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The toolbox of *Auricularia auricula-judae* dye-decolorizing peroxidase – Identification of three new potential substrate-interaction sites

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ABSTRACT

Dye-decolorizing peroxidases (DyPs) such as *Aau*DyPI from the fungus *Auricularia auricula-judae* are able to oxidize substrates of different kinds and sizes. A crystal structure of an *Aau*DyPI–imidazole complex gives insight into the binding patterns of organic molecules within the heme cavity of a DyP. Several small *N*-containing heterocyclic aromatics are shown to bind in the *Aau*DyPI heme cavity, hinting to susceptibility of DyPs to azole-based inhibitors similar to cytochromes P450. Imidazole is confirmed as a competitive inhibitor with regard to peroxide binding. In contrast, bulky substrates such as anthraquinone dyes are converted at the enzyme surface. In the crystal structure a substrate analog, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), binds to a tyrosine-rich hollow harboring Y25, Y147, and Y337. Spin trapping with a nitric oxide donor uncovers Y229 as an additional tyrosine-based radical center in *Aau*DyPI. Multi-frequency EPR spectroscopy further reveals the presence of at least one intermediate tryptophanyl radical center in activated *Aau*DyPI with W377 as the most likely candidate.
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Introduction

Hemoproteins are widespread within the large group of peroxide-dependent enzymes (E.C. classification 1.11). The catalytic cycle of these peroxidases and peroxygenases involving the oxidation of the heme molecule to the so-called Compound I and its subsequent reduction to the resting state has been extensively studied since the late 1960s [1–3]. This reaction sequence is not yet fully understood regarding crucial features such as the actual structure of the catalytic intermediates. It is, however, very well established that all heme peroxidases and peroxygenases employ modifications of this pathway for catalysis. In contrast, from the viewpoint of the substrate molecules to be oxidized the catalytic pathway of some peroxidase superfamilies is much less well understood.

DyP-type peroxidases (E.C. 1.11.1.19) for instance represent a rather young peroxidase superfamily, discovered some twenty years ago [4–6]. They are structurally unusual heme peroxidases possessing a distinct β-sheet fold in the distal region of the heme [7]. A quickly narrowing channel leads from the enzyme's surface to the heme cavity. In *Aau*DyPI, a DyP-type peroxidase produced by the jelly fungus *Auricularia auricula-judae*, the entrance to the heme cavity is defined by the sidechains of the catalytic acid-base pair (D168 and R332) and two hydrophobic residues (L357 and F359). D168 plays an important role in fungal DyPs as its marked flexibility appears to be essential in proton exchange during Compound I formation [8]; in addition, it functions as a width modulator of the heme chamber entrance allowing for the passage of small organic molecules [9]. In this respect, DyPs somewhat resemble another group of fungal heme enzymes, the unspecific

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peroxygenases (UPOs;[1] E.C. 1.11.2.1). As demonstrated in a recently published crystal structure, UPOs possess an almost identical distal acid-base pair, with aspartate replaced by glutamate, and a funnel-shaped heme-access channel [10,11]. The latter channel, however, is wider than in DyPs and is able to sequester even bulky polycyclic aromatic hydrocarbons such as anthracene and pyrene.

Regarding the oxidation of bulky substrates, DyPs take another route as their narrow heme-access channel clearly prevents on-site oxidation at the heme molecule. Compared to other heme peroxidases, DyPs such as *Aau*DyPI are particularly rich in amino acids with electron-rich aromatic side chains such as tyrosine and tryptophan. In fact, seven tyrosines and four tryptophans are incorporated in the *Aau*DyPI polypeptide comprising 448 amino acids. Furthermore, eight out of these eleven residues are surface-exposed to a considerable degree [12]. In view of the archetypical DyP-reaction – the oxidative cleavage of anthraquinone dyes – this clearly hints to the existence of tyrosine and/or tryptophan-based surface-exposed substrate-interaction sites in DyP-type peroxidases. In this respect, DyPs show unmistakable parallels to classical heme peroxidases such as lignin peroxidases (LiPs) or versatile peroxidases (VPs), which withdraw electrons from their substrates using surface-exposed tryptophanyl or tyrosyl radicals [13–15]. Using a combination of spin trapping and mass spectrometry, the first surface-exposed substrate-interaction site in DyPs was identified recently [9,12]. Y337 is conserved in fungal and in some bacterial DyPs and is located in a shallow cleft on the enzyme surface. Its aromatic ring is constrained in an upright position exposing one Cδ–Cε bond to the surrounding solvent [12]. Also, a 17 Å electron-transfer pathway was calculated leading from Y337 to the heme via L357 (part of the heme chamber entrance forming tetrad).

It appears that DyPs combine the two substrate-interaction strategies commonly found in heme peroxidases and peroxygenases. While it was not entirely clear whether *Aau*DyPI is indeed able to bind small aromatic molecules within its heme cavity, a mechanism for the conversion of bulkier substrates at substrate-interaction sites on the enzyme surface was confirmed experimentally. It is, however, questionable that Y337 represents the exclusive radical site in *Aau*DyPI given its richness in suitable tyrosine and tryptophan residues. Combining X-ray crystallography, UV–Vis and EPR spectroscopy, and a combination of spin trapping and mass spectrometry, we offer a more in-depth characterization of substrate interaction in DyP-type peroxidases to address these two issues.

Materials and methods

Chemicals

Diethylamine NONOate (DEA/NO) was purchased from Cayman Chemical. Imidazoles and triazole derivatives were from abcr, Acros, Aldrich, AlfaAesar, BASF, and Dr. Ehrenstorfer (metronidazole).

Enzyme crystallography

*Aau*DyPI was crystallized as reported elsewhere, however the protein concentration in the crystallization drops was elevated to 35 mg/ml [9]. The crystallization condition contained 30% methyl-2,4-pentanediol (MPD), 10% polyethylene glycol 4000, and 100 mM imidazole. The crystals belonged to the monoclinic space group $P2_1$ and grew as thin, often fan-like intergrown plates. Harvested crystals were directly flash-cooled in liquid nitrogen. Diffraction data were collected at the macromolecular beamline ID14-4 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. Data were indexed, processed, and scaled with XDS [16,17]. The structure was determined with the molecular replacement method using Phaser [18] and the PDB entry 4AU9 as search template [9]. The final model was obtained by consecutive building rounds in Coot [19] and refinement in REFMAC5 [20]. Data collection and processing statistics are given in Table 1. The atomic coordinates and structure factors have been deposited in the PDB database under the access code 4UZI. Figures were prepared with PyMOL [21], cavities were calculated with Hollow [22].

UV–Vis spectroscopy

UV–Vis spectra were all recorded on an UviLine 9400 spectrophotometer (Schott Instruments, Mainz, Germany) equipped with a detachable Peltier element (Secomam, Alès, France). The visible spectra of *Aau*DyPI complexes was recorded at ambient temperature in a range of 250–700 nm. Stock solutions of the ligands were prepared in a 5 mM sodium acetate buffer pH 6.8 dissolved in ultrapure water. Hydrophobic ligands were dissolved in dimethyl

Table 1
Data collection and refinement statistics.

Data collection	
Space group	$P2_1$
Unit-cell parameters (Å/°)	a = 67.33; b = 45.71; c = 140.47; β = 91.61
Beamline	ID14–4, ESRF
Wavelength (Å)	0.954
Temperature (K)	100
Number of crystals	1
Resolution range (Å)[a]	47.51–2.10 (2.22–2.10)
Total no. of reflections	179,568 (23,798)
No. of unique reflections	50,456 (7,771)
Completeness (%)	99.2 (95.8)
$I/\sigma(I)$	9.31 (2.72)
R_{merge}	0.106 (0.392)
Refinement	
R_{work}/R_{free}[b]	0.179/0.239
Molecules per asymmetric unit	2
Solvent content (%)	42
No. of amino acid residues	892
No. of heme molecules	2
No. of carbohydrates	9
No. of metal ions	$2K^+$
No. of ligands	4 imidazole, 2 HEPES, 8 MPD (racemic), 6 acetate, 1 oxalic acid, 1 formate, 1 glycolic acid
No. of water molecules	533
RMS deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.763
Ramachandran plot (%)	
Most favoured	95.9
Allowed	3.7
Disallowed	0.4
Average B-factors (Å^2)	
Main-chain atoms	20.7
Side-chain atoms	21.4
Water molecules	27.7
Ligands/hemes/carbohydrates/metals	36.8
Overall	22.3

[a] Values in parentheses belong to the highest resolution shell.
[b] R_{free} are calculated with 5% of the data.

[1] *Abbreviations used:* Aae, *Agrocybe aegerita*; Aau, *Auricularia auricula-judae*; cw-EPR, continuous wave electron paramagnetic resonance; DMP, dimethoxyphenol; DyP, dye-decolorizing peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hfc, hyperfine coupling; LC–MS: liquid chromatography–mass spectrometry; LiP, lignin peroxidase; MPD, methyl-2,4-pentanediol; RMS, root mean square; UPO, unspecific peroxygenase; VP, versatile peroxidase.

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