Crystallization and preliminary X-ray crystallographic studies of glutaredoxin 2 from *Saccharomyces cerevisiae* in different oxidation states

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Glutaredoxins are small (9–12 kDa) heat-stable proteins that are highly conserved throughout evolution; the glutaredoxin active site (Cys-Pro-Tyr-Cys) is conserved in most species. Five glutaredoxin genes have been identified in *Saccharomyces cerevisiae*; however, Grx2 is responsible for the majority of oxidoreductase activity in the cell, suggesting that its primary function may be the detoxification of mixed disulfides generated by reactive oxygen species (ROS). Recombinant Grx2 was expressed in *Escherichia coli* as a $6\times$His-tagged fusion protein and purified by nickel-affinity chromatography. Prior to crystallization trials, the enzyme was submitted to various treatments with reducing agents and peroxides. Crystals suitable for X-ray diffraction experiments were obtained from untreated protein and protein oxidized with *t*-butyl hydroperoxide (10 m*M*). Complete data sets were collected to resolutions 2.15 and 2.05 Å for untreated and oxidized Grx2, respectively, using a synchrotron-radiation source. The crystals belong to space group $P4_12_12$, with similar unit-cell parameters.

1. Introduction

Glutaredoxins (Grx) are small (9–12 kDa) heat-stable proteins with at least one cysteine in their active site that are highly conserved throughout evolution (reviewed by Holmgren, 1989). The ubiquitous distribution of glutaredoxins is probably related to the fact that these proteins are involved in many cellular processes, including protein folding, regulation of protein activity, reduction of dehydroascorbate, repair of oxidatively damaged proteins and sulfur metabolism (Holmgren, 1989; Rietsch & Beckwith, 1998).

There are five glutaredoxin genes (*GRX1–5*) in the yeast *Saccharomyces cerevisiae*. The Grx1 and Grx2 isoforms are dithiol proteins with a Cys-Pro-Tyr-Cys motif in their active site, whereas Grx3, Grx4 and Grx5 are monothiol proteins that contain Cys-Gly-Phe-Ser in their active site (Rodríguez-Manzaneque *et al.*, 1999; Bellí *et al.*, 2002). The importance of glutaredoxin is related to the fact that these proteins possess the ability to reduce disulfide bonds (Holmgren, 1989). Changes in the thiol–disulfide redox status of proteins are important not only for the reactivation of enzymes and for protein folding and stability, but also for the control of protein function (Ritz & Beckwith, 2001). *In vitro* studies have shown that both Grx1 and Grx2 can reduce mixed disulfides. However, despite the homology between Grx2 and Grx1 (64% identity and 85% similarity), Grx2 is responsible for the majority of oxidoreductase activity in the cell (Luikenhuis *et al.*, 1998; Grant, 2001). Grx2 performs the reversible disulfide-bond exchange reaction between its active site and the active site of the substrate protein *via* a monothiol mechanism, initiated by the nucleophilic N-terminal cysteine residue (Luikenhuis *et al.*, 1998; Collinson & Grant, 2003; Ritz & Beckwith, 2001).

It was recently proposed that Grx2 also possesses peroxidase and glutathione *S*-transferase activities (Collinson *et al.*, 2002; Collinson & Grant, 2003). Accordingly, the resistance of yeast to oxidative stress induced by hydroperoxides is affected by changes in the levels of glutaredoxins among other factors (Collinson *et al.*, 2002). Yeast strains with deletions in either the *GRX1* or *GRX2* genes are sensitive to hydroperoxides; however, the *GRX2* null mutant has higher hydrogen peroxide sensitivity, showing that Grx2 is probably the

main glutaredoxin involved in the detoxification of this oxidant in the cell (Collinson *et al.*, 2002; Wheeler & Grant, 2004). Both dithiolic glutaredoxins are capable of reducing hydroperoxides directly in a catalytic manner: their active sites act as an electron-transfer site and undergo a reversible oxidation of two vicinal protein thiol groups to a disulfide bridge (Collinson *et al.*, 2002). The resulting oxidized glutaredoxin is reduced by two GSH molecules and the resulting GSSG is reduced back by NADPH in a reaction catalyzed by glutathione reductase (Jordan *et al.*, 1997). Preliminary results from our group have indicated that Grx2 is also involved in another process: the deglutathionylation of the 20S proteasome. The converse process of deglutathionylation, glutathionylation, occurs avoiding the irreversible oxidation of the proteasome (Demasi *et al.*, 2003).

Recently, two different isoforms of Grx2 have been identified, one with a molecular weight of 15.9 kDa and the other of 11.9 kDa. Both isoforms are localized in the mitochondria, but the shorter isoform is also detected in the cytosolic fraction (Pedrajas *et al.*, 2002). The multitude of Grx2 functions can probably be related to the fact that this protein possesses these two isoforms.

At present, three-dimensional structures of glutaredoxins from only four sources have been determined: *Escherichia coli* Grx1 (Sodano *et al.*, 1991), Grx2 (Xia *et al.*, 2001) and Grx3 (Nordstrand *et al.*, 1999), bacteriophage T4 Grx (Eklund *et al.*, 1992), *Sus scrofa* Grx (Katti *et al.*, 1995) and *Homo sapiens* Grx (Sun *et al.*, 1998). These proteins share the same thioredoxin overall fold, despite variations in amino-acid sequences (Stefankova *et al.*, 2005). The glutaredoxin 2

from *E. coli*, the only Grx2 of known three-dimensional structure, is an atypical glutaredoxin with a molecular weight of 24 kDa, compared with the typical molecular weight of 9–12 kDa for other known glutaredoxins (Xia *et al.*, 2001). In this work, we present preliminary X-ray crystal analysis aiming towards the three-dimensional structure determination of the shorter *S. cerevisiae* Grx2 isoform, which may be helpful in comprehension of the molecular mechanisms by which Grx2 operates.

2. Methods

2.1. Cloning

The 432 bp *GRX2* gene was PCR amplified from *S. cerevisiae* genomic DNA of strain W303 using primers which, in addition to the open reading frame, contained cloning adaptors (*Nde*I and *Bam*HI restriction sites) to favour the gene transfer to expression plasmids. The PCR product and the expression vector pET15b were first digested with *Nde*I and then with *Bam*HI. The fragments generated by *Nde*I/*Bam*HI digestion containing the *GRX2* gene and pET15b were extracted from agarose gel by the Rapid Gel Extraction Concert kit (Invitrogen). After purification, the *GRX2* gene was ligated to the digested pET15b expression vector. The cloned gene sequence was confirmed by automated DNA sequencing and the resulting pET15b/*GRX2* was used to transform *E. coli* BL21 (DE3).
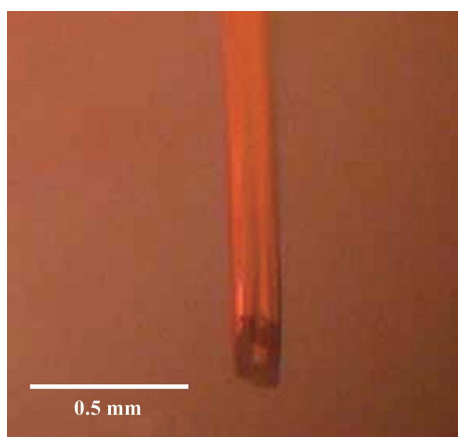
2.2. Expression and purification

E. coli BL21 (DE3) strain harbouring the pET15b/*GRX2* plasmid was grown (50 ml) overnight in LB medium containing 100 µg ml^{-1} ampicillin at 310 K and transferred to 1 l fresh LB/amp medium and cultured further at 310 K until the OD_{600} reached 0.6–0.8. Expression was induced with 0.5 m*M* isopropyl-β-D-thiogalactopyranoside and the cells were harvested after 4 h incubation at 310 K.
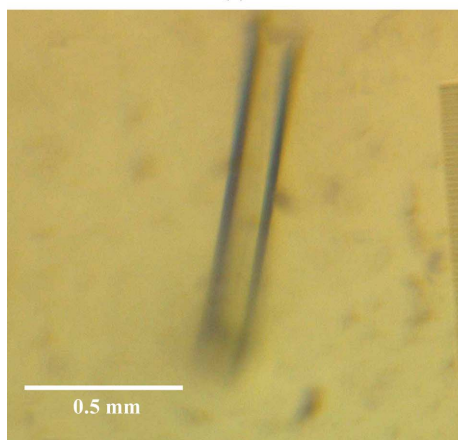
After cell lysis, the protein was purified by cobalt-affinity chromatography with an imidazole gradient (Talon metal-affinity resin from Clontech). We have obtained approximately 50 mg pure protein from 1 l cell culture. Protein purity was confirmed by SDS–PAGE and the purified protein was concentrated to 10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5.

2.3. Crystallization

Crystallization trials were executed using the hanging-drop vapour-diffusion method. Prior to crystallization, enzyme samples were submitted to treatment for 1 h at 310 K with hydrogen peroxide, *t*-butyl hydroperoxide, diamide and DTT. Initial screening was performed at 293 K using the commercially available Crystal Screen and Crystal Screen II kits from Hampton Research. The drops were prepared by mixing equal volumes (2 µl) of protein solution and reservoir solution. Promising crystals were identified in four conditions of the Crystal Screen kit: condition Nos. 10, 28, 32 and 47. The conditions were optimized by variation of precipitant or salt concentrations and pH. Crystals suitable for X-ray diffraction experiments were obtained from the untreated protein and from protein after oxidation with 10 m*M* *t*-butyl hydroperoxide. In both cases, the optimal condition was obtained with a reservoir solution consisting of 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6 and 0.2 *M* ammonium acetate. The crystals grown from the untreated sample reached dimensions of ~0.10 × 0.15 × 0.9 mm after one week (Fig. 1*a*), while crystals grown from the oxidized protein reached ~0.10 × 0.15 × 0.8 mm in about the same time (Fig. 1*b*).

Figure 1
Crystals of Grx2 from *S. cerevisiae*. (*a*) Untreated protein, (*b*) oxidized protein. Both Grx2 crystals were grown using 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6 and 0.2 *M* ammonium acetate.

Table 1
Data-collection parameters and crystallographic data statistics.

Values in parentheses refer to the highest resolution shell.

	Untreated protein	Oxidized protein
Wavelength (Å)	1.431	1.431
Temperature (K)	110	110
Space group	$P4_12_12$	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 47.66$, $c = 95.00$	$a = b = 47.63$, $c = 94.59$
Resolution range (Å)	42.60–2.15 (2.27–2.15)	42.56–2.05 (2.16–2.05)
Total reflections	68434	90136
Unique reflections	6439	7295
Completeness (%)	100.0 (100.0)	99.8 (99.3)
Multiplicity	10.6 (10.7)	12.3 (11.2)
R_{sym}† (%)	10.3 (31.2)	8.0 (29.4)
Average $I/\sigma(I)$	23.5 (7.3)	28.9 (7.8)

† $R_{sym} = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I \rangle_{hkl}| / \sum |\langle I \rangle_{hkl}|$.

2.4. Data collection and processing

The crystals, cryoprotected by a solution consisting of 30% PEG 4000, 0.1 M sodium acetate pH 4.6, 0.2 M ammonium acetate and 20% glycerol, were cooled to 110 K in a nitrogen-gas stream and X-ray diffraction data were collected using synchrotron radiation at the protein crystallography beamline D03B of the Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. LNLS D03B is a monochromatic beamline with maximum photon flux between 1.3 and 1.6 Å. The wavelength of the incident X-ray was set to 1.431 Å. A MAR CCD detector was used to record the oscillation data with $\Delta\varphi = 1°$. Data sets were processed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1993) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Glutaredoxin 2 from *S. cerevisiae* was submitted to crystallization trials after treatment with hydrogen peroxide, *t*-butyl hydroperoxide, diamide and DTT. Microcrystals were obtained from all samples, but to date crystals suitable for X-ray diffraction experiments only grew from the untreated protein and the sample after oxidation with 10 mM *t*-butyl hydroperoxide.

A crystal of untreated Grx2 diffracted to 2.15 Å resolution and belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 47.66$, $c = 95.00$ Å. The oxidized Grx2 crystal diffracted to 2.05 Å resolution and also belongs to space group $P4_12_12$, with similar unit-cell parameters. Table 1 summarizes the data-collection statistics. In order to estimate the number of molecules in the asymmetric unit, the Matthews coefficient V_M was calculated (Matthews, 1968). Both crystals presented one molecule per asymmetric unit with $V_M = 2.3$ Å^3 Da^{-1} and solvent contents of 45.8 and 45.0% for the native and oxidized protein, respectively.

Application of the molecular-replacement method using the program *AMoRe* (Navaza, 2001) indicated a probable solution when a polyalanine theoretical model constructed with the program *MODELLER* (Claude *et al.*, 2004) was used as the search model. The theoretical model was built based on the coordinates of a thiol-

transferase from *S. scrofa* (Katti *et al.*, 1995; PDB code 1kte; 36% sequence identity). Previous attempts to solve the structure using the 1kte coordinates as the search model for molecular replacement had failed. Currently, structure refinement is in progress.

We expect that knowledge of the three-dimensional structure of Grx2 in different redox states will contribute to the understanding of the catalytic mechanism of the enzyme, which is involved in several biological processes.

References

Bellí, G., Polaina, J., Tamarit, J., Torre, M. A., Rodríguez-Manzaneque, M. T., Ros, J. & Herrero, E. (2002). *J. Biol. Chem.* **277**, 37590–37596.
Claude, J. B., Suhre, K., Notredame, C., Claverie, J. M. & Abergel, C. (2004). *Nucleic Acid Res.* **32**, W606–W609.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Collinson, E. J. & Grant, C. M. (2003). *J. Biol. Chem.* **278**, 22492–22497.
Collinson, E. J., Wheeler, G. L., Garrido, E. O., Avery, A. M., Avery, S. V. & Grant, C. M. (2002). *J. Biol. Chem.* **277**, 16712–16717.
Demasi, M., Silva, G. M. & Netto, L. E. S. (2003). *J. Biol. Chem.* **278**, 679–685.
Eklund, H., Ingelman, M., Söderberg, B. O., Uhlin, T., Nordlund, P., Nikkola, M., Sonnerstam, U., Joelson, T. & Petratos, K. (1992). *J. Mol. Biol.* **228**, 596–618.
Evans, P. R. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by N. Isaacs, L. Sawyer & S. Bailey, pp. 114–122. Warrington: Daresbury Laboratory.
Grant, C. M. (2001). *Mol. Microbiol.* **39**, 533–541.
Holmgren, A. (1989). *J. Biol. Chem.* **264**, 13963–13966.
Jordan, A., Aslund, F., Pontis, E., Reichard, P. & Holmgren, A. (1997). *J. Biol. Chem.* **272**, 18044–18050.
Katti, S. K., Robbins, A. H., Yang, Y. & Wells, W. W. (1995). *Protein Sci.* **4**, 1998–2005.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Luikenhuis, S., Perrone, G., Dawes, I. W. & Grant, C. M. (1998). *Mol. Biol. Cell*, **9**, 1081–1091.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Navaza, J. (2001). *Acta Cryst.* D**57**, 1367–1372.
Nordstrand, K., Aslund, F., Holmgren, A., Otting, G. & Berndt, K. D. (1999). *J. Mol. Biol.* **286**, 541–552.
Pedrajas, J. R., Porras, P., Martinez-Galisteo, E., Padilla, C. A., Miranda-Vizuete, A. & Bárcena, J. A. (2002). *Biochem. J.* **364**, 617–623.
Rietsch, A. & Beckwith, J. (1998). *Annu. Rev. Genet.* **32**, 163–184.
Ritz, D. & Beckwith, J. (2001). *Annu. Rev. Microbiol.* **55**, 21–48.
Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A. & Herrero, E. (1999). *Mol. Cell. Biol.* **19**, 8180–8190.
Sodano, P., Xia, T. H., Bushweller, J. H., Bjornberg, O., Holmgren, A., Billeter, M. & Wulthrich, K. (1991). *J. Mol. Biol.* **221**, 1311–1324.
Stefankova, P., Maderova, J., Barak, I., Kollarova, M. & Otwinowski, Z. (2005). *Acta Cryst.* F**61**, 164–168.
Sun, C., Berardi, M. J. & Bushweller, J. H. (1998). *J. Mol. Biol.* **280**, 687–701.
Wheeler, G. L. & Grant, C. M. (2004). *Physiol. Plant.* **120**, 12–20.
Xia, B., Vlamis-Gardikas, A., Holmgren, A., Wright, P. E. & Dyson, H. J. (2001). *J. Mol. Biol.* **310**, 907–918.