Crystallization and X-ray diffraction studies of a two-domain laccase from *Streptomyces griseoflavus*

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Laccase (EC 1.10.3.2) is one of the most common copper-containing oxidases; it is found in many organisms and catalyzes the oxidation of primarily phenolic compounds by oxygen. Two-domain laccases have unusual thermostability, resistance to inhibitors and an alkaline optimum of activity. The causes of these properties in two-domain laccases are poorly understood. A recombinant two-domain laccase (SgfSL) was cloned from the genome of *Streptomyces griseoflavus* Ac-993, expressed in *Escherichia coli* and purified to homogeneity. The crystals of SgfSL belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 74.64$, $b = 94.72$, $c = 117.40$ Å, $\beta = 90.672^\circ$, and diffraction data were collected to 2.0 Å resolution using a synchrotron-radiation source. Two functional trimers per asymmetric unit correspond to a Matthews coefficient of 1.99 Å³ Da⁻¹ according to the monomer molecular weight of 35.6 kDa.

1. Introduction

Laccase (benzenediol:oxygen oxidoreductase) belongs to the family of multicopper oxidases, which have applications in the food industry, organic synthesis, cosmetics and medicine (Rodríguez Couto & Toca Herrera, 2006). Laccases have been found in fungi (Bollag & Leonowicz, 1984), plants (McDougall, 2000; Kiefer-Meyer *et al.*, 1996), insects (Dittmer & Kanost, 2010) and bacteria (Suzuki *et al.*, 2014; Hullo *et al.*, 2001). Their active centre contains four copper ions: the blue type 1 mononuclear copper centre (T1) and the trinuclear copper cluster (T2/T3), where the latter consists of a mononuclear type 2 copper centre (T2) and a binuclear type 3 copper centre (T3) (Solomon *et al.*, 1996). T1 acts to receive electrons from the reducing substrate, while T2/T3 serves as a binding site for molecular oxygen and its reduction to water (Bento *et al.*, 2005). Typical laccase molecules consist of three domains; the T1 centre is located in cupredoxin domain 3 and the T2 and T3 copper-binding centres are located between the first and third domains.

Along with typical three-domain laccases (3DLacs), bacteria produce two-domain laccases (2DLacs), which are also referred to as small laccases (SLACs). They consist of two cupredoxin domains. SLAC was first identified in the genomic sequence of *Streptomyces coelicolor* and lacks the second domain of typical 3DLacs. All 2DLacs showed properties that differed from those of three-domain laccases: they were resistant to the inhibitor NaN₃ and were active in the alkaline pH range. The mechanisms that provide these properties of 2DLacs are unknown. The crystal structures of two-domain bacterial laccases have been solved for the enzymes from

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Table 1

SgfSL production information.

Source organism	<i>S. griseoflavus</i> VKM Ac-993
DNA source	<i>S. griseoflavus</i> VKM Ac-993
Forward primer	5'-AGTGGATCCATGGACAGACGCGGTTTCAAC
Reverse primer	3'-TCAAAGCTTTTCAGTGCCTGCTCCTGG
Cloning vector	pQE-30
Expression vector	pQE-30
Expression host	<i>E. coli</i> M15 (pRep4)
Complete amino-acid sequence of the construct produced	HHHHHHGSMDDRRGFNRRVLLGGVAATTSLSLIAPE-AVSAPESAGTAAAGAAPAGGEVRRVTMYAER-LAGGQMGYGLEKKGASIPGPLIELNEGDTLHV-EFENTMDVPVSLHVLHGLDYEISSDGTKQNKSH-VEPGGTRTYTWRTHEPGRADGTWRAGSAGYW-HYHDHVVGTEHGTGGIRNGLYGPVIVRRKGDV-LPDATHTIVFNDMTINNRPAHTGPNFEATVGD-RVEIVMITHGEYYTTFHMHGRWADNRTGMLT-GPDDPSQVIDNKICGPADSFGFQIAGEGVGA-GAWMYHCHVQSHSDMGVGLFLVKKPDGTIPG-YDPQEHAAH

S. coelicolor (Skálová *et al.*, 2009; PDB entry 3cg8), *S. viridosporus* (Majumdar *et al.*, 2014; PDB entry 3tas), *Amycolatopsis* sp. (Majumdar *et al.*, 2014; PDB entry 3t9w), *S. vizeus* (Gunne & Urlacher, 2012; PDB entry 4m3h), *Nitrosomonas europaea* (Lawton *et al.*, 2009; PDB entry 3g5w), *S. viridochromogenes* (Trubitsina *et al.*, 2015; PDB entry 4n8u) and a metagenomic laccase (Komori *et al.*, 2009; PDB entry 2zwn).

Here, we describe the expression, purification, crystallization and preliminary X-ray analysis of a 2DLac from *S. griseoflavus* (SgfSL). The amino-acid sequence of SgfSL has high identity to the SLAC from *S. coelocolor* and 2DLac from *S. viridochromogenes* (87 and 84%, respectively). However, despite the high sequence homology, the thermal stability of SgfSL (353 K) is lower than that of 2DLac from *S. viridochromogenes* (371 K) (Trubitsina *et al.*, 2015). We believe that comparative structural analysis of SgfSL and the laccase from *S. viridochromogenes* will identify the structural basis of the thermal stability of these laccases.

2. Materials and methods

2.1. The microorganism and the cloning, recombinant expression and purification of SgfSL

S. griseoflavus strain VKM Ac-993 was obtained from the All-Russian Collection of Microorganisms (VKM; <http://vkm.ru>). The strain was grown on YEME medium and its genomic DNA was purified from the biomass using a Genomic DNA Purification Kit (Fermentas). Primers for PCR were constructed based on the predicted copper-oxidase sequence from the genome of *S. griseoflavus* Tu4000 (NCBI Reference Sequence WP_004922484.1). The DNA fragment encoding SgfSL was amplified by the PCR technique with primers 5'-AGTGGATCCATGGACAGACGCGGTTTCAAC and 3'-TCAAAGCTTTTCAGTGCCTGCTCCTGG using restriction sites for BamHI before the start codon and HindIII after the stop codon (start and stop codons are underlined and restriction sites are in italics). The PCR product encoding the SgfSL sequence with signal peptide (SP) was digested with BamHI and HindIII and ligated into a pQE-30 vector using

Table 2

Crystallization.

Method	Hanging drop
Plate type	Linbro
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	7
Buffer composition of protein solution	0.1 M NaCl, 0.05 M H ₃ BO ₃ -NaOH pH 9.0
Composition of reservoir solution	0.3 M NaCl, 0.01 M Tris-HCl pH 8.0, 27.5% (w/v) PEG 4K
Volume and ratio of drop	5 µl (4:1)
Volume of reservoir (ml)	0.3

T4-DNA ligase. The resulting plasmid pQE-993 consisted of an N-terminal histidine tag, an N-terminal SP and the mature part of SgfSL.

Escherichia coli strain M15 (pRep4) (Qiagen) transformed with the pQE-993 plasmid was grown at 310 K with shaking at 250 rev min⁻¹ to a cell density of $A_{600} = 0.8$. The production of SgfSL was then induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG; 0.1 mM). Together with IPTG, CuSO₄ was added to a final concentration of 0.25 mM and the culture was incubated for 17 h at 291 K with shaking at 50 rev min⁻¹. The cells were collected by centrifugation at 5000g for 15 min, suspended in 10 ml 20 mM phosphate buffer pH 7.4 containing 0.5 M NaCl and 1 mM imidazole (buffer A) and disrupted by sonication. Cell debris was removed by centrifugation (30 min at 15 000g) and the cell extract was loaded onto a 5 ml HisTrap column (GE Healthcare) equilibrated with buffer A. After loading, the column was washed with four volumes of the same buffer and the protein was eluted with buffer A with 0.3 M imidazole. Active fractions containing the enzyme were collected and loaded onto a HiLoad 16/60 Superdex 200 column equilibrated with 20 mM sodium acetate buffer pH 5.0, 0.1 M NaCl. Details of the cloning and recombinant expression of SgfSL are summarized in Table 1.

2.2. Crystallization

Crystallization experiments were performed at 291 K using the hanging-drop vapour-diffusion method on siliconized glass cover slides in Linbro plates. Drops were made by mixing 4 µl SgfSL at a concentration of 7 mg ml⁻¹ in 0.1 M NaCl, 0.05 M H₃BO₃-NaOH pH 9.0 with 1 µl reservoir solution. The reservoir solution consisted of 0.3 M NaCl, 0.01 M Tris-HCl pH 8.0, 27.5% (w/v) polyethylene glycol (PEG) 4K (condition No. 1.5 of MemGold from Molecular Dimensions). Crystals appeared after 4–5 d and grew to maximum dimensions of 0.05 × 0.1 × 0.7 mm within 2–3 weeks. Details of the crystallization are summarized in Table 2.

2.3. Data collection and processing

For diffraction data collection, a single crystal was flash-cooled after soaking in a solution consisting of 30% PEG 4K, 0.1 M Tris-HCl pH 8.0 prior to data collection. X-ray diffraction data were collected from a single crystal on beamline BL14.1 at BESSY II, Berlin, Germany. The intensities were indexed, integrated and scaled using the XDS

Figure 1

(a) UV–visible absorption spectrum of SgfSL. The maximum at 592 nm corresponds to the type 1 copper site and the shoulder around 330 nm corresponds to the type 3 copper centre. The inset shows an enlargement of the spectrum at 300–700 nm. (b) 12% SDS–PAGE of SgfSL after nickel-affinity and gel-filtration chromatography. Molecular-weight markers (lane *M*) are shown together with the sizes (in kDa) of specific bands.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline BL14.1, BESSY II
Wavelength (Å)	0.91841
Temperature (K)	100
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	345
Rotation range per image (°)	0.1
Total rotation range (°)	140
Exposure time per image (s)	15
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 74.64$, $b = 94.72$, $c = 117.40$, $\alpha = \gamma = 90$, $\beta = 90.672$
Mosaicity (°)	0.276
Resolution range (Å)	50.0–2.0 (2.1–2.0)
Total No. of reflections	411089 (57278)
No. of unique reflections	110108 (14992)
Completeness (%)	99.5 (99.9)
Multiplicity	3.73 (3.82)
$\langle I/\sigma(I) \rangle$	9.09 (2.37)
$R_{\text{r.i.m.}}$	0.114 (0.669)
Overall B factor from Wilson plot (Å ²)	31.39

(Kabsch, 2010). Details of the data-collection and processing statistics are summarized in Table 3. Experimental phasing, model fitting and refinement are in progress.

3. Results and discussion

A comparison with the amino-acid sequences of other *Streptomyces*-derived 2DLac enzymes characterized previously showed a high degree of sequence identity with SgfSL. The amino-acid sequence of SgfSL shares 90% identity with Ssl1 from *S. sviveus*, 75% with EpoA from *S. griseus*, 87% with SLAC from *S. coelicolor* and 90% with SilA from *S. ipomoeae*. All examined sequences contain two cupredoxin domains and complete sets of copper-coordinating ligands. Despite the high sequence homology of SgfSL and other characterized two-domain laccases, SgfSL has a different thermal stability to

the laccases from the *Streptomyces* genus (data not shown). Comparative analysis of the structures of these laccases and subsequent mutational analysis of SgfSL may determine the structural basis of the thermal stability of two-domain laccases.

The SgfSL preparation appeared blue in colour owing to the presence of a T1 copper-centre chromophore with an absorption peak at 590 nm (Fig. 1a). In the absorption spectrum there is a shoulder at 330 nm owing to the presence of a T3 copper centre. Based on its amino-acid sequence, the calculated molecular weight of SgfSL is 35.6 kDa. The apparent molecular weight of SgfSL determined by SDS–PAGE of the protein is 40 kDa (Fig. 1b). The crystals of SgfSL were clearly blue in colour (Fig. 2), belonged to space group $P2_1$ and diffracted to 2.0 Å resolution (Fig. 3). Data statistics are summarized in Table 3.

The initial phases of the structure factors were obtained by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007). The three-dimensional structure of laccase from *S. coelicolor* determined at 2.68 Å resolution (PDB entry 3cg8) was used as the starting model for calculation of the

Figure 2
Crystals of SgfSL.

Figure 3

X-ray diffraction pattern: concentric rings indicate resolution ranges and the high-resolution diffraction pattern is magnified.

Figure 4

Electron-density map around the T3 copper centre calculated with coefficients $(2|F_{\text{obs}}| - |F_{\text{calc}}|)$ with $\delta = 1.6$.

model phases. The top molecular-replacement models were assessed using Z-score and log-likelihood gain (LLG) statistics. Two trimeric molecules were located per asymmetric unit, giving a Z-score of 21.61 (LLG of 914.10), which corresponds to a Matthews coefficient of $1.99 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 38.3% (Matthews, 1968).

The models were initially subjected to crystallographic refinement using *REFMAC5* (Murshudov *et al.*, 2011). Manual rebuilding of the models was carried out in *Coot* (Emsley *et al.*, 2010). An example of an electron-density map is shown in

Fig. 4. The overall structure of the SgfSL functional trimer resembles the SLAC structure. Figures were prepared using *PyMOL* (<http://www.pymol.org>). Refinement of the structure is currently in progress and the structure will be published in the future.

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