Oxidation and S-Nitrosylation of Cysteines in Human Cytosolic and Mitochondrial Glutaredoxins

EFFECTS ON STRUCTURE AND ACTIVITY*

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Glutathione (GSH) is the major intracellular thiol present in 1–10 mM concentrations in human cells. However, the redox potential of the 2GSH/GSSG (glutathione disulfide) couple in cells varies in association with proliferation, differentiation, or apoptosis from −260 mV to −200 or −170 mV. Hydrogen peroxide is transiently produced as second messenger in receptor-mediated growth factor signaling. To understand oxidation mechanisms by GSSG or nitric oxide-related nitrosylation we studied effects on glutaredoxins (Grx), which catalyze GSH-dependent thiol-disulfide redox reactions, particularly reversible glutathionylation of protein sulfhydryl groups. Human Grx1 and Grx2 contain Cys-Pro-Tyr-Cys and Cys-Ser-Tyr-Cys active sites and have three and two additional structural Cys residues, respectively. We analyzed the redox state and disulfide pairing of Cys residues upon GSSG oxidation and S-nitrosylation. Cytosolic/nuclear Grx1 was partly inactivated by both S-nitrosylation and oxidation. Inhibition by nitrosylation was reversible under anaerobic conditions; aerobically it was stronger and irreversible, indicating inactivation by nitration. Oxidation of Grx1 induced a complex pattern of disulfide-bonded dimers and oligomers formed between Cys-8 and either Cys-79 or Cys-83. In addition, an intramolecular disulfide between Cys-79 and Cys-83 was identified, predicted to have a profound effect on the three-dimensional structure. In contrast, mitochondrial Grx2 retains activity upon oxidation, did not form disulfide-bonded dimers or oligomers, and could not be S-nitrosylated. The dimeric iron sulfur cluster-coordinating inactive form of Grx2 dissociated upon nitrosylation, leading to activation of the protein. The striking differences between Grx1 and Grx2 reflect their diverse regulatory functions in vivo and also adaptation to different subcellular localization.

Glutathione (GSH)7 is the major intracellular thiol in human cells with up to 10 mM concentration (1, 2). It has numerous functions in providing cells with electrons for reduction, is important in detoxification, and serves as a major cellular antioxidant. The redox state of the 2GSH/GSSG (glutathione disulfide) couple is determined by both the concentration of GSH as a squared function and the level of GSSG. In proliferating cells the GSH pool is highly reduced, whereas it becomes more oxidized during growth arrest either because of differentiation or contact inhibition. During severe oxidative stress the ratio of GSSG to GSH increases strongly; upon induction of apoptosis GSH is exported from cells and the GSSG concentration increases (3). Two other important messengers in redox signaling are H2O2, for instance as signal-transducing messenger following growth factor stimulation (4, 5), and nitric oxide (NO), which can signal through S-nitrosylation of protein sulfhydryl groups (6).

Glutaredoxins (Grxs) are GSH-dependent oxidoreductases that belong to the thioredoxin (Trx) family of proteins (7). Grxs catalyze thiol-disulfide redox reactions utilizing two distinct mechanisms. The reduction of protein disulfides and low molecular weight disulfides occurs in the dithiol mechanism, which requires both active site cysteines, whereas the reduction of glutathione-mixed disulfides is catalyzed in the monothiol mechanism, which requires only the more N-terminal-located active site cysteine residue (8, 9). Grxs are involved in many different cellular processes, e.g. DNA synthesis (10), sulfur assimilation (11, 12), defense against oxidative stress (13–15), apoptosis (16), cellular differentiation (17), regulation of transcription factor binding activity (18), and redox regulation via reactive oxygen species.

Mammalian cells contain two dithiol Grxs that differ in size, subcellular localization, and catalytic properties. The cytosolic Grx (Grx1) has been extensively studied (19–28), and three-dimensional structures have been determined for the reduced, the oxidized, and the GSH mixed disulfide intermediates (29–31). The more recently discovered second mitochondrial Grx (Grx2) contains the active site sequence Cys-Ser-Tyr-Cys

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7 The abbreviations used are: GSH, glutathione (reduced); GSSG, glutathione disulfide; Grx, glutaredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase; GSNQ, S-nitrosogluthathione; DT, dithiothreitol; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; PBS, phosphate-buffered saline; HED, hydroxyethyl disulfide; NESI-MS, nano-electrospray ionization mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time of flight.
Instead of the Grx consensus sequence Cys-Pro-Tyr-Cys (32, 33), Grx2 has a high affinity for glutathionylated proteins and the unique ability to use reducing equivalents from both GSH and thioredoxin reductase (TrxR) (34). Grx2 promotes cell survival upon treatment with reactive oxygen species-generating apoptotic stimuli. Silencing of Grx2 expression by RNA interference dramatically sensitized HeLa cells to cell death induced by oxidative stress (35), and overexpression attenuated oxidative stress-induced apoptosis by preventing cardiolipin oxidation, cytochrome c release, and caspase activation (36). Grx2 was recently described as the first iron-sulfur protein from the thioredoxin family of proteins (37). Spectroscopic analysis revealed a $[2\text{Fe}-2\text{S}]$ cluster bridging two molecules of Grx2. This 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 non-covalently to the proteins and in equilibrium with glutathione in solution (38, 39). Because holo-Grx2 is enzymatically inactive, it was proposed to serve as a redox sensor for the activation of the protein during oxidative stress (37, 38).

When compared with their bacterial counterparts, the two mammalian Grxs contain additional cysteine residues outside their active site, which are not conserved between Grx1 and Grx2 and were suggested to be involved in regulation of activity (19–21, 25, 40, 41). Grx1s from bovine, rabbit, and pig contain two cysteines (Cys-79 and Cys-83, human Grx1 numbering) C-terminal of the active site, whereas human Grx1 also contains a third cysteine (Cys-8) N-terminal of the active site (Fig. 1). Grx2s from human, mouse, and rat contain two additional cysteines, one located N-terminal (Cys-28, human Grx2 numbering starting at exon II) and the other one C-terminal (Cys-113) to the active site thiol/disulfide pair (42) (Fig. 1).

Grx1 and Grx2 have been implicated in major cellular processes that require strict redox regulation. Oxidation with $\text{H}_2\text{O}_2$ or GSSG has been shown to partially inactivate Grx1 (28, 43), whereas the activity of Grx2 was not affected by treatment with GSSG (32). In the present study, we have analyzed the thiol redox state and the molecular mechanism of oxidation of both Grx1 and Grx2 following treatment with GSSG and S-nitroso-glutathione (GSNO) to understand how glutaredoxins behave upon oxidative conditions. Our results revealed remarkable differences between the two human Grxs that reflect different functions in redox signaling and protection against oxidation.

**Experimental Procedures**

**Materials**—NAP-5 and HiTrap chelating columns were purchased from GE Healthcare. Bovine serum albumin, NADPH, GSH, GSSG, yeast glutathione reductase, reduced and oxidized DTT, SIN-1, and 5-IAF were from Sigma, HED was from Aldrich. GSNO was prepared as previously reported by the reaction of acidified sodium nitrite and glutathione (44).

**Protein Expression and Purification**—Human Grx1 and Grx2 (exon II–IV) were expressed as His-tagged proteins in the BL21-Codon Plus (DE3)-pRIL Escherichia coli strain (Stratagene) and purified on HiTrap chelating columns (GE Healthcare) as previously described (32). The protein concentrations of Grx1 and Grx2 were determined spectrophotometrically using molar extinction coefficients of 3160 and 6880 M$^{-1}$ cm$^{-1}$ at 280 nm, respectively (45).

Recombinant rat TrxR was a kind gift from Olle Rengby and Elias Arner from the Department of Medical Biochemistry and Biophysics, Karolinska Institutet, and expressed and purified as described previously (46). The concentration of TrxR was determined from the reduction of 5 mM DTNB, using the relationship that 1200 A$_{412}$ units ml$^{-1}$ correspond to 1 mg ml$^{-1}$ protein (19). Recombinant human Trx was expressed and purified according to Ren et al. (47).

**Reduction, Oxidation, and S-Nitrosylation of Human Glutaredoxins**—The proteins were reduced by incubating Grx (50–125 $\mu$m) with either 10 mM DTT or 10 mM GSH for 30 min at 37 °C followed by desalting on NAP-5 columns (Sephadex G25) equilibrated with 50 mM Tris-HCl, 1 mM EDTA, pH 7.5. Oxidized Grx proteins were prepared by incubating the reduced proteins with either 10 mM GSSG or 10 mM oxidized DTT for 30 min at 37 °C, followed by desalting on NAP-5 columns.

To study S-nitrosylation, freshly prepared reduced Grx in phosphate-buffered saline (PBS), pH 7.4, 1 mM EDTA were incubated with 10 equivalents of GSNO for 1 h at 37 °C either aerobically or in an anaerobic work station (MK3; Don Whitley Scientific, Shipley, UK), and Grx was isolated by chromatography on a NAP-5 column equilibrated with the buffer described above. S-nitrosylation of Grx was confirmed by analyzing the UV-visible spectra of the proteins, because nitrosylated samples exhibit an additional absorbance peak at 335 nm. For quantification, an extinction coefficient of 920 M$^{-1}$ cm$^{-1}$ (335 nm) for nitrosothiols was used (44). For denitrosylation of proteins, nitrosylated samples were incubated with 100 molar equivalents of DTT for 60 min at 37 °C followed by desalting on NAP-5 columns.

**Enzymatic Assays**—Enzymatic activity of oxidized and reduced glutaredoxins was determined using hydroxethyl disulfide (HED) as substrate (19). Briefly, 0.7 mM HED was added to a mixture containing 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1 mg ml$^{-1}$ bovine serum albumin, 0.2 mM NADPH, 1 mM GSH, and 6 $\mu$g ml$^{-1}$ glutathione reductase. After 3 min of

**FIGURE 1. Primary structure alignment of human glutaredoxins.** Cysteine residues are highlighted. The secondary structures, extracted from the NMR structure for Grx1 (Protein Data Bank code 1jhb) (31) and the crystal structure for Grx2 (2fls) (3) are shown above and below the sequences, respectively. In addition, the fragments resulting from trypsin digestion of the proteins (tr) are indicated.
preincubation, Grx1 or Grx2 were added to the sample cuvettes and the same volume of buffer to the reference cuvette. The decrease in absorbance at 340 nm was followed using a Shimadzu UV-2100 spectrophotometer. Activity was expressed as μmol NADPH oxidized/min using a molar extinction coefficient of 6200 M⁻¹ cm⁻¹.

To study the reaction between GSSG-treated Grx1 and the thioredoxin system, 20 μM GSSG-treated Grx1 in argon-purged PBS, pH 7.4, 1 mM EDTA, and 200 μM NADPH in a final volume of 500 μl were incubated with 40 nM TrxR. The oxidation of NADPH was followed at 340 nm subtracting the identical reference lacking Grx1.

**Analysis of Protein Thiol Content**—The number of free thiols in Grx proteins was determined spectrophotometrically with DTNB as described by Ellman (48). In brief, the reduced or oxidized proteins were added to a mixture containing 6 mM guanidine hydrochloride in 0.1 M Tris-HCl, pH 7.5, and 1 mM DTNB at room temperature, and the absorbance at 412 nm was determined. Thiols were calculated using a molar extinction coefficient of 13600 M⁻¹ cm⁻¹ (48).

Alkylation using fluorescent 5-(iodoacetamido)-fluorescein (Sigma–Aldrich) was performed as described by Lillig et al. (49). Briefly, oxidized Grx1 and Grx2 (20 μM) in 100 mM KPO₄, pH 8.0, were incubated with the reductants 5 mM GSH, 5 mM DTT, 275 nm TrxR, and 3.2 mM NADPH or 275 nm Trx, 275 nm TrxR, and 3.2 mM NADPH for 1 h at 37 °C in an argon atmosphere. The samples were then alkylated in the dark with 6.5 mM 5-(iodoacetamido)-fluorescein (dissolved in N,N-dimethyl formamide) for 30 min at room temperature before separation by non-reducing SDS-PAGE (8–16%, Ready Gels; Bio-Rad). Fluorescence was analyzed on a UV table followed by protein staining with Coomassie Brilliant Blue.

**Determination of the Number of Disulfides in the Active Site of Nitrosylated Grx1**—The method is based on the use of NADPH, GSH, and glutathione reductase to specifically reduce the disulfide in the active site of Grx-S₂ (Reactions 1 and 2).

\[
\text{Grx-S}_2 + 2 \text{GSH} \rightarrow \text{Grx-(SH)}_2 + \text{GSSG} \\
\text{REACTION 1}
\]

\[
\text{NADPH} + H^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2 \text{GSH} \\
\text{REACTION 2}
\]

The final volume of 500 μl of deoxygenated and argon-purged PBS, pH 7.4, 1 mM EDTA contained 0.1 mg ml⁻¹ bovine serum albumin, 0.2 mM NADPH, 1 mM GSH, and 6 μg ml⁻¹ GR. The reaction was started by adding Grx1 to the reaction cuvette and buffer to the reference cuvette. NADPH oxidation was followed at 340 nm, and an extinction coefficient of 6200 M⁻¹ cm⁻¹ was used for calculating the number of disulfide bonds in the active site.

**Mass Spectrometry**—Human Grx1 and Grx2 were reduced with DTT or oxidized with GSSG as described above. For analysis by nano-electrospray ionization mass spectrometry (nESI-MS), the proteins were alkylated 30 min at room temperature with 25-fold excess of iodoacetamide before digestion for 1 h at 37 °C with trypsin (a ratio of 1:10 trypsin:Grx) in a buffer containing 50% acetonitrile and 50 mM ammonium bicarbonate (50). In a separate experiment the oxidized and reduced proteins were denatured for 20 min at 95 °C in 8 M urea, followed by digestion with trypsin according to the manufacturer’s recommendations (Promega). For mass determination of thiol-alkylated full-length Grx2, 70 μM untagged protein purified as described in Ref. (32) were reduced with 2 mM DTT or oxidized with 2 mM GSSG, alkylated with 25 mM iodoacetamide, and diluted 10-fold with 30% formic acid. The protein was desalted by two rounds of 10-fold concentrating and 10-fold diluting the sample in 30% formic acid using Nanosept Spin Columns ( Pall).

For nESI-MS, aliquots of protein or tryptic digests were desalted using reverse-phase ZipTip columns (C18; Millipore), eluted in 60% acetonitrile containing 1% acetic acid and analyzed by nano-electrospray mass spectrometry (Q-TOF; Micromass). For identification of fragments and assignment of disulfide bonds, the resulting mass spectra were processed using the MaxEnt3 software (Micromass) and the data (singly charged ions) compared with theoretical tryptic peptide mass maps. For MALDI-TOF mass spectrometry, the desalted samples were eluted in 75% acetonitrile containing 1% trifluoroacetic acid, mixed 1:1 (v/v) with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid, and analyzed using the Voyager DE-PRO system (Applied Biosystems).

**RESULTS**

**Effect of Thiol Oxidation on Grx1 and Grx2**—Following overexpression and purification, freshly prepared Grx1 and [Fe,S]-free Grx2 did not contain any free thiols as determined using DTNB in 6 M guanidine HCl. To analyze the effect of oxidation and reduction of the Cys residues on the enzymatic activity, we reduced the proteins with DTT (100 molar equivalents) followed by treatment with GSSG (100 equivalents) and finally re-reduction with DTT. After each step, aliquots of the desalted Grx1 and Grx2 were examined for catalytic activity using the GSH-dependent reduction of the GSH β-mercaptoethanol mixed disulfide (HED assay).

For Grx1, we found a decrease in activity after oxidation with GSSG of ~25% (Fig. 2) and upon treatment with H₂O₂ of ~80% (not shown), which is in agreement with earlier observations (32). Full activity was regained by re-reduction with DTT (>100-fold molar excess for 60 min), suggesting that the partial inactivation was caused by disulfide formation. In contrast, the activity of Grx2 was not affected by treatment with oxidants or reductants (Fig. 2).

Why were Grx1 and Grx2 differently affected by oxidation of their cysteine residues? To answer this question, we investigated the redox state of the cysteiny1 groups in both proteins. First, we analyzed reduced and oxidized proteins using thiol-specific alkylation with the fluorescent probe 5-(iodoacetamide)-fluorescein followed by non-reducing SDS-PAGE (Fig. 3). In addition, we have determined the number of free thiols spectrophotometrically using DTNB in 6 M guanidine HCl (Fig. 4). Oxidation of Grx1 by GSSG led to a complex mixture of monomers, dimers, and oligomers while only a small fraction of the protein kept free thiols (Fig. 3A, lane 1; 0.4 thiols/monomer.

**Oxidation and S-Nitrosylation of Human Glutaredoxins**
when probed with DTNB, Fig. 4). The physiological reductant GSH partially reduced Grx1; the number of free thiols increased to 2.5/monomer. The amount of dimeric Grx1 was reduced and no oligomers could be detected (Fig. 3A, lane 2).

Incubation of oxidized Grx1 with TrxR, both in the absence and presence of Trx, decreased the amount of oligomeric protein, indicating the reduction of some intermolecular disulfides by TrxR (Fig. 3A, lanes 4 and 5). Reduction of the protein with DTT resulted in complete reduction (4.9 thiols/monomer) and monomerization of the protein (Fig. 3A, lane 3). A kinetic analysis revealed that the incomplete reduction of GSSG-treated Grx1 by TrxR occurs with an initial rate of 4.2 s\(^{-1}\) and consumes a total of 1.6 mol NADPH/mol Grx1 (Fig. 3C).

GSSG-treated Grx2 monomers did not form intermolecular disulfides upon oxidation, as seen from the absence of dimers and oligomers (Fig. 3B, lane 1). The number of free thiols was reduced to 0.1/monomer, which could be explained by formation of an intramolecular disulfide (42). Although the active site in Grx2 was shown to be a substrate for both GSH and TrxR (34), neither of them nor Trx was able to fully reduce the protein (Fig. 3B, lanes 2, 4, and 5). The partial reduction deduced from the increased fluorescence labeling most likely reflected the reduction of the active site. Incubation of the oxidized protein with DTT gave the most complete reduction (Fig. 3B, lane 3), i.e. 3.6 free thiols/molecule (Fig. 4).

**Disulfide Pairing of Oxidized Grx1 and Grx2**—To identify intra- and intermolecular disulfides, we analyzed both the oxidized and the reduced proteins by MALDI-TOF and nESI mass spectrometry. For the analysis by nESI-MS, we pretreated the proteins with the thiol-specific probe iodoacetamide to confirm or exclude the presence of reactive thiol groups. Digestion of reduced Grx1 generated three cysteine-containing peptides: tf-1 (see Table 1 and Fig. 1) including Cys-8 was identified in three of three experiments, tf-3 including the active site cysteines was identified by nESI-MS only, tf-9 including Cys-79 and Cys-83 by MALDI-TOF. The masses of the fragments and
their reactivity with iodoacetamide confirmed that all five Cys residues were present in the reduced state (Table 1). Digestion of oxidized Grx1 generated five cysteine-containing peptides, corresponding to mixed disulfides between GSH and Cys-8, Cys-79, and Cys-83, as well as disulfide formation between Cys-8 and Cys-79 or Cys-83, Cys-79 and Cys-83, and between the active site Cys residues. The lack of reactivity with iodoacetamide confirmed that all Cys-containing peptides were present in an oxidized state. The fragments containing Cys-8 and the active site were detected by both MALDI-TOF and nESI-MS and the various oxidized species of fragment tf-9 by either MALDI-TOF or nESI-MS.

Trypsin digestion of reduced Grx2 resulted in two fragments that represent the reduced active site (tf-2) and reduced Cys-113 (tf-I1) (Fig. 1, Table 1). Mass spectrometry of trypsin-digested oxidized Grx2 gave rise only to peptide tf-2, and the lack of reactivity with iodoacetamide confirmed the active site disulfide. Because we could not detect masses corresponding to peptides containing Cys-28 (Fig. 1, tf-I), or oxidized Cys-113 (tf-I1), we expressed and purified Grx2 without a His tag, alkylated the reduced and oxidized proteins with iodoacetamide, and directly analyzed the full-length protein by mass spectrometry. As depicted in Fig. 5, we recorded one mass for the oxidized and two masses for the partially reduced protein. The mass of the oxidized protein (Fig. 5, lower panel) corresponded to the mass of the protein without modification., e.g. a protein containing no free thiol groups. The two masses recorded for the reduced protein are in agreement with the addition of two and four acetal-midoieties, respectively. These results can only be explained by the formation of two intramolecular disulfides in Grx2, thus confirming previous results obtained with CNBr-digested His-tagged Grx2 (42). As one of these disulfides was formed in the active site of the protein, we can safely conclude that the second disulfide was formed between Cys-28 and Cys-113.

**Effect of Nitrosylation on Grx1 and Grx2**—Treatment of Grx1 with GSNO (10 equivalents) under aerobic conditions decreased its activity by almost 90%. The lost activity could not be regained, even by extensive treatment with reductants like DTT. In the absence of oxygen, treatment of Grx1 with GSNO decreased its activity by 30% and the enzyme regained full activity after reduction with DTT. The activity of the [Fe,S]-free, active Grx2 monomer was unaffected by pretreatment with GSSG or GSNO (Fig. 2).

The reaction of Grx1 with GSNO in the presence of oxygen decreased the number of free thiols to 0.3, but, unlike H₂O₂ or GSSG treatment, it did not lead to significant formation of dimers or oligomers (Fig. 6, inset). Furthermore, GSNO treatment of Grx1 in the presence and absence of oxygen induced a new broad absorption band ~335 nm (Fig. 6). This absorption band is characteristic for S-nitrosothiol groups, and the recorded absorbance corresponded to three (2.8 ± 0.1, Fig. 7) S-nitrosothiols. Because the active site Cys residues formed a disulfide (Fig. 7), we concluded that all three structural disul-
fides can react with GSNO to form S-nitrosothiol groups. The differential effect of GSNO in the presence or absence of oxygen prompted us to investigate whether Grx1 could be affected by nitration, e.g. through peroxynitrite generated from GSNO in the presence of oxygen. Therefore, we treated Grx1 with SIN-1. This compound releases superoxide and nitric oxide simultaneously in the presence of oxygen, leading to the generation of peroxynitrite. Treatment of both reduced and oxidized human Grx1 led to a dramatic decline in the activity of ~90% that in both cases was not reversible (Fig. 2).

Treatment of reduced Grx2 with GSNO reduced the number of free thiols to 0.3, it did not induce S-nitrosylation of the protein, and had, as SIN-1 treatment, no effect on activity of the protein. On the other hand, when the enzymatically inactive, dimeric [2Fe2S] complex was treated with GSNO or an alter-
Oxidation and S-Nitrosylation of Human Glutaredoxins

inactivated by oxidation of protein thiols to disulfides, which is also consistent with previous observations (28, 43, 57). The lost activity of oxidized Grx1 was regained after treatment with an excess of DTT, but none of the physiological relevant disulfide reductants/reductases (TrxR, Trx, GSH, or Grx) were able to recover full Grx1 activity. Analysis of oxidized Grx1 by SDS-PAGE revealed a complex pattern of homodimers and oligomers formed by intermolecular disulfide bonds between Cys-8 and either Cys-79 or Cys-83, see Fig. 9. In addition, an intramolecular disulfide between Cys-79 and Cys-83 was formed; Cys-8 as well as Cys-79 and Cys-83 formed mixed disulfides with GSH. This glutathionylation could prevent formation of the inter- and intramolecular disulfides, but it might also serve as intermediate for their formation. The complexity of this mixture of redox forms in Grx1 complicates the separation of single redox forms considerably, and we can therefore only speculate which modification contributed to the inhibition of activity. Oligomerization as well as glutathionylation and nitrosylation could mask the active site and interfere with substrate binding. Structural changes could also inactivate the protein. Cys-8 is located in the N terminus and is solvent-exposed in the structure of reduced Grx1 (31). It contributes significantly to the tendency of Grx1 to precipitate in solution, and the corresponding Cys to Ser mutant improved this behavior (26). No structure of wild-type oxidized human Grx1 is available, but a crystal structure of the pig homologue with oxidized active site has been solved (29). This protein does not contain a Cys residue corresponding to Cys-8 in human Grx1, and both Cys residues corresponding to Cys-79 and Cys-83 are present in the thiol form. Cys-83 is located close to the active site in an area important for substrate binding and has previously been suggested to be a potential site for regulation (31); Cys-79 is located at the end of β-sheet 4. Both are solvent-exposed but separated by a distance of ~12 Å (ca-ca) in the structures of mammalian Grx1s (29, 31). Formation of a disulfide bond between these two cysteines would certainly have a profound effect on the structure of the protein. Although their redox state clearly influences the activity of Grx1, none of the additional cysteines is essential for activity, as demonstrated by a mutant lacking Cys-8, Cys-79, Cys-83, and the C-terminal active site residue Cys-26 (30).

Grx2 is a mitochondrial protein that is strongly implicated in redox control and thiol homeostasis upon oxidative stress. Short interfering RNA-mediated silencing caused hypersensitivity of HeLa cells toward the oxidative stress/apoptosis-inducing agents doxorubicin and phenylarsine oxide (35), while overexpression attenuated apoptosis induced by doxorubicin and 2-deoxy-D-glucose (36). The iron-sulfur cluster in holo-Grx2, which is coordinated by the two N-terminal active site residues of two Grx2 monomers and two GSH molecules bound non-covalently (37–39), renders the protein inactive through sequestration of the active site thiol groups. The GSH in the complex is in dynamic equilibrium with free GSH. As a consequence, the complex is destroyed by oxidation, thus leading to activation of Grx2 under oxidative conditions (37, 38). As demonstrated here, reactive nitrogen species effectively induce dissociation of the holo-complex as well. Because apo-Grx2 is not inactivated by nitrosylation or nitration, reactive nitrogen species represent a second mechanism of Grx2 activation.

The two additional Cys residues in Grx2 form a structural disulfide that was, during the course of our investigations, confirmed by x-ray crystallography (39) as well as by protein chemistry (42). This disulfide appears to be important for the structure of Grx2 under oxidative conditions ensuring a high pool of

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**FIGURE 8.** Dissociation of the holo-Grx2 complex induced by nitric oxide. Enzymatically inactive holo-Grx2 (solid line), reconstituted as described in reference (32), was treated with 10 molar equivalents of GSNO, resulting in enzymatically active apo-Grx2 (dashed line). Inset, kinetics of cluster disintegration followed at 428 nm.

**FIGURE 9.** Model for the disulfide pairing and nitrosylation of human Grx1 and Grx2 cysteine residues. The structural representations were generated using the Protein Data Bank entries 1jhb (Grx1) (31) and 2fts (Grx2) (39); active site Cys residues are highlighted. Grx1 residues Cys-23 and Cys-26 are always found as a disulfide in the active site, whereas the additional three Cys residues can undergo various oxidation events as indicated. Grx2 contains a disulfide in the active site or is present as a [FeS] cluster bridged dimer. In addition, a stable disulfide is generated between Cys-28 and Cys-113.
active protein. Along with the unique ability of Grx2 to use reducing equivalents from both GSH and TrxR (34), our results imply an important role for Grx2 in an environment with variable redox conditions and may thus reflect an adaption to its mitochondrial localization.

Grxs and Trx belong to the same family of proteins, and it is intriguing to note some close points of similarity between the human Grx and Trx systems. Similar to Grx1, mammalian cytosolic Trx1 contain two to three additional cysteines that upon oxidation form disulfide-bonded dimers, oligomers, and mixed disulfides with glutathione, which in turn inhibit the activity of protein (47, 58, 59). Trx1 was shown to undergo reversible S-nitrosylation, but controversially one study reports that nitrosylation is necessary for the anti-apoptotic function of Trx in endothelial cells (60), whereas another study suggests that S-nitrosylation induces dissociation of Trx1 from apoptosis signal-regulating kinase 1, thus inducing apoptosis (61). Similar to Grx1, Trx1 can form an intramolecular disulfide between two cysteinyl residues (Cys-62 and Cys-69) upon oxidation (62). Similar to the disulfide formed between Cys-79 and Cys-83 in Grx1, this additional disulfide must have a profound effect on the structure of the protein. It should be noted that both Trx1 and Grx1 are found extracellularly in plasma and are secreted proteins (63–66). The possibility that the disulfide is required for secretion should be investigated. Mitochondrial Trx2, which does not contain additional cysteinyl residues, is not affected by treatment with oxidants (67). As for Grx2, this was discussed to be a prerequisite for its mitochondrial localization.

In conclusion, we found large differences between human cytosolic Grx1 and mitochondrial Grx2 upon oxidation (Fig. 9). The three additional cysteinyl residues of Grx1 provide mechanisms for inactivation during oxidative and nitrosative stress, perhaps for secretion and avoiding planting destructive disulfides in other proteins. Iron sulfur cluster coordination involving the N-terminal active site cysteine offers a mechanism for inactivation during oxidative and nitrosative stress, perhaps for secretion and avoiding planting destructive disulfides in other proteins. Iron sulfur cluster coordination involving the N-terminal active site cysteine offers a mechanism for inactivation during oxidative and nitrosative stress, perhaps for secretion and avoiding planting destructive disulfides in other proteins. Iron sulfur cluster coordination involving the N-terminal active site cysteine offers a mechanism for inactivation during oxidative and nitrosative stress, perhaps for secretion and avoiding planting destructive disulfides in other proteins.
Oxidation and S-Nitrosylation of Human Glutaredoxins

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