiently

remove excessive $H_2O_2$, as well as decrease the LPO level, which is essential for maintaining the structure and functions of biological membranes.

**Evolution of thiol-containing oxidoreductases.** Some amino acids are very sensitive to molecular oxygen or its derivatives (ROS). Thus, His, Met, Cys, Trp, and Tyr are the residues most susceptible to oxidative modification. Oxidation of Cys residues in proteins is of particular importance, since thiol group (SH-) can undergo several steps of oxidative transformation, which might affect the structure and functions of proteins. It should be mentioned that in proteins, Cys residues exhibit different susceptibility to oxidation due to the physicochemical properties of thiol group and its microenvironment (neighboring amino acid residues) in a particular tertiary structure. In the Prx active site, Cys forms hydrogen bonds with Thr and Arg residues, which facilitates Cys-SH deprotonation. The dissociation constant (p*K*a) of the Cys residue in peroxidase is significantly lower (~7.3) than that of the free cysteine (~8.5). Hence, at physiological pH values, the sulfhydryl group of the active site Cys could undergo deprotonation and participate in reversible or irreversible oxidative transformations in response to changes in the intracellular redox state [39].

The primary product of Cys-SH oxidation is sulfenic acid (Cys-SOH), which is unstable and can react with thiol groups of other Cys residues with the formation of intra- or intermolecular disulfide bonds. In addition, Cys-SOH can interact with GSH, forming mixed glutathione-protein disulfides Cys-SSG (the so-called glutathionylation), or can be oxidized to sulfinic acid (Cys-$SO_2H$). Sulfinic acid can be either reduced to sulfhydryl with the help of sulfiredoxins and Trxs or undergo further irreversible oxidation to sulfonic acid (Cys-$SO_3H$), which often results in the inactivation of proteins or changes in their functions (see below). Moreover, Cys-SH and thiyl radical ($RS^{\bullet}$) can react with RNS and covalently attach nitrogen monoxide in the S-nitrosylation reaction with the formation of Cys-SNO. Stable and oxidation-resistant intramolecular disulfides are of particular interest among the oxidized forms of Cys, as they could undergo reversible reduction. It is likely that formation and cleavage of disulfide bonds has been used in the evolution for altering protein functions in order to adapt protein activity to the changing redox state of the environment. Such reactions are involved in the regulation of important metabolic processes in response to the environmental conditions and cell needs [40].

Reduction of protein disulfide bonds to sulfhydryl groups (SH-) occurs with the involvement of the Trx system, which consists of the substrate, GSH, FAD-dependent enzyme glutathione reductase (GR), Grx, thioredoxin reductase (TrxR), and Trx. It should be mentioned that in addition to the tripeptide glutathione, other thiol-based reducers have been found in live organisms, such as coenzymes (A, B, M), glutathione amide, glutathione spermidine, gamma-glutamyl cysteine, mycothiol, bacillithiol, ergothioneine, ovothiol, and trypanothione. However, GSH is the most common reducer found in all Kingdoms of life. Glutathione is a universal adapter molecule, while the GSH/Trx system serves as a central metabolic system in the majority of aerobic organisms, where it removes or modifies endogenous electrophilic compounds (ROS, RNS, carbonyls), as well as numerous xenobiotics. GSH is present at high concentrations in the cytosol (1-11 mM), nucleus (3-15 mM), and mitochondria (5-11 mM). It is likely that the GSH/Trx system has emerged in aerobic organisms at the very early stages of evolution, which partly explains its involvement in almost every cellular process [41].

Hence, the above-mentioned thiol-containing oxidoreductases participate in the structural and functional modification of various oxidized proteins, including regulatory ones, via the dithiol-disulfide exchange. The changes in the degree of Cys oxidation in regulatory proteins are the mechanism used by the cells for the modulation of different signaling pathways. Trx-like oxidoreductases play the most important role in the regulation of these processes. The Trx system has evolved as a key redox component in live organisms by participating in the reactions of dithiol-disulfide exchange with numerous proteins and controlling main signaling and catalytic functions of cells under different physiological conditions. Phylogenetic studies of the Trx-like proteins have shown that this system is of ancient origin and has played a key role as an electron donor regulating the antioxidant systems and alterations in protein functions in response to changes in the environment redox state since the emergence of life on Earth [42].

**Trx-like oxidoreductases.** Considering the fact that thiol-containing oxidoreductases include different types of enzymes, we have focused our attention on the families of Trx-like oxidoreductases: Trxs, Prxs, GPxs, and GSTs. Trx-like oxidoreductases are globular proteins with the characteristic thioredoxin fold consisting of four β-strands and three α-helices (β1-α1-β2-α2-β3-β4-α3). The structures of enzymes from the Trx, Prx, GPrx, and GSTs families are presented in Fig. 1. Although these families differ significantly in the primary structure (e.g., human Trx1 exhibits ~12% homology with Prx6 and ~8% homology with GPx1), they share the same thioredoxin fold, a highly conserved and evolutionary ancient three-dimensional structure [43] [similar fold is typical for other thiol-containing oxidoreductases, such as Grxs, protein disulfide isomerases (PDIs), and others]. According to some estimates, the thioredoxin fold first emerged almost 4 billion years ago in the last archaeal common ancestor (LACA), i.e., long before the Great Oxidation Event [43]. This implies that free-radical processes (most likely, those involving thiyl radicals) have occurred in the primary ocean in the absence of oxygenated atmosphere (Table 1).

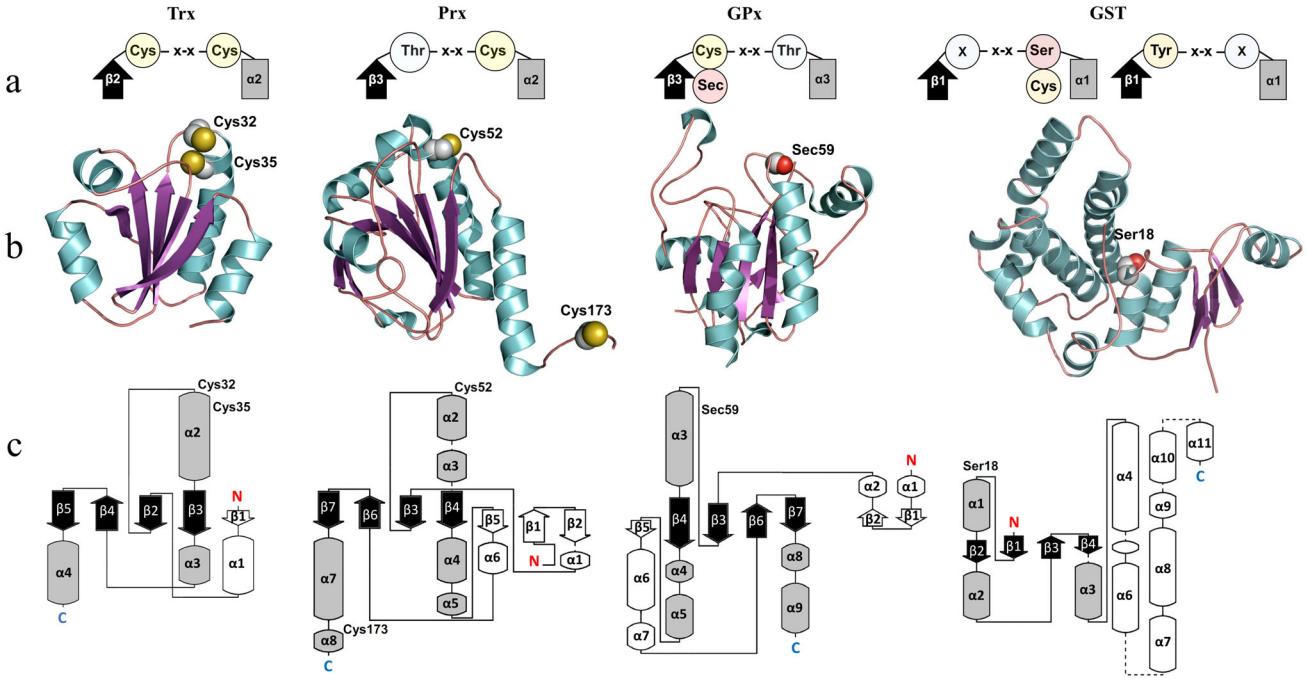


**Fig. 1.** The structure of thioredoxins (Trx), peroxiredoxins (Prx), glutathione peroxidases (GPx), and glutathione S-transferases (GST). a) Peroxidase catalytic site motifs. b) Schematic representation of the 3D structures of human Trx1, Prx1, GPx1, and GSTA1 (PyMOL v0.99). c) Organization of the secondary structure elements in the indicated enzymes; α-helices and β-strands in the thioredoxin fold are shown in gray and black, respectively.

The presence of conserved peroxidase site (motif) is typical for many Trx-like oxidoreductases. In Trxs, it contains the Cys-x-x-Cys sequence with two Cys residues separated by any two amino acids, while in Prxs, one of the cysteines is replaced with threonine (Thr-x-x-Cys). The Cys/Sec-x-x-Thr motif in the β3-strand (Fig. 1) is typical for GPxs. The presence of Sec increases the efficiency of peroxidase reaction by almost three orders of magnitude due to the high reactivity of this residue [44]. Some GSTs contain one or two Cys residues in the catalytic site; in most enzymes, this residue is replaced with serine (Ser/Cys-GSTs) or tyrosine (Tyr-GSTs) [45].

Thioredoxins. Bioinformatics studies have indicated that Trxs, which have originated approximately 4 billion years ago, are the most ancient enzymes among oxidoreductases. This could explain their involvement in numerous cellular processes associated with the reduction of oxidized cysteine; moreover, Trxs could be predecessors of Prxs, GPxs, and GSTs.

Trxs are a family of small proteins (~12 kDa) that catalyze redox reactions via dithiol-disulfide exchange with participation of two active site Cys residues separated by two amino acids (Fig. 1). Trxs are involved in the reduction of disulfide bonds in proteins and, hence, participate in multiple processes, such as (i) general cell metabolism (as substrates of ribonucleotide reductase in DNA synthesis and of 3-phosphoadenylyl sulfate reductase during sulfur assimilation); (ii) antioxidant defense (by ensuring reduction of antioxidant enzymes such as 2-Cys-PRXs; see below, and methionine sulfoxide reductase); (iii) cellular signaling in response to environmental stimuli (as regulators of enzymes); (iv) processes not directly associated with redox reactions (such as chaperon activity and restoration of the native structure of denatured proteins) [42].

Trxs are found in all living organisms, from archaea to humans. *Escherichia coli* genome encodes two Trxs, TRX1 and TRX2, and one TrxR (TRXR). Human genome contains two Trx genes, *TXN1* (cytosolic) and *TXN2* (mitochondrial), as well as two genes for TrxR, *TXNRD1* (cytosolic) and *TXNRD2* (mitochondrial) [41, 42]. Twenty Trx isoforms with the structures similar to those of both prokaryotic and eukaryotic enzymes have been found in plants [46]. Such a large number of Trx isoforms in plants is likely associated with the functioning of chloroplasts (photosynthesis) accompanied by the generation of high amounts of ROS. Despite the differences in the primary structure (for example, the sequence homology between the archaeal and human Trx1 proteins is only ~27%), all Trxs have a very conserved three-dimensional structure. Small differences in the α3-helix length are responsible for the protein structure stability [47].

The catalytic cycle of Trx includes three steps. (i) The thiol group of the N-terminal Cys in Trx attacks the disulfide bond with the formation of the disulfide bond with the target protein. (ii) Next, the thiol group of

the C-terminal Cys residue in Trx reduces disulfide bond in the Trx–target protein complex, and Trx is oxidized. (iii) Oxidized Trx is reduced by TrxR reductase in a NADPH-dependent reaction and the new cycle begins [46] (Fig. 2). Trxs play the most important role in the maintenance of redox homeostasis in animal cells. Mice lacking *TXN1* or *TXN2* genes die in the early embryogenesis [48, 49]. The main characteristics of human Trxs are presented in Table 2.

*Peroxiredoxins*. Prxs represent the most ancient family of peroxidases abundant in living organisms. Prxs have been found in all cell compartments and tissues (but mostly in the tissues of epithelial origin). The active site of Prxs contains conserved Cys residue involved in catalysis [69]. Modern classification of Prxs is based on the number of cysteines (one or two) in the active site and on the catalytic reaction properties. Peroxidatic cysteine residue ($C_P$) in the N-terminal region is required for catalysis and is typical for all Prxs (both 1-Cys and 2-Cys enzymes), while the additional resolving cysteine residue ($C_R$) is located in the C-terminal region of 2-Cys Prxs. Peroxidatic cysteine $C_P$ is surrounded by three amino acids conserved in all Prxs: Pro44, Thr48 and Arg127 (Prx1 numbering). Similar to all Trx-like enzymes, Prxs contain the thioredoxin fold (Fig. 1).

Similar to Trxs, the number of Prx isoforms have increased in the course of evolution, from bacteria and protists to multicellular organisms. Thus, bacteria have three Prx isoforms, yeast – 5, animals – 6, and plants – 9. It is likely that the increase of the number of cell compartments generating different forms of hydroperoxides has led to the specialization of Prxs against these peroxides and, respectively, to the increase in the number of Prx isoforms.

The efficiency of Prx-mediated catalysis ($k_{cat}$ in the range of ~$10^5$-$10^8$ $M^{-1}\cdot s^{-1}$) is lower than for CAT or selenium-containing GPxs [70]. It should be mentioned

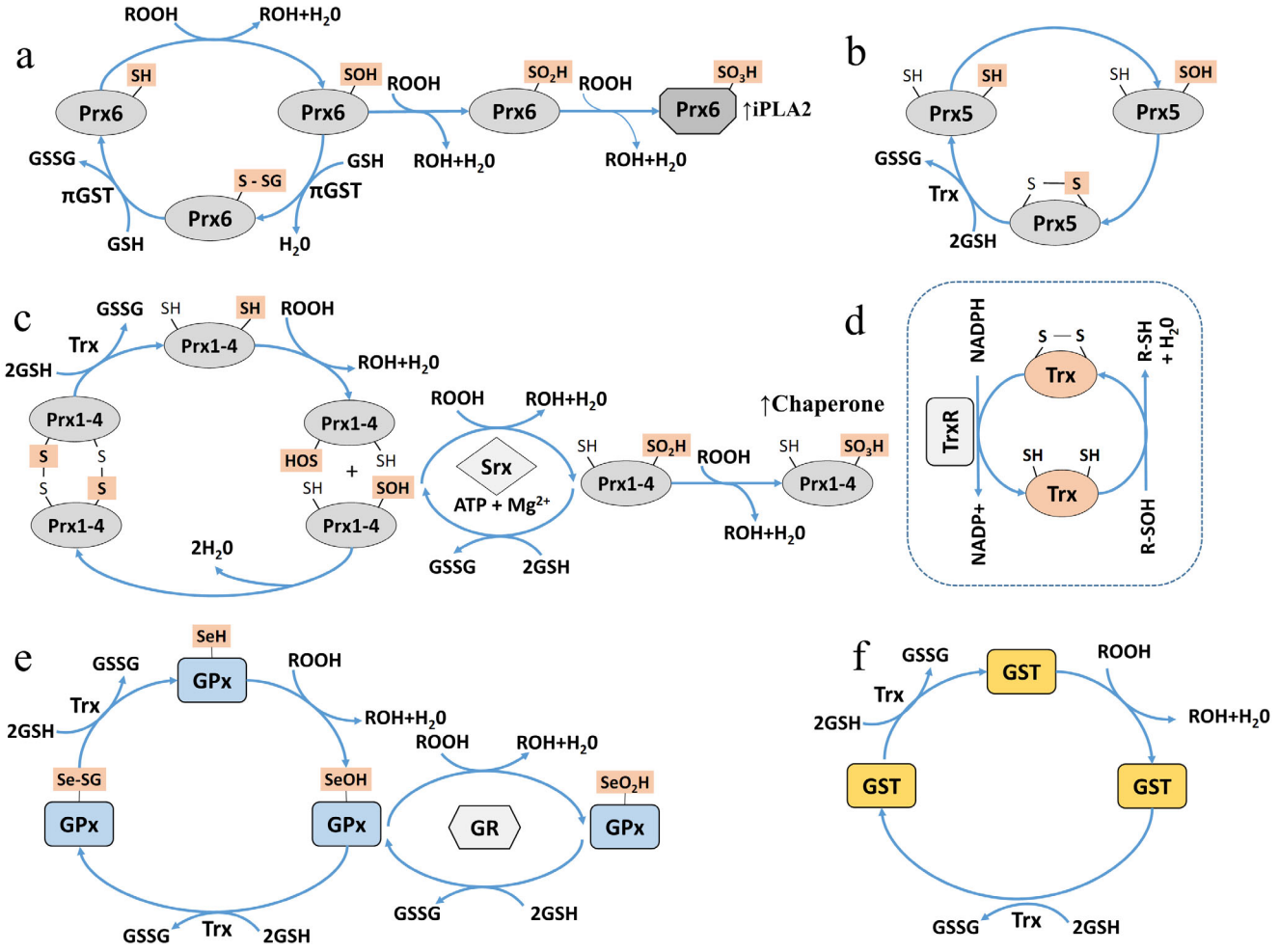


**Fig. 2.** The catalytic cycles of (a) 1-Cys Prxs (Prx6), (b) atypical 2-Cys Prxs (Prx5), (c) typical 2-Cys Prxs (Prx1-4), (d) Trxs, (e) selenium-containing GPxs, and (f) GSTs (GSTA1). ROOH, hydroperoxides; GSH, reduced glutathione; GSSG, oxidized glutathione; Srx, sulfiredoxin; GR, glutathione reductase; TrxR, thioredoxin reductase; πGST, glutathione S-transferase π; iPLA2, $Ca^{2+}$-independent phospholipase A2 activity of Prx6.

**Table 2.** Families of human antioxidant enzymes

| Properties | Trx | Prx | GST | GPx |
|---|---|---|---|---|
| Isoforms in humans | 2 isoforms: Trx1, Trx2 | 6 isoforms: Prx1-Prx6 | 17 cytosolic isoforms:<br>(α)/GSTA – 5,<br>(μ)/GSTM – 5,<br>(ω)/GSTO – 2,<br>(π)/GSTP – 1,<br>(σ)/GSTS – 1,<br>(θ)/GSTT – 2,<br>(ζ)/GSTZ – 1.<br>1 mitochondrial:<br>(κ)/GSTK – 1.<br>6 membrane-bound:<br>MGST1, MGST2, MGST3, LTC4, FLAP, PGES | 8 isoforms: GPx1-GPx8 |
| Cellular location | cytoplasm (Trx1), nucleus (Trx1), secreted (Trx1), mitochondria (Trx2) | cytoplasm (Prx1,2,5,6), endoplasmic reticulum (Prx4), mitochondria (Prx3,5), lysosomes (Prx6), nucleus (Prx1,2,6), secreted (Prx4,6) | cytoplasm/nucleus, mitochondria, microsomes (membrane-bound); in accordance with classification | cytoplasm (GPx1,4), endoplasmic reticulum (GPx4,8), mitochondria (GPx4), membrane-bound (GPx8), nucleus (GPx1,4), secreted (GPx3,5,6,7) |
| Tissue and organ location | in all tissues and organs | in all tissues and organs; predominantly in the epithelium | in all tissues and organs; large amounts are present in the intestine, liver, and lungs | GPx1 – in all tissues, predominantly in erythrocytes, liver, and kidneys;<br>GPx2 – intestine;<br>GPx3,8 - kidneys;<br>GPx4 – testes, retina;<br>GPx5 – epididymis;<br>GPx6 – olfactory epithelium;<br>GPx7 – esophageal epithelium |
| Main expression regulators | ARE (antioxidant-responsive element), Nrf2, NF-κB, Ref-1, AP-1 | ARE, Nrf2, NF-κB, HIF, AP-1, c-Myc, C/EBP, FOXO3 | ARE, XRE (xenobiotic-responsive element), GRE (glucocorticoid-responsive element), BBE (Barbie box element), AHR (aryl hydrocarbon receptor), MAF (macrophage-activating factor), NRL (neural retina leucine zipper), JNK, Fos, NF-κB | ARE, Nrf2, NF-κB, HIF, p63, AP-1, Sp1, NF-Y |
| Molecular weight | 12-18 kDa | 13-30 kDa | 23-28 kDa | 20-25 kDa |
| 3D structure | thioredoxin fold | thioredoxin fold | thioredoxin fold | thioredoxin fold |
| Quaternary structure | homodimers (Trx1,2) | monomer (Prx5)<br>homodimer (Prx1,2,3,4,6)<br>oligomers (Prx1,2,3,4,6) | homodimers/heterodimers for all isoforms | homotetramers (GPx1,2,3,5,6)<br>dimers (GPx4,7,8) |
| Peroxidase active site (motif) | **Cys**-X-X-**Cys** (Trx1,2) | Thr-X-X-**Cys** (Prx1-6) | **Tyr** (α, μ, π, σ)<br>**Cys** (ω)<br>**Ser** (κ, θ, ζ)<br>**Arg** (microsomal GST) | **Sec**-X-X-Thr (GPx1,2,3,4,6)<br>**Cys**-X-X-Thr (GPx5,7,8) |

**Table 2** (Contd.)

| Properties | Trx | Prx | GST | GPx |
|---|---|---|---|---|
| Reducing substrate | GSH (Trx1,2) | GSH (Prx1-6) | GSH | GSH (GPx1,3,4,7,8)<br>? (GPx2,5,6) |
| Enzyme reducer | TrxR | Trx, πGST, ERp46, Grx, PDI | – | Trx, Grx, PDI |
| Oxidizing substrate | $H_2O_2$<br>$ONOO^-$ | $H_2O_2$ (Prx1-6)<br>$ONOO^-$ (Prx2,5,6)<br>ROOH (Prx1-6)<br>LOOH (Prx6)<br>PLOOH (Prx6) | RX – xenobiotics<br>ROOH (α, μ, κ, θ...)<br>LOOH (α, μ, κ, θ...)<br>RCH2ONO2 (α...)<br>RCH2SCN (α...) | $H_2O_2$ (GPx1-8)<br>$ONOO^-$ (GPx1,4)<br>ROOH (GPx1,3,4)<br>LOOH (GPx1,3,4)<br>PLOOH (GPx3,4) |
| Peroxidase reaction rate ($H_2O_2$) | ~ $10^3$ $M^{-1}$ $s^{-1}$–$10^5$ $M^{-1}$ $s^{-1}$ | ~$10^5$-$10^8$ $M^{-1}$ $s^{-1}$ | ~ $10^5$-$10^6$ $M^{-1}$ $s^{-1}$ | ~ $10^7$-$10^8$ $M^{-1}$ $s^{-1}$ |
| Michaelis constant ($H_2O_2$) | 10-100 μM | 90-200 μM | >3 mM | 10 μM-500 μM |
| Michaelis constant (ROOH) | 6-20 μM | 80-120 μM | 0.05-3 mM | 0.05-1 mM |
| Number of partner proteins (https://thebiogrid.org/) | 130-233 | 130-240 | 3-148 | 3-51 |
| Participation in signaling pathways | ASK-1/AP-1, ERK, NF-κB, p53, ASK1/JNK/p38, KEAP1/Nrf2, MST1/FOXO3, PI3K, HIF1α, PI3K/PTEN/Akt/mTOR etc. | TLR4/NF-κB, ASK-1/AP-1, APE1/Ref-1, KEAP1/Nrf2, MST1/FOXO3, c-Jun/πGST, ERK/cyclin D1, JAK2/STAT3, JNK/p38, ERK1/2, HIF1α, C/EBPβ etc. | KEAP1/Nrf2, PI3K/AKT, HIF1α, ASK1/JNK/p38, MST1/FOXO3 | ASK-1/AP-1, KEAP1/Nrf2, PI3K/AKT/mTOR, NF-κB etc. |
| References | [41, 50-54] | [55-59] | [60-63] | [64-68] |

under normal conditions, the cellular concentration of $H_2O_2$ rarely exceeds 1-5 μM, but could reach up to 150 μM during the development of pathological processes. $H_2O_2$ concentrations above 200 μM cause apoptosis and cell necrosis [38]. Prx1-6 exhibit the maximum activity at the micromolar concentrations of hydroperoxides; the value of the apparent Michaelis constants (app. $K_m$) is in the range of 15-200 μM [71]. At the same time, CAT, myeloperoxidase, and the majority of GPxs exhibit the highest activity at the millimolar concentrations of hydroperoxide substrates (app. $K_m$ ~ 10-100 mM) [72]. Prxs have a wide substrate specificity. Due to their ability to reduce various inorganic ($H_2O_2$, peroxynitrite) and organic (alkyl hydroperoxides, phospholipid peroxides, etc.) hydroperoxides, Prx1-6 play an important role in the maintenance of cell redox homeostasis. The main characteristics of Prxs are presented in Table 2.

The catalytic cycle of Prxs includes three steps: (i) oxidation of peroxidatic Cys residue; (ii) formation of intramolecular or intermolecular disulfide bond; and (iii) reduction of the catalytic Cys (Fig. 2, a-c). The thiol group (SH-) of peroxidatic cysteine $C_P$ is reversibly oxidized to sulfenic acid (SOH-) in the course of peroxide substrate reduction. Depending on the peroxide concentration, sulfenic acid ($C_P$-SOH) can be oxidized further to sulfinic ($C_P$-$SO_2H$) and sulfonic ($C_P$-$SO_3H$) acids [59]. Oxidized peroxidatic Cys residue ($C_P$-SOH) is reduced to its reactive form $C_P$-SH with participation of GSH and Trx (see above). Sulfinic acid $C_P$-$SO_2H$ of the typical 2-Cys Prx is reduced to $C_P$-SOH by sulforedoxins (Srxs) or sestrins (SESNs) using the energy of ATP [73, 74], while $C_P$-$SO_3H$ is an irreversibly oxidized form. Deciphering the mechanism of $C_P$-SOH reduction has allowed to classify 2-Cys Prxs into typical and atypical enzymes. Atypical 2-Cys Prxs (Prx5) form the intramolecular bond within the same polypeptide chain, which makes their reaction mechanism closer to that of Trxs. Hence, Prx5 might be the most ancient representative of the Prxs family. It is likely that typical 2-Cys Prxs (Prx1-4), which form the disulfide bonds between the peroxidatic Cys

($C_P$) of one molecule and resolving Cys ($C_R$) of another molecule, have originated from atypical 2-Cys Prxs. The formed intermolecular or intramolecular disulfide bonds in 2-Cys Prxs are reduced with the participation of Trx and GSH. In the case of 1-Cys Prxs (Prx6), formation of the disulfide bond with GSH is catalyzed by glutathione-S transferase π (πGST) [75]. It is likely that the loss of resolving Cys ($C_R$) in Prx6 in the course of evolution was a secondary event, and the mechanism of catalysis by 1-Cys Prxs is the most recent.

Due to the kinetics of Prx1-6-mediated catalysis, which involves a slower reduction of sulfenic acid ($C_P$-SOH) in comparison with the $C_P$-SH oxidation, no oxidized Prxs ($C_P$-SOH) are accumulated in the cell [59]. Reduction of the oxidized Cys ($C_P$-SOH) in Prxs can result in the formation of intermolecular disulfide bonds not only with the protein reducers [Trx1, Trx2, PDI (ERp46), πGST], but also with other thiol-containing proteins (transcription factors, kinases, phosphatases, receptors, ion channels, and others), which, in turn, could modulate the activity of these proteins and affect numerous cellular processes [39, 58].

In addition to the peroxidase activity, Prxs have the chaperone, phospholipase, and signaling activities. Changes in the degree of peroxidatic Cys oxidation in the active site affect the properties and functions of Prxs in the cell [76]. Cysteine oxidation in the molecules of typical 2-Cys Prxs (Prx1-4) results in protein conformational changes and formation of circular oligomeric structures (toroids) with the chaperone activity. Due to the chaperone activity, typical 2-Cys Prxs prevent protein aggregation and facilitate restoration of protein native structure, thus promoting survival of cells under oxidative stress [55]. Oxidation of 1-Cys Prxs (Prx6) results in the activation of $Ca^{2+}$-independent phospholipase $A_2$ (iPLA2), which is active only under acidic conditions (in lysosomes and lamellar bodies at pH 4-5) and plays an important role in the phospholipid metabolism and transduction of intra- and intercellular signals [77]. In addition, extracellular forms of Prxs exhibit immunomodulating, signaling, and regulatory properties mediated through the Toll-like receptors [78, 79].

The physiological significance of Prxs has been demonstrated using Prx-deficient mice. The *PRDX1* gene knockout causes hemolytic anemia, increased oxidative tissue damage, and elevated number of malignant tumors. The *PRDX2* gene knockout results in erythrocyte damage, spleen pathology, and anemia development. The *PRDX3* knockout causes decrease of body mass. The *PRDX4* gene knockout results in the atrophy of testicles, oligozoospermia, and increased sensitivity of spermatogenic cells to oxidative stress. The *PRDX6* gene knockout results in the increased level of oxidative damage of proteins, tissues, and organs despite normal expression levels of other antioxidant enzymes [80]. No *PRDX5*-knockout animals have been produced; however, experiments in invertebrates indicated the involvement of this gene in the immune response [81]. Hence, Prxs are important components of the antioxidant, signaling, and regulatory systems of the organism.

*Glutathione peroxidases.* In contrast to Prxs (which contain one or two Cys residues in the active site), many GPxs are selenium-containing proteins. It is generally accepted that replacement of sulfur with selenium in the active site of GPx is associated with the fact that Sec has a lower dissociation constant (p*K*a = 5.47) and higher reduction potential compared to Cys. Due to its physiochemical properties, selenium is present in proteins at physiological pH in a form of selenolate (-$Se^-$). Selenolates are much more reactive than thiolates, which makes selenium-containing proteins more efficient catalysts. It should be mentioned that some TrxRs of the antioxidant systems also contain selenium. In particular, Trx and TrxR participate in the regeneration of Prxs [82].

Undoubtedly, selenium-containing GPxs have emerged in evolution after thiol-containing oxidoreductases, because biosynthesis of these enzymes requires special transcription and translation systems that include selenocysteine insertion sequence (SECIS, mRNA sequence that directs the cell to translate the UGA codon as Sec instead of using it as a stop codon), as well as specialized Sec-transporting tRNA [83]. GPxs, both selenium- and Cys-containing, are widely abundant in living organisms (from protists to humans). The number of selenium-containing proteins have increased in the course of evolution. Only ~20% prokaryotes use Sec in their proteins, while Sec-containing enzymes have been found in ~50% eukaryotes [84]. Humans have eight GPxs (GPx1-8), five of which (GPx1-4 and GPx6) contain Sec in the active site. Based on phylogenetic analysis, the GPx family consists of three evolutionary groups originating from a common Cys-containing predecessor: 1) GPx1/GPx2, 2) GPx3/GPx5/GPx6, and 3) GPx4/GPx7/GPx8. Cys-containing GPx7 and GPx8 have originated from a GPx4-like predecessor. GPx5 and GPx6 are likely the result of tandem duplication of the GPx3 gene. GPx1 and GPx2 are evolutionary branches of GPx3, GPx5, and GPx6 [85]. All GPxs are structurally similar and have the thioredoxin fold (Fig. 1c); they are mainly homotetrameric proteins (except monomeric GPx4).

GPxs are cell- and tissue-specific, which is likely associated with the specialization of different isoforms for the reduction of different hydroperoxides. GPx1 is located in the cytoplasm and mitochondria in almost all cells. GPx2 has been found in the colon epithelium, where it plays an important antioxidant role. GPx3 is secreted by the epithelium of renal tubules into the blood; a decrease in the content/activity of this enzyme increases the incidence of thrombosis. GPx4 is expressed in virtually all mammalian tissues; it is associated with the mitochondrial membrane and prevents oxidative damage of the mitochondria. GPx5 is secreted in the epididymis and protects

maturing spermatozoa from the action of ROS. GPx6 has been found in the olfactory epithelium, where it presumably plays an important role in the metabolism of odorants. Interestingly, human and porcine GPx6 enzymes have Sec in the active site, while the active site of mouse and rat GPx6 contains Cys. GPx7 and GPx8 are recently discovered Cys-containing GPxs of the endoplasmic reticulum that form complexes with PDIs and Trx in the reaction of dithiol-disulfide exchange, which prevents protein aggregation [86].

Typically, GPxs have a higher peroxidase activity and are more efficient catalysts than Trxs and Prxs. GPxs reduce hydrogen peroxide and organic hydroperoxides to water or corresponding alcohols, respectively, using GSH as an electron donor. The presence of Sec in the active site of GPxs allows efficient elimination of hydroperoxides with the rate constant of ~$10^7$-$10^8$ $M^{-1}\cdot s^{-1}$; moreover, such enzymes exhibit the maximum activity in a wide range of peroxide concentrations [87]. Substitution of Sec with Cys decreased the peroxidase activity of the recombinant enzyme by 2-3 orders of magnitude. The main characteristics of GPxs are presented in Table 2. Similar to the above-described oxidoreductase families, the catalytic cycle of GPxs includes three steps: (i) oxidation of peroxidatic Sec (-SeOH); (ii) formation of intermolecular disulfide bond with GSH (Se-SG); and (iii) reduction of Se-SG to the active Sec (-SeH) by the second GSH molecule. Elimination of peroxides by GPxs occurs via the ping-pong mechanism with participation of two substrates – oxidizer (peroxide/hydroperoxide) and reducer (GSH) [68].

It was found that the binding of GSH is mediated by three amino acids located in the vicinity of the GPx1 catalytic site. Interaction of GPx1 with hydrogen peroxide results in the oxidation of the -Se-H group in the enzyme active site to -Se-OH; next, the enzyme is reduced by a neighboring GSH molecule with the formation of the -Se-SG intermediate. The second GSH molecule interacts with the -Se-SG bond, which results in Sec reduction (-Se-H) and glutathione oxidation to GSSG. GSSG is reduced by the NADPH-dependent GR to two GSH molecules, which reenter the catalytic cycle (Fig. 2). The catalytic cycle of Cys-containing GPxs (NS-GPxs) is similar to that of 2-Cys Prxs with Trx and GSH as reducers, which once again corroborates the genetic association of these families.

The deficiency of selenium in the diet results in the decrease in the GPx content, which, in turn, lowers the resistance of an organism to oxidative stress and could result in the development of free radical-mediated pathologies. Reduced GPx1 activity as a result of selenium deficiency was detected in patients with cardiovascular diseases and cancer [88]. A decrease in the activity of GPx7 facilitates cell transformation and development of malignant breast tumors [89]. The physiological significance of some GPxs was demonstrated in the experiments in mice deficient by the respective genes. In particular, mice with one copy of the *GPX1* gene had normal phenotype and lifespan, while mice lacking both *GPX1* alleles demonstrated premature cataract development and defects in the proliferation of auxiliary muscle cells. Overexpression of *GPX1* protects mice from the development of oxidative stress; however, these animals were found to develop hyperglycemia, insulin resistance, and obesity [68]. Deficiency by the *GPX2* gene results in the development of inflammation of the intestinal mucosa, especially in the case of selenium-poor diet [65]. Mice with the knocked out mitochondrial and nuclear isoforms of *GPX4* die in early embryogenesis, while the knockout of the cytoplasmic *GPX4* does not cause any lethal consequences [64, 67]. The knockout of the *GPX5* gene results in the defects of general development and some disorders in the development of visual system in the offspring of *GPX5*-deficient males [66].

Hence, GPxs are important antioxidant enzymes that regulate redox homeostasis, cell signaling, apoptosis, and cell differentiation.

*Glutathione S-transferases*. GSTs are another group of important components of the hydroperoxide-reducing system in cells. These enzymes also have the thioredoxin fold and belong to the Trx-like proteins. GSTs participate in the early stages of detoxication of hydrophobic xenobiotics (RXs) by attaching GSH, which makes these compounds water-soluble. After a series of complex enzyme-mediated transformations, such modified molecules are converted into marcapturates that can be excreted from an organism: RX + GSH → GSR + HX [90]. GSTs provide cell resistance to antibiotics, herbicides, insecticides, and chemotherapy agents. Thus, GST overexpression was found in various drug-resistant cancer cells [63]. In addition to their detoxifying function, GSTs play an important role in the operation of the antioxidant system due to their ability to reduce organic hydroperoxide to alcohols using GSH as a reducer (see below). GSTs lack the Cys-x-x-Cys dithiol motif (typical for other Trx-like proteins) in the catalytic site. Amino acid residues essential for S-glutathionylation are in the so-called catalytic loop located after the first β-strand in the Trx-like domain of GST (Fig. 1).

GSTs are abundant in all Kingdoms of life, from bacteria to animals. They comprise a rather heterogenous enzyme family with a large number of isoforms (Table 2) differing in the substrate specificity and activity, which are results of gene duplication, genetic recombination, and accumulation of mutations in the course of evolution. For some members of the GST family, this functional heterogeneity is extended further via alternative splicing and point mutations (gene polymorphism), resulting in different substrate specificities of the new GST isoforms. The availability of a large number of GST isoforms is associated with the evolutionary adaptation to various environmental conditions [62].

All GSTs consist of two domains: conserved N-terminal (GSH-binding) domain common for all isoforms and variable C-terminal (substrate-binding) domain. Classification of GSTs is based on the differences in the amino acid sequence of the C-terminal domain. The GST family is subdivided into several subfamilies based on the subcellular location and the C-terminal domain sequence. Human GSTs form 7 subfamilies: one mitochondrial GST subfamily and six membrane-bound GST subfamilies [62]. The main characteristics of GSTs are presented in Table 2.

As mentioned above, GST are important antioxidant defense enzymes. Individual GST isoforms reduce various classes of organic compounds, such as nitrates ($RCH_2ONO_2$) and isothiocyanates ($RCH_2SCN$), as well as hydroperoxides of long-chain fatty acids (LOOHs) in the presence of GSH [90]. In latter case, the reaction proceeds in two stages [91], because the enzyme catalyzes only reduction of the lipoperoxide molecule:

$$LOOH + GSH \rightarrow LOH + GSOH, \quad (20)$$

while further (non-enzymatic) oxidation of another GSH molecule occurs spontaneously:

$$GSOH + GSH \rightarrow GSSG + H_2O. \quad (21)$$

The cumulative reaction is identical to the reaction catalyzed by GPxs:

$$LOOH + 2GSH \rightarrow LOH + GSSG + H_2O. \quad (22)$$

We were the first to establish [92, 93] that, similar to monomeric GPxs, GSTs reduce LOOH-acyls of oxidized lipids directly in the membranes, without prior hydrolysis by phospholipases. This discovery was later confirmed by other authors [94, 95]. Moreover, GSTs themselves are inhibited by the products of phospholipase-catalyzed hydrolysis (long-chain free fatty acids), while the cytosolic Se-containing GPxs, on the contrary, are fully resistant to the action of free fatty acids [96]. Based on these data, it can be suggested that under normal physiological conditions, when the activity of phospholipase $A_2$ is low, the level of LOOHs in biological membranes is controlled predominantly by GSTs that are capable of reducing membrane LOOHs directly. Development of various pathologies, e.g., ischemia, creates the conditions (acidosis, $Ca^{2+}$ release, and others) for the phospholipase $A_2$ activation, which should be accompanied by suppression of the GST activity due to accumulation of free fatty acids and involvement of cytosolic GPx in the detoxication process, which are insensitive to the products of phospholipase hydrolysis. At the same time, phospholipase $A_2$ predominantly hydrolyses oxidized fatty acid chains of phospholipids [97, 98], transforming them into hydroperoxides, which are substrates of Se-containing GPx. This confirms an important role of enzymatic hydrolysis in the repair of biological membranes after their free-radical damage. Hence, phospholipase $A_2$ is directly involved in the regulation of free-radical oxidation of lipids in cells by making oxidized fatty acids of membrane lipids accessible for the reduction with cytosolic GPxs. It was established that low-molecular-weight hydrophilic and nucleophilic organic hydroperoxides and $H_2O_2$ are the preferred substrates of the Se-containing GPx from bovine erythrocytes, while the non-selenium GST from porcine liver prefers lipophilic and electrophilic hydroperoxides with large hydrocarbon radicals [99]. Both enzymes efficiently reduce hydroperoxides of polyene fatty acids; however, GST (which is uncapable of reducing $H_2O_2$) efficiently reduces LOOH-acyls of unsaturated phospholipids in biomembranes without their prior hydrolysis by phospholipase $A_2$ [98-100].

In addition to the direct involvement in the reduction of hydroperoxides, some GST isoforms participate in the antioxidant defense indirectly. In particular, GSTπ reduces oxidized peroxidatic Cys of 1-Cys Prxs (Fig. 2), thus acting as a reducer in the catalytic cycle of human Prx6. Another group of GST substrates is oxidized catecholamines that facilitate formation of superoxide anion radical. Conjugation of such compounds with glutathione prevents excessive $O_2^{\cdot-}$ production and ensures normalization of cell redox status [101].

In addition to the detoxification and antioxidant functions, GSTs regulate intracellular signaling by interacting with kinases and transcription factors (Table 2). For example, GSTA1-1 and GSTP1-1 bind to JNK1 kinase (enzyme involved in apoptosis initiation), thus preventing its activation under normal conditions. The complexes of JNK1 with GSTA1-1/GSTP1-1 dissociate with the increase in the ROS concentration in the cell, which triggers apoptosis. Similar situation was demonstrated for GSTM1-1 and pro-apoptotic kinase ASK1. Glutathionylation of Cys residues by GSTs is involved in the regulation of serine/threonine AMP-activated protein kinases (AMPKs) that play a key role in the control of cell energy balance. GSTs also indirectly modulate the activity of regulatory proteins through controlling the level of LPO products (acrolein, 4-hydroxy-nonenal), which affect the activity of cyclin-dependent kinases [60].

The physiological role of some GSTs was demonstrated in animal models deficient by the corresponding genes (*GSTA3*, *GSTA4*, *GSTM1*, *GSTP1*, GSTT1, *GSTO1*, and *GSTS1*). It was found that the knockouts of individual GST genes did not lead to any serious alterations in the mouse phenotype or fertility; however, such animals demonstrated an increased sensitivity to many xenobiotics [61]. In particular, mice with the *GSTP1-1* knockout were more sensitive to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which resulted in the premature degradation of dopaminergic neurons and striatum fibers [102]. The *GSTT1* knockout

also resulted in the increased sensitivity to 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), dichloromethane (DCM), and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [61, 103]. Homozygotic deletions of the *GSTM1* and *GSTT1* genes were found in humans, which resulted in the decrease of total GST activity in the tissues. Deletion of the *GSTT1* gene in humans is associated with leuko- and neutropenia. Furthermore, it was suggested that individuals with such homozygotic deletion have an increased risk of malignant neoplasms due to the reduced carcinogen-detoxifying capacity [102, 104].

## CONCLUSIONS

Prxs, GPxs, and GSTs are phylogenetically close families of enzymes. They are the most important regulators of redox homeostasis in the cell that neutralize a wide spectrum on inorganic and organic peroxides. The peroxidase activity of GPxs is higher than that of Prxs and GSTs. Prxs are more evolutionary ancient proteins, being the predecessors of GPxs. However, they have acquired additional functions in the course of evolution. In particular, 2-Cys Prxs (Prx1-4) prevent protein aggregation in the cells during thermal or oxidative stress due to their chaperone activity. The presence of phospholipase activity in Prx6 allows this enzyme to participate in the metabolism of membrane phospholipids, as well as in the regulation of cell differentiation, migration, and death. The ability of Prxs to form intermolecular disulfide bonds with important regulatory proteins (transcription factors, receptors) underlies the indirect effect of these enzymes on all main cellular processes. GSTs are the most multifunctional Trx-like proteins. They are the major players in xenobiotic detoxification and regulation of intracellular signaling; moreover, GSTs participate in the elimination of many hydroperoxides (especially those of lipid nature). Compared to Prxs and GSTs, GPxs are evolutionary younger and more specialized enzymes. Due to the presence of Sec in the active site, GPxs belong to the most efficient peroxidases utilizing various peroxide substrates, which makes them indispensable elements of the antioxidant defense. Prxs, GPXs, and GSTs are results of molecular evolution of Trx-like proteins, whose interrelated functions have not been fully explored yet.

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