CGFS-Type Monothiol Glutaredoxins from the Cyanobacterium *Synechocystis* PCC6803 and Other Evolutionary Distant Model Organisms Possess a Glutathione-Ligated [2Fe-2S] Cluster†

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ABSTRACT: When produced in *Escherichia coli*, the CGFS-type monothiol Grxs from this organism (EcGrx4p) and the model cyanobacterium *Synechocystis* (SyGrx3p) exist as a dimeric iron-sulfur containing holoprotein or as a monomeric apoprotein in solution. Spectroscopic and site-directed mutagenesis analyses show that the SyGrx3 holoprotein contains a subunit-bridging [2Fe-2S] cluster that is ligated by the catalytic cysteine located in the CGFS motif of each monomer and the cysteines of two molecules of glutathione. The biochemical characterization of several monothiol Grxs from the cyanobacteria *Gloeobacter violaceus* (GvGrx3p) and *Thermosynechococcus elongatus* (TeGrx3p), the yeast *Saccharomyces cerevisiae* (ScGrx3p, ScGrx4p, and ScGrx5p), the plant *Arabidopsis thaliana* (AtGrx5p), and human (HsGrx5p) indicate that the incorporation of a GSH-ligated [2Fe-2S] center is a common feature of prokaryotic and eukaryotic CGFS-active site monothiol Grxs. In light of these results, the involvement of these enzymes in the sensing of iron and/or the biogenesis and transfer of Fe-S cluster is discussed.

Glutaredoxins are thiol-disulfide oxidoreductases that use glutathione (γGlu-Cys-Gly) as the reducing agent to catalyze the reduction of disulfides (protein-S-S) or glutathione mixed disulfides (protein-S-SG) (*1*). Depending on their active site sequences, glutaredoxins (Grxs) are classified in two families (*1*, *2*): the dithiol Grxs recognizable by the highly conserved CXXC motif, and the monothiol Grxs in which the CGFS active site motif is nearly invariant. Unlike dithiol Grxs, monothiol enzymes are unable to deglutathionylate the small mixed disulfide between β-mercaptoethanol and glutathione (*1*). Monothiol Grxs are best understood in the yeast *Saccharomyces cerevisiae* that possesses three such enzymes: ScGrx3p, ScGrx4p, and ScGrx5p (*1*). ScGrx3p and ScGrx4p both harbor an additional thioredoxin domain at their N-terminus that is required for their nuclear localization (*1*). They operate in iron sensing through a physical interaction with the transcriptional iron regulator Aft1p, which is thereafter inactivated, in a way that is dependent on both the cysteinyl residue of their invariant CGFS motif and their glutathione-binding pocket (*3*). ScGrx5p, which contains a single Grx domain, was shown in vitro to efficiently reduce the rat carbonic anhydrase III. More interestingly, ScGrx5p is part of the mitochondrial machinery for iron-sulfur cluster biogenesis, in operating in the transfer of Fe−S cluster to target proteins (*1*).

By contrast, little is known concerning monothiol Grxs in photoautotrophic organisms (*4*), even in basic ones such as cyanobacteria that support a large part of the biosphere and perform the two Fe-S cluster-requiring (*5*) processes—photosynthesis and respiration (*6*)—in the same membrane system (*7*). Consequently, we have initiated the analysis of the single monothiol Grx of the model cyanobacterium *Synechocystis* PCC6803. We found that this protein SyGrx3p (Slr1846 in cyanobase http://www.kazusa.or.jp/cyano/cyano.html) overproduced in *Escherichia coli* exists not only as a monomeric apoprotein but also as a dimeric form that contains a subunit-bridging [2Fe-2S] cluster ligated by the cysteine of the *C*GFS conserved motif of two monomers and the cysteines of two glutathione molecules. These findings were confirmed through the overproduction in *E. coli* of the endogenous EcGrx4 monothiol enzyme, which was also analyzed through site-directed mutagenesis. We also analyzed other CGFS-type monothiol Grxs from various model organisms—either prokaryotic (the cyanobacteria *Gloeobacter violaceus*, GvGrx3p, and *Thermosynechococcus elongatus*, TeGrx3p) or eukaryotic (the yeast *Saccharomyces cerevisiae*, ScGrx3p, ScGrx4p, and ScGrx5p; the plant *Arabidopsis thaliana*, AtGrx5p; and human, HsGrx5p)—and found that the occurrence of the [2Fe-2S] cluster is an evolutionary conserved feature of these enzymes.

MATERIALS AND METHODS

Reagents. Restriction enzymes, isopropylthio-β-D-galactopyranoside, kanamycin, and Ni-NTA agarose resin were purchased from Invitrogen; other reagents were purchased from Sigma unless otherwise indicated. *S*-Acetamido glutathione and ±-threo-1,4-*S*-acetamido-2,3-butane-diol (alkylated dithiothreitol) were synthesized as follows: 30 mM

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reduced glutathione (GSH) or 15 mM dithiothreitol (DTT) were alkylated for 45 min at 30 °C in the dark with 60 mM iodoacetamide in 100 mM Tris-HCl pH 8.8. Alkylation efficiency was checked by quantification of free sulfhydryl (see below), and then the alkylation reaction was quenched by 115 mM DTT for 15 min. Nearly all GSH and DTT molecules were converted to *S*-acetamido GSH and alkylated DTT, respectively (not shown).

Plasmid Construction and Mutagenesis. The coding sequence for mature Grxs (lacking the mitochondrial or chloroplastic target sequence of the eukaryotic enzymes) were PCR amplified (Pfu DNA polymerase Promega) with appropriate oligonucleotide primers (Table SM1) and subsequently cloned in pET28b either as NdeI/BamHI or NcoI/BamHI DNA segment, depending on whether the protein was or was not tagged with 6xHis. EcGrx4p and SyGrx3p site-directed mutagenesis experiments were performed with the Quick Change mutagenesis kit (Stratagene), using two sets of complementary mutant oligonucleotides (Table SM2, Supporting Information). DNA sequences were verified on both strands using the Big Dye Kit (ABI Perkin-Elmer).

Recombinant Protein Expression and Purification. Expression and purification of recombinant proteins were performed in *E. coli* BL21(DE3) as described (*8*). Relevant protein fractions were pooled and desalted on a PD10 Sephadex G-25M column (Amersham Biosciences). Purity of the purified proteins was greater than 95% as determined by SDS-PAGE electrophoresis (data not shown).

Apoproteins Preparation and in Vitro Reconstitution of the Fe-S Cluster. Fe-S clusters were disassembled from Grx proteins by a 2 h treatment at 4 °C with 100 mM of both dithionite and EDTA. After desalting, 150 μM apoproteins were reduced by 5 mM DTT with or without 1.5 mM reduced GSH. Five molar excess of both iron ($FeCl_3$) and sulfide (Na_2S) were added under anaerobic conditions (in a glove box), and the reaction was incubated overnight. Reconstituted proteins were desalted and concentrated using Microcon-3K (Millipore). Reconstitution with *S*-acetamido glutathione (1.5 mM) was performed similarly, using a mix containing 1.5 mM alkylated DTT and 1.5 mM reduced GSH as control.

Analytical Methods. Gel-filtration analyses were performed on a Superdex-75 PC 3,2/30 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl pH 8.0, 2 mM DTT, and 150 mM KCl and calibrated with the Low Molecular Weight Gel Filtration calibration kit (Amersham Biosciences). Elution profiles were recorded at both 280 and 410 nm under a flow rate of 0.05 mL min^{-1}. UV−visible spectra were monitored between 260 and 800 nm with a Beckman DU 640B spectrophotometer. EPR spectra were recorded using a Bruker ESP 300E spectrometer equipped with a standard ER 4102 (Bruker) X-band resonator and an Oxford Instruments cryostat (ESR 900). Quartz tubes containing samples were frozen in a dry ice/ethanol bath and then in liquid nitrogen. Spectra were recorded at 16 K with at least four accumulations, using 10 G modulation amplitude, 0.2 mW microwave power, 9.4 GHz microwave frequency, and 100 kHz modulation frequency. The *g*-values were measured using a Bruker 035M NMR gaussmeter. Protein concentration was determined either by Bradford (Bio-Rad) or direct measurement at 205 nm. Titration of free sulfhydryl groups was performed with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (*9*). Briefly, 50−100 μg of protein was incubated for 10 min in 1 mL of 200 μM DTNB, 100 mM Tris-HCl pH 8.0. A molar extinction coefficient of 14 150 M^{-1} cm^{-1} was used to calculate the number of titrated sulfhydryl groups. Iron and acid-labile sulfide specifically bound to desalted proteins were determined according to Doeg and Ziegler (*10*) and Broderick et al. (*11*), respectively.

In Silico Methods. Mitochondrial targeting sequences and cleavage sites of eukaryotic monothiol Grxs were identified with MITOPROT (*12*). Monothiol Grxs were identified with BLASTP (NCBI) and aligned with ClustalW (EBI). Graphical representation of the consensus sequence pattern was generated with WebLogo (*13*) using 119 sequences of prokaryotic and eukaryotic Grxs. Three-dimensional visualization of conservation pattern was performed using Pymol (Delano scientific) and the 3D-structure of *E. coli* Grx4p as model (*14*).

RESULTS

SyGrx3p the CGFS-Type Monothiol Glutaredoxin of Synechocystis PCC6803 Assembles a [2Fe-2S] Cluster. For a better understanding of the *Synechocystis* PCC6803 protein *Sy*Grx3p (Figure 1A), which could rescue the biogenesis of the [Fe-S] cluster in the *S. cerevisiae* mutant lacking the *Sc*Grx5 monothiol glutaredoxin (*15*), we have produced the SyGrx3p protein in *E. coli* and subsequently purified it to homogeneity. Very interestingly, SyGrx3p exhibited a brown color irrespective of the presence or absence of the His-Tag used for facile purification (data not shown). Gel filtration chromatography showed that the purified SyGrx3p protein separated into two distinct fractions (Figure 1B, inset): an abundant colorless fraction of 15 ± 2 kDa and a minor fraction of 30 ± 2 kDa that contains the brownish chromophore. On reducing SDS-PAGE, both fractions resolved as a single peptide with an apparent molecular weight of 15 kDa, indicating that the 30 kDa brownish fraction contains the homodimer of SyGrx3p (data not shown). The UV−visible absorption spectrum of the monomeric fraction of SyGrx3p showed the expected strong peak at 280 nm (Figure 1B), while the spectrum of the dimeric form of SyGrx3p displayed a strong additional peak at 410 nm and shoulders at 320, 510, and 590 nm. These findings together with the involvement of monothiol glutaredoxins in Fe-S cluster biogenesis (*15*−*17*) prompted us to test whether the dimeric form of SyGrx3p assembles an Fe-S center. Indeed, colorimetric assays performed with unfractionated SyGrx3p samples containing a mixture of both the monomer and the low-abundant homodimer indicated that SyGrx3p contains iron (0.2 ± 0.02 mol/mol SyGrx3p) and acid-labile sulfide (0.3 ± 0.08 mol/mol SyGrx3p). Furthermore, in reductive conditions, upon dithionite addition, SyGrx3p exhibited an EPR signal centered at $g = 1.93$, the shape and *g*-values of which are consistent with the presence of [2Fe-2S] cluster (Figure 1C). This EPR spectrum suggests that the [2Fe-2S] cluster is in two conformational states, an axial form with $g_{\|} = 2.01$ and $g_{\perp} \approx 1.93-1.95$ and a rhombic form with *g*-values of 2.07, 1.93−1.95, and 1.85. Collectively, these findings indicate that the holoform of SyGrx3p is a dimeric protein containing one [2Fe-2S] cluster.

CGFS-Type Monothiol Glutaredoxins Define a New Family of [2Fe-2S] Proteins. That monothiol glutaredoxins from various prokaryotic and eukaryotic organisms were able to

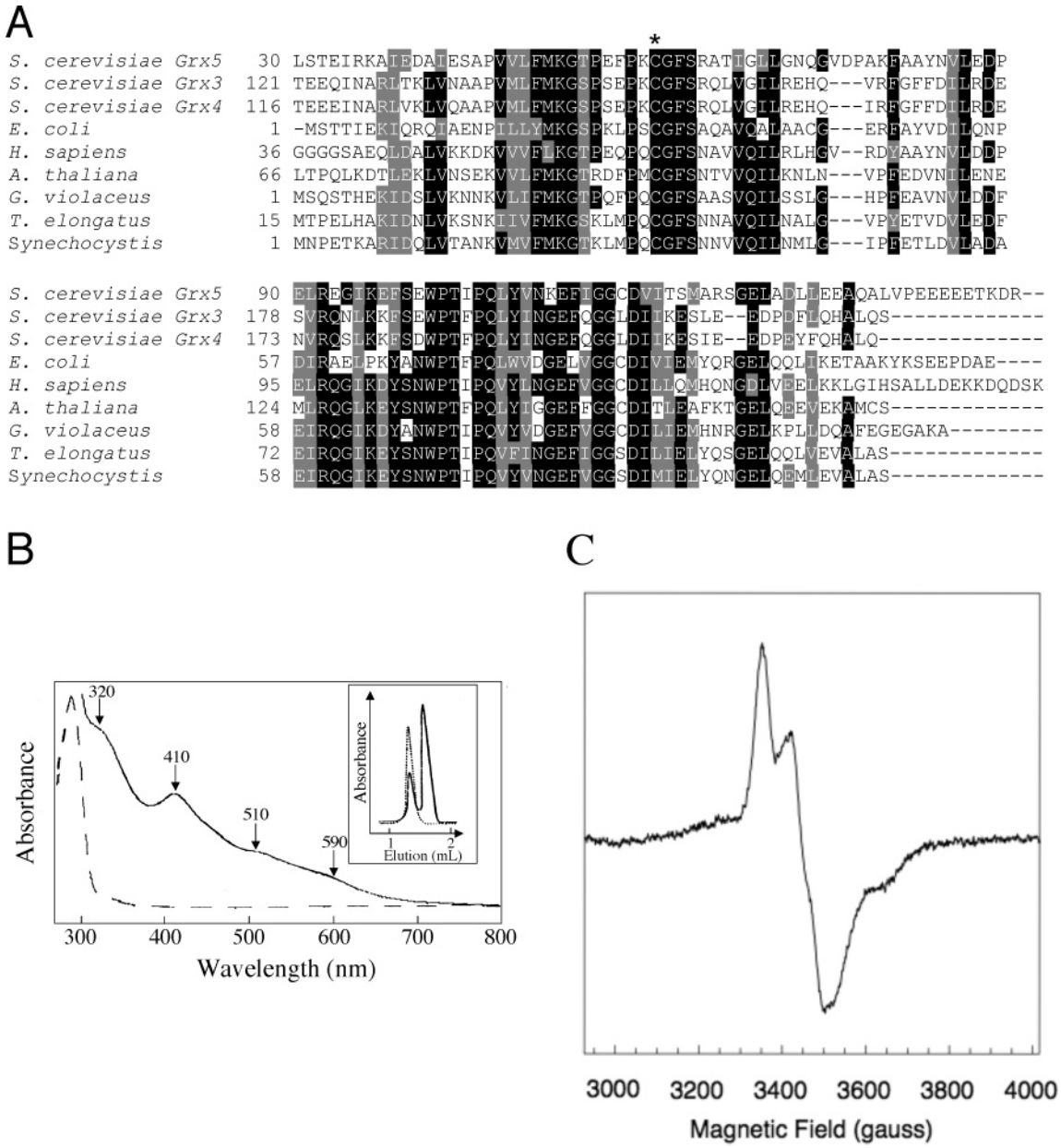


FIGURE 1: *Synechocystis* PCC6803 monothiol glutaredoxin SyGrx3p assembles a [2Fe-2S] cluster. (A) The sequences of CGFS-type monothiol glutaredoxins from the following model organisms were aligned with ClustalW: Sc, *S. cerevisiae*; Ec, *E. coli*; Hs, *H. sapiens*; At, *A. thaliana*; Gv, *G. violaceus*; Te, *T. elongatus*; and Sy, *Synechocystis* PCC6803. For the sake of clarity the plastid-targeting N-terminal sequence of eukaryotic Grxs to plastids were not represented. Amino acids conserved in more than 70% Grx sequences are highlighted in dark boxes, while conservative aa substitutions are shown in gray. The star shows the cysteine of the totally invariant CGFS motif. (B) UV−visible spectra of gel-filtration purified (inset) monomeric (dashed line) and dimeric (straight line) SyGrx3p protein fractions. Arrows indicate the absorption peaks and shoulders. Elution of the protein and the chromophore were recorded at 280 nm (straight line) and 410 nm (dotted line), respectively. (C) EPR spectrum of SyGrx3p (100 nmol) in the presence of dithionite.

rescue the biogenesis of Fe−S cluster in the ScGrx5p null mutant of yeast (*15*) suggests that the function of monothiol glutaredoxins has been evolutionary conserved and possibly their [2Fe-2S] cluster too. Consequently, we searched for the possible occurrence of a SyGrx3p-like [2Fe-2S] cluster not only in the ScGrx5p enzyme but also in other CGFS-type monothiol Grxs from prokaryotic (*E. coli*, and the cyanobacteria *T. elongatus*, *G. violaceus*) and eukaryotic (*S. cerevisiae*, *A. thaliana*, and *Homo sapiens*) model organisms (Figure 1A). As expected, all nine monothiol Grxs tested exhibited similar UV−visible absorption spectra with a maximum absorption peak at 410 nm (Figure 2A). Colorimetric assays indicated that they all contain iron and acid-labile sulfide (data not shown). EPR spectra (Figure 2B) were characteristic of a [2Fe-2S] cluster with mainly an axial symmetry with $g_{\parallel} \approx 2.01$ and $g_{\perp} \approx 1.93$ similar to the axial form detected in SyGrx3p. Collectively, these data indicate that CGFS-type monothiol Grxs defines a new family of [2Fe-2S] cluster-containing proteins.

The Cysteine of the CGFS Motif Is Essential for Both Fe-S Cluster Assembly and Protein Dimerization. In most cases, the [2Fe-2S] cluster is ligated on a protein by the thiol of four of its cysteines (*18*). We therefore decided to use site-directed mutagenesis (cysteine to serine substitution) to identify the Grx cysteines required for assembling the [2Fe-2S] cluster. We first mutagenized the cysteine residue of the

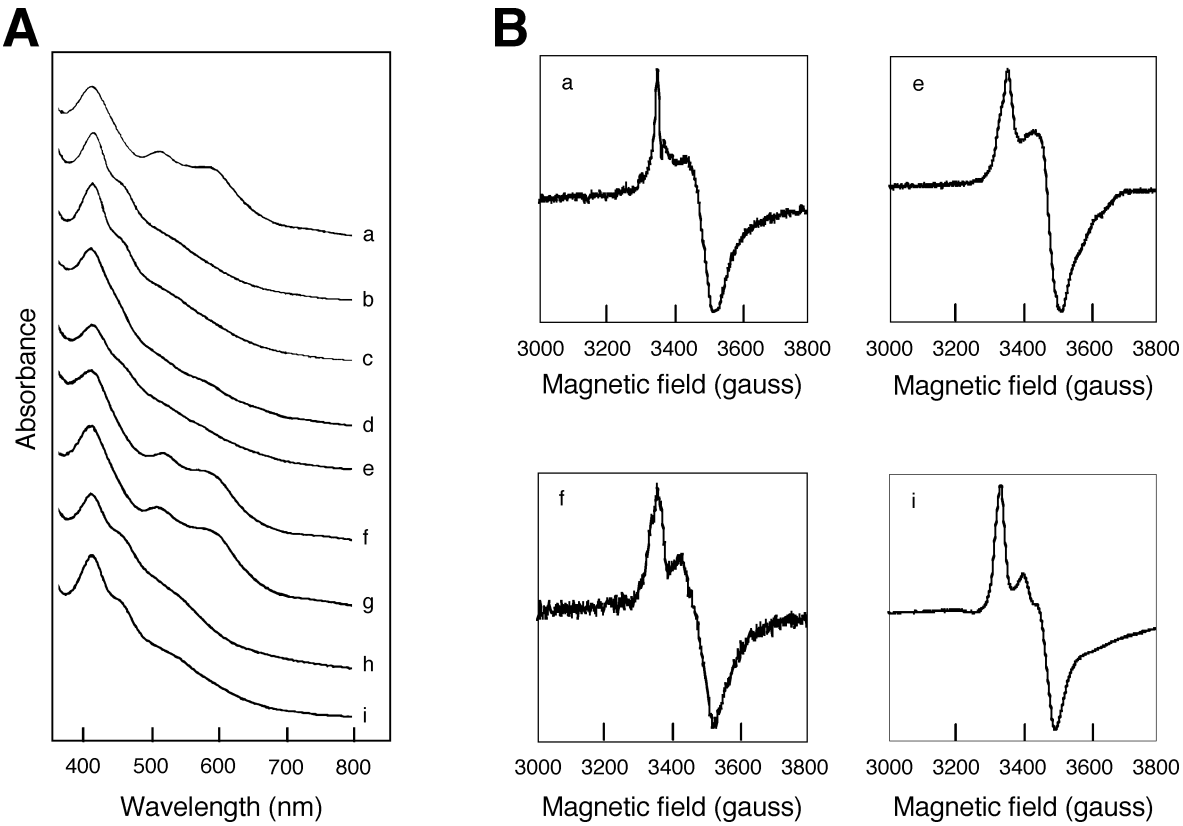


FIGURE 2: CGFS-type monothiol glutaredoxins make up a novel [2Fe-2S] protein family. (A) UV−visible spectra of 70 nmol of various CGFS-type monothiol Grx amplified in *E. coli*: a, SyGrx3p (*Synechocystis* PCC6803); b, ScGrx3p; c, ScGrx4p; d, ScGrx5p (*S. cerevisiae*); e, HsGrx5p (*H. sapiens*); f, AtGrx5p (*A. thaliana*); g, GvGrx3p (*G. violaceus*); h, TeGrx3p (*T. elongatus*); and i, EcGrx4p (*E. coli*). The ratio of dimer per monomer as obtained by gel filtration analyses are as follows: a: 30%; b, c, d, and e: 10%; f: 70%; and i: 90%. (B) EPR spectra of 100 nmol of ScGrx3p (a), HsGrx5p (e), AtGrx5p (f), and EcGrx4p (i).

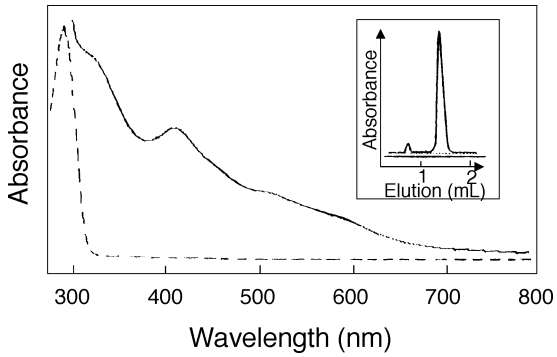


FIGURE 3: Unique cysteine of the SyGrx3p protein, located in the invariant CGFS motif, is essential for protein dimerization and ligation of the [2Fe-2S] cluster. UV−visible spectra of 70 nmol of dimeric SyGrx3p (straight line) and $SyGrx3_{C31S}$ (dashed line) purified by gel filtration. Inset: Gel filtration chromatogram of recombinant $SyGrx3_{C31S}$ recorded as described in Figure 1B.

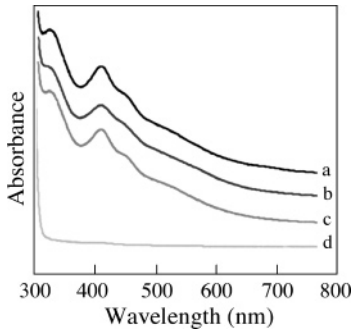


FIGURE 4: Only the cysteine of the CGFS invariant motif of the EcGrx4p protein, not the other two cysteines, is essential to ligating the [2Fe-2S] cluster. UV−visible spectra performed with 30 nmol of each wild type EcGrx4p (a), $EcGrx4_{C43S}$ (b), $EcGrx4_{C84S}$ (c), and $EcGrx4_{C30S}$ (d, the "CGFS" cysteine).

CGFS motif, which is the only strictly conserved cysteine in the nine Grxs studied (Figures 1A and SM1). Very interestingly, this cysteine (C31) is the unique cysteine of the SyGrx3p protein. As anticipated, the purified $SyGrx3_{C31S}$ mutant protein appeared to be monomeric and colorless, and its UV−visible spectrum missed the features above 280 nm (Figure 3). Moreover, EPR spectroscopy and colorimetric assay indicated that no iron atom was associated with the $SyGrx3_{C31S}$ mutant protein (data not shown). Collectively, these results show that the cysteine of the CGFS motif is crucial to both protein dimerization and [2Fe-2S] cluster assembly. This important finding was confirmed, and extended, through the C to S mutagenesis of the *E. coli* protein EcGrx4p that possesses three cysteines (Figure 1A). As expected (Figure 4), the C30S mutation of the CGFS motif abolished Fe−S cluster assembly, unlike the C43S and C84S mutations of the other two cysteines that have no counterpart in the SyGrx3p protein. Collectively, these findings demonstrate that the cysteine of the CGFS motif is crucial for the assembly of the [2Fe-2S] cluster of both the CGFS-type monothiol Grxs SyGrx3p and EcGrx4p of the evolutionary distant organisms *Synechocystis* and *E. coli*, respectively.

The Cysteinyl Residue of Glutathione Is Required for Anchoring the [2Fe-2S] Cluster onto Monothiol Glutaredoxins. Having shown that the monomer-bridging [2Fe-2S]

cluster of monothiol Grx is ligated by the cysteinyl residue of the CGFS motif of each monomer, we searched for the two missing anchoring cysteines (i.e., one per monomer). We thought about testing the cysteine of glutathione (GSH) because it interacts with Grx (GSH reduces Grx). Therefore, we performed in vitro assays of Fe-S cluster-reconstitution on apo-SyGrx3p in the presence or absence of reduced GSH, under anaerobic conditions because the Fe-S cluster is sensitive to oxygen degradation. In absence of GSH, SyGrx3p failed to reconstitute its [2Fe-2S] cluster (Figure 5). By contrast, in the presence of reduced GSH the [2Fe-2S] cluster was readily reconstituted onto the dimeric SyGrx3p, with the stoichiometry of 1 mol each of Fe and S^{2-} atoms per mole of SyGrx3p monomer (Figure 5, and data not presented), showing that the reconstitution was very effective. This explains why the reconstituted Fe-S cluster-containing SyGrx3p protein exhibited similar UV−visible and EPR spectra with similar *g*-values and the same "axial over rhombic forms" ratio as the native SyGrx3p protein sample (compare Figure 5 with Figure 1), but with more intense UV−visible peaks at 410 nm ($\epsilon = 4541\ M^{-1}\ cm^{-1}$), 510 nm ($\epsilon = 2600\ M^{-1}\ cm^{-1}$), and 590 nm ($\epsilon = 1995\ M^{-1}\ cm^{-1}$) because it is a pure holoprotein, unlike the native SyGrx3p fraction that contains both holoprotein and apoprotein. All nine monothiol Grxs presently studied behaved similarly (data not shown), indicating that CGFS-type Grxs require GSH to coordinate their monomer-bridging [2Fe-2S] cluster. To show that the free thiol group of GSH is required to ligate the [2Fe-2S] cluster, we compared the level of Fe-S cluster reconstitutions performed either with GSH (positive control) or with *S*-acetamido GSH (a-GSH), the thiol of which is blocked by alkylation. First, we verified that the GSH-requiring Fe-S cluster reconstitution on SyGrx3p performed very well in the presence of alkylated DTT (a-DTT, Figure 5), showing that alkylation per se does not impair Fe-S cluster reconstitution. Then, we showed that the substitution of GSH by a-GSH decreased the level of Fe-S cluster reconstitution down to the basal value obtained in absence of either GSH (Figure 5) or the crucial cysteine of the invariant motif CGFS ($SyGrx3_{C31S}$ mutant, Figure 5). Collectively, these data show that both the "CGFS" cysteine and the free thiol group of GSH are required to ligate the monomer-bridging [2Fe-2S] cluster onto monothiol Grxs.

The SyGrx3P Amino Acids K23, T71, and D86 Are Crucial to Both Glutathione Binding and Fe-S Cluster Assembly. To further validate that GSH-binding and Fe-S cluster assembly are interdependent features of monothiol Grxs and to identify some of the GSH-anchoring amino acids, we identified evolutionary conserved amino acids (aa)[11], mutagenized these aa in the SyGrx3p protein, and subsequently analyzed both GSH binding and Fe-S cluster assembly. Therefore, 119 putative monothiol Grxs from prokaryotic and eukaryotic phyla were aligned and used to generate a LOGO pattern (Figure SM1, Supporting Information), the conserved aa of which were positioned on the 3D structure of the *E. coli* Grx4p protein (*14*). Interestingly, the conserved aa located at the surface of the protein surface cluster into two large overlapping hydrophobic areas (Figures 6A and SM2). The

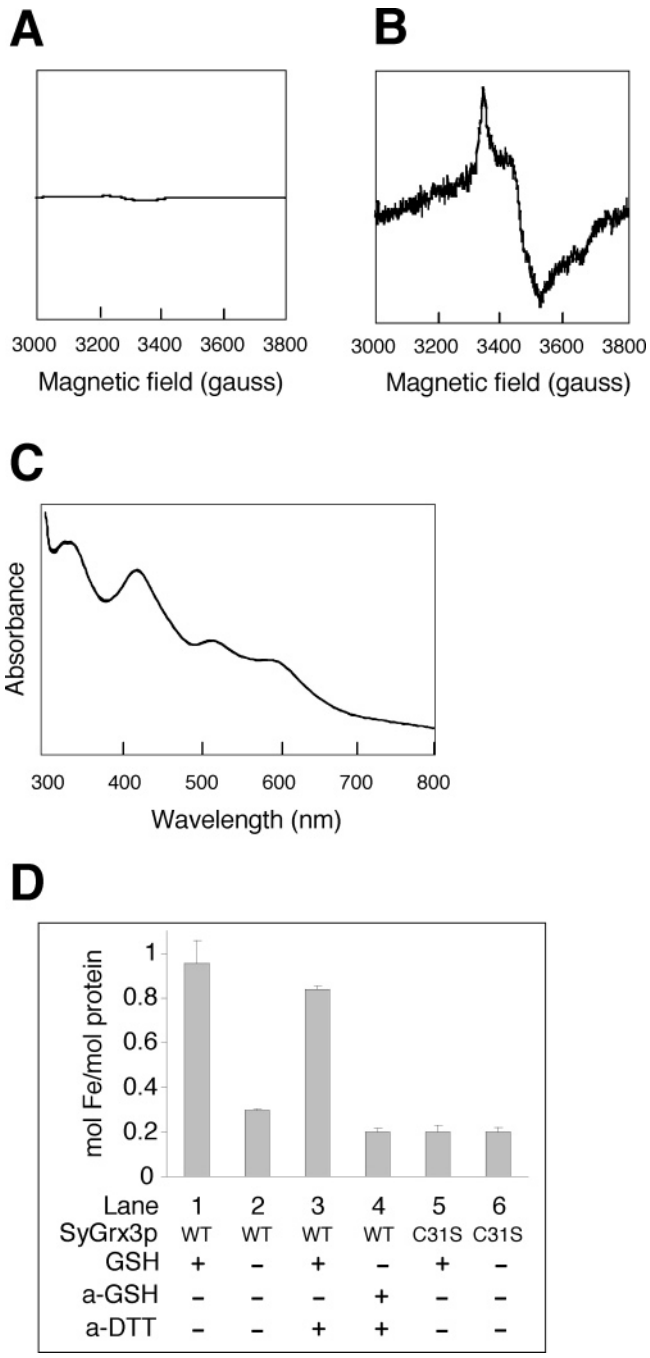
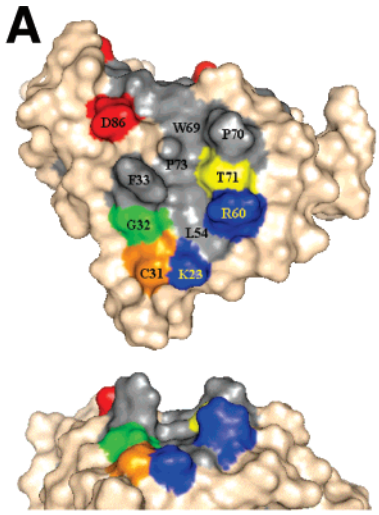


FIGURE 5: Cysteinyl residue of glutathione is required to anchor the [2Fe-2S] cluster onto the SyGrx3p protein. EPR spectra of 30 nmol of SyGrx3p reconstituted without (A) or with (B) glutathione (GSH) recorded at 16 K after reduction by dithionite. (C) UV-absorption spectrum performed on SyGrx3p reconstituted with GSH. (D) Analysis of Fe-S cluster reconstitution by iron quantification (*10*) performed on 10 nmol of wild type SyGrx3p (lanes 1 to 4) or mutant $SyGrx3_{C31S}$ (lanes 5 and 6) with GSH (lanes 1 and 5), without GSH (lanes 2 and 6), with GSH and alkylated DTT (a-DTT; lane 3), or with a-DTT and *S*-acetamido glutathione (a-GSH; lane 4).

first area encompasses the SyGrx3p aa K23, C31, G32, F33, L54, R60, W69, P70, T71, P73, and D86 (Figure SM1) structures as a groove that begins by K23 and both C31 and G32 of the CGFS motif and ends up by D86 and both W69 and P70 of the nearly invariant WPT(I/F)PQ(L/V) heptapeptide (Figures SM1 and 6A). The groove comprises D86 and F33 on one side; P70, T71, and R60 on the other side (Figure 6A); and L54, P73, and W69 at its bottom. The C31 cysteine

[1] Abbreviations: aa, amino-acid; DTT, dithiothreitol; a-DTT, alkylated DTT; EPR, electron paramagnetic resonance; Grx, glutaredoxin; GSH reduced glutathione; a-GSH alkylated glutathione; WT, wild type.



B

Mutant classes	SyGrx3p	Solubility	Color	Fe-S reconstitution
-	WT	+	Brown	+++
I	L54T	+	Brown	++
	R60Q	+	Brown	++
	P70G	+	Brown	NT
II	D86N	+	Light brown	+
	K23Q	+	None	-
III	C31S	+	None	-
	T71V	+	None	-
IV	F33V	-	NT	NT
	S34A	-	NT	NT

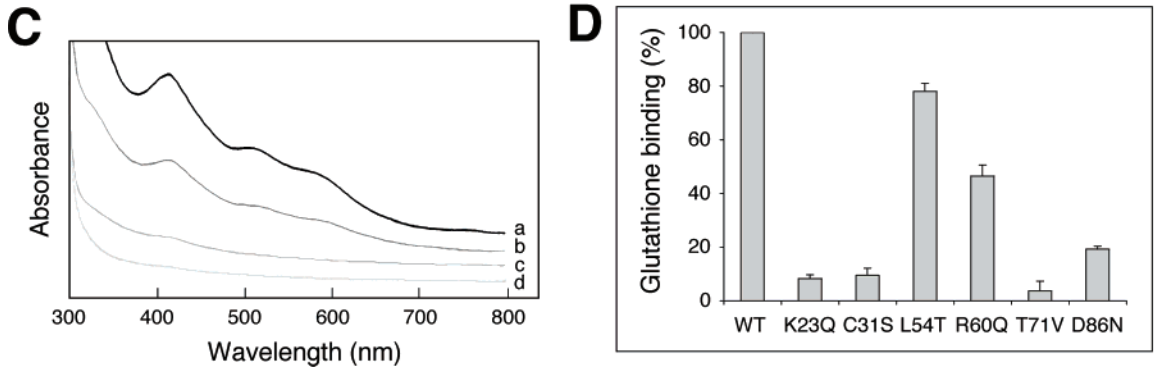


FIGURE 6: Structural requirement for glutathione binding and Fe-S cluster assembly. (A) Top and axis views of the conserved GSH-binding site of Grx shown in the SyGrx3p protein, using the following amino acid color code: red, acidic; purple, amides; blue, basic; dark, hydrophobic; yellow, alcoolic; green, glycine; orange, cysteine. (B) Biochemical phenotypes of SyGrx3p with or without (WT) the indicated mutation. The level of Fe-S cluster reconstitution is represented as follows: +++ (strong), ++ (moderate), + (poor), − (none), and NT (not tested). (C) UV−visible absorption spectra of 15 nmol of SyGrx3p proteins (WT, a), $SyGrx3_{D86N}$ (b), $SyGrx3_{K23Q}$ (c), and $SyGrx3_{T71V}$ (d). (D) Quantification of GSH molecules bound (*9*) onto reconstituted SyGrx3p proteins expressed in percentage of that obtained with the WT protein.

of the CGFS motif is located at the border of the groove, slightly offset of the axis (Figure 6A). The second area, encompassing the SyGrx3p aa V13, Y66, Y76, V77, E80, F81, V82, D86, I87, L9,1 and E96 (Figure SM1), forms a flat hydrophobic surface surrounded by the three acidic residues D86, E96, and E80 (Figure SM2).

On the basis of sequence conservation between monothiol and dithiol Grxs, it has been recently proposed that the Grx groove is involved in GSH recruitment (*14*). Therefore, we mutagenized the relevant aa of the groove so as to interfere with GSH binding, but not with the overall Grx structure (Figure 6A,B), thereby precluding mutation of G32, W69, and P73. The nine SyGrx3p mutants we constructed could be divided in four groups, based on their solubility and color (Figure 6B). Group I mutants, L54T, R60Q, and P70G, displayed a brown color and UV−visible spectrum very similar to that of the wild type protein (Figure 6C). The single member group II mutant D86N exhibited a lighter brown color and a smoother UV−visible spectrum than WT or group I mutants (Figure 6C). Group III mutants, K23Q, C31S, and T71V, lacked both the brown color and the UV−visible peaks (Figure 6C). Group IV mutants, being insoluble, were therefore not studied further.

In vitro assays were then used to test GSH-binding and Fe-S cluster-reconstitution. As expected, we found (Figure 6B,D) that both features were either unaffected (group I mutants L54T and R60Q) or simultaneously impaired strongly (group II, D86N) or totally (K23Q, C31S, and T71V, group III mutants), respectively. Together, these results show that aa crucial to GSH binding (K23, T71, and D86 located in the Grx groove) are critical to Fe-S cluster assembly as well and that the contrary is also true (see the C31 aa). This shows that GSH binding and Fe-S cluster assembly are interdependent, suggesting that they might occur concomitantly.

DISCUSSION

As little is known concerning CGFS-type monothiol glutaredoxin in photosynthetic organisms, we have analyzed the SyGrx3p protein of the model cyanobacterium *Synechocystis* PCC6803. We found that SyGrx3p overproduced in *E. coli* exists not only as a monomeric apoprotein but also as a dimeric form that contains a subunit-bridging [2Fe-2S] cluster. By contrast, we have seen no evidence that the two dithiol Grxs of *Synechocystis* possess an Fe-S cluster (data not shown), in agreement with the presence of a proline residue in their CPxC motif, a feature which in human and poplar dithiol Grxs has been proposed (*19*) and actually shown (*20*) to prevent Fe−S cluster assembly, likely through reducing the flexibility of the Grx protein backbone. Return-

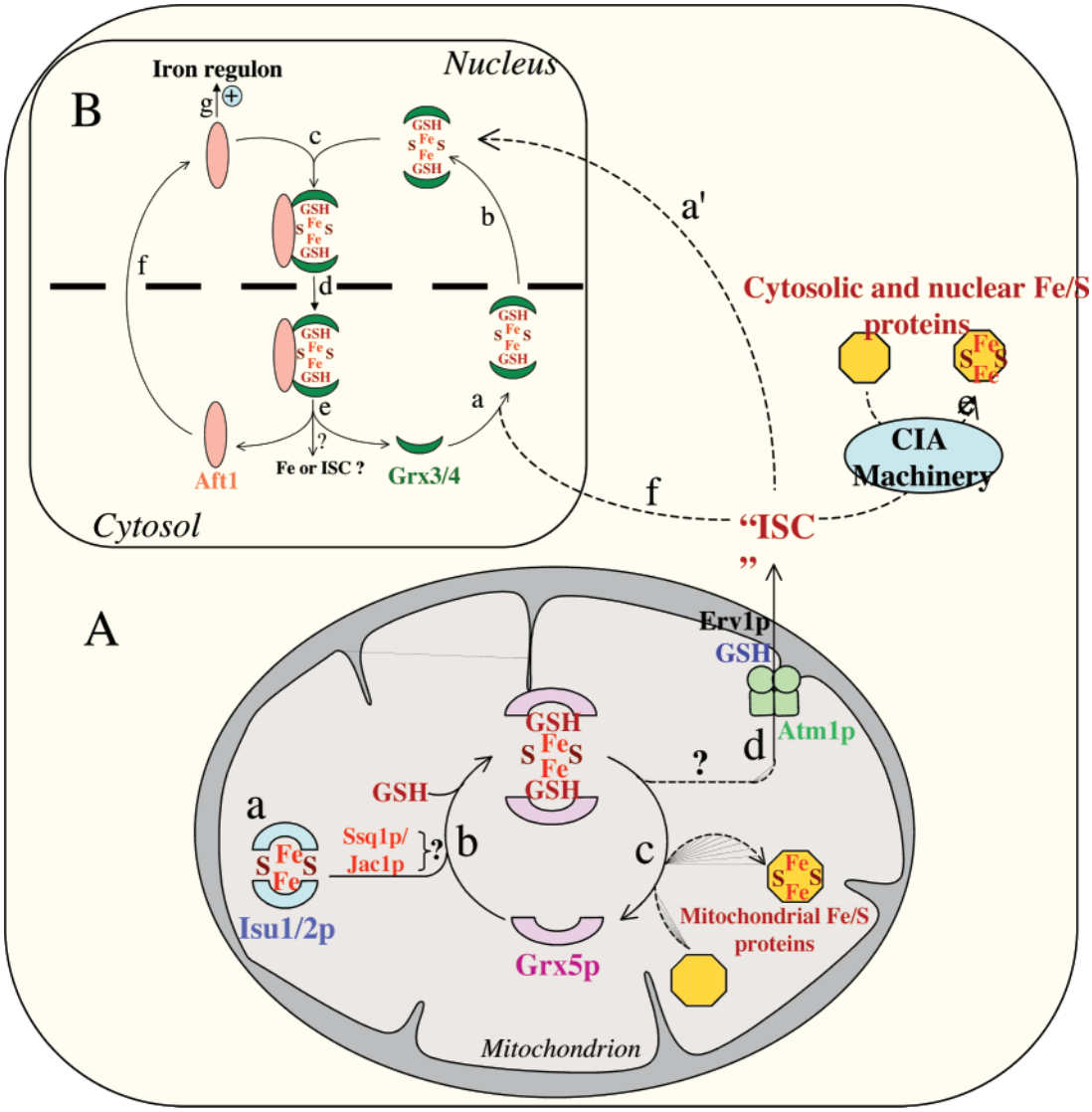


FIGURE 7: Model for maturation of Fe-S cluster-containing proteins (A) and regulation of iron homeostasis (B) in yeast cells. (A) Fe-S clusters (ISCs) assembled onto the scaffold proteins Isu1p/2p (a) are transferred onto ScGrx5p in a GSH-dependent manner (b) and, subsequently, to mitochondrial targets (c) and to the ISC-export machinery comprising the membrane protein Erv1p and the ABC transporter Atm1p (d), the homologues of which translocate GSH-metal complexes (*24*−*26*). This process likely involves the Ssq1p chaperone and Jac1p co-chaperone, the absence of which mimic that of ScGrx5p (*27*). Cytoplasmic ISCs can be transferred by the CIA machinery onto cytosolic and/or nuclear proteins (e) including ScGrx3p/4p (f) that modulate the transcriptional activity of the iron regulator Aft1p. We favor the possibility that the ISC cluster of ScGrx3p and ScGrx4p originate from the mitochondrial rather than the cytoplasmic (CIA) machinery, because the former but not the latter operates in Aft1p-mediated iron regulation (*28*, *29*). (B) When cellular iron is abundant, a GSH-associated ISC is transferred directly (a) or indirectly (a′) onto ScGrx3p and ScGrx4p (b) that associate with Aft1p inside the nucleus (c) and promotes translocation of Aft1p into the cytoplasm (d) where it can no longer be active. Under iron limitation, the Aft1p complex with ScGrx3p or/and ScGrx4p is disassembled (e), liberating free Aft1p that can relocate to the nucleus (*30*, *31*) (f) where it activates iron-uptake genes (g).

ing to the SyGrx3p protein we found that its Fe-S cluster is ligated by the unique *c*ysteine of each SyGrx3p monomer, which is located in the *C*GFS motif, and the cysteine of two molecules of glutathione (the γGlu-Cys-Gly tripeptide). That we observed the same features following the overproduction of the endogenous *E. coli* monothiol protein EcGrx4p rules out the hypothetical possibility that they were artifacts resulting from the overproduction of a foreign protein. Indeed, we verified that the EcGrx4p cysteine (C30) of the CGFS-motif is required for the ligation of the [2Fe-2S] cluster, unlike the two others cysteines C43 and C84 that have no counterparts in the SyGrx3p protein. Moreover, we showed that the SyGrx3P amino acids K23, C31, and T71, strictly conserved in monothiol Grxs (*14*), are crucial not only to glutathione binding but also to Fe-S cluster assembly, two features which appeared to be interdependent and might therefore occur concomitantly. These findings are consistent with the fact that the counterpart of K23 and C31 in the structurally similar human dithiol HsGrx2p protein, i.e., K34 and C37, were recently found to operate in the binding of the GSH molecules that ligate the Fe [2Fe-2S] cluster (*19*).

We also showed that CGFS-type monothiol Grxs from evolutionary-distant model organisms possess a [2Fe-2S] cluster, namely the two cyanobacteria *G. violaceus* (GvGrx3p protein) and *T. elongatus* (TeGrx3p); the yeast *S. cerevisiae* (ScGrx3p, ScGrx4p, and ScGrx5p); the plant *A. thaliana* (AtGrx5p); and human (HsGrx5p). The occurrence of a GSH-ligated [2Fe-2S] cluster in CGFS-type monothiol Grxs

makes sense in the view of the very recent findings that the same is true in structurally similar but biochemically different (*1*) dithiol enzymes of the CSYC-type (*21*) and CGYC-type (*20*), which are absent in many organisms such as cyanobacteria. In addition, the presently reported occurrence of a similar [2Fe-2S] cluster in the CGFS-type monothiol enzymes SyGrx3p and ScGrx5p is consistent with the previous finding that the *Synechocystis* SyGrx3p protein can rescue the Fe-S cluster-biogenesis default of the *S. cerevisiae* mutant lacking the endogenous ScGrx5p mitochondrial enzyme (*15*). Furthermore, our results also shed light on the role of GSH in the maturation of Fe-S cluster-containing proteins (*22*) and regulation of iron homeostasis (*3*). First, the cysteine of the CGFS motif of ScGrx5, presumably involved in the ligation of the presently described Fe-S cluster, appeared to be required for the ScGrx5-dependent biogenesis of Fe-S enzymes operating in the biosynthesis of leucine, lysine, and glutamic acid (*1*). Second, the cysteine of the CGFS active motif and the GSH-binding pocket of ScGrx3p and ScGrx4p, presumably anchoring their presently described Fe-S cluster, were found to modulate the activity of the iron regulatory transcription factor Aft1p that interacts with both ScGrx3p and ScGrx4p (*3*).

In conclusion, we have characterized CGFS-type monothiol Grxs as a new family of proteins containing a GSH-ligated Fe-S cluster. These findings shed light on the role of CGFS-type monothiol Grxs in Fe-S cluster biogenesis and/or Fe regulation, not only in model organisms but also in humans where a deficiency of HsGrx5p generates anemia (*23*). Indeed, monothiol Grxs may act as Fe-S cluster donors to Fe-S cluster-requiring proteins and/or as Fe-S cluster-dependent sensors informing iron regulators of the iron status of the cell. A model of the function of monothiol Grxs and glutathione in these processes in *S. cerevisiae*, where they are best understood, is depicted in Figure 7. It is based on the presently reported findings that monothiol Grxs contain a GSH-ligated Fe-S cluster. Interestingly, SyGrx3p, the presently studied single monothiol Grx of *Synechocystis*, may combine both functions, and the same can be true for its bacterial orthologs proteins which also appeared to be unique in their respective host organisms.

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SUPPORTING INFORMATION AVAILABLE

Two tables showing the oligonucleotides used in this work as well as two figures showing (i) the sequence conservation pattern of monothiol glutaredoxins and (ii) the view of the second conserved hydrophobic area exposed on the SyGrx3p surface. This material is available free of charge via the Internet at http://pubs.acs.org.

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