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Crystallization and initial X-ray analysis of phenoxazinone synthase from *Streptomyces antibioticus*

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Phenoxazinone synthase, an oligomeric multicopper oxidase produced by *Streptomyces antibioticus*, is responsible for the six-electron oxidative coupling of two molecules of 4-methyl 3-hydroxy-anthraniloyl pentapeptide to form the phenoxazinone chromophore of the antineoplastic agent actinomycin D. Spectroscopic studies have shown that the enzyme contains one type I (blue) and three to four type II copper centers. However, the exact arrangement of the copper centers in this multicopper oxidase is unknown. As a first step towards determining the three-dimensional structure of the enzyme, phenoxazinone synthase has been crystallized. The hexameric form of phenoxazinone synthase was purified from 72 h cultures of *S. lividans* containing the plasmid pIJ702. Purified hexamers were concentrated to 75 mg ml^{-1} and used to grow two forms of crystals. Data collected from the two crystal forms were processed in two separate space groups. Crystals of both forms were grown at 288 K using the sitting-drop vapour-diffusion method. Native data sets extending to resolutions of 3.35 and 2.30 Å have been collected and processed in space groups *R*32 and *P*1, respectively.

1. Introduction

Phenoxazinone synthase (PHS), produced by *Streptomyces antibioticus*, catalyzes the oxidative coupling of two molecules of 4-methyl 3-hydroxyanthraniloyl pentapeptide to form actinomycinic acid, the penultimate intermediate in the biosynthesis of the antineoplastic agent actinomycin D (Barry *et al.*, 1989). Phenoxazinone synthase can be isolated in two forms, a low-activity dimeric form and a high-activity hexameric form. The dimeric and hexameric forms of PHS are the predominant forms isolated from 12 and 72 h cultures, respectively (Choy & Jones, 1981). These two oligomeric states are distinct molecular entities and are not related by a simple association/dissociation equilibrium. The reason for the difference in enzymatic activity between the dimeric and hexameric forms of PHS is unknown.

The formation of phenoxazinone chromophore involves the six-electron oxidative coupling of two 2-aminophenol molecules with the concomitant reduction of molecular oxygen to water (Barry *et al.*, 1989). Spectroscopic studies have suggested that PHS contains a total of 4–5 Cu atoms per molecule, one of which forms a type I (blue) copper site; the remaining Cu atoms are presumed to be present in type II copper sites or to be bound as inactive copper (Freeman *et al.*, 1993). These data, combined with the lack of an absorption band at 330 nm, suggest that PHS does not contain a type III binuclear copper center, which is found in other members of the multicopper oxidase family of enzymes (Solomon *et al.*, 1996). In order to determine the number and arrangement of the Cu atoms within the enzyme, we have initiated structural characterization of PHS. Here, we report the crystallization and preliminary X-ray analysis of the hexameric form of PHS.

2. Materials, methods and results

2.1. Expression and purification

Cultures of *S. lividans* containing the plasmid pIJ702, which contains a 2.45 kbp insert coding for phenoxazinone synthase, were maintained as previously described (Jones & Hopwood, 1984). *S. lividans* spores were grown following previously established protocols (Jones & Hopwood, 1984). Six 1 l baffled flasks containing 250 ml NZ-amine media (Gallo & Katz, 1972) with 50 µg ml^{-1} thiostrepton were inoculated with *S. lividans* spores and incubated for 48 h in a 303 K rotary shaker (250 rev min^{-1}). Mycelia from the NZ-amine cultures were harvested at 3000*g* for 10 min. The harvested mycelia were washed twice with 0.9%(*w*/*v*) NaCl and used to inoculate six 500 ml baffled flasks containing 200 ml GGA media (Gallo & Katz, 1972) with 50 µg ml^{-1} thiostrepton. The GGA cultures

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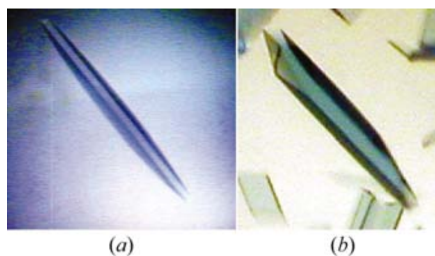
were incubated for 72 h in a 303 K rotary shaker (250 rev min^{-1}). These cultures were harvested at 4500g for 10 min, washed three times with 0.9%(w/v) NaCl and stored at 277 K until used for purification.

All purification steps were carried out at 277 K and were based on a modification of previously described procedures (Barry *et al.*, 1989). Mycelia were resuspended in minimum volume of 50 mM Tris pH 7.6, 1 mM phenylmethylsulfonyl fluoride. Lysozyme was added to a final concentration of 0.2 mg ml^{-1} and the solution was incubated at 295 K for 10 min and sonicated (15 s bursts) on ice while stirring for approximately 2 min. The sonicated solution was centrifuged at 10 000g for 20 min and the supernatant was retained. Nucleic acids were precipitated with streptomycin sulfate and the solution was centrifuged at 20 000g for 20 min. The supernatant was retained and ammonium sulfate was added slowly to 25% saturation. The solution was centrifuged (20 000g, 15 min) and the supernatant was retained. Additional ammonium sulfate was slowly added to the solution until 65% saturation was reached and the solution was centrifuged (20 000g, 15 min). The resulting pellet containing PHS was dissolved in 10 mM sodium phosphate pH 6.0 and dialyzed extensively in this buffer. The PHS solution was concentrated to 5 ml, loaded onto a short hydroxylapatite column equilibrated with 10 mM sodium phosphate pH 6.0 and washed with two bed volumes of 10 mM sodium phosphate pH 6.0 and two bed volumes of 50 mM sodium phosphate pH 6.0. PHS was eluted with 100 mM sodium phosphate pH 7.4. Fractions were analyzed for activity (Barry *et al.*, 1989) and pooled. Fractions showing activity were dialyzed for 24 h against 100 mM sodium phosphate pH 6.0, 500 μM copper (II) chloride followed by extensive dialysis against 100 mM sodium phosphate pH 6.0, 300 mM NaCl. The solution was concentrated to approximately 2 ml and loaded onto an Ultrogel AC34 column (120 × 1.5 cm) equilibrated with 100 mM sodium phosphate pH 6.0, 300 mM NaCl. Fractions were analyzed using native polyacrylamide gel electrophoresis. Fractions showing activity and containing the hexameric form of PHS were combined and dialyzed against 10 mM sodium phosphate pH 6.0. This procedure yields over 50 mg of pure protein. The dialyzed PHS solution was concentrated to approximately 75 mg ml^{-1} using the calculated extinction coefficient of 88 200 M^{-1} cm^{-1} at 280 nm, corresponding to an $A_{0.1\%}$ of 1.31.



(*a*) (*b*)

Figure 1
Crystals of phenoxazinone synthase. Crystals of the *R*32 (*a*) and *P*1 (*b*) space groups both show the characteristic blue colour of multicopper oxidases containing type I copper centers.

Table 1
Data-collection statistics for the two PHS crystal forms.

Values in parentheses are for the outer shell.

	Form 1	Form 2
Space group	*R*32	*P*1
Unit-cell parameters (Å, °)	$a = b = 294.2$, $c = 109.5$, $\alpha = \beta = 90.0$, $\gamma = 120.0$	$a = 109.5$ $b = 163.5$, $c = 164.4$, $\alpha = 117.0$, $\beta = 95.7$, $\gamma = 107.2$
Resolution limits (Å)	51.5–3.35 (3.53–3.35)	55.0–2.30 (2.42–2.30)
Total No. reflections	167349 (21984)	854627 (97894)
No. unique reflections	26066 (3783)	357511 (43794)
Redundancy	6.4 (5.8)	2.4 (2.2)
Completeness (%)	100.0 (100.0)	86.3 (86.3)
Average $I/\sigma(I)$	4.4 (2.2)	11.3 (2.6)
$R_{\rm merge}$† (%)	16.3 (34.5)	5.8 (28.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \overline{I(hkl)}| / \sum_{hkl}\sum_i I_i(hkl)$.

2.2. Crystallization

All crystallization trials were carried out using the sitting-drop vapour-diffusion technique. Initial trials were conducted using ammonium sulfate screens and Hampton Research Crystal Screen Kits I and II (Aliso Viego, CA, USA) at 295 K. Two initial conditions were identified and optimized by varying the protein concentration, incubation temperature, buffer concentration and the use of additives. Optimal conditions for crystal growth were obtained by mixing 4 μl drops of PHS (75 mg ml^{-1}) with 4 μl of a reservoir solution containing 200 mM citric acid pH 5.0, 260–280 mM ammonium sulfate and 15%(v/v) glycerol (condition 1). The plates were stored at 288 K. Crystals suitable for X-ray analysis grew within 12–14 d and were approximately 0.6 mm in length (Fig. 1*a*). Crystallization condition 1 yielded crystals that were assigned to the trigonal *R*32 space group, with unit-cell parameters $a = b = 294.2$, $c = 109.5$ Å, $\alpha = \beta = 90.0$, $\gamma = 120.0°$ (form 1). The Matthews coefficient (Matthews, 1968) for these crystals was calculated based on two subunits per asymmetric unit and was determined to be 3.2 Å^3 Da^{-1}, with a solvent content of 61.8%.

X-ray quality crystals were also grown by mixing 4 μl drops of PHS (75 mg ml^{-1}) with 4 μl of a reservoir solution containing 100 mM sodium citrate pH 5.0, 6.0%(v/v) 2-propanol, 12%(w/v) polyethylene glycol 4000 (PEG 4K) and 15%(v/v) glycerol (condition 2). The plates were stored at 288 K. Crystals suitable for X-ray analysis grew within 7–10 d and were approximately 0.5 mm in length (Fig. 1*b*). Similar conditions have been used to crystallize the spore-coat laccase CotA from *Bacillus subtilis* (Enguita *et al.*, 2002). Crystals grown under these conditions belong to the triclinic *P*1 space group, with unit-cell parameters $a = 109.5$, $b = 163.5$, $c = 164.4$ Å, $\alpha = 117.0$, $\beta = 95.7$, $\gamma = 107.2°$ (form 2). The Matthews coefficient (Matthews, 1968) for these crystals was calculated based on 12 subunits per asymmetric unit and determined to be 2.9 Å^3 Da^{-1}, with a solvent content of 56.6%.

2.3. Data collection, processing and initial phasing

Crystals from both conditions were looped out of the mother liquor and soaked in a cryoprotectant solution consisting of 100 mM sodium citrate pH 5.0, 8%(v/v) 2-propanol, 12%(w/v) PEG 4K and 30%(v/v) glycerol for 2 min. Crystals were cryocooled in a nitrogen-gas steam at 110 K. Data from both crystal forms were collected on a Rigaku R-AXIS IV^{++} image-plate area detector (The Woodlands, Texas, USA) using Cu $K\alpha$ radiation from a Rigaku RU-200HB rotating-anode X-ray generator (50 kV, 100 mA). The X-ray source was equipped with an Osmic confocal mirror assembly. The diffraction data were integrated with *MOSFLM* (Leslie, 1992) and scaled with *SCALA* (Evans, 1997). Data-processing statistics are provided in Table 1.

Initial phases for phenoxazinone synthase were obtained using the molecular-replacement method with the three-dimensional structure of CotA from *B. subtilis* as the

search model (Enguita *et al.*, 2003). Despite the relatively low homology between the PHS and CotA sequences (31% identity), a clear molecular-replacement solution was obtained using *CNS* (Brünger *et al.*, 1998) and the *R*32 data. The rotation function produced two clear peaks and the translation search resulted in two large peaks that were approximately twice as large as the nearest two peaks. Two molecules of CotA were iteratively placed in the asymmetric unit. The addition of the second CotA molecule doubled the monitor value of the translation function. Initial rigid-body refinement of the two CotA molecules using *CNS* (Brünger *et al.*, 1998) resulted in an *R* factor of 48.9% and a free *R* factor of 49.3%. The electron-density map generated from the *R*32 data shows a clear distinction between solvent and protein as well as the expected hexameric arrangement of the individual subunits. Data collected from form 1 crystals were also integrated using space group *C*2, which produced a lower $R_{\rm merge}$ (11.0%). However, no molecular-replacement solution was found using the *C*2 space group.

Altering the residues of the CotA molecules to reflect the sequence of PHS, the addition of the type I copper center and repeated rounds of refinement using *CNS* (Brünger *et al.*, 1998) produced a working model of PHS with an *R* factor of 34.2% and a free *R* factor of 38.9%. The PHS dimer comprising the *R*32 asymmetric unit was used to generate the complete PHS hexamer using *XtalView* (McRee, 1999). The PHS hexamer with the Cu atoms removed was used as a search model for a molecular-replacement search conducted with the *P*1 data and the program *MOLREP* (Vagin & Teplyakov, 1997). The results of the molecular-replacement search showed two hexamers in the unit cell related by a twofold non-crystallographic symmetry axis. The packing of the hexamers in the *P*1 cell was similar to the packing in the *R*32 cell. The initial values of the *R* factor and free *R* factor after *CNS* (Brünger *et al.*, 1998) rigid-body refinement were 51.5 and 51.4%, respectively. Rigid-body refinement conducted with each of the 12 subunits treated as individual rigid bodies lowered the *R* factor and free *R* factor to 45.2 and 45.5%, respectively. The $mF_{\rm o} - DF_{\rm c}$ electron-density maps calculated using the *P*1 data and refined PHS hexamer clearly showed electron density for the missing Cu atoms in all 12 subunits.

3. Discussion

The growth of X-ray quality crystals of phenoxazinone synthase was highly dependent on the concentration of the protein. A protein concentration of 35 mg ml^{-1} yielded crystals that were too small for X-ray analysis. Increasing the protein concentration to 75 mg ml^{-1} greatly increased the size and quality of the crystals and yielded large crystals with the characteristic blue colour of the type I copper center present in multicopper oxidases. The blue colour of the crystals observed in both crystal forms was stable and remained after a number of weeks.

Form 2 crystals diffracted to a higher resolution than form 1 crystals (2.30 compared with 3.35 Å). In addition, $R_{\rm merge}$ for the form 2 crystals was significantly lower than $R_{\rm merge}$ for the form 1 crystals assigned to space group *R*32. Although data from form 2 crystals were collected to a higher resolution, the hexameric arrangement of the individual protein molecules within the *P*1 cell is not constrained by crystallographic symmetry and may not be exact. Therefore, the molecular-replacement search using CotA and the subsequent refinement of the initial model were carried out using the data from form 1 crystals. The resulting model packed into well defined hexamers with a clear solvent–protein interface. The electron-density maps calculated without Cu atoms contained strong electron density for the Cu atoms and well defined density for all residues near the copper sites. Once refined to an acceptable *R* factor, the *R*32 model was used to determine the packing arrangement of the *P*1 cell. The results of the molecular-replacement search showed two hexamers related by a twofold non-crystallographic symmetry axis. The electron-density maps calculated using the *P*1 model clearly showed electron density for the missing Cu atoms, clear density for all copper ligands and improved electron density for missing loops. Model building and structural refinement are currently under way using the *P*1 data.

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