Homoprotocatechuate 2,3-Dioxygenase from Brevibacterium fuscum
A DIOXYGENASE WITH CATALASE ACTIVITY*

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Homoprotocatechuate 2,3-dioxygenase (2,3-HPCD) cleaves the aromatic ring of its substrate with insertion of both atoms of oxygen from O2 to form α-hydroxy-α-carboxymethyl cis-muconic semialdehyde. The enzyme has been purified from the Gram-positive bacterium Brevibacterium fuscum and characterized. The enzyme appears to have a range of quaternary structures with predominant components of α4 and α6 (α subunit M, = 42500 ± 1500) and binds 1 Fe(II)/subunit. Although the substrate Km values are similar to those of other Fe(II) ring cleaving dioxygenases, the turnover number is lower by 90–97% and the enzyme exhibits much higher stability to metal chelators and H2O2. The stability to H2O2 is shown to derive from an endogenous catalase activity of 2,3-HPCD (stoichiometry: 2 H2O2 → 2 H2O + O2) that is novel for dioxygenases. H2O2 is a mixed-type inhibitor of the dioxygenase activity, suggesting that dioxygenase and catalase activities are both catalyzed by the enzyme, but at distinguishable sites. In contrast, catecholic substrates, including homoprotocatechuate and p-nitrocatechol, are nonessential activators of the catalase activity. The plot of 1/v of catalase activity versus 1/[H2O2] is parabolic in the absence of catecholic substrates and linear in their presence, indicating that these reactions proceed by different mechanisms. A mechanism for catalase activity is proposed in which 2 H2O2 molecules bind simultaneously to the iron to account for the observed parabolic kinetic plot. Electron transfer between the peroxides mediated by the iron would yield 2 H2O and O2. Catecholic substrates are proposed to modify this reaction by excluding one H2O2 from the Fe(II), thereby causing the kinetic plots to appear linear. Electron donation by the catecholic substrates would facilitate O–O bond cleavage of H2O2, but outer sphere electron transfer from a second H2O2 in another step would be necessary to complete the reaction. p-Nitrocatechol is shown to bind differently to 2,3-HPCD than to other Fe(II) ring cleavage dioxygenases. Possible explanations for this observation are considered in the context of the proposed catalase and normal dioxygenase mechanisms which may also have bearing on the unique catalase activity and low dioxygenase turnover number of the enzyme.

Homoprotocatechuate 2,3-dioxygenase (2,3-HPCD; EC 1.13.11.15)1 catalyzes the cleavage of O2 and insertion of both oxygen atoms into the organic substrate (HPCA) to form α-hydroxy-α-carboxymethyl cis-muconic semialdehyde. The enzyme is widely distributed in bacteria and represents a focal point in the degradation pathways of more complex aromatic compounds (1, 2). Following ring cleavage, the product is further metabolized to CO2 and the tricarboxylic acid cycle intermediates, succinate and pyruvate. The enzyme was first isolated from Pseudomonas ovalis and proposed to contain mononuclear Fe(II) (3–5). As such, it represents a typical Fe(II) type or extradiol catecholic dioxygenase. However, the family of 2,3-HPCD enzymes subsequently isolated from different types of bacteria has proven to be much more diverse than any other type of dioxygenase enzyme. For example, the enzymes isolated from Bacillus and recently from Arthrobacter were found to contain active site Mn(II) and represent the only examples of dioxygenases that utilize this metal (6, 7). Moreover, a unique Mg(II)-containing 2,3-HPCD has recently been reported (8).

The investigation of the mechanism of Fe(II) type dioxygenases in general has been slowed by a lack of convenient spectroscopic probes. Also, the enzymes are relatively unstable and readily lose activity during turnover or when exposed to chelating or oxidizing reagents. For example, the Fe(II) is easily oxidized upon exposure to H2O2, leading to inactivation of the enzyme. Such oxidation by H2O2 has not been observed for the Mn(II)-containing enzyme which has served as a way to differentiate the iron and manganese-containing systems (5–7).

In past studies, we have gained some structural and mechanistic insight into the Fe(II) dioxygenases through the use of NO binding as an analog for O2 binding. The nitrosyl complex exhibits an intense EPR signal with g values near g = 4 and 2 characteristic of an S = 3/2 spin system. The observation of superhyperfine interactions from both hydroxyl oxygens of 17O-labeled substrates bound to the nitrosyl adduct have demonstrated that the Fe(II) must have three coordination sites that can be occupied by exogenous ligands and that catecholic substrates chelate the iron when they bind to the enzyme (9–11). Recent CD/magnetic CD (12) and EXAFS (13, 14) studies of catechol 2,3-dioxygenase have shown that the iron is 5-coordinate both before and after substrate binding. The EXAFS studies indicate that upon binding NO the iron becomes 6-coordinate. Recently, the crystal structure of the Fe(II) ring cleaving dioxygenase 2,3-dihydroxybiphenyl 1,2-dioxygenase has been solved (15, 16). The structure also shows a 5-coordinate iron site with 2 His, 1 Glu, and 2 vicinal solvent ligands. The ligands to the iron determined from this structure are conserved in other Fe(II) dioxygenases, suggesting that this

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* This work was supported in part by National Institutes of Health Grant GM24689. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported in part by National Institutes of Health Training Grant GM07323.

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1 The abbreviations used are: 2,3-HPCD, 3,4-dihydroxyphenylacetate 2,3-dioxygenase; HPCA, 3,4-dihydroxyphenylacetate; pNC, p-nitrocatechol; ICPES, inductively coupled plasma emission spectroscopy; EXAFS, extended x-ray absorption fine structure; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
iron ligation is a general characteristic (7).

When considered together, these studies suggest that the substrate displaces two iron ligands as it binds, probably the bound solvents. NO (and presumably \( \text{O}_2 \)) occupies a different site that results in expansion of the coordination sphere as it binds. Based on these studies, we have proposed the mechanism illustrated in Scheme 1 in which both the catechol and dioxygen bind directly to the iron (1, 14, 17). The iron acts to facilitate redistribution of negative charge density from the aromatic substrate to the oxygen. Subsequent nucleophilic attack by the resulting bound superoxide on the Fe(II) for NO increases by 3 or more orders of magnitude (17). How-

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade or better and used without further purification unless otherwise stated. Water was deionized and glass distilled. Catechol 2,3-dioxygenase was purified as described previously (9).

Growth of Microorganism—Brevibacterium fuscum (ATCC 15993) was grown in medium containing p-hydroxyphenylacetate as the sole carbon source. The media contained (in g/liter) 3 (NH\(_4\))\(_2\)SO\(_4\), 1.6 K\(_2\)HPO\(_4\), 2.5 NaCl, 0.27 MgSO\(_4\)\(_7\)H\(_2\)O, 0.05 Fe(NH\(_3\))\(_2\)(SO\(_4\))\(_2\), 0.5 yeast extract, and 2 p-hydroxyphenylacetate, pH 7.0. Starter cultures of 100 ml and then 1 liter in this medium were grown for successive 24-h periods with vigorous shaking (200 rpm, 30 °C). This culture was then added to 15 liters of medium in a carboy and grown for 36 h with forced aeration, with feedings of 30 g of p-hydroxyphenylacetate in 100 ml of buffer at pH 7.0 after 15 and 30 h of growth. The cells were harvested by ultrafiltration and centrifugation, frozen on dry ice, and stored at −80 °C.

Purification of 2,3-HPCD—All purification steps were conducted at 4 °C unless stated otherwise. Buffer B is 50 mM MOPS, pH 7.0, 2 mM cysteine, and 100 \( \mu \text{M} \) Fe\(^{2+}\) (NH\(_3\))\(_2\)(SO\(_4\))\(_2\). 100 g of cell paste were resuspended in 200 ml of buffer B, 5 \( \mu \text{M} \) phenylmethylsulfonyl fluoride, and then approximately 1 mg each of deoxyribonuclease and ribonuclease were added. The cells were disrupted by sonication (25 min) while maintaining the temperature below 10 °C, and then the suspension was centrifuged 45 min at 39,800 \( \times \) g (4 °C) (standard centrifugation conditions). The resulting crude extract was precipitated with addition of chilled acetone to 45% v/v with continuous stirring. The slurry was immediately centrifuged, and then the supernatant was loaded onto a 120-ml fast-flow DEAE-Sepharose column (Pharmacia Biotech Inc.) equilibrated in degassed buffer B plus 50% aceton. The column was washed with 10 volumes of degassed buffer B, and 10 volumes of buffer B plus 0.2 M NaCl. A 600 \( \times \) 600 ml gradient of 0.2 M NaCl to 0.5 M NaCl was used to elute 2,3-HPCD. Active fractions of >15% peak activity were pooled and brought to a concentration 0.5 M (NH\(_4\))\(_2\)SO\(_4\). The pool was then loaded onto a 60-ml phenyl-Sepharose CL-4B column (Pharmacia Biotech Inc.) equilibrated in buffer B plus 0.5 M (NH\(_4\))\(_2\)SO\(_4\). The column was washed with 10 volumes of buffer B, 0.5 M (NH\(_4\))\(_2\)SO\(_4\), and the protein was eluted with 500 ml of buffer B, 0.2 M (NH\(_4\))\(_2\)SO\(_4\). Active fractions of >15% peak activity were pooled and concentrated by ultrafiltration. The concentrated protein was loaded onto a 50-ml Sepharose 550 column equilibrated in buffer B and eluted with buffer B. The active fractions of >10% peak activity were loaded onto a fast protein liquid chromatography 8-ml MonoQ column equilibrated in buffer B. The protein was eluted with a 80-ml total volume gradient of 0.0 to 0.8 M NaCl in buffer B. Active fractions of >10% peak activity were pooled and concentrated by ultrafiltration. The concentrated protein was frozen rapidly in liquid N\(_2\), and stored at −80 °C. The enzyme was stable for several months when stored in this manner.

Partial Purification of the Endogenous Heme Catalase—The purification of the endogenous heme catalase of B. fuscum followed the same steps of the 2,3-HPCD purification described above through the 120-ml fast-flow DEAE-Sepharose column. The active fractions of heme catalase, which eluted after 2,3-HPCD, from the DEAE column were pooled and brought to a concentration of 0.2 M (NH\(_4\))\(_2\)SO\(_4\). The pool was then loaded onto a 60-ml phenyl-Sepharose CL-4B column (Pharmacia Biotech Inc.) equilibrated in buffer B plus 0.2 M (NH\(_4\))\(_2\)SO\(_4\). The column was washed with 10 volumes of buffer B, 0.2 M (NH\(_4\))\(_2\)SO\(_4\), and the protein was eluted with 500 ml of buffer B, 0.2 M (NH\(_4\))\(_2\)SO\(_4\). Active fractions of >15% peak activity were pooled and concentrated by ultrafiltration. The heme catalase was characterized by UV-visible and EPR spectroscopy and steady state kinetics as described for 2,3-HPCD.

Protein Purity, Molecular Weight, and Composition—SDS-polyacrylamide gel electrophoresis (PAGE) was performed by standard techniques to determine subunit molecular weight and purity (18). 20 \( \mu \text{g} \) of protein was loaded in 25 \( \mu \text{l} \) of loading buffer (20% SDS, 200 mM dithiothreitol, 50 mM MOPS, pH 7.0, bromphenol blue) and heated to 100 °C for 5 min. Samples were briefly centrifuged before loading. Molecular weight was determined using Sigma Mark VII and Sigma high molecular weight standards as markers. Sedimentation equilibrium ultracentrifugation, nondenaturing PAGE, and two-dimen-
sional gel electrophoresis were conducted as described previously (19–21).

Metal Content and Amino Acid Analysis and Sequencing—Amino acid analysis of reduced and carboxymethylated protein was conducted

![Diagram](http://example.com/diagram.png)
Catalase Activity of Homoprotocatechuic 2,3-Dioxygenase

Purification of 2,3-HPCD from B. fuscum

| Purification step | Vol. (ml) | Activity (units/ml) | Protein (mg) | Specific activity (units/mg) | Yield | Purification factor |
|-------------------|----------|---------------------|--------------|----------------------------|-------|-------------------|
| Cell supernatant  | 146      | 5.5                 | 3,314        | 0.24                       | 100   | 1                 |
| 45% acetone supernatant | 210    | 4.2                 | 919          | 0.96                       | 110   | 4                 |
| DEAE pool        | 202      | 7.1                 | 339          | 4.22                       | 180   | 17                |
| Phenylnitrocellulose | 85     | 8.4                 | 79           | 9.03                       | 89    | 37                |

a 23 °C, air saturated buffer.

b On the basis of 97 g (wet weight) of cells.

c A fast protein liquid chromatography Mono-Q column is often utilized as the final step to remove a minor protein contaminant (λ_{max} ≈ 380 nm) but specific activity is not substantially improved by this step.

as described previously (20, 22) following hydrolysis of duplicate samples for 24 and 48 h, respectively (Microchemical Facility, University of Minnesota). Serine and threonine quantitations were extrapolated to time 0 to correct for loss during hydrolysis. Tryptophan was determined by derivative analysis of the UV spectrum using the tyrosine content determined by amino acid analysis as a reference (20, 23). Peptide sequencing was performed using protein electrophorased into a poly(vinylidene fluoride) membrane after SDS-PAGE as described previously (Microchemical Facility, University of Minnesota) (24). Metal content was determined by inductively coupled plasma emission spectroscopy (ICPES, Soil Sciences Center, University of Minnesota) using samples for which the protein content had been determined by quantitative amino acid analysis (20).

Enzyme Assays—Enzyme concentrations were determined by the method of Bradford (25) using bovine serum albumin as the standard or by the measure of A_{280} (ε = 0.9 cm⁻¹ mg⁻¹ at pH 7.0, determined using samples standardized by quantitative amino acid analysis). Dioxygenase activity was measured polargraphically using an O₂-sensitive electrode as described previously (26). Standard assay conditions were 50 mM MOPS, pH 7.5, 1 mM HPCA. Dioxygenase activity was also measured spectrophotometrically by following the rate of product formation (λ_{max} = 380 nm; ε_{380} = 36,000 cm⁻¹ M⁻¹ at pH 7.5) (4).

Catalase activity was measured by two methods. Standard assay conditions were 50 mM MOPS, pH 7.5, 10 mM H₂O₂. The first method was the colorimetric assay of Hildebrandt et al. (27). This method requires the accumulation of timed aliquots which complicates the determination of initial velocity. In the second method, polargraphic measurement using the O₂-sensitive electrode was used to measure O₂ generation. This was the most sensitive method for measuring catalase activity. The base-line H₂O₂ reaction at the electrode surface was measured and subtracted from the enzymatic O₂ generation rate. Rates in the nanomol/min range can be detected and quantitated easily; hence, most of the kinetic studies were completed using this assay method. The stoichiometry of H₂O₂ utilized for each O₂ generation was determined using O₂ polargraphy as described above and also by manometry.

Kinetic data was analyzed using a nonlinear regression program developed by Neil C. Millar (King's College, London, UK) which fit the data to a hyperbolic function. Unless stated otherwise, the double reciprocal plots shown here were constructed from the best fit parameters determined by the nonlinear analysis and do not represent linear regression fits to the reciprocal data.

2,3-HPCD Turnover Velocity, Stoichiometry, and Complex Formation—pNC turnover velocity was measured both polargraphically as described above and spectrophotometrically by following product formation at 330 nm (ε_{330} = 19,000 cm⁻¹ M⁻¹). The enzyme was found to lose activity rapidly during pNC turnover (see "Results"). The stoichiometry of pNC turnovers per enzyme active site before inactivation was determined by addition of aliquots of pNC to a known amount of enzyme until the initial velocity (ΔOD_{330}/s) was zero. Component analysis of the final optical spectrum was used to determine the stoichiometry of product formed per iron site based on the known spectra of the substrate and product. The stoichiometry was also measured polargraphically by monitoring the total utilization of O₂ for a given number of iron sites in the presence of pNC. An aerobic 2,3-HPCD-pNC complexes for optical studies were formed under argon in a serum stoppered vial. The complexes were transferred with a gastight syringe to an anaerobic cuvette. Component analysis using spectra of pNC under different pH, solvent, and buffer conditions with and without added FeII(S) was used to determine the relative amounts of mono and diamic and pNC bound in the enzyme active site.

Anaerobic Sample Preparation—Samples were made anaerobic by repeated cycles of evacuation and flushing with argon that had been passed over a column of BASF Inc. copper catalyst at 170 °C to remove residual oxygen. Samples initially containing H₂O₂ or O₂ were allowed to react to completion (5-10 min) before degassing.

EPR Measurements and Procedures—EPR spectra were recorded with a Varian E-109 X-band instrument equipped with an Oxford ESR-910 liquid helium cryostat. Data were digitally recorded and analyzed as previously reported (17, 28). Temperature and g value calibrations were as described previously (29, 30). Spin quantitations were performed by single or double integration of the first derivative spectra by the method of Aasa and Vännögård (28) as described previously using a 1 mm copper perchlorate standard (17, 29). EPR spectra of spin S = 3/2, and spin S/2 complexes were analyzed according to the spin Hamiltonian equations, respectively:

\[
\hat{H}_a = g_B \mu_B \hat{S} \cdot \hat{H} + D(S_z^2 - 5/4) + E(D(S_z^2 - S_z^2)) \\
\hat{H}_e = g_e \mu_e \hat{S} \cdot \hat{H} + D(S_z^2 - 3/12) + E(D(S_z^2 - S_z^2))
\]

where D and E/D are zero field-splitting parameters and the other parameters have their usual definitions. The value of g, is generally assumed to be 2.0, but small deviations from this value sometimes occur due to symmetry perturbations. The term E/D is a measure of the departure of the electronic environment of the iron from axial symmetry and can assume values between 0 and 1/3 (31, 32), the extreme values representing the axial and completely rhombic cases, respectively. The absolute value of E/D can be calculated from the g values and is used as a convenient method to compare spectra of samples with S > 1/2.

Preparation of Enzyme Nitric Oxide Complexes—NO was added by slowly bubbling NO gas, pretreated by passage over NaOH, through the sample under argon flow. The samples were flushed with argon after NO addition to remove excess NO from the head space. The samples were then transferred by a gastight syringe to an EPR tube under argon. The samples were frozen by slow immersion in liquid N₂. EPR samples which were later assayed for activity were thawed under argon. The samples were transferred by a gastight syringe to a serum stoppered vial under argon. NO was removed from the sample by repeated cycles of evacuation and flushing with argon as described above.

RESULTS

Purification and Characterization of 2,3-HPCD—2,3-HPCD was purified to apparent homogeneity by standard precipitation and chromatography techniques as described under "Experimental Procedures." The results of a typical purification procedure are shown in Table I. The structural and kinetic characteristics of the enzyme are summarized in Tables II and III. The purified enzyme migrated as a single band during electrophoresis under denaturing conditions, suggesting that the purification procedure results in a homogeneous protein (Fig. 1). A subunit molecular weight of 42,500 ± 1,500 was estimated from SDS-PAGE. A single N-terminal amino acid sequence was found for the protein in the band extracted from the SDS-PAGE gel (Table III), suggesting that there is only a single subunit type. An iron stoichiometry of 0.9 iron per 42,500 molecular weight was determined by metal analysis using ICPES (see Table II). A holoenzyme molecular mass of 220,000 ± 11,000 Da was determined using sedimentation equilibrium ultracentrifugation. The measured values for subunit and holoenzyme M, and the iron content predict a quaternary structure of α5Fe5; however, pentameric quaternary structures are...
Subunit structure
Subunit molecular mass (Da) 42,500 ± 1500

Molecular mass (Da) 220,000 ± 500
Iron content 0.9/42,500 Da

pH optimum 8.5

Dioxygenase activity
- Specific activity (units/mg) 14
- K_m catecholic substrate (μM) 15
- K_m O_2 (μM) 60
- kcat (s^-1) 10

Catalase activity
- kcat (s^-1) with saturated pNC 0.1
- K_m H_2O_2 (μM), with saturated pNC 0.3

Table II

Physical properties of B. fuscum 2,3-HPCD and P. putida 2,3-CTD

|                  | B. fuscum 2,3-HPCD (ATCC 15933) | P. putida 2,3-CTD (ATCC 23973) |
|------------------|----------------------------------|---------------------------------|
| Subunit structure|                                  |                                 |
| Subunit molecular mass (Da) | 42,500 ± 1500 | 35,155 ± 200 |
| Molecular mass (Da) | 220,000 ± 500 | 140,620 ± 1500 |
| Iron content | 0.9/42,500 Da | 1/subunit |
| pH optimum | 8.5 | | |

Dioxygenase activity
- Specific activity (units/mg) 14
- K_m catecholic substrate (μM) 15
- K_m O_2 (μM) 60
- kcat (s^-1) 10

Catalase activity
- kcat (s^-1), with saturated pNC 0.1
- K_m H_2O_2 (μM), with saturated pNC 0.3

Table III

Amino acid composition of B. fuscum 2,3-HPCD

| Amino acid | Yield | Subunit | Total enzyme |
|-----------|-------|---------|--------------|
| Ala       | 1.54  | 27      | 108          |
| Arg       | 1.49  | 26      | 104          |
| Asx       | 2.78  | 48.5    | 194          |
| CM-Cys    | 0.21  | 3       | 12           |
| Glx       | 2.27  | 39.5    | 158          |
| Gly       | 1.85  | 32      | 128          |
| His       | 0.89  | 15.5    | 62           |
| Ile       | 1.02  | 18      | 72           |
| Leu       | 1.72  | 30      | 120          |
| Lys       | 0.88  | 15      | 60           |
| Met       | 0.22  | 4       | 16           |
| Phe       | 0.92  | 16      | 64           |
| Pro       | 0.98  | 17      | 68           |
| Ser       | 0.76  | 13      | 52           |
| Thr       | 1.55  | 20      | 80           |
| Trp       | 0.98  | 17      | 68           |
| Tyr       | 1.57  | 27.5    | 110          |
| Val       | 1.72  | 30      | 120          |
| Total residues | 374   | 1,496   |
| Total M_r | 41,900| 167,000 |

a Rounded to the nearest half residue. Based on the assumption that Met and CM-Cys are recovered in 100% yield.
b Trp was estimated by derivative analysis of the UV spectrum using tyrosine content determined by amino acid analysis as a reference.
c Estimated from SDS-PAGE.
d Preliminary results from sequencing studies of the cloned gene show a M_r of 41370 (Y.-Z. Wang and J. D. Lipscomb, unpublished results).
e See Ref. 33.
f See Ref. 34.
g Determined by sedimentation equilibrium ultracentrifugation.
h Reaction conditions for kinetic parameters were 50 mM MOPS buffer, pH 7.5 at 23°C.
i In the absence of catecholic substrates, the kcat of H_2O_2 could not be determined due to the complexity of the reaction (see text).

Table IV

Enzymatic properties of 2,3-HPCD and 2,3-CTD

| Subunit molecular mass (Da) | B. fuscum 2,3-HPCD (ATCC 15933) | P. putida 2,3-CTD (ATCC 23973) |
|----------------------------|----------------------------------|---------------------------------|
| 42,500 ± 1500              |                                  |                                 |
| 220,000 ± 500               |                                  |                                 |

Fe(II) dioxygenases, it is not rapidly inactivated upon exposure to H_2O_2. This resistance to H_2O_2 inactivation is also observed for the Mn(II) containing 2,3-HPCD and has been used in the past to differentiate Fe(II) and Mn(II) containing enzymes in general (5, 6, 39, 40).

One interpretation of these results is that the iron contained in 2,3-HPCD is adventitious, and the activity is actually associated with the another metal bound to the enzyme. Indeed, metal analysis does reveal the presence of a small amount of manganese (−0.04/subunit). However, the fact that iron is present in approximately the same concentration as the sub-
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Catalase Activity of Homoprotocatechuate 2,3-Dioxygenase

The initial velocity of the dioxygenase activity was measured polarographically as described under "Experimental Procedures." Incubation conditions were 50 mM aerobic MOPS buffer, pH 7.0, 10 μM 2,3-HPCD at 4°C with the different reagents at the concentrations listed below. The reaction conditions were 50 mM aerobic MOPS buffer, pH 7.0, 600 μM HPCA and 0.07 μM 2,3-HPCD at 23°C after 5 min of incubation with the different reagents.

| Table IV | Effects of metal chelators or oxidizing or reducing agents on enzymatic activity |
|----------|--------------------------------------------------------------------------------|
| Reagent  | % Activity | Fe(II) 2,3-HPCD | Mn(II) 2,3-HPCD | Fe(II) 2,3-PCD |
| None     | 100        | 100            | 100             |
| H2O2a    | 97         | 98             | 0               |
| K3Fe(CN)6b | 65       | 95             | 0               |
| Ascorbatec | 102      | 105            | 110             |
| o-Bathophenanthroleld | 97               | Inhibited      | 0               |
| EDTAHf   | 100        |                | 0               |

a See Ref. 6.
b See Ref. 38.
c See Ref. 17.
d The following concentrations of H2O2 were used: Fe(II) 2,3-HPCD, 2 mM; Mn(II) 2,3-HPCD, 10 mM; Fe(II) 2,3-PCD, 1 mM.
e The following concentrations of K3Fe(CN)6 were used: Fe(II) 2,3-HPCD, 10 mM; Mn(II) 2,3-HPCD, 5 mM; Fe(II) 2,3-PCD, 1 mM.
f The following concentrations of ascorbate were used: Fe(II) 2,3-HPCD, 8 mM; Mn(II) 2,3-HPCD, 5 mM; Fe(II) 2,3-PCD, 1 mM.
g The following concentrations of o-bathophenanthroline were used: Fe(II) 2,3-HPCD, 1 mM; Mn(II) 2,3-HPCD, 5 mM; Fe(II) 2,3-PCD, 1 mM.
h The following concentrations of EDTA were used: Fe(II) 2,3-HPCD, 1 mM; Fe(II) 2,3-PCD, 8 mM.

units, whereas manganese is present at great stoichiometric amounts, strongly suggests that iron is the active site metal. Moreover, dialysis or gel filtration results in a slow loss of Fe(II) and activity, whereas there is no such correlation with the Mn(II) content (data not shown). Results from spectroscopic investigations also suggest that iron is the active site metal as shown below.

Nitrosyl Complexes of 2,3-HPCD—The enzyme as purified has a small EPR signal at g = 4.3 ( ~ 0.05 spins per subunit) which arises from the middle Kramers doublet of an S = 5/2 spin system (E/D ~ 0.3), probably attributable to adventitiously bound Fe(III) (Fig. 2, insets). When the O2 analog nitric oxide is added to the anaerobic enzyme, an intense EPR spectrum from an S = 3/2 spin system (g = 4.04, 3.96, 2.0; E/D = 0.005; D > 10 cm−1) is observed (Fig. 2A). Our past studies show that this signal arises from the complex of Fe(II) in the active site with NO (17, 19, 29, 41). Formation of the 2,3-HPCD-HPCA-NO complex results in a large change of the EPR spectrum (Fig. 2B) in which a new majority S = 3/2 species (g = 4.15, 3.85, 1.99; E/D = 0.025, D > 10 cm−1) is observed. In all other dioxygenase enzymes that we have studied, a change of this magnitude has been associated with direct ligation of one or both substrate hydroxyl groups to the iron at a different ligand position than that of NO (17, 29, 35, 42). A minority species with an EPR spectrum identical to that of the 2,3-HPCD-NO complex is also observed. However, we believe that this spectrum has a different origin for reasons that will be discussed below.

Quantitation of the EPR signal from the 2,3-HPCD-NO complex with and without substrate indicates that the species represents approximately 0.9 spin/subunit, which correlates well with the iron/subunit quantitation. Thus, the EPR signal that is perturbed by substrate originates from the Fe(II)-NO complex, rather than any metal present in trace amounts, strongly supporting the proposal that the required active site metal is iron.

Catalase Activity—Stability against oxidation by H2O2 is unprecedented for an Fe(II) dioxygenase enzyme, so the effects of H2O2 were probed further. It was observed that the addition of H2O2 to the enzyme resulted in vigorous evolution of gas, suggesting that the enzyme was acting as a catalase to generate O2 and water from H2O2. For a comparison with 2,3-HPCD, catechol 2,3-dioxygenase was incubated with 1 mM H2O2. The enzyme was inactivated in <1 min, and no catalase activity was observed. A colorimetric assay (27) was used to demonstrate that H2O2 was catalytically degraded during incubation with 2,3-HPCD (Fig. 3). Both manometric and polarimetric measurements of O2 production showed that it was generated in the expected 1:2 ratio relative to the H2O2 utilized. The initial velocity of O2 generation was found to be linearly dependent on the amount of enzyme in the reaction mixture (Fig. 3, inset), showing that the catalytic agent is either the enzyme itself or something added with the enzyme.

Both the retention of dioxygenase activity and the production
HPCA and H2O2 to the enzyme solution under initially anaerobic conditions. The usual yellow aromatic ring cleavage product resulting from O2 insertion was formed at a rate which was constant for at least 4 min. The stability of the iron site to H2O2 was also demonstrated by the observation that the enzyme incubated with 10 mM H2O2 for 10 min showed only a slight decrease in the S = 3/2 spectrum following degassing and exposure to NO (compare Fig. 2, A and C).

In addition to reactions involving the active site Fe(II), there are other possible origins of the catalase activity associated with 2,3-HPCD. For example, because the enzyme is purified in the presence of low concentrations of exogenously added Fe(II) and cysteine, adventitious metal clusters bound to the protein surface or a metal complex free in solution might catalyze the degradation of H2O2 via Fenton or a related chemistry. If this were the case, however, scavengers for activated oxygen species should inhibit the chemistry, which is not observed (Table V). Also, heat-denatured 2,3-HPCD had no catalase activity. Another possible source of the catalase activity is the endogenous catalase of B. fuscum. To investigate this possibility, we isolated and partially characterized this catalase. The enzyme contains an easily detectable heme cofactor, and it is readily separated from 2,3-HPCD by the procedures described under "Experimental Procedures." The EPR and optical spectra of 2,3-HPCD show no evidence for a heme enzyme in the concentration necessary to account for the observed catalase activity of 2,3-HPCD.

It is also possible to distinguish between the endogenous catalase and 2,3-HPCD using steady state kinetics. For the endogenous catalase, the double reciprocal plot of the initial velocity as a function of H2O2 is linear as expected for an enzyme with a ping pong mechanism (Fig. 4). In marked contrast, the double reciprocal plot for 2,3-HPCD catalase activity is parabolic ($k_{cat} = 0.1 \text{s}^{-1}$, Table I), indicating that two enzymes disproportionate H2O2 by distinct mechanisms (see "Discussion"). Taken together, these results strongly suggest that active 2,3-HPCD is necessary for catalase activity and that any activated oxygen intermediates that might be formed in the catalase reaction are contained within the enzyme active site.

Common Origin of the Catalase and Dioxygenase Activities—If the catalase and dioxygenase activities originate from the same active site, then it is likely that each would be affected by the substrate of the other. The mixed type H2O2 inhibition of dioxygenase activity is illustrated in Fig. 5. K0 values of 130 $\mu M$ and 470 $\mu M$ ($\alpha = 3.6$) were determined for H2O2 binding to free and substrate saturated enzyme, respectively (Fig. 5, inset) (43).

The effect of HPCA on catalase activity is more difficult to assess because O2 produced by the catalase reaction can be immediately utilized by the dioxygenase reaction. Under the reaction conditions described in Table VI, a rate of O2 production of 3.0 units/ml from H2O2 turnover was observed in the absence of HPCA. Addition of HPCA to the catalase reaction resulted in a rate of O2 consumption of 5.2 units/ml. The dioxygenase activity in the presence of H2O2 was directly measured to be 13 units/ml (representing an 86% inhibition) by optically monitoring the rate of formation of yellow product. The rate of dioxygenase product formation is equivalent to the rate of O2 utilization by the dioxygenase reaction. If the presence of HPCA had no effect on the catalase reaction, then the measured rate of O2 utilization with both HPCA and H2O2...
present should at least equal the rate of O₂ utilization measured optically minus the rate of O₂ utilization in the absence of HPCA (calculated to be 10 units/ml). As shown in Table VI, the rate of O₂ utilization was considerably lower (5.2 units/ml). Thus, the rate of O₂ production must have increased to 8 units/ml, nearly a 3-fold activation of the catalase activity in the presence of HPCA.

Effect of p-Nitrophenol on Dioxygenase and Catalase Activities—The effect on catalase activity of the binding of catecholic molecules to the active site iron can be evaluated in more detail using the competitive inhibitor pNC (Kᵢ = 14 μM). pNC is also a substrate for the enzyme (see below), but it is turned over slowly in comparison to HPCA, and therefore, the O₂ uptake reaction is negligible compared to the O₂-evolving catalase reaction. The EPR spectrum of 2,3-HPCD-pNC-NO complex (Fig. 6A) shows that an S = 3/2 species with E / D = 0.03 similar to that of the 2,3-HPCD-HPCA-NO complex is formed. The EPR spectrum was observed to change to that of the 2,3-HPCD-HPCA-NO complex when a saturating amount of HPCA was added (Fig. 6B), suggesting that the substrate displaces pNC and probably binds to the same site on the Fe(II).³ The quantitation of the characteristic S = 3/2 spectra shown in Fig. 6A and B, indicates that the total reducing equivalents of the enzyme are not changed when the complex with pNC is formed anaerobically in the absence of H₂O₂.

As shown in Fig. 7, pNC is a saturable activator of the catalase activity. Note that in the presence of pNC, the double reciprocal plot of initial velocity as a function of H₂O₂ appears linear rather than parabolic (Fig. 4, inset). The kinetic parameters are also changed (Kₑ = 0.3 ± 1; Kᵢ = 580 μM, Table II). This suggests that pNC causes the enzyme to utilize a different mechanism for the disproportionation of H₂O₂ (see "Discussion"). These results clearly show that catecholic substrate and inhibitor molecules activate catalase activity, thereby strongly supporting the conclusions that: (i) 2,3-HPCD is responsible for the catalase activity and (ii) the catecholic molecule and the H₂O₂ can gain simultaneous access to the active site iron. Similar activation was not observed for monodentate substrate analogs such as p- or m-hydroxyphenylacetate but was observed for other substrates such as 4-sulfonyl-catechol, protocatechuate, and catechol.

Binding and Turnover of pNC—At neutral pH in aqueous solution, only one of the hydroxyl groups of pNC is ionized, giving rise to a characteristic optical spectrum maximizing at 426 nm. This spectrum is not altered substantially when pNC binds to most Fe(II) dioxygenases such as catechol 2,3-dioxygenase under anaerobic conditions, showing that the pNC binds as a monoanion to these enzymes. In contrast, when an anaerobic 2,3-HPCD-pNC complex is formed, the optical spectrum of pNC is markedly perturbed (Fig. 8). Component analysis of the spectrum shows that pNC binds to the enzyme in both the dianion (maxima of 410 and 510 nm, −½ of the total) and the monoanion (−½ of the total) forms. The monoanion component has spectroscopic features which are slightly different than the monoanionic pNC in water but very similar to the monoanionic form in 50% ethanol solution with added Fe(II). This is consistent with the direct binding of the monoanionic form of pNC to the Fe(II) in the active site.

Upon exposure to O₂, the 2,3-HPCD-pNC complex was observed to turn over to yield the ring cleavage product of pNC with peaks at 330 and 390 nm (specific activity = 0.11 unit/mg, −1% of that with HPCA) (Fig. 8, inset). This is the expected result for the case in which cleavage occurs between the 2 and 3 ring carbons (44). Interestingly, pNC turnover rapidly inactivates the enzyme for further ring cleavage of pNC. This inactivation occurs much more rapidly than the usual slow turnover of H₂O₂.

³ Note that the small EPR signal with an E/D of 0.005 intensifies upon the formation of the 2,3-HPCD-HPCA-NO complex with concomitant displacement of pNC. This demonstrates that the signal with an E/D of 0.005 results also from a 2,3-HPCD-HPCA-NO complex and not the substrate free 2,3-HPCD-NO complex.

Table VI

| Substrates present | Net rate of O₂ consumption or production | Rate of ring cleavage reaction |
|--------------------|----------------------------------------|-------------------------------|
| HPCA               | 93.3 ± 3.1                             | Consumed                      |
| H₂O₂               | 3.0 ± 0.3                              | Produced                      |
| HPCA and H₂O₂      | 5.2 ± 0.2                              | Consumed                      |
| HPCA and H₂O₂ (calculated) | 10⁻       | Consumed                      |

a Not applicable.
b Represents 86% inhibition of HPCA ring cleavage activity by H₂O₂.
c Calculated 2.7 fold activation, see text.
inactivation often observed during normal substrate turnover by Fe(II) dioxygenases (1, 17, 29). As shown in Fig. 8, inactivation required approximately two turnovers per Fe(II) site. It is interesting to note, however, that the enzyme which is inactive for pNC turnover is only partially inactivated for ring cleavage of HPCA and retains considerable catalase activity (Table VII). Moreover, the pNC activation of the catalase activity is not completely eliminated.

Oxidation of the Active Site Iron During pNC Turnover—When pNC was added to the enzyme and bubbled 10 min with 100% O2 before degassing and adding NO, no S = 3/2 EPR spectrum was observed, suggesting that the iron was oxidized to Fe(III) (Fig. 6C). Accordingly, the Fe(III) signal from the middle Kramers doublet of the 5/2 spin system became much larger relative to that observed for 2,3-HPCD alone. However, when HPCA was added anaerobically to the oxidized 2,3-HPCD-pNC complex followed by NO addition, an EPR signal characteristic of Fe(II) 2,3-HPCD-HPCA-NO and a radical signal at g = 2 were observed (Fig. 6D and inset). Although there are many possible origins of the radical, its appearance upon addition of HPCA is consistent with single electron transfer from HPCA to the Fe(III) to yield an HPCA radical and Fe(II).

This suggests that HPCA is capable of rereducing at least a portion of the active site iron, which would account for the observed HPCA turnover after complete iron oxidation by pNC.

Because pNC both oxidizes the enzyme during turnover and activates the catalase reaction, it is possible that the oxidized form of the enzyme is solely responsible for catalase activity. This is not the case, however, because the oxidation is a time-dependent process, whereas the highest activation is observed in the initial velocity of kinetic experiments immediately after mixing the enzyme, pNC, and H2O2. Nevertheless, aerobic preincubation with pNC does result in a decrease in both the absolute catalase activity and the extent of activation by pNC (Table VII and Fig. 7), thus the redox state of the iron does affect the reaction. This suggests two possibilities: (i) in the presence of a catechol, both Fe(II) and Fe(III) are capable of catalase activity (Fe(III) has a lower rate of turnover), or (ii) H2O2 is able to rereduce the Fe(III) when pNC is bound. Studies have been initiated to resolve these possibilities.

**DISCUSSION**

In this study, we have shown that 2,3-HPCD isolated from the Gram-positive bacterium B. fuscum shares many properties with the other well characterized members of the extradiol catecholic dioxygenase class. Notably, the enzyme utilizes mononuclear Fe(II) as the essential active site metal, and it allows binding of substrates and O2 analogs to this metal. Nevertheless, B. fuscum 2,3-HPCD has several unique characteristics, including a turnover number 10-30-fold lower than other Fe(II)-dependent dioxygenases, the ability to bind at least one diagnostic substrate as a dianion, and the presence of
catalase activity. These characteristics allow a new examination of the current proposal for the O₂ activation and insertion mechanism of Fe(II) dioxygenases which is discussed here.

Catalase Activity—Although the catalase activity of 2,3-HPCD is unprecedented for a non-heme mononuclear Fe(II) enzyme, it is a commonly encountered reaction for heme containing enzymes and proteins including the large family of prototypical catalases (45, 46), as well as for catalases containing a dimanganese cluster (47) and many nonphysiological metal chelates (47–50). We believe that none of these types of metal containing systems is the source of the observed 2,3-HPCD catalase activity for the following reasons: (i) the catalase activity requires nonadenatured, holo-2,3-HPCD; (ii) the activity depends linearly on the amount of enzyme present; (iii) H₂O₂ affects dioxygenase activity and HPCA affects catalase activity, both in a saturating manner; (iv) added iron and a variety of activated oxygen scavengers do not affect either dioxygenase or catalase activity; and (v) neither the steady state kinetics of the catalase activity nor the spectroscopic features of the 2,3-HPCD preparation are consistent with heme or dimanganese cluster containing catalases. Moreover, the unique stability of 2,3-HPCD in the presence of H₂O₂ implies that it must have developed a method to deal with this strong oxidant. The presence of endogenous catalase activity is the most reasonable solution to this problem, although controlled access of H₂O₂ to the active site presents a possible alternative. However, this alternative is unlikely because a range of substrates, inhibitors, and small molecules rapidly enter the active site and bind to the iron. Also, the crystal structure of the similar 2,3-dihydroxybiphenyl 1,2-dioxygenase shows no evidence for a barrier to the active site (15, 16). Considered together, these results make it very likely that 2,3-HPCD does have catalase activity.

General Considerations of the Catalase Reaction—In the case of heme catalases, the reaction proceeds through a high valent intermediate, considered to be an oxo-Fe(IV) heme cation radical, generated as a result of the reaction of H₂O₂ with the Fe(III) state of the heme to yield water as a by-product. Then, a second H₂O₂ molecule reduces this high valent intermediate to give a second molecule of water and O₂. Some nonheme metal chelate systems are thought to catalyze reactions with catalase stoichiometry by a variation of a Fenton chemistry. In Fenton chemistry as originally envisioned, H₂O₂ reacts with Fe(II), forming OH•, water, and Fe(III). The freely dissociable hydroxyl radical reacts with a second H₂O₂, forming water and O₂. Finally the O₂ reduces the iron, forming Fe(II) to start another cycle (51). It is possible, that at least in some systems, an oxo-Fe(IV) species is formed rather than a diffusible OH• (50–52). Note that the Fenton system invokes 1 e⁻ rather than 2 e⁻ transfer reactions as proposed for the heme and manganese-containing catalases. The 2,3-HPCD catalase activity described here is most active when the iron is reduced, thus it is unlikely that a species equivalent to the heme oxo-Fe(IV) cation radical is formed because this would require a 3 e⁻ oxidation. One e⁻ transfer, Fenton-like, chemistry in the active site must be considered; however, the active site iron coordination, the nature of the steady state kinetics observed, and parallels that can be drawn with the dioxygenase reaction mechanism suggest an alternative possibility.

A Proposal for the Mechanism of 2,3-HPCD Catalase Activity—Scheme 2 shows one possible mechanism for catalase activity that can be compared with the proposal for dioxygenase activity shown in Scheme 1 (see the Introduction). The parabolic double reciprocal plot of 2,3-HPCD catalase activity (Fig. 8).

![Image](56x373 to 300x554)

**TABLE VII**

| Treatment of enzyme | pNC in assay | % Remaining | % Iron as Fe(II)¹ |
|---------------------|-------------|-------------|-----------------|
|                     |             | Dioxygenase activity | Catalase activity |
| None                | No          | 100         | 100             |
| None                | Yes         | 100.4       | 278.7           |
| Anaerobic preincubation with pNC | Yes | 91.8       | 183.7           |
| Anaerobic preincubation with pNC + 100% O₂ | Yes | 29.6       | 178.6           |

¹The percent Fe⁺⁺ remaining was estimated from the double integration of the S = 3/2 EPR signal of the Fe⁺⁺ 2,3-HPCD · NO complex and substantiated by the change in the height of the 4.3 EPR signal of the middle doublet of the S=5/2 spin system. The error is approximately 10%.

²pNC was included in the reaction mixture at the concentration which results from pNC carryover in the pNC preincubation experiment.

³Addition of HPCA and NO to degassed enzyme after complete oxidation of the iron by pNC and O₂ results in the return of the S = 3/2 signal of the Fe(II) · NO complex (see Fig. 7).
Scheme 2. Proposal for the mechanism of catalase activity of 2,3-HPCD. The kinetic data suggests that two $\text{H}_2\text{O}_2$ molecules are present in the active site before the reaction occurs. The placement of these molecules in the scheme show is hypothetical (see text).

Scheme 3. Proposal for the mechanism of catalase activity of 2,3-HPCD when catecholic substrates and inhibitors are present. The Fe(III)-O$^-$ species is shown to illustrate the number of reducing equivalents present. There is no direct evidence for this species.
to the oxo-iron species would then yield the second H$_2$O, O$_2$, and restore the Fe(II) catecholic substrate complex. This mechanism would give the observed linear 1/$vi$ versus 1[H$_2$O$_2$] plot because the two H$_2$O$_2$ bind on different sides of a chemical step.

This mechanism suggests that the rate of the catalase reaction will increase as the catecholic substrate becomes more electron donating. Indeed, HPCA, which binds as an anion (perhaps a dianion) to the iron and has an electron donating substituent, is a potent activator of the catalase activity. In contrast, pNC with an electron withdrawing substituent, would not be expected to as effectively transfer charge to facilitate either O$_2$ activation in the dioxygenase mechanism or O–O bond cleavage in the catalase reaction. Accordingly, pNC was shown here to be a very slow substrate and a less effective activator of the catalase reaction than HPCA.

Inhibition of the Dioxygenase Reaction by H$_2$O$_2$—We show here that H$_2$O$_2$ acts as a mixed type inhibitor of dioxygenase activity with respect to HPCA. This is the expected result under the models proposed in Schemes 1 and 3 because H$_2$O$_2$ would bind in the O$_2$ binding site, a site required for dioxygenase activity but noncompetitive with respect to HPCA. Scheme 2 implies that H$_2$O$_2$ should also compete directly with HPCA binding. However, given the high binding affinity of the enzyme for HPCA, H$_2$O$_2$ would be a very poor competitive inhibitor and would act primarily as a noncompetitive inhibitor relative to the catecholic substrate. A “pure” noncompetitive inhibitor which binds equally to the free and substrate bound forms of the enzyme would give a family of double reciprocal plots that intercept on the x axis. However, this is not expected to be the case if the charge donation to the iron from the bound catecholic ligand changes the affinity of the iron for H$_2$O$_2$ as suggested by the mechanisms shown in Schemes 1 and 3. The observed intercept near the y axis shows that H$_2$O$_2$ binds more tightly to the substrate free form of the enzyme ($\alpha > 1$). This is in accord with our previous studies which indicate that the binding of catecholic substrate greatly increases the affinity of the enzyme for small electrophilic molecules that bind in the O$_2$ binding site (9,17). Conversely, the more nucleophilic H$_2$O$_2$ molecule (with respect to binding) might be expected to bind somewhat less well to the enzyme substrate complex than to free enzyme. The proposed mechanism predicts that competitive inhibition between H$_2$O$_2$ and O$_2$ should be observed, but the production of O$_2$ during the catalase reaction has prevented us from testing this directly.

Enzyme Inactivation during Turnover by Substrates and pNC—During turnover of substrates, 2,3-HPCD is slowly inactivated as observed for all other Fe(II) type dioxygenases. This inactivation has been difficult to approach experimentally because it appears to occur through a number of mechanisms including reversible oxidation of the iron and irreversible alteration of the enzyme that prevents rereduction of the iron. The inactivation by pNC is different because it occurs much more rapidly, within approximately two turnovers, to quantitatively yield Fe(III) in the active site. In the context of Scheme 1, the most obvious route to inactivation during turnover is loss of O$_2$ from the enzyme-substrate-O$_2$ complex to leave inactive Fe(III) in the active site. The O$_2$ might escape the active site to yield an enzyme that could be reactivated by reduction or it might react directly or indirectly with the enzyme to permanently inactivate it. The low turnover number of pNC suggests that it does not react rapidly with the iron-bound superoxide intermediate proposed in Scheme 1. Therefore, dissociation of O$_2$ becomes a kinetically competitive process. We have previously observed O$_2$ release from protoconate 2,3-dioxygenase and oxidation of the active site iron when slow substrates or tight binding inhibitors are bound (1,53). This may also be the case for the slow pNC turnover by 2,3-HPCD, but no O$_2$ has yet been detected, perhaps indicative of a reaction with the enzyme.

Significance of Perturbations in the pNC Spectrum—Analysis of the 2,3-HPCD-pNC complex indicates that pNC binds as a mixture of monoanion and dianion forms. For all other known pNC complexes of Fe(II) containing dioxygenases, only monoanionic binding is observed. These enzymes also have much higher turnover numbers and are much more resistant to inactivation during pNC turnover. In the case of one such enzyme, catechol 2,3-dioxygenase, we have shown that the normal substrate binding mode is the chelated monoanion, resulting in one weak and one strong O–Fe bond (14). This serves to increase the electrophilicity of the ring relative to the dianion form and to direct the iron-bound superoxide attack to the position of highest positive charge, adjacent to the strongly bound oxygen, thereby determining the site of ring cleavage. The electrophilicity of the ring is also increased by transfer of charge density from the ring onto the bound O$_2$, which in turn, increases the nucleophility of the bound superoxide for attack on the ring. If all substrates bind like pNC to 2,3-HPCD, then the significantly decreased electrophilicity of the ring might contribute to the much lower observed turnover number of this enzyme.

It is curious that 2,3-HPCD is unique among Fe(II) dioxygenases in exhibiting catalase activity because the general active site characteristics of this class seem to be highly conserved on the basis of amino acid sequence comparisons (7). In the context of Schemes 2 and 3, it is possible that the catalase activity depends upon the electron donation of the group binding in the substrate binding site. When this binding is asymmetric due to chelation of a monoanion, the usual case for Fe(II) dioxygenases, there may be insufficient charge donation to allow heterolytic O–O bond cleavage of peroxide. As a result the Fe(II)H$_2$O$_2$ may simply undergo the first step in Fenton chemistry by homolytic cleavage to yield OH· and Fe(III), thereby inactivating the enzyme. This proposal is analogous to the “push-pull” mechanism cited for heme peroxidases in which heterolytic cleavage is promoted by a strong electron donating trans heme ligand and by the presence of a strong base near the distal peroxide oxygen (54,55). Accordingly, 2,3-HPCD may also differ from dioxygenases lacking catalase activity by the fortuitous presence of a base in the active site pocket near the H$_2$O$_2$ binding site. This would promote ionic rather than the radical chemistry in the peroxide bond breaking step, thereby preventing the formation of potentially damaging radical species.

Conclusion—The most important structural and electronic features of our model for the Fe(II) dioxygenase mechanism are the presence of distinct sites on the iron for substrate and O$_2$ binding, the ability of the iron to facilitate electron transfer between these two substrates, and asymmetry of the substrate chelation which promotes rapid reaction and specificity of ring cleavage. We believe that the first two elements also facilitate catalase activity by this enzyme. The last element might generally inhibit catalase activity in most Fe(II) dioxygenases, but not in 2,3-HPCD, where symmetric binding appears to be allowed. This means that the enzyme is kinetically a poor dioxygenase but a competent catalase. In previous studies of chemical model systems which activate peroxide for either
dismutation to $O_2$ and $H_2O$ (52) or for oxidation of phenols (50), a similar role for iron in using two or more coordination sites and facilitating electron transfer between substrates has been invoked. The 2,3-HPDC catalase described here appears to be an excellent system for investigation of this interesting and important chemistry.

Acknowledgments—We thank Dr. Jeffrey T. Bolin for valuable discussions of Fe(II) dioxygenase active site structure prior to publication. We also thank Drs. Lawrence Que, Jr., and Lawrence P. Wackett for useful discussions.

REFERENCES

1. Lipscomb, J. D., and Orville, A. M. (1992) Metal Ions Biol. Systems 28, 243–298
2. Chapman, P. (1972) in Degradation of Synthetic Organic Molecules in the Biosphere, Proceedings of a Conference, pp. 17–55, National Academy of Science, Washington, D.C.
3. Adachi, K., and Takeda, Y. (1964) Biochim. Biophys. Acta 39, 483–493
4. Kita, H. (1965) J. Biochem. (Tokyo) 58, 116–122
5. Ono-Kamimoto, M. (1973) J. Biochem. (Tokyo) 74, 1049–1059
6. Que, L., Jr., Widom, J., and Crawford, R. L. (1981) J. Biol. Chem. 256, 10941–10944
7. Boldt, Y. R., Sadowsky, M. J., Ellis, L. B. M., Que, L., Jr., and Wackett, L. P. (1995) J. Biological Chem. 270, 1225–1232
8. Glibelo, A., Ferrer, E., Martin, M., and Garrido-Pertierra, A. (1994) Biochem. J. 301, 145–150
9. Arédo, D. M., Orville, A. M., and Lipscomb, J. D. (1985) J. Biol. Chem. 260, 14035–14044
10. Arédo, D. M., and Lipscomb, J. D. (1986) J. Biol. Chem. 261, 2170–2178
11. Harpel, M. R., and Lipscomb, J. D. (1990) J. Biol. Chem. 265, 22187–22196
12. Matrouk, P. A., Orville, A. M., Lipscomb, J. D., and Solomon, E. I. (1991) J. Am. Chem. Soc. 113, 4033–4036
13. Bertini, I., Briganti, F., Mangani, S., Nötting, H. F., and Scozzafava, A. (1994) Biochemistry 33, 10777–10784
14. Shu, L., Choi, Y.-M., Orville, A. M., Miller, M. A., Lipscomb, J. D., and Que, L., Jr. (1995) Biochemistry 34, 6649–6659
15. Sugiyama, K., Senda, T., Narita, H., Yamamoto, T., Kimbara, K., Fukuda, M., Yano, K., and Mitsui, Y. (1995) Proc. Jpn. Acad. 71, 22–35
16. Bolin, J. T., Han, S., and Eltsi, L. D. (1995) Science 270, 976–980
17. Wodzel, S. A., Dege, J. E., Perkins-Olson, P. E., Jarez-Garcia, C. H., Crawford, R. L., Münck, E., and Lipscomb, J. D. (1993) J. Bacteriol. 175, 4414–4426
18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Fox, B. G., Fröland, W. A., Dege, J., and Lipscomb, J. D. (1989) J. Biol. Chem. 264, 10023–10033
20. Jollie, D. R., and Lipscomb, J. D. (1993) J. Biol. Chem. 268, 21853–21863
21. Hedrick, J. L., and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155–164
22. Lundell, D. J., and Howard, J. B. (1978) J. Biol. Chem. 253, 3422–3426
23. Ragone, R., Colonna, G., Balestriere, C., Servillo, L., and Irace, G. (1984) Biochemistry 23, 1871–1875
24. Matsuda, H., and Nakagawa, H. (1987) J. Biol. Chem. 262, 10035–10038
25. Bradford, M. M. (1976) Anal. Biochem. 265, 248–254
26. Whittaker, J. W., Orville, A. M., and Lipscomb, J. D. (1990) Methods Enzymol. 188, 82–88
27. Kojima, Y., Itada, N., and Hayaishi, O. (1961) J. Biol. Chem. 236, 1039–1049
28. Walling, C. (1975) Accounts of Chemical Research 8, 125–131
29. Hamilton, G. A., and Friedman, J. P. (1963) J. Am. Chem. Soc. 85, 1008–1009
30. Tanaka, K., and Makino, R. (1983) J. Magn. Reson. 53, 374–379
31. Blumberg, W. E. (1967) in Magnetic Resonance in Biological Systems (Ehrenberg, A., Malmström, B. G., and Vännärg, T., eds) pp. 119–133, Pergamon Press, New York
32. Blumberg, W. E., and Pesach, J. (1973) Ann. N. Y. Acad. Sci. 222, 539–560
33. Nozaki, M., Kato, H., Sano, I., and Senoh, S. (1978) Biochim. Biophys. Acta 220, 213–223
34. Nakai, T., Kugamiyama, H., Nozaki, M., Nakazawa, T., Inouye, S., Ebina, Y., and Nakazawa, A. (1983) J. Biol. Chem. 258, 2923–2928
35. Harpel, M. R., and Lipscomb, J. D. (1990) J. Biol. Chem. 265, 6301–6311
36. Bolin, J. T., Han, S., and Eltis, L. D., (1995) J. Biol. Chem. 270, 22187–22196
37. Whiting, A. K., Boldt, Y. R., Hendrich, M. P., Wackett, L. P., and Que, L., Jr. (1996) Biochemistry 35, 160–170
38. Senoh, S., Kita, H., and Kamimoto, M. (1966) in Biological and Chemical Aspects of Oxygenases (Bloch, K., and Hayaishi, O., eds) pp. 378–389, Maruzen Co., Ltd., Tokyo
39. Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801–2807
40. Arciero, D. M., Chen, V. J., Kriauciunas, A., Harpel, M. R., Fox, B. G., Münck, E., and Lipscomb, J. D. (1992) Biochemistry 31, 4602–4612
41. Gelasco, A., and Pecoraro, V. L. (1993) J. Biol. Chem. 268, 8596–8607
42. Segel, I. H. (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems, J ohn Wiley & Sons, New York
43. Tyson, C. A. (1975) J. Biol. Chem. 250, 1765–1770
44. Valentine, J. S. (1994) in Bioinorganic Chemistry (Bertini, I., Gray, H. B., Lippard, S. J., and Valentine, J. S., eds) pp. 253–314, University Science Books, Mill Valley, CA
45. Schwob, M., and Chance, B. (1976) in Th e Enzymes (Boyer, P. D., ed) 2nd Ed., Vol. 13, pp. 363–408, Academic Press, New York
46. Persson, P. J., and Dismukes, G. C. (1994) J. Am. Chem. Soc. 116, 898–903
47. Goldstein, A., and Pecoraro, V. L. (1993) J. Am. Chem. Soc. 115, 7928–7929
48. Sawyer, D. T., Kang, C., Libbert, A., and Redman, C. (1993) J. Am. Chem. Soc. 115, 5817–5819
49. Siegel, E. B. (1976) Biochemistry 15, 844–4426
50. Matsuda, H., and Nakagawa, H. (1987) J. Biol. Chem. 262, 248–254
51. Walling, C. (1975) Accounts of Chemical Research 8, 125–131
52. Hamilton, G. A., and Friedman, J. P. (1963) J. Am. Chem. Soc. 85, 1008–1009
53. Wolfga, S. A. (1989) Structural and Mechanistic Studies of Protocatechuate 2,3-Dioxygenase from Bacillus macerans. Ph.D. Thesis, University of Minnesota, Minneapolis
54. Persox, T. L., and Kraut, J. (1980) J. Biol. Chem. 255, 8199–8205
55. Adachi, I., Nagano, S., Ishimori, K., Watanabe, Y., Morishima, I., Egawa, T., Kitagawa, T., and Makino, R. (1993) Biochemistry 32, 241–252