# Purification and characterization of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB 13259 and cloning and sequencing of its *catA* gene

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A method was developed for the purification of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB 13259 that had been grown in the presence of benzyl alcohol. The enzyme has very similar apparent $K_m$ (1–2 $\mu$M) and $V_{max}$ (13–19 units/mg of protein) values for the intradiol cleavage of catechol, 3-methylcatechol and 4-methylcatechol and it is optimally active at pH 9. Cross-linking studies indicate that the enzyme is a homodimer. It contains 0.6 atoms of Fe per subunit. The enzyme was crystallized with 15% (w/v) poly(ethylene glycol) 4000/0.33 M $CaCl_2$/25 mM Tris (pH 7.5) by using a microseeding technique. Preliminary X-ray characterization showed that the crystals are in space group $C2$ with unit-cell dimensions $a = 111.9$ Å, $b = 78.1$ Å, $c = 134.6$ Å, $\beta = 100$ °. An oligonucleotide probe, made by hemi-nested PCR, was used to clone the gene encoding catechol 1,2-dioxygenase (*catA*). The deduced 282-residue sequence corresponds to a protein of molecular mass 31539 Da, close to the molecular mass of 31558 Da obtained by electrospray MS of the purified enzyme. *catA* was subcloned into the expression vector pTB361, allowing the production of catechol 1,2-dioxygenase to approx. 40% of the total cellular protein. The deduced amino acid sequence of the enzyme has 56% and 75% identity with the catechol 1,2-dioxygenases of *Arthrobacter* mA3 and *Rhodococcus erythropolis* AN-13 respectively, but less than 35% identity with intradiol catechol and chlorocatechol dioxygenases of Gram-negative bacteria.

## INTRODUCTION

Many aromatic compounds can be degraded aerobically by micro-organisms [1,2]. Almost invariably such compounds are first converted into more reactive dihydroxylated intermediates such as catechol or protocatechuate and then subjected to intradiol or extradiol ring cleavage by molecular oxygen. With intradiol (*ortho*) ring cleavage the reaction occurs between vicinal hydroxy groups, whereas with extradiol (*meta*) cleavage, the reaction is adjacent to one hydroxy group [3]. Both types of enzyme use non-haem iron as the sole cofactor: in the intradiol-cleavage enzymes the cofactor is in the ferric state, whereas in the extradiol-cleavage enzymes it is in the ferrous state [4].

There are three main types of non-haem iron intradiol-cleavage enzymes: catechol 1,2-dioxygenases, protocatechuate 3,4-dioxygenases and chlorocatechol 1,2-dioxygenases. Catechol 1,2-dioxygenases are, with one or two possible exceptions [5,6], dimers of either identical or non-identical subunits [7], with molecular mass of 30.5–34 kDa [8,9]. The substrate specificities of the catechol 1,2-dioxygenases and chlorocatechol 1,2-dioxygenases and of the protocatechuate 3,4-dioxygenases are mutually exclusive: the first two types of enzyme catalyse the intradiol oxygenation of catechol and some of its derivatives but do not oxygenate protocatechuate, whereas protocatechuate 3,4-dioxygenase catalyses the intradiol oxygenation of protocatechuate but not catechol. Catechol 1,2-dioxygenases also perform extradiol cleavage of *meta*-substituted substrates such as 3-methylcatechol or 3-methoxycatechol [10,11]. Chlorocatechol 1,2-dioxygenases, by contrast, do not generally perform significant extradiol cleavage of aromatic substrates [12].

Until recently, much the best characterized intradiol-cleavage enzymes, both genetically and biochemically, have been those of several Gram-negative bacteria. However, catechol 1,2-dioxygenases from the Gram-positive bacteria *Arthrobacter* sp. and *Rhodococcus erythropolis* have now been cloned and sequenced and they share 56% sequence identity with each other; however, they have a much lower degree of identity with catechol 1,2-dioxygenases from Gram-negative bacteria [9,13]. Only the enzyme from *R. erythropolis* has also been purified and some evidence indicates that it may differ from catechol and chlorocatechol 1,2-dioxygenases from Gram-negative bacteria in not having a dimeric structure [5].

The present paper describes the characterization, cloning and sequencing of the catechol 1,2-dioxygenase from *Rhodococcus rhodochrous*, National Collections of Industrial and Marine Bacteria (NCIMB) 13259, a Gram-positive bacterium that grows well on styrene and various other aromatic compounds [14]. The results strengthen the conclusion of Murakami et al. [13] that there is a subfamily of catechol 1,2-dioxygenases in Gram-positive bacteria that is clearly distinct from the two subfamilies of catechol and chlorocatechol 1,2-dioxygenases in Gram-negative bacteria.

## EXPERIMENTAL

### Chemicals

Chemicals were AnalaR grade or better and were obtained from BDH Chemicals (Poole, Dorset, U.K.), except for those listed below or in the appropriate part of the text. 4-Methylcatechol and 3-methylcatechol were from Aldrich (Gillingham, Dorset, U.K.); Hybond-N hybridization membranes were from Amer-

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sham International (Little Chalfont, Bucks., U.K.); dithiothreitol and rabbit muscle fructose 1,6-bisphosphate aldolase were from Boehringer (London, U.K.); boric acid, *N*,*N*″-methylenebisacrylamide, SDS and Tris-washed phenol were from Fisons Scientific Equipment (Loughborough, Leics., U.K.); SeaKem GTG agarose was from FMC Bioproducts (Rockland, ME, U.S.A.); nutrient broth, yeast extract, agar and bactotryptone were from Merck (Darmstadt, Germany); *Vent* DNA polymerase was from New England Biolabs (Beverly, MA, U.S.A.); DEAE-Sephacel, phenyl-Sepharose, Sephadex G-25 and low-molecular-mass standards for SDS/PAGE were from Pharmacia (Milton Keynes, Bucks., U.K.); all restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were from Promega (Southampton, Hants., U.K.); heparin, ferrozine, Neocuprine, catechol, protocatechuic acid, lysozyme, RNase, low-melting-point agarose, Coomassie Brilliant Blue G250, ovalbumin and phenol/chloroform/3-methylbutan-1-ol were from Sigma (Poole, Dorset, U.K.).

### Growth of bacteria and preparation of extracts

*R. rhodochrous* NCIMB 13259 was grown at 30 °C on complex medium containing 26 g/l of nutrient broth, 0.9 g/l L-glutamic acid·HCl, 2 g/l $KH_2PO_4$, 1 g/l $(NH_4)_2SO_4$ and 0.4 g/l $MgSO_4$, $7H_2O$, adjusted to pH 7 with KOH [15]. Small volumes of up to 20 ml were inoculated with single colonies and left without shaking for 2 days at 30 °C. A 2 ml portion of the starter culture was then added to 200 ml of medium and grown at 30 °C in 500 ml conical flasks for 2 days on a rotary shaker. The culture was then transferred to 4 litres of medium and grown in 10-litre flasks, with mixing by magnetic stirrers and aeration at 4 l/min. After 24 h, 2.1 ml of benzyl alcohol was added to give a final concentration of 2 mM. A further 2.1 ml of benzyl alcohol was added 4 h later. After a total of 30 h the cultures were centrifuged at 2740 ***g*** for 10 min in a Beckman JA-10 rotor; the bacteria were then washed in buffer containing 1 g/l $(NH_4)_2SO_4$ and 2 g/l $KH_2PO_4$, adjusted to pH 7 with KOH. The bacteria were then pelleted at 14000 ***g*** for 10 min in a Beckman JA-14 rotor and either stored at −20 °C or used immediately. Bacteria (15–25 g) were suspended in 50 mM Tris/HCl buffer, pH 7.5, to a final concentration of 20 g (wet wt) of cells per 50 ml and then broken with a prechilled French press (Aminco; SLM Instruments, Urbana, IL, U.S.A.) at 117 MPa. The cellular debris was removed by centrifugation at 25000 ***g*** for 30 min and then at 100000 ***g*** for 90 min.

### Purification of catechol 1,2-dioxygenase

All purification steps were done at 4 °C. The extract was loaded at 60 ml/h on a DEAE-Sephacel column (10 cm × 2.6 cm) that had been equilibrated with buffer A (50 mM Tris/HCl, pH 7.5, containing 0.18 M NaCl). The column was then washed at 60 ml/h for approx. 7 column vol. with buffer A. Catechol 1,2-dioxygenase was eluted with a 300 ml positive salt gradient of buffer A/buffer B (50 mM Tris/HCl, pH 7.5, containing 0.4 M NaCl). The flow rate was 20 ml/h and those fractions (5 ml) containing 50 % or more activity of that in the peak fraction were pooled. The ion-exchange pool was brought to 15 %-satd. $(NH_4)_2SO_4$ by the addition of 8.2 g of $(NH_4)_2SO_4$ per 100 ml of eluate. The pool was then loaded at 60 ml/h on a phenyl-Sepharose column (10 cm × 2.6 cm) that had been pre-equilibrated with buffer C [50 mM Tris/HCl, pH 7.5, containing 0.18 M NaCl and 15 %-satd. $(NH_4)_2SO_4$]. The column was washed for 1 column vol. with the same buffer at 60 ml/h; the catechol 1,2-dioxygenase was then eluted with a negative $(NH_4)_2SO_4$ gradient (300 ml) of buffer C/buffer A. The flow rate was 20 ml/h and all fractions (5 ml) containing 50 % or more of that in the peak fraction were pooled. The volume of the phenyl-Sepharose pool was decreased by adding 7.9 g of $(NH_4)_2SO_4$ per 100 ml, re-binding the pool to 1–2 ml of phenyl-Sepharose gel and then eluting in 5–10 ml of Buffer A. Further concentration was done in Centricon-30 ultrafiltration units (Amicon). Protein was then either used immediately or stored at −20 °C. Catechol 1,2-dioxygenase required for crystallography was purified further by Mono Q FPLC with a 0.2–0.4 M NaCl gradient in 50 mM Tris, pH 7.5. Only those fractions with 50 % or more activity of the peak fraction were pooled.

### Protein estimation

Routine protein estimations were done by the method of Bradford [16]. Concentrations of purified catechol 1,2-dioxygenase were estimated spectrophotometrically on the basis of the number of Tyr, Trp and Cys residues known to be present in the protein [17].

### Determination of molecular mass

The subunit molecular mass was determined by electrospray MS with a VG platform quadrupole mass spectrometer fitted with a pneumatically assisted electrospray source and controlled with the VG Mass Lynx software (VG Biotech Ltd., Altrincham, Cheshire, U.K.). The carrier solvent [acetonitrile/water (1:1, v/v)] infusion was controlled at a flow rate of 10 μl/min with a Harvard syringe pump (Harvard Apparatus, South Natic, MA, U.S.A.). Capillary voltages were between 2.8 and 3.2 kV, extraction cone voltages were 20–30 V and the focusing cone voltage was offset by +10 V. The source temperature was set at 65 °C and the nebulizing gas flow at 10 l/h. Before use the instrument was calibrated over the molecular mass range of interest with horse heart myoglobin. Samples (10–20 μl) were desalted by ultrafiltration with HPLC-grade water, diluted with an equal volume of 4 % (v/v) formic acid in acetonitrile and then injected into the instrument. The raw data were processed with the MAXENT deconvolution program with a peak width of 1 Da and a channel resolution of 1 Da.

Native molecular mass values were determined with a Superose 12 gel-permeation column operated at 1 ml/min on a Pharmacia FPLC system using 50 mM Tris/HCl (pH 8)/0.2 M NaCl. The column was calibrated by determining the elution volumes of several standard proteins and then calculating the elution volume of each protein standard with respect to the elution volume of Blue Dextran.

### Electrophoresis

Purified fractions were monitored for purity by SDS/PAGE [18]. Cross-linked proteins were separated with SDS/PAGE in sodium phosphate buffers [19]. A Bio-Rad Mini-Protean II apparatus was used throughout and all proteins were stained with Coomassie Brilliant Blue G250. All procedures and reagents were as described previously [20].

### Determination of quaternary structure by chemical cross-linking

Purified catechol 1,2-dioxygenase and aldolase, which was used as a control, were chemically cross-linked, in separate reactions, with the bisimidoesters dimethylpimelimidate and dimethylsuberimidate. A double-strength stock solution of the cross-linking reagent was prepared immediately before use [0.2 M NaCl/0.1 M triethanolamine/HCl (pH 8)/40 mM bisimidoester, adjusted to pH 8 with 5 M NaOH]. Proteins were placed in 0.2 M NaCl/0.1 M triethanolamine/HCl (pH 8) by ultrafiltration and the

concentration was adjusted to 2 mg/ml, so that after addition of the cross-linking reagent the protein concentration was approx. 1 mg/ml. The reaction was performed in a volume of 100 $\mu$l and continued for 1 h at room temperature, whereupon the samples were analysed by sodium phosphate SDS/PAGE [19].

### Assay of catechol 1,2-dioxygenase activity

Intradiol catechol 1,2-dioxygenase activity was routinely assayed spectrophotometrically with a Pye Unicam 8730 spectrophotometer (Cambridge, U.K.) in 1 ml quartz cuvettes at 27 °C by measuring the increase in $A_{260}$ for the products from all substrates except protocatechuic acid, whose presumptive product was monitored at 270 nm. Reactions were performed in 50 mM sodium/potassium phosphate buffer, pH 7, with various amounts (1–20 $\mu$l) of enzyme, and were initiated by the addition of 10 $\mu$l of 10 mM substrate. Extradiol activity was monitored under the same conditions except that the increase in product absorbance was monitored at 375 nm. One unit of enzyme activity is defined as the amount of enzyme needed to produce 1 $\mu$mol of product/min.

The $\Delta\epsilon_{260}$ (substrate minus product) values were determined by enzymically converting a known amount of catechol to product [21]. The $\Delta\epsilon_{260}$ (substrate minus product) values ($mM^{-1} \cdot cm^{-1}$) for various catechols are as follows: catechol, 14.9; 3-methylcatechol, 15.9; 4-methylcatechol, 13.2.

Initial velocities used in determining kinetic coefficients were measured spectrophotometrically with air-saturated buffer (100 mM Tris/HCl, pH 9) at 27 °C. The data were analysed by the direct linear method with the ENZPACK computer program [22].

### Iron analysis

The iron content of the purified enzyme was measured with a Perkin–Elmer Cetus Atomic absorption spectrophotometer with an oxy-acetylene flame.

### Preparation of peptides

Protein solutions were made 50 mM $NH_4HCO_3$ (pH 7.8)/2 M guanidine/HCl by ultrafiltration and incubated at 37 °C for 15 min. The denatured protein was diluted with 2 vol. of distilled water and digested with *Staphylococcus aureus* V8 protease for 5 h at 37 °C with a protease-to-protein ratio of 1:50 (w/w). Peptides were then snap-chilled over a mixture of methanol and solid $CO_2$ and freeze-dried. Peptidic digests were dissolved in 0.1 % (v/v) trifluoroacetic acid in water and separated by HPLC with a Waters Bondapak $\mu$18 column (Millipore, Milford, MA, U.S.A.) with the following gradient: 100 % A [0.1 % (v/v) trifluoroacetic acid]–50 % B [0.1 % (v/v) trifluoroacetic acid in 90 % (v/v) acetonitrile]. The flow rate was 1 ml/min and the peptides were detected at both 220 and 280 nm.

### N-terminal and peptide sequencing

N-terminal and peptide sequences were determined by liquid-pulse Edman degradation on an Applied Biosystems 477A sequencer.

### Crystallography

Crystals were grown with the sitting-drop vapour-diffusion technique [23] in 24-well sitting-drop trays (NBS Biologicals, Hatfield, U.K.). Each reservoir was filled with 1 ml of precipitant solution [24]; 4 $\mu$l was then transferred from each reservoir to the corresponding well where it was mixed with 4 $\mu$l protein (25 mg/ml). The plates were covered with 'crystal clear' tape (Manco, Westlake, OH, U.S.A.) and maintained at 18 °C. To obtain crystals that diffracted consistently, microseeding was used. After equilibrating the reservoir and well solutions for 3–5 days a crystal, or a crystal fragment obtained from a previous experiment, was washed briefly in 10 $\mu$l of reservoir solution and then transferred to 10 $\mu$l of fresh reservoir solution where it was crushed with a needle. The microcrystals were diluted 1:20 with reservoir solution and then introduced into each well with a human hair [25]. X-ray diffraction data were collected on a Siemens Xentronics area detector and analysed with XDS software [26].

### Molecular biological techniques

Transformation and other cloning procedures were done by using standard techniques [20]. Restriction enzymes and DNA ligase were obtained from Promega and from New England Biolabs, and the reactions were performed as recommended by the manufacturers, with DNA fragments that had been treated with the Wizard clean-up kit (Promega).

Genomic DNA was purified essentially as described [27]. Cultures (200 ml) of *R. rhodochrous* were centrifuged in a Beckman JA-20 rotor at 2000 ***g*** for 10 min. The pellets were washed twice with 25 ml of TE buffer [10 mM Tris/1 mM EDTA (pH 8)], resuspended in 6 ml of TE buffer and incubated at 37 °C for 1 h with 12 mg of lysozyme. The lysate was then extracted with an equal volume of Tris-saturated phenol and the solution was mixed gently for 0.5 h before centrifugation in a Beckman JA-20 rotor at 2000 ***g*** for 10 min. Two further organic extractions were performed on the aqueous layer with phenol/chloroform/3-methylbutan-1-ol. Finally the aqueous layer was retained and the DNA was concentrated by precipitation with ethanol.

Preparative amounts of plasmid DNA were obtained with Qiagen (Hilden, Germany) midi-preps.

Radioactive labelling was performed with the Random Primed DNA labelling kit (USB, Cleveland, OH, U.S.A.) and unincorporated nucleotides were removed with Chroma Spin + TE-10 columns (Clontech Laboratories, Palo Alto, CA, U.S.A.). Before use, all probes were denatured at 80 °C for 10 min.

Southern blots and colony lifts were performed with Hybond-N membranes (Amersham) [20]. DNA was cross-linked to the membranes by exposure to a UV transilluminator for 2 min.

DNA samples were separated electrophoretically with 0.5–1.0 % agarose/TAE [40 mM Tris/acetate buffer/1 mM EDTA (pH 8.0)] gels. The requisite bands were excised with a scalpel under UV and extracted with Spin-X centrifuge filters (Costar, Cambridge, MA, U.S.A.).

Automated DNA sequencing was done with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit by using 200 ng of synthetic oligonucleotide primers, 1 $\mu$g of DNA and 5 % (v/v) DMSO. PCR amplification was performed with 30 cycles of denaturation at 95 °C for 1 min and annealing at 55 °C for 1 min, followed by a 2 min extension at 72 °C. The extension products were separated on 24 mm 4.75 % (w/v) polyacrylamide gels with an ABI DNA sequencer (model 373A).

### Synthesis of an oligonucleotide probe by hemi-nested PCR

Three pools of degenerate oligonucleotides were designed on the basis of the N-terminal sequence of catechol 1,2-dioxygenase from *R. rhodochrous* and the sequence of a *S. aureus* V-8 peptide (both underlined in Figure 2) and a strongly conserved hexapeptide (RPAHXH) identified from amino acid sequence alignments [9]. The three oligonucleotides were Oligo1F [5′-

GGGAATTCAA(C/T)GCIGCIACIGA(C/T)AA(G/T)TT-(C/T)AA-3′], Oligo2R [5′-GGGGATCC(G/A)TCIGCIGG-(G/A)AT(C/T)TT(G/A)TA-3′] and Oligo1R [5′-GGGGATC C(A/G)TGIAI(A/G)TGIGCIGGIC-3′] respectively. Each primer had a GG tail and either a *Bam*HI or an *Eco*RI restriction site (underlined) to facilitate cloning. To increase the specificity, the two primer half-pairs (oligo1F-oligo1R and oligo1F-oligo2R) were used in two successive PCRs, in which 1 % of the product from the outer half-pair reaction was used to prime the inner half-pair reaction. PCR amplification was done with a mixture of 1 $\mu$g of each primer, 4 % (v/v) DMSO, 200 ng DNA or 1 $\mu$l from a previous PCR, 6 mM $MgCl_2$ and 1 $\mu$l of *Taq* DNA polymerase. PCR consisted of 30 cycles of denaturation at 95 °C for 1 min and annealing at 40–54 °C for 4 min, followed by a 45 s extension at 72 °C. The amplified fragment was cloned into pBluescript SK+ (Promega Corp, Madison, WI, U.S.A.) and sequenced.

### Genomic Southern hybridization and colony hybridizations

DNA was blocked by incubation in prehybridization buffer (6 × SSC/50 $\mu$g/ml heparin) (20 × SSC stock was 3 M NaCl/0.3 M sodium citrate) for 2 h at 68 °C before hybridization with $^{32}$[P]dATP-labelled probe in 6 × SSC/50 $\mu$g/ml heparin/0.5 % SDS at 68 °C for 6 h. The blots were then given a series of stringent washes [20] and autoradiographed.

### Sequencing and overexpression of the *R. rhodochrous* gene for catechol 1,2-dioxygenase

The gene encoding catechol 1,2-dioxygenase (*catA*) from *R. rhodochrous* was amplified with *Vent* DNA polymerase by using two primers, CTDE1 (5′-GGAATTCCATATGACCACCACC-GAAAACCCCAC-3′) and CTDE2 (5′-GAAGATCTCAGGC-CTCGGGGTCGAGCGCGAA-3′). To facilitate cloning, the oligonucleotide CTDE1 had an *Nde*I restriction site (underlined) and a seven-base tail; this was followed by the 5′ end of the gene. The oligonucleotide CTDE2 had a *Bgl*II restriction site (underlined) and a two-base tail; this was followed by the 3′ end of the gene. PCR consisted of 25 cycles of denaturation at 95 °C for 1 min and annealing at 54 °C for 1.5 min, followed by a 45 s extension at 72 °C. The amplicon was cloned into the T7 expression plasmid pTB361.

## RESULTS AND DISCUSSION

### Purification of catechol 1,2-dioxygenase

The procedure that was developed for the purification of catechol 1,2-dioxygenase gave at least 10 mg of protein from 15–20 g of cells, representing more than 40 % of the starting enzyme (Table 1). The purification was completed within 30 h and after the final phenyl-Sepharose step the enzyme seemed virtually homogeneous by SDS/PAGE and Coomassie staining (Figure 1). Earlier attempts at purifying catechol 1,2-dioxygenase from *R. rhodochrous* included a lengthy gel-permeation step; subsequent Mono Q FPLC showed two protein and activity peaks. However, when the gel-permeation step was discontinued as a purification step, catechol 1,2-dioxygenase was eluted from Mono Q FPLC in a single peak (results not shown). Maltseva et al. [28], in their work on the chlorocatechol 1,2-dioxygenase from *R. erythropolis* 1CP, reported a similar effect after gel-permeation chromatography.

The purification was repeated several times, including some times on a larger scale giving up to 60 mg of catechol 1,2-dioxygenase from 90–140 g wet weight of cells. To handle the larger amount of material, the size of the ion-exchange column was increased to 12 cm × 5 cm and was operated throughout at 80 ml/h with a larger gradient volume of 1 litre. The phenyl-Sepharose column was increased to 17 cm × 2.6 cm but was operated at the same flow rate as in the small-scale preparation.

**Table 1 Purification of catechol 1,2-dioxygenase from *R. rhodochrous***

The protein concentration of the phenyl-Sepharose pool was not determined (n.d.).

| Step | Volume (ml) | Total activity (units) | Total protein (mg) | Activity (units/mg of protein) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Extract | 80 | 339 | 968 | 0.3 | 100 | 1 |
| DEAE-Sephacel | 100 | 243 | 59 | 4.3 | 72 | 13 |
| Phenyl-Sepharose | 36 | 149 | n.d. | n.d. | n.d. | n.d. |
| After concentration on phenyl-Sepharose | 9 | 146 | 10 | 14.3 | 43 | 42 |

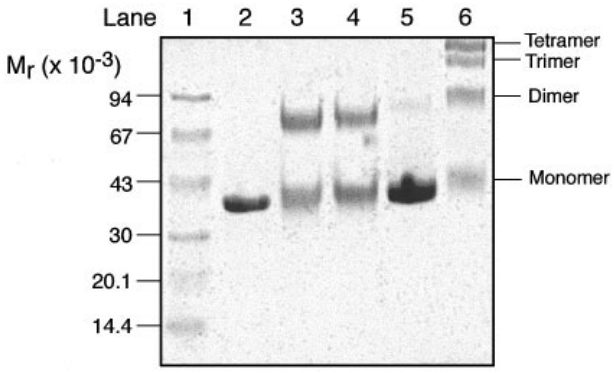


**Figure 1 Sodium phosphate SDS-PAGE of catechol 1,2-dioxygenase from *R. rhodochrous* cross-linked with dimethylpimelimidate and dimethylsuberimidate**

Catechol 1,2-dioxygenase from *R. rhodochrous* was cross-linked in separate experiments as described in the Experimental section and then analysed by sodium phosphate SDS/PAGE. Lane 1, molecular mass standards (indicated in kDa at the left); lane 2, catechol 1,2-dioxygenase; lane 3, catechol 1,2-dioxygenase treated with dimethylpimelimidate; lane 4, catechol 1,2-dioxygenase treated with dimethylsuberimidate; lane 5, aldolase; lane 6, aldolase treated with dimethylsuberimidate.

### Preliminary X-ray crystallographic analysis

Catechol 1,2-dioxygenase was crystallized in 15 % (w/v) poly-(ethylene glycol) 4000/0.33 M $CaCl_2$/25 mM Tris/HCl (pH 7.5). After microseeding, orange/brown rectangular plates appeared within 3–7 days, reaching a maximum size of 0.5 mm down the longest axis. Similar crystals were obtained from catechol 1,2-dioxygenase that had been expressed in *Escherichia coli* XL2-
Blue(pPDS2). However, crystals obtained with catechol 1,-2-dioxygenase which had been overexpressed in *E. coli* JM109(DE3)(pPDSX4CTD) produced only twinned needles.

A partial native data set (70 % complete) was collected on a Siemens Xentronics area detector mounted on a rotating anode with the use of Cu K$\alpha$ radiation of wavelength 1.5418 Å. The single crystal was mounted in a quartz capillary along with a small amount of mother liquor and the data were collected at room temperature; 400 frames with a $\phi$ rotation of 0.25° per frame yielded 10897 unique reflections to a resolution of 3.5 Å. Data processing with XDS gave unit-cell dimensions of $a =$

111.9 Å, $b = 78.1$ Å, $c = 134.6$ Å, $\beta = 100°$ in the monoclinic space group $C2$. A calculated Matthews number of 2.3 gives four subunits per asymmetric unit (16 in the unit cell) with a proposed solvent content of 47% [29].

### Molecular mass determination and quaternary structure

The presence of a single band on SDS-PAGE (Figure 1) and the unambiguous N-terminal sequence data (Figure 2) suggest that catechol 1,2-dioxygenase from *R. rhodochrous* consists of a single polypeptide species. The subunit molecular mass value of catechol 1,2-dioxygenase was estimated to be 31 558 ± 4 Da (mean ± S.D.) by electrospray MS, very close to the molecular mass of 31539 Da calculated from the deduced amino acid sequence. However, SDS/PAGE gave a value of 39800 ± 635 (mean ± S.D., $n = 5$). The decreased mobility of catechol 1,2-dioxygenases during SDS/PAGE has been observed by other workers [9] but the reason for this is unknown.

The apparent native molecular mass of *R. rhodochrous* catechol 1,2-dioxygenase was 120 kDa when measured on Superose 12 FPLC, which suggests that this enzyme is a tetramer. With three possible exceptions [5,6,30], all the catechol and chlorocatechol 1,2-dioxygenases characterized so far have been dimers. Aoki et al. [5] noted that the catechol 1,2-dioxygenase that they had isolated from *R. erythropolis* AN-13 had a molecular mass that varied with the salt concentration. When gel-permeation chromatography was done with 0.2 M NaCl at pH 8, they obtained the monomer, whereas when salt was omitted they obtained the tetramer. We investigated the quaternary structure of *R. rhodochrous* catechol 1,2-dioxygenase by chemically cross-linking the subunits with bisimidoesters, which are bifunctional reagents that react specifically with the amino groups of proteins [31]. Two bands were visible after electrophoresis (Figure 1 lanes 3 and 4) corresponding to the monomer and dimer, suggesting that catechol 1,2-dioxygenase is a dimer with an implied native molecular mass of 63 kDa and not 120 kDa. Both the gel-permeation chromatography and the cross-linking experiments were done at 0.2 M NaCl and pH 8, so it is unlikely that the discrepancy was due to different oligomeric states. Dorn and Knackmuss [10], in their work on the catechol 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase from *Pseudomonas* B13, obtained similarly high molecular masses when these were estimated by gel-permeation chromatography but they were able to demonstrate that catechol 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase elution volumes could not be correlated with the log of the molecular masses because the ellipticity of these proteins differed too much from the calibration proteins; perhaps this is also the explanation in the present case.

### Absorption spectra and the iron-to-subunit stoichiometry

The absorption spectrum of catechol 1,2-dioxygenase had maxima at approx. 220 and 280 nm. At higher concentrations of protein, another maximum at 426 nm was also evident and this is a characteristic of all non-haem iron intradiol-cleavage enzymes [4]. The molar absorption coefficient at 426 nm was 1850 $M^{-1} \cdot cm^{-1}$ with respect to the subunit concentration.

Iron quantification of two different protein preparations by atomic absorption spectrometry resulted in iron-to-subunit stoichiometries that were significantly less [0.60, 0.65 (Fe per subunit)] than the expected stoichiometry of one Fe atom per subunit. Very similar values were obtained when the Fe content was quantified colorimetrically (results not shown). The observed Fe-to-subunit ratios for the *R. rhodochrous* catechol 1,2-dioxygenase are presumably due to heterogeneous mixtures of dimers, ranging from those with their 'full' complement of iron to those with no iron at all. Because the observed value might have been a reflection of inadequate availability of iron during bacterial growth, a chelated metals solution was incorporated into the

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      -35                    -10_
  1   TGTTGACAATCACCTGTGACTGGACGCACAGTGTGTGTACGCACTGCGAT

 51   ACGGAAGTACGCACAGCGTACAACCCGAGTGGCGAGAGCGCCAGCCCCGC

101   GATCACCACCCACAGCTTTNGCGACCACCGTTCGAGCCTCGCCGAGGAGG

151   ACACATGACCACCACCGAAAACCCCACCGCCCACGCCTCGGGCAACGCCG
            T  T  T  E  N  P  T  A  H  A  S  G  N  A  A

201   CCACCGACAAGTTCAAGAGCGAGCGGGTCTCGTCGGACACGTCGGTCGAGC
        T  D  K  F  K  S  E  R  V  S  S  D  T  S  V  E  R

251   GCGCCGCTGCCATCTACAAGGACGTGCTCGGTGCGTTCGCCGAGATCAT
        A  A  A  I  Y  K  D  V  L  G  A  F  A  E  I  I

301   CCACAAGCACCAGGTCACCTACGACGAGTACCGGGTGCTCAAGCAGTGGA
        H  K  H  Q  V  T  Y  D  E  Y  R  V  L  K  Q  W  M

351   TGATCGACGTCGGCGAGTACGGCGAGTGGCCGCTGTGGCTGGACGTCTTC
        I  D  V  G  E  Y  G  E  W  P  L  W  L  D  V  F

401   GTCGAGCACGAGATCGAGGAAGTGCACTACAACCGCAAGCACTTCTCCGG
      V  E  H  E  I  E  E  V  H  Y  N  R  K  H  F  S  G

451   CACCAAGGGCAGCATCGAGGGCCCCTACTACGTGCCGGACTCGCCGAAGC
        T  K  G  S  I  E  G  P  Y  Y  V  P  D  S  P  K  L

501   TGCCGGCCAAGTGCACCATGCCGATGCGCGAGCAGGACAAGGCCGCCGAG
        P  A  K  C  T  M  P  M  R  E  Q  D  K  A  A  E

551   GCGCTGATCTTCAAGGGCCAGGTCACCGACCTCGAGGGCAAGGGCCTGCA
      A  L  I  F  K  G  Q  V  T  D  L  E  G  K  G  L  Q

601   GGGCGCCACCGTCGAGCTGTGGCACGCCGACAAGGAGGGCTACTACTCGC
        G  A  T  V  E  L  W  H  A  D  K  E  G  Y  Y  S  Q

651   AGTTCGCCCCCGGCATCCCCGAGTGGAACCTGCGCGGCACCATCGAGTGC
        F  A  P  G  I  P  E  W  N  L  R  G  T  I  E  C

701   GACGACGAGGGCAACTTCGAGATCACCACGCTCAAGCCCGCGCCCTACAA
      D  D  E  G  N  F  E  I  T  T  L  K  P  A  P  Y  K

751   GATCCCGGCCGACGGCCCGACCGGCTGGTTCATCGACTCCTACGGCGGCCA
        I  P  A  D  G  P  T  G  W  F  I  D  S  Y  G  G  H

801   CCCGTGGCGTCCCGCCCACCTGCACCTCATGGTGAAGGCTCCGGGCAAG
        P  W  R  P  A  H  L  H  L  M  V  K  A  P  G  K

851   CGCGCCATCACCACCCAGCTCTACTTCCAGGGCGGCGAGTGGGTCGAGGA
      R  A  I  T  T  Q  L  Y  F  Q  G  G  E  W  V  E  D

901   CGACGTCGCGACCGCGACCAAGCCCGAGCTGATCCTGGACCCGCAGCCGA
        D  V  A  T  A  T  K  P  E  L  I  L  D  P  Q  P  N

951   ACGCCGACGGCGTCGACGAGGTGACCTACAACTTCGCGCTCGACCCCGAG
        A  D  G  V  D  E  V  T  Y  N  F  A  L  D  P  E

1001  GCCTGAGCACTCCTGTTGAACCGGGTCCACGAGTGGTGACAAATGCCGGT
      A  *

1051  TGTCCGCCAAGGGCACCGGCATTCGTCGTCGCGGAAGCCCGGCTGTTGCC

1101  CCGCATCACCCGCCCGAAATCCGATTTGTTGAACTTGTTCGATGTCCCGA

1151  CCCCCGACCCTGAAATTCGTTCTCGATCCGATACGACGAATCGTCGAACT

1201  TTCCGCTCCTTGCGTTTCCCCAC
```

**Figure 2 Partial nucleotide sequence of the *Xma*I insert of the recombinant plasmid pPDS2 and the deduced amino acid sequence of catechol 1,2-dioxygenase of *R. rhodochrous***

Regions of amino acid sequence confirmed from amino acid sequencing experiments are underlined. Putative −35 and −10 promoter sequences, together with a putative ribosome-binding site (RBS), are doubly underlined. Two inverted repeat structures are shown in bold. The stop codon is indicated with an asterisk.

**Table 2 Determination of kinetic constants of catechol 1,2-dioxygenase from *R. rhodochrous***

$K_{cat}$ and $K_{cat}/K_m'$ values were calculated on the basis of one active site per subunit and a subunit molecular mass of 31539 Da. Values of $v$ are shown as means ± S.D. for $n$ values shown in parentheses.

| Substrate | $K_m'$ ($\mu$M) | $V_{max}$ (units/mg of protein) | $K_{cat}$ ($s^{-1}$) | $10^{-6} \times K_{cat}/K_m'$ ($mM^{-1} \cdot s^{-1}$) |
|---|---|---|---|---|
| Catechol | 1.1 | 19 ± 1.3 (4) | 11.6 | 9.7 |
| 3-Methylcatechol | 1.7 | 15 ± 4.2 (3) | 9.5 | 5.3 |
| 4-Methylcatechol | 1.2 | 13 ± 1.2 (4) | 6.3 | 5.7 |
| Protocatechuate | – | 0 | 0 | – |

growth medium; however, this had no effect on the iron-to-subunit stoichiometry of the enzyme purified from the resulting bacteria [0.60 (Fe per subunit)]. A shortfall of iron has been reported for catechol 1,2-dioxygenase and chlorocatechol 1,2-dioxygenase from *Pseudomonas* [7,12]. To try to increase the iron content of the chlorocatechol 1,2-dioxygenase from *Pseudomonas*, the enzyme was incubated with various $Fe^{2+}$ and $Fe^{3+}$ reagents, but without any effect [12]. The observed deficit might be because the intradiol-cleavage enzymes are synthesized faster than the iron can be mobilized. It is not known whether the marginally lower iron content [0.5 (Fe per subunit] found in the catechol 1,2-dioxygenase over-expressed in *E. coli* (DE3) (the present study) is significant in this regard.

### Kinetics and stability of catechol 1,2-dioxygenase

Catechol 1,2-dioxygenase was optimally active at pH 9.0, which is similar to the catechol 1,2-dioxygenases that have been isolated from *Rhizobium leguminosarum* [32] and *Rhizobium trifolii* [33] and marginally higher than the catechol and chlorocatechol 1,2-dioxygenases from pseudomonads [7,12,34] and the enzymes from *A. calcoaceticus* and *Frateuria* [6,35], which are optimally active at pH 7.5–8.0.

Rhodococcal catechol 1,2-dioxygenase did not discriminate greatly between catechol, 3-methylcatechol and 4-methylcatechol as substrates but it did not oxygenate protocatechuate at a detectable rate (Table 2).

No significant loss of activity was observed after incubating the enzyme at room temperature for 30 min in 100 mM Tris/HCl at pH 5, 7.5 or 9.0; indeed the enzyme could be stored in 100 mM Tris/HCl, pH 7.5, for several weeks at 4 °C with no loss of activity. However, the enzyme was rapidly inactivated in 100 mM Tris/NaOH, pH 11.2. The stability of the rhodococcal enzyme below pH 7 contrasts with other catechol 1,2-dioxygenases and chlorocatechol 1,2-dioxygenases, which tend to be unstable below approx. pH 7 [10,36].

Some of the non-haem iron intradiol-cleavage enzymes also cleave outside the vicinal hydroxy groups of 3-substituted substrates under certain conditions [11]. Rhodococcal catechol 1,2-dioxygenase was assayed for extradiol activity with catechol, 3-methylcatechol and 4-methylcatechol but no such activity could be detected. The activity with substituted catechols such as 3-methylcatechol, and the absence of any extradiol activity, show that the specificity of the rhodococcal catechol 1,2-dioxygenase is more like that of the chlorocatechol 1,2-dioxygenases than of the catechol 1,2-dioxygenases from Gram-negative bacteria, and it is similar to the specificity of the catechol 1,2-dioxygenase isolated from the aniline-assimilating bacterium *R. erythropolis* [5].

### Cloning and nucleotide sequence of the *R. rhodochrous catA* gene

The 560 bp product of the hemi-nested PCR was in agreement with the value predicted from peptide sequence alignments; the PCR product was therefore cloned in pBluescript SK+. Restriction analysis of 50 *E. coli* DH5$\alpha$ clones revealed eight recombinants, each containing a 560 bp insert. Four foreign fragments were sequenced, three of which were found to encode the same core region of a gene encoding catechol 1,2-dioxygenase. One of the recombinant plasmids (pP29) hybridized to a single *Sma*I fragment of approx. 3.5 kb. To give fragments with suitable 5′ overhangs, the requisite *Xma*I 3.5 kb band was cloned in pBluescript SK+. Three *E. coli* XL2-Blue clones hybridized strongly to the labelled probe. Restriction analysis of all three showed that each contained the same-sized insert of approx. 3.5 kb. Automated sequencing reactions were performed on one of the clones (pPDS2) and confirmed that the *Xma*I insert contained the gene encoding catechol 1,2-dioxygenase. At the same time, in-frame initiation and termination codons were discovered, suggesting that the entire gene had been cloned. The sequence (Figure 2) contains within it an open reading frame encoding a catechol 1,2-dioxygenase with 282 amino acid residues and a molecular mass of 31539 Da, close to the molecular mass of 31558 Da obtained by electrospray mass spectroscopy.

The deduced amino acid sequence of catechol 1,2-dioxygenase from *R. rhodochrous* was compared with other non-haem iron intradiol-cleavage enzymes. The closest alignment is with the catechol 1,2-dioxygenases from the Gram-positive bacteria *Arthrobacter* mA3 [9] and *R. erythropolis* [13], which contain 282 and 285 residues and are 56% and 75% identical with the *R. rhodochrous* enzyme respectively. There is less than 35% identity with the sequences of catechol 1,2-dioxygenases and chlorocatechol 1,2-dioxygenases from Gram-negative bacteria, which contain 251–260 and 302–311 residues respectively [13].

### Overexpression of *R. rhodochrous* catechol 1,2-dioxygenase

Catechol 1,2-dioxygenase was expressed in *E. coli* XL2-Blue-(pPDS2) in the absence of isopropyl $\beta$-D-thiogalactoside; it had the same specific activity as the wild-type enzyme. This suggests that the gene encoding this enzyme was being expressed from its own promoter. Within the upstream region, more than 100 nt from the ATG coding initiation start site, were putative −10 and −35 promotor sequences, 18 nt apart (Figure 2). Both bear a strong resemblance to the consensus sequences for those *E. coli* promoters recognized by RNA polymerases containing $\sigma^{70}$-like subunits [37]. Catechol 1,2-dioxygenase is expressed constitutively in *E. coli* but is induced in *R. rhodochrous* [14], which suggests that its expression is under negative control. In contrast, the genes encoding several bacterial catechol 1,2-dioxygenases and chlorocatechol 1,2-dioxygenases seem to be positively regulated by the LysR family of DNA-binding proteins [38,39], which recognize $T(N)_{11}A$ and $G(N)_{11}A$ motifs within inverted repeat structures straddling promoter regions [40]. Both motifs, together with their inverted repeats, are found upstream of the rhodococcal gene, the first one, $G(N)_{11}A$, starting within the putative −10 region (Figure 2). This is in complete contrast with positively regulated genes, where the DNA-binding motifs normally overlap, to some extent, the −35 consensus site. The relative spatial difference between positive and negative regulatory sites within *E. coli* $\sigma^{70}$ promoters was noted by Collado-Vides et al. [41], who found that approx. 60% of the activator sites that were studied touched the −40 position, whereas none touched the −10 position. In contrast, 49% of repressible sites have operators overlapping the +1 site.

The putative Shine–Dalgarno sequence (AGGAGG) in the rhodococcal gene (Figure 2) is identical with the proposed consensus sequence for *Streptomyces* genes (a/gGGAGG) [42]. *Rhodococcus* and *Streptomyces* are closely related Gram-positive genera [43]. McLaughlin et al. [44] proposed that the Shine–Dalgarno sequences of Gram-positive bacterial mRNA species are typically able to form strong complexes with the 16 S mRNA, whereas *E. coli* sequences are more variable. The results accumulated from the apparent Shine–Dalgarno sequences of 44 *Streptomyces* genes, however, suggest that the strength of *Streptomyces* Shine–Dalgarno sequences vary as much as those from *E. coli* [45], with both types of bacterium tolerating relatively poor Shine–Dalgarno sequences [42]. Expression analysis of *E. coli* JM109(DE3)(pPDSX4CTD) revealed that catechol 1,2-dioxygenase was expressed maximally after 4 h of induction with isopropyl $\beta$-D-thiogalactoside, to approx. 40 % of the cellular protein as estimated by densitometry of SDS/PAGE gels.

## Conclusions

On the basis of its amino acid sequence, number of amino acid residues, stability and lack of discrimination between differently substituted catechols, the catechol 1,2-dioxygenase from *R. rhodochrous* is remarkably similar to the catechol 1,2-dioxygenase from *R. erythropolis* [5,13]. Unfortunately, the enzyme from *Arthrobacter* [9] has not to our knowledge been purified and therefore characterized biochemically; however, the close amino acid sequence identity between the enzymes from *R. erythropolis* and *Arthrobacter* led Murakami et al. [13] to suggest that the catechol 1,2-dioxygenases from Gram-positive bacteria might form a new subdivision within the intradiol cleavage family of enzymes, and our results substantially extend and strengthen that conclusion.

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