Human mitochondrial glutaredoxin 2 (GLRX2), which controls intracellular redox balance and apoptosis, exists in a dynamic equilibrium of enzymatically active monomers and quiescent dimers. Crystal structures of both monomeric and dimeric forms of human GLRX2 reveal a distinct glutathione binding mode and show a 2Fe-2S-bridged dimer. The iron-sulfur cluster is coordinated through the N-terminal active site cysteine, Cys-37, and reduced glutathione. The structures indicate that the enzyme can be inhibited by a high GSH/GSSG ratio either by forming a 2Fe-2S-bridged dimer that locks away the N-terminal active site cysteine or by binding non-covalently and blocking the active site as seen in the monomer. The properties that permit GLRX2, and not other glutaredoxins, to form an iron-sulfur-containing dimer are likely due to the proline-to-serine substitution in the active site motif, allowing the main chain more flexibility in this area and providing polar interaction with the stabilizing glutathione. This appears to be a novel use of an iron-sulfur cluster in which binding of the cluster inactivates the protein by sequestering active site residues and where loss of the cluster through changes in subcellular redox status creates a catalytically active protein. Under oxidizing conditions, the dimers would readily separate into iron-free active monomers, providing a structural explanation for glutaredoxin activation under oxidative stress.

Glutaredoxins (GLRXs)\(^4\) are evolutionarily conserved, glutathione-dependent oxidoreductases that are critically involved in the maintenance of cellular redox homeostasis (1, 2). The enzymes belong to the thioredoxin-fold superfamily of proteins and catalyze a broad spectrum of thiol-disulfide reactions using two distinct reaction mechanisms that require either 1 or 2 cysteines in a Cys-X-X-Cys active site motif.

Reduction of protein and low molecular weight disulfides proceeds through a dithiol mechanism that results in an intramolecular disulfide that is reduced by two molecules of glutathione through a glutathione-protein mixed disulfide intermediate. In contrast, glutathionylation and de glutathionylation reactions require only the more N-terminal cysteine. In these reactions, a GLRX-glutathione mixed disulfide occurs that is either formed or reduced by one molecule of glutathione (1).

To date, three low molecular weight GLRXs with 15–34% sequence identity and distinct active site motifs have been described in mammalian cells. The well studied cytosolic glutaredoxin 1 (GLRX1) is a 12-kDa protein that contains the classical Cys-Pro-Tyr-Cys motif. GLRX1 supplies electrons to ribonucleotide reductase, catalyzes disulfide-dithiol exchanges in proteins or small molecules such as dehydroascorbate, and is intimately involved in transcriptional regulation, cellular differentiation, and apoptotic processes (1). Glutaredoxin 2 (GLRX2) is a 16-kDa protein directed either to the nucleus or to the mitochondria through differential splicing of the first exon (3). Human GLRX2 contains a \(^{37}\text{CSYC}^{40}\) active site motif with a serine residue replacing the conserved proline and has several biochemical properties that distinguish it from GLRX1, perhaps as a result of this substitution (4). First, GLRX2 catalyzes more efficiently monothiol-mediated protein de glutathionylation reactions due to its high affinity for glutathione-protein mixed disulfides and has been shown to catalyze the reversible glutathionylation of complex I and other proteins in the inner mitochondrial membrane (5). Second, GLRX2 is not inhibited by oxidation of structural cysteine residues (3). Third, in addition to receiving reducing equivalents from glutathione, GLRX2 can also receive electrons from thioredoxin reductase (4). More recently glutaredoxin 5 (GLRX5), with only one cysteine residue in the active-site motif, has been characterized in the yeast *Saccharomyces cerevisiae*, with homologs found in organisms from bacteria to humans (6–9). It is of particular interest that GLRX5 is targeted to mitochondria like GLRX2 and that GLRX5 participates in the biogenesis of iron-sulfur clusters (10, 11). An alignment of these three human isoforms is provided in Fig. 1.

Previous experimental results provide substantial evidence for a critical role of GLRX2 as a sensor of the mitochondrial redox status. The small interfering RNA-mediated down-regulation of mitochondrial GLRX2 resulted in dramatically increased sensitivity toward apoptotic stimuli such as doxorubicin or phenylarsine oxide (12), whereas overexpression of GLRX2 prevented cardiolipin oxidation, cytochrome c release,
and induction of apoptosis (13). Importantly, mitochondrial GLRX2 exists in two distinct states \textit{in vivo}: the catalytically competent monomeric form and a recently discovered inactive dimeric state that contains an iron-sulfur cluster. Under conditions of oxidative stress, there is a transition from the inactive dimer to active monomeric GLRX2 initiated by decreased GSH/GSSG ratios (14). In this instance, loss of the cluster through change of the redox status creates a catalytically active protein. An in-depth spectroscopic characterization of dimeric human GLRX2 by Lillig \textit{et al.} (14) identified the presence of Fe(III) in tetrahedral sulfur coordination and additional parameters consistent with a $[2\text{Fe}-2\text{S}]^{2+}$ cluster coordinated by 4 cysteine residues. The same study determined the stoichiometry to be two iron and two sulfide atoms per dimer, suggesting that the cluster served to mediate dimerization. However, there are conflicting reports of the residues responsible for coordinating the iron-sulfur cluster. Based upon mutagenic data, it was proposed that 2 cysteines outside the active site coordinate the cluster (14). In contrast, Feng \textit{et al.} (15) presented NMR data suggesting that poplar glutaredoxin C1 coordinates an iron-sulfur cluster through the N-terminal active-site cysteines and two glutathione molecules.

We have determined the crystal structures of human GLRX2 in monomeric and dimeric forms, which reveal novel details of glutathione binding and provide clear proof for iron coordination through 2 active-site cysteines.

\section*{EXPERIMENTAL PROCEDURES}

\textbf{Expression, Purification, and Characterization of Recombinant Human GLRX2—}A template plasmid encoding for full-length human GLRX2 was obtained from Invitrogen. Two constructs lacking the N-terminal mitochondrial targeting sequence (comprising GLRX2 core residues 56–164 and 41–164) were cloned into pNic28-Bsa4, a pET21a-derived sequence (comprising GLRX2 core residues 56–164 and additional 15 amino acids) corresponds to a clone used in previous biophysical characterization (3, 14). Expression plasmids were transformed into a phage-resistant derivative of BL21(DE3) carrying a plasmid for rare codon expression. Proteins from pNic28-Bsa4, a pET21a-derived expression vector with a tobacco etch virus cleavable N-terminal His$_6$ tag. Since the length of the mature protein after mitochondrial import is unknown, two clones were constructed. The shorter clone was designed to coincide with the N terminus of human GLRX1, whereas the longer construct (containing an additional 15 amino acids) corresponds to a clone used in previous biophysical characterization (3, 14). Expression plasmids were transformed into a phage-resistant derivative of BL21(DE3) carrying a plasmid for rare codon expression. Protein expression was induced by adding 1 mM isopropyl-$\beta$-thio-$\gamma$-galactopyranoside to cultures grown in TB (Terrific Broth) medium to $A_{600}$ = 3.4, and then cultures were further incubated at 18 °C overnight. Cell pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, protease inhibitors (Complete, Sigma) and lysed using a high pressure homogenizer. Cell debris and nucleic acids were removed by the addition of 0.15% polyethyleneimine followed by centrifugation for 30 min at 40,000 × g, and the supernatant was further clarified by filtration.

The proteins were purified using nickel-affinity chromatography (GE Healthcare), and monomeric and dimeric GLRX2 were separated by gel filtration chromatography with a Superdex 200 column (GE Healthcare) equilibrated in 10 mM HEPES, pH 7.5, 500 mM NaCl, and 5% glycerol. Eluted proteins were analyzed by SDS-PAGE and concentrated in an Amicon 5K centrifugal concentrator. Monomeric and dimeric GLRX2 were analyzed by UV-visible spectroscopy (Labtech, Nanodrop 1000 spectrophotometer), and the molecular mass was verified by electrospray mass ionization-time-of-flight mass spectrometry (Agilent LC/MSD time-of-flight).

\textbf{Aerobic Crystallization of Reduced Monomeric GLRX2 in Complex with Glutathione—}Reduced glutathione to a final concentration of 10 mM was added to the dimeric fraction of GLRX2Δ1–55 prior to concentration. Crystals in the form of thin rods were grown by vapor diffusion at 20 °C in a sitting drop consisting of 50 nl of protein (18 mg/ml) and 100 nl of precipitant solution containing 0.1 M citrate, pH 3.5, 25% polyethylene glycol 3350. The crystal was transferred to a cryoprotectant prepared from precipitant solution supplemented with 10% (v/v) 1,2-propanediol before flash-cooling in liquid nitrogen.

\textbf{Anaerobic Crystallization of dimeric GLRX2 with an Iron-Sulfur Cluster—}Dimeric GLRX2Δ1–40 for anaerobic crystallization was purified as described above except all buffers contained 10 mM reduced glutathione and were degassed and purged with argon (14). The protein was concentrated to 11.5 mg/ml and stored under nitrogen. A sitting drop consisting of 1 µl of protein and 1 µl of well solution was equilibrated against well solution containing 1.6 M MgSO$_4$, 100 mM MES, pH 6.5, in a glove box under argon to prevent oxidation of the iron-sulfur cluster. Under these conditions, brown, hexagonal plate-shaped crystals appeared and grew to a maximum dimension of ~75 µm over 6 weeks. A single crystal was transferred to a cryoprotectant of 1.7 M sodium malonate, pH 7.0, before flash-cooling in liquid nitrogen.

\textbf{Data Collection, Phasing, and Refinement—}A fluorescence scan was performed on the crystal grown anaerobically to identify the metal atoms present, and data sets for monomeric and dimeric crystal forms of GLRX2 were collected at the Swiss Light Source beamline X10SA. For the monomer, initial phases were calculated by molecular replacement using PHASER (16) and the lowest energy model from the NMR structure 2CQ9. The dimeric crystal form was solved by molecular replacement using PHASER and the monomer built from the x-ray data (2FLS) with ligand and solvent atoms removed. In both cases, before refinement commenced, 5% of the data were flagged for calculation of $R_{free}$. Statistics for the final models, which are the result of alternating rounds of model building and refinement performed with the programs COOT (17) and REFMAC5 (18), are given in Table 1.

The model for monomeric GLRX2 includes 102 protein residues, one glutathione molecule, and 53 water molecules. The model for the dimeric form includes 220 protein residues (two monomers), an inorganic 2Fe-2S cluster, two glutathione molecules, and 107 water molecules. All residues are in most favored and additional allowed regions of a Ramachandran plot calculated by PROCHECK (19). The protein residues in the models are numbered to be consistent with previous literature and thus are offset –40 residues from the gene-derived sequence.

\footnote{T. Abe, H. Hirotta, F. Hayashi, and S. Yokoyama, unpublished results.}
RESULTS AND DISCUSSION

Biochemical Characterization—To provide insight into glutathione binding and coordination of the iron-sulfur cluster in human GLRX2, we have crystallized both monomeric and dimeric forms of the protein in the presence of reduced glutathione. Two truncated versions of GLRX2, containing the core sequence without the mitochondrial targeting signal, were expressed and purified. Both constructs behaved identically during purification, and the additional residues at the N terminus of the longer construct were disordered and not visible in the electron density maps. Molecular masses were confirmed by mass spectrometry, indicating the presence of one intramolecular disulfide bond.

TABLE 1

|                     | Monomeric GLRX2 (2FLS) | Dimeric GLRX2 (2HT9) |
|---------------------|------------------------|----------------------|
| Data collection     | P3_21                  | P6_3                 |
| Cell dimensions     | a, b, c (Å)            | 60.2, 60.2, 68.0     | 111.8, 111.8, 51.6 |
| α, β, γ (*)         | 90, 90, 120            | 90, 90, 120          |
| Resolution (Å)      | 41.2-0.05 (2.16-2.05)  | 97.1-1.9 (2.0-1.9)   |
| Rmerge/Rfree (%)    | 0.12 (0.81)            | 0.06 (0.42)          |
| Mean l/σ (l)        | 13.5 (2.5)             | 13.1 (2.4)           |
| Completeness (%)    | 100 (100)              | 91.7 (87.1)          |
| Redundancy          | 5.9 (5.8)              | 3.9 (3.1)            |

Refinement

| Resolution (Å)      | 41.2-0.05 (2.16-2.05)  | 97.1-1.9 (2.0-1.9)   |
| No. reflections     | 8589                   | 25063                |
| Rmerge/Rfree (%)    | 0.190/0.231            | 0.149/0.178          |
| No. atoms           | Protein 786             | 1801                 |
|                     | Ligand/ion 20           | 44                   |
|                     | Water 53                | 107                  |
| r.m.s. deviations*  | bond length (Å) 0.017   | 0.015                |
|                     | bond angle (*) 1.50     | 1.45                 |

* r.m.s., root mean square.

Monomeric and dimeric GLRX2 were separated by gel filtration chromatography and analyzed by UV spectroscopy (see supplemental materials). Both fractions generated one band of ~16 kDa when analyzed by SDS-PAGE gels. Measurement of specific activity indicated that the majority of enzymatic activity was contained within the monomeric fraction. The dimeric fraction of GLRX2 was visibly brown and showed strong absorbance peaks at 320 and 420 nm, consistent with protein containing an iron-sulfur complex (14). Crystals that were grown anaerobically exhibit strong fluorescence emission at a wavelength in agreement with the presence of iron. Attempts to crystallize the monomer fraction from gel filtration chromatography were abandoned once it was apparent that the dimer dissociated into monomers during aerobic crystallization.

Structure of Monomeric Human GLRX2—Aerobic crystallization resulted in dissociation of the dimeric GLRX2 to a reduced monomer in complex with glutathione. GLRX2 adopts the fold of the thioredoxin superfamily, a central mixed β-sheet sandwiched between α-helices. GLRX2 contains 4 cysteines, organized in two pairs that are in close proximity to each other. The first pair (Cys-37, Cys-40) is reduced and is embedded in the conserved cysteine-rich motif that is known to be involved in catalysis. The second pair (Cys-28, Cys-113) forms a disulfide bond and is on the distal side of the molecule away from the catalytic pair. This second pair is completely conserved in GLRX2 proteins and not found in GLRX1 or GLRX5 enzymes (Fig. 1). Importantly, the cystine bridge formed between these residues likely increases protein stability by anchoring the N- and C-terminal helices. Furthermore, this internal disulfide provides an explanation why GLRX2 is resistant to oxidation of structural cysteines (3) since there are no free cysteines to further oxidize. Recently, the apo-structure of human GLRX2 was determined by NMR (2CQ9),5 and comparison with our crystal structure in complex with GSH indicates that the largest differences occur in the loop region preceding helix 2. This region contains the N-terminal catalytic cysteine; thus, the differences are likely due to glutathione binding.

Glutathione is bound to the active site of the GLRX2 monomer. The glutathione nestles into a groove on the surface of GLRX2, and each residue in the glutathione tripeptide makes several polar contacts with the protein (Fig. 24). The glutamate carboxylate forms hydrogen bonds with backbone amides of Ala-94 and Thr-95 as well as the side-chain hydroxyl of Thr-95. The cysteine main chain has two interactions with the backbone nitrogen and oxygen of Val-81, and the glycine carboxylate is engaged in polar interactions with the side chains of Lys-34 and Gln-69. In addition, the aliphatic portion of the glutathione glutamate packs against the pheno-
lic ring of Tyr-39, and there are three ordered water molecules mediating interactions between the ligand and protein. Although the glutathione is in a conformation where it could form a disulfide with the protein, the distance between sulfur atoms is \( >2.8 \, \text{Å} \), indicating that this bond is likely to be reduced. By comparison, the distance between sulfur atoms in the structural disulfide is \( <2.1 \, \text{Å} \). Glutathione bound in this orientation would probably prevent glutathionylated substrates from binding under reducing conditions and might explain the observed lower turnover compared with human GLRX1 (3). This ligand binding behavior may also be the reason for the observed affinity of GLRX2 for GSH-Sepharose, a characteristic that distinguishes GLRX2 from other glutaredoxins (20).

A search of the Protein Data Bank for glutaredoxins with a glutathione bound retrieved the following three structures: human glutaredoxin 1 (1B4Q) (21), Escherichia coli glutaredoxin 1 (1GRX) (22), and E. coli glutaredoxin 3 (3GRX) (23). All are NMR structures of a glutathione-protein mixed disulfide with the N-terminal cysteine in the catalytic motif. For the *E. coli* enzymes, this covalent bond is the main interaction between glutathione and the protein, although GLRX3 has two additional polar contacts: the glutathione glutamate nitrogen to a threonine hydroxyl and the glutathione cysteine oxygen to a valine main-chain amide. Comparison of human GLRX1 (21) and GLRX2 indicates that the interaction between the glutathione glutamate carboxylate and the main-chain amide is preserved, but no side-chain interaction occurs in this area, and in GLRX1, the glutathione glycine carboxylate interacts only with the side chain of Arg-67. Perhaps the most important difference in ligand binding between the two human structures is that the Glu-Cys peptide bond of glutathione is flipped, with the peptide bond in the GLRX1 structure having an opposite orientation than in the other glutaredoxin structures.

At present, no structural information is available for the monothiol active-site GLRX5 or any of its orthologs. Although GLRX5 has only a single cysteine in its Cys-Gly-Phe-Ser active-site motif, it has been shown to have thiol reductase activity (11), and it has been further demonstrated that a 2nd cysteine,
Cys-117, is essential for the catalytic activity. Ala-94 in GLRX2, the residue equivalent to Cys-117 in human GLRX5, maps to a region on the surface of GLRX2 adjacent to the active site, indicating that the reaction for GLRX5 likely proceeds through a dithiol mechanism with the second Cys residue located outside the classical Cys-X-X-Cys active-site motif.

**Structural Organization of Dimeric GLRX2 Containing a [2Fe-2S]^{2+} Cluster**—The GLRX2 dimer is composed of two GLRX2 protein chains, an inorganic 2Fe-2S cluster, and 2 glutathione molecules (Fig. 2B). The non-catalytic cysteine pair has been proposed to mediate dimerization by coordinating the iron-sulfur cluster for the following reasons; this extra pair is not conserved in other GLRXs, only GLRX2 is known to form an iron-sulfur-containing dimer, and mutation of these residues abrogated dimer formation during recombinant bacterial expression (14). In contrast to this prediction, the dimeric crystal structure reveals that the cluster is coordinated through Cys-37, the N-terminal cysteine in the active-site Cys-Ser-Tyr-Cys motif. Although the cluster itself is planar, each iron atom is tetrahedrally coordinated by Cys-37, two sulfur atoms, and a cysteine contributed by glutathione. The two inorganic sulfides bridge both iron atoms (Fig. 2C). Due to steric requirements, the side chains of Ser-38 and Tyr-39 adopt different rotamers when the cluster is bound. The tyrosine side chain adopts a high energy conformation (chi2 ~100°), shielding one side of the cluster from solvent and from oxidative degradation.

Although the glutathione must move ~1.5 Å to accommodate the cluster, it remains tightly bound as the polar interactions with the protein are largely unchanged, and additional interactions are found with the second protein chain. In the glutathione molecule most closely associated with chain B, additional interactions are found with the Ser-36 main chain and the Ser-38 side chain on monomer A. The glycine carboxylate for glutathione associated with chain A is in an alternate conformation where it loses its contacts with Lys-34 and Gln-69, whereas the cysteine sulfur forms an additional contact with the hydroxyl of Ser-38 on chain B. Thus, movement of the side chain of Ser-38 is important not only to accommodate the cluster but also because it forms additional interactions with the glutathione from the adjacent monomer. It is likely that a proline at this position, as occurs in GLRX1, will discourage cluster coordination by reducing the flexibility of the protein backbone, and furthermore, the proline side chain would not be able to form favorable interactions with the neighboring glutathione molecule.

**Biological Implications of Mitochondrial GLRX2**—The physiological role of mitochondrial GLRX2 is not fully understood at the present time; however, the crystal structures explain several observed features. Previous studies (12, 14) have revealed that GLRX2 is central in mitochondrial redox regulation. An RNA interference–induced GLRX2 knockdown sensitized mitochondria toward oxidative damage and apoptosis (12), indicating that GLRX2 could be an interesting intervention point in cancer therapy for sensitizing cells for apoptotic stimuli. It is conceivable that GLRX2 has several roles due to its enzymatic function in glutathionylation and deglutathionylation reactions. In the mitochondria, free protein thiols are present in higher concentration than GSH. Because of their higher abundance, protein thiols are primary scavengers for reactive oxygen species–induced oxidative damage, and as a consequence, are glutathionylated (24). It is likely that GLRX2 has a direct role in “protein repair” by restoring the free thiol state. A further striking feature of GLRX2 is that it appears to be destined to function enzymatically primarily under oxidative conditions. First, high GSH/GSSG ratios will drive the GLRX2 equilibrium into a quiescent dimeric Fe-S-bound form (12, 14), which can readily be activated as soon as the GSH/GSSG ratio drops under oxidative conditions. Second, GSH appears to be a direct inhibitor, as demonstrated by the non-productive complex of monomeric GLRX2 with reduced glutathione. Third, uncharacteristic of other GLRXs, GLRX2 can be reduced directly by thioredoxin reductase (4), indicating a role under increased oxidative stress. It therefore seems that this tight control of GLRX2 activity, through sequestration into a resting dimeric state and inhibition by GSH, coupled with its intrinsically lower activity, is of major biological importance for mitochondrial homeostasis. Clearly, further experiments are necessary to dissect the role of GLRX2 in mitochondrial redox control and to investigate possible interactions with other mitochondrial thiol scavengers such as lipoic acid, which has also been shown to be involved in mitochondrial redox control (25–27). Of particular relevance, dihydrolipoamide can supply electrons to GLRX2 (28), indicating a connection between lipoic acid or its derivatives and GLRX2 in redox control. Thus, GLRX2 is likely implicated in aging mechanisms as mitochondrial oxidative stress and redox control are central to neurodegenerative and metabolic diseases (29–31). It is interesting to observe that biological functions of GLRXs include repair, signaling, and assembly functions. At present, it cannot be excluded that GLRX2 has a function in iron-sulfur cluster biogenesis, as has been recently shown for GLRX5 (6). GLRX2 is the first member of the mitochondrial glutaredoxins to have its crystal structure determined, and it would therefore be interesting to investigate and compare the structure with the monothiol active site GLRX5 that is involved in the maturation and assembly of Fe-S cluster proteins.

**Acknowledgments**—Collaboration with the Swiss Light Source synchrotron facility and staff as well as data collection by Frank von Delft is gratefully acknowledged. The Structural Genomics Consortium is a registered charity (Number 1097737) funded by the Wellcome Trust, GlaxoSmithKline, Genome Canada, the Canadian Institutes of Health Research, the Ontario Innovation Trust, the Ontario Research and Development Challenge Fund, the Canadian Foundation for Innovation, Karolinska Institutet, Vinnova, the Knut and Alice Wallenberg Foundation, and the Swedish Strategic Research Foundation.

**Note Added in Proof**—While in press, a recent biochemical study confirmed the structural conclusions drawn in this study on GLRX2 Fe-S coordination (Berndt, C., Hudemann, C., Hanschmann, E. M., Axelsson, R., Holmgren, A., and Lillig, C. H. (2007) Antioxid. Redox Signal. 9, 151–157).

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