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Isolation and partial characterization of an extradiol non-haem iron dioxygenase which preferentially cleaves 3-methylcatechol

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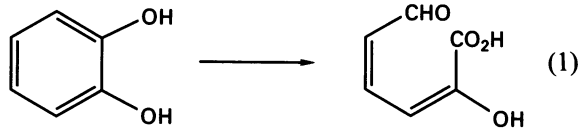
A purification procedure has been developed for an extradiol dioxygenase expressed in *Escherichia coli*, which was originally derived from a *Pseudomonas putida* strain able to grow on toluidine. Physical and kinetic properties of the enzyme have been investigated. The enzyme has a subunit M_r of 33500 ± 2000 by SDS/polyacrylamide-gel electrophoresis. Gel filtration indicates a molecular mass under non-denaturing conditions of 120000 ± 20000. The *N*-terminal sequence (35 residues) of the enzyme has been determined and exhibits 50% identity with other extradiol dioxygenases. Fe(II) is a cofactor of the enzyme, as it is for other extradiol dioxygenases. The reactivity of this enzyme towards catechol and methyl-substituted catechols is somewhat different from that seen for other catechol 2,3-dioxygenases, with 3-methylcatechol cleaved at a higher rate than catechol or 4-methylcatechol. K_m values for these substrates with this enzyme are all around 0.3 μM. The enzyme exhibits a bell-shaped pH profile with pK_a values of 6.9 ± 0.1 and 8.7 ± 0.1. These results are compared with those found for other extradiol dioxygenases.

INTRODUCTION

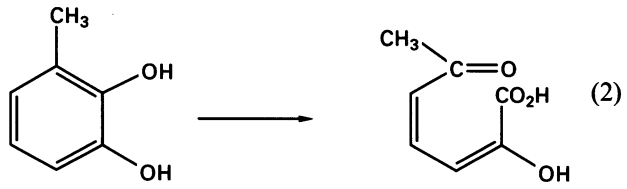
Dioxygenases incorporate both atoms of dioxygen into organic substrates [1]. They are involved in a variety of reactions, including the cleavage of aromatic rings. The best characterized of the non-haem iron dioxygenases are the bacterial enzymes which cleave the double bonds of aromatic compounds adjacent to, or in between, hydroxyl groups. These include the catechol dioxygenases [2], which divide into Fe(III)-containing enzymes which cleave in an intradiol fashion and Fe(II)-containing enzymes which cleave in an extradiol fashion [3].

Catechol 2,3-dioxygenase (or metapyrocatechase), which contains Fe(II) as a cofactor [3], cleaves catechol in an extradiol fashion as shown in eqn. (1). This enzyme is obtained from a *Pseudomonas putida* strain (A.T.C.C. 23973) grown on benzoate as the sole carbon source and inducer [4].



(1)

Catechol 2,3-dioxygenase is also capable of cleaving substituted catechols, such as 3-methylcatechol and 4-methylcatechol, in a proximal extradiol fashion as indicated in eqns. (2) and (3).



(2)

(3)

Recently, McClure & Venables have described a *Pseudomonas putida* strain, UCC2, able to utilize *m*- or *p*-toluidine as sole carbon and nitrogen source [5,6]. Strain UCC2 expresses a novel extradiol dioxygenase which is plasmid encoded [6]. A DNA fragment containing the structural gene for this dioxygenase has been cloned into vectors for direct expression in *Escherichia coli* ([6]; N. C. McClure, unpublished work). We report here the purification (from *E. coli*) and partial characterization of this non-haem iron dioxygenase which preferentially acts on 3-methylcatechol rather than catechol, and is thus referred to as 3-methylcatechol 2,3-dioxygenase throughout. Comparisons are made to the well-characterized catechol 2,3-dioxygenase from *Pseudomonas putida* (A.T.C.C. 23973).

MATERIALS AND METHODS

Strains, media and growth

3-Methylcatechol 2,3-dioxygenase was obtained from *E. coli* JM107 [7] containing pNMN24 (a gift from Dr. N. C. McClure), which is an expression vector containing the structural gene encoding for this enzyme (N. C. McClure, unpublished work). Plasmid-bearing *E. coli* cells were grown at 37 °C in Luria broth [8] supplemented with 100 μg of ampicillin/ml.

Catechol 2,3-dioxygenase was obtained from *Pseudomonas putida* A.T.C.C. 23973. *P. putida* A.T.C.C. 23973 cells were grown using benzoate as inducer and major carbon source as previously described [4].

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Table 1. Purification details for the isolation of the 3-methylcatechol 2,3-dioxygenase from *E. coli*

The Table is based on an extraction from 50 g (wet weight) of cells. Specific activity is expressed as the number of μmol of product produced/min per mg of protein at 25 °C and pH 7.00. A detailed description of the procedure can be found in the Materials and methods section. This Table can be compared with that previously reported for catechol 2,3-dioxygenase in [4].

Purification step	Total protein (mg)	Specific activity (munits)	Recovery (%)	Purification (fold)
Supernatant after cell lysis	4180	245	100	–
After $(NH_4)_2SO_4$ and dialysis	3370	275	90	1
After DE52 ion exchange	915	870	80	3
After S-300 gel filtration	55	7630	40	30

Enzyme isolation

The isolation procedure used was identical for both cell types and is substantially different to previously published methods. Frozen cells (50 g) were resuspended in 500 ml of acetone/phosphate buffer [0.05 M-phosphate buffer, pH 7.50, containing 10% (v/v) acetone] at 4 °C. A low concentration of an organic solvent (acetone or ethanol) is known to protect Fe(II)-dioxygenases from inactivation by oxidation [9]. Lysozyme (Sigma) was added to approx. 0.2 mg/ml and a small amount (1 mg) of DNAase (Sigma) was also added. The mixture was incubated on ice for 30 min, then centrifuged at 15000 *g* for 10 mins to remove cell debris. The supernatant was brought to 70% $(NH_4)_2SO_4$ saturation and centrifuged at 39000 *g* for 15 min. The resulting precipitate was resuspended in a minimum volume of acetone/phosphate buffer. Insoluble material was removed by centrifugation at 39000 *g* for 15 min. The resulting supernatant was dialysed against acetone/phosphate buffer overnight at 4 °C. Dialysed material was loaded on to a DE52 (Whatman) column (30 cm × 3 cm) equilibrated with acetone/phosphate buffer. The column was then washed with 1 column volume of acetone/phosphate buffer. Enzyme was eluted using a linear gradient between acetone/phosphate buffer and the same buffer containing 5% $(NH_4)_2SO_4$. Fractions containing active enzyme were combined and brought to 70% $(NH_4)_2SO_4$ saturation. After centrifugation at 39000 *g* for 15 min the resulting precipitate was dissolved in acetone/phosphate buffer and concentrated to < 5 ml with an Amicon concentrator. Portions (1 ml) of this solution were passed through a Sepharose S-300 gel filtration column (150 cm × 3 cm) in acetone/phosphate buffer. Fractions containing active enzyme were combined and concentrated to 5 ml using an Amicon concentrator. Fully purified enzyme showed a single band (M_r 33500 ± 2000) on a Coomassie-stained SDS/polyacrylamide gel. Further details of the purification procedure are given in Table 1.

Molecular mass determination

Subunit molecular masses were determined using SDS/PAGE (12% acrylamide). Native molecular masses were determined using a Sepharose S-300 column (150 cm × 3 cm) calibrated with the following molecular mass markers (Sigma): β-amylase (200000), alcohol dehydrogenase (150000), bovine serum albumin (66000), carbonic anhydrase (29000), myoglobin (17500) and cytochrome *c* (12500).

N-Terminal sequencing

The *N*-terminal sequence was determined using an Applied Biosystems 477 sequencer by the WELMET protein characterization facility at the University of Edinburgh.

Fe(II) replacement and oxidation

Fe(II) was removed from the enzyme (with a concomitant loss of activity to less than 5% of the original value) by treatment with EDTA (BDH AnalaR). EDTA (100-fold molar excess) was added to a 10–50 μM enzyme solution, which was then dialysed overnight against 0.05 M-phosphate buffer, pH 7.50, with no acetone present. Reactivation was achieved by addition of ferrous sulphate (10-fold molar excess) to the inactive enzyme which was then re-dialysed overnight, this time against acetone/phosphate buffer.

The oxidation of the Fe(II) cofactor was achieved by titration of an enzyme solution (10–50 μM) with either potassium ferricyanide or H_2O_2 until a complete loss of activity was achieved. Re-reduction was carried out by the addition of a 100-fold molar excess of sodium dithionite.

Kinetic studies

All kinetic experiments were carried out in 0.20 M-phosphate buffer at pH 7.00 and at 25 ± 0.1 °C (except for the pH profile experiments which were carried out in phosphate buffer between pH 5.00 and pH 9.00). All

Table 2. Substrates and reaction products, and spectroscopic values used in monitoring enzyme activity

The molar absorption coefficients were determined in 0.20 M-phosphate buffer, pH 7.00.

Substrate	Product	Wavelength monitored (nm)	ε ($M^{-1} \cdot cm^{-1}$)
Catechol	Hexa-2,4-dienedioic acid	375	48400
3-Methylcatechol	2-Hydroxy-6-oxohepta-2,4-dienoic acid	390	19800
4-Methylcatechol	2-Hydroxy-5-methyl-6-oxohexa-2,4-dienoic acid	380	33200

measurements were made under steady-state conditions with the substrates catechol, 3-methylcatechol and 4-methylcatechol. Enzyme activities were measured by following the formation of reaction products using Pye–Unicam SP800 and Perkin–Elmer λ9 spectrophotometers at the wavelengths shown in Table 2. Experimental data were fitted to a non-linear least-squares program and also represented as Lineweaver–Burk plots to yield K_m values.

Modification with diethylpyrocarbonate

Protein to be modified (30 μM) was treated with a 20-fold molar excess of diethylpyrocarbonate (Sigma) using published procedures [10,11]. The diethylpyrocarbonate was standardized before use with imidazole. Bovine serum albumin was used as a control protein to verify successful modification of histidine residues by monitoring the increase in absorbance at 238 nm (ϵ 2750 $M^{-1} \cdot cm^{-1}$ [11]).

RESULTS

Molecular mass determination

Purified 3-methylcatechol 2,3-dioxygenase showed a single band on SDS/PAGE corresponding to a subunit M_r of 33500±2000. The native molecular mass determined by gel filtration was found to be 120000±20000, consistent with the native enzyme being a tetramer of identical subunits.

N-Terminal amino acid sequence

The *N*-terminal sequence (35 residues) of the 3-methylcatechol 2,3-dioxygenase has been determined (Fig. 1) and shows around 50% identity to two published sequences of catechol 2,3-dioxygenases [12,13] deduced from the nucleotide sequences of the metapyrocatechase genes on the TOL plasmid of *P. putida* mt-2 [12] and the NAH7 plasmid of *P. putida* PpG7 [13].

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 1                5                10
Gly-Val-Leu-Arg-Ile-Gly-His-Ala-Ser-Leu-

11               15               20
Arg-Val-Met-Asp-Ile-Ala-Ala-Ala-Val-Lys-

21               25               30
His-Tyr-Glu-Glu-Val-Leu-Gly-Leu-Lys-Thr-

31               35
Val-Met-Lys-Asp-Asp
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Fig. 1. *N*-Terminal sequence (35 residues) of the 3-methylcatechol 2,3-dioxygenase expressed in *E. coli*

Residues underlined are identical to those found in the two published catechol 2,3-dioxygenase sequences deduced from the nucleotide sequences of the metapyrocatechase genes from the TOL plasmid of *P. putida* mt-2 [12] and the NAH7 plasmid of *P. putida* PpG7 [13].

Fe(II) replacement and oxidation

3-Methylcatechol 2,3-dioxygenase in acetone/phosphate buffer can be stored for a period of days at 4 °C without any marked loss of activity. Treatment of the enzyme with EDTA caused a reversible loss of enzymic activity. Activity was completely restored by incubation of the enzyme with excess ferrous sulphate.

Treatment of the enzyme with oxidants, ferricyanide and H_2O_2, also resulted in a loss of activity. The oxidation with ferricyanide was found to be reversible, with 60% of the original activity restored by reduction with sodium dithionite. Enzyme treated with H_2O_2 could not be reactivated at all by addition of sodium dithionite.

Kinetic studies

Steady-state kinetic measurements were carried out on both 3-methylcatechol 2,3-dioxygenase (from *E. coli*) and catechol 2,3-dioxygenase (from *P. putida* A.T.C.C. 23973) using the following substrates: catechol, 3-methylcatechol and 4-methylcatechol. Typical saturation behaviour was observed in all cases, allowing determination of K_m and relative $k_{cat.}$ values (Table 3). The relative $k_{cat.}$ values for the 3-methylcatechol 2,3-dioxygenase as isolated from *E. coli* are identical (within experimental error) with those for the same enzyme from *P. putida* UCC2 [6]. The dependence of the reaction rate on pH for 3-methylcatechol 2,3-dioxygenase (under saturating conditions) gave a bell-shaped curve (Fig. 2). The pK_a values from this curve were found to be 6.9±0.1

Table 3. Kinetic properties of 3-methylcatechol 2,3-dioxygenase and catechol 2,3-dioxygenase

All values were determined from steady-state kinetic measurements at 25±0.1 °C, in 0.20 M-phosphate buffer, pH 7.00. For the purposes of comparison all the $k_{cat.}$ values have been normalized to a value of 100 for catechol as substrate.

	Enzyme			
	3-Methylcatechol 2,3-dioxygenase		Catechol 2,3-dioxygenase	
Substrate	K_m (μM)	$k_{cat.}$ (relative)	K_m (μM)	$k_{cat.}$ (relative)
Catechol	0.36(±0.08)	100	2.6(±1.0)	100
3-Methylcatechol	0.35(±0.09)	175	2.8(±0.4)	31
4-Methylcatechol	0.30(±0.15)	40	2.3(±0.6)	56

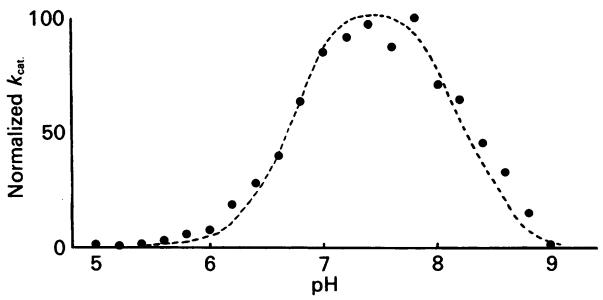

Fig. 2. pH-dependence of the catalytic activity of 3-methylcatechol 2,3-dioxygenase

The kinetic determinations at each pH value were determined under saturating conditions. The $k_{cat.}$ values were normalized to 100 for the value at pH 7.5, which corresponds to the pH optimum.

and 8.7 ± 0.1 and the pH optimum was around 7.5. Similarly, catechol 2,3-dioxygenase gave a bell-shaped curve, but with pK_a values of 6.2 ± 0.1 and 8.5 ± 0.2 and a pH optimum of 7.0. The possibility that the lower pK_a value might to be due to an active-site histidine was investigated by attempted modification of this residue with diethylpyrocarbonate. This would be expected to inactivate the enzyme by removing the ability of such a histidine to act as an active-site base. However, treatment of the enzyme with diethylpyrocarbonate (even in large excess, in which control experiments with bovine serum albumin show complete modification of all accessible histidines) had no effect on the enzymic activity.

DISCUSSION

We have described a purification procedure for a 3-methylcatechol 2,3-dioxygenase from *E. coli* cells containing pNMN24 (N. C. McClure, unpublished work). The isolation procedure allows purification of the enzyme to > 90% homogeneity (Table 1) within 4–5 days. After purification the enzyme has a native molecular mass of 120000 ± 20000, and consists of a single subunit type (M_r 33500 ± 2000), consistent with the enzyme existing as a tetramer of identical subunits. This is similar to the catechol 2,3-dioxygenase from *P. putida*, which has a molecular mass of 140000 and consists of four identical subunits [3]. It is therefore likely that these enzymes have similar quaternary structures.

The *N*-terminal amino acid sequence of the 3-methylcatechol 2,3-dioxygenase (Fig. 1) shows around 50% identity to the published sequences of two catechol 2,3-dioxygenases [12,13], indicating that, although the enzymes may have similar quaternary structures, there are marked differences in primary structure. Both of the catechol 2,3-dioxygenase sequences begin with Met-Asn-Lys [12,13]; these three residues are absent from the 3-methylcatechol 2,3-dioxygenase expressed in *E. coli*. This may be due to proteolysis of the enzyme during extraction from *E. coli*. The DNA sequence encoding the 3-methylcatechol 2,3-dioxygenase is currently being determined (N. C. McClure, personal communication); this will eventually allow the deduction of the entire amino acid sequence and hence confirm the presence or absence of these three residues.

Enzyme activity can be removed by treatment with EDTA and restored on incubation with ferrous ions, clearly demonstrating that activity is dependent on the presence of iron and also that metal removal and replacement is fairly easily achieved. Oxidation of the Fe(II) cofactor by ferricyanide also results in a loss of enzymic activity. The fact that activity is restored following re-reduction indicates that Fe(II) is essential for enzyme function, with the oxidized Fe(III) state being inactive. The irreversible loss of activity following treatment of the enzyme with H_2O_2 suggests that this oxidant has effects other than a simple one-electron oxidation of the Fe(II) centre.

There are marked differences between the kinetic properties of 3-methylcatechol 2,3-dioxygenase and catechol 2,3-dioxygenase (Table 3). For example, there are differences in the substrate specificity as deduced from the relative rates of cleavage for the two enzymes. This can be partly explained by considering the catabolic pathway in which 3-methylcatechol 2,3-dioxygenase functions. The 3-methylcatechol 2,3-dioxygenase originates from *P. putida* UCC2, which can utilize *m*- or *p*-toluidine as the sole carbon and nitrogen source. Oxidative deamination of toluidine leads to methyl-substituted catechols (as opposed to catechol) as ring-cleavage substrates [5]. Thus the enzyme might be expected to cleave a methyl-substituted catechol in preference to catechol itself. Another marked difference between the two enzymes is the fact that there is a 10-fold difference in substrate K_m values (Table 3), with values for 3-methylcatechol 2,3-dioxygenase in the region of 0.3 μM and for catechol 2,3-dioxygenase around 3 μM. This difference in K_m values corresponds to a difference in binding energy of around 5–6 kJ·mol^{-1}. The K_m values for the three substrates with 3-methylcatechol 2,3-dioxygenase are surprisingly low, in fact a K_m of 0.3 μM is lower than any other published K_m value for a catechol dioxygenase, whether of the extradiol or intradiol type (see table of values in [3]). This low K_m value implies a significant difference in the nature of the active site in 3-methylcatechol 2,3-dioxygenase compared to that of catechol 2,3-dioxygenase.

The dependence of 3-methylcatechol 2,3-dioxygenase enzyme activity on pH gave a bell-shaped curve (Fig. 2) with pK_a values of around 6.9 and 8.7 and a pH optimum of about 7.5. The two hydroxyl groups of catechol show pK_a values which are too high to account for these values, and we therefore ascribe them to protein-based protonations/deprotonations. The lower pK_a value of 6.9 might be due to an active site base, perhaps histidine. This possibility was investigated by the attempted modification of such a histidine with diethylpyrocarbonate. Treatment of the enzyme with diethylpyrocarbonate, however, had no discernable effect on activity. This failure to inactivate the enzyme with diethylpyrocarbonate does not in itself negate the role of an active site base, it may simply reflect the inaccessibility of such a residue to the modifying agent. Alternatively this pK_a might be the result of the protonation of some other active site residue such as a ligand to the iron.

From our studies on 3-methylcatechol 2,3-dioxygenase we drew the following conclusions. (i) The enzyme is a tetramer of identical subunits. (ii) The *N*-terminal amino acid sequence (35 residues) of the enzyme shows around 50% identity with published sequences of catechol 2,3-dioxygenases. (iii) Fe(II) is an essential cofactor of the enzyme. (iv) The K_m for catechol is the lowest seen for

any of the catechol dioxygenases to date. (v) The dependence of enzyme activity on pH follows a classic bell-shaped curve with a pH optimum of around 7.5.

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REFERENCES

1. Hayashi, O. (ed.) (1974) Molecular Mechanisms of Oxygen Activation, Academic Press, New York
2. Nozaki, M., Kotani, S., Ono, K. & Senoh, S. (1970) Biochim. Biophys. Acta **220**, 213–223
3. Nozaki, M. (1988) in Metalloproteins: Chemical Properties and Biological Effects (Otsuka, S. & Yamanaka, T., eds.), pp. 206–218, Elsevier, Amsterdam
4. Nozaki, M. (1970) Methods Enzymol. **17**, 522–525
5. McClure, N. C. & Venables, W. A. (1986) J. Gen. Microbiol. **132**, 2209–2218
6. McClure, N. C. & Venables, W. A. (1987) J. Gen. Microbiol. **133**, 2073–2077
7. Yanisch-Perron, C., Vieira, J. & Messing. J. (1985) Gene **33**, 103–119
8. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Nozaki, M., Kagamiyama, H. & Hayaishi, O. (1963) Biochem. Z. **338**, 582–590
10. Miles, E. W. (1977) Methods Enzymol. **47**, 431–442
11. Jackman, M. P., McGinnis, J., Powls, R., Salmon, G. A. & Sykes, A. G. (1988) J. Am. Chem. Soc. **110**, 5880–5887
12. Nakai, C., Kagamiyuma, H. & Nozaki, M. (1983) J. Biol. Chem. **258**, 2923–2928
13. Ghosal, D., You, I.-S. & Gunsalus, I. C. (1987) Gene **55**, 19–28

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