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Crystallization and preliminary X-ray studies of the glutaredoxin from poplar in complex with glutathione

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A monocysteinic mutant of poplar glutaredoxin (C30S) has been overproduced and purified. The protein has been crystallized in complex with glutathione using the hanging-drop vapour-diffusion technique in the presence of PEG 4000 as a precipitating agent. A native data set was collected at 1.55 Å resolution. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 45.7, b = 49.1, c = 104.8 Å. Isomorphous crystals of a selenomethionine derivative were grown under the same conditions. Three data sets were collected at 1.73 Å using the FIP synchrotron beamline at the ESRF. The positions of the Se atoms were determined and model rebuilding and refinement are in progress.

1. Introduction

Glutaredoxins (GRXs), also called thiol-transferases, are low-molecular-weight proteins (generally between 80 and 100 amino acids) which belong to the protein disulfide oxidoreductase class. The major function of proteins of this class is to selectively reduce disulfide bridges on other proteins (glutaredoxin or thioredoxin) or alternatively to oxidize close cysteine residues in order to create new disulfide bridges (protein disulfide isomerase) (Holmgren, 2000). These reactions are needed either for the regulation of enzymatic activities or for protein folding in cells. The best known member of this class is thioredoxin (TRX), also a small protein (typically ~110 amino acids), for which numerous structural models are available. One of the major differences between TRXs and GRXs is their mode of reduction. In plants, depending on its subcellular compartmentation, TRX is reduced either by the photosynthetic electron flow *via* ferredoxin and an iron–sulfur protein, ferredoxin thioredoxin reductase (chloroplast system), or by NADPH and a flavoenzyme, NADPH thioredoxin reductase (cytosolic and mitochondrial systems) (Meyer *et al.*, 1999). In contrast, GRX is reduced by glutathione, which is itself kept reduced by NADPH and the flavoenzyme glutathione reductase. Another difference relates to the active-site sequence, which is generally YCPYC in GRX rather than the usual WCGPC of TRX. The redox potentials of the disulfide bridge of the GRXs characterized so far are generally more positive by ~50 mV than those of TRXs (Åslund *et al.*, 1997; Prinz *et al.*, 1997). As a consequence, GRXs are considered to be weaker reductants than TRXs. Furthermore, GRX is able to function as a monocysteinic enzyme, while TRX is inactive in this configuration (Yang *et al.*, 1998).

Several three-dimensional structures of GRXs are currently available, but proteins from only four sources have been studied so far: the *Escherichia coli*, bacteriophage T_4, pig and human enzymes. All available structures of the *E. coli* GRXs have been solved by NMR [PDB codes 1qfn (Berardi & Bushweller, 1999), 1grx (Bushweller *et al.*, 1992), 1egr (Sodano *et al.*, 1991) and 1ego (Xia *et al.*, 1992) for Grx 1, 1g7o (Xia *et al.*, 2001) for Grx2 and 3grx (Nordstrand *et al.*, 1999) and 1fov (Nordstrand *et al.*, 2000) for Grx3]. NMR structures are also available for T_4 GRX (1de1 and 1de2; Wang & Wishart, unpublished results) and human GRX [1b4q (Yang *et al.*, 1998) and 1jhb (Sun *et al.*, 1998)]. Only four X-ray structures are currently available, one of *E. coli* Nrdh, a GRX homologue of only 81 amino acids (1h75; Stehr *et al.*, 2001), two of the T_4 GRX (1aaz and 1aba; Eklund *et al.*, 1992) and one of the pig enzyme (1kte; Katti *et al.*, 1995). All models show a similar sequence of secondary elements. The active site is consistently situated at the beginning of the first α-helix. In all sequences, the amino acids responsible for the binding of glutathione are conserved. When the GRX models are compared with those of TRX, it is apparent that the short GRXs lack the first β-strand and α-helix found at the N-terminus of TRX, but that the two proteins share the same overall fold, named the TRX fold.

The poplar GRX studied here is an interesting model both biologically and structurally. It possesses only a low sequence identity with the GRX for which a three-dimensional structure is currently available; next to the unusually long Grx2, it is the longest GRX isolated so far (112 amino acids; the next

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longest is the mammalian type with 106 residues; Rouhier *et al.*, 2002). This is the first GRX from a plant that has been crystallized so far and the first X-ray structure of a GRX in complex with glutathione. Interestingly, this protein has been demonstrated to be an efficient donor to a peroxiredoxin, an enzyme which is able to selectively remove various hydroperoxides (Rouhier *et al.*, 2001, 2002*b*). This property has not been reported in other species so far; the various peroxiredoxins studied instead use TRX as an electron donor. It is thus of great interest to study the molecular interaction between the two proteins in this unique system. We have recently reported the crystallization and preliminary X-ray data of the peroxiredoxin component (Echalier *et al.*, 2002). This paper reports parallel work on GRX, the other partner in this model protein–protein interaction.

2. Material and methods

2.1. Cloning, expression and purification

The cloning, expression and purification of wild-type and C30S mutant poplar GRX has been described in Rouhier *et al.* (2002, 2002*a*). Typically, 100 mg of homogeneous protein was obtained and was stored frozen in 1 ml aliquots in 30 m*M* Tris–HCl containing 1 m*M* EDTA and 14 m*M* β-mercaptoethanol at a concentration of 15 mg ml^{-1} at 252 K. The purity of the fractions was monitored by SDS–PAGE.

For the production of the selenomethione-substituted GRX, a special strain which was deficient in methionine synthesis [BL21(DE3)Met^{-}] was cotransformed by pET-Grx and pSBET. One ampicillin- and kanamycin-resistant colony was amplified to 1 l in M9 medium supplemented with all 19 regular amino acids and selenomethionine each at 50 mg ml^{-1}. When the culture reached the exponential stage, protein synthesis was induced by the addition of 100 µ*M* IPTG. The subsequent purification procedure was identical to that for the native protein. Selenomethionine substitution was checked by electrospray mass-spectrometry analysis. A mass increase of 47.1 Da was observed compared with the native protein, as expected from the presence of one SeMet residue per molecule.

2.2. Site-directed mutagenesis and preparation of the mixed glutaredoxin–glutathione complex

In order to isolate a stable mixed disulfide intermediate between GRX and glutathione, the second active-site cysteine has been replaced by Ser using the PCR strategy described in Rouhier *et al.* (2002*a*). The C30S mutated protein (40 mg ml^{-1}) was mixed with an equal volume of 100 m*M* phosphate buffer containing 1 m*M* EDTA at pH 8.0. The sample was reduced with 100 m*M* DTT for 15 min at 277 K. Glutathione disulfide (GSSG) was subsequently added to a concentration of 500 m*M*. After 30 min incubation at 277 K, excess GSSG was removed by gel filtration using a Sephadex G25 column pre-equilibrated with 50 m*M* phosphate buffer pH 6.0. The concentration was determined by UV absorption at 276 nm, using a molar extinction coefficient of 4350 M^{-1} cm^{-1}.

Table 1
Statistics of the X-ray diffraction data for the SeMet GRX crystals.

Values in parentheses refer to the highest resolution shell.

	Peak	Edge	Remote
Wavelength (Å)	0.9802	0.9804	0.9724
Resolution (Å)	1.73 (1.77–1.73)	1.73 (1.77–1.73)	1.73 (1.77–1.73)
Observed reflections	143400	167434	169462
Unique reflections	46336	46285	46954
Completeness (%)	96.1 (92.0)	96.4 (89.6)	97.9 (93.0)
Multiplicity	3.09	3.62	3.61
R_{sym} (%)	4.1 (13.3)	4.0 (19.8)	4.7 (36.6)
$I/\sigma(I)$	18.2 (3.4)	17.0 (2.3)	13.7 (2.0)
Mean figure of merit before solvent flattening	0.386		
Mean figure of merit after solvent flattening	0.899		

2.3. Crystallization

Crystallization experiments were carried out using the hanging-drop vapour-diffusion method (McPherson, 1999). The search for initial crystallization conditions was performed using Hampton Research Crystal Screen kits I and II (Jancarik & Kim, 1991; Cudney *et al.*, 1994) and Clear Strategy Screens (Molecular Dimensions Ltd). The wells contained 800 µl of precipitant solution and the drops were composed of 2 µl of reservoir solution and 2 µl of protein solution at various concentrations. The most promising crystallization conditions were refined by variation of protein or precipitant concentrations, pH or the ratio of protein to reservoir in the droplet. Finally, the best crystals were obtained at a protein concentration of 6.5 mg ml^{-1} from a solution containing 28% PEG 4000 and 0.2 *M* $(NH_4)_2SO_4$ at 285 K.

2.4. Data collection

X-ray diffraction experiments were carried out at 100 K. Since the crystallization solution was suitable to provide cryoprotection, the crystals were quickly washed in the reservoir solution and immediately flash-frozen in a nitrogen-gas stream at 100 K. The data sets for the native protein or for the selenomethionyl derivative were collected on beamline BM30A (FIP; Roth *et al.*, 2002) at the ESRF, using a MAR Research 345 image-plate detector. For the latter data set, three wavelengths were chosen from the fluorescence spectrum corresponding to the peak, inflection point and high-energy remote. The data were processed using *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997). Statistics of the data are presented in Table 1.

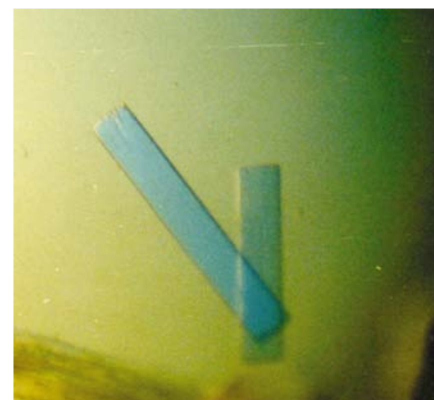

Figure 1
Crystals of C30S GRX in complex with glutathione (approximate dimensions 0.5 × 0.1 × 0.02 mm).

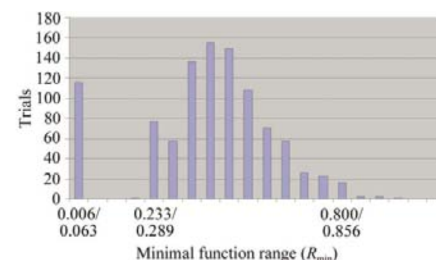

Figure 2
Histogram of the number of trials and their corresponding minimal function values R_{min} obtained with *Shake-and-Bake* (*SnB*). Using 700 reflections selected in the resolution range 25–1.66 Å and 230 invariants, a maximum of four sites were searched for 1000 trials of 20 cycles each. Two sites were found in 118 successful trials. The best value of R_{min} is 0.006.

3. Results and discussion

In our hands, crystallization trials using standard procedures failed to produce crystals from the wild-type protein or for several variants of the active site. However, the use of a monocysteinic mutant (C30S) allowed us to obtain a stable mixed disulfide complex (C30S–glutathione) that crystallized within 1 d under the conditions described above (Fig. 1). Crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 45.7, b = 49.09, c = 104.81 Å (Table 2). A native data set was collected at 1.55 Å resolution. Molecular-replacement trials using the program *AMoRe* and performed using the coordinates of the closest GRXs as search models were unsuccessful. An SeMet-substituted protein was therefore prepared and crystallized under the same conditions. Crystals only appeared after one month, but could be obtained within one week by the use of microseeding techniques. Three data sets were collected at three wavelengths to 1.73 Å resolution (Table 1). A search for the positions of two Se atoms per asymmetric unit was performed with the data set collected at the peak of the selenium K edge using either the Patterson method (*CNS*; Brünger *et al.*, 1998) or direct methods (*Shake-and-Bake* version 2.1; Weeks & Miller, 1999) (Fig. 2). Both methods unambiguously allowed the identification of the same two sites, as expected from the presence of one molecule of SeMet per molecule and two molecules per asymmetric unit. Peak-wavelength structure factors were phased with *SHARP* (de La Fortelle & Bricogne, 1997) to 1.73 Å resolution and the phases were improved by solvent flattening using *DM* (Collaborative Computational Project, Number 4, 1994) and *SOLOMON* (Abrahams & Leslie, 1996). Automatic model building with *ARP*/*wARP* (Perrakis *et al.*, 1999) led to the repositioning of 95 of 112 residues, with R and $R_{\rm free}$ values of 20 and 25% at the beginning of the refinement process. Model completion and refinement are currently in progress.

Table 2
Crystallization conditions of the GRX–glutathione complex and X-ray characterization.

Protein concentration (mg ml^{-1})	6.5
Crystallization solution	28% PEG 4000, 0.2 M $(NH_4)_2SO_4$
Crystal dimensions (mm)	0.5 × 0.2 × 0.02
Unit-cell parameters (Å)	a = 45.7, b = 49.09, c = 104.81
Space group	$P2_12_12_1$
Molecules per asymmetric unit	2
V_M (Å^3 Da^{-1})	2.49
Solvent content (%)	49
Diffraction limit (Å)	1.55

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