Overproduction, Purification, and Characterization of Chlorocatechol Dioxygenase, a Non-Heme Iron Dioxygenase with Broad Substrate Tolerance†

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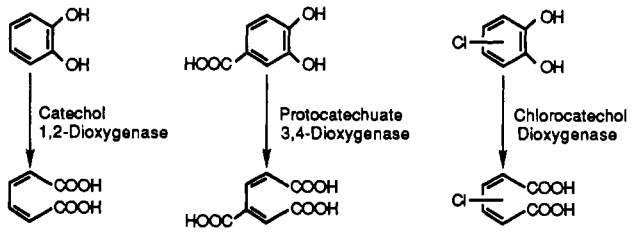
ABSTRACT: We show here that purified chlorocatechol dioxygenase from *Pseudomonas putida* is able to oxygenate a wide range of substituted catechols with turnover numbers ranging from 2 to 29 s^{-1}. This enzyme efficiently cleaves substituted catechols bearing electron-donating or multiple electron-withdrawing groups in an intradiol manner with k_{cat}/K_M values between 0.2×10^7 and 1.4×10^7 M^{-1} s^{-1}. These unique catalytic properties prompted a comparison with the related but highly specific enzymes catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase. The chlorocatechol dioxygenase gene (*clcA*) from the *Pseudomonas* plasmid pAC27 was subcloned into the expression vector pKK223-3, allowing production of chlorocatechol dioxygenase to approximately 7–8% of total cellular protein. An average of 4 mg of purified enzyme has been obtained per gram of wet cells. Protein and iron analyses indicate an iron stoichiometry of 1 iron/57.5-kDa homodimer, α_2Fe. The electronic absorption spectrum contains a broad tyrosinate to iron charge transfer transition centered at 430 nm (ϵ = 3095 M^{-1} cm^{-1} based on iron concentration) which shifts to 490 nm (ϵ = 3380 M^{-1} cm^{-1}) upon catechol binding. The resonance Raman spectrum of the native enzyme exhibits characteristic tyrosine ring vibrations. Electron paramagnetic resonance data for the resting enzyme (g = 4.25, 9.83) is consistent with high-spin iron (III) in a rhombic environment. This similarity between the spectroscopic properties of the Fe(III) centers in chlorocatechol dioxygenase and the more specific dioxygenases suggests a highly conserved catalytic site. We infer that the unique catalytic properties of chlorocatechol dioxygenase are due to other characteristics of its substrate binding pocket.

Widespread use of halogenated organic compounds in such products as pesticides, refrigerants, fire retardants, and paints presents persistent problems in the environment, particularly because many of these materials are resistant to both chemical oxidation and biological degradation (Ghosal et al., 1985). A number of these halogenated hydrocarbons have been shown to be degraded and used as sources of carbon and energy by specialized strains of aerobic and anaerobic bacteria (Ghosal et al., 1985; Frantz & Chakrabarty, 1986; Reineke & Knackmuss, 1988). Bacterial strains have been isolated that degrade pollutants such as the herbicide 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (Sangodkar et al., 1988), 2,6-dichlorotoluene (Vandenbergh et al., 1981), and mono- and polychlorinated biphenyls (Khan et al., 1988; Quensen et al., 1988), as well as other chlorinated hydrocarbons (Ghosal et al., 1985; Frantz & Chakrabarty, 1986; Reineke & Knackmuss, 1988).

Certain strains of *Pseudomonas* have been found that can utilize chlorobenzoate as a sole source of carbon and energy. In the case of *Pseudomonas putida*, the ability to grow on chlorobenzoate is encoded on the plasmid pAC27 (Chatterjee et al., 1981; Chatterjee & Chakrabarty, 1984). This plasmid contains genes for three enzymes that catalyze reactions analogous to three reactions in the chromosomally encoded degradative pathway of benzoate. The three plasmid-encoded enzymes have been shown to be necessary for the utilization

Scheme I



of chlorobenzoate. One of these enzymes, chlorocatechol dioxygenase, catalyzes a reaction analogous to those of catechol 1,2-dioxygenase (CTD)[1] and protocatechuate 3,4-dioxygenase (PCD) (Scheme I). Such halocarbon-degrading enzymes are of interest due to their unique catalytic properties and to their potential application in bioremediation processes.

The catechol dioxygenases are a class of bacterial non-heme iron enzymes that catalyze the addition of both atoms of molecular oxygen to 1,2-dihydroxybenzene (catechol) or its derivatives with subsequent cleavage of the aromatic ring (Scheme I). Aromatic ring-cleaving enzymes such as the catechol dioxygenases play a central role in metabolism of aromatic compounds and thus are ubiquitous in microorganisms. Indeed, the intradiol-cleaving catechol 1,2-dioxygenase

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[1] Abbreviations: CCD, chlorocatechol dioxygenase [also referred to as catechol 1,2-dioxygenase II (Ngai & Ornston, 1988)]; CTD, catechol 1,2-dioxygenase; PCD, protocatechuate 3,4-dioxygenase; 4NC, 4-nitrocatechol; TCC, tetrachlorocatechol; ES, enzyme–substrate; EP, enzyme–product; EI, enzyme–inhibitor; LB, Luria–Bertani medium; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Bis-Tris Propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

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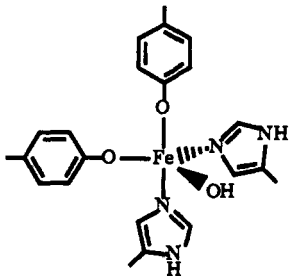
(CTD) and protocatechuate 3,4-dioxygenase (PCD) enzymes have been isolated from a number of different bacteria and studied extensively since their discovery in the 1950s (Nozaki, 1979; Que, 1980, 1989; Bull & Ballou, 1981; Lipscomb et al., 1987). Spectroscopic studies of these enzymes indicated an active-site iron coordinated by two tyrosines, two histidines, and one hydroxide, and this coordination environment was



verified by the solution of the X-ray crystal structure of *Pseudomonas aeruginosa* PCD (Ohlendorf et al., 1988). The active-site iron in these intradiol-cleaving enzymes has been shown to be in the Fe(III) state in the native enzyme and in all spectroscopically characterized ES and EI complexes. The CTD and PCD enzymes are chromosomally encoded and catalyze analogous reactions in two converging branches of the β-ketoadipate pathway. A chlorocatechol dioxygenase has been previously identified in *Pseudomonas* sp. B13 (Dorn & Knackmuss, 1978) but has not been purified and characterized.

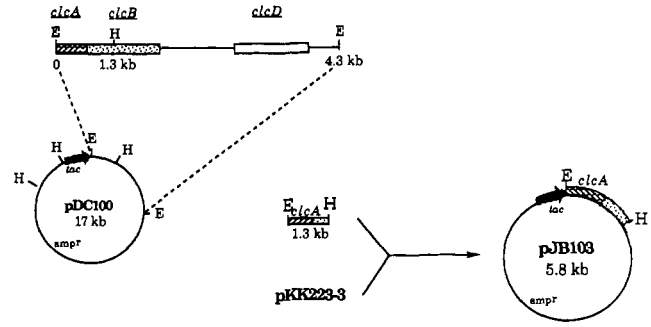
We report here the purification and properties of the chlorocatechol dioxygenase encoded on the *Pseudomonas* plasmid pAC27. A striking difference is observed in the substrate specificity and catalytic efficiency of purified chlorocatechol dioxygenase when compared to those of other catechol dioxygenases. Unlike the latter enzymes, CCD oxygenates a wide range of substituted catechols with rates of the same order of magnitude as for its physiological substrates, the chlorocatechols. The intriguing difference between the catalytic properties of chlorocatechol dioxygenase (CCD) and those of CTD and PCD implies a fundamental difference in the active site of CCD. The purified enzyme, like the CTD from *P. putida* (Nakai et al., 1988), exhibits an unexpected stoichiometry of 1 iron/homodimer, and the spectroscopic properties of CCD are indicative of an Fe(III) center similar to that in CTD and PCD. We infer that the principal differences between these related enzymes are in the substrate binding pocket.

Experimental Procedures

Materials. The expression vector pKK223-3 was obtained from Pharmacia. Catechol, tetrachlorocatechol, protocatechuate, 4-methylcatechol, 3-methoxycatechol, and 4-nitrocatechol were obtained commercially and used without further purification. The 4-halocatechols, 3-chlorocatechol, 3,5-dichlorocatechol, 3,5-dibromocatechol, and 4,5-dichlorocatechol were prepared by W. Pieken and J. W. Kozarich (Pieken, 1990) and used without further purification. The 3-methylcatechol was obtained from M. Harpel and J. D. Lipscomb and recrystallized from toluene prior to use. All other chemicals were obtained commercially and used without further purification. Membrane dialysis tubing was obtained from Spectrum Medical Industries, Inc. Columns and resins were obtained from Pharmacia.

Subcloning and Transformation. The plasmid pDC100, which contains genes for three enzymes in the chlorobenzoate degradative pathway (Frantz et al., 1987), was obtained from K. L. Ngai. The *clcA* gene was separated from the *clcB* and

Scheme II



clcD genes by cutting pDC100 with *Eco*RI and *Hin*dIII, gel-purifying approximately 2 μg of the resulting 1.3-kb fragment, and ligating it into the multiple cloning site of pKK223-3 that was previously cut by *Eco*RI and *Hin*dIII (Scheme II). The identity of the new construct (pJB103) was verified by restriction mapping, and it was used to transform *Escherichia coli* W3110. Transformants were selected by growth in LB medium containing 100 mg/L ampicillin. Gene manipulation procedures were those of Maniatis et al. (1982).

Purification. Freshly transformed cells (<2 days old) were used to prepare overnight inoculates in LB medium containing 100 mg/L ampicillin. Cultures were grown in 2-L Erlenmeyer flasks containing 700 mL of LB medium and 70 mg of ampicillin. Each flask was inoculated with 3.5 mL of an overnight culture of the transformed cells and grown with shaking at 37 °C to mid log phase (OD_{600} = 0.5, approximately 3 h). At this time the cultures were induced by addition of 700 μL of 1 M IPTG (isopropyl β-D-thiogalactopyranoside) and grown in the presence of 100 mg/L $FeSO_4 \cdot 7H_2O$ for 2 h more at 37 °C with shaking. Cultures were harvested by centrifugation at 8000*g* and 4 °C. The cell paste was stored at −70 °C. Typically, 3–4 g of cells were obtained/L of culture.

All purification steps were performed at 4 °C unless otherwise noted, while all pH values reported here were measured at room temperature. Approximately 35 g of frozen cells were suspended in 200 mL of cold buffer A (50 mM Tris hydrochloride, pH 8.0), and 0.5 mL of 250 mM PMSF (phenylmethanesulfonyl fluoride) in ethanol was added. Cells were lysed by sonication (Branson Cell Disruptor 200 sonicator), 0.8 mL of 250 mM PMSF was added, and the cell debris was pelleted by centrifugation at 12000*g*. To the supernatant was added 18 mg of protamine sulfate in extraction buffer. The resulting suspension was centrifuged at 20000*g* for 30 min. The supernatant was made 40% saturated in ammonium sulfate and then centrifuged at 18000*g* for 15 min. The supernatant from this step was brought to 70% saturation in ammonium sulfate and centrifuged at 18000*g* for 30 min. The 40–70% pellet was resuspended in a minimum volume of buffer B (20 mM Tris hydrochloride, pH 8.0) and loaded onto a Sephacryl S200 HR column (2.6 × 40 cm) that had been equilibrated with buffer B. The protein was eluted with buffer B, and the fractions that showed CCD activity were combined and dialyzed against 8 L of buffer B to remove residual ammonium sulfate. The brown solution was loaded onto a Pharmacia DEAE-Sepharose column (2.6 × 30 cm) preequilibrated with buffer B. After the column was washed with 150 mL of buffer B, the dioxygenase was eluted with a 500-mL gradient from 0.0 to 0.4 M NaCl in buffer B. Fractions with specific activities greater than 1.0 unit/mg were combined, diluted 1:1 with buffer B, and loaded onto a TSK-DEAE column (1.6 × 50 cm). After the column was washed with 200 mL of buffer B, the enzyme was eluted with a 1000-mL gradient from 0.0 to 0.20 M NaCl in buffer B, and fractions

with specific activities greater than 5.0 units/mg were pooled and concentrated by using Amicon Centriprep concentrators. The concentrated protein was loaded onto a Pharmacia Sephacryl S-200 HR gel-filtration column (2.6 × 40 cm) equilibrated with buffer C (50 mM Tris hydrochloride and 0.15 M NaCl, pH 8.0) and eluted with the same buffer. The purified enzyme was concentrated to ~25 mg/mL, quick-frozen in liquid nitrogen, and stored at −70 °C.

Protein Determinations. Protein concentrations were determined by the A_{280}/A_{205} method of Scopes (1974). The ϵ_{280} was also calculated on the basis of the number of tryptophan, tyrosine, and cysteine residues in the protein by using the method of Gill and von Hippel (1989).

Iron Analysis. The iron content of the purified enzyme was determined by the ferrozine method (Fish, 1988). The protein was hydrolyzed in acidic permanganate solution as described prior to determination by ferrozine. All glassware was acid-washed to avoid contaminating iron.

Enzymatic Activity Assay. Enzymatic activity was measured spectrophotometrically at 25 °C by monitoring the change in absorbance at 260 nm for all catechols except protocatechuic acid, which was monitored at 290 nm. Assays were performed in 1-mL quartz cuvettes containing 980 μL of 33 mM Tris hydrochloride, pH 7.5, and 20 μL of 10 mM substrate. Reaction was initiated by addition of enzyme. Due to its availability and low cost, catechol was used routinely during purification to monitor dioxygenase activity. The $\Delta\epsilon_{\text{substrate}\rightarrow\text{product}}$ values for various catechols were measured by enzymatically converting a known amount of the catechol to product as described previously (Dorn & Knackmuss, 1978b).[2] Observation of the characteristic strong muconate absorption band at approximately 260 nm confirmed the identity of these products as *cis,cis*-muconates (Dorn & Knackmuss, 1978b; W. Pieken, personal communication). Ratios of extradiol to intradiol cleavage were estimated as previously described (Fujiwara et al., 1975). Dialysis experiments on the EI complex were conducted under nitrogen, allowing 24 h to reach equilibrium between buffer changes and using Spectra Por 4 membrane dialysis tubing (MW cutoff = 12000–14000).

Effect of pH on Activity and Stability. The pH optimum for chlorocatechol dioxygenase activity in air-saturated buffer ($[O_2]$ = 255 μM) was determined by performing enzyme assays at various pH values from 6.5 to 9.5. Buffers were chosen that had adequate buffering capacity through a portion of this range, and at several pH values the enzyme was assayed with more than one buffer to examine the influence of buffer type on activity. The buffers and pH ranges used were as follows: MES, pH 5.5–6.5; Bis-Tris Propane, pH 6.5–7.0 and 8.5–9.5; Hepes, pH 7.0–7.5; and Tris, pH 7.5–8.5. All assays were performed as described above. The pH stability of CCD was determined by storing the enzyme at various pH values for 1 h at 4 °C prior to diluting and assaying the enzymatic activity in the usual manner at pH 7.5.

Molecular Weight and pI Determination. The apparent subunit molecular weight was determined by SDS–PAGE. Following incubation at 100 °C for 3 min in denaturing (SDS and β-mercaptoethanol) loading buffer (Dreyfuss et al., 1984), the enzyme and molecular weight standards were electrophoresed in a 12.5% polyacrylamide gel. The apparent holoenzyme molecular weight was determined by gel filtration on a Pharmacia Superose-12 column, with a standard curve constructed by using Bio-Rad gel-filtration standards. The isoelectric point of the enzyme was determined under nondenaturing conditions by using the Pharmacia Phast system for isoelectric focusing. The gel was stained for protein with Coomassie Blue.

Spectroscopy. Electronic absorption spectra were recorded at 25 °C on a Perkin-Elmer 320 UV/visible spectrophotometer. Electron paramagnetic resonance spectra were recorded on a Varian E-109 spectrometer operating at Q-band (35 GHz) employing an immersion cryostat. EPR samples were frozen by slow immersion in liquid nitrogen. Resonance Raman spectra were recorded at 20 °C with a SPEX double-grating monochromator with a photomultiplier tube detector. Samples were contained in a rotating sample cell that was cooled by a stream of air while in the laser beam to prevent excessive heating.

Kinetics. Steady-state kinetics were performed at 25 °C as described above but with varying substrate and enzyme concentrations. In the enzyme concentration range used in these assays, enzyme activity is directly proportional to the enzyme concentration. The Michaelis constants were determined by fitting the kinetic data with a nonlinear least-squares fitting program derived from that of Cleland (1979). Substrates that exhibited substrate inhibition at high substrate concentrations (3-chloro-, 4-chloro-, 4-bromo-, 4-fluoro-, 3-methoxy-, 4-methyl-, and 3-methylcatechol) were fit to

$$v = \frac{VA}{K + A + A^2/K_i} \quad (1)$$

where v is the reaction velocity, V is the maximal velocity, K is the Michaelis constant, A is the substrate concentration, and K_i is the inhibition constant [for a review of enzyme kinetics, see Dixon and Webb (1979)]. Data for substrates that showed typical Michaelis–Menten kinetic behavior (catechol, 3,5-dichloro- and 3,5-dibromocatechol) were fit to eq 2 with the parameters defined as above.

$$v = \frac{V}{1 + K/A} \quad (2)$$

Inhibition data for tetrachlorocatechol, 4,5-dichlorocatechol, and 4-nitrocatechol were plotted as double-reciprocal plots at various inhibitor concentrations. 4-Nitrocatechol and 4,5-dichlorocatechol were shown to be competitive inhibitors as described by

$$v = \frac{V}{1 + K_{app}/A} \quad (3)$$

where

$$K_{app} = K(1 + I/K_I) \quad (4)$$

and K_{app} is the apparent Michaelis constant, I is the inhibitor concentration, and K_I is the inhibition constant. Tetrachlorocatechol exhibits a more complex mixed inhibition, and the inhibition constants were determined from

$$v = \frac{V_{app}}{1 + K_{app}/A} \quad (5)$$

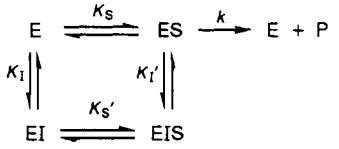
where

[2] The $\Delta\epsilon_{260}$ (substrate → product) values in M^{-1} cm^{-1} were determined to be as follows: catechol, 16000; 3-chlorocatechol, 16290; 4-chlorocatechol, 14480; 4-fluorocatechol, 16620; 4-bromocatechol, 9535; 3,5-dichlorocatechol, 12570; 3,5-dibromocatechol, 6325; 3-methylcatechol, 17400; 4-methylcatechol, 13460; and 3-methoxycatechol, 9668. The $\Delta\epsilon_{260}$ (substrate → product) for the slow substrates could not be determined in the same manner due to incomplete turnover of the catechol; the maximal activity for these substrates was calculated by assuming $\Delta\epsilon_{260} \geq 10000$ M^{-1} cm^{-1}.

$$V_{app} = \frac{V}{1 + I/K_I'} \quad (6)$$

$$K_{app} = K\frac{1 + I/K_I}{1 + I/K_I'} \quad (7)$$

and K_I and K_I' are defined as



The K_M for O_2 in the presence of catechol was determined by measuring the apparent V_{max} with varying concentrations of O_2 in the buffer. Buffers with various [O_2] were prepared by mixing known amounts of O_2-saturated buffer and air-saturated buffer. The O_2-saturated buffer was assumed to be 1276 μM in O_2, while the air-saturated buffer was taken to be 255 μM in O_2 on the basis of oxygen solubility tables (Hitchman, 1978). These O_2 concentrations are for 25 °C, 760 Torr total pressure, and 33 mM Cl^- in the buffer.

Results

Our initial attempts to purify CCD produced in *E. coli* strains containing the pDC100 expression vector (Frantz & Chakrabarty, 1987) were complicated by the difficulty in separating the *clcA* and *clcB* gene products, which are both overproduced by this vector (Frantz & Chakrabarty, 1987; Broderick and O'Halloran, unpublished results). The subcloning of only the *clcA* gene into pKK223-3 (Scheme II) resulted in the improved expression vector pJB103 and a simplified purification of chlorocatechol dioxygenase. Since the *clcA* gene termination codon and the *clcB* gene initiation codon overlap, a portion of the *clcB* gene was also cloned into pKK223-3. This did not cause problems in the purification. The purification scheme for chlorocatechol dioxygenase from *P. putida*, overproduced by pKK223-3 in *E. coli* W3110, is summarized in Table I. CCD constitutes approximately 7–8% of total cellular protein in this system as estimated from the specific activity of the crude extract. The entire purification, from cell lysis to freezing of purified enzyme, was typically completed within 120 h. The purified enzyme was judged to be >95% homogenous as determined by SDS–PAGE. Previous attempts at purification using Tris hydrochloride buffers at pH 7.5 resulted in purified enzyme that had 40% lower specific activity but a similar iron content to that of the enzyme purified at pH 8.0 (Broderick and O'Halloran, unpublished results).

The dimer molecular weight of the native enzyme, predicted on the basis of the DNA sequence (Frantz & Chakrabarty, 1987), is 57 507 Da, yet the enzyme migrates in gel-filtration chromatography with an apparent molecular weight of 64 600 Da. Denaturation and electrophoresis of the enzyme results in a single band that migrates with an apparent molecular weight of 32 000. Quantitation of the iron content of purified *P. putida* chlorocatechol dioxygenase from different runs consistently results in an iron ratio of 1.04 ± 0.15 iron/holoenzyme. Thus the apparent molecular composition of chlorocatechol dioxygenase from *P. putida* is α_2Fe. Although 1 iron/homodimer seems unusual, there is precedence for this stoichiometry in other catechol dioxygenases (Nakai et al., 1990; Chen & Lovell, 1990).

The isoelectric point of the holoenzyme was determined to be 5.8. CCD shows maximum stability when stored at pH > 7.2 and begins to lose activity quickly (<1 h) when stored at 4 °C below pH 7.0. The enzyme exhibits maximal activity in the pH range 7.5–7.9, with activity dropping off sharply below pH 7.0. The effect on the enzymatic reaction of various buffers used for these pH dependence studies, including MES, Bis-Tris Propane, Hepes, and Tris, is small or nonexistent, as evidenced by the continuity of the activity vs pH curve.

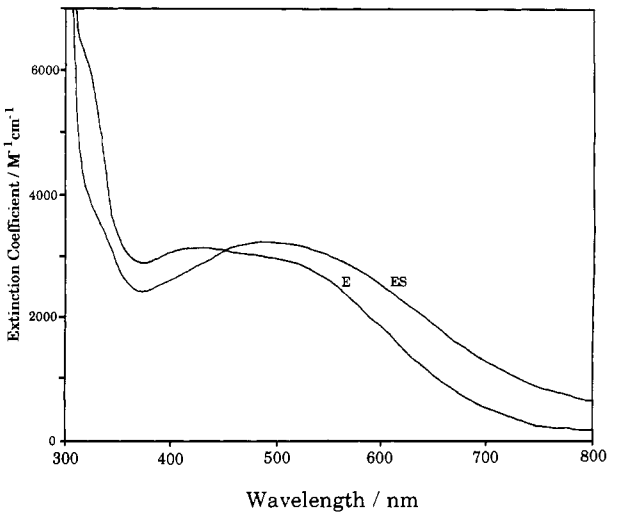


FIGURE 1: Electronic absorption spectra of chlorocatechol dioxygenase. The electronic absorption spectrum of the native enzyme (E) was recorded at room temperature on a 0.13 mM solution of the enzyme in 37 mM Tris hydrochloride and 50 mM NaCl buffer at pH 7.6. The spectrum of the enzyme–catechol complex (ES) was recorded as above but under nitrogen in the presence of 3.0 mM catechol.

The electronic absorption spectra for the native enzyme and the anaerobic enzyme–catechol complex are shown in Figure 1. The native enzyme exhibits a broad absorption band centered at 430 nm (ϵ = 3095 M^{-1} cm^{-1} based on iron concentration). Anaerobic addition of catechol increases absorbance in the long-wavelength region and red-shifts the maximum to 490 nm (ϵ = 3380 M^{-1} cm^{-1} based on iron concentration). The extinction coefficients of native enzyme at 280 and 205 nm were determined to be 1.1 ± 0.1 and 31.0 ± 0.5 $(mg/mL)^{-1}$ cm^{-1}, respectively, by the method of Scopes (1974). The extinction coefficient at 280 nm was also calculated on the basis of the tyrosine, tryptophan, and cysteine content of the enzyme by the method of Gill and von Hippel (1989), resulting in an ϵ_{280} of 1.13 $(mg/mL)^{-1}$ cm^{-1}, in excellent agreement with the empirical value.

The 35-GHz EPR spectrum of native chlorocatechol dioxygenase in frozen buffer at 4.2 K is shown in Figure 2. Resonances are centered at g = 9.83 and 4.25 and are characteristic of high-spin Fe(III) in a rhombic environment, similar to those observed for other catechol dioxygenases. In EPR spectra of other catechol dioxygenases (Que et al., 1976; Kent et al., 1987), the low-field signal has been attributed to g_y of the lower Kramer's doublet, while the $g \sim 4.3$ signal was assigned to the middle Kramer's doublet. This signal for CCD is clearly anisotropic at Q-band, with g_x = 4.21, g_y = 4.18, and g_z = 4.32, while at 9.5 GHz only a single signal is observed at g = 4.25 with a low-field signal at g = 9.79 (Figure 2, inset).

The resonance Raman spectrum of native chlorocatechol dioxygenase (λ_{ex} = 647.1 nm) is shown in Figure 3. Resonances attributable to tyrosine ring vibrations are observed at 1604 and 1507 cm^{-1} (C–C stretch), 1266 cm^{-1} (C–O stretch), and 1183 cm^{-1} (C–H stretch). These vibrational bands are similar to those observed for the other catechol dioxygenases, where the broad C–O stretching vibration was shown to consist of two separate C–O stretches (Que & Epstein, 1981). Upon anaerobic addition of catechol to CCD

Table I: Purification of *P. putida* Chlorocatechol Dioxygenase

fraction	volume (mL)	activity (units/mL)	total activity (units)	protein (mg/mL)	specific activity (units/mg)	yield (%)	purification (x-fold)
crude extract	208	9.72	2020	16.5	0.590	100	1.00
40–70% $(NH_4)_2SO_4$	36.0	46.8	1690	73.5	0.637	83.3	1.10
Sephacryl S-200 column 1	176	8.74	1540	13.4	0.654	76.1	1.11
DEAE-Sepharose	55.5	23.3	1290	9.76	2.39	63.9	4.10
TSK-DEAE	87.0	12.1	1050	1.78	6.80	52.1	11.5
Sephacryl S-200 column 2	8.4	91.6	770	11.7	7.83	38.1	13.3

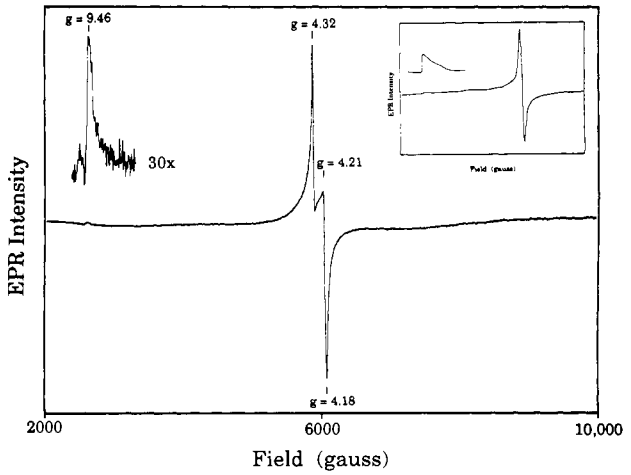


FIGURE 2: EPR spectra of chlorocatechol dioxygenase. The spectrum of approximately 1.4 mM enzyme was recorded at 4.2 K. Instrumental parameters were as follows: field center, 6000 G; scan range, 8000 G; scan time, 4 min; receiver gain, 40; modulation amplitude, 8.0; microwave frequency, 35.33 GHz; microwave power, 0.079 mW. The spectrum shown is an average of 100 scans. Inset: X-band EPR spectrum of 0.57 mM CCD. Instrumental parameters were as follows: field center, 1150 G; scan range, 1400 G; scan time, 4 min; receiver gain, 160; modulation amplitude, 1.6; microwave frequency, 9.238 GHz; microwave power, 0.2 mW.

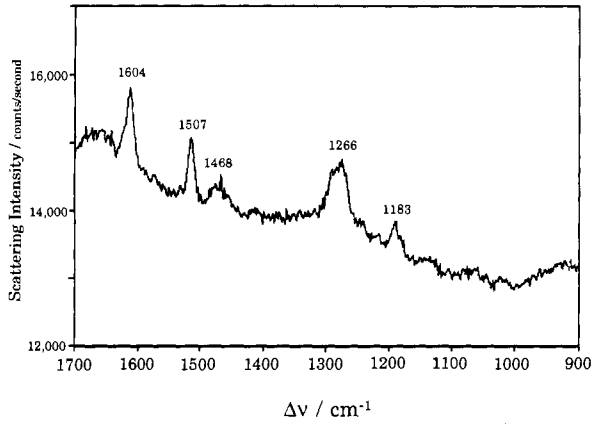


FIGURE 3: Resonance Raman spectrum of chlorocatechol dioxygenase. The spectrum was recorded at 20 °C in a spinning sample tube containing 1.6 mM enzyme in 20 mM Tris hydrochloride buffer at pH 7.8. Instrumental parameters were as follows: $\lambda_{excitation}$, 647.1 nm; laser power at sample, 65 mW; slit width, 300 μm; scan speed, 0.2 Å/s; dwell time, 5 s.

and excitation at 514.5 nm, the enzyme tyrosine vibrational bands disappear and new vibrational bands appear at 1477, 1318, 1257, and 1148 cm^{-1} (Figure 4), which are attributable to the catechol bound at the active site (Felton et al., 1978; Que & Heistand, 1979). The region around 1300 cm^{-1} has been shown to be indicative of the ionization state of the catechol (Que & Heistand, 1979); in particular, peaks near 1260 and 1320 cm^{-1} observed for the CCD–catechol complex are characteristic of the dianion form of catechol.

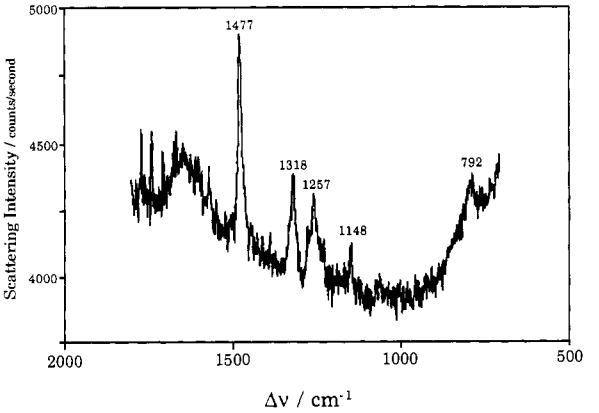


FIGURE 4: Resonance Raman spectrum of the anaerobic CCD–catechol complex. The spectrum was recorded at 20 °C in a spinning sample tube containing 1.4 mM chlorocatechol dioxygenase and 12.5 mM catechol in 50 mM Tris hydrochloride and 150 mM NaCl buffer at pH 7.8. Instrumental parameters were as follows: $\lambda_{excitation}$, 514.5 nm; laser power at sample, 170 mW; slit width, 200 μm; scan speed, 0.1 Å/s; dwell time, 2 s.

The highest specific activities of purified CCD were observed with 3-methyl- and 4-methylcatechol (29.8 and 22.9 units/mg, respectively, in air-saturated buffer at 25 °C), while the specific activities with the physiological substrates 3-chloro- and 4-chlorocatechol were significantly lower (10.2 and 6.25 units/mg, respectively). In contrast, specific activities of 20–60 units/mg are observed for the catechol 1,2-dioxygenases with catechol as substrate (Dorn & Knackmuss, 1978; Nakagawa et al., 1963; Kojima et al., 1967; Patel et al., 1976; Nakai et al., 1979, 1988) and protocatechuate dioxygenases with protocatechuate as substrate (Fujisawa & Hayaishi, 1968; Yoshida et al., 1976; Bull & Ballou, 1981; Whittaker & Lipscomb, 1984; Ohlendorf et al., 1987). Despite this somewhat low specific activity for its physiological substrates, CCD is able to oxygenate catechol and a number of substituted catechols with specific activities ranging from 2.40 to 29.8 units/mg (4-bromocatechol and 3-methylcatechol, respectively) and thus exhibits a significantly broadened substrate tolerance relative to other purified intradiol catechol dioxygenases.

In addition, CCD is distinct from CTD in its specificity for intradiol vs extradiol cleavage of 3-substituted catechols. Fujiwara et al. (1975) found that catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1 catalyzed both the intradiol and extradiol oxygenation of 3-methylcatechol and 3-methoxycatechol, with 5.9% of the 3-methylcatechol product and 20% of the 3-methoxycatechol product resulting from extradiol cleavage. In the case of CCD, we have found that less than 0.5% of the 3-methoxycatechol product and less than 0.07% of the 3-methylcatechol product result from extradiol cleavage.

The K_M for O_2 with catechol as substrate was estimated to be 5000 μM by extrapolating reaction velocities to infinite oxygen concentration. Because the concentration of oxygen in oxygen-saturated buffer at 760 Torr and 25 °C is only 1276 μM, it was not possible to measure the rate of turnover with

Table II: Kinetic Parameters for *P. putida* Chlorocatechol Dioxygenase

substrate	k_{cat}[a] (s^{-1})	$V_{max,rel}$[b]	K_M[a] or K_I[c] (μM)	k_{cat}/K_M[a] ($s^{-1}\ \mu M^{-1}$)	pK_I
catechol[d]	7.5 (0.2)[e]	1.00	4.7 (0.5)	1.6 (0.2)	9.75[f]
3-chlorocatechol[g]	9.8 (0.5)	1.31	0.8 (0.1)	12 (2)	8.40[f]
4-chlorocatechol[h]	5.99 (0.07)	0.80	0.44 (0.04)	14 (1)	9.00[f]
4-fluorocatechol[i]	10.9 (0.4)	1.45	3.5 (0.4)	3.1 (0.5)	9.28[j]
4-bromocatechol[k]	2.30 (0.06)	0.31	0.25 (0.03)	9 (1)	9.01[j]
3,5-dichlorocatechol[d]	6.7 (0.6)	0.89	0.6 (0.2)	11 (5)	7.87[j]
3,5-dibromocatechol[d]	3.5 (0.2)	0.47	0.30 (0.09)	12 (4)	7.58[j]
4,5-dichlorocatechol	<0.20	<0.03	[0.012(0.005)][c]		8.49[j]
tetrachlorocatechol	<0.03	<0.004	[0.008(0.003)][c]		5.80[f]
protocatechuate	0.0	0.0	[66(16)][c]		8.90[l]
3-methylcatechol[m]	28.6 (0.6)	3.81	12.2 (0.7)	2.3 (0.2)	9.75[f]
4-methylcatechol[n]	21.9 (0.5)	2.92	3.1 (0.3)	7.1 (0.9)	9.95[f]
3-methoxycatechol[o]	7.9 (0.2)	1.05	3.5 (0.3)	2.3 (0.3)	9.28[p]
4-nitrocatechol	0.0	0.0	[0.10(0.03)][c]		6.78[q]

[a] Measured in air-saturated buffers at 25 °C. [b] Relative to V_{max} with catechol. [c] K_I values (given in brackets) as determined by inhibition of catechol turnover. [d] Substrates exhibited standard Michaelis–Menten saturation kinetics. [e] The k_{cat} for catechol corresponds to a specific activity of 7.83 units/mg. [f] Dorn and Knackmuss (1978b). [g] Substrate inhibition constant $K_{I,S}$ = 150 ± 90 μM. [h] Substrate inhibition constant $K_{I,S}$ = 4000 ± 3000 μM. [i] Substrate inhibition constant $K_{I,S}$ = 400 ± 100 μM. [j] This work. [k] Substrate inhibition constant $K_{I,S}$ = 400 ± 100 μM. [l] Walsh and Ballou (1983). [m] Substrate inhibition constant $K_{I,S}$ = 4300 ± 900 μM. [n] Substrate inhibition constant $K_{I,S}$ = 14000 ± 12000 μM. [o] Substrate inhibition constant $K_{I,S}$ = 6000 ± 2000. [p] Rosenblatt et al. (1953). [q] Pichet and Benoit (1967).

$[O_2]$ close to the K_M for oxygen.

Tetrachlorocatechol, 4,5-dichlorocatechol, and 4-nitrocatechol were found to be competitive inhibitors of catechol turnover with very small K_I values,[3] while protocatechuic acid behaves as a competitive inhibitor with a relatively large K_I. In addition, tetrachlorocatechol and 4,5-dichlorocatechol, but not 4-nitrocatechol and protocatechuic acid, appear to be turned over by CCD at very low rates. Catechols tested for activity with CCD are listed in Table II, along with k_{cat} and K_M or K_I values, $V_{max,rel}$ (relative to catechol), and the specificity constant, k_{cat}/K_M (Fersht, 1985). Two other substituted catechols not listed, ethyl 3,4-dihydroxybenzoate and 3,4-dihydroxybenzylamine hydrobromide, were found to be weak competitive inhibitors of catechol turnover, with the latter showing approximately the same level of inhibition as protocatechuate and the former showing a somewhat stronger inhibition. In contrast to what has been found for PCD (Bull & Ballou, 1981), chloride in the range from 0 to 200 mM was not found to inhibit the reaction catalyzed by CCD.

Discussion

Chlorocatechol dioxygenase from *P. putida* has been overexpressed in *E. coli* and purified to homogeneity. The purification of this enzyme allows for the first time the systematic study of the physical properties of a catechol dioxygenase that exhibits a broader substrate tolerance than catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase, its likely evolutionary relatives (Neidle et al., 1988).

Previous attempts to purify chlorocatechol dioxygenase from *Pseudomonas* strains resulted in reports of an enzyme with broad substrate tolerance but low catalytic efficiency. While the former property has been verified for this enzyme, the latter was likely an artifact of the procedures used in purification. Dorn and Knackmuss (1978a) reported partial purification of catechol 1,2-dioxygenase (pyrocatechase I) and chlorocatechol dioxygenase (pyrocatechase II) from *Pseudomonas* sp. B13 grown on 3-chlorobenzoate as a sole carbon source. While the catechol 1,2-dioxygenase was purified 29-fold, the specific activity of the chlorocatechol dioxygenase was increased only 1.4-fold, reportedly due to its instability with respect to chromatography and dialysis. Ngai and Ornston (1988) reported the purification of chlorocatechol dioxygenase from *P. putida* plasmid pAC27. Although the enzyme was pure according to SDS–PAGE and amino-terminal sequencing, it had a specific activity of only 0.19 unit/mg. This specific activity with catechol as a substrate is significantly lower than that reported here for the same enzyme (7.83 units/mg) and apparently results from the use of buffers containing 10 mM sodium ethylenediamine, 1 mM $MnSO_4$, and 1 mM β-mercaptoethanol (BME) during purification. We have found that storing CCD in this buffer at 4 °C over a period of 5 days results in a 70% loss of activity, compared to only a 20% loss when stored in 33 mM Tris, pH 7.5. The BME appears to be the most damaging component of this buffer, as addition of 1 mM BME to Tris buffer results in 60% inactivation over the course of 5 days. The combined presence of a reducing agent (BME), a chelating agent (ethylenediamine), and an Fe(II) analogue [Mn(II)] probably inactivates the enzyme by cofactor removal.

Iron Content of CCD. With the exception of substrate tolerance, the physical properties of *P. putida* chlorocatechol dioxygenase, including the iron content, are indistinguishable from those of the *P. putida* catechol 1,2-dioxygenase (Nakai et al., 1988). The unexpected stoichiometry of 1 iron/homodimer in these *P. putida* dioxygenases has also been reported for two of the three isozymes of CTD from *P. arvilla* C-1 (Nakai et al., 1990), and in the catechol 1,2-dioxygenase from *Rhizobium leguminosarum* biovar *viceae* USDA 2370 (Chen & Lovell, 1990). In contrast, most catechol dioxygenases studied to date consist of 1 iron/$\alpha\beta$ heterodimer or 2 irons/$\alpha\alpha$ homodimer.

In attempts to increase the iron content and specific activity of CCD, we have added Fe(II) or Fe(III) to the assay buffer, incubated the enzyme with Fe(III) followed by gel filtration, and anaerobically incubated CCD with Fe(II) and various reducing agents followed by gel filtration and air oxidation (Fujiwara & Nozaki, 1973). None of these methods have

[3] Inhibition assays were done with 20–2000-fold excess of inhibitor over enzyme. However, because the inhibition constants for 4,5-dichlorocatechol, tetrachlorocatechol, and 4-nitrocatechol are of the same order of magnitude as the concentration of enzyme used in the inhibition assays, these inhibitors could be treated as high-affinity inhibitors. Using the graphical method of Dixon (1972) for treating high-affinity competitive inhibitors results in K_I values of 0.03 μM (4,5-dichlorocatechol), 0.005 μM (tetrachlorocatechol), and 0.04 μM (4-nitrocatechol), which are in general agreement with results obtained from eqs 3–7. In addition, the K_I' value determined for tetrachlorocatechol varies systematically from 0.192 μM for [TCC] = 0.02 μM to 1.67 μM for [TCC] = 2.0 μM. This systematic variation may indicate some deviation from classical mixed inhibition. The binding of TCC to CCD is currently under further investigation.

Table III: Comparison of Normalized V_{max} Values[a] for Oxygenation of Substituted Catechols by the Catechol Dioxygenases

catechol	*P. putida* CCD[b]	*P. putida* CTD[c]	*P. putida* PCD[d]	*P. arvilla* CTD[e]	*Pseudomonas* B13 CTD[f]	*Pseudomonas* B13 CCD[f,g]	*A. eutrophus* CTD[f]	*A. eutrophus* CCD[h]
catechol	1.00	1.0	0.004	1.0	1.0	1.0	1.0	1.0
3-chloro	1.31				0.007	1.05	0.002	1.24
4-chloro	0.80	0.036		0.020	0.113	0.96	0.070	1.22
4-fluoro	1.36				0.30	1.48	0.229	
4-bromo	0.29							
3,5-dichloro	0.83				0	0.36		1.81
4,5-dichloro	I[i]							
tetrachloro	I[i]				0	0	0	
3,5-dibromo	0.44							
PCA	0.0	0	1.0					
3-methyl	3.54	0.008	0.004	0.054	0.110	3.37	0.071	1.67
4-methyl	2.73	0.90	0.002	0.946	0.920	3.16	0.413	
3-methoxy	0.98	0.008			0.032	2.61		
4-nitro	I[i]	0						

[a] The V_{max} values are given relative to the V_{max} of catechol for the CTD and PCD enzymes and relative to the V_{max} of protocatechuic acid for PCD. [b] This work. [c] Nakai et al. (1988). [d] Fujisawa and Hayaishi (1968). [e] Fujiwara et al. (1975). [f] Dorn and Knackmuss (1978b). [g] Relative rates were determined by using the crude cell extract. [h] Pieper et al. (1988). [i] I indicates catechols that behaved as competitive inhibitors of catechol oxidation.

resulted in an increase in the specific activity of CCD. In addition, the catechol 1,2-dioxygenase from *P. putida* exhibits a specific activity similar to that for other catechol dioxygenases (31.6 units/mg) despite its "low" iron stoichiometry. These results suggest that the lower specific activity of CCD (7.83 units/mg) is not due merely to an insufficient iron complement but rather to an interaction with substrate that is fundamentally different than that of the more specific CTD and PCD enzymes.

The unusual stoichiometry of 1 iron/homodimer in CCD raises some intriguing possibilities. One is that an alternate metal ion occupies the second binding site in place of iron. This seems unlikely since analysis of purified CCD holoenzyme by inductively coupled plasma atomic emission reveals Zn/dimer and Mn/dimer mole ratios of less than 0.05 and 0.009, respectively. Another possibility is that the single iron atom is bound at the subunit interface with ligands contributed from both subunits. While this cannot be ruled out, it seems unlikely on the basis of the homology of the CCD amino acid sequence with that of the iron-binding portion of the β-subunit of *P. aeruginosa* PCD (Ohlendorf et al., 1988; Frantz & Chakrabarty, 1987; Broderick and O'Halloran, unpublished results). Alternatively, binding of one iron atom to the dimer may decrease the metal ion affinity of the other iron-binding site.

Catalytic Properties of CCD. The catechol 1,2-dioxygenases and the protocatechuate dioxygenases, including the CTD from *P. putida*, catalyze the oxygenation of catechol and protocatechuate, respectively, at rates that are 10–1000-fold faster than the turnover rate for most halogenated or otherwise substituted catechols and protocatechuates, the exceptions being 4-fluorocatechol and 4-methylcatechol for the CTD enzymes (see Table III). In the case of chlorocatechol dioxygenase, a number of substituted catechols are cleaved with rates of the same order of magnitude as the physiological substrates, while the specific activity with the physiological substrates is 3–10-fold lower than that of the CTD and PCD enzymes.

The efficiency of an enzyme, and its ability to discriminate between two competing substrates, can be gauged by k_{cat}/K_M ratios. If all substrates were available to the enzyme simultaneously, that with the highest k_{cat}/K_M value would be turned over at the highest rate (Fersht, 1985). The k_{cat}/K_M can also be reduced to an apparent second-order rate constant for reaction of free enzyme with free substrate (Fersht, 1985). The k_{cat}/K_M values for oxygenation of all of the mono- and disubstituted catechols by CCD are between 10^6 and 10^7 M^{-1} s^{-1}. These rates approach the diffusion-controlled limits for bimolecular reactions (10^8–10^9 M^{-1} s^{-1}) and thus are characteristic of highly efficient catalysis. CCD exhibits the greatest specificity for the chlorinated and brominated substrates, including the dihalogenated substrates (with the exception of 4,5-dichlorocatechol, vide infra), as shown in Table II. Chlorocatechol dioxygenase is the only physically characterized member of the catechol dioxygenase family that has been shown to exhibit both a relatively high activity across a broad range of substrates (Table III) and an efficient oxygenation of mono- and dihalogenated catechols.

A chlorocatechol dioxygenase has also been found to be encoded on the *Alcaligenes eutrophus* JMP134 plasmid pJP4 (Don et al., 1985) as part of the 2,4-dichlorophenoxyacetic acid (2,4-D) degradative pathway. Pieper et al. (1988) have reported the K_M and relative V_{max} values for the partially purified enzyme. As can be seen in Table III, the relative V_{max} values of the chlorinated catechols are all high in comparison to that of catechol, indicating a broad substrate tolerance. However, the relative specificity constants [as estimated from $V_{max,rel}/K_M$] vary widely for the substrates, with those for 4-chlorocatechol and 3,5-dichlorocatechol being approximately 45-fold greater than that for catechol and approximately 50-fold greater than that for 3-methylcatechol. This is in contrast to the narrow range of specificity constants observed for the *P. putida* CCD, which, as shown in Table II, vary by less than a factor of 10, between 1.6 and 14 μM^{-1} s^{-1}. The high efficiency of the *P. putida* CCD over a much broader range of substituted substrates suggests unusual steric and electronic properties of the substrate recognition environment. Despite these differences in the catalytic properties, the *P. putida* and *A. eutrophus* enzymes exhibit a high degree of sequence homology, with 60% identical residues (Ghosal & You, 1988) and 77% similar residues (Broderick and O'Halloran, unpublished results).

Iron Coordination Environment in CCD. The remarkable catalytic properties of this member of the intradiol dioxygenase family of enzymes prompted a detailed spectroscopic examination of the CCD active site. Results obtained from electronic absorption, EPR, and resonance Raman spectroscopies indicate the presence of high-spin Fe(III) in a rhombic environment with tyrosine coordination. The visible spectrum of native CCD is similar in both λ_{max} and extinction coefficient to those reported for the CTD and PCD enzymes. The spectrum of the CCD–catechol complex, however, lacks the distinct long-wavelength band observed (680–720 nm, ϵ = 1700–2500 M^{-1} cm^{-1}) in CTD–catechol and PCD–protocatechuate spectra (Kojima et al., 1967; Walsh & Ballou, 1983; Walsh et al.,

1983; Whittaker et al., 1984). EPR spectra of native CCD are characteristic of non-heme Fe(III) in a rhombic environment. The X-band (9.5 GHz) spectrum exhibits resonances at $g = 4.25$ and $g = 9.79$ (Figure 2, inset), similar to spectra reported for other catechol dioxygenases. At Q band (35 GHz), however, the $g \sim 4.3$ resonance of CCD is resolved into its three components, resulting in $g_x = 4.21$, $g_y = 4.18$, and $g_z = 4.32$, and the low-field signal is observed at $g = 9.83$. This is the first reported resolution of the components of the native enzyme $g \sim 4.3$ signal in a catechol dioxygenase.

Resonance Raman spectra of native CCD exhibit tyrosine ring vibrations characteristic of Fe(III)–tyrosinate sites in proteins in general and catechol dioxygenases in particular. The C–O stretching vibration at 1266 cm^{-1} is broad; this has been shown for the other dioxygenases to result from the unresolved vibrations of two different tyrosine ligands (Que et al., 1980; Que & Epstein, 1981). The band at 1468 cm^{-1} likely results from a non-resonance-enhanced C–H deformation of the protein backbone (Keyes et al., 1978; Felton et al., 1978). The resonance Raman spectrum of the CCD–catechol complex is very similar to the spectrum of the ES complex of CTD (Que & Heistand, 1979). In the latter case comparison of ES and model complex spectra led to the conclusion that the dianion form of catechol bound to the active site. This evidence suggests that substrate bound to the CCD active site is also in the dianion form.

This similarity between the resonance Raman spectra of the CCD–catechol and CTD–catechol complexes indicates a similar mode of substrate binding in the two enzymes. Several differences are observed in comparison to the resonance Raman spectrum of the PCD–catechol complex. In addition, the CCD (data not shown) and CTD (Tyson, 1975) complexes with the inhibitor 4-nitrocatechol show similar visible spectra that are nearly identical with the visible spectrum of 4-nitrocatechol chelated to Fe(III) (Tyson, 1975). These CCD- and CTD–4-nitrocatechol spectra are distinct from the visible spectrum of the PCD–4-nitrocatechol complex, which resembles the visible spectrum of (free) 4-nitrocatechol dianion (Tyson, 1975). These results suggest that 4-nitrocatechol chelates the active-site iron in the case of CCD and CTD but not in the case of PCD. Thus it appears that the iron center in CCD interacts with substrates and inhibitors in a way that is similar to that of CTD but different from that of PCD.

Enzyme–Inhibitor Complexes. Unlike the 3,5-dihalocatechols, 4,5-dichlorocatechol is not turned over by CCD at a significant rate, and thus the relative positioning of ring substituents is a critical determinant in the ability of CCD to turn over a given catechol. Such a substitution-position effect can be attributed to steric and/or electronic effects of the substrate, or possibly to the electronic properties of the product. 4,5-Dichlorocatechol is a potent competitive inhibitor (Table II) of catechol turnover and thus is not sterically hindered from entering the active site. This competitive inhibition may be attributed to a tightly bound but unreactive enzyme–inhibitor complex or to an enzyme–product complex with a very small dissociation constant. Indeed, product dissociation was shown to be the rate-limiting step in substrate turnover for PCD (Bull et al., 1981) and CTD (Walsh et al., 1983). The inhibitors tetrachlorocatechol and 4-nitrocatechol also apparently bind the active site quite tightly or produce tight-binding products, as evidenced by the very small K_I values (Table II). Exhaustive dialysis of the CCD–tetrachlorocatechol complex under nitrogen results in recovery of native enzyme activity, demonstrating that the inhibition is not due to removal of the Fe(III) cofactor.

While detailed correlations of steady-state kinetic parameters with substrate properties such as $\sigma\rho$ parameters are not helpful without additional knowledge of specific steps in the enzymatic reaction, several features of the mechanism are apparent from this study. First, the efficiency of the enzyme is high even when two moderately electron withdrawing groups are present on the substrate. Second, the trends in physical properties of those substituted catechols that act as substrates can be compared with those that act as inhibitors. One observation is the trend in pK_I values for substrates versus inhibitors (Table II). All catechols that are substrates have pK_I values greater than 7.5, while two of the three tight-binding inhibitors (i.e., $K_I < 1$ μM) have pK_I values less than 7.0. This suggests the possibility of inhibition due to catechol chelation of the active-site iron. Such resistance to intradiol dioxygenation for chelated catecholates has been reported in model reaction studies (Lauffer et al., 1981; White et al., 1984; Que et al., 1987) but is in contrast to recent EXAFS evidence that the "slow substrate" homoprotocatechuic acid chelates the active-site iron of PCD (True et al., 1990).

The trends in oxidation potentials of the catechols are consistent with the trends in reactivity. Catechols containing strong electron-withdrawing groups such as nitro or multiple halogens tend to have less positive oxidation potentials than less substituted catechols. Thus, if the enzymatic mechanism requires reduction of the active-site iron by the substrate prior to oxygenation, catechols such as tetrachlorocatechol and 4-nitrocatechol may be unable to carry out this first step. In the case of lipoxygenase, another non-heme iron oxygenase, Nelson (1988) has shown that catechols with more positive oxidation potentials can reduce the Fe(III) in lipoxygenase, while those with lower oxidation potentials cannot.

Protocatechuate inhibition of CCD differs in several respects. On the basis of its pK_{a_1} value alone, protocatechuate should be a reasonable substrate for CCD; however, it is a weak competitive inhibitor with $K_I = 66$ μM. 4-Chlorocatechol has a lower pK_{a_1} and yet is the best substrate in this group. This type of inhibition is consistent with the idea that the substrate binding site of CCD has been under an evolutionary pressure to discriminate against protocatechuate since the chromosomally encoded protocatechuate dioxygenase enzyme is already geared for this particular substituted catechol. Such discrimination would prevent crosstalk between the parallel sections of the β-ketoadipate and haloaromatic degradatory pathways shown in Scheme I. Furthermore, Table III indicates that the *P. putida* CTD enzyme also discriminates against protocatechuate.

Despite the numerous spectroscopic, kinetic, and structural studies of the catechol dioxygenases, the intimate mechanism of enzymatic oxygen activation remains unclear. The absence of evidence for an Fe(II) intermediate led Que and co-workers to suggest that the iron center served merely as a Lewis acid that could ketonize the catechol and therefore "activate" this substrate for direct reaction with oxygen. More recently, on the basis of small molecule model studies, Funabiki et al. (1986, 1987) and Que (Cox & Que, 1988) have suggested the possibility that the "activated" ES complex may be an Fe(II)–semiquinone species. Cox and Que (1988) favor a mechanism in which oxygen reacts directly with the semiquinone radical, without direct interaction of the dioxygen with Fe(II), and the extended Hückel calculations of Funabiki et al. (1990) support this argument. Alternatively, as has been suggested by Funabiki et al. (1987a,b), involvement of an Fe(II)–semiquinone intermediate could lead to activation of oxygen by Fe(II) prior to the reaction of oxygen and substrate.

If an Fe(II)–semiquinone intermediate is shown to exist in the enzymatic reaction pathway, then the question remains whether dioxygen reacts with this intermediate by attacking the substrate radical or the Fe(II). Chlorocatechol dioxygenase, with its slower turnover rate and relaxed specificity toward substituted substrates, may be well suited to further mechanistic studies of this family of enzymes.

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