Human Mitochondrial Glutaredoxin Reduces S-Glutathionylated Proteins with High Affinity Accepting Electrons from Either Glutathione or Thioredoxin Reductase*

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Glutaredoxins catalyze glutathione-dependent thiol disulfide oxidoreductions via a GSH-binding site and active cysteines. Recently a second human glutaredoxin (Grx2), which is targeted to either mitochondria or the nucleus, was cloned. Grx2 contains the active site sequence CSYC, which is different from the conserved CPYC motif present in the cytosolic Grx1. Here we have compared the activity of Grx2 and Grx1 using glutathionylated substrates and active site mutants. The kinetic studies showed that Grx2 catalyzes the reduction of glutathionylated substrates with a lower rate but higher affinity compared with Grx1, resulting in almost identical catalytic efficiencies (kcat/Km). Permutation of the active site motifs of Grx1 and Grx2 revealed that the CSYC sequence of Grx2 is a prerequisite for its high affinity toward glutathionylated proteins, which comes at the price of lower kcat. Furthermore Grx2 was a substrate for NADPH and thioredoxin reductase, which efficiently reduced both the active site disulfide and the GSH-glutaredoxin intermediate formed in the reduction of glutathionylated substrates. Using this novel electron donor pathway, Grx2 reduced low molecular weight disulfides such as CoA but with particular high efficiency glutathionylated substrates including GSSG. These results suggest an important role for Grx2 in protection and recovery from oxidative stress.

Thiol groups are central to most redox-sensitive processes in the cell, and their redox state controls cellular processes like growth, differentiation, and apoptosis. The intracellular thiol homeostasis is maintained by the thioredoxin and glutaredoxin systems, which both utilize reducing equivalents from NADPH to reduce protein and low molecular weight disulfides (1, 2). The thioredoxin system is composed of thioredoxin reductase (TrxR), in mammalian cells a selenocysteine-containing, dimeric nicotinamide nucleotide disulfide oxidoreductase (3), which reduces thioredoxin (Trx) (Reaction 1), the major protein disulfide reductase with a large number of functions in mammalian cells (4).

\[
\begin{align*}
\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ & \rightarrow \text{Trx}-(\text{SH})_2 + \text{NADP}^+ \\
\text{REACTION 1}
\end{align*}
\]

The glutaredoxin system consists of glutathione reductase, a dimeric enzyme with similarities to TrxR, which catalyzes the reduction of glutathione disulfide (GSSG) to glutathione (GSH), which in turn reduces glutaredoxin (Grx) (Reactions 2 and 3) (5). Reduced glutaredoxin acts as a disulfide reductase but is active in particular in the reduction of S-glutathionylated substrates (6, 7).

\[
\begin{align*}
\text{GSSG} + \text{NADPH} + \text{H}^+ & \rightarrow 2 \text{GSH} + \text{NADP}^+ \\
\text{REACTION 2}
\end{align*}
\]

\[
\begin{align*}
\text{Grx-S}_2 + 2 \text{GSH} & \rightarrow \text{Grx}-(\text{SH})_2 + \text{GSSG} \\
\text{REACTION 3}
\end{align*}
\]

Glutaredoxins and glutathione reductase share a similar three-dimensional structure, known as the thioredoxin fold (8), and both use two redox-active cysteines in a conserved CXXC active site motif to reduce protein disulfides and low molecular weight disulfides. These reactions are initiated by a nucleophilic attack of the N-terminal CXXC thiolate on the disulfide substrate, resulting in a mixed disulfide intermediate, which subsequently is reduced by the C-terminal CXXC cysteine, releasing the reduced substrate (4). Grx-catalyzed reduction of S-glutathionylated substrates, also called glutathione-mixed disulfides, proceeds by a monothiol pathway, requiring only the N-terminal active site cysteine (Reaction 4) (9). In these reactions a glutathione-glutaredoxin intermediate is formed that is reduced by a second molecule of GSH.

\[
\begin{align*}
\text{Grx} + \text{RS-SG} + \text{GSH} & \rightarrow \text{RSH} + \text{GSSG} \\
\text{REACTION 4}
\end{align*}
\]

Complete Trx and the Grx systems in mammalian cells are present both in the cytosol and the mitochondria (10–13). Whereas the cytosolic TrxR and Trx display high similarity to their mitochondrial counterparts (14–17), there are surprisingly large differences between the cytosolic and mitochondrial glutaredoxins (12, 13). The two Grxs show only 34% sequence identity, and the proteins differ both in size and active site sequence. The 12-kDa cytosolic Grx1 has been extensively studied (18–29), and three-dimensional structures have been determined for the reduced form and the glutathione-mixed

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were used as templates with two complementary primers containing – for Grx1 and Grx2 (exons II before they were cloned into the NdeI-BamHI sites of the pET15b vector.

Expression and Purification of Recombinant Grx1, Grx2, Grx2C40S, and Grx2C40S Mutants—The Grx1P23S, Grx2C40S, and Grx2C40S mutants were prepared using the QuickChange site-directed mutagenesis kit according to the manufacturer’s recommendations using Pfu polymerase and DpnI. The cDNAs for Grx1 and Grx2 (exons II–IV), subcloned into the pGEM-T vector, were used as templates with two complementary primers containing the desired mutation (Grx1P23S, 5'-CTACTAAGGCCCACCTGCTCTTAC-TGCCAGGGAGG-3'; Grx2C40S, 5'-CATGCTGTCCTCTCATCATAAGG-GAAAAGG-3'; Grx2CR2S, 5’-CTCAAAAAATCGTGGCCATCGTACAAGGCCACCTGCTCTTAC-TGCCAGGGAGG-3'). The constructs were verified by DNA sequence analysis by KI-seq, the core facility unit at the Karolinska Institute, before they were cloned into the NdeI-BamHI sites of the pET15b vector for protein expression.

Preparation of Glutathionylated RNase (RNase-SG) and Glutathionylated BSA (BSA-SG)—RNase-SG was prepared essentially as described previously (42). BSA-SG was prepared by reduction of the protein for 30 min at 37 °C with 5 mM dithiothreitol followed by desalting on a NAP-5 column. The reduced protein was incubated overnight with a 100-fold molar excess of GSSG followed by desalting on a NAP-5 column and dialysis against 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 200 mM NaCl. The amount of bound glutathione was 6–8 molecules per molecule of RNase and 0.9–1.7 molecules per molecule of BSA, quantified by complete reduction of the glutathionylated proteins as described previously (42).

Reducing Activities Using Electrons from Glutathione—Grx activities in the HED assay were determined as described previously (18). Briefly 0.7 mM HED was added to a freshly prepared mixture of 100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1 mM MgCl2, 200 μM NADPH, 1 mM GSH, 6 μg/ml glutathione reductase. After 3 min of preincubation, glutathione was added to the sample cuvettes, and buffer was added to the reference cuvette. The decrease in absorbance at 340 nm was followed using a Shimadzu UV-2100 spectrophotometer. Activity was expressed as μmol of NADPH oxidized/min using a molar extinction coefficient of 6200 M⁻¹cm⁻¹. One unit was defined as the oxidation of one μmol of NADPH/min.

The Km value for glutathione was determined with the procedure described above using 0.5–4 mM GSH and 1.25 mM Grx1, 10 mM Grx2, or 5 mM Grx2C40S. Activity and substrate specificity toward glutathionylated substrates were determined using either 0.1–3 mM HED, 0.25–8 μM RNase-SG, or 3.1–45 μM BSA-SG in a mixture of 100 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM GSH, 0.1 mg/ml BSA, 240 μM NADPH, and 6 μg/ml yeast glutathione reductase. The reaction was started by the addition of either 0.25 or 40 mM glutathionylated substrate and an equal amount of buffer to the reference cuvette. Grx activity was determined from the decrease in absorbance at 340 nm. Three independent experiments were performed at each substrate concentration, and the apparent Km and kcat values were calculated by non-linear regression using the program GRAPPA.

Reduction of Glutathionylated Substrates for Thioredoxin Reductase—NADPH-dependent reduction of Grx2 by TrxR was performed in a mixture containing 100 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM MgCl2, 200 μM NADPH, and 10 μM Grx2. The reaction was started by the addition of either 48 mM bovine TrxR1 or 77 nM bovine TrxR2. The amount of NADPH oxidized was determined from the decreased absorbance at 340 nm. The reaction was stopped by the addition of 100 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM GSH, 1 mg/ml bovine serum albumin, and 8 μg/ml yeast thioredoxin reductase (TrxR2 was a preparation available in the laboratory, purified from calf liver mitochondria by a procedure similar to that for TrxR1.

Purification of TrxR1 and TrxR2—Bovine TrxR1 was purified from calf liver as essentially as described earlier (39, 40). The mitochondrial TrxR2 was a preparation available in the laboratory, purified from calf liver mitochondria by a procedure similar to that for TrxR1.

Preparation of Glutathionylated RNase (RNase-SG) and Glutathionylated BSA (BSA-SG)—RNase-SG was prepared essentially as described previously (42). BSA-SG was prepared by reduction of the protein for 30 min at 37 °C with 5 mM dithiothreitol followed by desalting on a NAP-5 column. The reduced protein was incubated overnight with a 100-fold molar excess of GSSG followed by desalting on a NAP-5 column and dialysis against 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 200 mM NaCl. The amount of bound glutathione was 6–8 molecules per molecule of RNase and 0.9–1.7 molecules per molecule of BSA, quantified by complete reduction of the glutathionylated proteins as described previously (42).
of NADPH at 340 nm in a coupled system with 1 mM GSH and glutathione reductase; HED and GSH in the reaction mixture give rise to β-ME-SG-mixed disulfide during an initial preincubation for 3 min (18). Grx catalyzes the reduction of β-ME-SG by GSH, which is the rate-limiting reaction. To increase sensitivity, reactions are run at a high pH (8.0) with excess HED (0.7 mM). We have analyzed the kinetic properties of Grx1 and Grx2 using two glutathionylated proteins, RNase-SG and BSA-SG, as well as β-ME-SG as substrates in the presence of 1 mM GSH (18). The reactions followed the same pH dependence for both Grx1 and Grx2 with a maximum rate at pH 8.5 (data not shown). This is in agreement with the proposed mechanism with the rate-limiting step being the reduction of the Grx-SG intermediate by a second molecule of glutathione reductase; HED and GSH in the reaction mixture.

The reaction was performed at pH 7.0 in a mixture containing glutathione reductase, NADPH, GSH, and either RNase-SG, BSA-SG, or HED. The reaction was started by addition of Grx, and activity was determined from the decrease in absorbance at 340 nm. For more details see “Experimental Procedures.”

| Protein     | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (μM⁻¹ s⁻¹) |
|-------------|----------|-------------|----------------------|
| Grx1        | 4.1      | 6.79        | 1.66 × 10⁶           |
| Grx1P23S    | 0.61     | 2.25        | 3.69 × 10⁹           |
| Grx2        | 0.77     | 1.89        | 2.46 × 10⁹           |
| Grx2S38P    | 2.7      | 4.77        | 1.78 × 10⁹           |
| Grx2C40S    | 2.5      | 3.71        | 1.48 × 10⁹           |

| Protein     | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (μM⁻¹ s⁻¹) |
|-------------|----------|-------------|----------------------|
| Grx1        | 2.16     | 293         | 1.36 × 10²           |
| Grx2        | 5.90     | 71.3        | 1.21 × 10⁴           |
| Grx2C40S    | 0.95     | 37.6        | 3.96 × 10⁴           |

**Human Grx2 Is a Substrate for Thioredoxin Reductase**—The significant differences between Grx1 and Grx2 in structure and catalytic properties prompted us to analyze whether oxidized Grx2 is substrate for thioredoxin reductase. Since Grx2, like other glutaredoxins, contains a disulfide in the active site after purification and storage (data not shown), we measured the amount of NADPH oxidized in single turnover experiments (Reaction 5).

\[
\text{TrxR} \quad \text{Grx2-S₃ + NADPH + H}^+ \rightarrow \text{Grx2-(SH)₃ + NADP}^+ 
\]

**Reaction 5**

Surprisingly both the cytosolic and the mitochondrial isoforms of mammalian TrxR were able to reduce human Grx2 (Fig. 2), whereas oxidized human Grx1 was not a substrate for either of the TrxR isoforms, confirming previous results (5). The reduction of Grx2 by TrxR was also coupled to the reduc-
tion of RNase-SG (Fig. 3). The efficient reduction of RNase-SG by Grx2 in this reaction demonstrated that Grx2 used reducing equivalents directly from NADPH via TrxR to reduce glutathionylated substrates. We analyzed the kinetics of this reaction, and the $K_m$ of TrxR for Grx2 was estimated to be 22 $\mu$M, and the $k_{cat}$ was estimated to be 822/min, representing about one-third of the $k_{cat}$ of TrxR for the previously established substrate Trx (40).

To investigate the catalytic mechanism of the reduction of Grx2 by TrxR we used the monothiol active site mutant Grx2C40S in a coupled reaction with either RNase-SG or Cys-SG. Surprisingly Grx2C40S was also a substrate for TrxR, yielding higher activity compared with wild type Grx2 (Fig. 4). Together these results demonstrate that TrxR can reduce both the active site disulfide in human Grx2 and the mixed disulfide formed between glutathione and Grx2 (Fig. 5, reactions 3a and 3b).

**Reduction of Low Molecular Weight Disulfides Independent of GSH**—The new electron transfer pathway via TrxR allowed us to investigate substrates for Grx2 in a non-glutathione-dependent manner. The reduction of Grx2 by TrxR was coupled to the reduction of the physiologically relevant CoA-disulfide, CoA-SG, and GSSG as well as the artificial model disulfide HED. By adjusting the concentration of reagents, apparent second order rate conditions were created under which Grx2 slowly reduced both the CoA-disulfide and HED. However, the second order rate constant for reactions involving the glutathione-mixed disulfides was almost 1000-fold higher (Table III). These results demonstrate that reduced Grx2 can reduce low molecular weight disulfides as expected, although the capacity to reduce S-glutathionylated substrates via a GSH-binding site is the hallmark of a glutaredoxin. They also demonstrated that Grx2 might use reducing equivalents from TrxR to efficiently reduce GSSG creating a potentially important rescue system in mitochondria.

**DISCUSSION**

The major differences in primary structure between cytosolic Grx1 and mitochondrial Grx2 are also reflected in their catalytic activities as revealed in this study. Thus, mitochondrial Grx2 has a high affinity for S-glutathionylated substrates, and this is to a large extent dependent on the substitution of the Pro residue in the classical CPYC active site sequence by a Ser residue. Quite unexpected was the discovery that Grx2 is a direct substrate for thioredoxin reductase and thus acts as a thioredoxin, being able to accept electrons from both TrxR and TrXR2 as well as from GSH. This is a first example of a cellular glutaredoxin being able to cross-react this way. Previous studies have shown that in *E. coli*, yeast, or mammalian cells there is no cross-reactivity. How can these results be reconciled in the light of the localization of the Grx2 isoforms in mitochondria or the nucleus?

Our results clearly demonstrate that Grx2 reduced glutathionylated substrates with lower rates than Grx1 and *E. coli* glutaredoxins (42, 45, 47), but the higher affinity results in the same or even slightly higher catalytic efficiency ($k_{cat}/K_m$). Recognizing S-glutathionylated proteins in the mitochondria or at the nuclear membrane with high affinity may be important. The Grx2C40S mutant, unable to form an intramolecular disulfide in the active site, confirmed that Grx2 followed the usual glutaredoxin catalytic mechanism in that only the active site thiol closer to the N terminus was required for reduction of S-glutathionylated substrates. The higher $k_{cat}$ observed for this mutant, compared with the wild type enzyme, is similar to earlier observations for the corresponding mutant proteins of Grx1 from both human and pig (21, 27) but in contrast to the monothiol mutants of *E. coli* Grx1 and -3 and phage T4 Grx1, which exhibited significantly decreased activity (9, 44, 48). The higher turnover has been explained by the absence of the intramolecular side reaction in which the Grx forms an intramolecular disulfide bond releasing GSH (27), although it is not possible to predict this behavior today a priori. Furthermore the active site cysteines have been suggested to form a thiol-thiolate hydrogen bond network, which stabilizes the more N-terminal active site thiolate in the reduced form of the proteins (48–51). Thus, the higher turnover for the Cys to Ser monothiol mutants could be due to a stronger thiolate-hydroxyl hydrogen bond, which decreases the pK_a of the thiol closer to the N terminus, increasing its leaving group ability, a key factor of the rate-limiting step in the reduction of protein-glutathione-mixed disulfides (6, 51).
The dipeptide sequence within the CXXC motif is characteristic for individual members of the thioredoxin superfamily, and its importance has been studied earlier in several mutant variants (51–55). Previous studies indicate that the proline residue in the CPYC motif favors formation of hydrogen bonds between the more N-terminal cysteine thiolate and the amide protons of the tyrosine and the C-terminal cysteine, which stabilize the thiolate and decrease its pKa value (51). Replacement of this Pro with Ser, as in the active site of Grx2, most likely affects these interactions, changing the pKa of the thiol and thereby the redox properties of the protein (53, 55, 56). The Grx1P23S and the Grx2S38P mutants clearly demonstrate that a Pro to Ser exchange decreases the activity of the glutaredoxin but increases their affinity for glutathionylated substrates.

Obviously the fact that Grx2 is a substrate for thioredoxin reductase should make it particularly suited to remove and control the level of S-glutathionylated proteins and low molecular weight disulfides following overproduction of reactive oxygen species during oxidative stress. The standard redox potential for GSH/GSSG is \(-0.24\) V (57), and the actual potential within the cell depends upon the intracellular or intraorganellar concentrations of GSH and GSSG. At sufficiently oxidizing conditions, either by efflux of GSH (58) or consumption, the active site in glutaredoxin will not be reduced by GSH, and if this takes place in mitochondria the electron transport chain may be blocked, leading to apoptosis (59). The direct reduction via thioredoxin reductase may then rescue the redox balance. The only previous example of this interaction comes from virus-infected E. coli where bacteriophage-coded T4 Grx1 (4) is an equally good substrate for E. coli TrxR1 as the host Trx1. The specificity of the electron transport to the virus ribonucleotide reductase suggested that the mechanism aided in making the virus production of deoxynucleotides maximally effective (60).

The Km value of TrxR1 for Grx2 was higher than the Km determined for Trx (2.5 M) but lower than the Km reported for protein disulfide isomerase (35 M) (40, 61). Since TrxR1 and TrxR2 from various species reveal almost identical kinetic parameters (14–16), one can expect human mitochondrial TrxR2 to reduce Grx2 with an efficiency similar to that of the bovine TrxR1. Activity with the monothiol active site mutant Grx2C40S, in a coupled assay with Cys-SG, demonstrated further that TrxR can support both monothiol and dithiol reactions catalyzed by Grx2. This suggests that the glutaredoxin-GSH-mixed disulfide is attacked by the selenolate in TrxR, forming a mixed disulfide intermediate, releasing the glutathione moiety (Fig. 5, reaction 3b), revealing a new glutathione-independent path of electron flow from NADPH to various Grx substrates. This electron pathway is similar to the one suggested for the thioredoxin glutathione reductase enzymes where the monothiol or dithiol Grx domain accepts electrons from either the TrxR domain or from GSH (62–64). Using TrxR as an electron donor, we found that Grx2 could reduce both

![Fig. 3. Reduction of Grx2 by TrxR coupled to the reduction of RNase-SG.](image)

![Fig. 4. Reduction of Grx2 or Grx2C40S by TrxR coupled to the reduction of Cys-SG.](image)
Fig. 5. Suggested mechanism for reduction of the intramolecular and the glutathione-mixed disulfide in Grx2 by TrxR. Reduction of glutathione-mixed disulfides by Grx2 results in a glutathione-Grx2 intermediate (reaction 1), which subsequently can be reduced by the C-terminal active site cysteine, releasing glutathione (reaction 2). The disulfide formed in the active site of Grx2 is then attacked by the selenolate in the active site of TrxR (reaction 3a). Enzymatic activity of the Grx2C40S mutant, unable to form the intramolecular disulfide, indicates that also the glutathione-Grx2 intermediate can be attacked by the TrxR selenolate (reaction 3b). The covalent intermediate between Grx2 and TrxR formed in both reactions 3a and 3b is then reduced by the cysteine in the active site of TrxR (reaction 4), yielding reduced Grx2. Oxidized TrxR is reduced by NADPH (reaction 5).

Table III
Reduction of low molecular weight disulfides by Grx2 using electrons from TrxR and NADPH

| Low molecular weight substrate | Second order rate constant, $k_2$ |
|-------------------------------|----------------------------------|
| HED                           | $7.4 \pm 0.3$                   |
| CoA-disulfide                 | $14.7 \pm 1.9$                  |
| CoA-SG                        | $(22.3 \pm 1.2) \times 10^3$    |
| Cys-SG                        | $(13.0 \pm 0.9) \times 10^3$    |
| GSSG                          | $(17.9 \pm 0.7) \times 10^3$    |

Fig. 6. Alternative electron pathways for reduction of glutathionylated proteins or low molecular weight disulfides by human Grx2. DHA, dehydroascorbate; GR, glutathione reductase.

Reducation of low molecular weight disulfides by Grx2 using electrons from TrxR and NADPH

The reaction was performed at pH 7.4 in a mixture containing NADPH, TrxR1, and Grx2 and started by addition of the various low molecular weight disulfides. Activity was determined from the decrease in absorbance at 340 nm, and the second order rate constants were determined from three independent experiments. For further details see "Experimental Procedures."

hydroxyethyl disulfide and CoA-disulfide apart from their glutathione-mixed disulfides. Although reduction of the intramolecular disulfides was less efficient, it clearly demonstrates that Grx2 is able to reduce low molecular weight disulfides independently of GSH. CoA, which preferentially is compartmentalized to mitochondria and known to undergo thiol/disulfide exchange reactions with GSH and GSSG both in vitro and in vivo, could be a potential substrate for Grx2 in vivo (65–67). The redox state of CoA certainly affects many fundamental processes such as the oxidation and activation of fatty acids, suggested to have key roles in the mitochondrial membrane permeability transition (68), and the activity of the pyruvate and $\alpha$-ketoglutarate dehydrogenase complexes. The relevance of glutathionylation/deglutathionylation reactions has been intensively discussed in the last years (69–73). It has been suggested to be not only a way to protect critical thiols from irreversible oxidation but also as a common mechanism for redox regulation (74–76). Recent studies imply an important role also in mitochondria since the mitochondrial redox status modulates the activity of the $\alpha$-ketoglutarate dehydrogenase, succinate dehydrogenase, and NADH-ubiquinone oxidoreductase (complex I) (59, 77, 78). The fact that cytosolic Grx1 facilitates reactivation of the inactivated enzymes suggests a central role for glutaredoxins during oxidative conditions. Under normal conditions the reduction of Grx by GSH is fast. However, the rate of reduction falls substantially as the [GSH]/[GSSG] decreases as discussed above or when the intracellular pH falls (43). During these conditions, the importance of alternative pathways for reduction of Grx becomes evident. The feature of Grx2 to use reducing equivalents from TrxR, whose activity is not sensitive for either changes in the glutathione redox buffer or decreases in the intracellular pH (79), facilitates an efficient backup system that enables reactivation of inactivated enzymes and reduction of GSSG to restore the redox equilibrium (Fig. 6). Together these results suggest an important role for Grx2 in protection and recovery from oxidative stress. In fact, Grx2 may be a key regulator of apoptosis via the mitochondrial checkpoint.

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