THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 40, Issue of October 4, pp. 37590–37596, 2002
Printed in U.S.A.

Structure-Function Analysis of Yeast Grx5 Monothiol Glutaredoxin Defines Essential Amino Acids for the Function of the Protein*

Received for publication, February 19, 2002, and in revised form, July 16, 2002
Published, JBC Papers in Press, July 22, 2002, DOI 10.1074/jbc.M201688200

Gemma Bellí‡, Julio Polaina§, Jordi Tamarit‡, María Angeles de la Torre‡, María Teresa Rodríguez-Manzaneque‡, Joaquim Ros‡, and Enrique Herrero‡¶

From the ‡Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, Rovira Roure 44, 25198-Lleida, Spain and §Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Cientificas, 46980-Paterna, Valencia, Spain

Grx5 defines a family of yeast monothiol glutaredoxins that also includes Grx3 and Grx4. All three proteins display significant sequence homology with proteins found from bacteria to humans. Grx5 is involved in iron/sulfur cluster assembly at the mitochondria, but the function of Grx3 and Grx4 is unknown. Three-dimensional modeling based on known dithiol glutaredoxin structures predicted a thioredoxin fold structure for Grx5. Positionally conserved amino acids in this glutaredoxin family were replaced in Grx5, and the effect on the biological function of the protein has been tested. For all changes studied, there was a correlation between the effects on several different phenotypes: sensitivity to oxidants, constitutive protein oxidation, ability for respiratory growth, auxotrophy for a number of amino acids, and iron accumulation. Cys^{60} and Gly^{61} are essential for Grx5 function, whereas other single or double substitutions in the same region had no phenotypic effects. Gly^{115} and Gly^{116} could be important for the formation of a glutathione cleft on the Grx5 surface, in contrast to adjacent Cys^{117}. Substitution of Phe^{50} alters the β-sheet in the thioredoxin fold structure and inhibits Grx5 function. None of the substitutions tested affect the structure at a significant enough level to reduce protein stability.

Glutaredoxins are thiol oxidireductases that catalyze redox reactions involving reduced glutathione as a hydrogen donor for the reduction of protein disulfides (dithiol mechanism of action) or glutathione-protein-mixed disulfides (monothiol mechanism of action) (see Refs. 1 and 2 for review). Previously described glutaredoxins are small proteins (about 10 kDa) with a conserved active site that includes two cysteine residues (Cys-Pro-Tyr-Cys). Site-directed mutagenesis (3–5) has demonstrated that both cysteine residues in the active site are required for the dithiol reaction. In contrast, the amino-terminal cysteine is sufficient to catalyze the deglutathionylation of the reduced glutathione-mixed disulfides that are formed under oxidative stress conditions (5).

Three-dimensional structures of oxidized and reduced forms of viral, bacterial, and mammalian glutaredoxins and also of reduced glutathione-glutaredoxin complexes have been identified using x-ray crystallography (6, 7) or nuclear magnetic resonance spectroscopy (8–14). These studies have revealed which residues, apart from those at the active site, are important for stable interactions between glutathione and the glutaredoxin molecule (10, 13, 14). Dithiol glutaredoxins are members of the thioredoxin superfamily (15, 16) along with at least five other classes of proteins that interact with cysteine-containing substrates (thioredoxins, DbsA, protein disulfide isomerases, glutathione *S*-transferases, and glutathione peroxidases). This superfamily shares a structural motif (called the thioredoxin fold or αβα fold) formed by a four or five-stranded β-sheet (with parallel and antiparallel strands) surrounded by three or more α-helices distributed on either side of the β-sheet (15, 16). Thioredoxins share with glutaredoxins the ability to reduce disulfides, although the former directly use NADPH as hydrogen donor (1).

Dithiol glutaredoxins participate in a large number of functions in prokaryotic and eukaryotic cells, including the activation of ribonucleotide reductase (17) and 3′-phosphoadenylylsulfate reductase (18), reduction of ascorbate (19), regulation of the DNA binding activity of nuclear factors (20), and neuronal protection against dopamine-induced apoptosis (21, 22). A family of three *Saccharomyces cerevisiae* proteins (Grx3, Grx4, and Grx5) has been described (23) that has significant homology with dithiol glutaredoxins, preferentially at the carboxyl-terminal region of the molecules. The absence of any of these proteins leads to a decrease in cellular glutaredoxin activity, even though they do not contain the conserved active site of classic dithiol glutaredoxins. Instead, these proteins contain the conserved Cys-Gly-Phe-Ser motif at the amino-terminal region (23). This is the only cysteine residue found in Grx3 and Grx4, whereas Grx5 has an additional cysteine at the carboxyl-terminal moiety. From these data, it has been proposed that Grx3, Grx4, and Grx5 constitute a family of monothiol glutaredoxins in yeast (23). However, although there is a high degree of homology among them, these three proteins seem to carry out different cellular functions: the absence of Grx5 causes dramatic sensitivity to oxidants and growth defects in minimal medium, whereas no clear phenotypes are observed when Grx3 or Grx4 is absent. More recently, it has been shown that Grx5 is located at the mitochondria and involved in the biogenesis of iron/sulfur clusters (24). Accumulation of cellular iron when Grx5 is absent could lead to protein oxidation and sensitivity to external oxidants. Available data about Grx3 and Grx4 indicate that they are not located in the mitochondria (24).

Proteins homologous to yeast monothiol glutaredoxins exist in all types of organisms from bacteria to humans (23, 25, 26). The human homologue (PICOT[1] protein) has been proposed as

* This work was supported by Grants PB97-1468-CO2-01 (to E. H.), BIO2000-1297-C02-01 (to J. P.), and PB97-1456 (to J. R.) from the Spanish Ministry of Education and Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 34-973-702409; Fax: 34-973-702426; E-mail: enric.herrero@cmb.udl.es.

[1] The abbreviations used are: PICOT, protein kinase C-interacting cousin of thioredoxin; YPD, 1% yeast extract, 2% peptone, and 2% dextrose.

a negative regulator of protein kinase C-θ in the pathway leading to activation of the activator protein 1 and nuclear factor κB transcription factors (27). The conserved region has been termed PICOT homology domain, and in Grx5, it corresponds to the majority of the peptide (23, 25). Human PICOT, yeast Grx3 and Grx4, and other eukaryotic homologous proteins possess amino-terminal extensions of PICOT homology domain. These extensions have signatures characteristic of thioredoxins or dithiol glutaredoxins that do not encompass the oxidoreductase active site (25). All these observations support the differential roles displayed by monothiol glutaredoxins regardless of their structural similarities.

In this work, we show that Grx5 defines a ubiquitous family of proteins whose members are present in most types of organisms and are characterized by the presence of a thioredoxin fold structure. We also demonstrate the essential biological roles of a number of conserved amino acid residues, such as a cysteine located at the previously proposed active site in the amino-terminal region and a pair of glycines in the carboxyl-terminal region.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Amino Acid Replacements—CML235 (*MAT***a** *ura3-52 leu2Δ1 his3Δ200*) was used as wild-type strain. MML19 is an isogenic Δ*grx5*::*kanMX4* derivative of CML235 (23). MML160 was obtained from the latter by chromosomal integration of the YIplac211 vector (integrative, *LEU2* marker) (28). MML161 was constructed similarly, although a YIplac211-derived plasmid (pMM25) with *GRX5* expressed under its own promoter was integrated at *LEU2*. Other strains listed in Table I resulted from integration of pMM25-derived plasmids carrying the indicated point mutations at the mutant *leu2* locus of MML19.

The following plasmids contain the cloned *GRX5* open reading frame (with the type of mutation in the translation product indicated in parentheses) without further upstream or downstream sequences, under the control of the doxycycline-regulatable $tetO_7$ promoter in plasmid pCM190 (29): pCM319 (wild-type *GRX5*), pMM176 (F50E), pMM113 (C60S), pMM155 (G61V), pMM127 (G115V), and pMM112 (G116V). These plasmids were then transformed into strain CML276 (30), which carries the doxycycline-inducible *tetR′-SSN6* repressor gene, to determine the stability of the Grx5 wild-type protein and the amino acid-substituted derivatives.

Point mutations in the *GRX5* open reading frame that yielded the different amino acid replacements were constructed by the ExSite method (31), using either pMM25 or pCM319 DNA as a template. Oligonucleotides for PCR amplification were designed 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 it. Successful introduction of the mutations was confirmed by DNA sequencing.

Growth Conditions and Determination of Sensitivity to Menadione—Cells were usually grown at 30 °C in rich YPD medium. Plasmid-bearing transformants were grown in synthetic complete medium (32) without the selective auxotrophic requirement. Plates of synthetic defined medium (0.67% yeast nitrogen base, 2% glucose, and auxotrophic requirements) were used to test mutant growth. Cells growing exponentially in YPD medium at 30 °C (about 2×10^7 cells/ml) were treated with menadione (10 mM) to determine sensitivity to it. After treatment, 1:5 serial dilutions were made, and drops were spotted onto YPD plates. Growth was recorded after 2 days of incubation at 30 °C.

Analytical Methods—Protein carbonyl levels in crude cell extracts were quantified according to the dinitrophenylhydrazine derivatization method (23). Total iron cell content was determined under reducing conditions, after acid digestion of cells using 3% nitric acid (33). Mean cell volumes were determined in nonfixed cells using a Coulter Z2 counter to calculate cell iron concentration.

Determination of Protein Stability—Exponentially growing cells that overexpressed *GRX5* under the control of the $tetO_7$ promoter were added to doxycycline (20 μg/ml) to interrupt gene expression. At successive times, samples were taken, and total cell extracts were prepared (24). Western analyses were carried out using a polyclonal antibody prepared against *Escherichia coli*-expressed full-length Grx5 protein at a 1:2500 dilution (24). In each experiment, equal amounts of total cell protein (60 μg) were run in parallel for each sample. The relative level of Grx5 was determined from the intensity of the Grx5 band signal, following quantification with the Lumi-Imager equipment (Roche Molecular Biochemicals) software.

Grx5 Structure Prediction—Protein structures related to Grx5 were identified by applying the GenThreader fold recognition method (34). Two structures offering maximal probability of correct match were selected and collected from the Protein Data Bank (Protein Data Bank accession numbers 1KTE and 3GRX). They respectively corresponded to pig liver thioltransferase (7) and *E. coli* Grx3 glutaredoxin (14). Different models of Grx5 based on these protein structures were obtained using the Swiss-Model server (35, 36). Protein structures were analyzed applying the Swiss-PDB Viewer program (35).

Protein Sequence Analyses—Grx5 amino acid sequence was compared with proteins from the Institute for Chemical Research (Kyoto University, Kyoto, Japan) and Swiss Protein Databases using FASTA analysis provided by the two servers. Multiple sequences were aligned using the ClustalW program (37) and the tools provided by the European Bioinformatics Institute. Internal gaps were not eliminated, and the Blosum80 matrix option was used for alignment.

RESULTS

Yeast Grx5 Is a Member of a Ubiquitous Family of Proteins Sharing the PICOT Domain—Yeast Grx5 has been characterized as a monothiol glutaredoxin-like protein whose amino acid sequence displays extensive homology (particularly at what have been designated its amino-terminal and carboxyl-terminal regions) with a family of proteins whose members are present in all living organisms from bacteria to humans (23, 25). The carboxyl-terminal region also has significant homology with classic dithiol glutaredoxins (23). To extend these initial studies, the Institute for Chemical Research and Swiss Protein Databases were searched for proteins with the highest homology with Grx5 (*E* value cutoff, $1 \times e^{-10}$), using FASTA analysis. We only considered proteins that retained the putative active site CGFS sequence in the amino-terminal region (23) for comparison. The 35 protein sequences with the highest similarity score with Grx5 were then aligned using the ClustalW program (Fig. 1). A putative Grx5 homologue from *Candida albicans*, as deduced from the genome sequence of the latter organism, was also included for comparison. Extensions at the amino-terminal and carboxyl-terminal ends (that are present only in some of the family members (see below)) were omitted for the alignment. The existence of two amino-terminal and carboxyl-terminal regions with extensive homology (23) (separated by a less well-conserved region with a slightly variable length) was confirmed in this extended study.

Most multicellular eukaryotic members of the Grx5 family have large amino-terminal extensions. This is also the case for the *S. cerevisiae* Grx3 and Grx4 glutaredoxins and for one of the two sequences in fission yeast (Fig. 1). This amino-terminal extension includes a highly conserved duplication of the region shown in Fig. 1 in the cases of human and rat species and in one of the two mouse species (Q9JLZ2M) (Ref. 25 and this study). Interestingly, the *Arabidopsis thaliana* protein Q9ZPH2A (but not other members of the same protein family in this plant species) contains three conserved domains in tandem, but only the most carboxyl-terminal of these is shown in the Fig. 1 alignment. On the other hand, *S. cerevisiae* Grx5, the *C. albicans* Grx5 homologous protein, the other fission yeast protein, and all bacterial members of the family have shorter versions of the protein without amino-terminal extensions.

The domain shown in the alignments is almost totally coincident with the PICOT homology domain region named after the human Grx5 homologue (25, 27). Our study shows that this domain may be shared by proteins from prokaryotes (both Archaea and bacteria) and eukaryotes. These proteins may have divergent functions and different cellular locations.

A Thioredoxin Fold Structure Is Predicted for Grx5 Glutaredoxin—Grx5 has sequence similarity with dithiol glutaredoxins, mostly at the carboxyl-terminal moiety (23). The three-

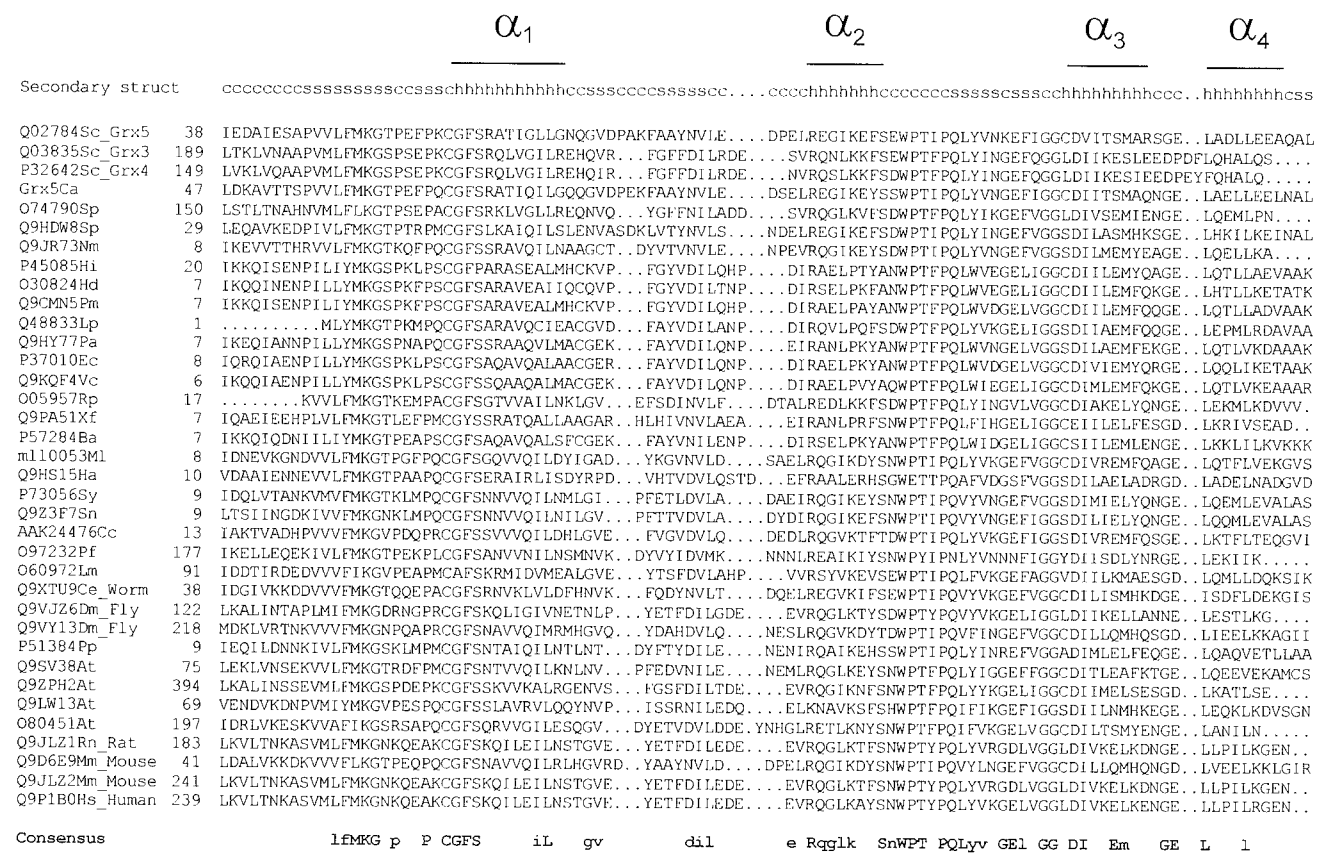


FIG. 1. **ClustalW alignment of proteins displaying homology to *S. cerevisiae Grx5*.** Amino-terminal and carboxyl-terminal extensions were not considered for the alignment. Protein species are named according to their Swiss-Prot accession number, except for ml10053, which corresponds to the Institute for Chemical Research accession number. The *number* at the *left* of the sequences indicates the position of the first amino acid residue shown. The *top row* indicates the predicted secondary structure. The four predicted α-helix regions are named according to the nomenclature for thioredoxin fold structure (15). Abbreviations for organisms were as follows: *Sc*, *S. cerevisiae*; *Ca*, *C. albicans*; *Sp*, *Schizosaccharomyces pombe*; *Nm*, *Neisseria meningitidis*; *Hi*, *Haemophilus influenzae*; *Hd*, *Haemophilus ducreyi*; *Pm*, *Pasteurella multocida*; *Lp*, *Legionella pneumophila*; *Pa*, *Pseudomonas aeruginosa*; *Ec*, *E. coli*; *Vc*, *Vibrio cholerae*; *Rp*, *Rickettsia prowazekii*; *Xf*, *Xylella fastidiosa*; *Ba*, *Buchnera aphidicola*; *Ml*, *Mesorhizobium loti*; *Ha*, *Halobacterium* sp.; *Sy*, *Synechocystis* sp.; *Sn*, *Synechococcus* sp.; *Cc*, *Caulobacter crescentus*; *Pf*, *Plasmodium falciparum*; *Lm*, *Leishmania major*; *Ce*, *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster*; *Pp*, *Porphyra purpurea*; *At*, *A. thaliana*; *Rn*, *Rattus norvegicus*; *Mm*, *Mus musculus*; and *Hs*, *Homo sapiens*.

dimensional structure of a number of dithiol glutaredoxins was already known, from either x-ray crystallography or NMR spectroscopy studies. Structures in the Protein Data Bank were used to construct a three-dimensional model for Grx5. Two protein structures, pig liver thioltransferase (1KTE) (7) and *E. coli* Grx3 glutaredoxin (3GRX) (14), yielded useful models for Grx5. Amino acid sequences of these two proteins show 28% and 29% identity to Grx5, respectively. Models based both individually and simultaneously on 1KTE and 3GRX yielded almost identical results for a large, carboxyl-terminal part of the protein, which represented about two-thirds of the sequence. Differences in the amino-terminal part were attributed to the poor quality of the 3GRX-based model because homology of *E. coli* Grx3 to Grx5 is low in this region. The 1KTE structure (Fig. 2*A*) was therefore finally taken as the basis for the Grx5 model (Fig. 2*B*), which is considered to provide a valid representation of the Grx5 protein structure. The proposed model shows an $\alpha\beta\alpha$ fold typical of thiol oxidoreductases and other enzymes from the superfamily (15). Four α-helix segments are predicted: amino acids 61–71 (α_1), 92–98 (α_2), 117–125 (α_3), and 129–135 (α_4) (Fig. 1, *top row*). For comparison, Fig. 2*C* shows the structure of phage T4 glutaredoxin (1ABA) (6), which is one of the simplest (structurally speaking) representatives of the thioredoxin fold superfamily. The model shows that the similarity between Grx5 and other proteins from the superfamily extends to the amino-terminal moiety of the molecule.

Cys^{60}, but Not Cys^{117}, Is Essential for the Biological Activity of Grx5—Grx5 contains two cysteine residues at positions 60 and 117. The first is part of a conserved sequence common to all members of the family (Ref. 23; Fig. 1). It is exposed at the surface of Grx5 between a β-strand and α_1 (Fig. 3). Cys^{117} is only present in certain family members (Fig. 1), although many dithiol glutaredoxins also contain a cysteine residue in an equivalent position (23). This cysteine is the first α_3 residue and follows two glycine residues that are conserved in all the dithiol and monothiol glutaredoxins (Fig. 3; Ref. 23). Cys^{60} and Cys^{117} were separately substituted by serine residues, and the biological effects of these mutations were studied after reintroducing the respective mutant Grx5 forms into a strain that was devoid of the wild-type version. Initially, two phenotypes were studied that had been shown to be affected in null *grx5* mutants (23): sensitivity to oxidative stress induced by menadione, and defective growth in synthetic defined medium. Cells with the C60S mutation in Grx5 behaved like those without the protein, whereas those with the C117S change did not differ with respect to wild-type cells (Fig. 4*B*). Therefore, the absence of Cys^{60} annuls the biological activity of Grx5, whereas Cys^{117} is not essential for it. These results demonstrate that Cys^{60} is the active residue for the oxidoreductase reaction and confirm observations for two dithiol glutaredoxins, pig liver thioltransferase (4) and *E. coli* Grx3 (14), where the cysteine residues at

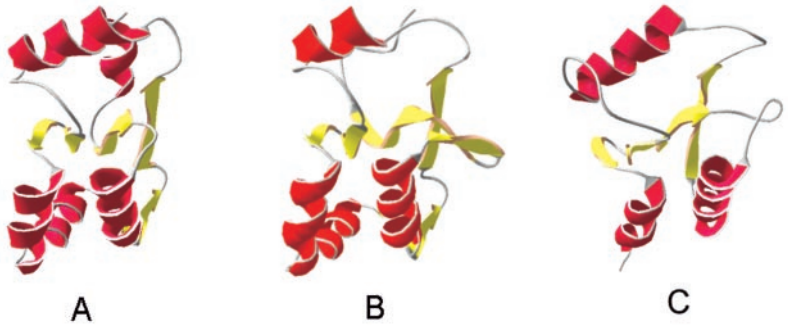


FIG. 2. **Structure of *S. cerevisiae* Grx5 protein.** Model for Grx5 three-dimensional structure (*B*) based on the structure of pig liver thioltransferase (*A*). The latter was determined by x-ray crystallography (7). The structure of a simple thioredoxin fold, that of phage T4 glutaredoxin (6), is also shown (*C*). β-Strands are represented as *yellow arrows*, α-helices are represented as *red spirals*, and loops are colored *gray*.

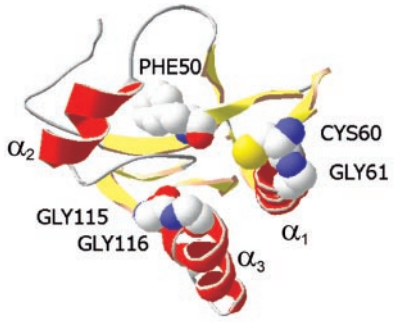


FIG. 3. **Location of essential residues in Grx5**. Secondary structure elements are indicated as described in the Fig. 2 legend. α-Helix regions are numbered according to Ref. 15. The α_4 region is positioned toward the bottom of the model, almost at a right angle with respect to α_3.

equivalent positions to Grx5 Cys^{117} were shown not to be required for the glutaredoxin activity.

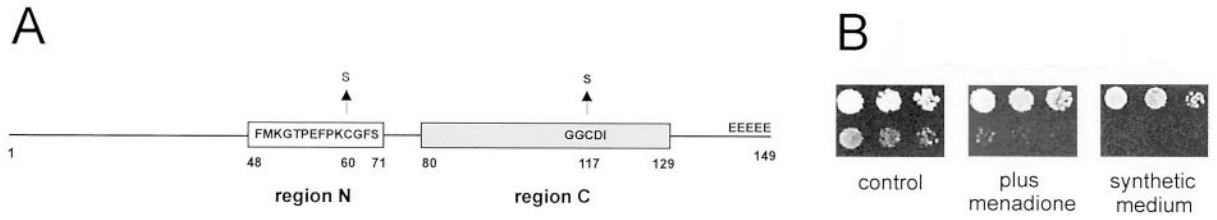
A Number of Conserved Residues Are Important for Grx5 Activity—Besides Cys^{60} and Cys^{117}, other residues are also conserved in the Grx5 sequence as revealed by comparison with the other family members. We introduced a number of single and double point mutations into *GRX5* (Fig. 4*A*; Table I) that changed the amino acid residues in the putative active site region. Changes were also introduced in other amino acids that were presumably important for maintaining the three-dimensional structure of Grx5. Thus, according to the proposed model (Fig. 3), the F50E mutation could alter a β-strand that is part of the active site cleft of Grx5. The G115V and G116V changes alter a glycine pair that is conserved in all dithiol and monothiol glutaredoxins (23). This pair is probably important for the proper orientation of α_3 relative to α_1 and Cys^{60} (Fig. 3). Of the single and double amino acid changes in the conserved CFGS region, only the G61V change caused biological inactivation of Grx5 (Fig. 4*C*). In contrast, the F50E and the single G115V and G116V mutations annulled the biological activity of Grx5. Less bulky side chains were also used for Gly^{61} or Gly^{115} substitutions. In both cases, introduction of an alanine residue maintained the wild-type phenotype, whereas serine disrupted the biological activity of the protein (Fig. 4*C*). We concluded that some but not all of the conserved residues in the Grx5 family are essential for the activity of the protein.

Loss of *GRX5* causes other phenotypic effects besides those described above, including the accumulation of cellular iron and the constitutive carbonylation of cell proteins (23, 24). Increased iron concentration in *grx5* cells has been associated with a rise in the number of protein carbonyl groups, which is an indicator of oxidative damage to protein (24). Thus, we determined whether the mutations that caused an increase in menadione sensitivity or inhibited growth in minimal medium also resulted in the previously mentioned phenotypes. In fact, all the Grx5 mutants studied that were hypersensitive to oxidative stress contained higher levels of iron than wild-type cells or mutants not affected in oxidative stress sensitivity (Fig. 5). Amino acid substitutions that caused increased intracellular iron resulted in an increase of about 50% in total carbonyl groups in cell proteins, whereas amino acid changes such as C117S did not affect other cell phenotypes (Table II).

Grx5 Stability Is Not Affected in the Mutants—It is expected that a significant change in the structure of Grx5 due to amino acid substitutions could alter the protein stability. Changes in the half-life of Grx5 would therefore be indicative of significant modifications in protein tertiary structure. To determine the half-life of Grx5, we used the doxycycline-regulatable tTA activator/Ssn6 repressor dual system (30) that allows strong promoter repression upon antibiotic addition. In fact, Northern analysis (data not shown) demonstrated that in CML276 cells (where the chromosomal-integrated *SSN6* repressor gene is activated by doxycycline) transformed with pCM319 plasmid carrying the *tet-GRX5* construction (overexpressed in the absence of the antibiotic), *GRX5* expression was rapidly inhibited (in less than 10 min) upon doxycycline addition. Under these conditions, wild-type Grx5 had a half-life of about 4 h (Fig. 6, *A* and *B*). This value was consistent with our previous observations showing that a strain in which the endogenous *GRX5* promoter had been substituted for the *tetO* promoter displayed the *grx5*-null phenotype about 12 h after inhibition of *GRX5* expression (24). Amino acid substitutions that altered Grx5 activity did not significantly affect the stability of the protein (Fig. 6*B*). This also applied for the F50E change that disrupted one of the β-strands in the region close to the active site. We conclude that the amino acid changes studied in this work did not alter the tertiary structure of Grx5 at a significant enough level to negatively affect the protein stability.

DISCUSSION

Higher eukaryotes have cytosolic dithiol glutaredoxins, which are required for maintaining the reduced status of protein thiol groups and for the activity of specific proteins (1, 2, 17–22). Furthermore, a mitochondrial dithiol glutaredoxin has recently been described in human cells (38, 39). Its existence reveals the importance of glutathione as a hydrogen donor for protein disulfide groups not only in the cytoplasm, but also in other cellular compartments. *S. cerevisiae* cells contain two dithiol glutaredoxins (Grx1 and Grx2) that are located at the cytosol and are highly homologous to other prokaryotic and eukaryotic members of the family (40, 41). However, no typical dithiol glutaredoxin seems to exist in yeast mitochondria. A family of three proteins (Grx3, Grx4, and Grx5) has recently been described in yeast. They all have glutaredoxin signatures



C

Mutation	Sensitivity to menadione	Growth in synthetic medium
GRX5	-	+
F50E	+	-
P58V	-	+
K59Q	-	+
C60S	+	-
G61V	+	-
G61A	-	+
G61S	+	-
F62S	-	+
P58V, K59Q	-	+
P58V, F62S	-	+
G115V	+	-
G115A	-	+
G115S	+	-
G116V	+	-
C117S	-	+

FIG. 4. **Functional analysis of residues essential for the biological activity of Grx5.** A scheme of Grx5 is shown in *A*, with the two amino-terminal and carboxyl-terminal regions defined on the basis of homology to dithiol glutaredoxins (23). Sequences from the specific regions where mutations were introduced are shown in detail, together with the polyglutamic acid tail at the carboxyl-terminal end. *B*, growth of wild-type (MML161, *top spots*) and Δ*grx5* (MML160, *bottom spots*) cells in YPD medium before treatment (*left panel*) and after 45 min of treatment (*middle panel*) with 10 mM menadione. *Right panel* shows growth in synthetic defined medium. *C*, summary of the growth characteristics of strains (see Table I for strain name) carrying different amino substitutions in Grx5. Menadione treatment was as described in *B*; + denotes absence of growth after treatment with the drug, such as in the case of the Δ*grx5* mutant in *B*; − denotes a growth pattern comparable with that of wild-type cells. For growth in SD medium, the + and − symbols describe growth patterns similar to those shown in *B* for wild-type and mutant cells, respectively.

but contain a single cysteine residue at the conserved putative active site (23). Based on this and the fact that both single mutants and combinations of double mutants display reduced glutaredoxin activity in cell extracts, we classified them as monothiol glutaredoxins. Grx5 is mitochondrially located and is involved in the maturation of Fe/S cluster-containing proteins at the organelle matrix (24). Defects in *grx5*-null mutants are common to mutants in other genes involved in Fe/S cluster assembly (24, 42) and include sensitivity to oxidants, auxotrophy for amino acids whose biosynthesis requires Fe/S-containing enzymes, respiratory defects, and iron accumulation. The function of yeast Grx5 is different from that of its human homologue, the PICOT protein, which has been proposed as a modulator of the protein kinase C-θ pathway (25, 27). Yeast Grx3 and Grx4 are not mitochondrial, and their absence does not cause the phenotypes observed in *grx5* cells. On the other hand, sequence alignment reveals that Grx3 and Grx4 are closer relatives to PICOT than Grx5. This raises the possibility, which has yet to be investigated, of a functional relationship between Grx3/Grx4 and the PICOT protein. Taken together, the above observations show a spatial and functional separation between yeast monothiol glutaredoxins.

Here we have centered our attention on the Grx5 structure-function relationship. From studies with dithiol glutaredoxin mutants in which one of the two Cys residues in the active site was eliminated, it has been concluded that monothiol glutaredoxins are active against mixed disulfides involving glutathione and protein sulfhydryls (5, 43). A three-dimensional model of Grx5, based on the known structure of a number of dithiol glutaredoxins, is proposed. Grx5 has an obvious thioredoxin fold structure. Cys^{60} (in the conserved P*X*CGFS region) lies opposite the Gly^{115} and Gly^{116} residues conserved in both monothiol and dithiol glutaredoxins. As in the dithiol molecules (14, 44), this glycine pair forms a loop that could confer flexibility for the appropriate positioning of α_1 relative to α_3 (Fig. 3). Thus, the two α_1 and α_3 regions form the glutathione cleft with the β-sheet at the bottom. From structural studies involving dithiol glutaredoxins, we can also deduce that other conserved residues in Grx5 are important for the stabilization of glutathione at the active site groove and its interaction with Cys^{60}. Asp^{118} is present at the α_3 region of Grx5 and is conserved in both glutaredoxin families (Ref. 23 and this work). It has been proposed that this residue establishes an ionic interaction with the α-amino group of the glutamic acid residue of glutathione (3, 13, 14). Lys^{20} of Grx5 is also conserved in both types of glutaredoxins, and its amino group could interact electrostatically with the α-carboxylate of the carboxyl-terminal glycine of glutathione (13, 14). Stabilization of glutathione in the cleft of

TABLE I
Yeast strains and inferred secondary structure at the amino acid substitution positions

All strains are isogenic to wild-type *S. cerevisiae* CML235. The strains listed were derived from MML19 by insertion of the corresponding plasmids. Plasmids (YIplac211 vector or derivatives with wild-type or *GRX5* mutants) were integrated at the chromosomal *LEU2* locus after transformation with DNA that had been linearized by digestion at the single *Eco*RV site within the plasmid *LEU2* gene. The amino acid replacements introduced in the *GRX5* translation product are indicated. The secondary structure elements at the amino acid substitution positions are inferred from the model in Fig. 3.

Strain	Plasmid	Point mutation	Inferred secondary structure at position
MML160	YIplac211	No insert	
MML161	pMM25	Wild-type *GRX5*	
MML163	pMM27	C60S	Coil
MML165	pMM28	C117S	Helix
MML219	pMM76	F62S	Helix
MML221	pMM77	P58V	Coil
MML223	pMM78	K59Q	Coil
MML225	pMM79	G116V	Coil
MML273	pMM88	P58V K59Q	Coil
MML274	pMM90	P58V F62S	Coil, helix
MML276	pMM92	G115V	Coil
MML322	pMM124	G61V	Helix
MML374	pMM174	F50E	Beta
MML421	pMM202	G115A	Coil
MML423	pMM203	G115S	Coil
MML425	pMM204	G61A	Helix
MML427	pMM205	G61S	Helix

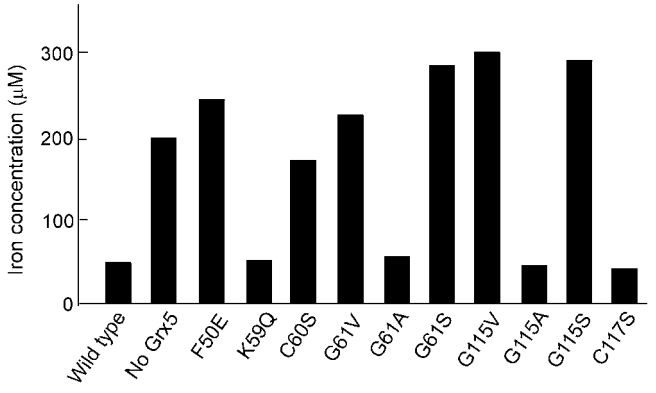


FIG. 5. **Iron concentration in Grx5 mutants with different amino acid substitutions.** Cultures of cells carrying the wild-type gene (MML161) or the null Δ*grx5* mutation (MML160) or the mutations that result in the indicated substitutions (see Table I for strain name) were grown exponentially in YPD medium at 30 °C, and total iron content per cell was determined (32). Mean cell volume was measured in parallel to calculate iron concentration. Results for each strain are the mean of three independent experiments; the differences between the individual experiments were <20%.

TABLE II
Protein carbonyl content in Grx5 mutants with different amino acid substitutions

Values (± S.D.) are the mean of at least three independent experiments and are normalized with respect to wild-type (MML161) cells. They correspond to nanomoles of carbonyl groups per milligram of cell protein in exponentially growing cultures in YPD medium at 30 °C. Absolute carbonyl content in MML161 cells was 0.70 ± 0.02 nmol/mg protein. Strains with the respective amino acid substitutions were those indicated in Table I.

Grx5 amino acid substitution	Relative carbonyl content
None	1.00
No Grx5	1.48 ± 0.07
F50E	1.42 ± 0.03
C60S	1.59 ± 0.04
G61S	1.52 ± 0.05
G115V	1.55 ± 0.04
C117S	1.06 ± 0.02

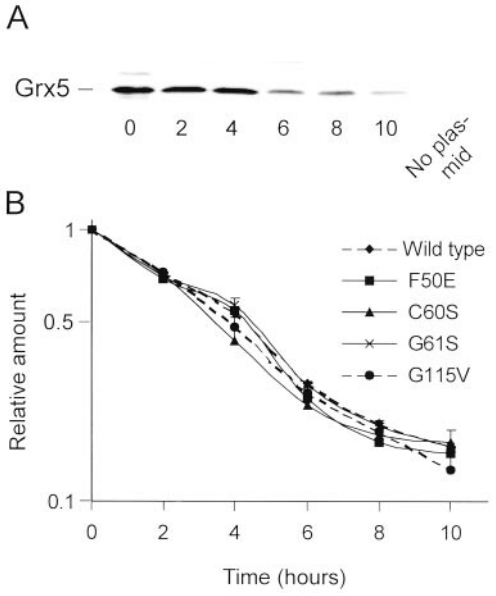


FIG. 6. **Half-life of Grx5.** CML276 cells transformed with plasmids that expressed the wild-type *GRX5* gene or different mutant derivatives under the control of the $tetO_7$ promoter were grown in YPD medium at 30 °C. At time 0, doxycycline (20 μg/ml) was added to repress Grx5 synthesis. *A*, Western blot analysis of Grx5 in CML276 cells transformed with pCM319. The same amount of total cell protein was loaded for each sample. The *left-most run* corresponds to CML276 cells expressing only the chromosomal *GRX5* gene under its own promoter. *B*, quantification of Grx5 levels in samples taken at successive intervals after doxycycline addition from Western blot analyses similar to that shown in *A*. Transformants that expressed the indicated Grx5 forms were used. At least two independent experiments were carried out for each transformant.

E. coli Grx3 glutaredoxin could also involve ionic interaction with an Arg^{40} residue (14). This residue is not present in all dithiol glutaredoxins, but an equivalent Arg^{92} residue is present in the α_2-helix of Grx5 and is conserved in all the monothiol glutaredoxins analyzed. It may therefore also contribute to glutathione stabilization. A coiled region following the α_2-helix (Fig. 1) contains a number of conserved residues that could contribute to stabilization of glutathione-mixed disulfide through hydrogen bonds, by analogy to equivalent residues in the *E. coli* Grx1 dithiol glutaredoxin (10).

To test the validity of our three-dimensional model for Grx5, we introduced a number of amino acid substitutions in some of the conserved residues and tested their effect on the biological activity of the protein. Replacing Cys^{60} with a serine residue totally eliminated activity, as did changing the following residue (Gly^{61}). However, the substitution of other residues in the PKCGFS region had no effect on the phenotype. Only the introduction of bulkier valine or serine side chains (but not alanine) to replace glycine at position 61 seems to inhibit the formation of glutathione mixed disulfide with Cys^{60}. The substitution of either Gly^{115} or Gly^{116} for valine or serine residues has the same effect as a *grx5*-null mutation. This supports the role proposed above for this glycine pair in the formation of the glutathione cleft. Each substitution would alter the orientation of the α_3-helix relative to α_1 and the active cysteine in position 60, whereas a bulky side chain would impede access of the glutathione molecule to the active site. We also hypothesized that changing Phe^{50} for a residue such as glutamic acid would alter the β-sheet structure that delimits the glutathione cleft and would make it difficult to appropriately position glutathi-

one relative to Cys^{60}. In fact, this was the case: the F50E change annulled the Grx5 activity.

Grx5 contains a second cysteine at position 117, which is not required for the protein biological activity. This carboxyl-terminal cysteine residue is also present at equivalent positions in many, but not all, monothiol and dithiol glutaredoxins; Grx3 and Grx4, for instance, do not possess it. Mutation of this cysteine in *E. coli* Grx3 has no effect on enzyme activity, and it has been proposed that the residue could have a regulatory role on the interaction of glutaredoxin with a second glutathione molecule necessary in the dithiol mechanism of action (14). There is, however, no evidence for such regulatory role in Grx5.

We have determined the half-life of the Grx5 protein using the *tet* promoter to conditionally express *GRX5*. The same result was obtained for the wild-type strain and for the different mutants, which is an argument against major alterations in the three-dimensional structure of the protein, even in the case of amino acid replacements that cause loss of activity.

The absence of Grx5 causes a number of phenotypic effects that are all closely related (24). Thus, the primary defect in the assembly of Fe/S clusters would lead to (i) an inability to synthesize a number of amino acids, (ii) respiratory growth defects, and (iii) an accumulation of iron in the cell. As a consequence of the latter, there is an accumulation of reactive oxygen species in the cells, which in turn increases the level of protein carbonyl groups and makes cells more sensitive to external oxidants. Those amino acids substitutions that affect the biological activity of Grx5 alter all the indicated phenotypes in a similar way, including the ability for respiratory metabolism (growth on glycerol medium). This confirms that all phenotype defects can be traced to the same loss of function. This is independent of the fact that some substitutions (such as F50E, G115S, and G116S) are expected to hinder the accessibility and stabilization of glutathione at the active site, whereas others (C60S and G61V) do not affect the access of the glutathione moiety of mixed disulfides to the active site but would affect the attack on the disulfide bond. Grx5 may be part of a multiprotein complex involved in the mitochondrial assembly of Fe/S clusters (24). The polyglutamic acid tail at the carboxyl-terminal end (Fig. 4) could be important for interactions with other proteins of the complex. The mutants used in this work, among others, may facilitate the study of biochemical reactions involving monothiol glutaredoxins and the specific role of Grx5 in Fe/S cluster assembly.

Acknowledgments—We appreciate the assistance of Lidia Piedrafita, Vanessa Guijarro, and Anna Valls throughout this work.

REFERENCES

1. Holmgren, A. (1989) *J. Biol. Chem.* **264,** 13963–13966
2. Holmgren, A., and Aslund, F. (1995) *Methods Enzymol.* **252,** 283–292
3. Nikkola, M., Gleason, F. L., Saarinen, M., Joelson, T., Björnberg, O., and Eklund, H. (1991) *J. Biol. Chem.* **266,** 16105–16112
4. Yang, Y., and Wells, W. W. (1991) *J. Biol. Chem.* **266,** 12759–12765
5. Bushweller, J. H., Aslund, F., Wuthrich, K., and Holmgren, A. (1992) *Biochemistry* **31,** 9288–9293
6. Eklund, H., Ingelman, M., Söderberg, B.-O., Uhlin, T., Nordlund, P., Nikkola, M., Sonnerstam, U., Joelsson, T., and Petratos, K. (1992) *J. Mol. Biol.* **228,** 596–618
7. Katti, S. K., Robbins, A. H., Yang, Y., and Wells, W. W. (1995) *Protein Sci.* **4,** 1998–2005
8. Sodano, P., Xia, T., Bushweller, J. H., Björnberg, O., Holmgren, A., Billeter, M., and Wüthrich, K. (1991) *J. Mol. Biol.* **221,** 1311–1324
9. Xia, T., Bushweller, J. H., Sodano, P., Billeter, M., Björnberg, O., Holmgren, A., and Wüthrich, K. (1992) *Protein Sci.* **1,** 310–321
10. Bushweller, J. H., Billeter, M., Holmgren, A., and Wüthrich, K. (1994) *J. Mol. Biol.* **235,** 1585–1597
11. Ingelman, M., Nordlund, P., and Eklund, H. (1995) *FEBS Lett.* **370,** 209–211
12. Aslund, F., Nordstrand, K., Berndt, K. D., Nikkola, M., Bergman, T., Ponstingl, H., Jörnvall, H., Otting, G., and Holmgren, A. (1996) *J. Biol. Chem.* **271,** 6736–6745
13. Sun, C., Berardi, M. J., and Bushweller, J. H. (1998) *J. Mol. Biol.* **280,** 687–701
14. Nordstrand, K., Aslund, F., Holmgren, A., Otting, G., and Berndt, K. D. (1999) *J. Mol. Biol.* **286,** 541–552
15. Martin, J. L. (1995) *Structure* **3,** 245–250
16. Ferrari, D. M., and Söling, H. D. (1999) *Biochem. J.* **339,** 1–10
17. Holmgren, A. (1979) *J. Biol. Chem.* **254,** 3672–3678
18. Lillig, C. H., Prior, A., Schwenn, J. D., Aslund, F., Ritz, D., Vlamis-Gardikas, A., and Holmgren, A. (1999) *J. Biol. Chem.* **274,** 7695–7698
19. Wells, W. W., Xu, D. P., Yang, Y. F., and Rocque, P. A. (1990) *J. Biol. Chem.* **265,** 15361–15364
20. Bandyopadhyay, S., Strake, D. W., Mieyal, J. J., and Gronostajski, R. M. (1998) *J. Biol. Chem.* **273,** 392–397
21. Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) *J. Biol. Chem.* **276,** 1335–1344
22. Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., and Barzilai, A. (2001) *J. Biol. Chem.* **276,** 21618–21626
23. Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A., and Herrero, E. (1999) *Mol. Cell. Biol.* **19,** 8180–8190
24. Rodríguez-Manzaneque, M. T., Tamarit, J., Bellí, G., Ros, J., and Herrero, E. (2002) *Mol. Biol. Cell* **13,** 1109–1121
25. Isakov, N., Witte, S., and Altman, A. (2000) *Trends Biochem. Sci.* **25,** 537–539
26. Rahlfs, S., Fischer, M., and Becker, K. (2001) *J. Biol. Chem.* **276,** 37133–37140
27. Witte, S., Villalba, M., Bi, K., Liu, Y., Isakov, N., and Altman, A. (2000) *J. Biol. Chem.* **275,** 1902–1909
28. Gietz, R. D., and Sugino, A. (1988) *Gene* (*Amst.*) **74,** 3065–3073
29. Garí, E., Piedrafita, L., Aldea, M., and Herrero, E. (1997) *Yeast* **13,** 837–848
30. Bellí, G., Garí, E., Aldea, M., and Herrero, E. (1998) *Yeast* **14,** 1127–1138
31. Weiner, M. P., and Costa, L. (1995) in *PCR Primer: A Laboratory Manual* (Dieffenbach, C. W., and Dveksler, G. S., eds), pp. 613–621, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
32. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Fish, W. W. (1988) *Methods Enzymol.* **158,** 357–364
34. Jones, D. T. (1999) *J. Mol. Biol.* **287,** 797–815
35. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18,** 2714–2723
36. Guex, N., Diemand, A., and Peitsch, M. C. (1999) *Trends Biochem. Sci.* **24,** 364–367
37. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
38. Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobson, G., Ljung, J., Johansson, M., and Holmgren, A. (2001) *J. Biol. Chem.* **276,** 26269–26275
39. Gladyshev, V. N., Liu, A., Novoselov, S. V., Krysan, K., Sun, Q. A., Kryukov, V. M., Kryukov, G. V., and Lou, M. F. (2001) *J. Biol. Chem.* **276,** 30374–30380
40. Luikenhuis, S., Dawes, I. W., and Grant, C. M. (1997) *Mol. Biol. Cell* **9,** 1081–1091
41. Grant, C. M. (2001) *Mol. Microbiol.* **39,** 533–541
42. Lill, R., and Kispal, G. (2000) *Trends Biochem. Sci.* **25,** 352–356
43. Herrero, E., and Ros, J. (2002) *Methods Enzymol.* **348,** 136–146
44. Berardi, M. J., and Bushweller, J. H. (1999) *J. Mol. Biol.* **292,** 151–161

PROTEIN STRUCTURE AND FOLDING:

Structure-Function Analysis of Yeast Grx5 Monothiol Glutaredoxin Defines Essential Amino Acids for the Function of the Protein

Gemma Belli?, Julio Polaina, Jordi Tamarit, Mari?a Angeles de la Torre, Mari?a Teresa Rodri?guez-Manzaneque, Joaquim Ros and Enrique Herrero

J. Biol. Chem. 2002, 277:37590-37596.