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Site-Directed Mutagenesis as a Tool to Characterize Specificity in Thiol-Based Redox Interactions Between Proteins and Substrates

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1. Introduction

Redox pathways are involved in several processes in biology, such as signal transduction, regulation of gene expression, oxidative stress and energy metabolism. Proteins are the central mediators of electron transfer processes. Many of these proteins rely on non-proteinaceous redox cofactors (such as NAD^+; FAD; heme; or Cu, Fe or other transition metals) for their redox activity. In contrast, other proteins use cysteine residues for this property (Netto et al., 2007). The amino acid cysteine has low reactivity for redox transitions (Winterbourn and Metodiewa, 1999; Wood et al., 2003; Marino and Gladishev, 2011). However, protein folding can generate environments in which cysteine residues are reactive. Examples are reduction (or isomerization or formation) of disulfide bonds, reduction of methionine thioesther-sulfoxide, degradation of peptide bonds, peroxide reduction, and others (Lindahl et al., 2011).

Glutathione (GSH) is by far the major non-proteinaceous thiol in cells that plays a central role in several redox processes, such as xenobiotic excretion and antioxidant defense. GSH is composed of three amino acids: glutamate, cysteine and glycine. GSH synthesis is performed in two steps, which occur mainly in liver cells. In the first step, the γ-glutamylcysteine synthetase enzyme catalyses the rate-limiting step, with the formation of an unusual peptide bond between the gamma-carboxyl group of the side chain of glutamate and the primary amino group of a cysteine in an ATP-dependent reaction. Then GSH synthetase catalyzes the formation of a peptide bond between the carboxyl group of cysteine (from the dipeptide γ-glutamylcysteine) with the amino group of a glycine. This tripeptide is considered to be the major redox buffer in mammalian cells, and it is a substrate of two relevant groups of enzymes: glutathione transferases and glutathione peroxidases. It is thought that most healthy cells have higher GSH/GSSG (glutathione disulfide) ratios than sick ones (Berndt et al., 2007; Jacob et al., 2003; Jones, 2006).

Besides glutathione, there is a high number of Cys-based (see Table 1 in the chapter by Figurski et al. for amino acid abbreviations) redox proteins. These Cys-based proteins are very versatile. The oxidation states of their sulfur atoms can vary from +6 to -2 (Jacob et al., 2003). One of the most widespread functions of Cys-based proteins is the catalysis of thiol-

disulfide exchange reactions, by which these enzymes control the oxidation state (dithiol or disulfide) in their targets/substrates (Netto et al., 2007). Thioredoxins and glutaredoxins (also known as thioltransferases) are disulfide reductases, whereas protein disulfide isomerases are also involved in the oxidation of dithiols and/or the shuffling of disulfides. Furthermore, Cys-based proteins can also control the levels of other eletrophiles, such as peroxides (in the cases of peroxiredoxins and GSH peroxidases), xenobiotics (GSH transferases) and sulfoxides (methionine sulfoxide reductases). Therefore, this large repertoire of proteins, together with GSH, is part of a complex network that, in a dynamic fashion, controls intracellular redox balance.

The classical view is that the reactivity of a cysteine sulfhydryl group is related to its pK_a, since its deprotonated form (thiolate = RS^-) is more nucleophilic and, therefore, reacts faster than the equivalent protonated form (R-SH). According to this view, the lower the pK_a of a thiol, the higher will be the availability of the more nucleophilic species, the thiolate. The sulfhydryl groups of most cysteines (either linked to a polypeptide backbone or the free amino acid) possess low reactivity, which has been related to the fact that their pK_a values are around 8.5 (Benesch and Benesch, 1955). In contrast, most redox proteins possess a reactive cysteine that is stabilized in the thiolate form by a basic residue - in most cases by a lysine, histidine or arginine residue (Copley et al., 2004).

However, a decrease in the pK_a value of several orders of magnitude would give rise to an increase in thiolate concentration, with a maximum increase of one order of magnitude (Ferrer-Sueta et al., 2011). However, as an example of Cys-based redox proteins, peroxiredoxins reacts one to ten million times faster with peroxides than the corresponding reaction with the free amino acid cysteine (Winterbourn and Hampton, 2008). Therefore, factors other than thiolate availability should be taken into account. Indeed the stabilization of the transition state by active-site residues was recently proposed to be the catalytic power of peroxiredoxins. Site-directed mutagenesis was employed to test these hypotheses (Hall et al., 2010; Nagy et al., 2011).

It is clear that Cys-based proteins present reactive Cys residues to specific reactions, most of them being the nucleophilic substitution (S_N2) type. Indeed peroxiredoxins are effective in reducing peroxides; but they are poor in reducing other eletrophiles, such as chroloamines (Peskin et al., 2007). In contrast, glutaredoxins are powerful GSH-dependent disulfide reductants. In spite of the fact that their reactive Cys residues have low pK_a values (<4.0), these oxido-reductases are unable to reduce O-O bonds (Discola et al., 2009).

In line with the observation that Cys-based circuits display high specificity, a new concept of oxidative stress was proposed by Jones (2006). Since several antioxidant interventions failed to have therapeutic effects, it was thought that oxidative stress leads to alterations of discrete pathways, rather than to an overall redox imbalance. Therefore, perhaps an antioxidant intervention would be more effective if it were directed to specific pathways, *i.e.*, the oxidative stress would be better defined as a disruption of a specific pathway (Jones, 2006). For instance, some signal transduction pathways are activated by oxidized, but not by reduced, thioredoxin (Trx) (Berndt et al., 2007), *e.g.*, only reduced Trx1 binds Ask-1, thereby inhibiting the kinase activity of Ask-1, whereas oxidation of Trx1 leads to dissociation of the complex and activation of Ask-1, which can trigger apoptosis (Saitoh et al., 1998). Another example is the activation of NF-κB. Binding of subunit p50 to its target sequence in DNA requires the reduction of a single cysteinyl residue in the nucleus by Trx1 (Matthews et al.,

1992; Hayashi et al., 1993). Also circadian cycles depend on Cys-based redox signaling (O'Neill et al., 2011). The specificity of these pathways involves protein–protein interactions. The identification of the amino acids involved is relevant for the comprehension of pathophysiological phenomena.

2. Approach

Our group has followed an approach for studying Cys-based redox systems that involves multiple methodologies. Initially we decided to study yeast thiol-based systems, such as the thioredoxin and glutaredoxin ones. Yeast is a convenient system because it is very amenable to genetic manipulation. We obtained from *EUROSCARF* (http://web.unifrankfurt.de/fb15/mikro/euroscarf/complete.html) a collection of about four thousand strains, each one with a single deletion of a specific gene. In the case of the cytosolic thioredoxin system from *Saccharomyces cerevisiae*, we have elucidated the three-dimensional structures of all proteins by NMR (*i.e.*, ScTrx1 and ScTrx2), in collaboration with the group of Dr. Almeida (Pinheiro et al., 2008; Amorim et al., 2007), as well as by crystallography, *i.e.*, ScTrxR1, also known as Trr1 (Oliveira et al., 2010). We have also elucidated structures of the yeast glutaredoxins (Discola et al., 2009). With this information, together with available public structural, biochemical, and enzymatic data, we were able to generate a hypothesis about the mechanistic aspects of these redox pathways that could be tested by site-directed mutagenesis.

Recently we have done this approach with the bacterial thiol-based systems, as a consequence of our participation in the genome sequencing project for the phytopathogen *Xylella fastidiosa* (Simpson et al., 2000). Again site-directed mutagenesis was employed to test a hypothesis generated by experimental work. The hypothesis proposed the involvement of amino acid residues in catalysis. The interpretation of the data was not always straightforward, probably because amino acids can interact with other residues in a protein to give unpredictable effects. In the following sections, we will describe what we have learned in different thiol-based systems, using site-directed mutagenesis to test hypotheses.

3. Characterization of Cys-based proteins

3. 1. Cys-based proteins from *Saccharomyces cerevisiae*

3.1.1 Molecular aspects of specific redox protein-protein interactions in the cytosolic thioredoxin system

Thioredoxin appears to be an ancient protein, since it is widespread among all living organisms. These small proteins (12–13 kDa) possess disulfide reductase activity endowed by two vicinal cysteines present in a CXXC residue motif - typically CGPC. The cysteines are used to reduce target proteins that are recognized by other domains of thioredoxin polypeptide. The reduction of target proteins results in a disulfide bridge between the two cysteines from the thioredoxin CXXC motif, which is then reduced by thioredoxin reductase using reducing equivalents from NADPH. Some of the target proteins of thioredoxin include ribonucleotide reductase (important for DNA synthesis), methionine sulfoxide reductase, peroxiredoxins and transcription factors such as p53 and NF-*k*B (reviewed by Powis and Montfort, 2001). Therefore, the thioredoxin system is composed of NADPH, thioredoxin reductase and thioredoxin.

Proteins endowed with thioredoxin reductase activity are also widespread and comprise enzymes with different redox centers. Thioredoxin reductase enzymes belong to the nucleotide pyridine disulfide oxidoreductase family, which includes glutathione reductase, alkyl hydroperoxide reductase F (AhpF), and lipoamide dehydrogenase (Williams et al., 2000). Constituents of this family are homodimeric flavoproteins that also contain one or two dithiol-disulfide motifs - CXXXXC and/or CXXC. Thioredoxin reductase catalyzes the disulfide reduction of oxidized thioredoxin, using NADPH via the FAD molecule and the redox-active cysteine residues (Waksman et al., 1994). Initially thioredoxin reductases were divided into two sub-groups (low and high molecular weight TrxR) based on the absence or presence of a dimerization domain (Williams et al., 2000). However, there are some thioredoxin reductase enzymes with distinct extra domains that do not fit well into these two classes. Therefore, based on structural and biochemical considerations, we proposed that thioredoxin reductases should be divided into five sub-classes. In spite all these differences, all thioredoxin reductases share a common core, containing two domains (a NADPH-binding domain and a FAD-binding domain) and two redox centers: a FAD molecule and a dithiol-disulfide group (Oliveira et al., 2010).

Cytosolic thioredoxin system from yeast *Saccharomyces cerevisiae* is composed of one low molecular weight thioredoxin reductase (yTrxR1) and two thioredoxin enzymes (yTrx1 and yTrx2). Interestingly, most thioredoxin systems are composed of only one thioredoxin. yTrx1 and yTrx2 share 78% amino-acid identity and were initially considered as fully redundant enzymes. We are investigating whether these two oxido-reductases have specific roles. The expression of yTrx2 is highly inducible by peroxides in a process mediated by Yap1, whereas expression of yTrx1 is more constitutive (Kuge and Jones, 1994; Lee et al., 1999). The relevance of the cytosolic thioredoxin system from yeast can be attested to by the fact that deletion of ScTrxR1 gene renders yeast inviable (Giaever et al., 2002). Some of their targets include at least three peroxiredoxins (Tsa1, Tsa2 and Ahp1), methionine sulfoxide reductase, ribonucleotide reductase, a Cys-based peroxidase involved in the oxidative stress response (Gpx3-Orp1) (Fourquet et al., 2008) and the system involved in sulfate assimilation (PAPS). yTrx1 and yTrx2 present specificity towards their targets, *i.e.*, they cannot reduce all disulfide bonds. Some protein-protein interactions should occur in order for a protein disulfide reduction to take place. Once yTrx1 (or yTrx2) reduces a target disulfide bond, it gets oxidized. While studying the reduction of yTrx1 (or yTrx2) by yTrxR1, we observed that this flavoprotein exhibited remarkable specificity, *i.e.*, yTrxR1 only reduces yeast thioredoxins (yTrx1 and yTrx2), but not mammalian or bacterial thioredoxins (Oliveira et al., 2010). yTrxR1 can also reduce yeast mitochondrial thioredoxin (yTrx3). Probably this species-specificity phenomenon involves recognition of certain amino acid residues by yTrxR1 through protein-protein interactions.

The identification of protein–protein interactions is a major challenge in cell biology. The interactions for the various pathways are specific, directing signals to specific targets. Thiol-based systems are emerging as relevant pathways in signal transduction. Because there are several thiol-disulfide oxido-reductases in each genome, it is reasonable to think that each one of them interacts with different partners. Although all thioredoxin reductases catalyze the same overall reaction (*i.e.*, reduction of thioredoxin at the expense of NADPH), apparently the species-specificity phenomenon is restricted to the low molecular weight enzymes. This is probably because high molecular weight thioredoxin reductases have an external selenocysteine residue (a cysteine analog with a selenium instead of the sulfur atom
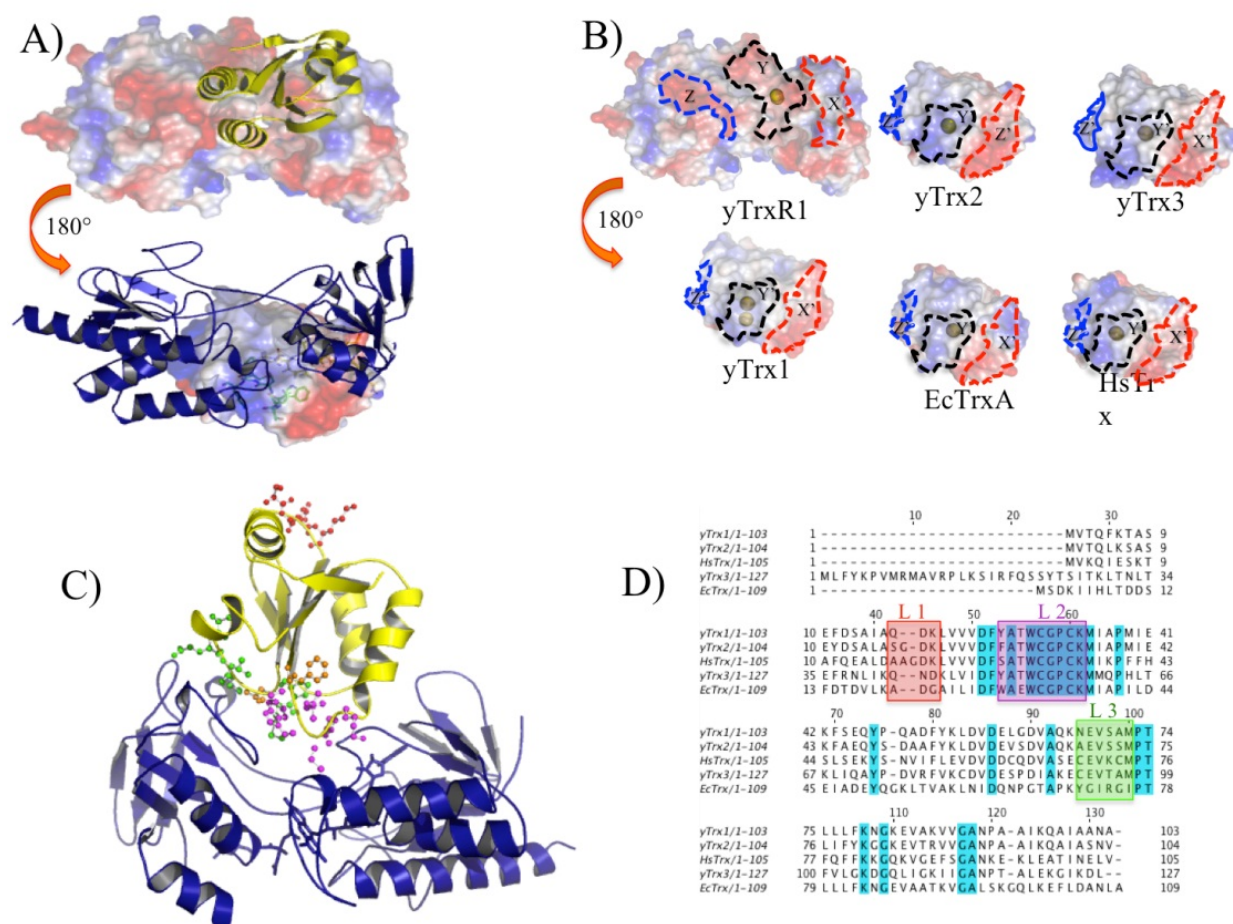
Fig. 1. **Structural features involved in the species–specificity phenomenon**. (A) Theoretical representation of the yTrx-yTrxR complex. The electrostatic surface of yTrxR1 is depicted at the top (Red = negatively charged atoms; blue = positively charged atoms; white = no charge) and yTrx1 is represented by the cartoon (yellow). At the bottom is shown the yTrxr1 electrostatic surface and yTrxr1 (cartoon-blue). (B) Comparison of electrostatic surfaces among five distinct thioredoxin enzymes, three of them from yeast. yTrx = thioredoxin from *S. cerevisiae*; EcTrxA = thioredoxin A from *E. coli*; and HsTrx = thioredoxin from *H. sapiens*. (C) Loop 2 (grape) and loop 3 (green) are in close proximity to thioredoxin reductase. (D) Amino-acid alignment among five thioredoxin enzymes. Three loops are candidates for physical interaction with thioredoxin reductase. Since the loop 3 (L3) amino-acid sequences display higher variability than the loop 2 (L2) sequences, loop 3 is implicated in the species–specificity phenomenon.

in the side chain) that can reduce target substrates with low physical interaction. Therefore, species specificity probably requires extensive protein-protein interactions. As mentioned above, thioredoxin reductase 1 from *S. cerevisiae* (yTrxR1 = yTrr1) can reduce cytosolic and mitochondrial thioredoxins from yeast; but it cannot reduce thioredoxin from *Escherichia coli* or from *Homo sapiens*.

Since thioredoxins present high sequence similarity, we are interested in identifying factors involved in species–specific interactions. As mentioned above, we obtained the structures of all proteins belonging to the cytosolic thioredoxin system from the yeast *S. cerevisiae* (yTrx1 = PDB 2I9H; yTrx2 = PDB 2HSY; yTrxR1 = PDB 3ITJ). This allowed us to test models for protein–protein interactions. The analysis indicated that complementary electrostatic surfaces between yTrxR1 and yTrxs are partially responsible for the species-specificity phenomenon (Fig. 1A). Furthermore, residues that belong to loop 3 appear to be directly related to protein-protein interactions (Fig. 1B). Indeed site-directed mutagenesis was a valuable tool for testing hypotheses raised by crystal structure analysis and by biochemical assays (Oliveira et al., 2010).

3.1.2 Aspects involved in the high oxido-reductase activity of yeast Glutaredoxin 2 in comparison with yeast Glutaredoxin 1

Like thioredoxin, glutaredoxin enzymes are thiol-disulfide oxido-reductases, whose genes are widespread among both eukaryotic and prokaryotic genomes. These small, heat stable enzymes are ubiquitously distributed and endowed with disulfide reductase activity (Discola et al., 2009). In the case of the yeast *S. cerevisiae*, eight isoforms have been identified so far. Three of them are dithiolic glutaredoxins, with two vicinal cysteines in a CXXC motif (mostly CPYC). The other five are monothiolic enzymes. They are characterized less well and will not be considered here.

yGrx1 and yGrx2 are the two major dithiolic glutaredoxins from *S. cerevisiae*. They display high amino acid sequence similarity to each other (85%). These enzymes can reduce disulfide bonds through two distinct mechanisms. In the most studied one, a mixed disulfide between a target protein and GSH is reduced by the monothiolic mechanism. In this case, only the N-terminal Cys of the CXXC motif takes part (Figure 2, reactions f and g). The most used assay to measure glutaredoxin activity, the HED (β-hydroxyethyl disulfide) assay, operates through this monothiolic pathway. Alternatively, a glutaredoxin with two Cys residues can reduce disulfides through the dithiolic pathway (Figure 2, reactions a–e).

The monothiolic pathway has received increased attention because it appears to control the levels of glutathionylated enzymes in cells. Glutathiolation is an emerging post-translational modification. This modication protects reactive Cys residues from irreversible oxidation to the sulfinic (RSO_2H) or sulfonic (RSO_3H) states. In analogy to phosphatases, glutaredoxins catalyze removal of the glutathionyl moiety and thereby regulate signaling processes (Gallogly and Mieyal, 2007). Like phosphorylation and other post-translational modifications, glutathionylation is reversible. To evaluate if a specific glutathiolation event is regulatory, some criteria were proposed, such as (1) a change in the activity of the target protein; (2) occurrence in response to a stimulus; or (3) occurrence at a physiological GSH/GSSG ratio (normally high in the cytosol), with both the modification and its

disappearance being fast (Gallogly and Mieyal, 2007). Some proteins that fulfill these criteria are actin (Wang et al., 2001; Wang et al., 2003), Ras (Adachi et al., 2004) and Protein Tyrosine Phosphatase (Kanda et al., 2006).
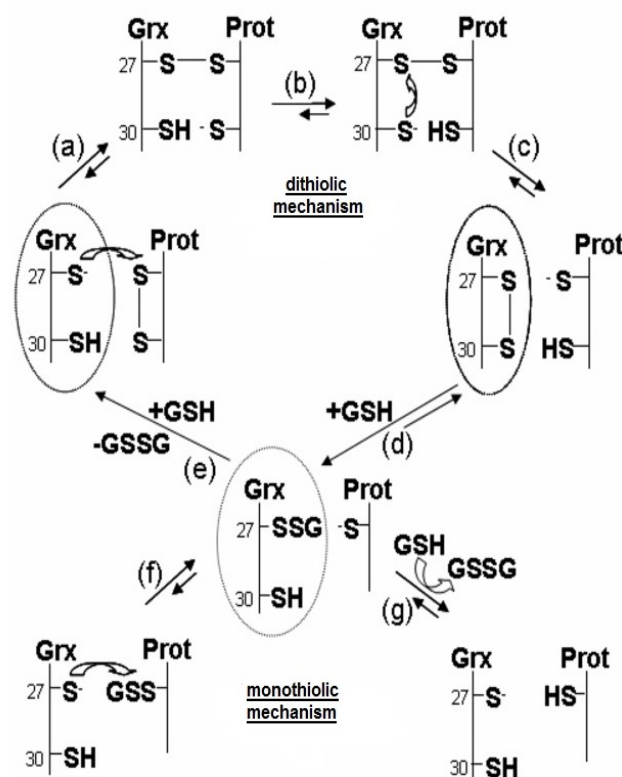

Fig. 2. **Mechanisms of disulfide reduction by glutaredoxin**. Dithiolic: Reactive Cys (in thiolate form) from glutaredoxin (Grx) performs a nucleophilic attack (S_N2 type) on a disulfide of a target proteins, leading to the formation of a mixed disulfide (reaction a); a thiolate is formed in the second Cys (reaction b) and this thiolate performs a nucleophilic attack (S_N2 type) on the mixed disulfide, generating a intramolecular disulfide on glutaredoxin (reaction c). Reduction of glutaredoxin takes places by two consecutive reactions with GSH (reactions d and f). Monothiolic: Reactive Cys (in thiolate form) from glutaredoxin (Grx) performs a nucleophilic attack (S_N2 type) on a mixed disulfide between GSH and a target protein, leading to the formation of glutathiolated glutaredoxin (reaction f); reduction takes place by reaction with a second GSH molecule (reaction g). Reaction g is considered the rate-limiting step in the monothiolic pathway (Srinivasan et al., 1997).

In collaboration with Dr. Demasi, our group has shown that the proteasome is also post-translationally modified by glutathiolation in response to oxidative stress (Demasi et al., 2001; Demasi et al., 2003) and also that glutaredoxin can reduce the mixed disulfide bond between the proteasome and GSH (Silva et al., 2008). Site-directed mutagenesis of Cys residues in the 20S proteasome is underway in order to clarify mechanistic details of this process. Therefore, it is relevant to comprehend features that control the deglutathionylase activity of glutaredoxins (Figure 2, reactions f and g) to better appreciate the function of this post-translational modification in cell biology.

In this regard, it was relevant to observe that the two main dithiolic glutaredoxins from yeast display markedly distinct monothiolic (HED assay) specific activities (Discola et al., 2009). Although these two enzymes share a high degree of similarity in their amino acid sequences, yGrx2 is two orders of magnitude more active than yGrx1 (Discola et al, 2009). These data are consistent with results from studies with knockout strains (*i.e.*, strains with null alleles) that indicate that yGrx2 accounts for most of the oxido-reductase activity observed in yeast extracts (Luikenhuis et al., 1998). In an attempt to gain insights on this phenomenon, our group obtained two crystallographic structures of yGrx2 (intramolecular disulfide = PDB 3D4M; mixed disulfide with GSH = PDB 3D5J, both of which are related to the short form yGrx2) and compared them with the crystal structures of yGrx1 (reduced = PDB 2JAD; mixed disulfide with GSH = PDB 2JAC) available in the literature (Håkansson and Winther, 2007). The overall structures are highly similar (Fig. 3A). However, differences in the active sites were hypothesized to be involved in the distinct catalytical efficiencies between yGrx1 and yGRx2 (Fig. 3B). In order to obtain the structures of these complexes, it was necessary to mutate the C-terminal Cys (Cys30) to Ser in order to slow reaction d (reverse) (see d in Fig. 2). The analysis of the structures of yGrx1C30S and yGrx2C30S (short isoform) in complex with GSH revealed that the distances between Ser30 (Cys 30 in both yGrx1 and yGrx2, short isoform, wild-type proteins) and the reactive Cys (Cys47) are markedly distinct (3.47 Å in yGrx1C30S and 5.14 Å in yGrx2C30S).
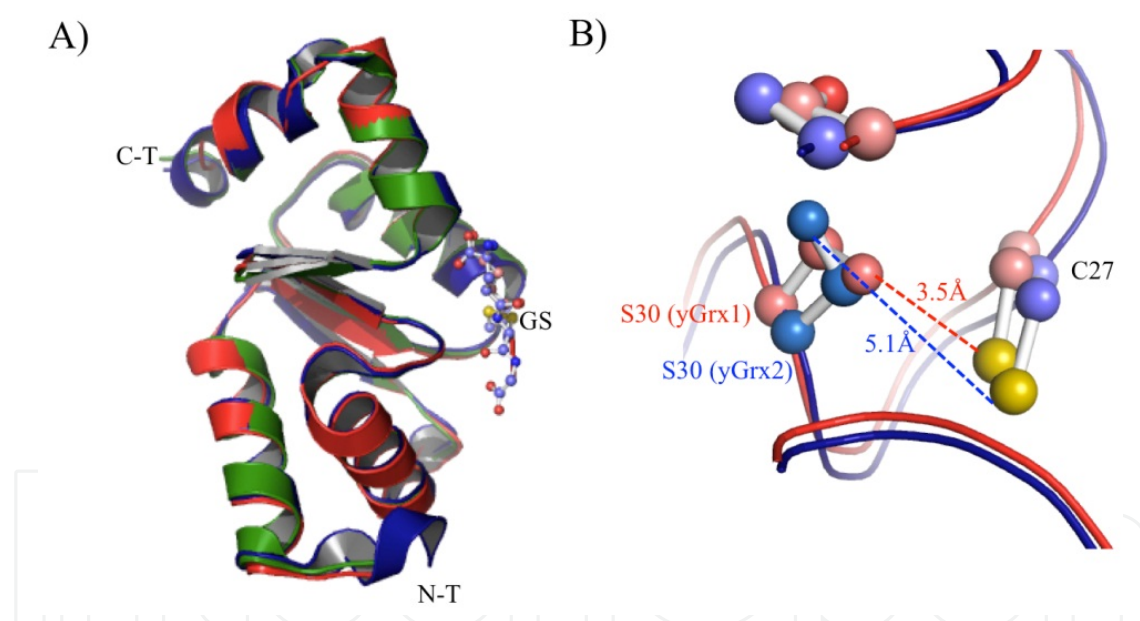

Fig. 3. **Crystal structures of dithiolic glutaredoxins from yeast**. (A) Cartoon representation of overall glutaredoxin structures. (Red= yGrx1C30S, PDB code 2JAC; Green = yGrx2 disulfide, PDB code 3D4M; Blue = yGrx2C30S, PDB code = 3D5J); (B) Active sites of the complex with glutathione. The distances between the resolving Cys and the reactive cysteine (sulfur atoms colored in yellow) are shown by dashed lines. Colors are defined in A.

In principle, any factor that would slow reaction d (reverse) (see d in Fig.2) should favor reaction g and, consequently, the monothiolic activity, which is the mechanism by which the HED assay operates. According to this hypothesis, anything that increases the distance between the two sulfur atoms of the CXXC motif should favor the monothiolic activity and, consequently, the rates in the HED assay. Accordingly, Ser23 is in close proximity to Ser30

in yGRx2 (short isoform). This interaction between two serine residues is probably stabilizing a configuration, in which the distances between the two sulfur atoms would be high in the wild-type yGrx2, thereby accounting for its high catalytical efficiency (Discola et al., 2009). In contrast, yGrx1 has an Ala residue at position 23; and this side chain cannot make a salt bridge. In this case, the distances for residues 27 and 30 (cysteines in the wild-type protein) are short. A short distance between the sulfur atoms favors the dithiolic mechanism over the monothiolic mechanism (Fig. 2). This hypothesis was tested by site-directed mutagenesis. Indeed the relevance of a serine at position 23 for the monothiolic activity was demonstrated (Discola et al., 2009).

Probably biochemical and structural features other than a serine/alanine residue at position 23 are involved with the higher catalytical efficiency of yGrx2 over yGrx1. Indeed Ser89 in yGrx2 (short isoform) and Asp89 in yGrx1 were recently implicated in the different catalytical properties of these two oxido-reductases (Li et al., 2010). Ser89 is involved in the binding of GSH in glutaredoxin (Discola et al., 2009). The authors also employed site-directed mutagenesis to show that their hypothesis was correct (Li et al., 2010).

Since glutathionylation is emerging as a key concept in redox signaling, it is reasonable that the combined approach of biochemical and structural assays together with site-directed mutagenesis will be followed to establish the involvement of other factors in this post-translational modification.

3.1.3 Site-directed mutagenesis to characterize residues that allow reduction of 1-Cys peroxiredoxin by ascorbate

Peroxiredoxins are ubiquitous, Cys-based peroxidases, whose importance is underlined by their high abundance and their involvement in multiple cellular processes probably related to their capacity to decompose hydroperoxides (Rhee and Woo, 2011; Wood et al., 2003). Indeed several groups have shown independently that peroxiredoxins compete with heme-peroxidases and Se-GSH peroxidases for hydroperoxides (Horta et al., 2010; Ogosucu et al., 2007; Parsonage et al., 2005; Toledo Jr et al., 2011). As a consequence of their high abundance and reactivity, peroxiredoxins are major sinks for peroxides (Winterbourn and Hampton, 2008).

A peroxiredoxin can be classified as a 1-Cys or 2-Cys Prx, depending on the number of Cys residues that participate in the catalytic cycle (Rhee and Woo, 2011; Wood et al., 2003). For most 2-Cys Prxs, the biological reductant is thioredoxin. For 1-Cys Prxs, the situation is far more complex. In many cases the identity of the reductant is not known. Our group has shown that ascorbate can support the peroxidase activity of 1-Cys Prx. This represented a change of the thiol-specific antioxidant paradigm (Monteiro et al., 2007).

Since 1-Cys and 2-Cys Prxs share amino acid sequence similarity, we asked ourselves which amino acids are responsible for the ability of 1-Cys Prx enzymes to accept ascorbate as the electron donor. Through a multiple approach involving amino acid sequence alignment, mass spectrometry and enzymatic assays, we postulated that two features are required: (1) the absence of a Cys involved in disulfide formation (resolving Cys) and (2) the presence of a His residue fully conserved in 1-Cys Prxs and absent in the 2-Cys Prx counterparts. By site-directed mutagenesis, we were able to engineer a 2-Cys Prx to be reducible by ascorbate by taking into account the two factors described above (Monteiro et al., 2007). Further

studies are underway in order to comprehend the physiological significance of ascorbating acting as a reducing agent for 1-Cys Prx.

3.2 Cys-based proteins from bacteria

3.2.1 Residues of Peroxiredoxin Q involved in redox-dependent secondary structure change

Our group is also interested in the analyses of Cys-based proteins from bacteria, as a consequence of our participation in the genome-sequencing project of the bacterium *Xylella fastidiosa*. *X. fastidiosa* is a gram-negative bacterium that is the etiologic agent of several plant diseases, such as Citrus Variegated Chlorosis, which imposes great losses in orange production in Brazil (Lambais, 2000). *X. fastidiosa* also causes Pierce disease in grapevines, phony peach disease, and leaf scorch diseases in almond and oleander (Hendson et al., 2001).

Animal and plant hosts generate oxidative insults against pathogens, such as *X. fastidiosa*, in an attempt to avoid infection. The oxidants include hydrogen peroxide, organic hydroperoxides, and peroxynitrite (Koszelak-Rosenblum et al., 2008; Tenhaken et al., 1999; Wrzaczek et al., 2009). To counteract this host response, bacteria present a large repertoire of antioxidants, including Cys-based peroxidases (Horta et al., 2010). Therefore, in principle, any intervention that results in the decrease of antioxidants from pathogens can have a therapeutic property. Indeed the mechanism of action of several antibiotics is based on the generation of oxidants (Kohanski et al., 2010).

After completion of the genome-sequencing project of *X. fastidiosa* (Simpson et al., 2000), we decided to characterize Cys-based peroxidases from this plant pathogen. Analysis of the *X. fastidiosa* genome revealed the presence of five genes that encode proteins potentially involved in hydroperoxide decomposition: one catalase, one glutathione peroxidase (GPx), one organic hydroperoxide resistance protein (Ohr) and two peroxiredoxins (AhpC and PrxQ), both of which probably display the 2-Cys Prx mechanism (Horta et al., 2010). All of them, except GPx protein, were identified in the whole-cell extract and extracellular fraction of the citrus-isolated strain 9a5c (Smolka et al., 2003). We decided to characterize peroxiredoxins from *X. fastidiosa*.

As noted above (Section 3.1.3), peroxiredoxins can be classified into two groups, 2-Cys Prxs and 1-Cys Prxs, depending on the mechanism of catalysis. Besides this mechanistic classification, others were proposed that are based on amino acid sequence similarity. Later structural features were incorporated into the classification proposals. They provided insights on the evolution of proteins within the Trx superfamily, which includes the Prxs (Copley et al., 2004; Nelson et al., 2011). Adopting the classification described in Copley et al. (2004) for Prx classes, class 1 is the most ancestral, but the least characterized of all 4 classes. The other 3 classes of Prxs were derived from those of class 1. We therefore decided to investigate a class 1 Prx, peroxiredoxin Q (XfPrxQ) from *X. fastidiosa* (Horta et al., 2010).

Historically all classes of Prx have been considered only moderately reactive. The reason was that their catalytic efficiencies (k_{cat}/Km) toward hydroperoxides, as determined by steady-state kinetics, were in the 10^4-10^5 M^{-1} s^{-1} range. In contrast, selenocysteine-containing GPx (10^8 M^{-1} s^{-1}) and heme-containing catalases (10^6 M^{-1} s^{-1}) presented considerably higher

values (Wood et al., 2003). More recently, with the development of new assays, Prx enzymes were considered as reactive as selenium- and heme-containing proteins (Ogusucu et al., 2007; Parsonage et al., 2005; Trujillo et al., 2007). At that time, only class 3 Prx enzymes and class 4 Prx enzymes (composed mostly of typical 2-Cys Prx enzymes, but also some 1-Cys Prx proteins) were analyzed by these assays. Consequently, the catalytic efficiencies for enzymes of the other Prx classes remained to be determined. Through a competitive-kinetics approach (Toledo et al., 2011), we demonstrated that the second-order rate constants of the peroxidase reactions of XfPrxQ with hydrogen peroxide and peroxynitrite lay in the order of 10^7 and 10^6 M^{-1} s^{-1}, respectively. These reactions are as fast as the most efficient peroxidases. Furthermore, the catalytic cycle of XfPrxQ was elucidated by multiple approaches, such as X-ray crystallography, circular dichroism, biochemical assays, mass spectrometry, and site-directed mutagenesis. Using data obtained by site-directed mutagenesis, we were able to propose a model for the redox-dependent structural changes in PrxQ proteins (Fig. 4) that was consistent with all of our data and data from the literature (Horta et al., 2010). Site-
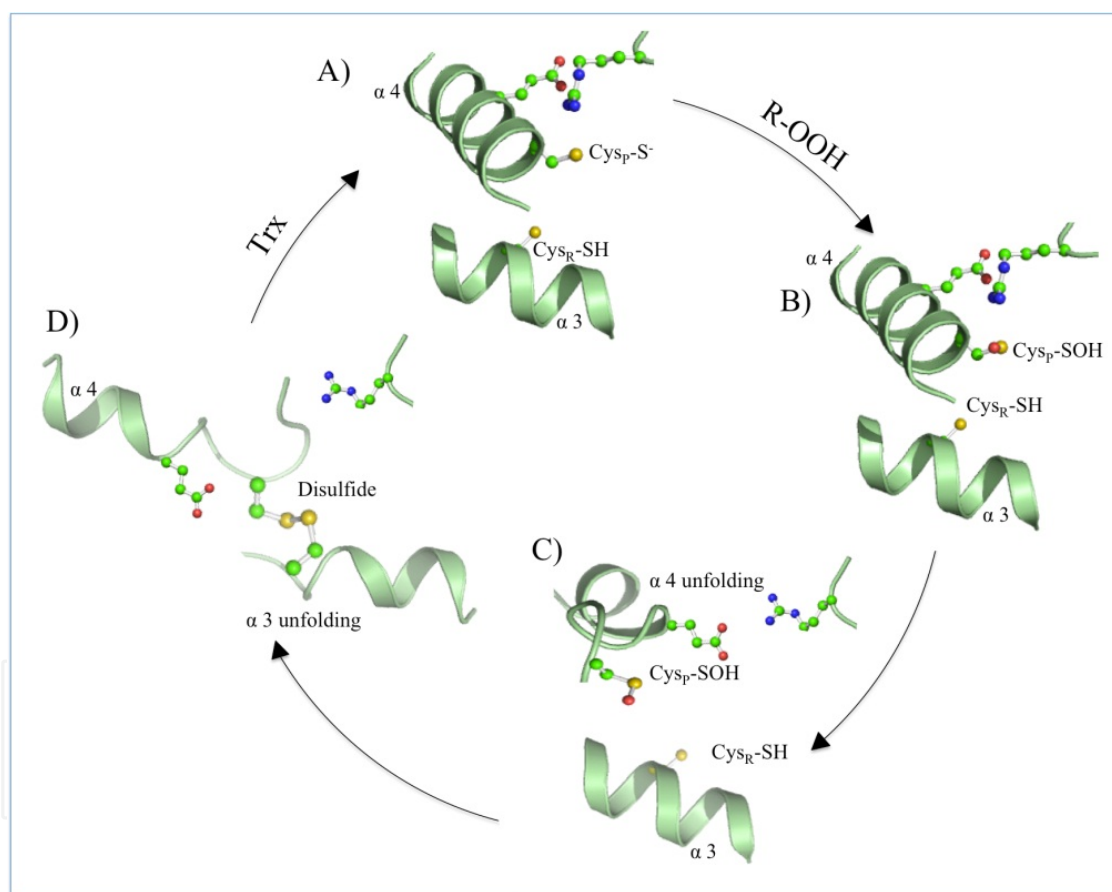

Fig. 4. **Model for the redox-dependent conformational changes in XfPrxQ**. Proposed sequence of structure snapshots along the catalytic cycle of the PrxQ subfamily proteins. The protein is represented in cartoon (light green) and residues are represented as sticks. Atoms are colored as follow: C=green, O=red, N=blue and S=orange. Peroxidatic and resolving cysteines are indicated as Cys_P and Cys_R, respectively. (A) Reduced species based on the crystal structure of XfPrxQ C47S. (B) and (C) Hypothetical conformational intermediates based on circular dichroism data (Horta et al., 2010). (D) Oxidized species based on the crystal structure of Bcp from *X. campestris* (PDB code = 3GKK).

directed mutagenesis revealed that Cys47 is the center responsible for the changes in secondary structure measured by circular dichroism (Horta et al., 2010).

3.2.2 Site-directed mutagenesis as a valuable tool to characterize a new antioxidant protein: Organic Hydroperoxide Resistance Protein

During the annotation of the *X. fastidiosa* genome (Simpson et al., 2000), the function of one gene caught our attention: *ohr*. What was reported at that time was that the deletion of *ohr* gene in *X. campestris pv. Phaseoli* rendered those cells sensitive to the oxidative insult by organic hydroperoxides, but not by hydrogen peroxide (Mongkolsuk et al., 1998). Furthermore, the transcription of the *ohr* gene was specifically induced by organic hydroperoxides, such as *tert*-butyl-hydroperoxide (Mongkolsuk et al., 1998). Therefore, this gene was named *ohr* (organic hydroperoxide resistance gene). However, the biological activity for the corresponding protein was not known.

Alignment of the deduced amino acid sequences of putative Ohr proteins from several bacteria revealed the presence of two fully conserved Cys residues. Therefore, we hypothesized that the *ohr* gene probably encodes a Cys-based, thiol-dependent peroxidase. In order to test this hypothesis, recombinant Ohr was obtained by cloning and expressing the *ohr* gene from *X. fastidiosa*. Indeed it displayed a thiol-dependent peroxidase (Cussiol et al., 2003). Remarkably, the peroxidase activity of Ohr was specifically supported by dithiols, such as DTT, but not by monothiols, such as 2-mercapthoethanol. In contrast, both mono- and dithiols support the enzymatic activity of peroxiredoxins and glutathione peroxidase. Furthermore, Ohr shows high activity towards organic hydroperoxides. Site-directed mutagenesis of the two conserved Cys residues unequivocally revealed that the most N-terminal one (Cys62) is the redox center (Cussiol et al., 2003). Another major achievement was the elucidation of the X-ray structure of Ohr (Oliveira et al., 2006). It showed a unique structure. The "Ohr fold" was quite different from the thioredoxin fold that is present in peroxiredoxin and the GSH peroxidases. At the same time, the structure and biochemical activity of the Ohr from *Pseudomonas aeruginosa* was elucidated (Lesniak et al., 2002). Essentially it has the same features described above.

In contrast to peroxiredoxins and GSH peroxidases, in which the reactive Cys residue is solvent-exposed, the reactive Cys residue in Ohr is buried in the polypeptide chain. The microenvironment where the reactive Cys is located is surrounded by several hydrophobic residues that probably confer to Ohr higher affinity for organic hydroperoxides. Proteins with folds similar to Ohr are only present in bacteria. This fact might indicate that this peroxidase is a target for drug development. Another unique property of Ohr is that only lipoamide, and neither GSH nor thioredoxin can support its Cys-based peroxidase activity (Cussiol et al., 2010). Due to these distinct properties, we are searching for Ohr inhibitors. Therefore, we are pursuing the characterization of enzyme-substrate interactions. We found in the active site of Ohr an electronic density of the polyethylene glycol molecule. Polyethylene glycol is a polymeric compound with elongated shape that was used in the crystallization trials. Since peroxides derived from fatty acids present an elongated structure and fit very well into this electronic density, we proposed that this kind of substrate may be the physiological target of the Ohr enzyme. Amino acid residues possibly involved in enzyme–substrate interactions were identified (Oliveira et al., 2006). Currently site-directed mutagenesis of these residues is underway in order to test the hypothesis that they are

involved in the binding of organic hydroperoxides. This information will be relevant in the search for Ohr inhibitors. We have already found some chemicals that can inhibit Ohr and also inhibit the growth of of *X. fastidiosa*.

4. Concluding remarks

In the characterization of Cys–based proteins, the involvement of amino acids in catalysis was analyzed by several enzymatic and biochemical assays, as well as by X-ray crystallography. Using an approach that combined these methodologies with site-directed mutagenesis allowed several hypotheses to be raised. Research is currently testing them.

5. References

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