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Review

Monothiol glutaredoxins: a common domain for multiple functions

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Abstract. Monothiol glutaredoxins with the CGFS sequence at the active site are widespread among prokaryotes and eukaryotes. Two subclasses exist, those with a single glutaredoxin domain and those with a thioredoxin-like region followed by one or more glutaredoxin domains. Studies in *Saccharomyces cerevisiae* have demonstrated the role of the Grx5 protein in the biogenesis of iron-sulfur clusters. Grx5 homologues in other eukaryotes could carry out similar functions. Two *S. cerevisiae* monothiol glutaredoxins with the thioredoxin-like extension, Grx3 and Grx4, are modulators of the transcriptional activator Aft1, which regulates iron uptake in yeast. The human PICOT protein is a Grx3/Grx4 homologue with the same hybrid primary structure that regulates protein kinase C activity and may participate in physiological processes such as control of cardiac function. Therefore, monothiol glutaredoxins share a common basic structural motif and biochemical mechanism of action, while participating in a diversity of cellular functions as protein redox regulators.

Keywords. Glutaredoxin, redox regulation, glutathione, oxidative stress, iron-sulfur cluster, transcription regulator, signal transduction.

Introduction

Aerobic life generates highly oxidant molecular species as a consequence of incomplete reduction of molecular oxygen to water. These reactive oxygen species (ROS) include, in addition to hydrogen peroxide, superoxide radical, hydroxyl radical and singlet oxygen [1]. They react with proteins, lipids and nucleic acids, modifying them and causing different degrees of toxicity. Oxidative stress may be provoked not only by ROS generation during aerobic respiration and other metabolic processes but also by external oxidants.

Cysteine is a key amino acid for protein folding, metal coordination and enzyme catalysis due to the redox properties of its thiol group [2–4]. In parallel, it is especially sensitive to ROS action [5]. ROS modification of the free thiol of the cysteine molecule leads to increasingly oxidized forms, from disulfide bond to sulfenic (-SOH), sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acid. Disulfide bonds between protein cysteine residues can be intra or intermolecular, contributing to the tertiary and quaternary structure, respectively, of proteins. However, under certain conditions formation of a disulfide bond between two cysteine thiols can inactivate the protein due to alteration of the active protein structure or because specific oxidation of the thiol(s) is required for protein activity. On the other hand, mixed disulfides can be formed between a

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cysteine thiol of a protein and a different molecule, such as the tripeptide glutathione, L-γ-glutamyl-L-cysteinyl-glycine (GSH in its reduced form). This particular reaction is termed glutathionylation. Formation of disulfide bonds or of sulfenic acid derivatives is a reversible process, while formation of sulfinic and sulfonic acid derivatives has been considered to be irreversible: that is, it leads to definitive protein inactivation. However, in yeast cells a sulfiredoxin that is able to reduce sulfinic acid derivatives has recently been characterized [6]. Glutathionylation would be a protective mechanism on protein thiols under oxidative conditions, since it can be reversed [7–10]. Cysteine nitrosylation after oxidative stress (with formation of an –SNO group) is another way to reversibly protect thiol groups. Nevertheless, modifications in the redox state of protein cysteines should be contemplated not only as the final consequence of changes under oxidative conditions inside the cell but also as an active process that may regulate cellular functions [7, 8]. For instance, DNA binding activity of the transcriptional factors NF-κB and c-Jun is regulated by glutathionylation of cysteine residues [11, 12]. Also, glutathionylation of an essential cysteine of glyceraldehyde 3-phosphate dehydrogenase inactivates the enzyme in the yeast *Saccharomyces cerevisiae,* and it has been proposed that the glycolytic flux is regulated through this mechanism after oxidative stress [13]. Disulfide bond formation is important for redox sensors of oxidative stress such as the *Escherichia coli* Hsp33 [14] or OxyR regulators [15].

The redox state of protein cysteine thiols is regulated by the thioredoxin and glutaredoxin systems [16–19]. Thioredoxins are low molecular weight thiol-disulfide oxidoreductases with a conserved CGPC active site motif; they reduce protein disulfides and regenerate the active thioredoxin enzyme using thioredoxin reductase and electrons from NADPH [16, 17]. In plant chloroplasts, active thioredoxin is regenerated in the light by the photosynthetic electron transfer chain through ferredoxin and ferredoxin-thioredoxin reductase [20]. Glutaredoxins (Grxs) are also thiol-disulfide oxidoreductases, but they employ GSH as a reducing agent, the system being completed with NADPH and glutathione reductase [16, 18] (Fig. 1A). Depending on the active site structure, a division has been established between dithiol (CPY/FC motif) and monothiol (CGFS motif) Grxs [18, 21]. In addition, *E. coli* Grx2 has a hybrid structure between a classical dithiol Grx and a glutathione transferase, although the enzyme activity is characteristic of dithiol Grxs [22]. The increasing number of sequenced genomes is revealing the existence of Grxs with a primary structure that deviates from standard dithiol and monothiol Grxs [23–26], pointing to the complexity of the Grx family. Since they were first discovered, dithiol Grxs have been more thoroughly studied [18, 27]. Here we will focus our attention mainly on recently accumulated information about monothiol Grxs from both a structural and a functional point of view.

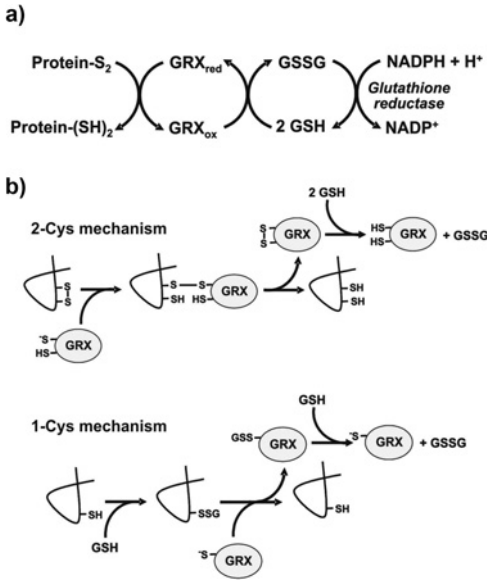

Figure 1. (a) Components of the glutaredoxin (Grx) system in the reduction of dithiol groups. These can consist of protein disulfides or of mixed disulfides between a protein and a glutathione molecule (GRX, glutaredoxin; GSH, reduced glutathione; GSSG, glutathione disulfide). (b) 2-Cys and 1-Cys mechanisms of action of Grxs for reduction of dithiol groups. Active cysteine residues in glutaredoxin proteins are indicated as SH or S^- depending on the redox state. See text for details.

Classic dithiol Grxs as thiol oxidoreductases

Dithiol Grxs were first characterized as reductants of ribonucleotide reductase in *E. coli* mutant cells that lacked thioredoxin activity [28], although subsequent studies revealed the existence of dithiol Grxs in bacteria, viruses and eukaryotes (reviewed in [18]). These Grxs are low molecular weight proteins (about 10 kDa) with the CP/YFC motif and a 'thioredoxin fold' protein structure. The thioredoxin fold domain is formed by a four- or five-stranded β-sheet flanked by three or more α-helices on either side of the β-sheet [29].

Biochemical studies on dithiol Grxs were facilitated by the design of an *in vitro* assay for measuring enzyme activity [30], which employs β-hydroxyethyl disulfide (HED) as a substrate. This standard assay measures the ability of the Grx enzyme to deglutathionylate the mixed disulfide formed between one GSH molecule and one mercaptoethanol moiety from HED. In addition, dithiol Grxs display dehydroascorbate re-

ductase (DHAR) activity in *in vitro* assays [31]. Dithiol Grxs can employ two different mechanisms for reducing disulfide bridges (Fig. 1B). The 2-Cys mechanism is shared with thioredoxins and requires both cysteine residues of the active site [31]. The most N-terminal cysteine forms a mixed disulfide between the Grx protein and the target protein, and the second cysteine is required for solving this intermediate dithiol bridge. GSH molecules restore the reduced active state of Grx. The 1-Cys mechanism of action requires only the most N-terminal cysteine of the active site [31] and is employed for reducing mixed disulfides between GSH and the target protein (Fig. 1B). Engineered Grxs in which the second cysteine of the active site has been substituted by a serine residue are active in the HED assay [31].

To date, the functional studies on dithiol Grxs have mainly focused on *E. coli*, *S. cerevisiae* and mammalian Grxs, although information is also emerging on the diversity of plant Grxs [23, 25, 32, 33]. Nevertheless, much less is known about the actual targets of Grxs than about the biochemistry and structure of these proteins. Among the three dithiol Grxs of *E. coli* (Grx1, Grx2, Grx3), one or more participates in the activation of ribonucleotide reductase and 3′-phosphoadenylysulfate (PAPS) reductase, an enzyme required for sulfate assimilation. They also participate in the reduction of both arsenate reductase and the transcriptional regulator OxyR involved in the oxidative stress response (reviewed in [18, 34]). It was recently shown that the three *E. coli* dithiol Grxs can stimulate the insertion of an iron-sulfur (Fe-S) cluster into the bacterial fumarate nitrate reductase regulator (FNR) apoprotein [35]. FNR acts as an oxygen sensor that regulates gene expression in response to oxygen availability, and its Fe-S cluster plays an essential role in controlling gene expression. At least under *in vitro* conditions, Grx1, Grx2 and Grx3 could regulate the reduced state of essential cysteine residues in the FNR apoprotein required for the Fe-S cluster insertion.

S. cerevisiae contains two classical dithiol Grxs (Grx1 and Grx2) [36]. While Grx1 is exclusively located in the cytosol, Grx2 is located in both the cytosol and mitochondria; alternative translation initiation from two in-frame ATG sites explains the existence of the two Grx2 isoforms [37]. Studies with the respective mutants [36] have demonstrated the participation of Grx1 and Grx2 in defense against external oxidants such as hydroperoxides or the superoxide generator menadione, although their functions are only partially overlapping in this respect. Both yeast Grxs are also active as glutathione peroxidases [38] and glutathione transferases [39]. A rice dithiol Grx that possesses GSH-dependent peroxidase activity has also been described [40].

Human cells contain two dithiol Grxs (reviewed in [19]). Grx1 is cytosolic and participates in such diverse functions as ribonucleotide and dehydroascorbate reduction, regulation of transcription factors and apoptosis. Grx2 has two isoforms derived from the same gene by alternative exon usage, and they are respectively targeted to the mitochondria and nucleus [41, 42]. Grx2 has an unusual motif (CSYC) at the active site, which results in lower activity in the HED assay, although the protein is effective in the deglutathionylation reaction [42]. Grx2 contains an Fe-S cluster that bridges two Grx2 monomers and acts as a sensor of oxidant conditions [43]. The position of the cluster in the Grx2 protein is controversial: while the original study [43] proposed that it is located independent of the active site, a more recent work [44] indicates that it is coordinated to an active site cysteine. Degradation of the cluster under oxidative stress results in formation of Grx2 monomers and consequent enzyme activation. These observations therefore relate Grx activity with responses to oxidative stress conditions, the Fe-S clusters acting as sensors of these conditions. Poplar Grx C1 has also been described to contain an associated Fe-S cluster. In this case the cluster is coordinated by the first cysteine of the Grx active site, bridging two enzyme subunits along with two cysteines from two GSH molecules [45]. Using a yeast heterologous system that expresses human forms of Cu/Zn-dependent superoxide dismutase SOD1, it was recently shown that amyotrophic lateral sclerosis-associated human SOD1 mutants are more easily reduced and destabilized by the *S. cerevisiae* dithiol Grxs than wild-type SOD1 [46]. This observation suggests that a correct balance of Grx activity is needed for controlling SOD1 stability and activity *in vivo*. In summary, although the possibility exists that dithiol Grxs are general protein redox regulators, increasing evidence supports the idea of more specialized functions for these proteins.

Monothiol Grxs: structurally similar to but biochemically different from dithiol Grxs

Analysis of the sequence of the *S. cerevisiae* genome revealed the existence of three open reading frames (ORFs) whose putative products share significant homology with *S. cerevisiae* Grx1 and Grx2 [21]. This homology was higher at the C-terminal moiety, while in the three putative proteins the CPY/FC active site signature of dithiol Grxs was substituted by the conserved sequence CGFS. Based on the homology, the three proteins were named Grx3, Grx4 and Grx5 in this initial study, and they were classified as

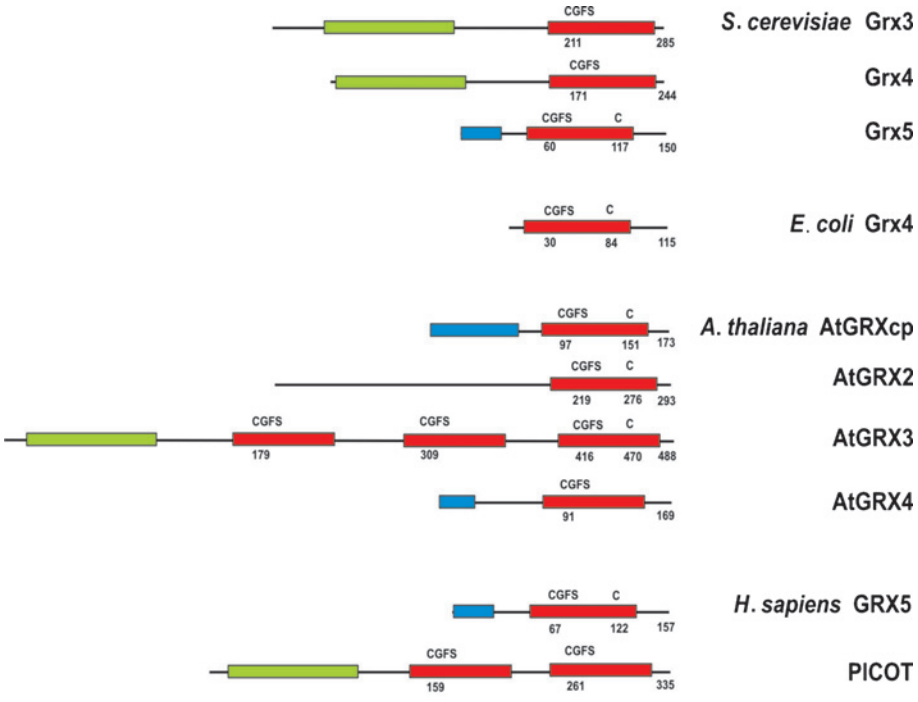

Figure 2. Structure of monothiol glutaredoxins from different organisms. The position and size of mitochondrial/plastid targeting sequences (blue boxes) and glutaredoxin (red) and thioredoxin-like (blue) domains is indicated. Numbers correspond to the position of the cysteine residue in the active site CGFS motif, the position of a partially conserved second C-terminal cysteine and the total length of the proteins. The proteins are named according to the most recent literature.

monothiol Grxs due to the presence of a single cysteine residue at the conserved putative active site [21]. BLAST analysis of completed genomes revealed the existence of homologues of the three yeast monothiol Grxs in bacteria (in particular in cyanobacteria and proteobacteria), protozoa, fungi, plants and animals; that is, they were widespread along the evolutionary scale [23, 25, 33, 47, 48]. Primary sequence comparison denotes a complex structural pattern for monothiol Grxs (Fig. 2). Thus, while *S. cerevisiae* Grx5 contains a single Grx domain, Grx3 and Grx4 have an additional thioredoxin (Trx)-like domain as an N-terminal extension of the Grx domain [47–49]; a non-conserved linker region connects the two domains. The Trx-like domain of Grx3 and Grx4 contains a WAD/EPCK sequence that is reminiscent of the authentic thioredoxin active site motif WCGPCK [50]. While all bacterial monothiol Grxs detected to date are of the single Grx domain type, in eukaryotes the Grx-type and the hybrid Trx-Grx-type monothiol Grxs coexist [48]. This is the case in human cells (Fig. 2), which contain the hGRX5 protein with a single Grx domain [51] and the so-called PICOT protein (for PKC-interacting cousin of thioredoxin, see below) [52] with a Trx-Grx structure. It is for this reason that the monothiol Grx domain has also been named the PICOT domain [52]. In fact, the human PICOT protein contains two Grx domains in tandem plus the N-terminal Trx-like domain (Fig. 2). This same complex pattern is present in plant monothiol Grxs with the CGFS motif [23, 25, 32, 33]: for instance, four different monothiol (CGFS) Grxs coexist in *Arabidopsis thaliana*, and one of these, AtGRX3, has three Grx domains in addition to the Trx-like domain (Fig. 2).

Only one monothiol Grx three-dimensional structure has been determined to date, that of *E. coli* Grx4 [53]. This protein has a single Grx domain, as occurs with *S. cerevisiae* Grx5 (Fig. 2). High resolution NMR of the bacterial Grx4 revealed a thioredoxin-fold protein structure, similar to dithiol Grx. The three-dimensional pattern of Grx4 confirmed that of the three cysteine residues in the protein, only the one in the CGFS motif could interact with GSH, therefore supporting the active site role of this motif [53]. The conservation of important amino acids of the *E. coli* Grx4 structure in other monothiol Grxs suggests that the Grx4 three-dimensional structure is representative of most monothiol Grxs. Besides the active site differences, other subtle differences exist between the spatial structures of dithiol and monothiol Grxs, which affect GSH binding [53]. For instance, although the Grx residues important for interaction with the C-terminal glycine of the GSH molecule are conserved between dithiol and monothiol Grxs, this conservation does not occur in the case of the charged residues of the dithiol Grxs, which are responsible of the interaction with the α-glutamyl residue of GSH.

The small but significant differences in GSH binding between dithiol and monothiol Grxs may explain the biochemical differences between the two types of proteins. In fact, several monothiol Grxs have been

purified from extracts of recombinant *E. coli* cells (Grx4 of *E. coli* [54], Grx5 of *S. cerevisiae* [55] and GLP-1 of *Plasmodium falciparum* [56]), and none of them are active in the HED assay, in contrast to all dithiol Grxs tested. This means that monothiol Grxs are unable to deglutathionylate the small mixed disulfide between β-mercaptoethanol and a glutathione moiety. The null activity of monothiol Grxs in the assay could be due to the null [54, 56] or low [55] affinity of the oxidized Grxs for GSH in *in vitro* studies, which correlates with the above-mentioned structural characteristics of the monothiol Grx proteins. A 1-Cys mechanism of action (Fig. 1B) could be hypothesized to explain the biochemical role of monothiol Grxs as deglutathionylating enzymes [57]. In fact, *S. cerevisiae* Grx5 is able to efficiently reduce rat carbonic anhydrase III *in vitro*, and its redox potential (–175 mV) places Grx5 at an intermediate position among thiol-disulfide oxidoreductases [55]. However, the differences in the HED assay between monothiol Grxs and 1-Cys derivatives of dithiol Grxs [31] have not been explained. Another open question is the nature of the protein physiologically acting as a reductant for the Grx. The low or null activity of GSH with the monothiol Grxs eliminates the possibility that GSH is the hydrogen donor. It was recently shown that *E. coli* Grx4 can be reduced *in vitro* by the bacterial thioredoxin reductase [54], and yeast Grx5 can also be reduced by *E. coli* thioredoxin reductase [55]. It is therefore possible that interplay between the thioredoxin and monothiol Grx systems exists through this reductase, at least in bacteria.

Another aspect to be solved concerning the mechanism of the monothiol Grx reactions is the possible role of a second cysteine residue in the Grx protein. Besides the cysteine in the CGFS motif, *E. coli* Grx4, *S. cerevisiae* Grx5 and other (but not all) monothiol Grxs possess another cysteine residue at a conserved position in the C-terminal part of the protein (Fig. 2). In Grx5, the *in vitro* deglutathionylation reaction begins with the formation of a mixed disulfide between the active site cysteine (Cys60) and GSH and results in the formation of a disulfide bond between Cys60 and the second cysteine (Cys117) [55]. Consequently, a mutant Grx5 lacking Cys117 is defective in the deglutathionylation reaction. Formation of the disulfide bond between both cysteines has also been observed in *E. coli* Grx4 [54]. Nevertheless, the physiological role of the C-terminal cysteine residue is not clear, as it is not required for the biological function of Grx5 *in vivo* (see below) [47].

Diverse subcellular locations of monothiol Grxs

Subcellular localization of the three monothiol Grxs of *S. cerevisiae* has been thoroughly studied. Comparison of the whole product of the *GRX5* ORF with the mature protein expressed in yeast cells revealed an N-terminal 28 amino acid-length region acting as a putative mitochondrial targeting sequence. Subsequent cellular subfractionation analyses confirmed that Grx5 localizes at the mitochondrial matrix and that a mutant lacking the N-terminal targeting region remains in the cytosol [58]. Interestingly, this cytosolic version of Grx5 did not rescue the phenotypic defects of a null *grx5* mutant (see below), demonstrating that *S. cerevisiae* Grx5 carries out its physiological functions exclusively at the mitochondria [58]. The *Schizosaccharomyces pombe* homologue of Grx5 also localizes at the mitochondria, as determined microscopically with a GFP-tagged version of the protein [59]. The completely sequenced genomes of eleven additional fungal species also contain an ORF coding for a mitochondrially located Grx5 homologue of the single Grx-domain type, as predicted by *in silico* analysis [26]. The GLP-1 protein of *P. falciparum* is also predicted to be mitochondrial, with a hypothetical mitochondrial targeting sequence cleaved at position 43 [56]. Therefore, the existence of a mitochondrially localized monothiol Grx with a single Grx domain could be a general rule among unicellular eukaryotes. The presence of Grx5 homologues in cellular organelles may also be common in plants. Among the four *A. thaliana* monothiol Grxs (Fig. 2), AtGRXcp was experimentally determined to localize at chloroplasts and plastids [60], while the AtGRX4 protein from the same species is predicted to be mitochondrial [23]. Both proteins contain a single Grx domain, with a 63-amino acid signal peptide in the case of AtGRXcp (Fig. 2). A third *A. thaliana* monothiol Grx, AtGRX2, does not contain an apparent organelle targeting sequence, although a homologue has been experimentally detected in maize chloroplasts [61]. This raises the possibility that both AtGRXcp and AtGRX2 are located in chloroplasts, where they could have overlapping functions (see below). Another genome that has been thoroughly studied *in silico* after genome sequencing completion, that of *Populus trichocarpa*, contains two single Grx domain homologues of Grx5 predicted to be mitochondrial and plastidic [25].

The two *S. cerevisiae* monothiol Grxs with a hybrid Trx-Grx structure (Grx3 and Grx4) are both localized at the nucleus [49, 62]. At least in the case of Grx3, this nuclear location is constitutive, as it is not affected by application of external stresses [49]. No consensus nuclear location signal is predicted in the Grx3

sequence, although it is the Trx-like region of the protein that is responsible for nuclear targeting [49]. The constitutivity of Grx3 nuclear localization contrasts with its homologue, the human PICOT protein, which translocates from the cytosol to the nucleus in response to oxidative stress [63]. In *S. pombe*, the only monothiol Grx with a Trx-Grx structure, Grx4, also has nuclear localization [59]. *S. pombe* cells have a third monothiol Grx with an atypical CPYS active site, named Grx3, which localizes at the nuclear rim and endoplasmic reticulum [62]. Other fungal species [24] and plant species [23, 25] also contain homologues of *S. cerevisiae* Grx3/Grx4 with the Trx-Grx hybrid structure. The subcellular location has not been experimentally determined for any of these proteins, and the significance of *in silico* prediction of nuclear localization of proteins is lower than for mitochondrial/plastidic proteins. However, the experimental observations in yeast and human cells suggest that monothiol Grxs may exist in different eukaryotic groups with constitutive or stress-induced nuclear location. An open question is whether constitutive cytosolic monothiol Grxs also exist.

Rice (*Oryza sativa*) has seven ORFs for monothiol Grxs (OsGRX1–7) [60], another example of the large number of individual species of monothiol Grxs coexisting in the cells of photosynthetic organisms. Additional diversity in rice cells could be generated by alternative splicing events that would create more than one isoform coded by a single gene, isoforms that could have diverse subcellular location [60], similar to some dithiol Grxs in yeast and human cells [37, 41, 42].

Monothiol Grxs and Fe-S cluster synthesis

Techniques for easily disrupting *S. cerevisiae* genes and consequently analyzing the phenotype of the resulting mutants are available; this explains why this yeast species is an extremely useful eukaryotic model for functional analysis of genes and their protein products [64]. The determination of the role of *S. cerevisiae* Grx5 in the formation of Fe-S clusters [21, 58] illustrates the above assertion. A null *grx5* mutant is hypersensitive to external oxidants and shows protein hypercarbonylation compared to wild-type cells, an indication of intracellular oxidative conditions above normal levels [21]. In addition, *grx5* mutant cells grow deficiently in synthetic minimal medium; this latter phenotype was taken as a selective condition to isolate genes that suppress the growth deficiency when overexpressed [58]. As a result, two genes involved in the synthesis of Fe-S clusters, *SSQ1* and *ISA2*, were isolated as suppressors. Both Ssq1 and Isa2, like Grx5, are located at the mitochondrial matrix [65]. The role of Grx5 in the biogenesis of the Fe-S clusters at the mitochondria was confirmed through the use of regulatable promoters expressing *GRX5:* switching off expression of the gene led to depletion of Grx5 and inactivation of enzymes containing Fe-S clusters such as aconitase and succinate dehydrogenase, with the concomitant accumulation of iron inside the cell [58]. The fact that biosynthesis of leucine, lysine and glutamic acid requires Fe-S enzymes explains the auxotrophic requirement of the *grx5* mutant and the growth defects in minimal medium. Characterization of selectable phenotypes for Grx5 function also allowed the determination that Cys60 in the CGFS motif, but not Cys117, is essential for the biological activity of the protein [47]; this establishes a discordance between *in vivo* data and those obtained *in vitro* (see above), which pointed to a role for Cys117 in the deglutathionylation activity of the enzyme. Perhaps Cys60 alone is required for Grx5 activity in the rather oxidant conditions of the mitochondrial matrix. In the absence of Grx5, a number of proteins became specifically glutathionylated, among them glyceraldehide 3-phosphate dehydrogenase [66], probably due to these same oxidant conditions.

Iron accumulation is characteristic of mutants in other yeast genes involved in the formation of the Fe-S clusters. This accumulation occurs mainly at the mitochondria [65, 67–69]. The disruption of iron homeostasis in Fe-S biogenesis mutants could be explained on the basis of the participation of a mitochondrial Fe-S protein as a sensor of iron content for the activation of the iron-uptake system. In fact, a mitochondrial iron signal has been shown to participate in cellular iron sensing [70], although the molecular nature of such a signal is still not known. The iron-uptake system depends on the transcriptional activators Aft1 and Aft2 and is activated under conditions of internal iron depletion [71–74]. In accordance with this, the *grx5* mutant and other mutants in Fe-S cluster biogenesis have constitutively up-regulated the Aft1/Aft2-dependent iron regulon [75–77]. The hyperoxidation of cell proteins and sensitivity to external oxidants in the absence of Grx5 could be a direct consequence of iron-mediated generation of ROS through the Fenton reaction. However, it was recently observed that in a *grx5* mutant, high levels of iron can displace manganese in the Mn-dependent Sod2 superoxide dismutase and therefore inactivate this mitochondrial enzyme [78, 79]; this may also help to explain the oxidant hypersensitivity phenotype of the mutant. Nevertheless, the synthetic lethality of the *grx5* and *grx2* mutations on one hand and of the *grx5* mutation with the *grx3 grx4* combination on the other [21] points to a more

complex relationship between monothiol and dithiol Grxs in *S. cerevisiae* cells, perhaps related to the sensitivity to oxidative stress (see below).

Synthesis of Fe-S clusters occurs exclusively at the mitochondrial matrix in yeast cells, independently of the final destination of the Fe-S clusters to mitochondrial, nuclear or cytosolic proteins (reviewed in [65, 67–69]). However, once the clusters have been exported from the mitochondria, specific proteins (the CIA machinery) are additionally required to assemble Fe-S clusters into apoproteins destined to the cytosol or the nucleus [67–69]. Whether Fe-S cluster biogenesis is exclusively mitochondrial in non-photosynthetic higher eukaryotes is still a matter of debate, although the participating components are evolutionarily conserved. Bacteria have three Fe-S biosynthetic systems, and one (the Isc system) functions constitutively and is the equivalent of the mitochondrial biosynthetic system [4]. Of the other two bacterial systems, the Nif system is responsible for the formation of Fe-S clusters for nitrogenase, while the Suf system is functional under oxidative stress and iron limitation [4].

In yeast cell mitochondria, Fe-S clusters are assembled on a scaffold formed by the Isu1/Isu2 proteins, using sulfur liberated from cysteine by the cysteine desulfurase activity of Nfs1 and iron imported from the cytosol [80]. By analogy to the bacterial Isc system, Isa1 and Isa2 could recruit iron to the Isu1/Isu2 scaffold [81], although it has been proposed that Yfh1 (homologue of the human frataxin protein) could also contribute to the process [65, 80]. Once assembled, the clusters are transferred to the target apoproteins at the mitochondrial matrix. Grx5 participates at these latter steps, together with the Hsp70 chaperone Ssq1 and its co-chaperone Jac1 [80]. This picture is, however, complicated by the fact that two-hybrid analyses show an *in vivo* interaction between Grx5 and Isa1 [48] and by results from *in silico* modeling of the Fe-S assembly process, which suggest participation of Grx5 at the initial assembly steps on the Isu1/Isu2 scaffold [82]. Independently of the step where Grx5 is acting, efforts are required to characterize the exact function of a thiol oxidoreductase such as Grx5 in the process. Protein cysteine residues involved in both the biogenesis of the clusters on the Isu1/Isu2 scaffold and the transfer of clusters to apoproteins require an appropriate redox state. By acting as a deglutathionylating enzyme, Grx5 could repair mixed disulfides formed between GSH and components of the Fe-S biosynthetic complex under the oxidant conditions of the mitochondrial matrix.

The above-mentioned mitochondrial sensor of iron content in *S. cerevisiae* cells [70] requires the entire mitochondrial machinery for Fe-S biogenesis as well as the Atm1 Fe-S transporter localized in the mitochondrial inner membrane. However, the cytosolic CIA machinery is not required for iron sensing [70]. This initially rules out the participation of a conventional cytosolic (or nuclear) Fe-S protein in transducing the iron signal and regulating the activity of Aft1/Aft2.

How conserved is the role of Grx5 along evolution? The homologues in other fungi are also predicted to be mitochondrial [26]. In the case of *S. pombe*, the *grx5* mutant grows slower than wild-type cells and is hypersensitive to external oxidants; there is also synthetic lethality between the *grx5* and *grx2* mutations [59], as occurs in *S. cerevisiae.* It is therefore probable that *S. pombe* Grx5 also participates in the formation of Fe-S clusters. However, some differences exist between the two yeast species: expression of *S. cerevisiae GRX5* and *S. pombe* $grx5^{+}$ is maximal at exponential phase [21, 83], but while *GRX5* is expressed constitutively and is not induced by external stresses [21], expression of fission yeast $grx5^{+}$ is induced by osmotic and nitrosative stresses in a process dependent on the transcription factor Pap1 [83]. Differences between *S. cerevisiae* and *S. pombe* may extend to the whole process of Fe-S cluster synthesis and thiol oxidoreductase requirement. Thus, in contrast to *S. cerevisiae,* the fission yeast requires mitochondrial forms of glutathione reductase or thioredoxin for the maintenance of Fe-S clusters in both mitochondrial and non-mitochondrial holoproteins [84]. However, the direct participation of these two types of enzymes in the biogenesis of the clusters has not been demonstrated.

The *S. cerevisiae grx5* mutant has been employed as a tool to analyze the functional conservation of monothiol Grxs. Thus, derivatives of nuclear Grx3 and Grx4 *S. cerevisiae* proteins have been engineered to target them to the mitochondria of Grx5-deficient cells using the mitochondrial targeting sequence of the endogenous Grx5 protein. Under these conditions, the two Trx-Grx-type proteins (Grx3/Grx4) rescue the defects of the *grx5* mutant, that is, they are able to participate in the biogenesis of the Fe-S clusters [49]. This is not the case for dithiol Grx2. Although the biological functions of Grx3 and Grx4 are totally different from Grx5 (see below), the above observation shows that yeast monothiol Grxs can substitute for each other provided that compartmental barriers are eliminated. Using the same strategy, it has been demonstrated that two bacterial monothiol Grxs (*E. coli* Grx4 and *Synechocystis* sp. GrxC) can also partially substitute for the biological function of Grx5 in *S. cerevisiae* mitochondria [51], which could be taken as proof for functional conservation. In the case of *E. coli* Grx4, a mutant lacking this protein is non-viable [54, 85],

pointing to functions for Grx4 in addition to its possible participation in the formation of Fe-S clusters, since *E. coli* mutants in most of the genes of the Isc system are viable [4]. Levels of *E. coli* Grx4 are maximal at stationary phase [54]. However, the fact that Grx4 is up-regulated upon iron depletion suggests that the protein may be involved in iron-dependent functions.

With respect to pluricellular eukaryotes, it was demonstrated using the same experimental approach as above [51] that the human (hGRX5) and chicken (cGRX5) homologues are also able to participate in the formation of Fe-S clusters once internalized into *S. cerevisiae* mitochondria. The gene coding for cGRX5 is expressed in diverse tissues in chicken embryos [50]. A thorough study carried out by Wingert et al. [86] demonstrates that deficiency of Grx5 in zebrafish embryos causes hypochromic anemia in *shiraz* mutants due to an inability to assemble Fe-S clusters and consequent inhibition of heme synthesis. The *shiraz* mutant phenotype was reproduced in wild-type embryos injected with *grx5* antisense RNA, and overexpression of *S. cerevisiae*, mouse or human RNAs coding for the respective Grx5 proteins could rescue this phenotype [86]. The study also showed that the *GRX5* gene is expressed in mouse embryos and therefore confirmed the conservation of Grx5 function in the assembly of Fe-S clusters in eukaryotes and how this relates to regulation of heme synthesis, at least in the zebrafish vertebrate model.

Two recent independent studies add new insights into the function of Grx5 homologues in plant cells. The AtGRXcp protein, which localizes at mitochondria and chloroplasts (see above), suppresses the phenotypes of an *S. cerevisiae grx5* mutant once targeted to the yeast mitochondria [60], as occurs with human hGRX5. The *AtGRXcp* gene is expressed in young cotyledons and green leaves, and *atgrxcp* mutant seedlings are hypersensitive to external oxidants [60]. This, together with the fact that AtGRXcp protein was originally identified as a Ca^{2+}/H^{+} antiporter regulator and modulator of Ca^{2+} transport [87], supports a connection between monothiol Grxs, synthesis of Fe-S clusters, oxidative stress and possibly also calcium signaling in plant cells. In a parallel study [88], it was shown that *A. thaliana* SufE protein is an activator of cysteine desulfurase NifS at chloroplasts in a manner that is dependent on cysteine residues of both SufE and NifS. The SufE/NifS pair acts at the initial steps of the chloroplast biosynthetic system for Fe-S clusters, which works autonomously from other plant cell compartments and resembles the bacterial Suf system [89]. The SufE activator of *A. thaliana*, as well as its homologue in rice, has a C-terminal sequence that is homologous to the evolutionarily conserved BolA sequence [88]. Using a bioinformatics approach, it has been predicted that BolA sequences, acting as reductases, interact with monothiol Grxs [90]. These observations therefore also point to a connection between Grx-mediated redox regulation and Fe-S synthesis in plant chloroplasts. However, mitochondria and chloroplasts could employ partially different strategies for Fe-S synthesis (the Isc and Suf systems, respectively), explaining why some phenotypes of a yeast *grx5* mutant, such as lysine auxotrophy, are only partially rescued by the plant AtGRXcp protein [60].

Monothiol Grxs and regulation of transcription factors

The cellular function of both Grx3 and Grx4 monothiol Grxs of *S. cerevisiae* has remained unknown until very recently. Their nuclear location suggested a function as regulators of nuclear proteins and, consequently, of gene expression. In accordance with this, Pujol et al. [91] recently demonstrated that both Grx3 and Grx4 regulate Aft1 nuclear localization, which implies that both Grxs share a function related to the regulation of cellular iron homeostasis. The Aft1 transcription factor, which regulates expression of the high-affinity iron-uptake regulon (see above), translocates to the nucleus under conditions of iron depletion [92]. Importantly, Aft1 transcriptional activity is precluded by its nuclear localization and not by iron availability. In this respect, the primary function of Grx3 and Grx4 appears to be the regulation of Aft1 nuclear localization. Grx3 and Grx4 both interact with Aft1 in the nucleus [91, 93]: more specifically, Aft1, Grx3 and Grx4 interact physically in the nucleus forming a functional complex [91]. Each monothiol Grx interacts with Aft1 in an independent manner, suggesting that Grx3 and Grx4 can function independently in Aft1 regulation. Interestingly, Aft1 binds to both the Grx and the Trx domain of each Grx; however, there exists evidence indicating that only the Grx domains drive Aft1 nucleo-cytoplasm translocation [91]. What is the biological meaning of Aft1 binding to both thioredoxin domains? This is a question that deserves further study in order to offer deeper insight into Aft1 cellular regulation.

The single *grx3* and *grx4* mutants do not present very significant or marked phenotypes other than up-regulation of the Aft1 regulon [91, 93]. However, the *grx3/grx4* double mutant shows a marked defect in cell cycle progression, intracellular iron accumulation and a clear sensitivity to specific oxidant agents, such as hydroperoxides [91]. Therefore, Grx3 and Grx4 have a redundant and additive function in cell growth

and oxidative stress protection. In fact, the absence of both Grx3 and Grx4 function constitutes a model for endogenous oxidative stress caused, at least partly, by iron homeostasis misregulation.

Despite the information available concerning Grx3 and Grx4 function in *S. cerevisiae,* there are still a number of questions that remain unanswered: is there another function for Grx3 and Grx4 and, more specifically, another function in the oxidative stress defense other than Aft1 regulation? What is the biochemical mechanism that Grx3 and Grx4 use to regulate Aft1?

The *S. pombe* Grx4 protein is a homologue of *S. cerevisiae* Grx3/Grx4 proteins and, as noted above, also has a nuclear location [59]. The target(s) of fission yeast Grx4 still remain unknown, although, in contrast to *S. cerevisiae GRX3* and *GRX4, grx4*$^+$ is an essential gene in *S. pombe*; this, however, could be explained in part by the functional redundancy observed for both Grx3 and Grx4 in budding yeast. In the case of *S. pombe*, *grx4*$^+$ as a single gene might account for all the regulatory function exerted in the nucleus. There are similarities among the Trx-Grx-type monothiol Grxs of budding and fission yeast. The *grx4*$^+$ product is likely to function as a monothiol Grx in fission yeast and is involved in the protection against oxidative stress [83, 94], similar to Grx3 and Grx4 in *S. cerevisiae.* It will be interesting to elucidate whether regulation of iron homeostasis and, consequently, of oxidative endogenous stress is a characteristic function of monothiol Grxs conserved throughout evolution.

The presence of monothiol Grxs in the nucleus suggests an essential function as regulators of the redox state of nuclear proteins, raising the question of which factor or factors regulate the activity of these nuclear monothiol proteins. In this respect, Grx4 interacts in the nucleus with the *S. cerevisiae* protein kinase piD261/Bud32 [62], a protein that has been related to such diverse functions as bud-site selection, telomere uncapping and elongation or modulation of transcription factors [95–97]. However, a link between *S. cerevisiae* Grx3/Grx4 and these functions has not been established. Bud32 phosphorylates Grx4, which suggests that the kinase might act as a regulator of Grx4 activity [62]. To date, the information about the regulatory proteins that modulate nuclear monothiol Grxs in different organisms is scarce and needs to be investigated further.

Monothiol Grxs and signal transduction

Thioredoxins and dithiol Grxs can act as redox regulators of signal transduction pathways [8–10]. The human PICOT protein with the complex Trx-Grx-type structure (see above and Fig. 2) is also a modulator of signal transduction. Initial studies detected its expression in T lymphocytes, where it acts as a negative regulator of protein kinase C-θ (PKCθ) and plays an important role in T cell receptor-induced activation [98]. After being translocated into the nucleus in response to stress [63], the PICOT protein interacts with PKCθ through the Trx-like domain and inactivates the kinase activity [98]. This transiently inhibits several PKC targets, such as the c-Jun N-terminal kinase and the transcription factors AP-1 and NF-κB.

The modulator role of PICOT is not restricted to T cells: PICOT may act as an endogenous negative regulator of cardiac hypertrophy through its PKC inhibitory activity [99]. Transgenic mouse cardiomyocites overexpressing PICOT displayed enhanced contractility associated with increased myofilament Ca^{2+} responsiveness. The transgenic mice exhibited increased ventricular function and decreased cardiac hypertrophy induced by pressure overload [99]. Therefore, PICOT might regulate PKC-mediated signaling pathways participating in the development of cardiac hypertrophy [100].

Yeast PKC participates in very different functions from mammalian PKC proteins, mainly in cell wall remodeling and cellular morphogenesis [101]. However, the above observations with mammalian PICOT proteins make us consider the possibility that the yeast homologues Grx3 and Grx4 might be regulators of signal transduction pathways in addition to their role in modulating Aft1 activity.

Other proteins that act as monothiol Grxs

The spectrum of thiol oxidoreductases acting through a monothiol mechanism is even more complex. Fungal cells have ORFs coding for Grx-like proteins with the putative active site sequence CPYS and an N-terminal transmembrane domain [26]. The *S. pombe* Grx3 protein, which is located at the nuclear rim and endoplasmic reticulum, is an example of such a Grx-like protein [59]. *S. cerevisiae* has two ORFs (*YBR024c* and *YDL010w*) coding for homologues of *S. pombe* Grx3 [26], and purified forms of both protein products display thiol oxidoreductase activity in the HED assay, activity that is dependent on the cysteine residue in the CPYS motif (our unpublished observations). These fungal Grx-like proteins therefore con-

stitute a new class of monothiol Grxs that biochemically differ from the CGFS monothiol Grxs and whose biological function as possible membrane-associated Grxs is unknown.

Most glutathione transferases (GSTs) act as detoxifiers of xenobiotics by conjugating a GSH molecule to the target molecule [102]. This nucleophilic attack by GSH requires a serine or tyrosine residue at the active site of the GST protein [102, 103]. However, Omega-class GSTs differ from most other GSTs by having a cysteine residue at the active site, which is essential for enzyme activity [104, 105]. Purified Omega-class GSTs from different origins do not have significant activity on standard GST substrates but are active as thiol oxidoreductases in the HED assay [104–106]. *S. cerevisiae* contains three Omega GSTs named Gto1, Gto2 and Gto3 [106]. Biochemical studies with Gto2 have shown that a single cysteine in the active site is sufficient for the GSH-dependent thiol oxidoreductase activity, indicating that the enzyme acts through a 1-Cys mechanism [106]. Substitution of the Gto2 active site motif with the active site of Grx5 eliminates the enzymatic activity on HED, while replacement of the Grx5 CGFS motif with the Gto2 active site restores some Grx5 activity on HED [106]. These observations emphasize the importance of the CGFS motif for the biochemical properties of monothiol Grxs.

Of the three Gto proteins of *S. cerevisiae*, only Gto1 has been studied with regard to biological function [107]. It is a peroxisomal GST (in contrast to cytosolic Gto2 and Gto3) whose absence causes dysfunction in the biosynthetic pathway of sulfur amino acids. It has been proposed [107] that Gto1 could be a redox regulator of cystathionine β-lyase, another peroxisomal protein required for transulfuration from cysteine to homocysteine in yeast cells. *S. cerevisiae* Gto1 therefore exemplifies an enzyme with monothiol Grx activity that has a subcellular location different from that of other monothiol Grxs described to date. However, the peroxisomal location of Gto1 is probably a recent evolutionary acquisition in yeast species closely related to *S. cerevisiae* [107, 108]. No examples of peroxisomal Omega-class GSTs exist in higher eukaryotes, reflecting the evolutionary plasticity of peroxisomes [108].

Conclusions

Monothiol Grxs with the CGFS active site share a common structural domain and probably also a common mechanism of enzyme action. The presence of an additional thioredoxin-like domain in some monothiol Grxs may be relevant for expanding the number of cellular compartments where they can be localized as well as for substrate specificity. The diversity of both the cellular compartments where monothiol Grxs carry out their activities and their protein targets helps to explain the broad spectrum of cellular functions in which they participate through their glutathionylating activity (Fig. 3): these include functions as diverse as synthesis of Fe-S clusters, activation of transcription factors and modulation of signal transduction pathways. Especially in plant cells, diversity in location and function result from the large number of monothiol Grxs encoded by each genome but may also be the consequence of post-transcriptional processes. While there is increasing evidence that participation of monothiol Grxs with a single Grx domain in Fe-S cluster biogenesis is evolutionarily conserved, at present there are no data supporting conservation of other specific functions. However, the diversity of organisms, both prokaryotic and eukaryotic, that contain different subclasses of monothiol Grxs points to important biological functions related to protein redox regulation and defense against oxidative stress in which these thiol oxidoreductases participate.

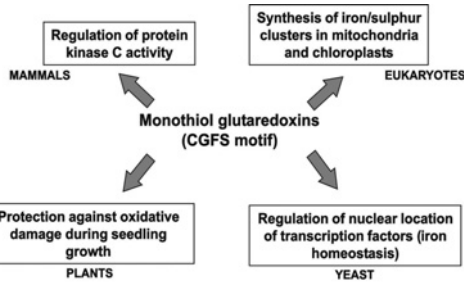

Figure 3. Summary of experimentally determined functions for monothiol glutaredoxins and the organisms in which these functions have been demonstrated.

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