SHORT COMMUNICATIONS

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Crystallization and preliminary X-ray diffraction analysis of glutathione peroxidase from human plasma.

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Abstract

The extracellular form of glutathione peroxidase from human plasma has been crystallized by the sitting-drop vapour-diffusion method. The crystals belong to tetragonal space group $I4_1$ with cell dimensions of $a = b = 83.1$ and $c = 131.0$ Å. They diffract beyond 2.9 Å resolution and have one dimer in the asymmetric unit. A self-rotation function analysis shows the possible (222) symmetry for the tetramers of this enzyme.

Introduction

Glutathione peroxidase (E.C. 1.11.1.9) belongs to the family of selenoproteins (Flohe, Gunzler & Schock, 1973) and plays an important role in the defense mechanisms of mammals, birds and fish against oxidative damage by catalyzing the reduction of a variety of hydroperoxides, using glutathione as the reducing substrate (Mills, 1957),

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG.$$

Three distinct species of glutathione peroxidase have been identified in mammals to date, the 'classical' cellular enzyme (cGSHPx),* the phospholipid hydroperoxide metabolizing enzyme (PHGPx) and the plasma enzyme (pGSHPx). Their primary structures are poorly related. It has been shown that they are encoded by different genes and that they have different enzymatic properties (Takahashi *et al.*, 1990; Schuckelt *et al.*, 1991; Yamamoto & Takahashi, 1993; Esworthy, Chu, Geiger, Girotti & Doroshow, 1993). cGSHPx and PHGPx occur as cytosolic or membrane-bound forms. cGSHPx can reduce hydrogen peroxide, organic hydroperoxides as well as lipid hydroperoxides, while PHGPx in particular reduces phospholipid and cholesterol hydroperoxides in biological membranes (Thomas, Maiorino, Ursini & Girotti, 1990). The crystal structure of bovine erythrocyte glutathione peroxidase has been determined and crystallographically refined at 2 Å resolution (Ladenstein *et al.*, 1979; Epp, Ladenstein & Wendel, 1983). The structural studies have provided the first view of the active site with its selenocysteine residue, which is involved in catalysis and probably shuttles between the selenol state and the selenenic acid state in the catalytic cycle. Because of the limited success of crystallographic studies involving GSH binding, specific enzyme–substrate/product interactions and their influence on catalysis have not been elucidated.

pGSHpx was first purified from human plasma and characterized by Broderick, Deagan & Whanger, (1987), Takahashi, Avissar, Whitin & Cohen, (1987) and Maddipati & Marnett (1987). Unlike the other two enzymes, the plasma enzyme is an extracellular glycosylated selenoprotein which is mainly synthesized and secreted from kidney (Avissar, Kerl, Baker & Cohen, 1994). Human pGSHPx exists in solution as a tetramer with a selenocysteine residue in each of the identical subunits. However, the biological function of pGSHPx still remains unclear. Enzymatic studies using GSH as the reducing substrate revealed human pGSHPx as an enzyme with low activity (Takahashi *et al.*, 1987; Maddipati & Marnett, 1987; Yamamoto & Takahashi, 1993; Esworthy *et al.*, 1993). It was shown to have a tenfold lower affinity for GSH than cGSHPx. Although pGSHPx can reduce hydrogen peroxide and organic hydroperoxides, it is approximately tenfold slower than cGSHPx at cellular levels of glutathione. pGSHPx may be able to react with certain phospholipid hydroperoxides, but the reactions were usually either incomplete or less effective than those catalyzed by PHGPx. Even though, all the peroxidase activity in human plasma can be attributed to pGSHPx (Maddipati, Gasparski & Marnett, 1987). However, due to the low levels of the reduced thiol in plasma (Frei, Stocker & Ames, 1988) and the low reactivity of pGSHPx, it is still difficult to assign a definite physiological role to this enzyme.

The primary structure of human pGSHPx shows only limited identity with the other two enzymes, 44% with human cGSHPx and 25% with pig PHGPx (Takahashi *et al.*, 1990; Schuckelt *et al.*, 1991). However, higher conservations in these three glutathione peroxidases are observed among the amino acids constituting the active site (Epp *et al.*, 1983). This suggests that the catalytic mechanisms of the three enzymes are, in principle, identical or at least very similar. The amino-acid variations in the active site, including two arginines which are involved in GSH binding, may account for the possible differences in substrate-binding mechanisms by comparing pGSHPx with the other glutathione peroxidases. It can be expected that the crystal structure of human pGSHPx could help to elucidate its substrate specificity and enzymatic nature and also to improve our understanding of the catalytic mechanism of glutathione peroxidases in general.

Materials and methods

pGSHPx was purified from human plasma (obtained from the Blood Bank, Lund University Hospital) using a modification of a previously described procedure (Huang & Åkesson, 1993).

*Abbreviations used: cGSHPx, cellular glutathione peroxidase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; pGSHPx, plasma glutathione peroxidase; ROOH, hydroperoxide; ROH, reduced hydroperoxide; GSH, reduced glutathione; GSSG, oxidized glutathione; PEG, polyethylene glycol; MPD, 2-methyl-2,4-pentanediol; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

In brief, it included ammonium sulfate fractionation followed by five chromatographic steps using columns with phenyl-Sepharose CL-4B, DEAE–Sephadex A-50, Sephadex G-200, phenyl-Sepharose CL-4B, and Mono-Q HR 5/5. Approximately 5 mg pure pGSHPx (purification 10 000- to 20 000-fold) was obtained from 9 l of human plasma. Videodensitometry scanning of SDS–PAGE gels indicated the protein to be at least 99% pure. The purified protein was kept in 10 m*M* Tris–HCl (pH 7.5), 1 m*M* dithiothreitol.

Crystallization conditions were screened extensively by the hanging-drop vapour-diffusion method in a pH range $4.5 < \text{pH} < 9.5$ (Carter & Carter, 1979). The most promising conditions were adapted to the sitting-drop vapour-diffusion method to obtain large crystals suitable for X-ray analysis. The initial droplet contained 3 µl of enzyme solution (10 mg ml^{-1}) and 3 µl of the precipitant reservoir solution [5% PEG 6000 and 10% MPD in 100 m*M* bis-Tris propane buffer (pH 6.5)]. To stabilize the crystals before sealing them in siliconized glass capillaries, they were treated with 8% PEG 6000 and 16% MPD in 100 m*M* bis-Tris propane buffer (pH 6.5).

A preliminary set of X-ray intensity data was collected to 3.0 Å resolution from a single crystal using an MAR research image-plate system on a Siemens/Mac Science rotating-anode generator operating at 45 kV and 90 mA with Cu $K\alpha$ radiation. The data were processed using the *MARXDS* program (Kabsch, 1988) and the *PROTEIN* package (Steigemann, 1974).

A rotation-function analysis was performed to check the local symmetry of the molecules in the unit cell. The native Patterson map was calculated on a 1 Å grid using intensity data ($F^2 > 2\sigma$) in the resolution range 10–4 Å. A self-rotation function (Rossmann & Blow, 1962) was calculated for twofold symmetry axes using the real-space search options *SELF* of the *X-PLOR* program suite (Brünger, 1991). From this map, 4000 highest peaks were selected to represent the second Patterson function. The Patterson space explored was a hollow sphere. Choosing an inner integration radius limit of 4.0 Å, the origin peak of the map was excluded. The outer radius limit was set to 20 Å.

Results and discussion

Single crystals were obtained at 293 K. They grew to maximum dimensions of $0.2 \times 0.3 \times 0.5$ mm within three weeks. Their shape is prismatic with a square cross section (Fig. 1). The crystals diffracted to beyond 2.9 Å and were very stable to X-ray exposure. The space group was determined from rotation photographs and examination of the collected data set using the auto-indexing routine of Kabsch (1988). Auto-indexing resulted in the choice of a tetragonal unit cell ($I4_1$ or $I4_122$). Inspection

Fig. 1. Tetragonal crystal of glutathione peroxidase from human plasma. Space group $I4_1$, $a = b = 83.1$ and $c = 131.0$ Å. The maximal dimension of the crystal is about 0.5 mm.

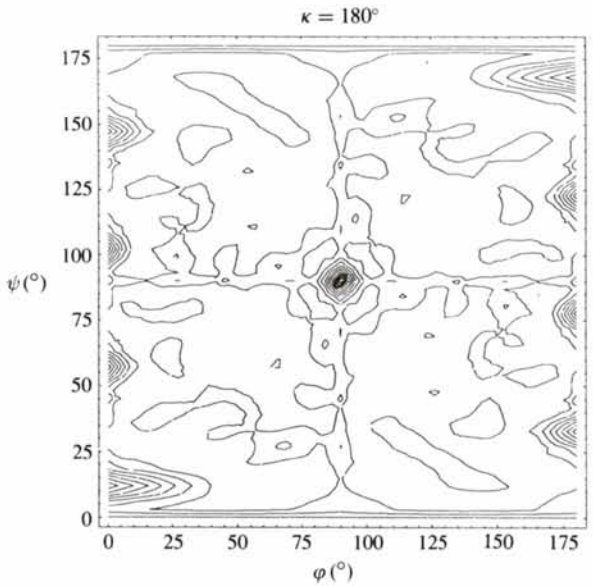

Fig. 2. Self-rotation function of human pGSHPx for twofold local axes at $\chi = 180°$. The self-rotation function was calculated in a spherical polar angle coordinate system (Rossmann & Blow, 1962) using data from 20 to 4 Å. The central peak corresponds to the crystallographic fourfold screw axis. Non-crystallographic twofold peaks at $\varphi = 0°$ and 180° are clearly indicated, local axis A ($\psi = 12°$, $\varphi = 0°$), local axis B ($\psi = 102°$, $\varphi = 0°$). The non-crystallographic peaks are 85.6% of the origin peak (the crystallographic fourfold). Height of origin peak, 29.4σ; height of non-crystallographic peak, 25.0σ; mean value of rotation function, 18.1σ. The map is contoured from 18.1σ in steps of 1.81σ.

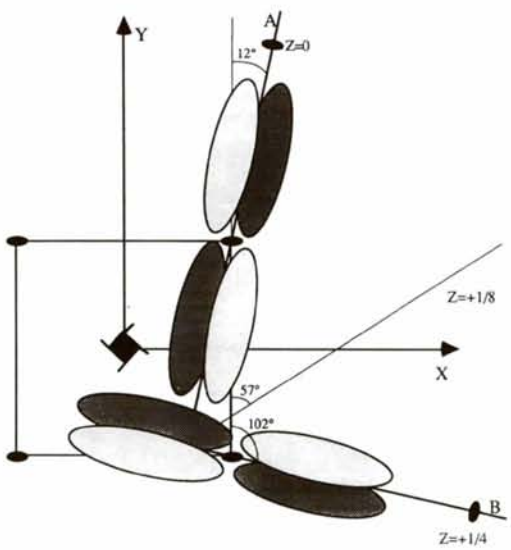

Fig. 3. Hypothetical packing model of pGSHPx tetramers in the tetragonal cell; for a description see *Results and discussion*.

of the systematic absences and the Laue symmetry on rotation photographs showed that the crystals belong to the tetragonal space group $I4_1$ with $a = b = 83.1$ and $c = 131.0$ Å. The final data set [$I_o > 2\sigma(I_o)$] has a merging R factor for symmetry-related reflections of 0.119 at 3.0 Å resolution. The completeness of the data with 7033 unique reflections was 74% (158–3 Å) and still 40% in the resolution shell 3.0–2.9 Å.

Human pGSHPx exists as a tetramer in its native state in solution (Takahashi *et al.*, 1987). The amino-acid sequence of this enzyme predicted from the cDNA sequence revealed a subunit molecular weight of 25 389 Da (Takahashi *et al.*, 1990). Assuming one dimer per asymmetric unit, the V_m value is 2.3 $Å^3\,Da^{-1}$, which is within the most probable range of values found for proteins (Matthews, 1968). Inspection of the self-rotation function (Fig. 2) indicated the presence of two non-crystallographic twofold rotation axes which are perpendicular to each other and also to the crystallographic fourfold screw axis. The local axes are inclined 12° (*A*) and 102° (*B*) against the crystallographic *Y* axis and lie in the *XY* plane. They are necessarily related by the crystallographic fourfold screw axis and show the same correlation height (25σ). These twofold local axes relate the monomers within pGSHPx dimers. A hypothetical packing model derived from the self-rotation results and geometric considerations is shown in Fig. 3. The pGSHPx tetramers are most likely formed by interaction of the local twofolds with the crystallographic twofolds, which are parallel to the crystallographic fourfold screw axis. The tetramers sitting at $Z = 0$, 1/4, 1/2, 3/4 would thus exhibit molecular (222) symmetry. Because of the symmetry relations in Patterson space, another twofold axis is generated which is 45° distant from either local axis *A* or *B*. This packing model has been partly verified by a preliminary cross-rotation search using the crystal structure of glutathione peroxidase from bovine erythrocyte as the search model, but further verification is needed by translation-function analysis. An attempt to solve the three-dimensional structure of this peroxidase by molecular replacement is under way.

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