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addenda and errata

Purification, crystallization and preliminary X-ray diffraction studies of the three components of the toluene 2,3-dioxygenase enzyme system. Corrigendum

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The article by Lee *et al.* (2005) [*Acta Cryst.* F**61**, 669–672] is corrected.

Fig. 1 of the article by Lee *et al.* (2005) was labelled incorrectly. The first component should be reductase and the second ferredoxin. A correct version of Fig. 1 is given below.

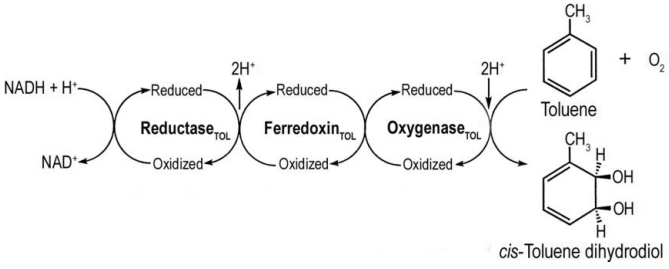

Figure 1
Dihydroxylation of toluene to *cis*-toluene dihydrodiol catalyzed by the three-component toluene dioxygenase enzyme system.

References

Lee, K., Friemann, R., Parales, J. V., Gibson, D. T. & Ramaswamy, S. (2005). *Acta Cryst.* F**61**, 669–672.

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Purification, crystallization and preliminary X-ray diffraction studies of the three components of the toluene 2,3-dioxygenase enzyme system

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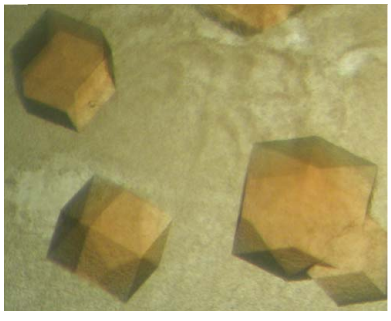
Pseudomonas putida F1 can grow with toluene as its sole source of carbon and energy. The initial reaction of the degradation of toluene is catalyzed by a three-component toluene dioxygenase enzyme system consisting of a reductase ($Reductase_{TOL}$), a ferredoxin ($Ferredoxin_{TOL}$) and a Rieske non-heme iron dioxygenase ($Oxygenase_{TOL}$). The three components and the apoenzyme of the dioxygenase (apo-$Oxygenase_{TOL}$) were overexpressed, purified and crystallized. $Reductase_{TOL}$ diffracts to 1.8 Å and belongs to space group $P4_12_12$, with unit-cell parameters $a = b = 77.1$, $c = 156.3$ Å. $Ferredoxin_{TOL}$ diffracts to 1.2 Å and belongs to space group $P2_1$, with unit-cell parameters $a = 30.5$, $b = 52.0$, $c = 30.95$ Å, $\beta = 113.7^\circ$. Apo-$Oxygenase_{TOL}$ and $Oxygenase_{TOL}$ diffract to 3.2 Å and belong to space group $P4_332$, with unit-cell parameters $a = 235.9$ Å and $a = 234.5$ Å, respectively.

1. Introduction

Toluene is used extensively as a gasoline component and as an industrial solvent (Greenberg, 1997). Unfortunately, this widespread use has resulted in the identification of toluene as a frequent pollutant of soils, groundwater and the urban atmosphere. Bacteria play a key role in the biodegradation of toluene. For example, *Pseudomonas putida* F1 can grow with toluene as its sole source of carbon and energy (Gibson *et al.*, 1968). This organism initiates the catabolism of toluene by the enantioselective addition of dioxygen to the aromatic nucleus to form *cis*-(1*R*,2*S*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol; Gibson *et al.*, 1970; Kobal *et al.*, 1973). The reaction is catalyzed by the three-component toluene dioxygenase enzyme system organized as shown in Fig. 1. The reductase ($Reductase_{TOL}$) is a flavoprotein (Subramanian *et al.*, 1981) which transfers electrons from NADH to a Rieske [2Fe–2S] ferredoxin ($Ferredoxin_{TOL}$; Subramanian *et al.*, 1985). The latter shuttles electrons to the dioxygenase ($Oxygenase_{TOL}$), which catalyzes the formation of *cis*-toluene dihydrodiol (Subramanian *et al.*, 1979). $Oxygenase_{TOL}$ is a Rieske non-heme iron dioxygenase and a member of the toluene/biphenyl family (Gibson & Parales, 2000). Previous studies have suggested that $Oxygenase_{TOL}$ has an $\alpha_2\beta_2$ subunit composition, with each α-subunit containing a Rieske [2Fe–2S] cluster and mononuclear iron at the active site (Jiang *et al.*, 1996). More recent work on the structure of naphthalene (Kauppi *et al.*,



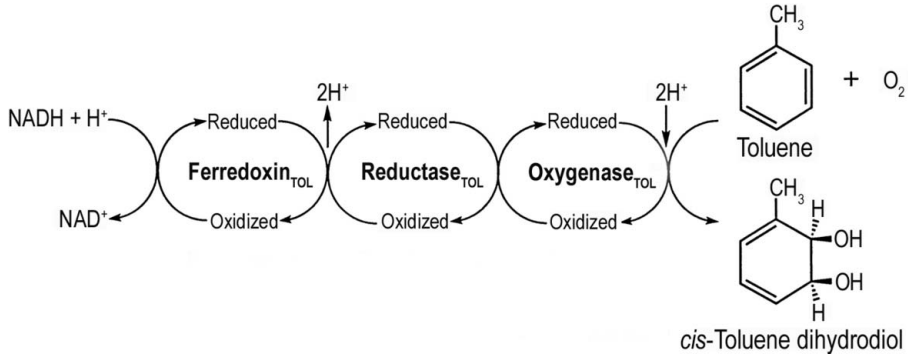


Figure 1
Dihydroxylation of toluene to *cis*-toluene dihydrodiol catalyzed by the three-component toluene dioxygenase enzyme system.

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1998), nitrobenzene (Friemann *et al.*, 2005) and biphenyl dioxygenases (Furusawa *et al.*, 2004) has shown that each dioxygenase component has an $\alpha_3\beta_3$ subunit. The α-subunits from each protein contain a Rieske [2Fe–2S] cluster coordinated to two histidine and two cysteine residues and a His,His-carboxylate facial triad (Kauppi *et al.*, 1998; Hegg & Que, 1997) which binds mononuclear iron at the active site. The genes encoding Reductase$_{TOL}$ (*todA*), Ferredoxin$_{TOL}$ (*todB*) and Oxygenase$_{TOL}$ (*todC1* and *todC2*) have been cloned and their respective nucleotide sequences determined (Zylstra & Gibson, 1989; Huang, 1991). To date, more than 30 Rieske non-heme iron oxygenase systems have been identified by sequence analysis and/or protein purification. However, three-dimensional structures are only available for a few of them. In this paper, we present the overexpression, purification and crystallization of the three components of the Oxygenase$_{TOL}$ enzyme system, along with X-ray diffraction data.

2. Materials and methods

2.1. Expression of the three components of the toluene dioxygenase enzyme system

Escherichia coli CGSC#7692 (pDTG601A) containing the cloned *todC1C2BA* genes that encode Oxygenase$_{TOL}$, Ferredoxin$_{TOL}$ and Reductase$_{TOL}$ from *P. putida* F1 were expressed under the control of a IPTG-inducible *tac* promotor carried on the expression vector pKK223-3 (Pharmacia) (Zylstra & Gibson, 1991). *E. coli* CGSC#7692 is a tryptophanase (*tnaA5*) negative strain, which does not produce indigo on expression of the toluene dioxygenase enzyme system. The tryptophanase-negative strain was necessary in order to be able to grow the cells in rich media that contain tryptophan. Tryptophanase cleaves tryptophan and produces indole. Indole is a good substrate for the enzyme system and results in indigo production, which ends up being toxic to the cell and results in lower yields. Cells were grown at 291 K in a 8 l fermentor inoculated with a 500 ml seed culture to an $OD_{600\,nm}$ of 2.4 in a medium containing yeast extract (80 g), tryptone (80 g), NaCl (40 g), tryptophan (0.4 g), ampicillin (0.4 g) and ferrous ammonium sulfate (0.4 g). Protein expression was induced with IPTG (1 g) for 3 h. Cells were harvested by centrifugation at 14 300*g* at 277 K for 10 min, suspended in BTGD buffer (50 m*M* Bis-Tris pH 6.8, 5% glycerol, 1 m*M* dithiothreitol; Lee *et al.*, 1997) and stored at 201 K until use.

2.2. Purification

All purification procedures were performed at 286 K using an automated FPLC system (Bio-Rad Laboratories, Hercules, CA, USA). Chromatography columns and column resins were from Pharmacia LKB.

Frozen IPTG-induced recombinant *E. coli* cells were thawed and DNase I was added to a final concentration of 1 µg ml^{-1}. The cells were broken by passage through a chilled French pressure cell at 137 MPa. The crude cell-free extract was obtained from the supernatant following ultracentrifugation at 146 000*g* for 1 h at 277 K.

2.2.1. Separation of Reductase$_{TOL}$, Ferredoxin$_{TOL}$ and Oxygenase$_{TOL}$. Crude cell extract (6.6 g) was applied onto a XK50/30 chromatography column with Q-Sepharose FF (5 × 25 cm) pre-equilibrated with BTDG buffer. The column was washed with 400 ml BTGD buffer containing 0.2 *M* KCl at a flow rate of 2 ml min^{-1} to elute unbound proteins. Bound proteins were eluted with a 2 l linear KCl gradient (0.2–0.5 *M*) in BTGD buffer at a flow rate of 2 ml min^{-1}. The proteins were eluted in the order Reductase$_{TOL}$, Ferredoxin$_{TOL}$ and Oxygenase$_{TOL}$.

2.2.2. Purification of Reductase$_{TOL}$. Fractions containing active Reductase$_{TOL}$ were pooled, concentrated and desalted by ultracentrifugation over an Amicon YM-30 membrane. After centrifugation, the supernatant was applied onto a Blue Sepharose CL-6B column (2.6 × 28 cm) pre-equilibrated with BTGD buffer. The column was eluted with BTGD buffer at a flow rate of 0.5 ml min^{-1}. Fractions containing Reductase$_{TOL}$ were pooled, concentrated and desalted by ultrafiltration over an Amicon YM-30 membrane. After centrifugation, the supernatant was applied onto a DEAE cellulose DE52 column (2.6 × 12 cm) pre-equilibrated with BTGD buffer. The bound protein was eluted with a 450 ml linear KCl gradient (0–0.5 *M*) in BTGD buffer at a flow rate of 0.5 ml min^{-1}. Fractions containing Reductase$_{TOL}$ were pooled, desalted, concentrated by ultrafiltration and centrifugated. The supernatant protein solution in BTGD buffer was rapidly frozen in liquid nitrogen and stored at 345 K.

2.2.3. Purification of Oxygenase$_{TOL}$. Fractions containing active Oxygenase$_{TOL}$ from the Q-Sepharose FF column were pooled and concentrated by ultrafiltration over an Amicon YM-100 membrane. Solid $(NH_4)_2SO_4$ was slowly added under stirring to a final concentration of ∼1 *M*. After centrifugation, the supernatant was applied onto a butyl Sepharose 4B column (2.6 × 26 cm) equilibrated with BTGD buffer containing 1 *M* $(NH_4)_2SO_4$. Oxygenase$_{TOL}$ was eluted with a 450 ml decreasing linear $(NH_4)_2SO_4$ gradient (1–0 *M*) followed by 150 ml BTGD buffer at a flow rate of 0.5 ml min^{-1}. Active fractions were pooled, desalted and concentrated by ultrafiltration over a YM100 membrane. The purified Oxygenase$_{TOL}$ in BTGD buffer was stored as described above.

2.2.4. Purification of Ferredoxin$_{TOL}$. Because the level of expression of Ferredoxin$_{TOL}$ was not sufficient in *E. coli* CGSC#7692 (pDTG601A), we used *E. coli* JM109 (pDTG614) (Zylstra & Gibson, 1991) for purification of Ferredoxin$_{TOL}$. The conditions for production of the cell extract and running the first Q-Sepharose FF column with the cell extract of *E. coli* JM109 (pDTG614) were the same as described in §2.2.1.

Fractions containing active Ferredoxin$_{TOL}$ from the Q-Sepharose FF column were pooled and concentrated by ultracentrifugation over an Amicon YM-10 membrane as described above. $(NH_4)_2SO_4$ was added under slow stirring to a final concentration of 1.6 *M*. The precipitate was centrifugated and the supernatant was applied onto a Octyl Sepharose column (2.6 × 16.5 cm) equilibrated with BTGD buffer containing 1.6 *M* $(NH_4)_2SO_4$. The column was eluted isoratically with a flow rate of 0.4 ml min^{-1}. Fractions containing Ferredoxin$_{TOL}$ were pooled, concentrated, washed with BTGD buffer by ultrafiltration and stored as described above.

2.3. Analytical procedures and enzyme assays

Samples of purified proteins were analyzed on 12.5% SDS–PAGE and by dynamic light scattering using a light-scattering apparatus from Protein Solutions. The fractions containing the active TDO components during purification were assayed with ^{14}C toluene as the substrate in the presence of the other two components as described previously (Jiang *et al.*, 1996). In addition, the active fractions containing Ferredoxin$_{TOL}$ and Oxygenase$_{TOL}$ components could be identified and pooled from their brown colour. Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. The activity of Oxygenase$_{TOL}$ was determined by measuring the rate of oxygen consumption with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) in a chamber containing 1 ml 50 m*M* MES buffer pH 6.8, 0.25 m*M* NADH, 0.61 µ*M* Reductase$_{TOL}$, 2.5 µ*M* Ferredoxin$_{TOL}$ and 0.1 µ*M* Oxygenase$_{TOL}$ by injection of 2 µl toluene

Table 1
X-ray data-collection statistics.

Values in parentheses indicate the statistics for the last resolution shell.

	Reductase$_{TOL}$	Ferredoxin$_{TOL}$	Apo-Oxygenase$_{TOL}$	Oxygenase$_{TOL}$
Space group	$P4_12_12$	$P2_1$	$P4_332$	$P4_332$
Unit-cell parameters (Å, °)	$a = b = 77.13$, $c = 156.38$	$a = 30.54$, $b = 52.09$, $c = 30.95$, $\beta = 113.67$	$a = b = c = 235.87$	$a = b = c = 234.53$
Beamline	X26A, BNL	ID14-1, ESRF	17-ID, APS	ID14-2, ESRF
Resolution (Å)	19.9–1.8 (1.86–1.8)	28.3–1.2 (1.26–1.20)	48.0–3.2 (3.37–3.2)	30.0–3.2 (3.37–3.2)
No. of observations	409869	91725	366056	886708
Unique reflections	44352	25558	37544	36887
Completeness (%)	99.0 (96.4)	98.2 (96.8)	100 (100)	99.9 (100)
R_{merge}†	7.6 (38.6)	8.8 (39.9)	12.3 (67.2)	11.8 (38.9)
$I/\sigma(I)$	14.6 (4.0)	3.7 (2.1)	5.6 (1.2)‡	6.0 (2.0)
Multiplicity	9.2 (7.0)	3.4 (3.0)	9.7 (8.5)	24.0 (24.7)

† $R_{merge} = \sum_h \sum_i I(h)_i - \langle I \rangle | / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of a reflection h, $\sum_h$ is the sum over all reflections and $\sum_h$ is the sum over I measurements of reflection h. ‡ $I/\sigma(I)$ is 2.00 for the apo-Oxygenase at 3.5 Å resolution.

(0.1 *M* dissolved in methanol) in the presence or absence of ferrous iron (0.125 m*M*) as described previously (Lee, 1999).

2.4. Crystallization

Crystallization was performed by the hanging-drop or sitting-drop vapor-diffusion method. In all the experiments except for Oxygenase$_{TOL}$ (see §3), 1.5–2 µl protein solution and 1.5–2 µl precipitant solution were equilibrated against 0.7–1 ml precipitant solution. The initial crystallization conditions were found using Hampton Crystal Screen (Hampton Research, USA) and Wizard I and II Screens (Emerald Biostructures, USA).

3. Results and discussion

The three components of the toluene dioxygenase enzyme system have been purified from wild-type *P. putida* strain F1 and characterized (Subramanian *et al.*, 1979, 1981, 1985). In this study, we have further optimized the purifications from recombinant *E. coli* cells to obtain large quantities of proteins for crystallization. The procedures described in §2 gave 25 mg Reductase$_{TOL}$, 19.2 mg Ferredoxin$_{TOL}$ and 380 mg Oxygenase$_{TOL}$, with purification folds of 66, 103 and 8, respectively. The preparations were homogenous when analyzed by SDS–PAGE and gave the same absorption spectra as reported previously (Subramanian *et al.*, 1979, 1981, 1985). The purified Oxygenase$_{TOL}$ yielded a specific activity of 2.15 µmol min^{-1} mg^{-1} (equivalent to a turnover of 2.7 s^{-1}) in the presence of ferrous iron in the assay mixture. However, the activity was negligible in the absence of added ferrous iron, indicating that labile mononuclear iron was lost during purification, as Oxygenase$_{TOL}$ retained the visible spectrum arising from Rieske [2Fe–2S] clusters. Thus, we referred to the initially purified protein as Oxygenase$_{TOL}$ apoenzyme (apo-Oxygenase$_{TOL}$).

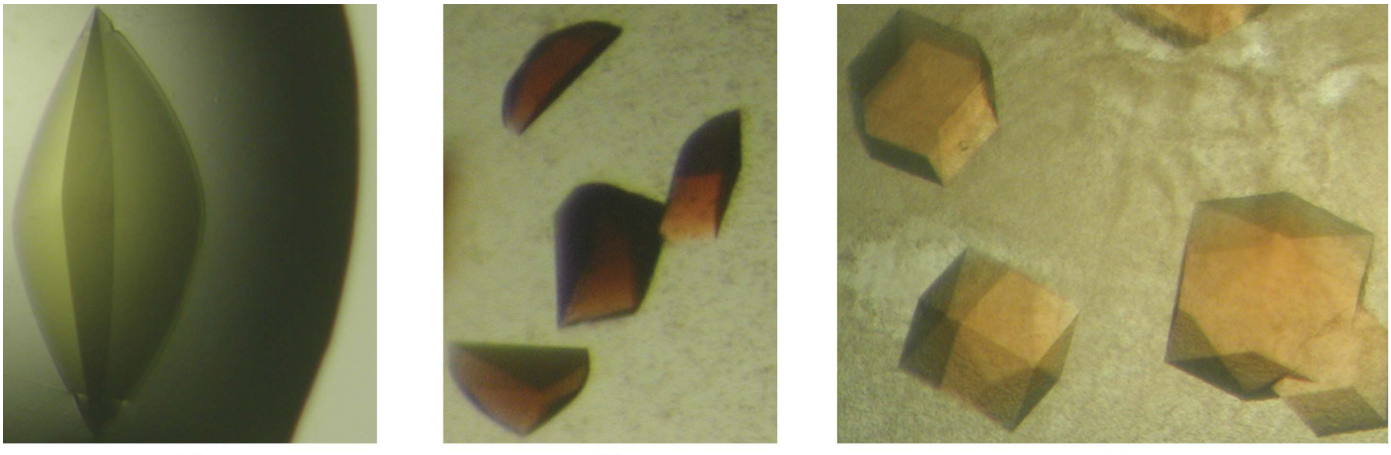
3.1. Crystallization

Photographs of the crystals of Reductase$_{TOL}$, Ferredoxin$_{TOL}$ and Oxygenase$_{TOL}$ are presented in Fig. 2.

Reductase$_{TOL}$ (25 mg ml^{-1} in half-strength BTGD buffer) initially crystallized in 0.1 *M* Tris pH 8.5, 1.5 *M* $(NH_4)_2SO_4$ and 12% glycerol at 286 K. These conditions were optimized to 1.4 *M* $(NH_4)_2SO_4$ and 0.1 *M* HEPES pH 7.7. Crystals (0.1 × 0.1 × 0.3 mm) were formed in two weeks.

Ferredoxin$_{TOL}$ (6 mg ml^{-1} in half-strength BTGD buffer) crystallized at 286 K in 38% PEG 8000, 0.1 *M* MES pH 6.1 or 37% PEG 8000, 0.1 *M* MES pH 5.8. Crystals (0.1 × 0.2 × 0.3 mm) formed within 3 d.

Apo-Oxygenase$_{TOL}$ (50 mg ml^{-1} in BTGD buffer) crystallized at 286 K in 40%(*w*/*v*) polyethylene glycol 600, 0.1 *M* sodium citrate pH 5.8 at 286 K. Cubic crystals (0.1 × 0.1 × 0.1 to 1.0 × 1.0 × 1.0 mm) were obtained within one week.



(*a*) (*b*) (*c*)

Figure 2
Photographs of crystals of Reductase$_{TOL}$ (*a*), Ferredoxin$_{TOL}$ (*b*) and Oxygenase$_{TOL}$ (*c*).

Co-crystallization experiments with $Fe(NH_4)_2(SO_4)_2$ were performed under strict anaerobic conditions as described for apo-Oxygenase$_{TOL}$. Cubic crystals (0.1 × 0.1 × 0.1 to 1.0 × 1.0 × 1.0 mm) were obtained within 24 h in 40%(*w*/*v*) polyethylene glycol 600, 0.1 *M* sodium citrate pH 6.1, 20–50 m*M* $Fe(NH_4)_2(SO_4)_2$ at 296 K.

3.2. X-ray diffraction study

Diffraction data were collected under liquid-nitrogen cryoconditions at 100 K. Data-collection statistics are summarized in Table 1.

Reductase$_{TOL}$ crystals were cryoprotected using reservoir solution supplemented with 10%(*v*/*v*) glycerol and flash-cooled by rapidly moving them into a cold nitrogen stream. X-ray diffraction data were collected at beamline X26A at Brookhaven National Laboratories (BNL), Upton, NY, USA using a ADSC Q4 CCD detector. The crystals diffracted to 1.8 Å and belong to space group $P4_12_12$, with unit-cell parameters $a = b = 77.13$, $c = 156.38$ Å. The asymmetric unit contain one molecule of Reductase$_{TOL}$, corresponding to a solvent content of 54%. The data were indexed, integrated, scaled and merged using *d*TREK* (Pflugrath, 1999).

Ferredoxin$_{TOL}$ crystals were soaked in a cryoprotection solution consisting of the reservoir solution with the addition of 25%(*v*/*v*) polyethylene glycol 400 for 10–30 s upon flash-cooling in liquid nitrogen. X-ray diffraction data were collected at beamline ID14-1 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France using an ADSC Q4 CCD detector. The crystals belongs to space group $P2_1$, with unit-cell parameters $a = 30.54$, $b = 52.09$, $c = 30.95$ Å, $\beta = 113.67^\circ$, and diffracted to 1.2 Å. These crystals have a solvent content of 35%. The data were indexed, integrated, scaled and merged using *MOSFLM* (Powell, 1999) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

The Oxygenase$_{TOL}$ apoenzyme crystals were flashed-cooled in liquid nitrogen after a 10–30 s soak in a cryoprotection solution consisting of the reservoir solution with the addition of 25%(*v*/*v*) glycerol. Crystals of Oxygenase$_{TOL}$ co-crystallized with $Fe(NH_4)_2(SO_4)_2$ were flashed-cooled in liquid nitrogen inside an anaerobic box after a 10–30 s soak in a cryoprotection solution consisting of the reservoir solution with the addition of 25%(*v*/*v*) glycerol. X-ray diffraction data were collected on beamline ID14-2 at the ESRF, Grenoble, France using an ADSC Q4 CCD detector and on beamline 17-ID (IMCA-CAT) at the Advanced Photon Source (APS), Argonne, IL, USA using an ADSC Q4 CCD detector. The apoenzyme and Oxygenase$_{TOL}$ co-crystallized with $Fe(NH_4)_2(SO_4)_2$ belong to space group $P4_332$. The asymmetric unit contain one $\alpha\beta$ heterodimer, with a solvent content of 84% as estimated by the program *TRUNCATE* (Collaborative Computational Project, Number 4, 1994). The data were indexed, integrated, scaled and merged using *MOSFLM* (Powell, 1999) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

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Purification, crystallization and preliminary X-ray diffraction studies of the three components of the toluene 2,3-dioxygenase enzyme system. Corrigendum

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The article by Lee *et al.* (2005) [*Acta Cryst.* F**61**, 669–672] is corrected.

Fig. 1 of the article by Lee *et al.* (2005) was labelled incorrectly. The first component should be reductase and the second ferredoxin. A correct version of Fig. 1 is given below.

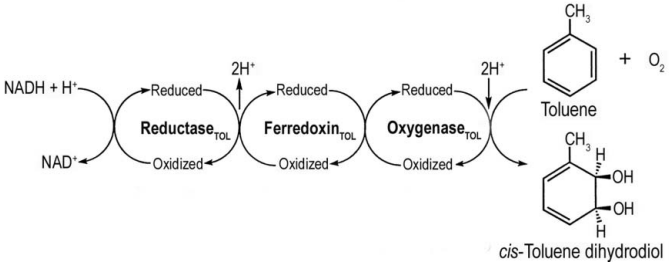

Figure 1
Dihydroxylation of toluene to *cis*-toluene dihydrodiol catalyzed by the three-component toluene dioxygenase enzyme system.

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