News & Views

How Does Iron–Sulfur Cluster Coordination Regulate the Activity of Human Glutaredoxin 2?

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ABSTRACT

Human mitochondrial glutaredoxin (Grx2) was described as the first iron–sulfur protein from the thioredoxin superfamily of proteins. The [2Fe–2S] cluster was proposed to serve as redox sensor for the activation of Grx2 during oxidative stress. The authors have demonstrated that the iron–sulfur cluster is complexed by the two N-terminal active site thiols of two Grx2 monomers and two molecules of glutathione that are bound noncovalently to the proteins and in equilibrium with glutathione in solution. When reduced glutathione becomes the limiting factor for cluster coordination, the holo-Grx2 complex dissociates, yielding enzymatically active Grx2. *Antioxid. Redox Signal.* 9, 151–157.

HUMAN GLUTAREDOXINS

GLUTAREDOXINS (GRXS) are members of the thioredoxin family of thiol–disulfide oxidoreductases, crucial for the maintenance of thiol redox homeostasis in the cell (9). First discovered as electron donor for ribonucleotide reductase (7), Grxs catalyze reactions in two distinct but functionally connected reaction mechanisms. The dithiol mechanism requires both active site cysteines of the Cys–Pro–Tyr–Cys active site, the monothiol mechanism requires only the more N-terminal thiol (2, 6, 8, 18, 23, 27).

Human cells contain two dithiol Grxs, the cytosolic Grx1 and the primary mitochondrial Grx2 (5, 17). Grx2 contains the unusual active site sequence Cys–Ser–Tyr–Cys, and is, due to its high affinity for protein glutathione-mixed disulfides, an efficient catalyst of protein de-glutathionylation. Moreover, the oxidized active site of Grx2, which in all other eukaryotic Grxs is exclusively reduced by GSH, is also a substrate for thioredoxin reductase (10). HeLa cells, in which the expression of Grx2 was silenced by RNA interference, were dramatically sensitized to cell death induced by oxidative stress-inducing apoptotic agents (15). The EC_{50} for the anticancer drug doxorubicin/adriamycin decreased 60-fold and the EC_{50} for phenylarsine oxide, which reacts specifically with vicinal dithiols, 40-fold. Corroboratively, overexpression of Grx2 decreased the susceptibility of HeLa cells to apoptosis induced by doxorubicin or the antimetabolite 2-deoxy-D-glucose (3). Grx2 prevented loss of cardiolipin, inhibited cytochrome *c* release, and caspase activation.

MITOCHONDRIAL GLUTAREDOXIN (GRX2)—THE FIRST IRON–SULFUR PROTEIN FROM THE THIOREDOXIN FAMILY OF PROTEINS

High-yield expression of Grx2 in *Escherichia coli (E. coli)* gave rise to a brown iron-containing protein (14). Spectroscopic analysis revealed the presence of a four cysteine-coordinated, nonoxidizable $[2Fe–2S]^{2+}$ cluster in the recombinantly expressed protein, that bridges two molecules of Grx2. Dimeric holo-Grx2 was enzymatically inactive, but cluster degradation and monomerization of Grx2 activated the oxidoreductase. Slow loss of the cofactor under aerobic conditions was prevented by the presence of reduced GSH; GSSG

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as well as one electron oxidants or reductants promoted monomerization and activation of Grx2. Co-immunoprecipitation of the [55]Fe isotope with Grx2 from two different human cell lines indicated the presence of the cluster *in vivo*. Grx2 contains two additional cysteinyl residues outside its active site. Exchange of the C-terminal active site cysteine for serine in Grx2 did not change the spectral properties of the protein expressed in *E. coli*, whereas replacement of the two cysteinyl groups outside the active site resulted in the expression of the active chromophore-free protein. Based on these results, we have proposed that the cluster is coordinated by the two nonactive site cysteinyl residues from each monomer (14).

Recently, human Grx2 has been used as an affinity tag for the purification of peptides, because Grx2 can bind to a glutathione Sepharose matrix (16). Moreover, a crystal structure of Grx2 became recently available (PBD code 2FLS, Structural Genomics Consortium, Oxford, England) that shows a noncovalent complex between glutathione and reduced Grx2. This unusual feature could be the basis for the stabilization of [2Fe–2S] Grx2 by GSH, and it prompted us to question our previous model of cluster coordination.
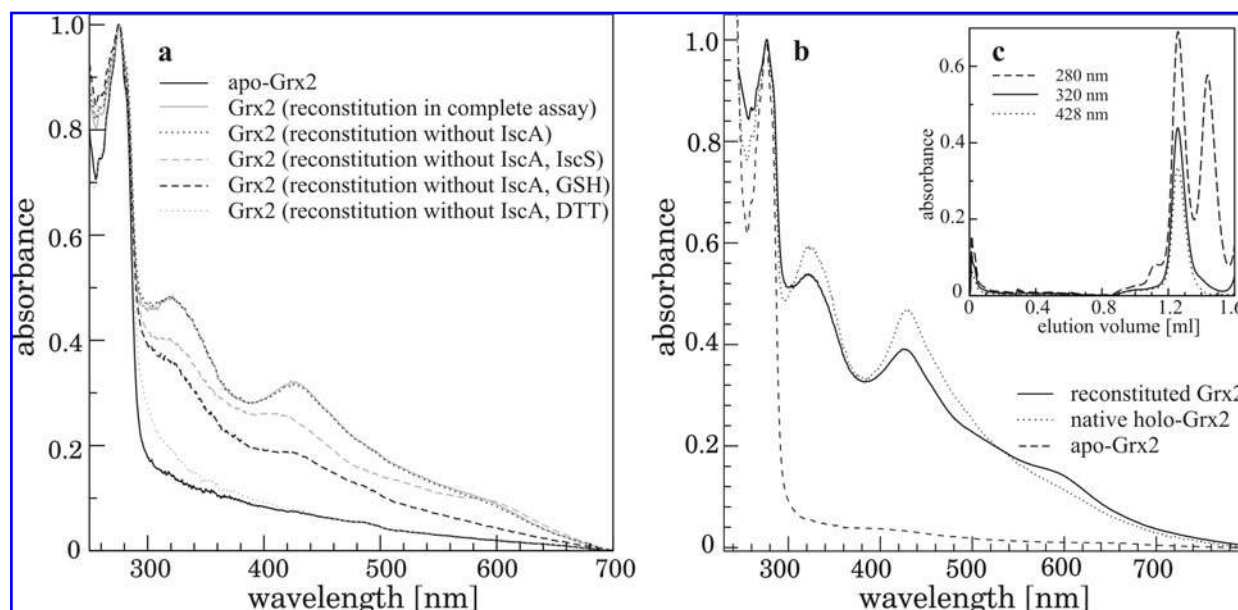
RECONSTITUTION OF HOLO-GRX2

Previously, analysis of iron–sulfur Grx2 depended on the purification of holo-Grx2 from a mixture of recombinantly expressed apo- and holo-protein by gel filtration chromatography in the presence of reduced glutathione, which stabilizes the holo-protein (14). This procedure proved to be inefficient,

because the holo-protein could not be stored for a longer period of time.

To overcome this problem, we have developed an assay for the reconstitution of holo-Grx2 *in vitro* based on the cysteine desulfurylase IscS and the [Fe–S] scaffold IscA from the iron–sulfur cluster synthesis machinery of *E. coli*. Interestingly, efficient reassembly of the Grx2 [2Fe–2S] cluster required not only iron, in the form of $Fe(NH_4)_2(SO_4)_2$, and sulfide, donated by IscS, cysteine, and DTT, but also glutathione. Addition of IscA did not stimulate or enhance cluster formation in our setup (Fig. 1a). After about 2 h, up to 80% of the initial apo-Grx2 had formed an iron–sulfur cluster-bridged dimer, as seen from gel filtration HPLC (Fig. 1c). The spectral properties of this dimer were very similar to those of the holo-protein isolated from *E. coli* (Fig. 1b).

GSH IS A PART OF THE HOLO-GRX2 COMPLEX

The strong stimulation of cluster formation in Grx2 by GSH prompted us to investigate whether GSH could be part of the holo-Grx2 complex. We have included radiolabeled GSH in our reconstitution assay, and after 2 h of incubation separated the assay mixture by gel filtration using prepacked Sephadex G25 columns (Fig. 2). The collected fractions were analyzed for radioactivity and their optical spectra were recorded. We found GSH bound to Grx2 that had assembled the iron–sulfur cluster (Fig. 2a), but not to protein that could not form the cluster, due to the lack of iron in the assay (Fig. 2b). We concluded that GSH is part of the holo-Grx2 complex but

FIG. 1. Reconstitution of [Fe–S] Grx2. (**a**) UV/VIS spectra after reconstitution of 100 μ*M* holo-Grx2 in the presence or absence of IscA, IscA and IscS, IscA and GSH, or IscA and DTT. (**b**) UV/VIS spectra of apo-Grx2 and dimeric holo-Grx2 after purification and separation by gel filtration in comparison to holo-Grx2 following reconstitution. (**c**) Demonstration of dimeric holo-Grx2 by gel filtration HPLC following reconstitution; the chromophore elutes exclusively with dimeric Grx2 (first peak, approx. 32 kDa).
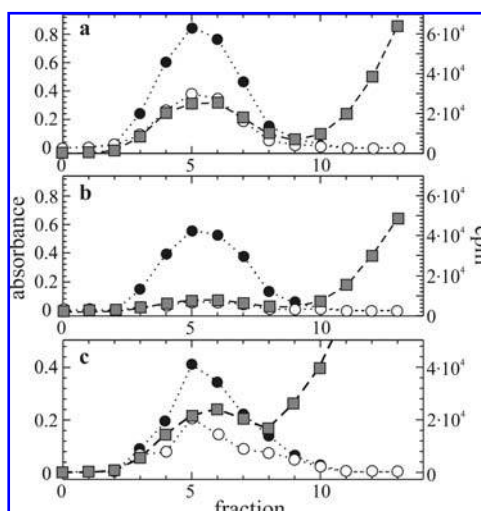
FIG. 2. GSH is part of the holo-Grx2 complex and in dynamic equilibrium with a pool of free GSH. Elution profiles from gel filtration chromatography following [Fe–S] reconstitution in the presence of (^{3}H)-GSH. Reconstitution in the presence (**a**) or absence of iron (**b**). Incubation of holo-Grx2 with one equivalent of (^{3}H)-GSH demonstrated the exchange of GSH in the holo-Grx2 complex for labeled GSH from the solution (**c**). *Filled circles, dotted lines*: absorbance at 280 nm (protein); *open circles, dotted lines*: absorbance at 320 nm ([Fe–S] cluster); *gray squares and dashed lines*: radioactivity (GSH).

does not form a particularly stable complex with apo-Grx2 in our assay.

Dimeric holo-Grx2 is specifically stabilized by reduced GSH (14), by approximately 9.3 kJ mol^{-1} as estimated from

the different rate constants of cluster disintegration (not shown). The reason for this stabilization could be that GSH in the holo-Grx2 complex is only weakly bound and needs to be in dynamic equilibrium with a pool of free GSH. To test this hypothesis, we incubated equal amounts of unlabeled holo-Grx2 with radiolabeled GSH for 4 h at room temperature and separated the mixture by gel filtration as before. As depicted in Fig. 2c, significant amounts of the GSH in the holo-Grx2 complex were exchanged for radiolabeled GSH while the iron–sulfur cluster remained intact (Fig. 2c).

CHARACTERIZATION OF CLUSTER COORDINATION BY SITE DIRECTED MUTAGENESIS OF GLUTAREDOXIN 2

Next, we used the new reconstitution protocol to analyze a series of Grx2 mutants for their ability to assemble the [2Fe–2S] cluster *in vitro.* First, we analyzed which of the four cysteinyl residues in Grx2 are necessary for cluster formation *in vitro* by exchanging them individually to seryl residues. We had previously shown that recombinantly expressed Grx2 mutants lacking either of the nonactive site cysteinyl residues (Cys28 or Cys113) did not contain the iron–sulfur cluster, while a mutant lacking the C-terminal active site residue (Cys40) was purified as a mixture of apo- and holo-protein (14). In our reconstitution assay, however, all of these mutants were able to assemble the cluster with spectral properties similar to those of the wild-type protein (Fig. 3). Only when we mutated the N-terminal active site Cys37, the protein lost its ability to form the [2Fe–2S] cluster-bridged dimer both during expression in *E. coli* and *in vitro* (Fig. 3).

Why were the Cys28 and the Cys113 mutants isolated from *E. coli* as apo-proteins? We have recognized the continuous
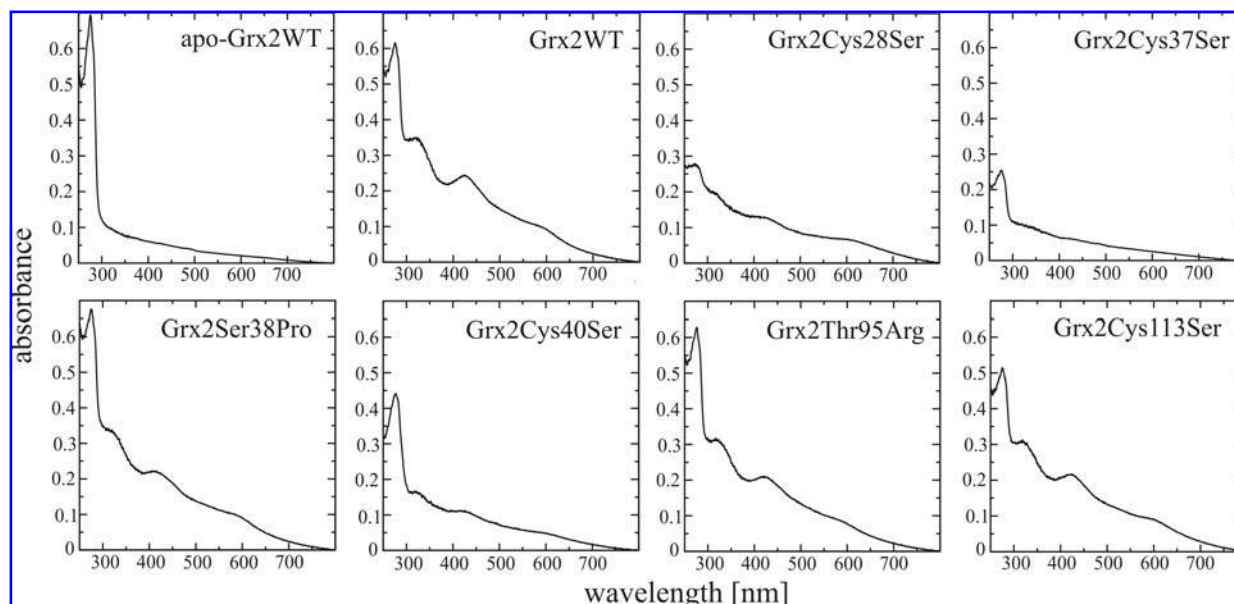
FIG. 3. UV-Vis spectra of Grx2 proteins altered by site directed mutagenesis following [Fe–S] reconstitution. 90 μ*M* of the respective apo-protein were used to assemble the [Fe–S] cluster.

aggregation of these mutants throughout the purification and reconstitution procedure (see, for instance, the decreased absorbance at 280 nm after reconstitution in Fig. 3). We compared the temperature-dependent aggregation of the Cys113Ser and the wild-type apo-proteins at 25 μM concentration following the increase in turbidity at 600 nm. While the wild-type protein showed no signs of aggregation up to 70°C, all of the mutant protein was aggregated at 53°C (T_m = 46°C, data not shown). The sensitivity of the active site structure in Grx2 to mutation of one of the nonactive site cysteinyl residues was also confirmed by their approximately 50% decreased activity in the Grx-specific HED assay (Table 1). These results indicate that the nonactive site cysteinyl residues are important for the structure of both apo- and holo-Grx2, but not for cluster coordination itself.

Human Grx2 has the unique ability to bind GSH noncovalently to its reduced active site. We aimed at specifically weakening this binding to analyze its effect on cluster formation and enzymatic activity. Based on the crystal structure of the complex between human Grx2 and GSH (2FLS), we selected to mutate Thr95, because this residue forms two hydrogen bonds and Van-der-Waals interactions with GSH. We decided to mutate Thr95 to Arg, because this mutation does not only destroy one of the hydrogen bonds, but our structural predictions indicate that its guanidino group could also interfere with the coordination of the carboxy and the amino groups of the Glu residue in GSH (not shown). The Thr95Arg mutant was purified from *E. coli* as iron–sulfur protein, and cluster reconstitution yielded a protein with spectral properties that were indistinguishable from that of wild-type Grx2 (Fig. 3). However, when we followed the degradation of the cluster under aerobic conditions, this mutant was much less stabilized by reduced GSH. This was not due to structural instability of the protein itself, which showed no difference in thermal stability to the wild-type protein (not shown). Moreover, the specific activity of the mutant in the HED assay, as well as its $V_{max,}$ increased considerably (about twofold). This was expected, because the complex between reduced Grx2 and GSH must represent an inhibitory dead-end complex for both monothiol and dithiol reactions. Further on, the mutation had almost no effect on the K_m-value for GSH (Table 1), which was also expected,

since in the HED assay K_m is a measure of the affinity of the Grx2–GSH mixed disulfide complex for GSH. We concluded that the Thr95Arg mutant specifically decreased the affinity of reduced Grx2 for GSH resulting in a decreased stability of the iron–sulfur cluster and an increased specific activity of the apo-protein.

CLUSTER COORDINATION IN OTHER GLUTAREDOXINS?

As a control, we have tried to assemble an iron–sulfur cluster in human Grx1, which contains the consensus active site sequence Cys–Pro–Tyr–Cys and has no functional homologs to the extra cysteinyl residues or to Thr95 of Grx2. As expected, no cluster was formed in the reconstitution assay (Fig. 4). Next, we investigated the role of the Ser to Pro exchange in the active site of Grx2 by expression of a Grx1 mutant containing the Cys–Ser–Tyr–Cys active site (Pro23Ser). To our surprise, this protein was already isolated from *E. coli* as a brownish iron–sulfur protein and the *in vitro* reconstitution assay yielded a protein with an optical spectrum typical for [2Fe–2S] proteins (Fig. 4). We also prepared the corresponding Grx2 mutant mimicking the Cys–Pro–Tyr–Cys active site of Grx1. This protein was isolated as a colorless apo-protein from *E. coli* (not shown) and, in agreement with earlier results (10), exhibited an about fourfold increased specific activity. Despite this, we were able to assemble the iron–sulfur cluster *in vitro* (Fig. 3). The Ser to Pro exchange in the active site of Grx2 is clearly the most important, but not the only factor enabling this unique Grx to bind the [2Fe–2S] cluster.
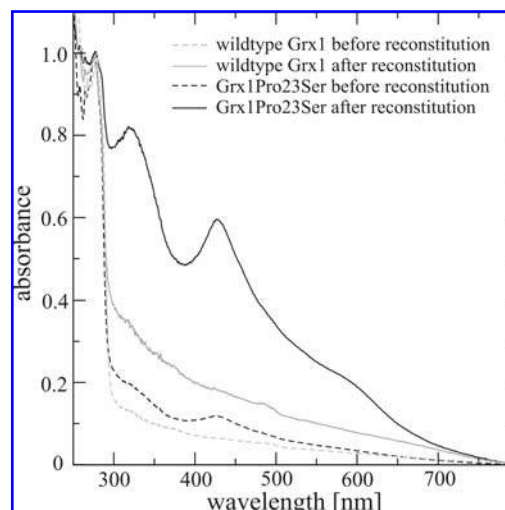
TABLE 1.   SPECIFIC ACTIVITY OF GRX2 MUTANTS

Protein	Specific activity in HED assay s^{-1}	K_m (GSH) mM	V_{max} s^{-1}
Wild type	4.46 ± 1.17	4.31	43.5
Cys28Ser	2.63 ± 0.6	n.d.*	n.d.
Cys40Ser	1.42 ± 0.21	n.d.	n.d.
Cys113Ser	2.36 ± 0.36	n.d.	n.d.
Ser38Pro	17.05 ± 2.66	n.d.	n.d.
Thr95Arg	8.87 ± 1.87	5.65	115.1

*n.d., not determined.

Apo-proteins were assayed as outlined in the appendix section.

FIG. 4. Iron–sulfur cluster assembly in wild-type and Pro23Ser (Cys–Ser–Tyr–Cys) human Grx1. 100 μM of apo-Grx1 and apo-Grx1Pro23Ser were incubated in [Fe–S] reconstitution mixture. Optical absorbance spectra were recorded to illustrate the formation of iron–sulfur proteins.

CONCLUSIONS AND OPEN QUESTIONS

In the present study, we found that the only essential thiol group for the coordination of the [2Fe–2S] cluster in human Grx2 was the N-terminal active site cysteine Cys37. We know from Mössbauer spectroscopy that the iron bound in Grx2 is in tetrahedral sulfur coordination (14). If only one cysteinyl residue from each monomer in the dimeric holo-complex is essential for cluster coordination, where do the other two sulfur atoms come from? We have demonstrated here, that GSH is part of the holo-Grx2 complex and we have learned from the results presented by Lundberg *et al.* (16) and the crystal structure of the protein that GSH can bind noncovalently to the reduced active site of Grx2. Taken all this into account, the cluster must be complexed by the N-terminal active site residue from two Grx2 monomers and two molecules of GSH bound noncovalently to each of the monomers in the holo-Grx2 complex.

This new configuration of cluster coordination is the basis for the molecular mechanism of both the stabilization of holo-Grx2 by reduced GSH and the enzymatic activation of Grx2 through redox-induced disassembly of its iron–sulfur cluster (Fig. 5). Newly translated apo-Grx2 that enters the mitochondrial matrix is, by the action of the mitochondrial iron–sulfur cluster assembly machinery (12, 13), loaded with its iron–sulfur cluster. The [2Fe–2S] Grx2 is enzymatically inactive, because cluster coordination involves the N-terminal active site residue, which is essential for activity (2, 11). The GSH coordinating the cluster is in constant exchange with the pool of free GSH (see Fig. 2c), which is why the cluster is stabilized by reduced GSH and destroyed by alterations in the redox environment (14). Cluster disassembly leads to enzymatic activation, because the active site becomes available for catalysis. Since Grx2 can also use electrons donated by thioredoxin reductase, for instance, to reduce GSSG, the enzyme can retain its activity under oxidizing conditions when the glutathione pool becomes oxidized (10). Once redox homeostasis is re-established (*i.e.*, sufficient amounts of reduced GSH become available), the cluster can be re-assembled and Grx2 is turned off.

The features that enable apo-Grx2 to coordinate its iron–sulfur cluster in cooperation with GSH lead to a profound decrease in enzymatic activity compared to other Grxs, because the complex between GSH and reduced Grx2 represents an inhibitory dead-end complex for both monothiol and dithiol reactions. We have analyzed two mutants affecting this binding, Ser38Pro and Thr95Arg, and both show a significantly increased enzymatic activity and efficiency (Table 1). On the other hand, it was the single Pro to Ser exchange in the active site that enabled Grx1 to complex the [2Fe–2S] cluster (Fig. 4). The presence of the Pro in the active site must be unfavorable for the conformation necessary to bind the [2Fe–2S] cluster and GSH, especially for the geometry of the thiol group of Cys37, which must point outwards to coordinate iron. However, Grx2 is clearly more adopted to accommodate an iron–sulfur cluster, because the Ser to Pro mutant was still able to bind the cluster *in vitro*.

It is tempting to speculate that more iron–sulfur Grxs will be identified in the future, because variations in the active site sequence Cys–Pro–Tyr–Cys are frequently found, especially in plant Grxs (22). The now emerging group of the so-called monothiol Grxs, that contain the active site sequence Cys–Gly–Phe–Ser, is involved in iron homeostasis in *E. coli* and yeast (4, 21). Recent studies indicate that this function might be conserved in higher eukaryotes as well (20, 25). However, *E. coli* Grx4 from this group could not complex an iron–sulfur cluster in our hands (not shown).

Grx2 may qualify as therapeutic target in diseases with impaired redox homeostasis. Now that we start to understand the molecular basis of the redox-induced activation of Grx2, we have to answer the questions why Grx2 sacrifices a high enzymatic activity for the ability to bind an iron–sulfur cluster and what the advantage of this dual functionality is for the cell.
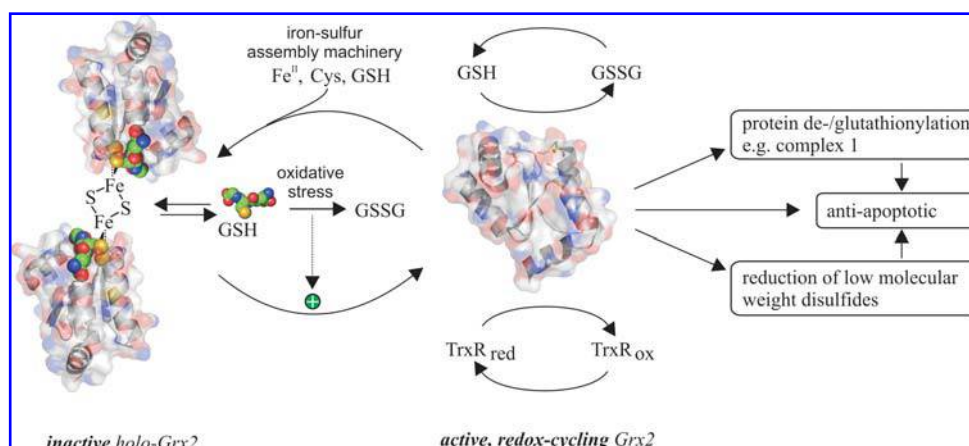
FIG. 5. Model for the regulation of Grx2 *in vivo*.   For details, see text.

ABBREVIATIONS

DTT: dithiotreitol, Grx: glutaredoxin, HED: hydroxyethyl disulfide.

APPENDIX

Materials and general methods

Chemicals were purchased from Sigma (Stockholm, Sweden), unless otherwise stated, and were of analytical grade or better. Materials for molecular biology were purchased from Fermentas (St. Leon–Rot, Germany). Plasmid DNA was purified from bacteria using the Mini- and Midiprep Kit from Qiagen (Hilden, Germany). SDS-PAGE was run using the Novex Mini-Cell and precasted NuPAGE gels (12% acrylamide, Bis-Tris, Invitrogen, Stockholm, Sweden), according to the manufacturers' instructions.

Plasmids, cloning and site directed mutagenesis

The plasmids for the expression of human Grx1, its Ser23Pro mutant, human Grx2, and its Cys28Ser, Ser38Pro, Cys40Ser, and Cys113Ser mutants were described previously (14, 17). *Escherichia coli isc*S and *isc*A were amplified from genomic DNA by PCR using the primer pairs 5′-CACACACCATATGTACG-GAGTTTATAGAGCAATGAAATTACC/5′-CACACACTCGAGATGATGAGCCCATTCGATGCTGTTC and 5′-CACACACATATGTCGATTACACTGAGCGACAGTG/5′-CACACAGGATCCTCAAACGTGGAAGCTTTCGCCG, respectively). Subsequently, *iscS* was cloned into the NcoI and XhoI sites of vector pET28a (Novagen, La Jolla, CA), *iscA* was cloned into the NdeI and BamHI sites of vector pET15b (Novagen). Site directed mutagenesis was performed using the Quick Change Site Directed Mutagenesis protocol (Stratagene, Darmstadt, Germany). Complementary oligonucleotide pairs harboring the desired nucleotide exchanges were used (Cys37Ser: 5′-CTCAAAAACATCCTcTTCTTACTGTACAATGGC, Thr95Arg: 5′-GGAGGTGCAcgTGACACTC) together with the pET15b expression plasmid harboring wild-type Grx2 cDNA (17) as template. All expression plasmids were verified by DNA sequencing.

Expression and purification of proteins

Wild-type and mutant human Grx proteins were expressed and purified as described previously (14). His tagged *E. coli* IscS and IscA were purified using Talon resin as suggested by the manufacturer (Novagen). Residual imidazole was removed by gel filtration using Sephadex G25 resin (Amersham) equilibrated with 50 mM sodium phosphate, 200 mM NaCl, pH 8.0.

Reconstitution assay

Prior to reconstitution, remaining [Fe–S] clusters were removed by treatment with 2 mM dithionite for at least 15 min. Remaining dithionite and free iron were removed using pre-packed Sephadex 25 gel filtration columns (PD10, Amersham, Upsalla, Sweden). Cluster reassembly was performed similar to the protocols described in references (1, 26). 50–200 μM Grx were incubated under argon atmosphere at room temperature in 50 mM sodium phosphate buffer (pH 8.0) containing 200 mM NaCl, 0.01 molar equivalents of *E. coli* IscS, 2 equivalents of Fe(NH$_4$)$_2$(SO$_4$)$_2$, 2.5 equivalents of cysteine, 0–1 mM GSH, 5 mM dithiothreitol (DTT), 10 μM pyridoxal phosphate, and, when indicated, 0.01 molar equivalents of *E. coli* IscA. After 2 h, the mixture was desalted using pre-packed Sephadex G25 gel filtration columns (NAP-5 or PD10, Amersham). UV-VIS spectra and the kinetics of the reconstitution and cluster degeneration were recorded using a Shimadzu UV-2100 spectrophotometer (Duisberg, Germany).

For the labeling of the holo-Grx2 complex, radiolabeled reduced glutathione (³H-glycine) was purchased from Perkin Elmer (Wellesley, MA). The reconstitution assay was set up as described above using 200 μM Grx2 in a volume of 1 ml including 600 μM GSH adjusted to a specific activity of 650 cpm nmol^{-1}. After 2 h, the reaction mixture was centrifuged at 20,000 g and separated by gel filtration chromatography (PD10 columns, Amersham). Fractions of 250 μl were collected and their optical absorption spectra as well as their radioactivity were recorded.

To analyze the exchange of GSH in the holo-Grx2 complex for GSH from a pool of free GSH, 200 μM reconstituted Grx2 were purified from free iron, cysteine, and GSH using Nanosep centrifuge devices (3K, Pall, Lund, Sweden). Afterwards the protein was incubated for 4 h with 200 μM radiolabeled GSH and the mixture was separated by gel filtration chromatography, as described above.

The formation of dimeric holo-Grx2 was confirmed by gel filtration HPLC (Amersham SMART system) using Superdex 75 beads (Amersham) equilibrated with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl.

Kinetic analysis

Grx2 activity was measured using a modified protocol of the hydroxyethylen disulfide (HED)-assay (19) in a total volume of 200 μl. Briefly, 50 μl HED (0.7 mM final concentration) were added to 100 μl of a freshly prepared substrate mix containing 100 mM Tris/HCl (pH 8.0), 1 mM EDTA, 0.1 mg ml^{-1} BSA, 1 mM GSH, 250 μM NADPH, and 6 μg ml^{-1} yeast glutathione reductase (final concentrations). After 5 min of pre-incubation at 30°C, different amounts of Grx in a volume of 50 μl (0, 10, 25, 50, 75, 100, 150, and 200 nM final concentration, respectively) were added. The decrease in absorbance at 340 nm was measured at 30°C in a 96-well plate using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). NADPH consumption was quantified based on 12 standards of NADPH from 0–250 μM concentration. K_m for GSH and V_{max} of wild type and Thr95Arg Grx2 were determined using the same assay containing 0.7 mM HED (about sevenfold K_m, (10)), varying concentrations of GSH (1–4 mM) and 20 or 40 nM of enzyme. Kinetic constants were determined by nonlinear regression from the average of three independent data sets, assuming Michaelis-Menten kinetics.

Calculations

The molecular interactions between GSH and reduced Grx2 in the published crystal structure (PDB code 2FLS) and the possible consequences of mutations were analyzed using Whatif (24). Nonlinear curve fittings for the determination of kinetic constants and derivatives of spectra were calculated using Grace (http://plasma-gate.weizmann.ac.il/Grace/) [October 13, 2006].

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