Chapter 6

Saccharomyces cerevisiae Peroxiredoxins in Biological Processes: Antioxidant Defense, Signal Transduction, Circadian Rhythm, and More

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Abstract

The yeast *Saccharomyces cerevisiae* is a model organism for biochemical and genetic studies, and several very important discoveries of fundamental biological processes have been conducted using this yeast as an experimental organism. An emerging concept, which is validated by several works using this organism, relies on the biological importance of oxidant species, specially the hydroperoxides. These molecules were formed during aerobic biological process and control several intracellular mechanisms such as a range of signaling pathways, cell cycle, programmed cell death, circadian rhythm, aging, and lifespan extension. Thereby, cellular homeostasis depends on a refined control of hydroperoxides levels and low-molecular-weight molecules in combination with antioxidant enzymes playing a role in this equilibrium. This proposal is focused on the *S. cerevisiae* peroxiredoxins and their role in peroxide decomposition, signal transduction, circadian clocks, and aging as model enzymes for the study and comprehension of these biological processes in living organisms, including humans.

Keywords: thiol-specific antioxidant protein, functional transitions, peroxidase, chaperone, overoxidation

1. Introduction

The use of *Saccharomyces cerevisiae* as a biological model in the field of oxidant species research represents a very important tool in an exciting area. Emerging concepts, validated by several

works, revealed the importance of oxidant molecules in biological processes, especially the hydroperoxides [1, 2]. These molecules are formed during several aerobic biological processes and, in adequate levels, are involved in a number of intracellular mechanisms, such as redox signaling pathways related to cell cycle progression, programmed cell death, circadian rhythm, aging, and lifespan extension [2–7]. However, the accumulation of these molecules can be harmful to the cells [3, 4]. In fact, highly deleterious radical species can be generated from hydroperoxides, such as hydroxyl radical (•OH), that is generated from hydrogen peroxide (H_2O_2) through Fenton and Harber-Weiss reactions. The •OH is able to oxidize carbohydrates, lipids, proteins, and DNA, being extremely toxic to cells. Thereby, cellular homeostasis depends on a refined control of hydroperoxides levels, and this role is played by both low-molecular-weight molecules, such as glutathione and ascorbic acid, as well as by antioxidant enzymes such as glutathione peroxidases (Gpxs), catalases (Cats), and peroxiredoxins (Prxs) [2, 4]. The latter ones have been subject of intense studies since works involving kinetic approaches indicate that the Prxs decompose more than 90% of cellular hydroperoxides [8, 9]. Additionally, to exert their biological functions, several Prxs are able to perform amazing structural switches, revealing an intricate puzzle among protein structure and function [10–12].

The first Prx described was a cytosolic enzyme identified in *S. cerevisiae* and received the name of "thiol-specific antioxidant protein 1" (Tsa1) [13]. Subsequently, a second homologue cytosolic isoform, named Tsa2, was identified and characterized. Currently, there are five Prx isoforms identified in this yeast. In mammals, there are six isoforms described, and as in other organisms, they are located in several cellular environments as cytosol, nucleus, peroxisome, mitochondria, endoplasmic reticulum, and even in the nucleus [14, 15]. Furthermore, these proteins are very abundant. For example, in *S. cerevisiae*, they can reach ~0.9% of total soluble proteins and can represent one of ten most expressed enzymes in bacteria and in mammal cells [16]. In human erythrocytes, PrxII is the third most abundant protein, only losing in concentration for globins and carbonic anhydrase, and its level is modulated during cell differentiation [17].

Besides the widespread cellular distribution and abundance, Prx stands out due to their highly efficient ability to decompose a wide variety of hydroperoxides (H_2O_2, nitrite peroxide, lipid peroxides, among others), with second order rates reaching ~10^6–10^8 M^{-1} s^{-1}) [18–21]. These characteristics place the Prx as one of the main modulators of hydroperoxides levels and, consequently, of the cellular processes mediated by them. The Prx enzymes are able to decompose hydroperoxides without any prosthetic group, but using only a highly reactive cysteine residue named peroxidatic cysteine (C_P) [5, 22]. All the Prxs described to date present a conserved motif containing the C_P (PXXXT/SXXC_P), which is oxidized to cysteine sulfenic acid (C_P-SOH) after hydroperoxide reduction [10]. This enzyme family is very heterogeneous, and different classifications have been proposed; the most currently used one subdivides these proteins in three large subclasses,1-Cys Prx, typical 2-Cys Prx, and atypical 2-Cys Prx, based in the number of cysteines involved in catalytic cycle and structural aspects (**Figure 1**). The 1-Cys Prxs are homodimeric proteins that present only one cysteine residue, the C_P, involved in hydroperoxide catalysis. 2-Cys Prxs may be monomeric (in the case of some atypical 2-Cys Prx) or homodimeric proteins and present a second cysteine residue,

named resolving cysteine (C_R), which condenses with C_P forming a disulfide bond as final product during the catalytic cycle. In typical 2-Cys Prx, the disulfide is intermolecular (e.g., between different monomers), while in atypical 2-Cys Prx, the disulfide is intramolecular (in the same monomer) [23].

Among the different Prx subclasses, the typical 2-Cys Prxs are the best studied, and, from this point on, our focus will be on this Prx subclass. After oxidation, the disulfide bond of the typical 2-Cys Prx is frequently reduced by the low-molecular-weight (~11 kDa) enzyme thioredoxin (Trx). The oxidized Trx is reduced by thioredoxin reductase (TrxR), which uses electrons from nicotinamide adenine dinucleotide phosphate (NADPH) *via* a flavin adenine dinucleotide (FAD) molecule. Together, Trx, TrxR, and NADPH are named thioredoxin system (Trx system) [21]. It is important to mention that all electron exchanges between the proteins are performed using catalytic cysteines [25] (**Figure 2**).

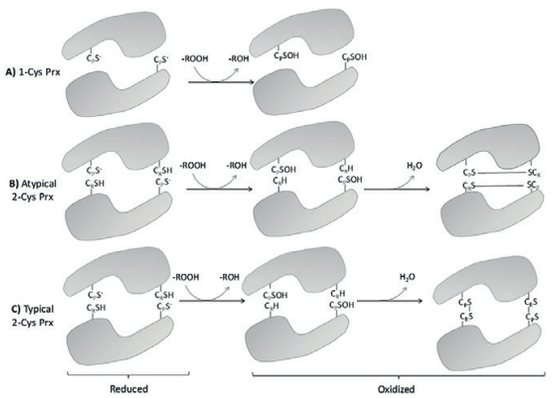

Figure 1. Prx subclasses in reduced and oxidized states. For all enzymes, the first step of the catalytic cycle is represented by the attack of the C_P-S^- over the O—O from hydroperoxide forming cysteine sulfenic acid (C_P-SOH) and releasing R—OH. (A) 1-Cys are dimeric enzymes containing only the peroxidatic cysteine, which is stable in oxidized state (C_P-SOH). (B) In the atypical 2-Cys Prx, the oxidized cysteine (C_P-SOH) formed after hydroperoxide decomposition condenses with the C_R-SH from the same monomer forming an intramolecular disulfide bond. (C) In typical 2-Cys Prx, the C_P-SOH condenses with C_R from the adjacent monomer forming an intermolecular disulfide.

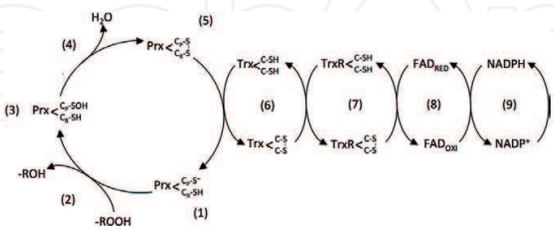

Figure 2. Hydroperoxide reduction steps by typical 2-Cys Prx and Trx system. The Prx C_P in thiolate form (**1**) attacks the hydroperoxide (**2**), releasing a water molecule in the case of H_2O_2 reduction, or an alcohol when the substrate is an organic hydroperoxide (the "R" represents the hydroperoxide radical). C_P is oxidized to cysteine sulfenic acid (**3**), releases a water molecule (**4**) and condenses with C_R forming an intermolecular disulfide (**5**), which is reduced by the enzyme Trx (**6**). Trx disulfide is reduced by the cysteines from TrxR enzyme (**7**) using electrons from NADPH (**9**) *via* a FAD molecule (**8**).

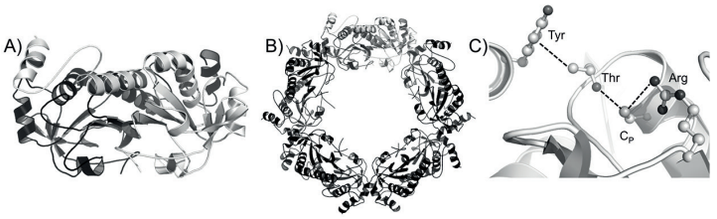

Figure 3. Quaternary structures of the typical 2-Cys Prx. (A) The yeast Tsa1 homodimer is represented in cartoon. (B) α2(5) decamer formed by the association of five homodimers. (C) Microenvironment of the CP in the active site. The Thr and Arg residues are involved in the thiolate (S−) stabilization. Additionally, the Thr residue is able to perform a CH-π interaction with the C atoms of a Tyr ring from the adjacent dimer. The proteins are represented in cartoon, and catalytic triad and the Tyr residue are represented in ball and stick. Figures were generated using the S. cerevisiae Tsa1 crystallographic coordinates (PDB: 3SBC) and Pymol software (http://www.pymol.org/).

Despite that the basic functional unit of the typical 2-Cys Prx is represented by a α(2) homodimer, studies using the Tsa1 and Tsa2 isoforms from *S. cerevisiae* revealed that this oligomeric state presents low peroxidase activity, and the highest reactivity of the typical 2-Cys Prx is reached when these proteins are found in a ring-shaped α2(5) decamers (association of five homodimers; **Figure 3A**). It is believed that the alternation between these two quaternary structures is responsible for the modulation of their peroxidase activity and may be involved in signal transduction (**Figure 3B**) [10]. Additionally, the typical 2-Cys Prx enzymes may also present other oligomeric states that will be discussed posteriorly.

The high reactivity of Prx over hydroperoxides is related to the maintenance of C_P in thiolate form (C_P-S^-), suitable for catalysis as a consequence of the microenvironment of the active site. The C_P thiolate is stabilized by polar interactions with a threonine (or a serine, in some cases) and an arginine residue (**Figure 3C**). These three residues (Thr, C_P, and Arg) are named catalytic triad and are widely conserved among all Prxs described to date [10]. During catalysis, a guanidine group of the Arg residue is able to perform a hydrogen bond with the proximal oxygen (O) of the hydroperoxide, allowing the nucleophilic attack of the C_P over the hydroperoxide [24]. The Oγ from Thr, in turn, would act as an acceptor of the hydrogen bond with the distal O from hydroperoxide, aiding the positioning of the molecule in a productive way to catalysis [24].

Typical 2-Cys Prxs, such as *S. cerevisiae* Tsa1 and Tsa2, are still able to perform additional structural and functional switches acting as peroxide sensors, molecular chaperones and are involved in several hydroperoxide-dependent signal transduction pathways, as it will be discussed further [20, 21]. Tsa1 and Tsa2 are also evolutionarily related to human PrxI and PrxII. In fact, Tsa1 presents 67% of identity and 77% of similarity with human PrxII, while Tsa2 presents 60% of identity and 76% of similarity with human PrxI, which places these proteins as important models to the study of the human Prx and the biological processes related to them.

2. Redox cycle and structural transitions

During the redox cycle, some typical 2-Cys Prxs are able to transit between different oligomeric species: α2(5) decamers (reduced enzyme) and α2 dimers (disulfide oxidized protein). Aiming to understand the details of the catalytic cycle and structural transitions, we have

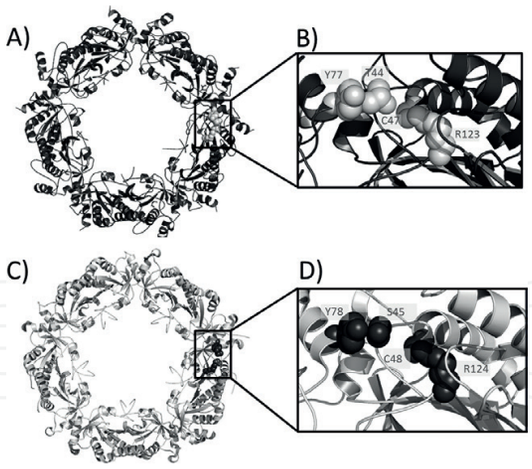

Figure 4. Tsa1 and Tsa2 interactions at dimer-dimer interface. Cartoon representations of quaternary structures of the Tsa1 and Tsa2. (A) Representation of theTsa1 decamer in cartoon. (B) Interaction between Thr44 and Tyr77 in Tsa1 decamer interface. (C) Tsa2 decamer in cartoon. (D) Interaction between Ser45 and Tyr78 in the Tsa2 decamer interface. In (B) and (D) the atoms are represented by spheres and colored as follow: O = red, C = yellow, N = blue. The gures were generated using the S. cerevisiae Tsa1 (PDB: 3SBC) and Tsa2 (PDB: 5DVB) coordinates and the Pymol software (http://www.pymol.org/).

determined the crystallographic structure of Tsa1 [21]. In fact, the analysis of the structure revealed an interaction of the Thr from the active site motif, at the dimer-dimer interface of the decamer. Recently, using different methodological approaches as site-directed mutagenesis, biochemical approaches, size exclusion chromatography, and structural analysis, we have demonstrated that a slight difference in the PXXXT(S)XXC_P is involved in decamers to dimers transitions [10]. While Tsa1 possess a Thr residue embedded in the conserved motif, in Tsa2, the Thr is naturally substituted by a Ser (**Figure 4**). In fact, the Tsa1 enzyme, containing Thr residue, transits between dimers (oxidized form) and decamers (reduced enzyme), but the Ser-containing enzyme Tsa2 is not able to dissociate in dimers. Indeed, the rearrangements as consequence of the redox states in the Tsa1 may cause hysteric hindrance of the Thr Oγ with the Tyr aromatic ring of the adjacent monomer, causing the decamer dissociation. Since Tsa2 presents a Ser residue, the hysteric clash probably is avoided. These characteristics may indicate an additional regulation of Prx quaternary structure, which may have implications in biological processes.

3. Prx overoxidation: structural and functional implications

During the typical 2-Cys Prx catalytic cycle under high levels of hydroperoxides, before disulfide formation, C_P-SOH can be attacked by another hydroperoxide molecule and becomes overoxidized to cysteine sulfinic acid (C_P-SO_2H) or sulfonic acid (C_P-SO_3H). The C_P overoxidation is related to spectacular functional and structural switch in typical 2-Cys Prx. As mentioned before, when the typical 2-Cys Prx are in reduced state (C_P-S^-), these proteins are decamers, but when are oxidized in disulfide, they can be dimers and/or decamers and are able to act as peroxidases (**Figure 5A** and **B**) [9, 11]. However, when the C_P is overoxidized, these enzymes are able to promote an intense oligomerization to form high-molecular-weight (HMW) spherical complexes (**Figure 5C**), with the concomitant inactivation of the peroxidase

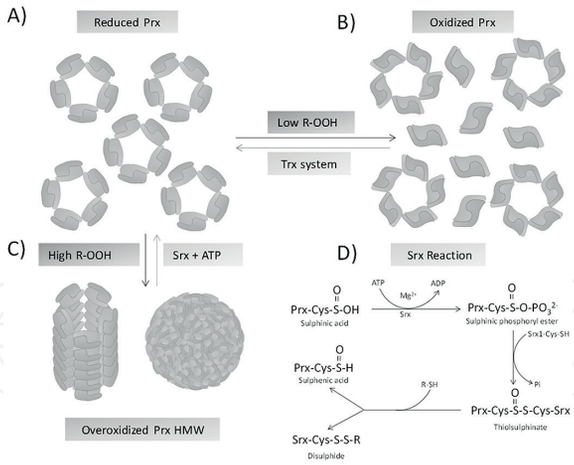

Figure 5. Typical 2-Cys Prx overoxidation and high-molecular-weight complex formation. The typical 2-Cys Prx in reduced form are presented as α2(5) decamers (A). In low concentrations of hydroperoxides, the C_P is oxidized in C_P-SOH, and the intermolecular disulfide is formed with C_R. The disulfide formation, in some cases, is able to destabilize the decamers, forming a mixture of decamers and dimers (B). The oxidized form is reduced by Trx system. When the typical 2-Cys Prx are challenged with high concentrations of hydroperoxides, the C_P can be overoxidized to C_P-SO_2H. The C_P overoxidation promotes the HMW complexes formation which presents chaperone properties (C). The C_P-SO_2H can be reduced by sulfiredoxin, in ATP and Mg^{2+} dependent manner (D).

activity. The HMW complexes formation was first reported in *S. cerevisiae* Tsa1 and Tsa2 by Jang and coworkers [11], and, posteriorly, very similar complexes were described to the human homologues typical 2-Cys Prxs (PrxI and PrxII) [26]. Using transmission electron microscopy (TEM), it was demonstrated that complexes are represented by heterogeneous spherical structures, which can reach 1 GDa, and biochemical approaches revealed that the complexes present an extraordinary chaperone holdase activity [9, 26]. Later on, similar spherical and another type of HWM complexes, represented by the stacking of several decamers (**Figure 5C**), were described for the plant chloroplastic 2-Cys Prxs, cyanobacterial Anabaena PCC7120 2-Cys Prx, among others [27–30]. The structural differences between the HMW complexes are not well understood to date.

A very important point relies on the fact that the Prx overoxidized species cannot be reduced by the Trx system, but some studies revealed that overoxidized typical 2-Cys Prx species could be regenerated to the reduced form *in vivo* [31]. Posteriorly, it was identified in *S. cerevisiae*, and after in human and other species, an enzyme named sulfiredoxin (Srx) which is able to reduce the C_P-SO_2H in a ATP and Mg^{2+} dependent reaction, but not C_P-SO_3H, suggesting that this oxidation state is refractive to reduction [31]. Curiously, the C_P-SO_2H reduction rates by Srx are very slow when compared to the disulfide reduction by Trx (~2 M^{-1} s^{-1} and ~10^6 M^{-1} s^{-1}, respectively) [32]. The biochemical steps of the C_P-SO_2H reduction by Srx are represented in **Figure 5D**. It is important to highlight that the Srx was identified in several eukaryotes, but few prokaryotes possess this enzyme, which may be an evolutionary sophistication of the 2-Cys Prx redox cycle [33]. In fact, to the majority of the prokaryotes, no homologous Srx gene was detected in their genomes, and the typical 2-Cys Prxs are much more resistant to overoxidation. Moreover, an additional classification can be done based on the C_P overoxidation susceptibility, and the 2-Cys Prx can be classified as sensitive or robust.

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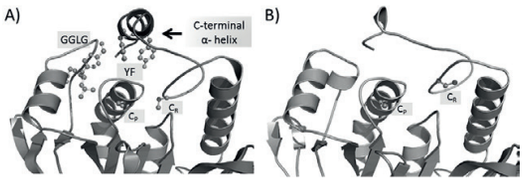

Figure 6. Structural comparison of the sensitive S. cerevisiae Tsa1 and the robust S. typhimurium AhpC. The comparison of Tsa1 (A) and AhpC (B) structures reveal the presence of the GGLG and YF motifs typically found in eukaryotes. The structures are represented in cartoon and the structural motifs as well the CP and CR are represented in ball and sticks. The figures were generated using the S. cerevisiae Tsa1 (PDB: 3SBC) and S. typhimurium AhpC (PDB: 4MA9) coordinates in Pymol software (http://www.pymol.org/).

The sensitive enzymes are present in eukaryotes and in some cyanobacteria, and the robust 2-Cys Prxs are exclusive to prokaryotes [34, 35]. The structural analyses of sensitive versus robust 2-Cys Prx revealed the presence of two motifs in the sensitive 2-Cys Prx. One is an insertion with conserved Gly-Gly-Leu-Gly, denominated GGLG motif (**Figure 6A**), and the other is an additional α-helix in C-terminal extension with a conserved Tyr-Phe sequence, the YF motif, both involved in C_P overoxidation susceptibility. **Figure 6** shows the comparison of *S. cerevisiae* Tsa1, a sensitive typical 2-Cys Prx, and *Salmonella typhimurium* (AhpC), a robust enzyme [34]. This difference is associated with important effects in redox cell signaling transduction and will be detailed in the next topics.

4. Typical 2-Cys Prx roles in redox signal transduction pathways

Increasing evidence shows the involvement of the typical 2-Cys Prx with the redox signal transduction pathways. Several antioxidant coding genes are activated by the transcriptional regulator activator protein 1 (AP1) which is considered as the major transcriptional activator of the antioxidant proteins in eukaryotes. It has been shown that the translocation of the homologue factor in budding yeast (YAP1) from cytosol to the nucleus may be controlled by 2-Cys Prx indirectly by the modulation of the cytosolic hydroperoxide levels [36]. In mammals, the PrxII is able to perform a physical interaction with the transcription factor STAT 3 (signal transducer and activator of transcription 3), which is able to activate the transcription of several genes involved in cell growth and apoptosis [37]. The authors demonstrated that PrxII can form mixed disulfides through C_P and cysteine residues of the DNA binding and trans-activating domains from STAT3, attenuating its transcriptional activity. Although the direct interaction of the typical 2-Cys Prx with target proteins is still an emerging area, this work reveals that the Prx may be an ultrasensitive hydroperoxide sensor that can form transient disulfides with unknown target proteins, which may have implications in biological processes. Additionally, the mammal PrxI can bind to several proteins including the tumor suppressor phosphatase and tensin homolog (PTEN), protecting it against suppression of its lipid phosphatase activity, which occurs under oxidative stress. On the other hand, PTEN deficiency causes decrease of PrxI, PrxII, PrxV, and PrxVI, suggesting that the Prxs and PTEN act together to maintain cellular antioxidant levels and suppress cancer-promoting pathways, such as the PI3K-Akt pathway [38].

Despite the importance of the physical interaction between typical 2-Cys Prx and biological targets, the indirect role in the regulation of the cell-signaling redox pathways is dependent of an intricate balance between peroxiredoxin, thioredoxin, and sulfiredoxin levels and their redox state. As an example, in yeast, the number of Tsa1 molecules per cell is estimated in 378,000 in aerobic conditions (log phase, SD medium), while its reductants represented by Trx and Srx molecules are much lower (~13,000 and 538 molecules/cell, respectively) [16]. In the case of Trx enzymes, additionally to the Prx reduction, these enzymes are involved in several biological processes as deoxyribonucleotide synthesis, repair of oxidatively damaged proteins, protein folding, sulfur metabolism, and activation of transcription factors among others [16]. The importance of Tsa1 reduction by Trx in redox signaling promoted by hydrogen peroxide may be significant in the cells since it produces oxidized Trx, and many signal transduction pathways are only activated by the reduced Trx enzyme [1, 39]. Because the oxidation of Trx by hydroperoxidesis is negligible, Prxs may act as a catalyst of this reaction in the cells [21].

The typical 2-Cys Prx inactivation by the C_P overoxidation combined with the low rates of the reduction of the C_P-SO_2H by sulfiredoxin (~2 M^{-1} s^{-1}) [32] is able to enhance levels of the reduced Trx to participate of other biological processes. In fact, it has been shown that the C_P overoxidation of the typical 2-Cys Prx from *Schizosaccharomyces pombe* (Tpx1) enhance the levels of the reduced Trx and allow the repair of damaged proteins increasing cell survival [40]. Accordingly, in mammals, only the reduced form of Trx is able to bind to the apoptosis signal regulating kinase (Ask-1), inhibiting the apoptosis, thus revealing a redox-dependent signal transduction pathway, which is induced by Trx oxidation [41]. Also in mammals, the activation of the nuclear factor kappa light chain enhancer of activated β cells (NF-kβ), a transcription factor that plays a central role regulating pathways of immune and inflammatory processes [42], is dependent on the reduction of a cysteine residue by Trx [43]. Additionally, Trx is involved in the reduction and activation of several transcription factors as the tumor-suppressor p53, the glucocorticoid and estrogen receptors, and c-Fos/c-Jun complexes [39].

Finally, the direct modulation of peroxides levels is an important role of the 2-Cys Prx enzymes in cell growth. It has been shown that PrxI and PrxII can eliminate the intracellular hydrogen peroxide generated by the receptors stimulation. Overexpression of PrxI and PrxII in culture cells dramatically reduces the intracellular hydrogen peroxide levels generated in response to platelet-derived growth factor (PDGF), epidermal growth factor (EGF), tirotropin (TSH), and TNF-related apoptosis inducing ligands (TRAIL). Furthermore, it has been shown that the expression of these proteins also led to a block of NF-κβ activity, which is induced by the extracellular addition of H_2O_2 or tumor necrosis factor α (TNF-α) [44]. It has also been shown that PrxII regulates different MAP kinases. Under stimulation of TNF, in which the activity of PrxII was blocked or partially abolished (knockout and RNAi), the activity of JNK and P38 MAP kinase was increased [45]. Due to the involvement of PrxI and PrxII in cell growth events, several studies have demonstrated that these isoforms have elevated levels in distinct types of cancers in different organs and tissues such as esophagus, pancreas, thyroid, lung, and breast [44–46]. The high expression of PrxI/PrxII is also associated to a more aggressive phenotype of cancer cells resistant to chemotherapy and radiotherapy [44–46].

Some authors argued that the typical 2-Cys Prx enzymes maintain hydrogen peroxide in appropriate levels to cell growth but not to apoptosis. However, Liu et al. [47] showed that the neoplastic cells of acute myeloid leukemia treated with an inhibitor of the PrxI and PrxII peroxidase activity demonstrated that the accumulation of intracellular H_2O_2 is related to the activation of the ERK1 and ERK2 (extracellular signal regulatory kinases). The kinases activation leads to an increase in the expression of the CCAAT-enhancer-binding proteins β (C/EBPβ). This condition resulted in cell differentiation and consequent tumor regression [47, 48], showing additional complexity of the neoplastic processes with the involvement of the Prx. Since there is a notable resemblance between human and yeasts typical 2-Cys Prx as also other proteins of these pathways, yeasts may be used to explore these mechanisms.

5. Prx structural switch and circadian rhythm

The circadian rhythm is a fundamental process considered to be a feature of almost all living cells. The organisms are able to exhibit cycles in their metabolism, physiology, and behavior, even when isolated from external stimuli, maintaining a 24-h period [49]. However, the molecular mechanisms which drive the circadian rhythm are not simple to elucidate, since the already identified clock genes and proteins are not very conserved across phylogenetic kingdoms [49–53]. A common model for molecular mechanism has been described for all organisms which had their circadian rhythm investigated, named transcription-translation feedback loop (TTFL) [49]. However, the TTFL components are not shared between organisms, suggesting independent evolutionary processes. Additionally, it was showed that nontranscriptional mechanisms are sufficient to sustain circadian timekeeping in the eukaryotic lineage, although they normally function in conjunction with transcriptional components [51].

Recently, it has been demonstrated that in human erythrocytes, a cell type without transcriptional activity, the PrxI and PrxII exhibit an approximate 24-h rhythm according to the C_P overoxidation. This characteristic is shared with several organisms, including *S. cerevisiae*, indicating that the typical 2-Cys Prxs constitute a universal rhythmic biomarker [52]. To reach this conclusion, the authors performed immunoblotting analyses using a Prx C_P-$SOH_{2/3}$ antibody and showed that 2-Cys Prx proteins from organisms of different domains have been oscillated to overoxidized Prx species, in constant conditions, exhibiting a circadian oscillation, probably reflecting an endogenous rhythm in the generation of reactive oxygen species (ROS; **Figure 7**) [51, 54–56]. Because all living organisms possess typical 2-Cys Prx enzymes that present remarkable conservation of the active site, the same antibody was able to detect overoxidized typical 2-Cys Prx in mice, fungi, plants, bacteria, and archaea. This indicates that the circadian clock mechanism is likely conserved across phylogenetic domains [54].

Yeast Tsa1 and Tsa2 isoforms exhibit relationship with the shorter period yeast respiratory oscillations, a cell-autonomous, temperature-compensated rhythm in oxygen consumption that synchronizes spontaneously when cells are grown at high density in aerobic, nutrient-limited, continuous culture [52]. Additionally, the yeast respiratory oscillation cycle shares

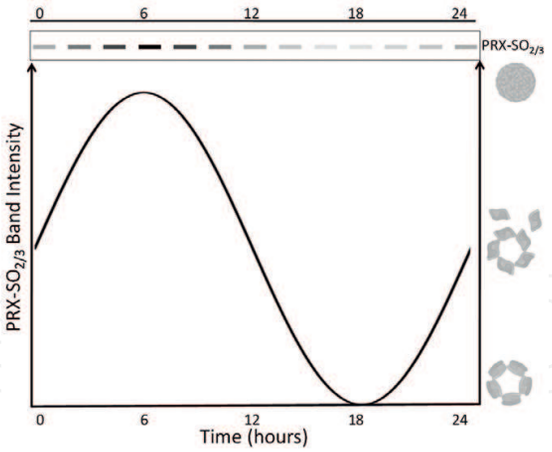

Figure 7. Redox circadian cycle of typical 2-Cys Prx. The circadian cycle of 2-Cys Prx could be detected by PRX-$SOH_{2/3}$ immunoblot. Western blot representation shows that overoxidized Prx has a circadian rhythm (upper part of the figure), and, consequently, the oligomeric state follows the redox state from Prx, alternating between dimers and decamers, in oxidative and reduced states, respectively, and HMW formation in overoxidized species (represented in the right side of the figure).

key features with the clock in mammalian cells, which may contribute to the elucidation about the origins of biological timekeeping [52].

Finally, it has been determined that the deregulation of the circadian rhythm is related to aging and genetic diseases [57]. Curiously, it has also been demonstrated that aging is related to the accumulation of the 2-Cys Prx overoxidized species in mammals [58]. Recently, a study involving the overoxidation of Tsa1 revealed that the chaperone activity detected in overoxidized species may be attributed to the association of this protein with the heat shock proteins Hsp70/Hsp104, revealing a pathway where the hydrogen peroxide is directly related to the aging process [12]. The authors also showed that the disaggregation process of the protein is dependent of Srx. Another study demonstrated that the presence of a mutant allele of Tsa1 resulted in accelerated aging in yeast [59]. One of the reasons for the involvement of these enzymes in the senescence process resides in the increase of the level of C_P overoxidation in Prx over time, even in the absence of oxidative stress [6]. In fact, this process also involves the caloric restriction, a well-known intervention that extends life span [60]. The caloric restriction elevates the level of Srx, which is responsible to reduce the hyperoxidized Tsa1, the inhibition of Tsa1 causes a profound genome instability, like chromosomal rearrangements and recombination, therefore increasing aging process [6, 61].

Another situation in which Prxs are involved is in the telomere length homeostasis [62]. The telomere dysfunction causes cellular senescence due to DNA damage [63]. The yeast mutant with *tsa1* gene deleted displayed reduction of telomere lengthening, which was not observed in conditions of low-oxidative exposure, probably due to the role of Tsa1 in hydroperoxide decomposition, avoiding DNA damage [62]. The understanding of the aging process and its implications in yeast can be used to extrapolate to higher eukaryotes. In fact, even in erythrocytes, the 2-Cys Prxs are related to the aging process. PrxII also has the ability to associate with the erythrocyte cell membrane through the N-terminal cytoplasmic domain of band 3

protein, after which PrxII undergoes a conformational change that does not entail the loss of its peroxidase function. This association may indicate a potential role of this Prx in the protection of membrane lipids against oxidative damage increasing life span [64]. Accordingly, a study carried out using mouse erythrocytes showed that the levels of overoxidized PrxII are due to autoxidation of hemoglobin and to PrxII degradation by the 20S proteasome. Approximately 1% of PrxII-SO_2H is degraded daily, leading to progressive loss of this enzyme which is directly related with the erythrocyte senescence [65]. Additionally, the aging process is directly related the genome instability. This instability is maintained in part by Prx action, and it is also involved in some diseases like cancer. Together, the data presented here reveal a cross talk of the 2-Cys Prx C_P overoxidation in circadian clocks, aging, and lifespan.

6. Methodologies to detect different redox species of the typical 2-Cys Prx

Several methodologies such as transmission electron microscopy (TEM), cryo-electron microscopy (Cryo-EM), size exclusion chromatography (SEC), mass spectrometry (MS), two-dimensional gel electrophoresis (2DGE), nonreducing SDS PAGE, and immunoblotting can be used to explore directly or indirectly the redox state of typical 2-Cys Prx [11, 66, 67]. However, for some experimental procedures, high-cost equipment and/or complex experimental procedures are necessary. Among these techniques, the nonreducing SDS PAGE, immunoblotting, and SEC are very good and cost-effective procedures, since no expensive equipment or complicated protocols are required. In this topic, these techniques and some experimental procedures will be discussed.

To access the formation of HMW complexes of purified 2-Cys Prx samples, the size exclusion chromatography (SEC) is the best choice. This methodology was used in the pioneer work performed by Jang and coworkers [11] using Tsa1 and Tsa2. In our lab, we performed a similar assay, using Tsa1, Trx system, and high concentration of cumene hydroperoxide (CHP) to promote the HMW complexes formation. Using SEC methodology, it is possible to separate several molecular species with mass range from ~45 kDa, correspondent to a dimer, followed by a ~200-kDa peak representing the decameric species, several oligomeric intermediates, and a prominent specie with more than 1000 kDa (**Figure 8**). These results are in accordance with structural analyses performed by transmission electron microscopy (TEM) by Jang and coworkers [11], using negative stain. These authors analyzed different fractions separated by SEC, and their results revealed three distinct oligomeric configurations: large spherical shaped particles, heterogeneous spherical particles, and ring-shaped structures, as represented in **Figure 8**. Currently, the cryo-electron microscopy development has provided pronounced advances to resolve complex protein structures in high resolution, such as the human Prx [67].

To reduced, oxidized, and overoxidized species from purified proteins samples *in vitro,* a simple nonreducing SDS PAGE (without DTT or another reductant) can be used to detect different redox species of the enzymes. In fact, the reduce Prx decamers and HMW complexes are held together by weak molecular forces as hydrophobic, van der Walls, and polar interactions, that are disrupted in SDS PAGE. As an example, on a nonreducing gel containing SDS,

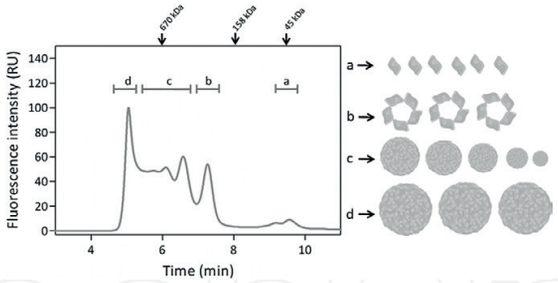

Figure 8. Overoxidized Tsa1 complexes analyzed by SEC. Tsa1 HMW species formation was analyzed by size-exclusion chromatography. The assay was performed overnight at 4°C in Hepes-NaOH 50mM (pH 7.4), DTPA 100 μM, sodium azide 1 mM, NADPH 1 mM, S. cerevisiae Prx 43.6 μM; S. cerevisiae Trx1 1 μM; S. cerevisiae TrxR1 0.3 μM and CHP 10 mM. The reaction was injected into the system containing a BioSep-SEC-S3000 column, eluted at a ow rate of 1 ml min−1 and monitored by tryptophan uorescence (excitation, 280 nm; emission, 340 nm). The elution pro le of the molecular standards thyroglobulin (bovine) (670 kDa), γ-globulin (bovine) (158 kDa), and ovalbumin (chicken) (44 kDa) were used to identify the 2-Cys Prx oligomers.

the Tsa1 is detected as a monomer (~25 kDa). The oxidized form is detected as ~50 kDa bands (dimer) as a consequence of the intermolecular disulfide bond that is formed between the C_P and C_R that is not disrupted in the gel. The overoxidized forms (Cys_P-SO_2H or Cys_P-SO_3H) can also be visualized as monomers, since the disulfide bond formation is not achievable [68, 69] (**Figure 9A** and **B**).

Figure 9B shows the SDS-PAGE result of *in vitro* procedure to perform Tsa1 overoxidation using growing concentrations of organic hydroperoxide (*t*-BOOH) and the Trx system (see the legend for detail). In this example, it is possible to verify the presence of the Tsa1 overoxidized species when high concentrations of *t*-BOOH were used (**Figure 9B**, upper panel). In **Figure 9C**, it is represented the probable quaternary structure present in the correspondent lane of the gel. In low concentrations of hydroperoxides, there are, predominantly, reduced Tsa1 in decameric

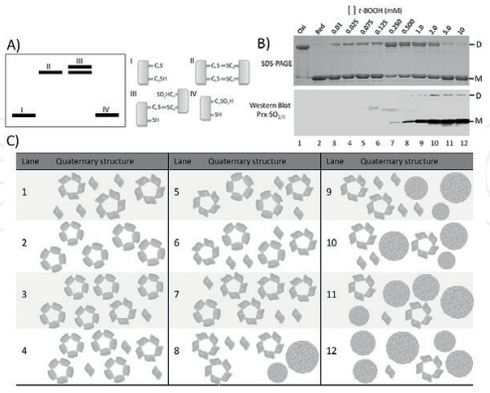

Figure 9. Redox state analyses by nonreducing SDS-PAGE and immunoblotting of the typical 2-Cys Prx. Diagram of the different 2-Cys Prx redox species in SDS-PAGE in nonreducing conditions by monomer or dimer formation (A). The Tsa1 overoxidation can be followed by SDS-PAGE in nonreducing conditions using *in vitro* approaches with Trx system in growing concentrations of hydroperoxides (B). In the example, the reaction was performed in a final volume of 50 μl at 30°C in Hepes-NaOH 50 mM (pH 7.0), DTPA 100 μM, sodium azide 1 mM, NADPH 150 μM, *S. cerevisiae* Tsa1 9.3 μM; *S. cerevisiae* Trx1 1 μM; *S. cerevisiae* TrxR1 0.3 μM and growing concentrations of *t*-BOOH (0.01, 0.025, 0.05, 0.75, 0.125, 0.25, 0.5, 1, 5, and 10 mM).The C_P overoxidized species can be observed in higher concentrations of *t*-BOOH. The oxidative state of Tsa1 induces the quaternary structural changes (C). The numbers in C demonstrate the possible structure of Tsa1 in different oxidative states (dimers, reduced decamers, oxidized decamers, and HMW complexes).

form, which is disrupted in SDS PAGE. At intermediate hydroperoxide concentrations, disulfide oxidized forms are detected, being represented by dimers and weak decamers (**Figure 9C**), that are detected as dimers in SDS PAGE. However, in high concentration of hydroperoxide, C_P overoxidation and HMW structure formations that, in the gel, are detected as monomers occur [11] (**Figure 9B**). To confirm the redox state result, a western blot analysis can be performed using an anti-$SO_{2/3}$ anti-body (**Figure 9B**, lower panel) [31, 70]. Additionally, the immunoblot technique can be used in nonpurified samples, as cell extracts, since antibodies to typical 2-Cys Prx are commercially available by several suppliers. Moreover, as mentioned before, the antibodies can be used in different species since the enzymes possess remarkable conservation [54].

7. Conclusions

S. cerevisiae is continuously used as a model organism by several researchers, being associated with significant advances in life sciences. In this chapter, we exposed several discoveries related with the role of the yeast Prx as a model in several studies related to hydroperoxide detoxification and signaling, and how these characteristics influence physiological processes like circadian rhythm and aging and diseases like cancer. All these features are related to the redox state of Prx and amazing functional and structural switches and the cross talk with different pathways that are regulated by hydroperoxide levels. Additionally, we present some practical approaches which can be easily implemented to Prx studies, like nonredox SDS-PAGE, size exclusion chromatography, and transmission electron microscopy. We believe that the use of these techniques may facilitate the study of these intricate enzymes for those interested in joining to this exciting research area.

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