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Biochemical Characterization of Yeast Mitochondrial Grx5 Monothiol Glutaredoxin*

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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 biochemically characterized previously. Grx5 contains a conserved cysteine residue (Cys-60) and a non-conserved one (Cys-117). 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 wild type Grx5. The pK_a values obtained by titration of mutant proteins with iodoacetamide at different pHs were 5.0 for Cys-60 and 8.2 for Cys-117. When Grx5 was incubated with glutathione disulfide, a transient mixed disulfide was formed between glutathione and the cystein 60 of the protein because of its low pK_a. Binding of glutathione to Cys-60 promoted a decrease in the Cys-117 pK_a value that triggered the formation of a disulfide bond between both cysteine residues of the protein, indicating that Cys-117 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 the 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 wild type Grx5 efficiently reduced a glutathiolated protein used as a substrate indicated that Grx5 may act as a thiol reductase inside the mitochondria.

Glutaredoxins (Grx)[1] are small proteins with thiol reductase activity that are required for maintaining protein cysteines in reduced form. In contrast to thioredoxins, glutaredoxins require the reduced form of glutathione, GSH, as the 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 already been biochemically characterized (9–11). On the basis of sequence analysis, a new family of monothiolic glutaredoxins has been described recently. 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 biochemically characterized properly.

Three monothiolic glutaredoxins are found in yeast (Grx3/4/5). No clear phenotypes have been described in yeast cells lacking Grx3 and Grx4 and, consistent with this, 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 or 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 (Cys-60) 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 (Cys-117). 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 thiol reductase. Also, the specific role of Grx5 in iron-sulfur biogenesis is still not clear. Shenton *et al.* (19) showed that, in cells lacking Grx5, the cytosolic enzyme glyceraldehyde-3-phosphate-dehydrogenase was glutathiolated, and they suggested that Grx5 could work as a deglutathiolase. However the recent finding that Grx5 is a mitochondrial enzyme (18) suggests that this glutathiolation may be related to the oxidative stress conditions generated by iron accumulation in Δ*grx5* cells rather 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 the cysteine pK_a value and redox potential. Based on these results, we propose a mechanism of action for the Grx5 protein. This is the first characterization of a monothiolic glutaredoxin and constitutes the first evidence that these proteins can work as thioloxidoreductases.

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[1] The abbreviations used are: Grx, glutaredoxin; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; HED, β-hydroxyethyl disulfide; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; WT, wild type; HPLC, High performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

EXPERIMENTAL PROCEDURES

Materials—GSH, 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 (National Institutes of Health, Bethesda, MD).

Strains and Plasmids—Plasmid pMM192 contains the *GRX5* open reading frame without the region coding from amino acids 2–29 (PCR-amplified from *S. cerevisiae* genomic DNA), cloned between the *Nde*I and *Bam*H 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 of 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 min 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 Ref. 2. Dehydroascorbate reductase activity and glutathione peroxidase activities were performed according to Refs. 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 dithiothreitol. Reduced and carboxymethylated Grx5 proteins 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. The reaction was stopped by the addition of 2 μl of 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 of reduced protein (150 μM concentration) with 0.5 mM GSSG for 30 min 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 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% was in the reduced form. WT-Grx5 glutathiolated at Cys-60 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 Cys-60 (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 solubilized 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). A 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/ionization, 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 reduced *S. cerevisiae* Grx5 and 50 μM oxidized *E. coli* Grx1 (and vice versa) in 0.1 M sodium phosphate buffer, 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 gas-tight 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 of 30–50% 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). 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 comparison with previous articles, original sequence number positions have been maintained throughout the text. The wild type and mutant Grx5 proteins were overexpressed in *E. coli* cells and purified by a two-step method that includes size exclusion and ionic exchange chromatography. The resulting proteins were >99% pure and showed an apparent molecular mass 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 catalyzed by dithiolic glutaredoxins. The most widely used form to asses glutaredoxin activity is the glutathione:HED transhydrogenase assay. In this assay, glutaredoxin catalyzes the reduction of a mixed disulfide between glutathione and HED (2). Dithiolic glutaredoxins lacking one of the two conserved cysteines are still capable of catalyzing this reaction (6). Glutaredoxin activity of WT Grx5, C117S Grx5, and C60S Grx5 was assayed with the HED assay. No activity could be detected

TABLE I
Biochemical characterization of Grx5 proteins

Protein	Free cysteines[a]	pK_a value		50% oxidation[b]	Redox potential[c]
		Cys-60	Cys-117		
				mM	
WT	2.2			0.098	−175 ± 3 mV
C60S	1.1		8.2 ± 0.1	1.34	
C117S	1.2	5.0 ± 0.1		0.206	

[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 20°C at pH 8.0.
[c] Redox potential determined by direct equilibria with Grx1 from *E. coli*.

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 Cys-60 and Cys-117 pK_a Value—Reactivity of thiol groups in proteins depends highly on its pK_a value. Active cysteines from dithiolic glutaredoxins have pK_a values close to 4 (9, 26, 27). 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 iodoacetamide at pH values between 3 and 11. Reaction was stopped at different times by the addition of 10 mM dithiothreitol, 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 Equation 1,

$$K_{app} = [Grx5_{cmc}]/(t \times [Grx5_o] \times [Grx5_{red}]) \quad \text{(Eq. 1)}$$

and plotted against pH (Fig. 1*B*) ($[Grx_0]$ = initial concentration of Grx5). Reaction rates showed a sigmoidal dependence on pH value at pH values of ~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 ionization of Cys-60 and Cys-117. Using the Henderson-Hasselbalch equation (28, 29), thiol pK_a values of 5.0 ± 0.1 and 8.2 ± 0.1 were calculated for Cys-60 and Cys-117, respectively (Table I).

Reaction of Grx5 with GSSG—Reactivity of reduced Grx5 with GSSG was tested at pH 8.0 in 0.1 M Tris-HCl buffer, because mitochondrial pH is close to this value (30). WT Grx5 was incubated with increasing concentrations of GSSG for 15 min at 20 °C, and the products of the reaction were separated by HPLC. Fig. 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 with peak 5 (reduced form), whereas only one additional glutathione molecule was present in both peak 3 and peak 4. Peak 1 corresponded to a protein glutathiolated at both cysteines, whereas 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

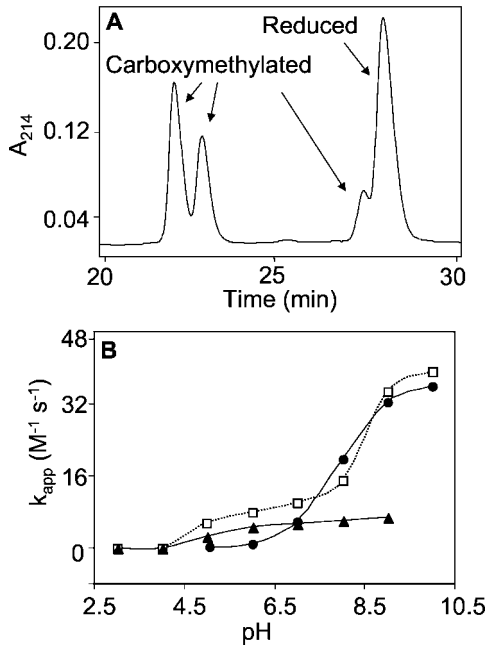

FIG. 1. **Determination of thiol pK_a values of Grx5.** *A*, separation by HPLC of reduced and carboxymethylated WT Grx5 protein. *B*, pH dependence of the second order rate constant (k_{app}) of the reaction between reduced WT (□), C60S (●), and C117S (▲) Grx5 proteins and iodoacetamide. The second order rate constant was calculated using Equation 1.

when the peak 2 protein was incubated with DTNB. (ii) Peak 2 presented a mass of 13,484 Da (as the reduced form). (iii) Peak 2 was also the major peak obtained when reduced Grx5 was incubated with several oxidants such as H_2O_2, cystine, or oxidized proteins. (iv) Peak 2 was the end product obtained either from peak 3 or peak 4 when these peaks were collected, dried, and solubilized at pH 8.0; and (v) reduced Grx5 (peak 5) was obtained by incubation of peak 2 with dithiothreitol. Concerning the monoglutathiolated forms of the protein, our results indicate that peak 3 corresponded to a protein glutathiolated at Cys-60, whereas peak 4 corresponded to a protein glutathiolated at Cys-117. According to the pK_a values previously calculated for Cys-60 and Cys-117, the pH dependence of the appearance of peaks 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, although in mutant proteins they were nearly the same (compare Figs. 1 and 3). Thus, introduction of the first glutathione in the WT protein may increase the reactivity of the Cys-117. This suggested the idea that one glutathione molecule could be transferred from Cys-60 to Cys-117 in the wild type protein during the reaction, and glutathiolation of Cys-117 would be 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 4 °C. Fig. 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 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 toward GSSG. When WT Grx5 and mutant proteins were incubated for 15 min at 20 °C with increasing GSSG concentrations at pH 8.0, oxi-

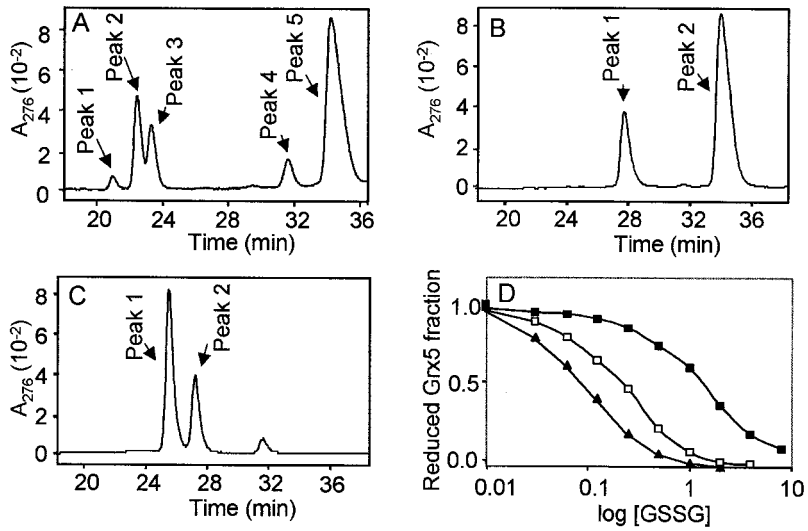

FIG. 2. **Reaction of reduced Grx5 with glutathione disulfide.** Reduced WT and mutant Grx5 proteins were incubated with increasing GSSG concentrations for 15 min at 20 °C in Tris-HCl buffer, pH 8.0. The HPLC separation profiles of the products of the reaction of WT Grx5 with 62.5 μM GSSG (*A*), C60S 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*.

TABLE II
Biochemical characterization of peaks obtained after incubation of Grx5 with GSSG

Protein	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	Cys-117 glutathiolated
Peak 4	31.4	13,779	0.78	Cys-60 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.[e]	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 solubilizing the corresponding peak in Tris-HCl buffer, pH 8.0.
[d] Redox state of the corresponding Grx5 protein. A difference of 305 and 610 Da would be expected upon addition of one and two glutathione molecules, respectively.
[e] Not determined.

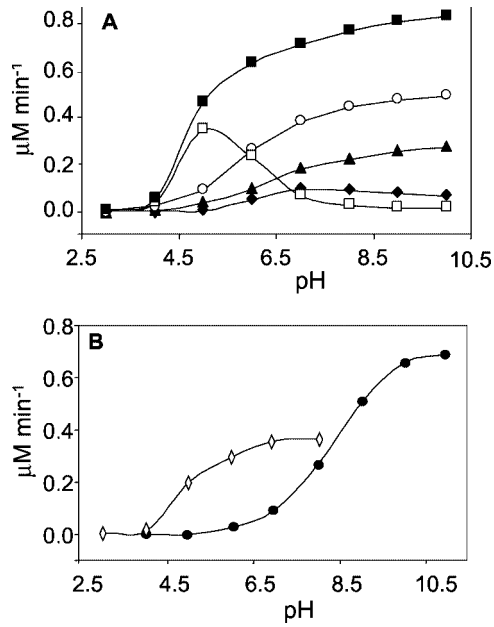

FIG. 3. **pH dependence of the rate of glutathiolation of Grx5.** Reduced WT and mutant proteins were incubated with 250 μM GSSG at different pHs for 5, 10, and 15 min, and the concentration of each product of the reaction was determined by HPLC. The rate of formation of the glutathiolated and oxidized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

dation of half of the WT protein required 99 μM GSSG, whereas oxidation of half of the C117S and C60S mutant proteins required GSSG concentrations of 206 μM and 1.34 mM, respectively (Fig. 2*D*). These results reinforced the idea that interaction between both cysteines occurred and that the presence of Cys-117 enhanced Grx5 reactivity. Nevertheless reactivity of Cys-117 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.* (31) suggested that GSH was unable to reduce monothiolic glutaredoxins. 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 min as described under "Experimental Procedures." This preparation was incubated with increasing amounts of GSH for 15 min 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. Fig. 5*B* shows that, even at the shorter incubation times (30 s), the reaction of Grx1 with GSH reached equilibrium. On the other hand, the reaction of Grx5 with GSH required 1 h to reach equilibrium. Thus, the reduction rate 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 thiol reductase activity. The absence of detectable HED activity in monothiolic glutaredoxins may thus be related to the inefficient 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.

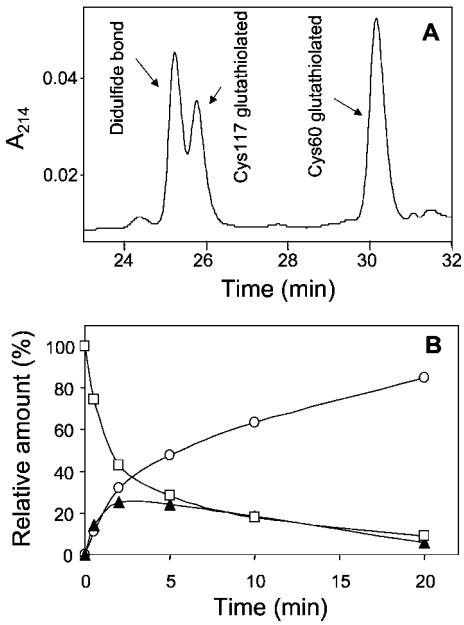

FIG. 4. **Transfer of glutathione from Cys-60 to Cys-117.** WT Grx5 glutathiolated at Cys-60, obtained as described under "Experimental Procedures," was incubated at 4 °C in 0.1 M Tris-HCl buffer, pH 8.0. Reaction was stopped at different times by the addition of trifluoroacetic acid, and the relative amount of the different products of the reaction was determined by HPLC separation (*A*). The relative amounts of oxidized Grx5 (○) and Grx5 glutathiolated at Cys-60 (□) or Cys-117 (▲) are shown in *B*.

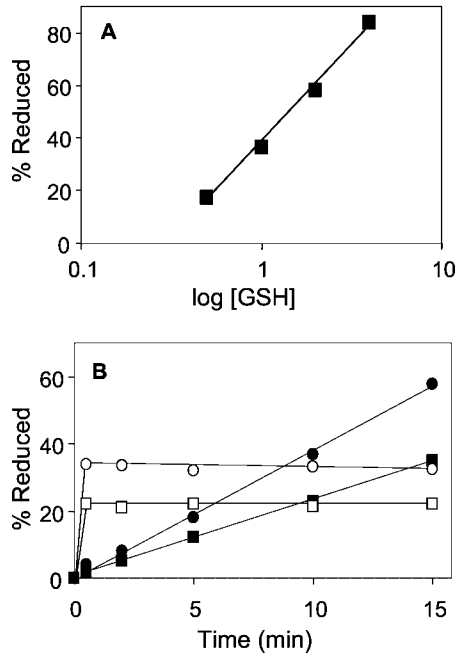

FIG. 5. **Comparative analysis of the reduction of *S. cerevisiae* Grx5 and *E. coli* Grx1 glutaredoxins by GSH.** *A*, oxidized WT Grx5 (■) was incubated with increasing amounts of GSH for 15 min in Tris-HCl buffer, pH 8.0, and the amount of reduced protein was determined by HPLC. *B*, rate of reduction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

HPLC separation and quantification of the four protein species was performed after incubation for 1, 2, 4, 8, and 12 h (Fig. 6). The redox equilibrium was obtained after 4 h of incubation as indicated by a stable ratio of the four protein species. The same results were obtained when oxidized Grx1 and reduced Grx5 were used as the starting material. As shown in Equation 2,

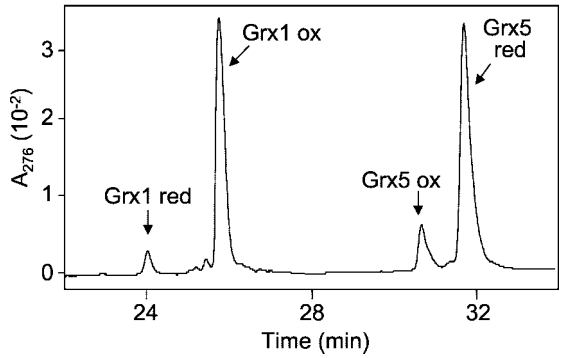

FIG. 6. **HPLC profile of the separation of the reduced (*red*) and oxidized (*ox*) *S. cerevisiae* Grx5 and *E. coli* Grx1.** Reduced Grx5 and oxidized Grx1 were incubated for 4 h at 25 °C in 200 μl of 100 mM sodium phosphate, pH 7.0, plus 1 mM EDTA. The proteins were separated by HPLC as described under "Experimental Procedures."

$$E^{\circ\prime}_{Grx5} = E^{\circ\prime}_{Grx1} - (RT/nF)\ln([Grx5_{ox}][Grx1_{red}]/[Grx5_{red}][Grx1_{ox}]) \quad \text{(Eq. 2)}$$

the standard state redox potential of Grx5 at 25°C ($E^{\circ\prime}_{Grx5}$) was calculated from a Nernst equation. 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 the 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.[2] Reduced and monoglutathiolated carbonic anhydrase were easily separated by HPLC. Furthermore, these forms of carbonic anhydrase 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 250 μM GSSG for 3 h 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 Fig. 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 members of a new family of proteins with glutaredoxin signature to be described. 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

[2] R. L. Levine, personal communication.

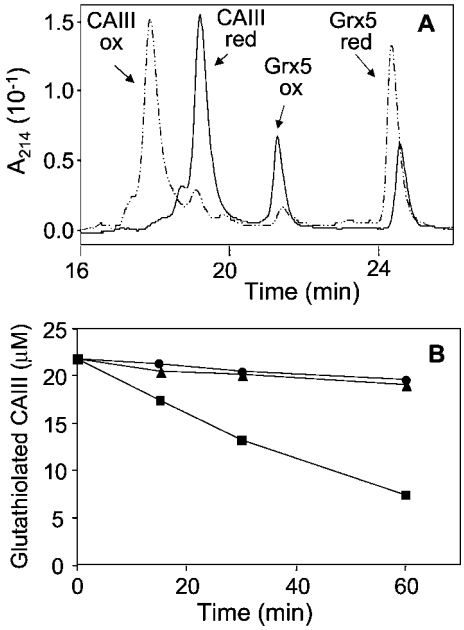

FIG. 7. **Reduction of carbonic anhydrase III by Grx5.** Glutathiolated rat carbonic anhydrase III was incubated with reduced Grx5 (either wild type or mutant proteins) at 20 °C in Tris-HCl buffer, pH 8.0. *A*, HPLC profile of the separation of the oxidized (*ox*) and reduced (*red*) forms of carbonic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

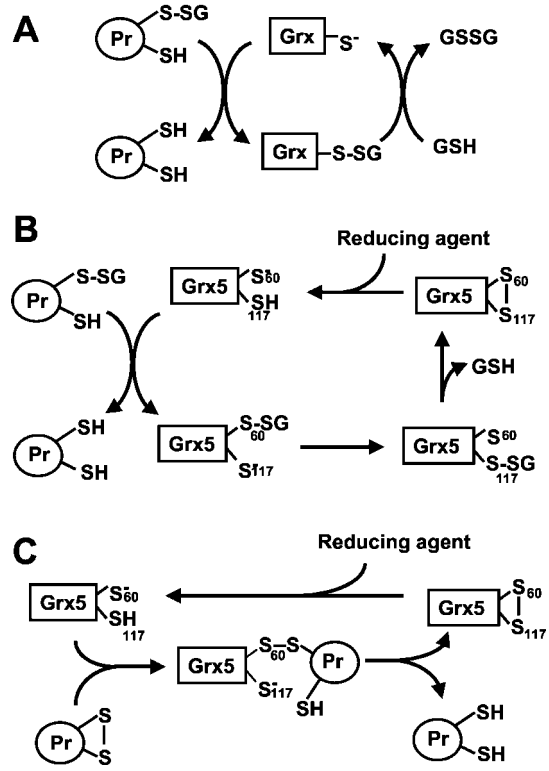

FIG. 8. **Proposed catalytic mechanism of action of Grx5.** *A*, mechanism of action of mutant dithiolic glutaredoxins lacking the C-terminal cysteine in the active site, as proposed by Bushweller *et al.* (6). *B*, proposed mechanism of reaction of Grx5 with glutathiolated proteins. *C*, hypothetic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

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 involved in the maturation of Fe-S cluster-containing proteins (18). The glutaredoxin-like protein GLP1 from *P. falciparum* has also been cloned and purified but 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.* (6) reported that mutant dithiolic glutaredoxins lacking the second conserved cysteine residue were still able to catalyze the reduction of the HED-GSH mixed disulfide. The mechanism proposed for this reaction (summarized in Fig. 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.* (31) purified and partially characterized PfGLP1 from *P. falciparum* and concluded that it could not be reduced by GSH. 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 thiol reductase that can participate in thiol redox reactions. Several pieces of evidence support this idea. First, Cys-60 presents a low pK_a, close to the pK_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; and finally, Grx5 has the ability to reduce a glutathiolated protein such as 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 Fig. 8*B*). First, a mixed disulfide will be formed between Cys-60 and glutathione. This would induce a decrease in the Cys-117 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, which would not be low enough to efficiently reduce the mixed HED-GSH disulfide. The redox potential of Grx5 (−175mV) 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 Cys-117 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. A 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 Cys-117 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 polypeptide chain and allow a member of a family of proteins to develop new specific functions in cell metabolism. The relevance of Cys-117 was not identified in a previous work (13) in which the functional complementation of Grx5 by several mutant forms of the protein (including the C117S variant) expressed in Δ*grx5* yeast cells was investigated. Although it is less efficient than WT Grx5, the C117S protein may display enough activity to suppress the severe growth defects found in a Δ*grx5* strain by the monothiolic mechanism described in Fig. 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 thiol reductase 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 the 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 be that Grx5 would act as a general mitochondrial thiol reductase. Its absence would affect the assembly of iron-sulfur centers more dramatically than any other biological process. However, it is important to note that Grx5 is not the most abundant thiol reductase in mitochondria, wherein 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 the recognition by Grx5 of specific regions in target proteins.

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Enzyme Catalysis and Regulation:

Biochemical Characterization of Yeast Mitochondrial Grx5 Monothiol Glutaredoxin

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