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Crystallization and preliminary X-ray analysis of a truncated mutant of yeast nuclear thiol peroxidase, a novel atypical 2-Cys peroxiredoxin

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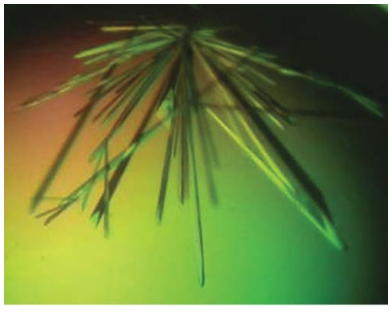


Saccharomyces cerevisiae nTPx is a thioredoxin-dependent thiol peroxidase that is localized in the nucleus. nTPx belongs to the C-type atypical 2-Cys peroxiredoxin family members, which are frequently called BCPs or PrxQs. A double mutant (C107S/C112S) of nTPx overexpressed in *Escherichia coli* was spontaneously degraded upon freezing and thawing and its truncated form (residues 57–215; MW = 17837 Da) was crystallized with PEG 3350 and mercury(II) acetate as precipitants using the hanging-drop vapour-diffusion method. Diffraction data were collected to 1.8 Å resolution using X-ray synchrotron radiation. The crystals belong to the trigonal space group $P3_2$, with unit-cell parameters $a = b = 37.54$, $c = 83.26$ Å. The asymmetric unit contains one molecule of truncated mutant nTPx, with a corresponding V_M of 1.91 Å^3 Da^{-1} and a solvent content of 35.5%.

1. Introduction

Peroxiredoxins (Prxs) are a large family of antioxidant enzymes which are abundant in several isoforms in yeast, plant and animal cells and in most eubacteria and archaea (Chae, Robinson *et al.*, 1994; Rhee *et al.*, 2001; Hofmann *et al.*, 2002). Prxs reduce deleterious H_2O_2 or alkyl hydroperoxides utilizing the thiol group of a cysteine residue (Wood, Schröder *et al.*, 2003) and in some cases are involved in the decomposition of highly toxic peroxynitrite (Bryk *et al.*, 2000). Some Prxs also control cytokine-induced peroxide levels, which mediate redox-sensitive signal transduction (Wood, Poole *et al.*, 2003). All Prxs belonging to the thioredoxin-fold superfamily contain a conserved 'peroxidatic' Cys (C_P) in the N-terminal portion and share the same peroxidatic active-site structure (Wood, Schröder *et al.*, 2003). The C_P residue is oxidized by peroxides to a cysteine sulfenic acid (C_P-SOH) intermediate. Prxs are classified into either the 2-Cys or 1-Cys types based on the occurrence of the so-called 'resolving' Cys (C_R; Rhee *et al.*, 2001; Hofmann *et al.*, 2002). In 2-Cys Prxs, C_P-SOH and C_R-SH react and form a disulfide (C_P-S-S-C_R). The stable disulfide form is then reduced by one of several cell-specific disulfide oxidoreductases [*e.g.* thioredoxin (Trx), tryparedoxin, AhpD or AhpF], completing the catalytic cycle. In 1-Cys Prxs, the sulfenic acid is directly recycled *via* oxidoreductases such as Trx and glutaredoxin. The 2-Cys Prxs have been subdivided into either 'typical' or 'atypical' types depending on the location of the C_R residue. In typical 2-Cys Prxs, the C_P-SOH reacts with the C_R residue located in the C-terminal arm of the other subunit. In contrast, the C_R residue in atypical 2-Cys Prxs resides within the same subunit. As the atypical 2-Cys Prxs have been further subdivided into L-, C- or R-type subfamilies, also depending on the spatial location of the C_R residue (Choi *et al.*, 2003), there are a total of five unique Prx subfamilies.

To date, five distinct Prxs have been found in the yeast *Saccharomyces cerevisiae* (Chae, Chung *et al.*, 1994; Park *et al.*, 2000). They include three thiol peroxidases (cTPx I, II and III) localized in the cytoplasm, one (mTPx) in the mitochondria and one (nTPx) in the nucleus. cTPx I, II and III are typical 2-Cys Prxs, while mTPx is a 1-Cys Prx. nTPx is particularly interesting as it contains an unusual C*xxxx*C motif that differs from the other four TPxs and the occurrence of nucleus-based Prxs is rather rare. A recent study on the intact forms of nTPx and three mutants (C107S, C112S, C107S/

C112S) indicated that nTPx acts as a Trx-linked alkyl hydroperoxide reductase in the nucleus of *S. cerevisiae* during its post-diauxic growth (Cha *et al.*, 2003). Cys107 and Cys112 have been identified as the C_P and C_R residues, respectively, designating nTPx as a member of the C-type atypical 2-Cys Prxs. The bacterial homologues of this class of atypical 2-Cys Prxs are frequently referred to as the bacterioferritin comigratory proteins (BCPs; Jeong *et al.*, 2000) and their plant homologues are named PrxQ (Kong *et al.*, 2000; Rouhier *et al.*, 2004). The C-type atypical 2-Cys Prxs are unique in that they function as monomers, while the other Prxs exist as functional dimers or redox-dependent oligomers (usually decamers; Wood *et al.*, 2002; Echalier *et al.*, 2005; Sarma *et al.*, 2005). At present, no structural information is available for this type of Prx, although 20 crystal structures of the other four Prx subfamily members in various redox states have been elucidated and deposited in the PDB (Berman *et al.*, 2000). We have attempted to crystallize intact nTPx and its mutant proteins, but only crystals of the C107S/C112S nTPx mutant in its truncated form (159 residues, MW = 17837 Da) have been obtained. This structure, mimicking the molecular organization of the reduced mature nTPx, should provide three-dimensional information relevant for all five Prx subfamilies, clarifying the structure–function relationships of the Prx family of proteins.

2. Materials and methods

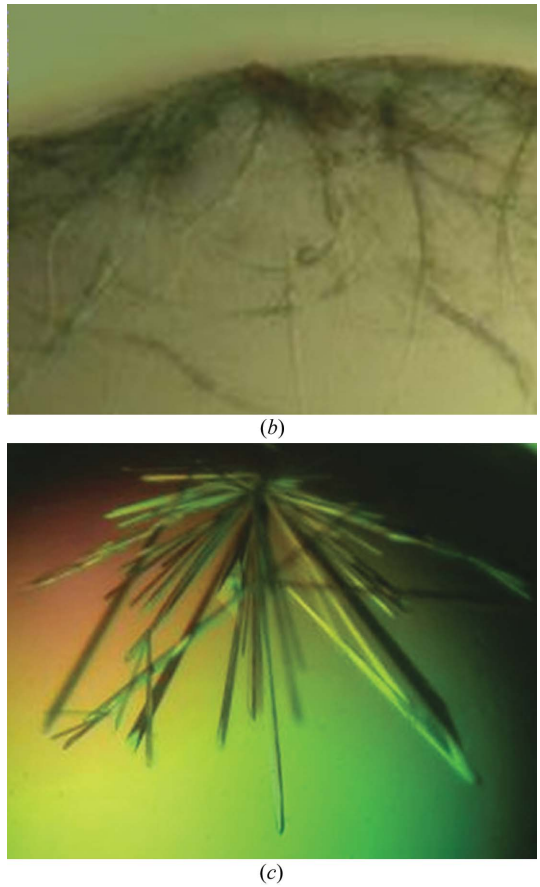
2.1. Overexpression and purification

The nTPx gene was amplified from yeast genomic DNA by the polymerase chain reaction (PCR) with two pairs of primers covering the whole coding sequence. The oligonucleotides 5′-CGATC-**CATATG**GGTGAAGCACTACGTAG containing the *Nde*I restriction-enzyme site (shown in bold) and 5′-CGC**GGATCC**TTATTC-TTCTTTAAACTTTTCAGCG containing the *Bam*HI site (bold) were used as the forward and reverse primers, respectively. The resulting PCR products were cloned into *Escherichia coli* expression vector pT7-7. The C107S/C112S mutant nTPx was generated by standard PCR-mediated site-directed mutagenesis with complementary primers containing a 1 bp mismatch that converts the codon for the respective cysteine to the codon for serine. The oligonucleotides 5′-CACGCCTGGT**TCT**ACTAGACAGGCC and 5′-GGCCTGTC-TAGT**AGA**ACCAGGCGTG (Ser codon shown in bold) were used as the forward and reverse primers, respectively, for the Cys107→Ser mutation and 5′-CTAGACAGGCC**TCT**GGATTTCGTGAC and 5′-GTCACGAAATCC**AGA**GGCCTGTCTAG were used for the Cys112→Ser mutation. The mutated PCR products were ligated into pT7-7 digested with *Nde*I and *Bam*HI. Transformed cells were cultured at 310 K overnight in LB medium supplemented with ampicillin (100 µg ml^{-1}) and then transferred to fresh medium at a ratio of 1:200. When the optical density of the culture at 600 nm reached 0.4, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 m*M*. After induction for 3 h, the cells were harvested by centrifugation at 2900*g* for 20 min at 277 K using a Hanil Supra 28K rotor and stored at 203 K until use. The frozen cells were suspended in 50 m*M* Tris–HCl pH 8.0 containing 2 m*M* phenylmethylsulfonyl fluoride and 1 m*M* EDTA and were lysed by sonication. After centrifugation at 39 000*g* for 30 min at 277 K using a Sorvall SS-34 rotor, the supernatants were loaded onto a DEAE cellulose column that had been previously equilibrated with 50 m*M* Tris–HCl buffer pH 7.4. The proteins were eluted with a linear gradient of NaCl from 0 to 500 m*M*. Protein-containing fractions were pooled and precipitated with 80% ammonium sulfate. The precipitate was dissolved in 50 m*M* Tris–HCl buffer pH 7.4 containing 200 m*M* NaCl and further purified by Sephacryl-100 column chromatography using the same buffer solution. Fractions containing the purified mutant nTPx were collected, dispensed in 1 ml aliquots and frozen at 203 K. Prior to crystallization, each sample containing ∼3 mg of protein was thawed, desalted and concentrated by ultracentrifugation with a Centricon YM-10 (Millipore) to ∼10 mg ml^{-1} in 50 m*M* Tris–HCl buffer pH 7.4 and stored at 283 K.

While analyzing the crystallographic data, it was found that the crystallized protein might not consist of the intact form but of a degradation product of mutant nTPx. Subsequently, the protein samples were analyzed by SDS–PAGE and matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI–TOF MS), which revealed that the mutant nTPx has been gradually but spontaneously degraded upon freezing and thawing. MALDI–TOF MS analyses were carried out using a Voyager DE-STR mass spectrometer (Applied Biosystems). Sinapic acid (10 mg ml^{-1}) dissolved in a 30%(*v*/*v*) acetonitrile solution containing 0.3%(*v*/*v*) trifluoroacetic acid was used as a matrix. 1 µl protein sample (1 µg µl^{-1}) was mixed with an equal volume of matrix solution and the mixture was allowed to dry at room temperature. Spectra were recorded from 300 laser shots (nitrogen laser, 337 nm) with an accelerating voltage of 20 kV in the linear mode. Calibration was performed with bovine insulin, *E. coli* Trx and horse apomyoglobin.

2.2. Crystallization and data collection

Crystallization of the nTPx mutant was carried out using the hanging-drop vapour-diffusion method. Initial searches for crystal-



(*b*)

(*c*)

Figure 1
Crystals of *S. cerevisiae* nTPx mutant. (*a*) Crystals from initial screens. (*b*) Crystals from optimized conditions.

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (1.86–1.80 Å).

X-ray wavelength (Å)	1.12714 (Pohang Light Source BL-6B)
Temperature (K)	100
Space group	$P3_2$
Unit-cell parameters (Å)	$a = b = 37.54$, $c = 83.26$
Resolution limit (Å)	1.8
No. of measured reflections	55862 (5457)
No. of unique reflections	11928 (1171)
Completeness (%)	98.1 (97.0)
$\langle I/\sigma(I)\rangle$	12.4 (3.09)
$R_{\rm merge}$† (%)	8.5 (32.1)
Redundancy	4.6 (4.6)

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of the reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over the i measurements of reflection h.

lization conditions were performed using 24-well Linbro tissue-culture plates (ICN) and the Wizard I screening system (Emerald BioSystems) at 283 K. Hanging drops were prepared by mixing equal volumes (2 µl) of protein solution (10 mg ml^{-1} in 50 m*M* Tris–HCl pH 7.4) and reservoir solution and were allowed to equilibrate against 0.5 ml reservoir solution.

To facilitate cryo-work with the crystals, 3 µl 40%(*w*/*v*) glucose was added to the hanging drops. Within about 1 min, crystals were mounted in nylon loops and flash-cooled at 100 K in a nitrogen-gas stream. X-ray diffraction data were collected at 100 K with a Bruker AXS Proteum300 CCD detector at the BL-6B experimental station of Pohang Light Source (PLS), South Korea. The synchrotron X-ray wavelength was 1.12714 Å. The crystal-to-detector distance was set to 150 mm and the exposure time per frame to 30 s for data collection. The crystal was rotated through a total of 150° with an oscillation range of 1.0° per frame. The raw data were processed and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant C107S/C112S mutant nTPx was overexpressed in *E. coli* in a soluble form with a yield of about 10 mg homogeneous protein per litre of culture. Initially, thin hairy tufts that barely looked like crystals appeared in the hanging drops with a reservoir solution containing a mixture of 20%(*w*/*v*) PEG 3000 and 200 m*M* NaCl as precipitant at 283 K in 20 d (Fig. 1*a*). Based on this observation, a systematic search for optimized crystallization conditions was performed, which concluded that addition of divalent metal ions dramatically improved the crystal quality. Finally, single crystals of decent quality, with maximum dimensions of 1.0 × 0.04 × 0.04 mm, were obtained using a mixture of 30%(*w*/*v*) PEG 3350 and 2 m*M* mercury(II) acetate in 100 m*M* Tris–HCl pH 7.9 at 283 K in 7 d (Fig. 1*b*). Diffraction data were collected to 1.8 Å resolution using synchrotron X-rays. A total of 55 862 measured reflections were merged into 11 928 unique reflections with an $R_{\rm merge}$ (on intensity) of 8.5%. The merged data set is 98.1% complete to 1.8 Å. The crystals belong to the trigonal space group $P3_1$ or $P3_2$, with unit-cell parameters $a = b = 37.54$, $c = 83.26$ Å. The data-collection statistics are summarized in Table 1.

If the asymmetric unit contains one molecule of intact mutant nTPx (calculated molecular weight 24 087 Da), the crystal volume per protein weight $V_{\rm M}$ and the corresponding solvent content would be 1.41 Å^3 Da^{-1} and 12.5%, respectively (Matthews, 1968). These values are extremely, if not impossibly, low for a protein, which led us to analyze the protein samples in detail. To our own surprise, the SDS–PAGE and MALDI–TOF MS analyses indicated that the mutant nTPx had spontaneously degraded upon freezing and thawing of the purified sample. Time-dependent proteolytic behaviour of mutant nTPx had not been previously recognized, since every protein sample used for biochemical studies had been freshly prepared prior to each experiment and had never been subjected to a freeze–thaw process. In fact, single crystals could be obtained only when protein solutions that had been allowed to age for a sufficient period of time after thawing were used for crystallization. The reason for this phenomenon could not be conjectured at the time but became obvious from the following retrospective analyses.

We analyzed two protein samples which had been stored under the same conditions (283 K in a cold chamber) after thawing them for different periods of time: one for a week and the other for two months. The one-week-old sample appeared as several bands on a SDS–PAGE gel under reducing conditions and resulted in a noisy mass spectrum with many prominent peaks. Major MALDI–TOF peaks appeared at 23 574, 23 081, 22 959, 22 764, 22 430, 22 132, 21 839 and 18 657, which correspond to the masses of the products cleaved off after residues 5, 9, 10, 13, 15, 17, 19 and 49, respectively. In contrast, the two-month-old sample from which single crystals were obtained gave a single MALDI–TOF peak at a molecular mass of 17 855, indicating that the sample was pure. The N-terminal sequencing analysis also confirmed that 56 N-terminal amino acid residues of the intact mutant nTPx were cleaved off. Thus, the truncated mutant nTPx contains 159 amino-acid residues (residues 57–215) with an expected mass of 17837. The presence of one such molecule in the asymmetric unit gives a $V_{\rm M}$ of 1.91 Å^3 Da^{-1} and a corresponding solvent content of 35.5%.

nTPx contains putative nuclear targeting signals that have to be cleaved off upon localization (Cha *et al.*, 2003). It remains to be determined whether the spontaneous degradation of mutant nTPx corresponds to a physiologically mature protein. The structure was solved in space group $P3_2$ by molecular replacement using a model built based on the X-ray structures of TPxB, a human 2-Cys Prx (PDB code 1qmv; Schröder *et al.*, 2000), and *S. pneumoniae* thiol peroxidase (PDB code 1psq); the details will be described elsewhere.

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