

Crystallization of *Pichia pastoris* lysyl oxidase

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A copper-containing amine oxidase (PPLO) from the yeast *Pichia pastoris* has been purified and crystallized in two forms. PPLO is a glycoprotein. The molecular mass from SDS–polyacrylamide gels is 112 kDa, consistent with 20% glycosylation by weight (the calculated molecular weight of the polypeptide is 89.7 kDa). Orthorhombic crystals belonging to space group $P2_12_12_1$, with unit-cell parameters $a = 163.7$, $b = 316.1$, $c = 84.0$ Å, diffract to 2.65 Å resolution. Monoclinic crystals belonging to space group $C2$, with unit-cell parameters $a = 248.4$, $b = 121.1$, $c = 151.8$ Å, $\beta = 124.6^\circ$, diffract to 1.65 Å resolution. Native data have been recorded from each crystal form at 100 K using synchrotron radiation. A self-rotation function for the monoclinic crystal form reveals the presence of a non-crystallographic twofold axis perpendicular to the crystallographic twofold axis, consistent with the presence of two dimers in the asymmetric unit.

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1. Introduction

Amine oxidases catalyze the oxidative deamination of organic amines to yield aldehyde, ammonia and hydrogen peroxide: $RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$. Copper-containing amine oxidases (CAOs) have been isolated from a wide range of organisms including bacteria, fungi, yeast, plants and mammals (Buffoni & Ignesti, 2000). To catalyze the two-electron transfer reaction, CAOs contain a novel redox cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ; Li *et al.*, 1998). TPQ is formed from the post-translational modification of a precursor Tyr residue (Mu *et al.*, 1992). CAOs perform both the initial oxidation of Tyr to TPQ and the catalytic oxidation of amines in the same active site. Crystal structures of CAOs from *Escherichia coli* (ECAO; Murray *et al.*, 1999), pea seedling (*Pisum sativum*; PSAO), *Arthrobacter globiformis* (AGAO) and *Hansenula polymorpha* (Li *et al.*, 1998) have been solved and refined (Kumar *et al.*, 1996; Li *et al.*, 1998; Parsons *et al.*, 1995; Wilce *et al.*, 1997). Mammalian CAOs have so far resisted crystallization. The sequences of the cited enzymes may be aligned to yield pairwise identities that range from 23 to 30% (Kuchar & Dooley, 2001). They also have similar tertiary and quaternary structures, indicating that they may have evolved from a common ancestor (Kuchar & Dooley, 2001). Typically, amine oxidases display relatively broad substrate specificities, which makes it difficult to determine their roles in many organisms. All CAOs of known structure are dimers of 70–90 kDa subunits. The largest of the known structures is that of the *E. coli*

enzyme, which has been described as resembling a mushroom with the N-terminal domains of the two subunits making up the stalk (Parsons *et al.*, 1995). The other structurally characterized CAOs lack the 'stalk' domain, but are otherwise closely similar to ECAO with regard to the overall fold. The active sites, characterized by the presence of the Cu atom and the TPQ cofactor, lie at the base of a deep and narrow channel. The distance from the reactive O atom of the TPQ to the protein surface is greater than 10 Å. A CAO from the yeast *Pichia pastoris* was originally characterized as a 'benzylamine oxidase/putrescine amine oxidase' on the basis of its identified substrate specificity (Green *et al.*, 1983). It was noted that this enzyme could also oxidize lysine and ornithine as well as spermine and spermidine, thus preventing a potential use in the quantitation of the diamines. It was subsequently shown that the *P. pastoris* CAO could oxidize the side-chain amino groups of lysine residues in synthetic peptides, elastin and collagen, with specificity and sensitivity to inhibitors similar to the mammalian enzyme lysyl oxidase (Kagan *et al.*, 1984; Tur & Lerch, 1988). Mammalian lysyl oxidases are also copper-containing amine oxidases, but have a lysyl-tyrosyl-quinone (LTQ) cofactor (Wang *et al.*, 1996) and lack sequence homology with the TPQ-containing enzymes, including the *P. pastoris* CAO. In view of its substrate specificity, the *P. pastoris* CAO has been called a 'lysyl oxidase' and is the first reported non-animal lysyl oxidase. *P. pastoris* lysyl oxidase (PPLO) is a TPQ-containing CAO. Despite sequence and

probably structural homology with other TPQ-containing CAOs, access to the active site in PPLO must differ from that in the structurally characterized CAOs, which cannot accommodate a peptidyl lysine residue (Kuchar & Dooley, 2001). At the amino-acid sequence level, the closest homologue of PPLO is human kidney diamine oxidase, with which PPLO is 30% identical (Kuchar & Dooley, 2001). We are seeking to determine the structure of PPLO in order to understand its ability to oxidize peptidyl lysine residues and to provide a model for the difficult-to-crystallize mammalian CAOs.

2. Experimental

2.1. Crystallization

The CAO from *P. pastoris* (PPLO) was cloned, expressed and purified using previously published methods (Dove *et al.*, 1996; Kuchar & Dooley, 2001). Clones with the correct vector were selected in Inv α' *E. coli* cells. These constructs were then incorporated into *P. pastoris* GS115 cells. Positive integrants were screened by PCR and the sequences were verified. Purification of PPLO was carried out as described previously (Dove *et al.*, 1996). Briefly, the yeast-extract mixture was treated with DEAE fast-flow media equilibrated in 20 mM KPO₄ buffer pH 7. Under these

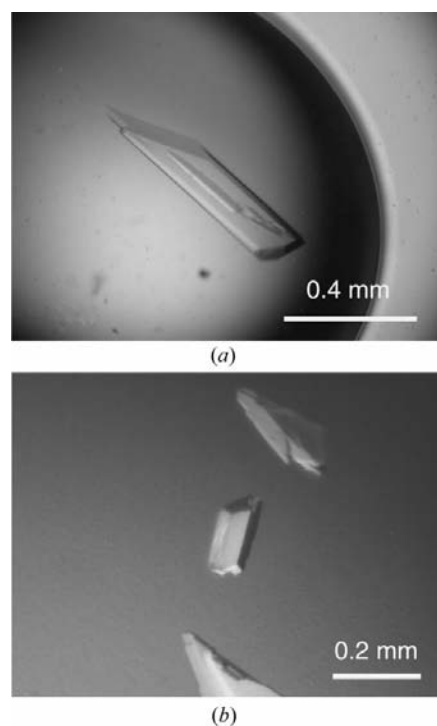


Figure 1
Crystals of PPLO. (a) Form 1, (b) form 2.

Table 1

Data-collection and processing statistics for PPLO.

Values in parentheses refer to the highest resolution shells.

Crystal	Form 1	Form 2
Space group	$P2_12_12_1$	$C2$
Unit-cell parameters (\AA , $^\circ$)	$a = 163.7, b = 316.1,$ $c = 84.0$	$a = 248.4, b = 121.1,$ $c = 151.8, \beta = 124.6$
Unit-cell volume (\AA^3)	4345969	3760290
Asymmetric unit volume (\AA^3)	1086492	940072
Beamline	SSRL 9-2	SSRL 9-1
Detector	ADSC Quantum 4	MAR345
Resolution (\AA)	2.65	1.65
No. of reflections	117862	419017
Completeness (%)	91.6 (81.9)	94.9 (91.2)
R_{merge} (%)	11.4 (32.0)	6.2 (42.0)
Average $I/\sigma(I)$	9.9 (3.0)	13.7 (2.2)
Data with $I > 3\sigma(I)$ (%)	78 (44)	72 (31)
Mean redundancy	3.8 (3.1)	2.8 (2.6)

conditions, the PPLO is quantitatively absorbed. The DEAE was then poured into a column and the PPLO eluted with 500 mM KPO₄ buffer and concentrated. The final purification step involved gel filtration using Sepharose-12. The enzyme was monitored for activity using a standard assay (Tabor *et al.*, 1954). Broad screening for suitable crystallization conditions was first conducted using commercial screens obtained from Hampton Research. Crystals of PPLO were grown using the vapour-diffusion method with hanging drops at 277 K. The stock solution contained 8 mg ml⁻¹ protein and was mixed in a 1:1 ratio with the well solution at the commencement of the crystallization experiment. Two different crystal forms have been obtained. The well solution for form 1 crystals was 30% polyethylene glycol monomethyl ether 2000, 200 mM ammonium sulfate and 100 mM sodium acetate at pH 5.0. Prior to flash-freezing, these crystals were successively transferred to solutions containing 15% glycerol in 5% increments. Form 2 crystals were grown over a well solution containing 25% polyethylene glycol 4000 and 175 mM ammonium sulfate. These crystals were dipped in well solution containing 15% MPD prior to flash-freezing. Crystals of PPLO are shown in Fig. 1.

2.2. X-ray analysis

Synchrotron data were recorded at beamlines 9-2 and 9-1 at SSRL using an ADSC Quantum 4 CCD and MAR345 image plates, respectively. Data were processed with either the *HKL* suite of programs,

DENZO and *SCALEPACK* (Otwinowski & Minor, 1997), or with *MOSFLM* (Leslie, 1992) and *SCALA*. A diffraction image from a form 2 crystal is shown in Fig. 2. Data-collection statistics for native crystals of the two forms are summarized in Table 1. The first form are orthorhombic, space group $P2_12_12_1$, with unit-cell parameters $a = 163.7, b = 316.1, c = 84.0 \text{ \AA}$. These crystals diffract to 2.65 \AA resolution. The most reasonable value of the Matthews coefficient (Matthews, 1968) is obtained for four monomers or two dimers in the crystallographic asymmetric unit. This would imply a solvent content of 50%, calculated if the molecular mass is 110 kDa. Form 2 crystals are monoclinic, space group $C2$, with unit-cell parameters $a = 248.4, b = 121.2, c = 151.8 \text{ \AA}, \beta = 124.6^\circ$. These crystals are also predicted to have two dimers in the

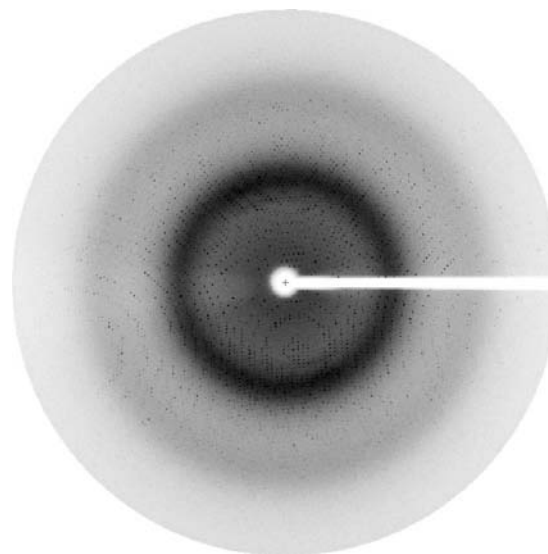


Figure 2
Diffraction image of form 2 crystals of PPLO recorded on beamline 9-1 at SSRL using a MAR345 image-plate scanner (wavelength of 0.9912 \AA). The resolution at the edge of the plate is 1.57 \AA .

asymmetric unit, but the solvent content of 45% is slightly lower. The appearance of the crystals and the quality of the diffraction were difficult to correlate. Some crystals which appeared to be perfect when viewed with polarized light gave diffraction patterns that indicated splitting or twinning of some kind and were discarded. The form 2 crystals could be indexed with an *I*-centered orthorhombic lattice with $a = 119.6$, $b = 150.8$, $c = 204.6$ Å, but the fit was poor at high resolution and merging statistics indicated that the crystals are monoclinic, space group *C2*. Self-rotation functions were calculated with *POLARRFN* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994) at various resolutions with data from both crystal forms. There is a significant peak corresponding to a local twofold axis of symmetry in the form 2 crystals (Fig. 3). This axis is parallel to the crystallographic *c* axis of the monoclinic cell or the *b* axis of the pseudo-orthorhombic cell. Attempts to solve the structure by both molecular-replacement and heavy-atom methods are in progress.

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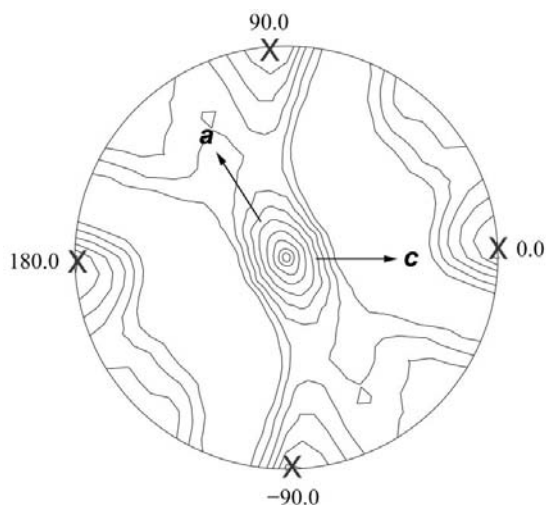


Figure 3

Stereographic projection of the self-rotation function calculated using data in the range 4.5–50 Å and an integration radius to 25 Å. Contours are shown at 0.5, 1.0, 1.5 σ etc. The orthogonalization convention placed *c* parallel to *x*, *a** parallel to *y* and *b* parallel to *z*. The polar angle ω is the angle from the pole (measured from the *z* axis), φ is the angle (values indicated) around the equator (measured from the *x* axis) and the section displayed is for the rotation angle $\kappa = 180^\circ$. The view is down *b* and the other cell directions are indicated. The crosses mark the self-rotation peaks.

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