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Received 22 August 2011

Accepted 17 September 2011

Thermostable multicopper oxidase from *Thermus thermophilus* HB27: crystallization and preliminary X-ray diffraction analysis of apo and holo forms

A thermostable multicopper oxidase from *Thermus thermophilus* HB27 (*Tth*-MCO) was successfully crystallized using the sitting-drop and hanging-drop vapour-diffusion methods. Crystallization conditions and preliminary X-ray diffraction data to 1.5 Å resolution obtained using synchrotron radiation at 100 K are reported. The crystals belonged to space group $C222_1$, with unit-cell parameters $a = 93.6$, $b = 110.3$, $c = 96.3$ Å. A monomer in the asymmetric unit yielded a Matthews coefficient (V_M) of $2.60 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 53%. An inactive enzyme form, apo-*Tth*-MCO, was also crystallized and diffraction data were collected to 1.7 Å resolution. In addition, a second inactive form of the enzyme, Hg-*Tth*-MCO, was obtained by soaking apo-*Tth*-MCO crystals with mercury(II) chloride and data were collected to a resolution of 1.7 Å.

1. Introduction

Multicopper oxidases (MCOs) catalyze four one-electron oxidations of reducing substrates coupled to the four-electron reduction of molecular oxygen to water. MCOs are a family of enzymes comprising laccases (EC 1.10.3.2), ferroxidases (EC 1.16.3.1), ascorbate oxidase (EC 1.10.3.3) and ceruloplasmin (Hoegger *et al.*, 2006). The catalytic motif in these proteins includes at least four Cu atoms, which are classified into three types of sites: type 1 copper (T1Cu), type 2 copper (T2Cu) and the type 3 binuclear T3'Cu–T3Cu cluster. The type 2 and type 3 sites form a trinuclear Cu cluster (TNC) where molecular oxygen is reduced to water (Quintanar *et al.*, 2007). These enzymes have been found and described in several fungi, plants and bacteria (Mayer & Staples, 2002; Valderrama *et al.*, 2003; Sharma *et al.*, 2007); however, in the case of thermophilic bacterial MCOs little is known about their functional properties (Hildén *et al.*, 2009). MCOs present low substrate specificity and have been used in a variety of biotechnological applications, including organic synthesis (Bernini *et al.*, 2011), biofuel cells (Service, 2002; Vincent *et al.*, 2005), textile dye bleaching (Rodríguez Couto & Toca Herrera, 2006) and bioremediation (Gullotto *et al.*, 2008). The most thoroughly studied thermoactive bacterial MCO is CotA from *Bacillus subtilis*. CotA is an enzyme with optimum ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] oxidation activity at 348 K and a $T_{1/2}$ of almost 2 h at 353 K (Martins *et al.*, 2002). *Thermus thermophilus* strain HB27 locus TT_C1370 encodes the only MCO in this organism (Henne *et al.*, 2004). The protein encoded by this gene has been isolated and found to present oxidase activity with an optimal temperature for ABTS oxidation at 365 K and a $T_{1/2}$ of over 14 h at 353 K (Miyazaki, 2005). Owing to this remarkable resistance to thermal denaturation, *T. thermophilus* HB27 multicopper oxidase (*Tth*-MCO), also named *T. thermophilus* HB27 laccase (EC 1.10.3.2; *Tth*-laccase; Miyazaki, 2005), may have potential in biotechnological applications and is an attractive target for protein engineering. Additionally, the presence of Cu atoms in the active sites of MCOs concentrates radiation damage and/or radiolysis effects in the vicinity, making them targets for radiation-dose X-ray-induced reduction studies.

When a crystal of a copper-containing protein is exposed to X-ray radiation, one of the first events to occur is photoreduction of the



metal atoms. This effect can be seen in the final crystallographic structure as a mixture of copper oxidation states (Macedo *et al.*, 2009). In addition, it has been suggested that the electrons generated during X-ray data collection are able to lead to the reduction of bound molecular oxygen to water in MCOs (Hakulinen *et al.*, 2006). Therefore, structural study of *T. thermophilus* multicopper oxidase in its apo, holo and apo loaded with mercury forms (*Tth*-MCO, apo-*Tth*-

MCO and Hg-*Tth*-MCO, respectively) offers an opportunity to obtain insights into the redox mechanism of this particular family of enzymes guided by their crystalline structures and the interaction of metal atoms with X-ray radiation.

2. Materials and methods

2.1. Gene cloning, protein expression and purification

T. thermophilus strain HB27 (Oshima & Imahori, 1974; DSM 7039, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) was grown as described elsewhere (Dominguez *et al.*, 2010) and genomic DNA was isolated using an UltraClean Microbial DNA-isolation kit (MO BIO). The gene encoded by locus TT_C1370 was obtained by amplification of genomic DNA using the primers 5'-CATATGCAAGGCCCTTCCTTCCCC-3' and 5'-CGAATTCCC-CACCTCGAGGACTCCCAT-3'. The amplified fragment was cloned into pGEM-T Easy vector (Promega) for sequence confirmation. One of the plasmids with the correct sequence was digested with *Nde*I and *Eco*RI and the released fragment of ~1.5 kb was cloned into the corresponding sites of pET32a(+) (Novagen), resulting in plasmid p*Tth*1. Electrocompetent *Escherichia coli* BL21 (DE3) cells were transformed with p*Tth*1 and grown on Luria-Bertani (LB) [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] agar plates containing 200 µg ml⁻¹ ampicillin and 20 µg ml⁻¹ tetracycline at 310 K. A single colony was picked and grown for plasmid isolation and insert verification by sequencing. Once validated, a sample of a liquid culture of exponentially growing BL21 (DE3)/p*Tth*1 was collected by centrifugation, resuspended in a sterile solution of 50% glycerol and maintained at 193 K. Aliquots of the frozen cell stock were thawed and inoculated into 30 ml LB medium containing 200 µg ml⁻¹ ampicillin and 20 µg ml⁻¹ tetracycline and incubated for 12 h at 303 K. Part of the culture (10 ml) was used to inoculate 11 LB medium with 200 µg ml⁻¹ ampicillin and 20 µg ml⁻¹ tetracycline at 303 K until the optical density at 600 nm reached 0.5. Expression of *Tth*-MCO was then induced by adding IPTG (final concentration 0.1 mM) to the medium and incubating for 24 h at 303 K. Cells were concentrated by centrifugation (7500g, 30 min, 277 K) and resuspended in 50 ml 20 mM Tris pH 8.0 containing Complete protease-inhibitor cocktail (Roche Molecular Biochemicals) and DNase (1 µg ml⁻¹). The cell suspension was sonicated on ice and the resulting lysate was heated at 338 K for 20 min. Cell debris was removed by centrifugation (15 300g, 35 min, 277 K) and the supernatant was dialyzed five times at 278 K against ten volumes of 20 mM Tris pH 8.0 with 0.1 mM CuSO₄ to obtain *Tth*-MCO and without CuSO₄ to obtain apo-*Tth*-MCO. Subsequent protein purifications were performed at room temperature and under the same conditions for the apoenzyme and the holoenzyme unless stated otherwise. The supernatant was loaded onto 25 ml SP Sepharose Fast Flow (GE Healthcare) pre-equilibrated with 20 mM Tris pH 8.0, 50 mM NaCl, and *Tth*-MCO was eluted with the same buffer at a flow rate of 1 ml min⁻¹. Fractions with oxidase activity were concentrated and dialyzed by ultrafiltration. Purified recombinant *Tth*-MCO showed a molecular mass of ~50 kDa on SDS-PAGE. Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a

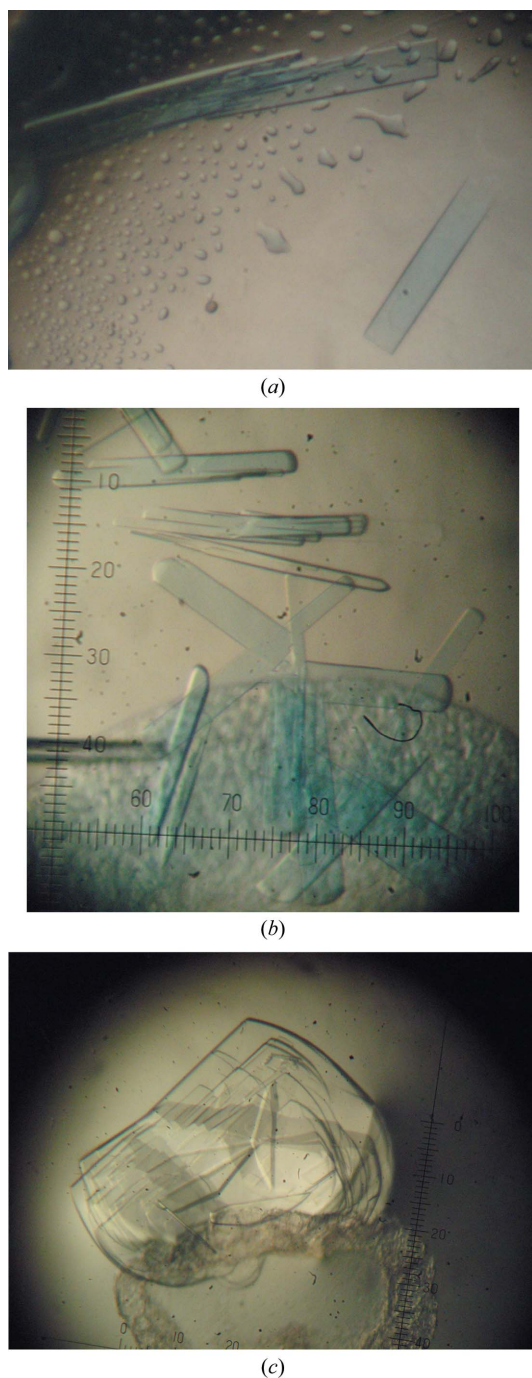


Figure 1 Crystals of *Tth*-MCO obtained by (a) the sitting-drop vapour-diffusion method and (b) the hanging-drop vapour-diffusion method (microseeding technique); (c) crystals of apo-*Tth*-MCO obtained by the hanging-drop vapour-diffusion method. All photographs were taken without a polarizer in order to obtain real colour images and all of the images were obtained at the same magnification. In the case of the plate-like crystals observed in (c) an acupuncture needle was used to separate and cut the crystals before mounting.

Table 1
Summary of crystallographic data.

Values in parentheses are for the highest resolution shell.

	<i>Tth</i> -MCO	<i>Tth</i> -MCO-SAD	Apo- <i>Tth</i> -MCO	Hg- <i>Tth</i> -MCO
Source	BNL NSLS beamline X6A	BNL NSLS beamline X6A	BNL NSLS beamline X6A	BNL NSLS beamline X6A
Wavelength (Å)	0.9795	1.3767	1.3747	0.8321
Space group	<i>C</i> 222 ₁	<i>C</i> 222 ₁	<i>C</i> 222 ₁	<i>C</i> 222 ₁
Unit-cell parameters (Å)	<i>a</i> = 93.6, <i>b</i> = 110.3, <i>c</i> = 96.3	<i>a</i> = 93.6, <i>b</i> = 110.3, <i>c</i> = 96.7	<i>a</i> = 93.0, <i>b</i> = 110.1, <i>c</i> = 96.3	<i>a</i> = 93.5, <i>b</i> = 110.2, <i>c</i> = 96.3
Resolution (Å)	23.0–1.50 (1.60–1.50)	35.0–2.00 (2.10–2.00)	26.0–1.70 (1.80–1.70)	20.0–1.70 (1.80–1.70)
No. of observed reflections	899414	493015	435926	542926
No. of unique reflections	78896 (10654)	34001 (4848)	53818 (7154)	54841 (8585)
Completeness (%)	94.7 (83.9)	99.6 (98.6)	98.9 (82.1)	94.6 (91.4)
Multiplicity	11.4 (9.9)	14.5 (14.1)	8.1 (6.6)	9.9 (10.3)
$\langle I/\sigma(I) \rangle$	19.4 (4.1)	23.6 (8.5)	18.5 (3.8)	9.1 (3.7)
$R_{\text{merge}}^{\dagger}$ (%)	8.6 (39.5)	8.1 (34.7)	9.0 (45.0)	13.3 (35.3)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

standard. The purification yield was approximately 6 mg purified protein from 1 l culture.

2.2. Protein crystallization

Initial crystallization conditions were obtained by the hanging-drop vapour-diffusion method using Crystal Screen and Crystal Screen 2 (Hampton Research). Protein droplets consisting of 1 μ l *Tth*-MCO (19.5 mg ml⁻¹ in 20 mM Tris pH 8.0) and 1 μ l reservoir solution (0.1 M HEPES pH 7.5, 70% MPD; condition No. 35 of Crystal Screen 2) were equilibrated against 1 ml reservoir solution at 278 K. Plate-like crystals appeared and grew to approximate dimensions of around 0.025 \times 0.1 \times 0.25 mm after ten months. After optimization, protein crystals with approximate dimensions of 0.025 \times 0.05 \times 0.1 mm were obtained by the sitting-drop vapour-diffusion method with a protein concentration of 30 mg ml⁻¹ in 20 mM Tris pH 8.0 using 1 + 1 μ l drops and 0.5 ml reservoir solution (0.1 M HEPES pH 7.5, 70% MPD) at a temperature of 278 K, with a growth time of two to three months (Fig. 1a). These crystals were suitable for X-ray analysis; however, they were also used as seeds. Using the microseeding technique, a large number of crystal nuclei were transferred using a horse hair into droplets consisting of 1 μ l protein solution (30 mg ml⁻¹ in 20 mM Tris pH 8.0) and 1 μ l reservoir solution (0.1 M HEPES pH 7.5, 70% MPD), which were equilibrated against 0.5 ml reservoir solution using the sitting-drop vapour-diffusion method or against 1 ml reservoir solution using the hanging-drop vapour-diffusion method. After a week small crystals of *Tth*-MCO were observed, but they required one to two months to reach approximate dimensions

of 0.025 \times 0.05 \times 0.1 mm (Fig. 1b). Apo-*Tth*-MCO crystals were obtained with a protein concentration of 30 mg ml⁻¹ in 20 mM Tris pH 8.0 using a reservoir solution consisting of 0.1 M HEPES pH 7.5, 70% MPD. Plate-like crystals appeared and grew to approximate dimensions of around 0.025 \times 0.1 \times 0.25 mm within three months (Fig. 1c). These apo-*Tth*-MCO crystals were separated using an acupuncture needle before mounting.

2.3. Data collection and crystallographic analysis

Data collection from the *Tth*-MCO, apo-*Tth*-MCO and Hg-*Tth*-MCO crystals was performed on beamline X6A of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL), USA using an ADSC Quantum 270 detector. X-ray diffraction data for *Tth*-MCO were collected from a single crystal at two different wavelengths, $\lambda = 0.9795$ and 1.3767 Å, with the latter being used for the single-wavelength anomalous dispersion (SAD) technique (*Tth*-MCO-SAD data set). Before choosing the wavelengths, an energy scan was performed on the sample in order to identify the energy of the anomalous peak for Cu in *Tth*-MCO (9.003 keV, 1.3767 Å). The crystal-to-detector distance was maintained at 180 mm with an oscillation range per image of 0.5°. For data collection under cryogenic conditions, crystals were soaked for a few seconds in a solution consisting of 0.1 M HEPES pH 7.5, 45% MPD. Crystals were loop-mounted and flash-cooled in a 100 K dry nitrogen stream. Diffraction images were integrated using *XDS* (Kabsch, 2010) and scaling was performed with *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). All of the *Tth*-MCO crystals belonged to a *C*-centred orthorhombic space group, with unit-cell parameters *a* = 93.6, *b* = 110.3, *c* = 96.3 Å, $\alpha = \beta = \gamma = 90^\circ$. A total of 899 414 reflections were integrated to a resolution of 1.5 Å and were merged to obtain 78 896 unique reflections with an overall R_{merge} of 0.086 and a completeness of 94.7%. Matthews coefficient calculations suggested that there was one molecule per asymmetric unit ($V_M = 2.60 \text{ \AA}^3 \text{ Da}^{-1}$, 53% solvent content). A data set ($\lambda = 1.3747 \text{ \AA}$) was collected to a resolution of 1.7 Å for the apo-*Tth*-MCO crystal; its unit-cell parameters were *a* = 93.0, *b* = 110.1, *c* = 96.3 Å, $\alpha = \beta = \gamma = 90^\circ$. The derivative Hg-*Tth*-MCO was obtained by soaking an apo-*Tth*-MCO crystal for 5 min in 5 mM HgCl₂ at 278 K and a data set ($\lambda = 0.8321 \text{ \AA}$) was collected to a resolution of 1.7 Å; the unit-cell parameters of the crystal were *a* = 93.5, *b* = 110.2, *c* = 96.3 Å, $\alpha = \beta = \gamma = 90^\circ$. Data-collection statistics are summarized in Table 1.

The most probable space group for all of the crystals analyzed was a *C*-centred orthorhombic space group. Assuming space group *C*222, the overall R_{merge} for the *Tth*-MCO data set (resolution limits 23.0–1.5 Å) was 0.093 (0.668 in the highest resolution bin). However, when

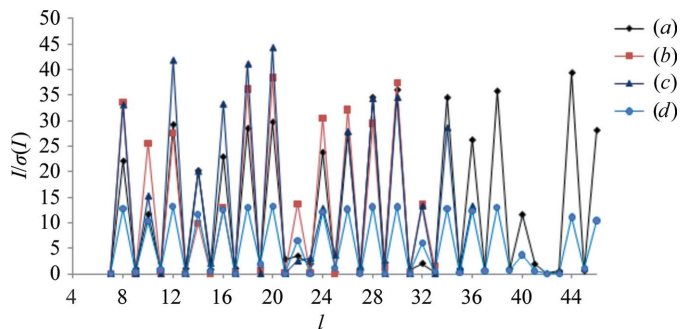


Figure 2
 $I/\sigma(I)$ versus 00*l* reflection plots for the (a) *Tth*-MCO, (b) *Tth*-MCO-SAD, (c) apo-*Tth*-MCO and (d) Hg-*Tth*-MCO data sets. All data sets were integrated in space group *C*222 in order to test the presence of axial reflections in *l* (00*l*: *l* = 2*n*). The presence of a 2₁ screw-rotation axis is clearly visible in the plot, supporting space group *C*222₁.

the same data set with the same resolution limits was integrated in space group $C222_1$ the corresponding value of R_{merge} was 0.086 (0.395 in the highest resolution bin). When all data sets were integrated in space group $C222$, one screw-rotation axis was clearly present in the $I/\sigma(I)$ versus axial reflection plot on l ($00l$: $l = 2n$); this general reflection condition is indicative of space group $C222_1$ (Fig. 2). Additionally, *POINTLESS* (Evans, 2006) clearly chose space group $C222_1$ with a probability of 97.5%.

The structure of *Tth*-MCO was determined by a combination of molecular replacement and single-wavelength anomalous dispersion (SAD) techniques using the Cu signal and assuming space group $C222_1$. Molecular-replacement trials were performed in *Phaser* (McCoy *et al.*, 2007) using the structure of CueO from *E. coli* (Roberts *et al.*, 2002; PDB entry 1kv7) as a model; this structure has a sequence identity of 31.5% to *Tth*-MCO. The phases obtained from molecular replacement were not sufficient for structure determination, but an attempt to solve the phase problem using a combination of molecular replacement and single-wavelength anomalous dispersion (SAD) was successful and two fully occupied and one partially occupied copper-ion positions were found using *Phaser* in MR-SAD mode (McCoy *et al.*, 2007). Initial construction of the protein model was carried out using *ARP/wARP* (Langer *et al.*, 2008). The apo-*Tth*-MCO and Hg-*Tth*-MCO structures were determined by molecular replacement using *Phaser* (McCoy *et al.*, 2007) with the *Tth*-MCO atomic coordinates as a template. Refinement is currently under way using the programs *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010).

HSP was supported by a PhD fellowship from CONACyT. ERP and BV acknowledge financial support from CONACyT projects 102370 and 128156, respectively. ERP gratefully acknowledges financial support of PAPIIT project IN204611. We thank the staff at BNL NSLS beamline X6A for data-collection facilities. Beamline X6A is funded by NIGMS (GM-0080) and the US Department of Energy (No. DE-AC02-98CH10886). The authors thank Biol. Sonia P. Rojas-Trejo, Biol. Guadalupe Paredes-Valdéz and Dr Hector Ayala-Castro for technical assistance.

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