EPR and Mössbauer Studies of Protocatechuate 4,5-Dioxygenase
CHARACTERIZATION OF A NEW Fe$^{3+}$ ENVIRONMENT*

(Received for publication, June 1, 1983)

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Protocatechuate 4,5-dioxygenase from Pseudomonas testosteroni has been purified to homogeneity and crystallized. The iron containing, extradiol dioxygenase is shown to be composed of two subunit types (α, $M_r = 17,700$ and β, $M_r = 13,800$) in a 1:1 ratio; such a composition has not been observed for other extradiol dioxygenases. The 4.2 K Mössbauer spectrum of native protocatechuate 4,5-dioxygenase prepared from cells grown in $^{57}$Fe-enriched media consists of a doublet with quadrupole splitting, $\Delta E_q = 2.22$ mm/s, and isomer shift $\delta = 1.28$ mm/s, demonstrating a high spin Fe$^{3+}$ site. These parameters, and the temperature dependence of $\Delta E_q$, are unique among enzymes but are strikingly similar to those reported for the reaction center of the photosynthetic bacterium Rhodopseudomonas spheroides (26, 27), suggesting very similar ligand environments. The Fe$^{3+}$ of extradiol dioxygenase can be oxidized, for instance by $H_2O_2$, to yield high spin Fe$^{3+}$ with EPR g values around $g = 6$ (and $g = 4.3$). In the oxidized state, protocatechuate 4,5-dioxygenase is inactive; the iron, however, can be reoxidized by ascorbate to yield active enzyme. Our data suggest that protocatechuate binds to Fe$^{3+}$; the spectra indicate that the ligand binding is heterogeneous. The Mössbauer spectra observed here are fundamentally different from those reported earlier (Zabinski, R. M., Moulton, E., Champion, P., and Wood, J. M. (1972) Biochemistry 11, 3212–3219). The spectra of the earlier (reconstituted) preparations, which had substantially lower specific activities, probably reflect adventitiously bound Fe$^{3+}$. We discuss here how adventitiously bound iron can be identified and removed.

The Fe$^{3+}$ which is present in native protocatechuate 4,5-dioxygenase and its complexes with substrates and inhibitors reacts quantitatively with nitric oxide to produce a species with electronic spin $S = 3/2$. The EPR and Mössbauer spectra of these complexes compare favorably with EDTA+Fe(II)NO. We have studied the latter complex extensively and have analyzed the Mössbauer spectra with an $S = 3/2$ spin Hamiltonian. EPR spectra show that protocatechuate 4,5-dioxygenase-NO complexes with substrates or inhibitors are heterogeneous and consist of several well defined subspecies. The data show that NO, and presumably also $O_3$, has access to the active site Fe$^{3+}$ in the enzyme-substrate complex.

An earlier Mossbauer investigation of $^{57}$Fe reconstituted Pseudomonas testosteroni (1, 2) as well as Pseudomonas species (3). An analogous step in aromatic metabolism by fluorescent pseudomonads is catalyzed by the intradiol cleaving enzyme protocatechuate 3,4-dioxygenase. The latter enzyme has recently been shown to possess a new class of iron center and a unique mechanism for substrate and oxygen activation by a variety of experimental approaches (4, 5). Similar details of the iron site structure and the mechanism of protocatechuate 4,5-dioxygenase are largely unexplored.

A number of other extradiol dioxygenases which catalyze the cleavage of catechol and its derivatives have been purified. Those isolated from pseudomonads reportedly contain ferrous iron (6, 7) as an essential metal cofactor, although this has only been directly demonstrated for catechol 2,3-dioxygenase (8). In contrast, the intradiol cleaving enzymes, protocatechuate 3,4-dioxygenase (4) and catechol 1,2-dioxygenase (9), contain only Fe$^{2+}$. This difference in the redox state of the iron is frequently cited as a fundamental distinguishing feature between the intra- and extradiol cleaving enzymes (10). An earlier Mossbauer investigation of $^{57}$Fe reconstituted P. testosteroni protocatechuate 4,5-dioxygenase (11) which showed that the enzyme did not fall into this general pattern was thus unexpected and is reinvestigated in this report.

Extradiol dioxygenases tend to present many more experimental obstacles than the intradiol enzymes. For example, they are quite labile and often undergo loss of activity during turnover (1, 12). They also lack the visible spectrum and other convenient spectroscopic features of intradiol dioxygenases. As a result, progress in the investigation of these enzymes in general, and of protocatechuate 4,5-dioxygenase in particular, has been slow. We have recently (13) found conditions which allow preparation of P. testosteroni protocatechuate 4,5-dioxygenase-NO complexes with substrates or inhibitors.

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†† Recipient of National Institutes of Health predoctoral traineeship in partial support of this work.

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The abbreviations used are: PCA, protocatechuic acid; SDS, sodium dodecyl sulfate.
Iron Environment of Protocatechuate 4,5-Dioxygenase

**Table I**

| Step                          | Volume | Activity | Total activity | Protein | Specific activity | Yield | Purification |
|-------------------------------|--------|----------|----------------|---------|------------------|-------|--------------|
| Crude extract supernatant*    | 670    | 258      | 173,000        | 44.0    | 5.85             | 100   | 1            |
| Heat step supernatant         | 555    | 360      | 200,000        | 30.0    | 12.0             | 116   | 2.1          |
| Protamine-SO₄ supernatant     | 530    | 374      | 198,000        | 21.2    | 17.6             | 115   | 3.0          |
| 48% (NH₄)₂SO₄ fractionation   | 315    | 572      | 180,000        | 15.6    | 36.7             | 104   | 6.3          |
| pellet, redissolved           |        |          |                |         |                  |       |              |
| Phenyl-Sepharose fractionation| 225    | 757      | 166,000        | 9.8     | 75.1             | 96.1  | 12.8         |
| DE52 fractionation            | 200    | 619      | 124,000        | 5.5     | 113              | 71.8  | 18.9         |
| Affinity fractionation        | 55.5   | 1410     | 78,300         | 9.9     | 142              | 45.4  | 24.6         |
| Fractogel HW-55F fractionation| 56.0   | 1510     | 86,900         | 7.3     | 212              | 50.3  | 37.2         |

* From 210 g of cells; wet weight.

Purification of Protocatechuate 4,5-Dioxygenase

**Results**

The specific activity of this protocatechuate 4,5-dioxygenase is discussed in the "Miniprint." Purification of protocatechuate 4,5-dioxygenase as described under "Materials and Methods." The crystals shown are approximately 0.01 mm across.

**Materials and Methods and Results**

**Purification of Protocatechuate, 4,5-Dioxygenase**—The purification of protocatechuate 4,5-dioxygenase as described under "Materials and Methods" is summarized in Table I. The entire procedure is carried out in buffer containing 10% glycerol, 2 mM cysteine, and 100 μM ferrous ion. Although the purification procedure can be carried out in the absence of iron and cysteine in the steps preceding DE52 chromatography, these stabilizers are required to maintain activity in the later steps. The affinity chromatography step proved to be an effective method to remove contaminating proteins which were not readily separated by the other procedures. Vanillate was used rather than PCA as an affinity ligand in preparing this column because vanillate is less readily oxidized. No metabolism of PCA linked to Sepharose has been observed. The specific activity of this protocatechuate 4,5-dioxygenase preparation (specific activity = 212) is approximately 15 times that previously reported (2, 11), and comparable to that reported for purified *P. species* protocatechuate 4,5-dioxygenase (specific activity = 168) (3). The stability of the purified enzyme is discussed in the "Miniprint."

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2 Portions of this paper (including "Materials and Methods," part of "Results," and Table II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, Route 210, 800 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1528, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
indications of protein purity detailed above, ultracentrifugation experiments showed that the preparation contained species of different molecular weights. For example, plots of $r^2$ versus $\ln$ (protein concentration) generated from sedimentation equilibrium data, were slightly curved. A similar conclusion could be drawn from gel electrophoresis experiments conducted under nondenaturing conditions which exhibited a broad, diffuse protein staining band. The specific stain for enzymatic activity described under “Materials and Methods” showed, however, that protocatechuate 4,5-dioxygenase was present throughout this band. Furthermore, when the gel containing the broad protein band was placed at the top of a slab gel containing SDS and electrophoresis conducted in a second dimension, only the protein staining bands associated with the subunits were observed. Together these data suggest that the molecular weight heterogeneity is observed because protocatechuate 4,5-dioxygenase can exist in several different forms in solution, all of which are composed of either the same subunits or subunits of similar molecular weights. Since we have no indication of families of $\alpha$ and $\beta$ subunits differing in amino acid compositions, the different forms of the enzyme probably result from a dynamic equilibrium of quaternary structures or from a differential interaction with the stabilizing agents (see below). Iron analysis of the enzyme treated with the subunits were observed. Together these data suggest that the iron content of the enzyme was monitored by EPR and the various indications of molecular weight heterogeneity cited above suggest that there is a significant contribution from higher polymers. We also cannot discount the presence of apoenzyme which would decrease the apparent iron to protein ratio, but this possibility is made less likely by the consistent, half-integer ratio observed, and the high specific activity of the preparation.

Protocatechuate 4,5-dioxygenase prepared as described here contains approximately equal amounts of Fe$^{2+}$ and Fe$^{3+}$. The latter is largely EPR silent. In the “Miniprint” under “Specific Removal of Ferric Iron” we discuss in detail how this dependence results when excited orbital like many high spin ferrous proteins, exhibits a temperature-dependent $\Delta E_0$. This dependence results when excited orbital states become thermally populated (24). In Table III we have...
High energy line. This suggests that a minor high spin ferric center iron, presence of a impurity contributes to the absorption of the low energy line. The samples contain about 0.2 mM 57Fe. An absorption line was more intense by about 5-10% than the reported for the reaction center of the photosynthetic bacterium. We noted, impurity may be observed. For reduced electronic system and sizable magnetic hyperfine interactions may be observed. For reduced Pseudomonas aeruginosa protocatechuate 3,4-dioxygenase such studies (26) have revealed the presence of internal fields up to 25 Tesla. We have studied the protocatechuate 4,5-dioxygenase sample in an external field of 6.0 Tesla. The spectrum was poorly resolved; it revealed, however, that the internal fields, at 4.2 K are not larger than about 1 Tesla. Moreover, the 6.0-Tesla spectrum of protocatechuate 4,5-dioxygenase was essentially the same as that observed for the R. sphaeroides reaction center.

EPR studies of protocatechuate 4,5-dioxygenase frequently reveal a signal around \( g = 6 \) which disappears after addition of ascorbate (see Fig. 5 below). We have added ascorbate to a preparation of protocatechuate 4,5-dioxygenase which exhibited a \( g = 6 \) resonance. The Mössbauer spectra revealed that the decrease of the \( g = 6 \) signal intensity was accompanied by an increase of the intensity of the ferrous doublet discussed here suggesting that the \( g = 6 \) signal species results from oxidation of the \( \Delta E_g = 2.23 \text{ mm/s} \) component.

Fig. 4B shows a Mössbauer spectrum measured at 4.2 K of the enzyme in the presence of protocatechuate. The spectrum consists now of a superposition of two doublets. The least squares fit shown in Fig. 4B yielded two doublets with \( \Delta E_g(1) = 2.33 \pm 0.05 \text{ mm/s} \) and \( \delta_{g}(I) = 1.27 \pm 0.03 \text{ mm/s} \), and \( \Delta E_g(II) = 2.80 \pm 0.05 \text{ mm/s} \) and \( \delta_{g}(II) = 1.22 \pm 0.03 \text{ mm/s} \).

![Fig. 4. Mössbauer spectra of 57Fe-enriched protocatechuate 4,5-dioxygenase.](image)

![Fig. 5. EPR spectra of native protocatechuate 4,5-dioxygenase.](image)

**Table III**

| \( T \) | Protocatechuate 4,5-dioxygenase | Reaction center |
|---|---|---|
| \( K \) | \( \text{mm/s} \) | \( \Delta E_g \) | \( \Delta E_g \) |
| 4.2 | 2.22 | 2.22 |
| 50 | 2.23 | 2.24 |
| 90 | 2.17 | 2.17 |
| 120 | 2.09 | 2.09 |
| 150 | 2.03 | 2.00 |
| 180 | 1.97 | 1.92 |

\(^a\) The uncertainties are about \( \pm 0.01 \text{ mm/s} \) for both proteins.

\(^b\) Taken from Fig. 3 of Bosso et al. (25).

listed the parameters obtained at various temperatures. The fits to the whole series of spectra revealed that the low energy absorption line was more intense by about 5-10% than the high energy line. This suggests that a minor high spin ferric impurity contributes to the absorption of the low energy line.

The Mössbauer parameters obtained here are quite different from those observed for Fe\(^{2+}\) enzymes in enzymes. We noted, however, a striking similarity of our parameters with those reported for the reaction center of the photosynthetic bacterium R. sphaeroides R-26 (25, 35). The parameters for \( \Delta E_g \) are compared in Table III. The isomer shift of the reaction center iron, \( \delta_{g} = 1.17 \text{ mm/s} \) (see Table I of Ref. 25) is also quite close to the value \( \delta_{g} = 1.28 \text{ mm/s} \) observed here (high spin ferrous compounds span the range 0.7 mm/s < \( \delta_{g} < 1.5 \text{ mm/s} \)).

In the absence of an applied magnetic field, no magnetic hyperfine interactions are observed for high spin Fe\(^{2+}\) compounds. A strong applied field, however, can polarize the electronic system and sizable magnetic hyperfine interactions may be observed. For reduced Pseudomonas aeruginosa protocatechuate 3,4-dioxygenase such studies (26) have revealed the presence of internal fields up to 25 Tesla. We have studied the protocatechuate 4,5-dioxygenase sample in an external field of 6.0 Tesla. The spectrum was poorly resolved; it revealed, however, that the internal fields, at 4.2 K are not larger than about 1 Tesla. Moreover, the 6.0-Tesla spectrum of protocatechuate 4,5-dioxygenase was essentially the same as that observed for the R. sphaeroides reaction center.

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The resolution does not allow us to quantitate the two species to better than ±5%; the fit displayed in Fig. 4B suggests that 60% of the total ferrous ion belongs to doublet I. The parameters obtained for site II suggest that substrate is bound to the Fe^{3+} site. The parameters of site I are rather close to those observed for the native enzyme suggesting uncomplexed enzyme. Two observations, however, argue against this interpretation. First, a 3-fold further increase in the substrate concentration did not result in any spectral changes. Secondly, reaction of the enzyme substrate complex with NO yields two major S = 3/2 species, both of which are different from the species observed when native enzyme is reacted with NO (see Fig. 6B below). We have also studied the sample at 150 K; at this temperature, the quadrupole splittings are $\Delta E_Q(II) = 2.10$ mm/s and $\Delta E_Q(II) = 2.65$ mm/s.

**EPR Spectra of Protocatechuate 4,5-Dioxygenase**—The relative concentration of ferric ion, which remains in preparations after Tiron treatment, is variable, but usually very low; the sample used for Mössbauer spectroscopy (Fig. 4; EPR spectrum shown in Fig. 5A) contained only 0.06 Fe^{3+}/(α/2β) structural unit. All of this Fe^{3+} appears to be EPR active. Since we have observed an approximate inverse correlation between the concentration of residual Fe^{3+} and specific activity, it seems very unlikely that ferric ion has a catalytic role. Nevertheless, the residual Fe^{3+} is tightly bound, and its reduction by mild reductants leads to activation of the enzyme, suggesting that at least a portion of the Fe^{3+} is the oxidized form of the active site ferrous iron. This interpretation would imply that the residual ferric ion concentration could be accounted for in terms of spontaneous oxidation of the Fe^{3+} occurring, for example, during handling procedures, purification, or freeze-thaw cycles. EPR spectra of a preparation of protocatechuate 4,5-dioxygenase which had retained a relatively large amount of Fe^{3+} after treatment to remove adventitious iron are shown in Fig. 5, B–G, and rough quantitations of the EPR active species of this sample are summarized in Table IV. Two types of iron are present (Fig. 5B): near axial type with g values near g = 6, and a rhombic type resonating near g = 4.3. Both sets of resonances are rather broad suggesting that the species are heterogeneous. Reduction of the sample by ascorbate (Fig. 5C) resulted in elimination of the EPR signal from the axial species as well as part of the signal from the rhombic species. Concurrently, the specific activity of the sample increased by 33%. Quantitation of the spectra suggested that the axial species accounted for approximately 16% of the total iron in the sample while the rhombic species accounted for approximately 25%, of which one third was reducible by ascorbate. In other samples, the concentration of the axial type ferric species ranged from 0 to about 16% while the rhombic species ranged from about 3 to 25% of the total. Ascorbate treatment failed to increase the specific activity of the samples with no g = 6 type ferric species, despite the fact that part of the rhombic species was always reduced. Anaerobic addition of PCA or any other substrate as well as most nonmetabolized substrate analogs caused the axial signal to disappear from the EPR spectrum and the rhombic signal to increase (Fig. 5D). A shoulder is apparent on the low field side of the g = 4.3 resonance shown in Fig. 5D, suggesting that the increase in signal is due to a new, slightly less rhombic, species. Since the substrate complex of the ascorbate-reduced enzyme failed to show a similar increase in the g = 4.3 signal (Fig. 5E), it is likely that the intensity increase around g = 4.3 originated from the ascorbate-reducible iron with g values near g = 6 in Fig. 5B. This implies some sort of interaction of the axial ferric iron with substrate. The observation that the rhombic ferric species is only partially reduced by ascorbate suggests that it is composed of two or more subspecies. We cannot eliminate the possibility that oxidation of some of the ferrous iron also contributes to one of these species. The properties of the iron components observed in protocatechuate 4,5-dioxygenase are summarized in Table V.

**Enzyme–Nitric Oxide Complexes**—During the past few years it has been shown that nitric oxide can complex with ferrous ion of several non-heme iron proteins (14, 29–31) to produce species with electronic spin S = 3/2. The identification of this spin state is firmly established by the observation of EPR resonances around g = 4 and 2. A similar EPR signal, with g values at g = 4.1, 3.9, and 2.0, has also been reported for an EDTA-Fe(II)-NO complex (30). The observed EPR signals of the S = 3/2 complexes can be described with the spin Hamiltonian:

$$H_s = D[S_i^2 - 5/4 + E/(2S_i^2 + S_j^2)] + g_\beta S_i \cdot H$$  \hspace{1cm} (1)

where $g_\beta = 2$ and $D$ and $E$ are parameters describing the zero field splitting. The NO complexes studied thus far have $D > 0$. Broad EPR signals near $g = 4.3$ are commonly observed in biological preparations. Signals of this type are observed for iron in solutions containing buffer ions, iron nonspecifically associated with proteins, and iron chelated by catechols as well as from active site iron in many proteins. Since the $g = 6$ type iron is, by contrast, rare in non-heme proteins and seldom, if ever, observed for nonactive site iron, we believe that the $g = 6$ type iron is the principal form of the oxidized active site iron of our enzyme. Nevertheless, the $g = 4.3$ signal increases in intensity when the enzyme is oxidized and decreases when the enzyme is rereduced; thus, it is possible that some of the oxidized active site iron resonates near $g = 4.3$. Since full activity is not recovered after rereduction, however, this signal may originate from enzyme molecules damaged in some way by the oxidation process.
were unsuccessful suggesting that there are at least three distinct enzyme-substrate complexes with NO. Exposure of the enzyme to NO results in formation of at least 14986 values of

\[ g = \text{constant} \]

components suggesting that all of the substrate and substrate analog complexes are comprised of multiple species. The

\[ \text{Component} \quad \text{Characteristic} \]

\[ \begin{align*}
\text{Fe}^{2+}, \Delta E_{q} = 2.2 \text{ mm/s} & \quad \text{Active form of active site iron.} \\
& \quad \text{Sensitive to substrate ligation.} \\
& \quad \text{Reacts with NO to give } S = 3/2 \text{ species.} \\
& \quad \text{Reduces by ascorbate to yield additional } g = 4.3 \text{ species.} \\
& \quad \text{Generated by oxidation of Fe}^{2+} \text{ by } H_{2}O_{2}. \\
\text{Fe}^{2+}, g = 6.4 \text{ and } 5.3 & \quad \text{Oxidized form of active site iron.} \\
& \quad \text{Reduces by ascorbate to active } Fe^{2+} \text{.} \\
& \quad \text{Reduction increases specific activity.} \\
& \quad \text{Generated as a consequence of oxidation of } Fe^{2+} \text{ by } H_{2}O_{2}. \\
\text{Fe}^{2+}, g = 4.3 & \quad \text{Component} \quad \text{Characteristic} \\
& \quad \text{Reduces by ascorbate to active } Fe^{2+} \text{.} \\
& \quad \text{Reduction increases specific activity.} \\
& \quad \text{Generated as a consequence of oxidation of } Fe^{2+} \text{ by } H_{2}O_{2}. \\
& \quad \text{Generated during inactivation by 4-methylcatechol.} \\
\end{align*} \]

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\[ E/D \]

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\[ E \]

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\[ I \]

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\[ N \]

\[ O \]

\[ P \]

\[ Q \]

\[ R \]

\[ S \]

\[ T \]

\[ U \]

\[ V \]

\[ W \]

\[ X \]

\[ Y \]

\[ Z \]

\[ a \]

\[ b \]

\[ c \]

\[ d \]

\[ e \]
FIG. 7. 4.2 K Mössbauer spectra of protocatechuate 4,5-dioxygenase-substrate-nitric oxide complex (upper spectrum) and EDTA-Fe(II) NO complex (lower spectrum) were recorded in fields of 60 milliTesla (A and B) and 5.0 Tesla (C) applied parallel to the observed γ-radiation. The solid lines in B and C are computer simulations using Equations 1 and 2. The parameters used to fit the EDTA-Fe(II) NO data and the estimated uncertainties are: \( D = +12(\pm 1) \text{ cm}^{-1}, E/D = 0.02; A_0 = -(32(\pm 2) \text{ MHz}; \Delta E_Q = -(1.67(\pm 0.03) \text{ mm/s}; \eta = 0.07; \text{ and } \delta_{\eta} = 0.66 \text{ mm/s.} \)

The spectra shown in Fig. 7 exhibit well developed magnetic patterns due to the presence of magnetic hyperfine interactions; for a discussion of such spectra see Refs. 45 and 46. In order to evaluate these spectra, we augment the Hamiltonian of Equation 1 by a term describing the magnetic hyperfine interactions, \( A_0 \bar{S} \cdot \bar{I}, \) a nuclear Zeeman term, \(-g_0\delta_0 \bar{H} \cdot \bar{I},\) and a term describing the interaction of the nuclear quadrupole moment \( Q \) with the electric field gradient tensor (principal axes components \( V_{xx}, V_{yy}, V_{zz}; \eta = (V_{xx} - V_{yy})/V_{zz}, \Delta E_Q = \langle e\Omega V_{zz}/2\rangle \text{ Hz}. \)

The theoretical curves shown in Fig. 7 were generated by computer simulations from the Hamiltonian \( \hat{H} = \hat{H}_0 + \hat{H}_n \) using the parameters listed in the legend to Fig. 7. The parameters quoted were determined in the same way as described elsewhere (46). The values for \( E/D \) and \( g_0 \) are obtained from EPR. \( A_0 \) is determined from the overall magnetic splitting. The sign of \( A_0 \) and the magnitude of \( D \) are obtained from the 5.0-Tesla spectrum of Fig. 7C. \( V_{xx} \) and \( V_{yy} \) and therefore \( \Delta E_Q \) and \( \eta \) are determined from the line positions of the spectra recorded in parallel (Fig. 7) and transverse (spectrum not shown) magnetic fields. A more detailed analysis revealed that the \( x \)-axis of the electric field gradient tensor is tilted by about 15° relative to the \( z \)-axis which defines the zero field splitting frame. Within the uncertainties the parameter set for the EDTA-Fe(II) NO complex fits the spectrum of Fig. 7A also. (Since we have not studied the enzyme complex in a strong applied magnetic field, the Mössbauer data give no information on the magnitude of \( D \). We know however, from EPR that \( D \) is comparable to that of the EDTA-Fe(II) NO complex.)

Inactivation of Protocatechuate 4,5-Dioxygenase—Inactivation of protocatechuate 4,5-dioxygenase by treatment with \( \text{H}_2\text{O}_2 \) (Fig. 8A), FeCN \(_6^2-\), or extended exposure to air results in EPR active species with major resonances near \( g = 6 \) and \( g = 4.3 \), similar to the minority species observed in native enzyme (Fig. 5B). As shown in Table IV, the total \( \text{Fe}^{3+} \) present after \( \text{H}_2\text{O}_2 \) treatment accounts for approximately 96% of the total iron in the sample. Under these conditions, the enzymatic activity and the EPR signal intensity of the \( S = 3/2 \) NO complex are correspondingly reduced. These results show that loss of activity occurs upon oxidation of the active site iron. Treatment of the inactivated enzyme with ascorbate restores approximately 50% of both the original activity and the \( S = 3/2 \) EPR signal from the NO complex (Fig. 8D).

Aerobic incubation of protocatechuate 4,5-dioxygenase with 4-methylcatechol or one of several other nonmetabolized substrate analogs yields an inactive enzyme exhibiting an EPR spectrum with a large signal near \( g = 4.3 \), this signal accounts for approximately 90% of the iron in the sample. The EPR spectra taken after the addition of NO to this sample exhibited an \( S = 3/2 \) signal with only 10% of the intensity that is observed when active enzyme is reacted with NO. Furthermore, the activity of the inactivated sample was not restored by treatment with ascorbate or \( \text{Fe}^{3+} \). Reductants with a lower redox potential than ascorbate were also ineffective.

**DISCUSSION**

**Fe\(^{2+}\) Active Site**—In this study we have demonstrated that the active site iron of native protocatechuate 4,5-dioxygenase is high spin ferrous. The observed Mössbauer parameters are unique among enzymes containing \( \text{Fe}^{2+} \). The Mössbauer spectra are, however, strikingly similar to those reported for the iron of the reaction center from *R. sphaeroides* R-26. The similarity between the two sites as expressed by the \( \Delta E_Q \) values (Table III) is also apparent when the spectra obtained in 6-Tesla applied fields are compared. The iron sites of both...
proteins exhibit unusually small internal magnetic fields at 4.2 K. The near identity of the Mössbauer spectra implies close structural similarities of the iron sites. Two recent extended x-ray fine structure studies (33, 34) of the R. sphaero-roides reaction center suggest an octahedral iron environment consisting mainly of nitrogen and oxygen ligands. Bunker et al. (34) suggest that the data are best fitted by assuming that four histidines and two oxygenous ligands are coordinated to the iron. Such an environment is in accord with the isomer shifts observed here.

Since we have only recently arrived at metal pure preparations, we have not yet performed systematic studies of the redox properties of the protocatechuate 4,5-dioxygenase. Our preliminary data suggest that oxidation of the iron yields a high spin ferric species with EPR resonances at g = 6, a signal which is rarely observed (4) for non-heme iron. The latter species is reductively readily by ascorbateg suggesting a site with a high redox potential, a feature which protocatechuate 4,5-dioxygenase shares with the reaction center (33). It is likely that valuable clues about the reaction center iron can be obtained from further studies of protocatechuate 4,5-dioxygenase. In particular, it would be interesting to learn whether the Fe(II) of the reaction center reacts with NO.

The Mössbauer spectra obtained here are fundamentally different from those observed by Zabinski et al. (11). Their spectra did not reveal any trace of high spin ferrous material. We have elaborated in the “Miniprint” on the instability of the protein and on its affinity for binding iron in multiple sites. We have described in detail how we improved the preparations by closely monitoring all iron environments with both Mössbauer and EPR spectroscopy. Our present preparations have specific activities higher by at least a factor of 15 than those of Zabinski et al. (11); moreover, their reported reconstitution procedures do not work with our preparation.

We have applied our preparative procedure to isolate protocatechuate 4,5-dioxygenase from the same strain of P. testosteroni and P. testosteroni 2,3-dioxygenase isolated from Bacillus macerans (36). That although the iron observed in the earlier study (11) was high spin ferric in character, it is entirely different from those observed for protocatechuate 4,5-dioxygenase; in particular, the 6.0-Tesla spectra of the two compounds (data not shown) differ substantially. Furthermore, the enzyme-substrate spectra are also quite unlike those of Fe(II)-EDTA.

So far a proper description of the electronic structure of the S = 3/2 complexes has not emerged in the literature. The S = 3/2 state could reflect either a high spin 3d^5 configuration, or an intermediate spin complex of ferric iron (with NO electronegative), or a ferrous complex with NO with three unpaired electrons in appropriate molecular orbitals. It is noteworthy that the electronic Zeeman term of EDTA-Fe(II)-NO is isotropic and that the magnetic hyperfine interaction seems to be isotropic as well (the Mössbauer data show that A, = A, to within a few per cent; A, cannot be determined better than ±20%). The isomer shift, 6Fe, = 2.06 mm/s, argues against a ferric configuration. This conclusion is suggested by comparison of our data with those reported by Fitzsimmons et al. (37) who have observed substantially smaller isomer shifts (6Fe = 0.45 mm/s); we have corrected their room temperature values to account for the second order Doppler shift for intermediately spin ferric Fe(III)(salen) complex (9, 10)(salen = N,N'-ethylenebis(salicyldenediene)amine).  

Structure—We have found that P. testosteroni protocatechuate 4,5-dioxygenase has a complex subunit structure, nominally (αβFe), than other extradiol dioxygenases. Both P. arvilla catechol 2,3-dioxygenase (23, 39) and Pseudomonas ovalis 3,4-dihydroxyphenylacetate 2,3-dioxygenase (38) reportedly have α, structures with 1-3 and 4-5 iron, respectively, depending on growth and purification conditions. The recently reported manganese containing 3,4-dihydroxyphenylacetate 2,3-dioxygenase from Bacillus brevis (61) also has an α, subunit structure. In contrast, the subunit structure of all well characterized catecholic intradiol dioxygenases is (αβFe), or (αβFe), where n values of 1, 4, 5, 8, and 10 have been reported (40, 42, 52). Among the protocatechuate 3,4-dioxygenases the α subunits are remarkably similar in terms of their physical properties and preliminary evidence suggests that the substrate-binding site is located on this subunit (5, 32). The α subunit of protocatechuate 4,5-dioxygenase has approximately the same size as these subunits; however, more physical and structural studies need to be done before a meaningful comparison can be made. Within the experimental uncertainties the β subunit is the same size as the single subunit of known extradiol dioxygenases. Since the amino acid sequences of catechol 2,3-dioxygenase (50) and protocatechuate 3,4-dioxygenase (47, 48) have recently been reported, we will be able to search for regions of homology in each of...
the protocatechuate 4,5-dioxygenase subunits. The apparent differences in the iron sites of catechol 2,3-dioxygenase and our enzyme, together with the fact that the gene of the former enzyme is plasmid borne may indicate, however, that the enzymes have evolved separately for some time.

The overall physical size and specific activity of our enzyme are very similar to those reported for protocatechuate 4,5-dioxygenase isolated from \textit{P. species} (3). The principal distinguishing characteristic is the requirement for ethanol as a stabilizing agent for the latter enzyme. We do not find ethanol to be an effective stabilizer for the \textit{P. testosteroni} enzyme. The two enzymes are inactivated by aerobic storage, \(\text{H}_2\text{O}_2\), metals such as Cu, and by substrate turnover as are most other known iron containing extradiol dioxygenases. The subunit structure of \textit{P. species} protocatechuate 4,5-dioxygenase has not been reported.

Our work was facilitated greatly by the recognition that the enzyme is much more stable if prepared from bacteria which were well out of log phase growth at the time of harvest. This suggests that some permanent change in the structure of the enzyme occurs during the post log phase period. It is possible that a post-translational modification of some sort takes place, but our efforts to identify a modified amino acid have thus far been unsuccessful.

The iron site of the native enzyme is apparently quite homogeneous. In contrast, the anaerobically formed substrate complex is markedly heterogeneous. Similar heterogeneities have also been observed for the anaerobic PCA complex with protocatechuate 3,4-dioxygenase (4, 41). Recent studies of the latter enzyme in the presence of transition state analogs suggest that only the initial, relatively weak complex is heterogeneous (41). A high affinity, homogeneous complex appears to form coincident with or after \(\text{O}_2\) binding. A similar mechanism would also be consistent with the properties of the extradiol protocatechuate 4,5-dioxygenase observed here.

\textbf{Inactivation by Oxidation}—The assignment of Fe\(^{2+}\) as the active site iron is supported by the observation that two fundamentally different inactivation processes lead ultimately to oxidation of the iron. EPR spectra of protocatechuate 4,5-dioxygenase recorded after treatment of the enzyme with \(\text{H}_2\text{O}_2\) or Fe(CN)\(_2\) clearly show that the Fe\(^{2+}\) has become oxidized as suggested by numerous less direct techniques for other extradiol dioxygenases similarly inactivated. The oxidized iron gives rise to EPR features in spectral regions characteristic of axial and rhombic environments showing that numerous species have been generated. Nevertheless, the iron appears to remain bound to the enzyme since we have not observed \(g = 6\) type signals in Fe/substrate/buffer solutions in the absence of enzyme. This type of inactivation, whether induced by oxidants or occurring spontaneously during purification, is reversible at least in part by the addition of mild reductants such as ascorbate. The inability of ascorbate to effect complete reactivation may indicate that the inactivation is progressive in nature and is only initiated by the oxidation. The second type of inactivation results when specific substrate analogs, such as 4-methylcatechol, interact with the enzyme under aerobic conditions. The \(g = 4.3\) type EPR spectrum of this form shows no evidence for protein-bound iron and is identical in shape to the spectrum of free Fe-catecholate complex. Since \(\text{Fe}^{2+}\) will exchange under anaerobic conditions with the active site iron, the iron dissociation constant must be relatively high compared to those of lumes and other iron containing proteins. Thus, it is possible that the inactivating inhibitors, which are good iron chelators, shift the equilibrium significantly in favor of iron release. We have observed that the free \(\text{Fe}^{2+}\)-inhibitor complexes oxidize rapidly under aerobic conditions. Since the resulting complexes have very low redox potentials and low dissociation constants, the inactivation process would be difficult to reverse. However, we have also observed that the inactivation proceeds much more rapidly with 4-methylcatechol than with other analogs which are equally effective as chelators. This suggests that the destabilization is initiated by binding of the inhibitor rather than dissociation of the iron.

\textbf{Mechanistic Implications}—Past studies have provided evidence that the presence of Fe\(^{2+}\) is essential for maintaining or restoring the activity of most extradiol catecholic dioxygenases. The present study shows that active protocatechuate 4,5-dioxygenase contains an Fe\(^{2+}\) site and that this site has a unique environment, distinct from that of \textit{P. arvilla} catechol 2,3-dioxygenase and other enzymes containing ferrous ion. The presence of Fe\(^{2+}\) in the extradiol cleaving enzymes provides a clear contrast to intradiol dioxygenases which perform an analogous reaction on the same substrate, but utilize exclusively Fe\(^{2+}\) throughout the catalytic cycle (4, 5). Our studies have shown that the iron of protocatechuate 4,5-dioxygenase remains ferrous in the substrate complex prepared anaerobically. Moreover, our data show that small molecules such as NO have access to the ferrous iron even when substrate is bound. We thus suspect that the metal ion is accessible to O\(_2\) as well, and a reaction mechanism in which \(\text{Fe}^{2+}\) is activated by binding directly to the Fe\(^{2+}\) should be considered. On the other hand, we have observed no evidence for such an oxo complex. Moreover, the characteristic reactions and components of other systems which activate oxygen by direct interaction with the metal, such as rapid autoxidation and the participation of low redox potential centers (49), appear to be absent.

\textbf{Acknowledgments}—We thank James W. Whittaker for synthesizing 4-sulfonyle catechol and protocatechuate. We also thank G. Casy, R. D. Kunjummen, and R. W. Salo for their assistance in preparation of the enzyme. We are grateful for the advice and help in crystallizing the enzyme extended by Dr. Douglas Rees and the many useful suggestions offered by Dr. James D. Howard.

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Iron Environment of Protocatechuate 4,5-Dioxygenase

**Supplementary Materials**

**Iron and Monooxygen Stages of Protocatechuate 4,5-Dioxygenase**

Characterization of a Mono Ferrous Ion Environment

David K. Arciero, John S. McCammon, and John A. W. Clark

**Materials and Methods**

Growth of the organism: Pseudomonas aeruginosa strain 171 was isolated by a colorimetric method from soil at 17°C. It was phototrophically grown on a carbon source of glucose and glutamate, or succinate and glutamate, or succinate and fumarate in a chemostat. The cells were harvested by centrifugation and the supernatant was removed.

Iron Environment of Protocatechuate 4,5-Dioxygenase: Divalent iron was supplied in the medium as Fe(NO)3...H2O. The cells were harvested from the chemostat and the supernatant was removed. The cells were then washed twice with phosphate buffer and resuspended in fresh buffer.

**Results**

**Table I**

|      | Native | Recombinant |
|------|--------|-------------|
| Yield | 17,702 | 33,708      |
| A280  | 1.78   | 1.81        |

**Discussion**

Iron-enriched ferrous protein preparations were obtained by protease digestion of the purified enzyme. The results showed that the enzyme is a dimer with a mol wt of about 150,000 daltons. The enzyme was found to be active in the presence of oxygen and in the absence of oxygen. The enzyme showed a single, well-defined peak in the EPR spectrum and was not affected by the presence of ferrous ions.

**Table II**

|      | Native | Recombinant |
|------|--------|-------------|
| Yield | 17,702 | 33,708      |
| A280  | 1.78   | 1.81        |

The enzyme was found to be active in the presence of oxygen and in the absence of oxygen. The enzyme showed a single, well-defined peak in the EPR spectrum and was not affected by the presence of ferrous ions. The enzyme was found to be active in the presence of oxygen and in the absence of oxygen. The enzyme showed a single, well-defined peak in the EPR spectrum and was not affected by the presence of ferrous ions.
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D M Arciero, J D Lipscomb, B H Huynh, T A Kent and E Münck

J. Biol. Chem. 1983, 258:14981-14991.

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