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Zo-peroxidase: Crystal structure and sequence of a highly-glycosylated peroxidase resistant to high concentrations of H_2O_2 from Japanese radish

Nizaá Jiménez-Arroyo, Paloma C. Gil-Rodríguez[1], Adelaida Díaz-Vilchis, Sonia P. Rojas-Trejo, Enrique Rudiño-Piñera*

Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Colonia Chamilpa, Cuernavaca, Morelos 62210, Mexico

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ABSTRACT

Understanding Peroxidase (PRXs) enzymatic diversity and functional significance from a three-dimensional point of view is a key point for structural and mechanistic studies. In this context, Zo-peroxidase (ZoPrx) a member of the class III peroxidases and secreted by plants, differs from all previously described PRXs because of its remarkable catalytic stability in the presence of hydrogen peroxide. In this work, we present the crystallographic structure of ZoPrx isolated from Japanese radish, at 2.05 Å resolution. The mature enzyme consists of a single monomer of 308 residues exhibiting the same fold as all previously described members of the plant PRXs superfamily. Furthermore, the enzyme contains a heme *b* group as the prosthetic group and two Ca^{2+} binding sites. Moreover, seven *N*-glycosylation sites were found in the structure, and 49 glycans bound to the two ZoPrx molecules found in the asymmetric unit are clearly visible in the electron density map. The comparison of ZoPrx coordinates with homologous enzymes revealed minor structural changes, in which the residue 177 appears to be responsible for enlarging the access to the heme cavity, the only structural finding which may be related to the H_2O_2 tolerance of ZoPrx and detected by X-ray crystallography. Because of its characteristics, ZoPrx has a broad range of potential applications from chemical synthesis to environmental biocatalysis, thus its aminoacidic sequence, partially completed using the electron density, and the three-dimensional structure itself, become a possible starting point to engineering heme-peroxidases to enhance oxidative stability.

1. Introduction

Peroxidases (PRXs, EC 1.11.1) are oxidoreductases present in bacteria, fungi, plants and animals. They use hydrogen peroxide (H_2O_2) to catalyze the oxidation of a wide variety of organic and inorganic substrates, from other proteins to small aromatic molecules, or from large polymers to inorganic ions. All PRXs studied to date share a similar catalytic cycle [1]; the general reaction goes as:

$$2RH + H_2O_2 \rightarrow 2R^{\bullet} + 2H_2O$$

PRXs have aroused interest from a biotechnological perspective for some time; these enzymes compel a large range of applications, from chemical synthesis to environmental biocatalysis. PRXs prosthetic group is one ferriprotoporphyrin IX commonly known as heme *b* [2]. According to their sequence identities and three-dimensional structures, heme peroxidases (hPRXs, EC 1.11.1.7) have been classified in two superfamilies. The first includes vertebrate enzymes and was named as the peroxidase-cyclooxygenase superfamily [3]. Meanwhile the second superfamily comprising bacteria, fungi and plant enzymes is known as the peroxidase-catalase superfamily. Additionally, to these superfamilies, three PRXs families are also described: di-heme peroxidases, dyp-type heme peroxidases and haloperoxidases [4].

The peroxidase-catalase superfamily is the most studied to date. Members of this superfamily, exhibit a conserved fold, contain as prosthetic group the heme *b* and are divided into three classes. The class I, integrated by prokaryotic intracellular enzymes (i.e. yeast cytochrome C peroxidase, CcP); class II that groups extracellular enzymes from fungi (i.e. lignin peroxidase, LiP); and the class III (POXs) which includes all secreted plant peroxidases (i.e. horseradish peroxidase isoenzyme C, HRPC) [1,2,5].

POXs are monomeric enzymes of ~ 300 amino acid residues [6], fundamentally involved in cell elongation, cell-wall formation and self-defense against various pathogen agents [7]. POXs also contain two hepta-coordinated calcium binding sites, four disulfide bridges, one salt

* Corresponding author.
E-mail address: rudino@ibt.unam.mx (E. Rudiño-Piñera).
[1] Current address: Case Western Reserve University, School of Medicine, Department of Physiology and Biophysics, Cleveland, OH, USA.

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bridge invariably localized on an aspartic acid and an arginine (Asp99 and Arg123 for HRPC) and a signal peptide for secretion [5]. All members of the superfamily have the same helical fold independent of the presence (class II and III) or absence (class I) of the disulfide bridges and structural calcium ions [8]. POXs are highly glycosylated; the *N*-glycans linked to these enzymes reach up to 30% of the total protein mass. As an example, HRPC and Banyan Peroxidase (BP) have 8 and 7 glycosylation sites, respectively, that display the Asn-X-Ser/Thr (X is not Pro) sequon, which occurs in loop regions toward the enzyme exterior [9].

Every POX studied so far is susceptible to inactivation by its co-substrate H_2O_2, even in the presence of reducing substrates, because of the low oxidative stability of these hPRXs against catalytic concentrations of H_2O_2, although their susceptibility level differs from enzyme to enzyme [1]. However, the Zo-Peroxidase (ZoPrx), a POX isoform isolated from roots of Daikon radish (Japanese radish *Raphanus sativus* L. var. *Longipinnatus*) has been described as naturally resistant to inactivation by H_2O_2 [10,11]. The mature enzyme consists of a single monomer of 308 residues exhibiting the same folding as the three members of the superfamily of plant PRXs. Additionally, it contains a heme *b* as the prosthetic group and two calcium binding sites within similar positions and deposited in the PDB (entries 6AS2, 1PA2, 1QO4 and 4CUO). In this work, we present the crystal structure of ZoPrx at 2.05 Å resolution and an analysis of its crystallographic structure. We emphasize on the putative role of the structure in regards the capacity of ZoPrx to resist inactivation by H_2O_2. In the structural determination, a complete sequence of ZoPrx was generated by fitting and refining the side chains into the electron density map following a mixed approach described below. The comparison of the ZoPrx three-dimensional structure with structurally homologous enzymes like *Arabidopsis* anionic peroxidase 2 (ATP A2, PDB entry 1PA2) and HRPC (PDB entry 1ATJ) point to the single variation of residue 177, located in the substrate access crevice near the prosthetic group, resulting in a potential bigger aperture toward the heme group reported in the available three-dimensional structures of POXs deposited in the PDB.

2. Materials and methods

2.1. Enzyme MS and crystallization

ZoPrx was isolated from fresh radishes as previously reported and its monomeric nature was tested by gel filtration analysis [11]. Mass spectrometry analysis and identification of the resultant sequences were carried out as described in Gil-Rodríguez et al. [10]. The SDS-PAGE bands of purified enzyme, used in this work, were digested with pepsin instead of trypsin.

Crystals were grown by hanging drop vapor-diffusion method after testing and generating several specific matrices based on *Crystal Screen I* and *II* kits from Hampton Research (Aliso Viejo, CA, USA) at 291 K. Drops were prepared manually in 24-well crystallization plates by mixing the enzyme (2 µl) at 50 mg ml^{-1} (in 10 mM sodium phosphate, pH 6.1) with the reservoir solution (2 µl) containing 0.1 M MES, pH 6.5% and 20% PEG 6000. Crystals suitable for data collection appeared after six weeks and were flash-cooled by immersion in liquid nitrogen exchanging water in the reservoir solution with 20% PEG 200 as cryoprotectant.

2.2. Data collection, data processing, and model refinement

A single diffraction data set was collected from a single crystal at 100 K on beamline X6A of the National Synchrotron Light Source (NSLS), using an ADSC Quantum 270 detector. The diffraction data set was indexed, integrated and scaled using the HKL-2000 suite [12]. Phases were determined by molecular replacement using the program MOLREP [13] with the coordinates from ATP A2 (PDB entry 1PA2) as a search model. The resulting model was improved by rigid-body refinement followed by restrained refinement with REFMAC [14]. The full sequence of ZoPrx was estimated by evaluating the fit of the model to the $2F_O - F_C$ electron-density map. Program COOT [15] was used for manual model building and addition of calcium ions, glycans and water molecules. Glycosylation sites were previously monitored using GlyProt [16]. All further refinement was performed with PHENIX [17] to a final R_{work}/R_{free} of 16.2/19.7%. Data collection and refinement statistics are summarized in Table 1.

Table 1
Summary of crystallographic data collection and refinement. Values in parentheses are for the highest resolution shell.

Parameters	ZoPrx PDB (6AS2)
Data collection statistics	
X-ray source	*BNL NSLS Beamline X6A*
Wavelength (Å)	*1.73*
Space group	$P12_11$
Unit-cell dimensions	
a, b, c (Å)	*59.1, 41.1, 137.8*
α, β, γ angles (°)	*90.0, 96.9, 90.0*
Resolution range (Å)	*30.0–2.05 (2.09–2.05)*
Unique reflections	*51,917*
Completeness (%)	*98.6 (99.2)*
Mosaicity	*0.3*
R_{sym} (%)[a]	*8 (50)*
Solvent content (%)	*53.0*
I/σ(I)	*21.6 (3.5)*
Multiplicity	*6.1 (6.4)*
Asymmetric unit content	*2 monomers*
Refinement statistics	
R_{work}/R_{free} (%)	*16.2/19.7*
B-value ($Å^2$)	
Protein	*30.7*
Water	*40.4*
Heme	*26.6*
Calcium ions	*25.7*
Glycans	*55.2*
PEG fragments	*55.3*
Wilson plot B-value ($Å^2$)	*30.8*
RMSD from ideal stereochemistry	
Bond lengths (Å)	*0.02*
Bond angles (°)	*1.75*
Coordinate error (Maximum-Likelihood Base) (Å)	*0.24*
Ramachandran plot (%)	
Most favored regions	*98.46*
Additional allowed regions	*1.37*
Disallowed regions	*0.17*

[a] $R_{sym} = \Sigma_{hkl} \Sigma_i |I_i(hkl) - (I(hkl))| \Sigma_{hkl} \Sigma_i I_i (hkl)$, where $I_i(hkl)$ and $(I(hkl))$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

2.3. Sequence-structure comparison with homologous enzymes

Initially, a preliminary sequence for ZoPrx based on known sequence segments of ZoPrx filling the unknown residues with ATP A2 sequence was used to search similar regions within available sequences to find homologous enzymes through the EBI NCBI-BLAST [18]. The PDB and the EBI's UniProt (http://www.uniprot.org) were employed to find protein sequences with at least 50% sequence identity with the preliminary sequence of ZoPrx. From the search in the PDB, all the enzymes (all been hPRXs) which had an identity greater than 40% (eight enzymes collectively referred as 8POXs) were selected. From the search within the UniProt, only the first 50 sequences (all been hPRXs) sharing an identity up to 58% were used. A multiple sequence alignment (MSA) was carried out using *MView* [19] with ZoPrx's preliminary sequence as a seed, the 8POXs from the PDB and the 50 hPRXs from UniProt were used to complete the ZoPrx sequence over the electron density map fitting. To identify residue 177 a simulated annealing omit map were performed using the final phases; additionally, residues Arg, Gln, His, Lys, Val, Ser and Thr were modeled and their $2F_O - F_C$ and F_O