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Crystallization of mutant forms of glutaredoxin Grx1p from yeast

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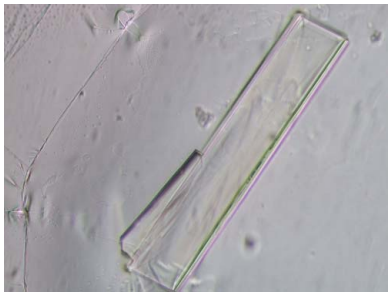
Glutaredoxin Grx1p from yeast was crystallized both as an independent protein and in a protein fusion with His-tagged yellow fluorescent protein (rxYFP). A glutathionylated C30S mutant of the 12 kDa Grx1p was crystallized in two different forms in PEG 4000 at low pH. These orthorhombic and monoclinic forms diffract to 2.0 Å (synchrotron radiation) and 2.7 Å (rotating-anode generator), respectively. In contrast, rxYFP-Grx1p formed crystals at high pH in $MgSO_4$ which diffract synchrotron radiation to 2.7 Å.

1. Introduction

Glutaredoxins (Grx) catalyse the glutathione (GSH) mediated reduction of disulfide bonds of intracellular proteins (for a review, see Fernandes & Holmgren, 2004). Glutathionylated cysteines in proteins can be reduced by Grx in a mechanism where the glutathionyl group is transferred from the target protein to the N-terminal of the two Grx active-site cysteines, which is reduced and regenerated by free GSH. Grx belongs to the thioredoxin superfamily (Sodano *et al.*, 1991) and several isoforms are found both in prokaryotes such as *Escherichia coli* and eukaryotes such as yeast and man. The published record of glutaredoxin crystal structures seems to be limited to the distantly related T4 glutaredoxin, which was solved more than a decade ago (Eklund *et al.*, 1992), but the small size of glutaredoxins have made them amenable to NMR studies, *e.g.* in the recently deposited (Berman *et al.*, 2000) NMR structures of glutaredoxin from *Populus tremula* × *tremuloides* (PDB codes 1z7r, 1z7p) and human glutaredoxin 2, which has been subject to both crystallographic (PDB code 2fls) and NMR (PDB code 2cq9) studies. In addition, glutaredoxins are found fused with peroxiredoxin in natural hybrid molecules found in some prokaryotes, exemplified by the structurally investigated *Haemophilus influenza* Prx5 (Kim *et al.*, 2003). A yeast Grx1p mutant C30S was recently described, along with a fusion protein consisting of an engineered yellow fluorescent protein mutant rxYFP from *Aequorea victoria* and the C30S Grx1p mutant (Bjornberg *et al.*, 2006; Ostergaard *et al.*, 2001). The mutation prevents disulfide formation between the active-site cysteines Cys27 and Cys30 and is thus particularly suited for determination of the redox potential for glutathionylation of Cys27. A structure of the glutathionylated protein will also be helpful to map essential residues in the active site and investigate their impact through site-directed mutagenesis. The rxYFP moiety in the fusion protein functions as a specific redox sensor for glutathione in its environment. The redox potential of the glutathione thiol–disulfide pair can be directly monitored through changes in fluorescence. We report the crystallization of the glutathionylated yeast Grx1p C30S mutant in two different crystal forms and of the rxYFP-C30S Grx1p fusion protein.



2. Methods

The glutathionylated yeast Grx1p C30S mutant and the His-tagged yellow fluorescent protein–Grx1p C30S fusion protein were cloned, purified and prepared as described previously (Bjornberg *et al.*, 2006). Briefly, both proteins were expressed in *E. coli* BL21 (DE3) and

extracted from the cells by ten freeze–thaw cycles. Grx1p C30S was purified on a Q-Sepharose column in 20 m*M* phosphate pH 7.8, 1 m*M* DTT, eluted with an NaCl gradient and finally concentrated to 16 mg ml^{-1} in 2 m*M* HEPES pH 7.5, 250 m*M* NaCl. The rxYFP-Grx1p C30S fusion protein was loaded onto His-bind resin (Novagen) in 50 m*M* phosphate pH 7.8, 20 m*M* imidazole, 0.3 *M* NaCl, eluted with 0.25 *M* imidazole in the same buffer and concentrated in 50 m*M* HEPES pH 7.0, 2 m*M* EDTA to a final concentration of 10 mg ml^{-1}. The sequence of Grx1p has been deposited as SWISS-PROT entry No. p25373. The sequence and structure of yellow fluorescent protein can be found in PDB entry 1h6r. No cleavage or tag removal was undertaken and the total sequences of the two crystallized derivatives are displayed in Fig. 1.

Crystallization was performed with the hanging-drop method at room temperature. Equal volumes of protein solution and precipitant were mixed and vapour-equilibrated against 0.5 ml precipitant solution. Initial screenings were performed with the sparse-matrix screens Crystal Screens 1 and 2 (Hampton Research) using 2 µl protein solution, while a larger drop size of 5 + 5 µl was used in the final optimized crystal incubations. In-house data were collected using a Rigaku RU-300 X-ray generator and a MAR 345 image-plate detector, while synchrotron data were collected at MAX-lab beam station 7-11 (Lund, Sweden) with a MAR CCD detector. Data were indexed, processed and reduced with *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The *CCP*4 version of *AMoRe* (Navaza, 1994) was used for molecular replacement and the *CCP*4 program *POLARRFN* was used to calculate the self-rotation function (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The His-tagged YFP-Grx1p C30S mutant was crystallized in $MgSO_4$ at alkaline pH. The best results were obtained by screening with a fine grid of $MgSO_4$ concentrations in the interval 1.5–1.75 *M* and 50 m*M* bicarbonate pH 9.75–10.25 (the position of the best results within this screen varied). A precipitate that formed upon mixing $MgSO_4$ and bicarbonate usually dissolved upon stirring and did not interfere with the crystallization experiments. The crystals obtained were of a cylindrical shape with a yellow hue (Fig. 2*a*). They appeared after approximately a week and were usually very small, but occasionally continued to grow for up to two months and in favourable cases reached a size of 0.5 × 0.1 mm.

Data could be collected at 100 K after flash-freezing in 35% glycerol. The resolution obtained in-house was only 3.4 Å, but this could be improved to 2.7 Å using synchrotron data (Table 1). Although the data set was almost 97% complete, there was only one observation on the *c* axis (parallel to the crystal cylinder axis aligned along the spindle axis). The indices for this reflection were 0 0 12 and were thus compatible with all hexagonal alternatives. Molecular replacement using *H. influenza* Prx5 (Kim *et al.*, 2003) glutaredoxin domain (PDB code 1nm3, at the time the most closely related crystallographic structure in the PDB) failed. However, molecular replacement with yellow fluorescent protein (PDB code 1h6r) (Ostergaard *et al.*, 2001) stripped of solvent molecules as a search model gave a much better solution with space group $P6_4$ than with any of the other hexagonal alternatives ($P6$, $P6_1$, $P6_2$, $P6_3$, $P6_5$). At 3 Å resolution there was only one solution, with an *R* value of 42% and a correlation coefficient of 61.8% and a calculated V_M (Matthews coefficient; Matthews, 1968) of 3.6 Å^3 Da^{-1} (corresponding to 66% solvent content), indicating that there is only a single molecule in the

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MSKGEELFTG VVPILVELDG DVNGHKFSVS GEGEGDATYG KLTLKFIVTT  50
GKLPVPWPTL VTTFAYGLQC FARYPDHMKR HDFFKSAMPE GYVQERTIFF 100
KDDGNYKTRA EVKFEGDTLV NRIELKGIDF KEDGNILGHK LEYNYNSHCV 150
YIVADKQKNG IKVNFKIRHN IEDGSVQLAD HYQQNTPIGD GPVLLPDNHY 200
LCYQSALSKD PNERRDHMVL LEFVTAAGIT HGMHELYKSG SGSGSGMVSQ 250
ETIKHVKDLI AENEIFVASK TYCPYSHAAL NTLFEKLKVP RSKVLVLQLN 300
DMKEGADIQA ALYEINGQRT VPNIYINGKH IGGNDDLQEL RETGELEELL 350
EPILANHHHHHH
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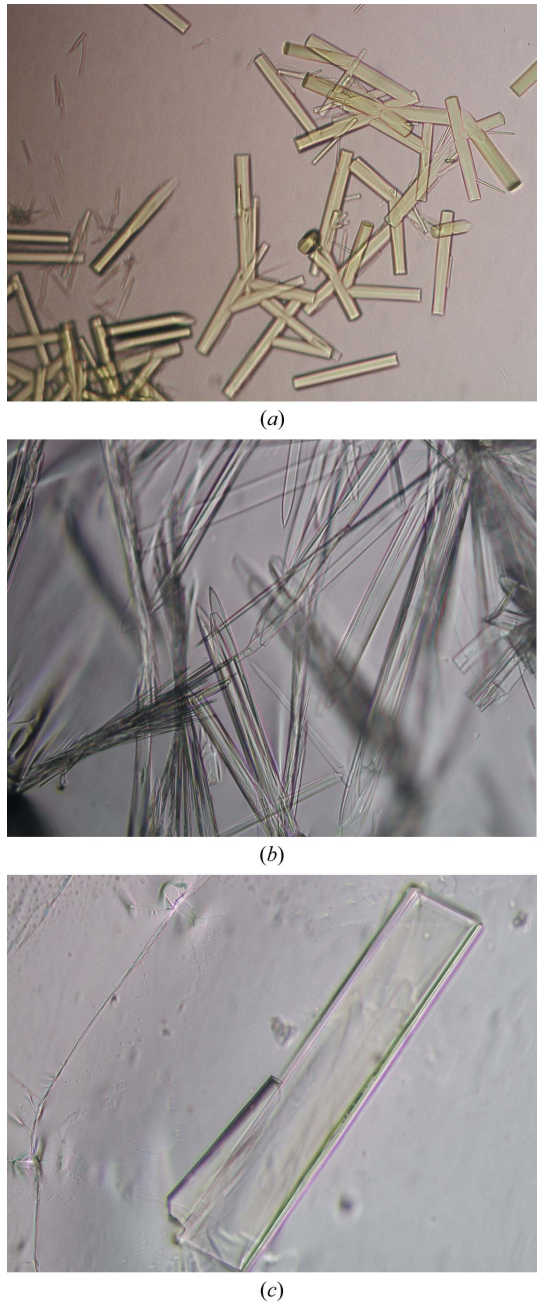
Figure 1
The total sequence of the rxYFP-Grx1p C30S fusion protein. The sequence of Grx1p C30S is shown on a blue background. Note the presence of a hexahistidyl tag in the fusion-protein sequence and the presence of an SGSGSGSG linker region between the rxYFP and Grx1p domains. The mutated residue is marked with an asterisk. The molecular weight of the Grx1p 30S protein is 12 kDa; that of the rxYFP-Grx1p C30S fusion protein is 41 kDa.



(*a*)

(*b*)

(*c*)

Figure 2
Crystals of (*a*) rxYFP-Grx1p C30S (hexagonal), (*b*) Grx1p C30S (orthorhombic) and (*c*) Grx1p C30S (monoclinic). The width of each photograph is approximately 1.3 mm.

Table 1
Data-collection statistics.

	rxYFP-Grx1p C30S fusion protein (hexagonal)	Glutathionylated Grx1p C30S (orthorhombic)	Glutathionylated Grx1p C30S (monoclinic)
Wavelength (Å)	1.115	1.115	1.542
Temperature (K)	100	100	298
Space group	$P6_4$	$C222_1$	$P2$
Unit-cell parameters (Å, °)	$a = b = 132.1$, $c = 58.7$	$a = 32.0$, $b = 78.5$, $c = 100.1$	$a = 64.7$, $b = 33.5$, $c = 78.3$, $\beta = 97.2$
Resolution	20–2.7 (2.85–2.7)	20–2.02 (2.13–2.02)	20.2–2.7
No. of unique observations	15714 (2216)	8364 (1036)	8962 (1313)
Completeness (%)	96.7 (95.1)	97.5 (84.5)	94.9 (96.9)
R_{sym}	0.097 (0.363)	0.059 (0.232)	0.143 (0.372)
Multiplicity	2.8 (2.4)	4.1 (4.0)	2.3 (2.3)
$I/\sigma(I)$	5.8 (2.1)	8.9 (3.1)	3.5 (0.9)
Wilson B (Å^2)	64.2	25.7	43.0

asymmetric unit. The solution packed well in the unit cell without overlap and phases calculated for this solution resulted in electron-density maps with interpretable β-strand segments (not shown). The search model comprises 63% of the total 41 kDa hybrid molecule. We intend to build the structure from these data and use the result to solve the structure of the other crystal forms (see below) through molecular replacement.

Glutathionylated glutaredoxin C30S mutant was crystallized in two different space groups, $C222_1$ and $P2$. Both are derived from Hampton Crystal Screen I solution No. 20 by optimizing the pH and precipitant concentration. Crystals obtained at pH 4–5 in 50 m*M* citrate and 10–22.5% PEG 4000 were flash-frozen in 50% sucrose and diffracted synchrotron radiation to 2.0 Å. The crystals were *C*-centred orthorhombic, with unit-cell parameters $a = 32.0$, $b = 78.5$, $c = 100.1$ Å. 20 of the 42 reflections along the l axis had intensities significantly above σ. These 20 reflections had $l = 2n$, which makes $C222_1$ the most likely space group. With one molecule per asymmetric unit, this gives a V_M (Matthews, 1968) of 2.5 Å^3 Da^{-1} and a solvent content of 52%. Originally, these crystals grew as cluster of needles, but after some optimization, *i.e.* increase in the pH, they grew as sword-shaped long and very thin plates of approximately 1 × 0.05 mm in size (Fig. 2*b*). The crystal used for synchrotron data collection was obtained at pH 4.75.

Glutathionylated Grx1p C30S also crystallized in a second form at a slightly higher pH of 4.5–6 in 50 m*M* acetate or bis-Tris buffer, again with PEG 4000 (10–20%) as precipitant. These crystals were produced by setting up a grid screen each time (24 wells) and crystals usually appeared in two or three of the drops, varying in each setup. The space group is $P2$ (out of seven unique reflections on the k axis, three had an odd k), with unit-cell parameters $a = 64.1$, $b = 33.4$, $c = 78.7$ Å, $\beta = 97.2°$. These crystals grew to an appreciable size; the specimen shown in Fig. 2(*c*) is approximately 1 × 0.3 × 0.1 mm. The self-rotation function gives no solution for threefold symmetry but two peaks for $\kappa = 180°$ perpendicular to the crystallographic twofold axis. This is consistent with a dimer in each asymmetric unit, although the functional unit of glutaredoxin is a monomer. Two molecules in the asymmetric unit would give a V_M (Matthews, 1968) of 3.4 Å^3 Da^{-1} (64% solvent content), which is within the normal range. These crystals cracked upon freezing in glycerol, sucrose, propanol or PEG 400. At room temperature, diffraction was initially observed to 2.5 Å, but the crystals suffered radiation damage. A freezing procedure for these crystals needs to be determined prior to synchrotron data collection.

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