Biochemical Characterization of Yeast Mitochondrial Grx5
Monothiol Glutaredoxin.

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Running title: Characterization of yeast Grx5.

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Summary

Grx5 is a yeast mitochondrial protein involved in iron-sulfur biogenesis that belongs to a recently described family of monothiolic glutaredoxin-like proteins. No member of this family has been previously biochemically characterized. Grx5 contains a conserved cysteine residue (Cys60) and a non-conserved one (Cys117). In this work we have purified wild type and mutant C60S and C117S proteins and characterized their biochemical properties. A redox potential of -175 mV was calculated for WT-Grx5. The \( pK_a \) values obtained by titration of mutant proteins with iodoacetamide at different pHs were 5.0 for Cys60 and 8.2 for Cys117. When Grx5 was incubated with glutathione disulfide a transient mixed disulfide was formed between glutathione and the cystein 60 of the protein due to its low \( pK_a \). Binding of glutathione to Cys60 promoted a decrease in Cys117 \( pK_a \) value that triggered the formation of a disulfide bond between both cysteine residues of the protein indicating that Cys117 plays an essential role in the catalytic mechanism of Grx5. The disulfide bond in Grx5 could be reduced by GSH but at a rate at least 20 times slower than that observed for reduction of glutaredoxin 1 from \( E. \ coli \), a dithiolic glutaredoxin. This slow reduction rate could suggest that GSH may not be the physiologic reducing agent of Grx5. The fact that WT-Grx5 efficiently reduced a glutathiolated protein used as a substrate, indicated that Grx5 may act as a thiolreductase inside the mitochondria.
Introduction

Glutaredoxins are small proteins with thiolreductase activity that are required for maintaining protein cysteines in reduced form. In contrast to thioredoxins, glutaredoxins require the reduced form of glutathione (GSH) as electron donor (1-3). Previously characterized glutaredoxins contain an active site that includes two conserved cysteine residues with two non-conserved residues between them (4-6). Mutagenic studies have shown that both residues are required for reducing protein disulfides. However, only the amino-terminal cysteine may be essential for the reduction of mixed disulfides of proteins with glutathione (6-8). In Saccharomyces cerevisiae five different glutaredoxins have been described. Two of them (Grx1-2) are classic dithiolic glutaredoxins containing both conserved cysteine residues and have been already biochemically characterized (9-11). On the basis of sequence analysis, a new family of monothiolic glutaredoxins has been recently described. These proteins are highly homologous to glutaredoxins but contain only one cysteine residue in its putative active site (12). Members of this family are found elsewhere, from bacteria to mammals, including human (13). To date, none of them has been properly biochemically characterized.

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1The abbreviations used are: GSH, Reduced Glutathione; GSSG, Glutathione disulfide; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; HED, β-hydroxyethyl disulfide; DTT, dithiothreitol; MOPS, morpholinopropane sulfonic acid; MES, morpholinoethane sulfonic acid; HPLC, High performance liquid chromatography; E°, standard state redox potential at 25°C.
Three monothiolic glutaredoxins are found in yeast (Grx3-5). No clear phenotypes have been described in yeast cells lacking Grx3 and Grx4 and consistently no specific role has been assigned to any of these proteins. In contrast, the absence of Grx5 induces severe growth defects (12). Cells lacking Grx5 are not able to grow on minimal medium nor in the presence of non-fermentable carbon-sources, they accumulate iron in the mitochondria and show decreased activities of iron-sulfur containing enzymes. These characteristics are common to other genes involved in the synthesis and assembly of Fe/S clusters such as SSQ1, JAC1, ATM1, NFU, YAH1, ARH1, ISU1-2 (14), ISA1-2 (15) NFS1, YFH1 (16) and ERV1 (17). Recently, we have shown that Grx5 is a mitochondrial protein involved in iron-sulfur biogenesis (18).

A three dimensional model of Grx5 was recently presented based on the known structure of several dithiolic glutaredoxins (13). Grx5 shows a classic thioredoxin fold structure, with the putative catalytic cysteine (Cys60) lying opposite to another conserved motif that could be involved in the formation of a glutathione cleft. Beside this motif, another non-conserved cysteine is found (Cys117). Site directed mutagenesis studies suggest that this cysteine is not essential for the biological activity of the protein (13).

Despite these observations there is no evidence that Grx5 works as a thiolreductase. Also, the specific role of Grx5 in iron-sulfur biogenesis is still not clear. Shenton et al. showed that in cells lacking Grx5 the cytosolic enzyme glyceraldehyde-3-phosphate-dehydrogenase was glutathiolated and suggested that Grx5 could work as a deglutathiolase (19). However the recent finding that Grx5 is a mitochondrial enzyme (18) suggests that this glutathiolation may be rather related to the oxidative stress conditions generated by iron accumulation in Δgrx5 cells than to the direct effect of Grx5, a mitochondrial protein, on glyceraldehyde-3-phosphate-dehydrogenase, a
cytosolic enzyme. In this work we address the biochemical characterization of Grx5, including determination of cysteine $pK_a$ value and redox potential. Based on these results we propose a mechanism of action for Grx5 protein. This is the first characterization of a monothiolic glutaredoxin and constitutes the first evidence that these proteins can work as thioloxidoreductases.

**Experimental Procedures**

*Materials-* GSH, glutathione disulfide (GSSG), cystine, dehydroascorbate, iodoacetamide, glutathione reductase, thioredoxin and trifluoroacetic acid were from Sigma. Glutaredoxin 1 (Grx1) from *Escherichia coli* was from Calbiochem and 2-hydroxyethyl disulfide (HED) was from Aldrich. Rat carbonic anhydrase III was a kind gift of Dr. Rod Levine (NIH, Bethesda).

*Strains and plasmids-* Plasmid pMM192 contains the *GRX5* open reading frame without the region coding from amino acid 2 to 29 (PCR-amplified from *S. cerevisiae* genomic DNA), cloned between the Nde I and BamH I unique sites of the *E. coli* expression vector pET-21a (Novagen). Point mutations in *GRX5* that yielded the different amino acid replacements were constructed by the ExSite method (20), using pMMM192 as template. Oligonucleotides for the introduction of the point mutations were designated in such a way that a restriction site that did not alter the translation product was introduced near to the desired point mutation and used as a marker for the DNA sequencing. Plasmids were maintained and amplified in *E. coli* BL21 cells (Novagen).

*Purification of Grx5 wild type and mutant proteins-* *E. coli* cells carrying the previously described plasmids coding for Grx5 wild type and mutant proteins were grown at 30°C in Luria-Bertani medium with 100 µg/ml ampicillin. When the $A_{600}$ reached a value of
0.4, expression of Grx5 was induced with 0.5 mM isopropyl thio-β-D-galactoside. After 4 h growth, the cells were centrifuged, washed twice with 50 mM Tris-HCl pH 8.0, and frozen in liquid nitrogen. Purification of the enzyme was made at 4 °C. The cells (3 g) were suspended in 5 ml of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl and 1 mM phenylmethanesulfonyl fluoride and sonicated. After centrifugation at 14,000 rpm for 30 min, the supernatant solution (5 ml, 35 mg of protein/ml) was applied on a Sephacryl S-100 HR column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl pH 8.0 plus 100 mM NaCl. After void volume, 4 ml fractions were analyzed for the presence of Grx5 by SDS-polyacrylamide gel electrophoresis. Fractions containing Grx5 were pooled and applied to a DEAE-15HR column (Waters Associates, Milford, MA) equilibrated with 50 mM Tris-HCl pH 8.0 and 100 mM NaCl. After a washing step of 20 min with the same buffer, elution was carried out by a linear gradient from 100 to 500 mM NaCl over 40 min at a flow rate of 5 ml/min. Grx5 eluted at 300 mM NaCl. Salt was diluted 30 times by several steps of concentration/dilution of the protein using an Amicon 8010 Ultrafiltration Cell. Protein was stored at -80°C at concentrations above 20 mg/ml. Protein was 99% pure as examined by SDS-polyacrylamide gel electrophoresis.

Analyses- Protein concentration was determined by the Bradford method (21). Titration of free sulfhydryl groups with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was performed as described (22). Briefly, 20-50 µg of protein were incubated for 10 minutes in a solution containing 200 µM DTNB in 100 mM Tris-HCl buffer pH 8.0 in a final volume of 0.1 ml. A molar extinction coefficient of 14,150 M⁻¹ cm⁻¹ was used to calculate the number of titrated sulfhydryl groups.

Activities- Reduction of the mixed disulfide formed between HED and glutathione (low molecular weight mixed disulfide reduction assay or HED assay) was assayed as
described in reference 2. Dehydroascorbate reductase activity and glutathione peroxidase activities were performed according to references 23 and 24.

_Determination of thiol pK_a value_- The rate of carboxymethylation of Grx5 was determined by incubation of reduced Grx5 (25 µM), either wild type or mutant proteins, with 0.6 mM iodoacetamide in 23 µl of 10 mM Tris, 10 mM potassium acetate, 10 mM MOPS, 10 mM MES and 0.2 M KCl at pH values between 3 and 10. At desired incubation times the reaction was stopped by the addition of 2 µl of 100 mM DTT. Reduced and carboxymethylated Grx5 were separated by HPLC in a DeltaPak HPI C18 column (Waters Associates, Milford, MA). Proteins were eluted by a linear gradient from 40 to 50% acetonitrile in 0.05% trifluoroacetic acid over 20 min at a flow rate of 0.2 ml/min. Proteins were detected and quantified from their corresponding peak areas at 276 nm.

_Reaction of Grx5 with GSSG_- Preparations (23 µl) containing 25 µM of either wild type or mutant proteins in 100 mM Tris-HCl pH 8.0 buffer were incubated with different concentrations of GSSG at 20°C in sealed tubes under nitrogen. Reaction was stopped by the addition of 2 µl 10% trifluoroacetic acid. The pH dependence of the rate of glutathiolation was assayed under the same conditions, except that GSSG was always present at 250 µM and Tris-HCl buffer was replaced by a mixture containing 10 mM Tris, 10 mM potassium acetate, 10 mM MOPS, 10 mM MES and 0.2 M KCl at pH values between 3 and 10. Reaction products were separated and quantified by HPLC as described above.

_Preparation of oxidized proteins_- The fully oxidized form of WT-Grx5 (disulfide bond) was prepared by incubation of 1 mg reduced protein (150 µM concentration) with 0.5 mM GSSG for 30 minutes at 20°C in Tris-HCl buffer pH 8.0. Excess glutathione was
removed by size-exclusion chromatography using a PD10 column (Amersham Biosciences) and by concentration of the protein using a Centricon 10K (Amicon). After this treatment HPLC analysis showed that 95% of the protein was in the oxidized form (disulfide bond, see results) and the remaining 5% in the reduced form. WT-Grx5 glutathiolated at Cys60 was obtained after incubation of 30 µg of reduced protein (90 µM concentration) with 1 mM GSSG at pH 5.0. This preparation was separated by HPLC and the peak corresponding to protein glutathiolated at Cys60 (see results) was collected and dried in a Speed Vac.

**Molecular weight determination of modified forms of Grx5**- Peaks obtained by incubation of Grx5 with GSSG were separated by HPLC as described above, collected, dried in a Speed Vac and solubilised in 20 µl of 0.1% trifluoroacetic acid. Proteins were mixed 1:1 with matrix solution (saturated 3,5-dimethoxy-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid). An 0.7 µl aliquot of this mixture was deposited onto a stainless steel MALDI probe and allowed to dry at room temperature. Samples were measured on a Bruker Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with the SCOUT source in positive ion linear mode using delayed extraction (200 ns). A nitrogen laser (337 nm) was employed for desorption/ionisation and the ion acceleration voltage was 20 kV. The equipment was externally calibrated employing protonated mass signals from horse cytochrome c.

**Determination of the redox potential**- The redox potential of Grx5 was determined by direct protein-protein redox equilibration (25). Briefly, 300 µl of a preparation containing 50 µM of reduced *S. cerevisiae* Grx5 and 50 µM oxidized *E. coli* Grx1 (and viceversa) in sodium phosphate buffer 0.1 M pH 7.0 plus 1 mM EDTA were incubated in small plastic tubes attached to a manifold and purged with a constant flux of moist nitrogen.
At different times, 23 µl of the preparation were removed through the septum of the tubes using a degassed 25 µl Hamilton gastight syringe and mixed with 2 µl of 10% trifluoroacetic acid to stop the reaction. Protein mixtures were analyzed by HPLC as described above except that a linear gradient from 30 to 50 % acetonitrile was used.

Results

Expression and purification of Grx5 proteins- In a previous work we had shown that Grx5 isolated from yeast cells was a processed form lacking the first 29 amino acids cleaved during import of the protein into mitochondria (18). In order to obtain enzymes resembling as much as possible the mature form of Grx5, all proteins used in this study were prepared without their mitochondrial targeting signals. It should be noted, however, that for a better comprehension and to compare with previous articles, original sequence number positions have been maintained all over the text. The wild type and mutant Grx5 proteins were overexpressed in E. coli cells and purified by a two step method including size exclusion and ionic exchange chromatography. The resulting proteins were >99% pure and showed an apparent molecular weight of 15.5 kDa. The same size was determined for the mature form present in yeast extracts detected by western blot (18). The theoretical mass of the protein (13,478 Da) was used for the determination of the molar concentration of the proteins. After purification, proteins were obtained in reduced form, as indicated by cysteine titration with DTNB (Table I).

Grx5 is not active in the HED assay- Several reactions can be catalysed by dithiolic glutaredoxins. The most widely used form to asses glutaredoxin activity is the glutathione:HED transhydrogenase assay. In this assay glutaredoxin catalyses the reduction of a mixed disulfide between glutathione and HED (2). Dithiolic glutaredoxins lacking one of the two conserved cysteines are still capable to catalyse this reaction
Glutaredoxin activity of WT-Grx5, C117S-Grx5 and C60S-Grx5 was assayed with the HED assay. No activity could be detected even when a wide range of pH values (7-9.5) and GSH concentrations (0.6-40 mM) were used. Additionally, dehydroascorbate reductase and glutathione peroxidase activities, which have been described for dithiolic glutaredoxins (23, 24), were also tested. None of the Grx5 variants showed detectable activity in these assays.

Determination of Cys60 and Cys117 $pK_a$ value- Reactivity of thiol groups in proteins highly depends on its $pK_a$ value. Active cysteines from dithiolic glutaredoxins have $pK_a$ values close to 4 (9, 26, 27). In order to determine the $pK_a$ value of both cysteine residues in Grx5 we measured the rate of alkylation of Grx5 with iodoacetamide at different pHs. This reaction occurs only when cysteines are in the ionized thiolate anion state (28). Thus, reduced WT, C117S and C60S Grx5 proteins were incubated with 0.6 mM iodacetamide at pH values between 3 and 11. Reaction was stopped at different times by addition of 10 mM DTT and samples were analyzed by HPLC. The concentrations of reduced (Grx5$_{red}$) and carboxymethylated glutaredoxin (Grx5$_{cmc}$) were calculated from the peak area of the HPLC profile (shown in Fig. 1 A). Plots of $1/[$Grx$_{red}$] versus time yielded straight lines indicating that the reaction of iodoacetamide with Grx5 follows a second order reaction with a single rate constant. The second order rate constants were calculated according to the equation $k_{app} = [Grx5_{cmc}]/(t \times [Grx5_o] \times [Grx5_{red}])$ and plotted against pH (Fig. 1 B) ([Grx$_o$] = initial concentration of Grx5). Reaction rates showed a sigmoidal dependence on pH value at pHs around 5 (WT and C117S) and 8 (WT and C60S). From these data it can be deduced that the increases in reaction rates at low and high pH were respectively a consequence of the ionisation of Cys60 and Cys117. Using the Henderson-Hasselbach equation (28) thiol $pK_a$ values of 5.0 $\pm$ 0.1 and 8.2 $\pm$ 0.1 were calculated for Cys60 and Cys117 respectively (Table I).
Reaction of Grx5 with GSSG: Reactivity of reduced Grx5 with GSSG was tested at pH 8.0 in 0.1M Tris-HCl buffer because mitochondrial pH is close to this value (30). WT-Grx5 was incubated with increasing concentrations of GSSG for 15 minutes at 20ºC and the products of the reaction were separated by HPLC. Figure 2 A shows that four new peaks corresponding to oxidized forms of the protein appeared. When the mutant proteins were incubated with GSSG, only one new peak appeared (Fig. 2 B and C). The characterization of these peaks is summarized in Table II. From mass spectrometry data it can be deduced that two additional glutathione molecules were present in peak 1 compared to peak 5 (reduced form) while only one additional glutathione molecule was present in both peak 3 and 4. Peak 1 corresponded to a protein glutathiolated at both cysteines while peak 2 was a protein presenting a disulfide bond between both cysteines. This was deduced from the following observations: i) no free thiols were detected when peak 2 protein was incubated with DTNB; ii) it presented a mass of 13,484 Da (as the reduced form); iii) it was also the major peak obtained when reduced Grx5 was incubated with several oxidants such as H₂O₂, cystine or oxidized proteins; iv) peak 2 was the end product obtained either from peak 3 or from peak 4 when these peaks were collected, dried and solubilized at pH 8.0; v) reduced Grx5 (peak 5) was obtained by incubation of peak 2 with DTT. Concerning the monoglutathiolated forms of the protein, our results indicate that peak 3 corresponded to a protein glutathiolated at Cys60 while peak 4 corresponded to a protein glutathiolated at Cys117. According to the pKₐ values previously calculated for Cys60 and Cys117, the pH dependence of the appearance of peak 3 and 4 was consistent with this assumption (Fig 3 A). This was confirmed by analyzing the rate of glutathiolation of C117S and C60S mutant proteins at different pHs (Fig. 3 B).

One interesting result was the observation that the rates of carboxymethylation and glutathiolation did not follow the same pH dependence in the WT protein, while in mutant proteins they were nearly the same (compare figures 1 and 3). Thus,
introduction of the first glutathione in the WT protein may increase the reactivity of the Cys117. This suggested the idea that one glutathione molecule could be transferred from Cys60 to Cys117 in the wild type protein during the reaction, being glutathiolation of Cys117 an intermediate step before formation of the disulfide bond. To test this hypothesis peak 4 was collected, dried, rehydrated with Tris-HCl buffer at pH 8.0, and incubated at 4ºC. Figure 4 shows the percentage of each peak found at different incubation times as determined by HPLC analysis. It can be observed that peak 3 appeared mainly at short incubation times, as it would be expected for an intermediary product of the transformation of peak 4 to peak 2.

Finally, it should be noted that the presence of both cysteines resulted in a higher reactivity of the protein towards GSSG. When Grx5-WT and mutant proteins were incubated for 15 minutes at 20ºC with increasing GSSG concentrations at pH 8.0, oxidation of half of the WT protein required 99 µM GSSG while oxidation of half of C117S and C60S mutant proteins required respectively GSSG concentrations of 206 µM and 1.34 mM (Fig. 2 D). These results reinforced the idea that interaction between both cysteines occurred, and that the presence of Cys117 enhanced Grx5 reactivity. Nevertheless reactivity of Cys117 alone (in the C60S protein) was very poor.

Reduction of Grx5 by GSH- On the basis of their results with Plasmodium falciparum GLP1 monothiolic glutaredoxin, Rahlfts et al. suggested that GSH was unable to reduce monothiolic glutaredoxins (31). This fact would explain the absence of activity of both Grx5 and GLP1 in the HED assay. From the above results it was clear that GSSG strongly reacted with Grx5 promoting the formation of a disulfide bond between both cysteines of the protein. To study whether this disulfide bond could be reduced by GSH, oxidized Grx5 was prepared by incubating 1 mg of protein with 0.5 mM GSSG for 30 minutes as described under Experimental Procedures. This preparation was
incubated with increasing amounts of GSH for 15 minutes at 20°C. Reduction of half of
the protein required 1.4 mM GSH (Fig. 5 A). In addition, we compared the rate of
reduction of the oxidized Grx5 with that of Grx1 from E. coli, a dithiolic glutaredoxin
active on the HED assay. Both proteins were incubated at fixed concentrations of 1 and
2 mM GSH for different times. Figure 5 B shows that even at the shorter incubation
times (30 seconds) the reaction of Grx1 with GSH reached the equilibrium. Instead,
reaction of Grx5 with GSH required 1 hour to reach the equilibrium. Thus, the rate
reduction of Grx5 was at least 20 times slower than that of Grx1. These results
indicated that reduction of Grx5 by GSH can be a limiting step for its thiolreductase
activity. The absence of detectable HED activity in monothiolic glutaredoxins may thus
be related to the unefficient reduction of these proteins.

*Determination of the redox potential of Grx5*- The redox potential of Grx5 was
determined by direct protein-protein equilibration with E. coli Grx1 (25). Reduced Grx5
and oxidized Grx1 were incubated at 25°C under anaerobic conditions. HPLC
separation and quantification of the four protein species was performed after incubation
for 1, 2, 4, 8 and 12 hours (Fig. 6). The redox equilibrium was obtained after 4 hours of
incubation, as indicated by a stable ratio of the four protein species. Same results were
obtained when oxidized Grx1 and reduced Grx5 were used as the starting material.
The redox potential of Grx5 was calculated from the following Nernst equation:

\[ E^{\circ}_{\text{Grx5}} = E^{\circ}_{\text{Grx1}} - \frac{RT}{nF} \ln \left( \frac{[\text{Grx5}_{\text{ox}}][\text{Grx1}_{\text{red}}]}{[\text{Grx5}_{\text{red}}][\text{Grx1}_{\text{ox}}]} \right) \]

The standard redox potential of E. coli Grx1 is -233 mV (25). Analysis of different
mixtures of oxidized and reduced Grx5 and Grx1 resulted in an standard redox
potential of -175 ± 3 mV for Grx5. This result placed Grx5 in an intermediate position
among thiol-disulfide oxidoreductases. Members of this family show very diverse redox
potentials that range from the oxidizing -124 mV of *E. coli* DsbA to the strong reducing -270 mV of *E. coli* thioredoxin. (25).

*Reduction of mixed disulfides in proteins by Grx5*- To test whether Grx5 could participate in deglutathiolation of cysteine residues, rat carbonic anhydrase III was used as a substrate. This protein contains five cysteines. Two of them, cys-186 and cys-181, can be easily glutathiolated *in vitro* when purified protein is incubated with GSSG (32). Glutathiolation has also been described to occur *in vivo* (33, 34). A mutant carbonic anhydrase with cysteine 181 substituted for serine was used for this study. When this mutant protein is incubated with GSSG only cysteine 186 becomes glutathiolated (R.L. Levine personal communication). Reduced and monoglutathiolated carbonic anhydrase were easily separated by HPLC. Furthermore, they did not interfere in the chromatographic separation with any of the forms of Grx5. The glutathiolated form of C181S carbonic anhydrase was prepared by incubating the purified protein with 250µM GSSG for 3 hours at 37°C. Excess glutathione was removed by extensive dialysis against 50 mM Tris-HCl buffer pH 7.5. Equimolar amounts of reduced WT-Grx5 and glutathiolated carbonic anhydrase were incubated at 20°C and separated by HPLC in a C18 column (Fig. 7 A). As shown in Figure 7 B carbonic anhydrase was deglutathiolated in a time dependent manner. Grx5 was converted to the complete oxidized form (disulfide bond). The same experiment was performed with C117S and C60S Grx5 proteins. Both mutants were almost unable to reduce carbonic anhydrase.

**Discussion**

Grx3, 4 and 5 from *S. cerevisiae* were the first described members of a new family of proteins with glutaredoxin signature. These proteins contain one conserved cysteine
residue at the putative active site (12), and they have been found in all types of organisms from bacteria to humans (13). Very few of them have been studied and only two of them have an assigned function. The human PICOT protein has been proposed to be a modulator of the protein kinase C-θ pathway (35). We have recently shown that Grx5 from yeast is located in the mitochondria and is involved in the maturation of Fe/S cluster-containing proteins (18). The glutaredoxin-like protein GLP1 from \textit{P. falciparum} has also been cloned and purified, but it has no specific assigned role (31).

Despite these observations, there was no consistent biochemical data supporting the involvement of monothiolic glutaredoxins in thiol redox reactions and consequently no mechanism of action had been proposed for the members of this family. Bushweller et al. described that mutant dithiolic glutaredoxins lacking the second conserved cysteine residue were still able to catalyse the reduction of the HED-GSH mixed disulfide (6). The mechanism proposed for this reaction (summarized in figure 8 A) involved the formation of a mixed disulfide between glutathione and the cysteine located at the active site. This mixed disulfide could be cleft by GSH yielding reduced glutaredoxin and GSSG. It has been suggested that monothiolic glutaredoxins could follow this same scheme (3). However, this was a controversial issue. First, Rahlfs et al. purified and partially characterized PfGLP1 from \textit{P. falciparum} and concluded that it could not be reduced by GSH (31). However, this was probably because PfGLP1 was already reduced after purification, as occurs with Grx5. Second, neither Grx5 nor PfGLP1 are active in the HED assay, although dithiolic glutaredoxins lacking the C-terminal cysteine are still active in this assay (31, 19).

The results from this work demonstrate that Grx5 is a thiolreductase that can participate in thiol redox reactions. Several evidences support this idea: first, Cys60 presents a low pK\textsubscript{a}, close to the pK\textsubscript{a} values of reactive cysteines in dithiolic glutaredoxins (9, 26, 27); second, Grx5 has the potential to form a mixed disulfide with
glutathione with high affinity; finally, Grx5 has the ability to reduce a glutathiolated protein such carbonic anhydrase, indicating that its redox potential is low enough to act as an electron donor in redox reactions involving oxidized proteins. We propose a mechanism of action for the reduction of mixed disulfides by Grx5 based on the reaction of Grx5 with GSSG (summarized in figure 8 B). First, a mixed disulfide will be formed between Cys60 and glutathione. This would induce a decrease in the Cys117 pK$_a$ value that will trigger the formation of a disulfide bond between both cysteines and yield reduced glutathione. However, it is not clear how Grx5 may be reduced in vivo because the reduction rate of Grx5 by GSH may not be fast enough to allow the efficient reduction of oxidized Grx5. Thus, involvement of other mitochondrial reducing agent(s) in this last step should be considered in further investigations. In this context it is interesting to note that *E. coli* thioredoxin efficiently reduces Grx5 (data not shown). Finally, it should be noted that the absence of activity of Grx5 in the HED assay may be a consequence of its inefficient reduction by GSH, but also of its redox potential that would not be low enough to efficiently reduce the mixed HED-GSH disulfide. The redox potential of Grx5 (-175 mV) is higher than that of dithiolic glutaredoxins, which range from -198 to -233 mV (25). However, it can be low enough to reduce other disulfide bonds, as indicated by our results with glutathiolated carbonic anhydrase.

Another important conclusion derived from this work is the relevance of Cys117 for Grx5 reactivity and the formation of a disulfide bond between both cysteine residues in the polypeptide chain. The influence of this cysteine residue on Grx5 reactivity is clearly observed in the experiments with carbonic anhydrase. The involvement of a second cysteine in the mechanism of action would allow Grx5 to perform the reduction of disulfide bonds in proteins, increasing the number of potential substrates. An hypothetic mechanism for this reaction, based on the mechanism of action of dithiolic glutaredoxins, is presented in Fig 8 C. Another interesting point arises from the observation that Cys117 is only conserved in about half of the monothiolic
glutaredoxins identified so far. As a consequence monothiolic glutaredoxins should be separated in two different classes depending on the presence of this second cysteine residue. The relevance of this non-conserved residue is a nice example of how a single mutation can modulate the reactivity of a polipeptide chain and allow a member of a family of proteins to develop new specific functions in cell metabolism. The relevance of Cys117 was not identified in a previous work where the functional complementation of Grx5 by several mutant forms of the protein (including the C117S variant) expressed in Δgrx5 yeast cells was investigated (13). Although being less efficient than WT-Grx5, the C117S protein may display enough activity to suppress the severe growth defects found in a Δgrx5 strain by the monothiolic mechanism described in figure 8 A.

Genetic and biochemical results obtained with yeast cells depleted in Grx5 have linked this protein to the process of iron-sulfur assembly (18). Now it is clear that its role may be related to its thiolreductase activity. However, its physiological substrate remains unknown. Several steps in the process of Fe/S assembly may require the presence of a thiol reductase. Recent works in this field indicate that the bacterial proteins IscU and IscA (homologous to Isa and Isu proteins in yeast) serve as scaffolds for the assembly of iron/sulfur clusters (36, 37). The first step in this process is a sulfur transfer from the cysteine desulfurase IscS (NifS in yeast) to IscU or IscA (38, 39). Later, iron is incorporated and a transient [2Fe2S] center is formed in IscA/U proteins. Although the exact mechanism is still controversial, it seems clear that reducing equivalents required for this process would be provided by the formation of a disulfide bond between two cysteines in IscA/U and/or IscS proteins (40, 41). Grx5 would be required for the reduction of these cysteine residues and constitute an essential enzyme for the turnover of the whole process. Another possibility may consider that Grx5 would act as a general mitochondrial thiolreductase, being one of the steps in iron-sulfur assembly more dramatically affected for its absence than any other biological process. However, it is important to note that Grx5 is not the most abundant thiolreductase in
mitochondria, where the presence of thioredoxin 3 and Grx2 have also been described (11, 42). Thus, a specific role for Grx5 seems quite possible. Further research will determine whether this specificity is a consequence of Grx5 redox potential or of the recognition by Grx5 of specific regions in target proteins.

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Figure legends

Figure 1. Determination of thiol pK_a values of Grx5. A, Separation by HPLC of reduced and carboxymethylated WT-Grx5 protein. B, pH dependence of the second order rate constant (k_app) of the reaction between reduced WT (□), C60S (●) and C117S (▲) Grx5 proteins and iodoacetamide. The second order rate constant was calculated using the equation given in results.

Figure 2. Reaction of reduced Grx5 with glutathione disulfide. Reduced WT and mutant Grx5 proteins were incubated with increasing GSSG concentrations for 15 minutes at 20ºC in Tris-HCl buffer pH 8.0. The HPLC separation profiles of the products of the reaction of WT-Grx5 with 62.5 µM GSSG (A), C60S-Grx5 with 500 µM GSSG (B) and C117S-Grx5 with 500 µM GSSG (C) are shown. The reduced fraction of WT (▲), C60S (■) and C117S (□) Grx5 proteins at each GSSG concentration is shown in D.

Figure 3. pH dependence of the rate of glutathiolation of Grx5. Reduced WT and mutant proteins were incubated with 250 µM GSSG at different pHs for 5, 10 and 15 minutes and the concentration of each product of the reaction determined by HPLC. The rate of formation of the glutathiolated and oxidized forms of the WT protein is shown in A while that of glutathiolation of C60S (●) and C117S (◊) proteins is shown in B. The different symbols in A correspond to peak 1 (♦), peak 2 (O), peak 3 (▲), peak 4 (□) and to the sum of these four peaks (■) (see also figure 2A).

Figure 4. Transfer of glutathione from Cys60 to Cys117. WT-Grx5 glutathiolated at Cys60, obtained as described in Experimental Procedures, was incubated at 4ºC in 0.1 M Tris-HCl buffer pH 8.0. Reaction was stopped at different times by the addition of trifluoroacetic acid and the relative amount of the different products of the reaction was
determined by HPLC separation (A). The relative amounts of oxidized Grx5 (O) and of Grx5 glutathiolated at Cys60 (■) or Cys117 (▲) are shown in B.

Figure 5. Comparative analysis of the reduction of *S. cerevisiae* Grx5 and *E. coli* Grx1 glutaredoxins by GSH. A, oxidized WT-Grx5 (■) was incubated with increasing amounts of GSH for 15 minutes in Tris-HCl buffer pH 8.0 and the amount of reduced protein determined by HPLC. B, rate of reduction of WT-Grx5 (filled symbols) and Grx1 (open symbols) when incubated at 1mM (■, □) and 2mM (●, O).

Figure 6. HPLC profile of the separation of the reduced and oxidized *S. cerevisiae* Grx5 and *E. coli* Grx1. Reduced Grx5 and oxidized Grx1 were incubated for 4 hours at 25ºC in 200 µl of 100 mM sodium phosphate pH 7.0 plus 1 mM EDTA. The proteins were separated by HPLC as described under Experimental Procedures.

Figure 7. Reduction of carbonic anhydrase III by Grx5. Glutathiolated rat carbonic anhydrase III was incubated with reduced Grx5 (either wild type or mutant proteins) at 20ºC in Tris-HCl buffer pH 8.0. A, HPLC profile of the separation of the oxidized and reduced forms of carbonic anhydrase III (CAIII) and WT-Grx5 after 5 minutes (dashed line) and 60 minutes (solid line) of incubation. B, rate of reduction of glutathiolated carbonic anhydrase by WT-Grx5 (■), C60S-Grx5 (●) and C117S-Grx5 (▲).

Figura 8. Proposed catalytic mechanism of action of Grx5. A, mechanism of action of mutant dithiolic glutaredoxins lacking the C-terminal cysteine in the active site, as proposed by Bushweller et al. (6). B, proposed mechanism of reaction of Grx5 with glutathiolated proteins. C, hypothetic mechanism of action for the reduction of protein disulfide bonds by Grx5 based on the mechanism of action of dithiolic glutaredoxins (described in reference 6).
### Table I

**Biochemical characterization of Grx5 proteins**

| Protein | Free Cysteines<sup>(a)</sup> | pK<sub>a</sub> value | 50% oxidation<sup>(b)</sup> | Redox Potential<sup>(c)</sup> |
|---------|-----------------------------|--------------------|-----------------------------|-----------------------------|
| WT      | 2.2                         | Cys60: 0.098 mM    | Cys117: 0.098 mM            | -175 ± 3 mV                 |
| C60S    | 1.1                         | 8.2 ± 0.1          | 1.34 mM                     |
| C117S   | 1.2                         | 5.0 ± 0.1          | 0.206 mM                    |

a. Number of free cysteine residues after purification, determined with DTNB  
b. GSSG concentration producing oxidation of half of the protein after incubation with Grx5 for 30 minutes at 20ºC at pH 8.0  
c. Redox potential determined by direct equilibria with Grx1 from *E. coli*
Table II  
*Biochemical characterization of peaks obtained after incubation of Grx5 with GSSG*

| Protein | Peak number | Retention time (a) | Mass (b) | Free cysteines (c) | Redox state (d) |
|---------|-------------|--------------------|----------|-------------------|-----------------|
| WT      | Peak 1      | 21.2               | 14,093   | 0.0               | Diglutathiolated |
|         | Peak 2      | 22.6               | 13,483   | 0.0               | Disulfide bond   |
|         | Peak 3      | 23.6               | 13,779   | 0.75              | Cys117 glutathiolated |
|         | Peak 4      | 31.4               | 13,779   | 0.78              | Cys60 glutathiolated |
|         | Peak 5      | 34.4               | 13,484   | 1.58              | Reduced |
| C60S    | Peak 1      | 27.8               | 13,763   | 0.0               | Glutathiolated   |
|         | Peak 2      | 34.2               | N.D. (e) | 0.85              | Reduced |
| C117S   | Peak 1      | 25.6               | 13,762   | 0.0               | Glutathiolated   |
|         | Peak 2      | 27.6               | N.D.     | 0.79              | Reduced |

a. Retention time in minutes.
b. Masses in Daltons, as determined by mass spectrometry.
c. Number of free thiols determined with DTNB after collecting, drying and solubilising in Tris-HCl buffer pH 8.0 the corresponding peak.
d. Redox state of the corresponding Grx5 protein. A difference of 305 Da and 610 Da would be expected upon addition of one and two glutathione molecules respectively.
e. Not determined
Figure 2

A

B

C

D

Reduced Grx5 fraction

\[ \log \text{[GSSG]} \]
Figure 3
Figure 4

(A) Time (min)

(B) Relative amount (%) vs. Time (min)

- Cys17 glutathiolated
- Cys60 glutathiolated
- Didulfide bond
Figure 5

A

% Reduced

log [GSH]

B

% Reduced

Time (min)
Figure 6
Figure 7

A

\[ A_{214} (10^{-1}) \]

\[
\begin{array}{c}
\text{CAIII ox} \\
\text{CAIII red} \\
\text{Grx5 ox} \\
\text{Grx5 red}
\end{array}
\]

\[
\text{Time (min)} \quad 16 \quad 20 \quad 24
\]

B

\[
\begin{array}{c}
\text{Glutathiolated CAIII (µM)} \\
0 \quad 5 \quad 10 \quad 15 \quad 20 \quad 25
\end{array}
\]

\[
\text{Time (min)} \quad 0 \quad 20 \quad 40 \quad 60
\]
Figure 8

A

\[
\begin{align*}
&\text{Pr} \quad \text{S-SG} \\
&\quad \text{SH} \\
&\text{Pr} \quad \text{SH} \\
&\text{Grx} \quad \text{S-SG} \\
&\quad \text{GSSG} \\
&\text{Pr} \quad \text{SH} \\
&\quad \text{Grx} \quad \text{S-SG} \\
&\quad \text{GSH} \\
\end{align*}
\]

B

\[
\begin{align*}
&\text{Pr} \quad \text{S-SG} \\
&\quad \text{SH} \\
&\text{Grx5} \quad \text{S-SG} \\
&\quad \text{S} \quad \text{S-} \\
&\text{Grx5} \quad \text{S-SG} \\
&\quad \text{S} \quad \text{S-} \\
&\text{Pr} \quad \text{SH} \\
&\quad \text{Grx5} \quad \text{S-SG} \\
&\quad \text{GSH} \\
\end{align*}
\]

C

\[
\begin{align*}
&\text{Grx5} \quad \text{S-SG} \\
&\quad \text{S} \quad \text{S-} \\
&\text{Grx5} \quad \text{S-SG} \\
&\quad \text{S} \quad \text{S-} \\
&\text{Pr} \quad \text{SH} \\
&\quad \text{Grx5} \quad \text{S-SG} \\
&\quad \text{GSH} \\
\end{align*}
\]

Reducing agent

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