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MÖSSBAUER AND EPR SPECTROSCOPY ON PROTOCATECHUATE 3,4-DIOXYGENASE FROM *PSEUDOMONAS AERUGINOSA*

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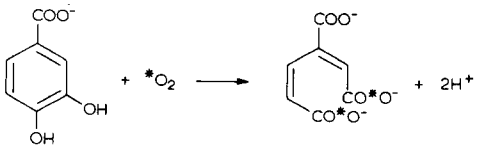
Summary

Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) from *Pseudomonas aeruginosa* has been investigated by EPR and Mössbauer spectroscopy. Low temperature Mössbauer data on the native enzyme (Fe^{3+}, $S = 5/2$) yields a hyperfine field $H_{sat} = -525$ kG at the nucleus. This observation is inconsistent with earlier suggestions, based on EPR data, of a rubredoxin-like ligand environment around the iron, i.e. a tetrahedral sulfur coordination. Likewise, the dithionite-reduced enzyme has Mössbauer parameters unlike those of reduced rubredoxin. We conclude that the iron atoms are in a previously unrecognized environment. The ternary complex of the enzyme with 3,4-dihydroxyphenylpropionate and O_2 yields EPR signals at $g = 6.7$ and $g = 5.3$; these signals result from an excited state Kramers doublet. The kinetics of the disappearance of these signals parallels product formation and the decay of the ternary complex as observed in the optical spectrum. The Mössbauer and EPR data on the ternary complex establish the iron atoms to be in a high-spin ferric state characterized by a large and negative zero-field splitting, $D = \simeq -2$ cm^{-1}.

Introduction

Protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* (EC 1.13.11.3) catalyzes the cleavage of protocatechuate to β-carboxy-*cis*,*cis*-muconate with the incorporation of two atoms of molecular oxygen.



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In the past decade, this enzyme has been studied extensively by Hayaishi and his co-workers [1]. Molecular weights of 700 000 and 660 000 have been estimated for the crystalline enzyme by sedimentation equilibrium and total amino acid composition, respectively [1]. Reportedly eight identical subunits, each containing one iron atom, comprise the holoenzyme [2]. Recently we have found that each of the subunits is a tetramer consisting of two different polypeptides in an $\alpha_2\beta_2$ structure [3].

The absorbance spectrum of the native enzyme is characterized by a broad band with λ_{max} centered near 450 nm [1], which shifts to 480 nm upon anearobic addition of substrate [2]. Addition of oxygen to the enzyme-substrate complex yields a transient species with λ_{max} near 520 nm; this spectrum has been associated with an enzyme-substrate-oxygen complex because its rate of formation is dependent upon oxygen concentration [4].

EPR spectroscopy has firmly established that the irons in the native enzyme are in a high-spin ferric state ($S = 5/2$). The enzyme exhibits a prominent feature near $g = 4.3$, typical of high-spin ferric iron in a "rhombic" environment [2,5]. By measuring the temperature dependence of the low temperature EPR signals, Blumberg and Peisach [5] were able to deduce the parameters (D,λ) describing the zero-field splitting of the spin sextet. The magnitude of the zero-field splitting of protocatechuate 3,4-dioxygenase was found to be quite similar to that of ferric rubredoxin and much larger than the values found for other iron proteins exhibiting a $g = 4.3$ resonance. On the basis of this observation, Blumberg and Peisach suggested an iron ligand environment in protocatechuate 3,4-dioxygenase similar to ferric rubredoxin, i.e. a tetrahedral arrangement of four cysteinyl sulfurs.

In this communication, we report the results of a combined EPR and Mössbauer investigation of ^{57}Fe-enriched protocatechuate 3,4-dioxygenase. Our results suggest that the iron coordination in this enzyme differs markedly from rubredoxin. In addition, our studies on the ternary enzyme-substrate-oxygen complex reveal a high-spin ferric ion characterized by a large and negative zero-field splitting, a species which has so far not been observed in biological systems. Our work has yielded some unique spectral parameters which should serve as useful guidelines for the design of inorganic models for these enzymes.

Materials and Methods

Proteins

Pseudomonas aeruginosa (ATCC 23975) was grown in a medium containing *p*-hydroxybenzoic acid as the sole carbon source in acid-washed 14 liter New Brunswick fermentors at 26° C. The medium contained, in a volume of 1 liter, *p*-hydroxybenzoic acid, 3.0 g; yeast extract, 0.1 g; $(NH_4)_2HPO_4$, 3.0 *g*; K_2HPO_4, 1.2 *g*; $MgSO_4 \cdot 7H_2O$, 0.1 *g*; NaCl, 1.2 *g*; and $^{57}FeCl_2$, 2.5 mg. The iron was obtained as 93% enriched $^{57}FeO_3$ from Union Carbide Corporation and reduced to iron metal which was subsequently dissolved in 1 M HCl. Oxygen tension was monitored with a New Brunswick dissolved oxygen analyzer. Aeration was increased from 300 cm^3/min at the time of inoculation to 600 cm^3/min as the rate of oxygen utilization increased. Stirring rate was increased from 300 rpm to 400 rpm, after 7 h of growth. 1.5 g of *p*-hydroxybenzoic acid was added per

liter of growth medium when the rate of O_2 utilization maximized (about 7 h) and after 3 additional hours of growth. The yield of cells was approximately 9 g wet weight per liter of medium when harvested at the cessation of O_2 uptake. Purification and crystallization of protocatechuate 3,4-dioxygenase was achieved via two procedures. The first procedure is that proposed by Fujisawa and Hayaishi except for the omission of the protamine sulfate step. In the second procedure the precipitate from the first ammonium sulfate precipitation in the former preparation was redissolved in 50 mM Tris · HCl, pH 8.5 (at 4°C), and fractionated on a Biogel P-300 column (8 cm × 60 cm) equilibrated in the same buffer. Fractions with a specific activity greater than 35 were pooled and loaded on a Whatman DE-52 DEAE cellulose column (4 cm × 24 cm) equilibrated in 50 mM potassium phosphate buffer, pH 7.5. The column was washed with 1 liter of the same buffer plus 0.08 M KCl followed by 500 ml of buffer plus 0.115 M KCl. The enzyme eluted in the second buffer. Fractions with a specific activity greater than 55 were pooled and crystallized as previously described. Both procedures resulted in electrophoretically homogeneous enzyme with a specific activity of 57 to 65 and an overall yield of about 40%.

Methods

EPR spectroscopy was performed at X-band on a Varian E-9 spectrometer, with provisions for 100 kHz modulation and operation in the temperature ranges 6—40°K using helium boil off gas as the coolant [6]. Temperatures were routinely monitored via a calibrated carbon resistor below the sample in the gas stream. The spectrometer conditions are given in the figure captions.

The Mössbauer spectrometer was of the constant accelerator type. A 30 mCi source of ^{57}Co in rhodium was used which gave a minimum observable line width of 0.24 mm/s. The system was calibrated with a metallic iron absorber. A Janis variable temperature cryostat was used for the measurements. The samples were inserted into the tail section from the top and the γ-rays from the ^{57}Co source passed horizontally through the sample via two pairs of mylar windows. A small permanent magnet made from Indox V wafers was placed around the tail section of the cryostat allowing measurements in either parallel or perpendicular magnetic fields of 600 gauss. During the course of this work we realized that the velocity transducer (Elscint MVT-3) was very sensitive to the presence of small (10 gauss) magnetic fields; therefore the unit had to be enclosed in an iron box.

Iodoacetamide treated protocatechuate 3,4-dioxygenase was made by dialyzing the enzyme vs. 50 mM Tris · HCl buffer, pH 8.5, containing 10 mM iodoacetamide at 4°C for 18 h followed by dialysis vs. the same buffer. Enzyme-substrate complex was made by evacuating the enzyme solution and flushing with O_2-free nitrogen. Substrate in a similarly degassed solution was added using a gas-tight syringe and the mixture was frozen. The enzyme-3,4-dihydroxyphenylpropionate-O_2 complex for the Mössbauer study was made by injecting substrate into enzyme solution saturated with O_2 at 3 atmospheres at 6°C and quickly freezing the mixture.

The EPR samples for studying the decay of the oxygenated intermediate were made as follows: protocatechuate 3,4-dioxygenase, 3,4-dihydroxyphenyl-

propionate, and buffer solution saturated with O_2 were incubated in the EPR tube at 6° C and observed in a Cary 14 spectrophotometer with the EPR tube positioned in the light beam. When the appropriate absorbance change occurred, the sample was frozen by rapid submersion in a stirred isopentane bath at 130° K; the samples freeze within 1—2 seconds of this treatment.

β-Carboxy-*cis*,*cis*-muconate was made passing 200 μM protocatechuate in 50 mM potassium phosphate buffer, pH 6.5, through a column (1 cm × 10 cm) of protocatechuate 3,4-dioxygenase covalently attached to CNBr-activated Sepharose.

Theoretical background

Most of the results discussed in this paper concern the high-spin ferric ion ($S = 5/2$). Although the theoretical framework underlying the evaluation of the data, the spin Hamiltonian, has been discussed extensively in the literature [7—10] we briefly present the relevant expressions. It is customary to describe EPR and Mössbauer data by the spin Hamiltonian

$$\mathcal{H} = \mathcal{H}_e + \mathcal{H}_{hf} \tag{1}$$

with

$$\mathcal{H}_e = D[S_z^2 - 35/12 + \lambda(S_x^2 - S_y^2)] + g_0\beta S \cdot H \tag{2}$$

and

$$\mathcal{H}_{hf} = A_0 S \cdot I + \mathcal{H}_Q \tag{3}$$

where

$$\mathcal{H}_Q = \frac{eQV_{zz}}{12}[3I_z^2 - 15/4 + \eta(I_x^2 - I_y^2)].$$

The electronic part of the spin Hamiltonian, $\mathcal{H}_e$, describes the zero-field splitting (D,λ) of the electronic spin sextet and the interaction of the electronic magnetic moment $g_0\beta S$ with an applied magnetic field H. To describe the Mossbauer data $\mathcal{H}_e$ has to be augmented by terms describing the magnetic hyperfine interaction $(A_0\ S \cdot I)$ and the electric quadrupole interaction $\mathcal{H}_Q(V_{xx}, V_{yy}$, and V_{zz} are the principal components of electric field gradient tensor. $\eta = (V_{xx} - V_{yy})/V_{zz}$ is the asymmetry parameter.) As usual for high-spin ferric compounds we take the electronic Zeeman interaction and the magnetic hyperfine interaction to be isotropic (g_0,A_0); this assumption holds quite well for Fe^{3+} compounds [9].

The zero-field splitting term in Eqn. 2 splits the ^6S ground state of the ferric ion into three Kramers doublets. For applied magnetic fields H such that $\beta H \ll |D|$ it is customary to describe the magnetic properties of each Kramers doublet by an effective $S' = 1/2$ spin Hamiltonian

$$\mathcal{H}_e^{(n)} = E^{(n)} + \beta S' \cdot \mathbf{g}^{(n)} \cdot H, \tag{4}$$

where $E^{(n)}$ gives the energy of the nth doublet. The g-tensor defined in Eqn. 4 is the quantity commonly reported from EPR experiments. It can be computed from Eqn. 2; the g-values depend essentially on λ, since $g_0 = 2.0$. For long elec-

tronic spin relaxation times (long compared to the nuclear precession time) one observes a magnetic Mössbauer spectrum for each Kramers doublet. As for the electronic Zeeman interaction we refer the magnetic hyperfine interaction to each doublet. Thus the Mössbauer spectrum for each Kramers doublet is described by (we drop the superscript (n))

$$\mathcal{H} = \beta S' \cdot \mathbf{g} \cdot H + S' \cdot \mathbf{A} \cdot I + \mathcal{H}_Q \tag{5}$$

The components of the effective A-tensor are related to the g-values [7] by

$$\frac{g_x}{A_x} = \frac{g_y}{A_y} = \frac{g_z}{A_z} = \frac{g_0}{A_0} \tag{6}$$

Thus, if the g-values are known (from EPR experiments) the A-tensor is known except for a scaling factor which can easily be determined from the total magnetic splitting of the Mössbauer spectrum.

In a Mössbauer experiment the effective hyperfine tensor associated with each doublet is measured. If the g-values of the doublet under consideration are known (usually the knowledge of the largest g-value is sufficient) A_0 can be computed from Eqn. 6. In the Mössbauer literature it is customary to quote the saturation field H_{sat} which is defined by ($g_g = 0.1806$)

$$H_{sat} = -5/2 \frac{A_0}{g_g \beta_n} \tag{7}$$

H_{sat} reflects the degree of covalent bonding of the ferric ion. Fe^{3+} compounds with an octahedral oxygen coordination typically yield $H_{sat} \simeq -550$ kG (for a review see [10]). The fields found for heme proteins range from −450 to −530 kG [9]. The smallest fields have been observed for compounds with a tetrahedral sulfur coordination: −370 kG for ferric rubredoxin [11,12] and an appropriate model complex for this protein [13]; $\simeq$ −360 kG for the ferric site in plant-type ferredoxins [14].

Results

1. Native protocatechuate 3,4-dioxygenase

An EPR spectrum of the native enzyme taken at 12°K is shown in Fig. 1. This spectrum closely resembles those reported by Blumberg and Peisach [5]. These authors have interpreted the EPR data in terms of the spin Hamiltonian Eqn. 2, using $\lambda = 0.29$. This choice of λ yields three almost equally spaced Kramers doublets; the middle doublet gives rise to resonances at $g_x = 4.23$, $g_y = 4.03$, and $g_z = 4.52$, i.e. the prominent signal around "$g = 4.3$". Moreover, Eqn. 2 predicts g-values for the lowest doublet at $g_x = 1.17$, $g_y = 9.56$, and $g_z = 0.72$. In the low-field region Blumberg and Peisach observed a ground doublet resonance at $g = 9.51$; we observed this resonance at $g = 9.35$ *. By mea-

* A detailed investigation of the features around $g = 9.35$ in some of our earlier preparations revealed that the signal reflects a heterogeneous environment (iron sites with slightly different values for λ). It had a width of about 100 gauss and various species could be discerned, but not resolved. Treatment of the enzyme with iodoacetamide sharpened up the signal consisting then essentially of two barely resolved peaks at $g = 9.4$ and $g = 9.0$. The iodoacetamide reaction has been shown to reduce aggregation of holoenzyme units without loss of activity [1]. This suggests that the iron environment is sensitive to surface effects and perhaps also to stacking of subunits in the holoenzyme. The "best" results, i.e. the sharpest features, were achieved by using polycrystalline material frozen in its mother solution (see Fig. 1); the width of the $g = 9.35$ resonance in Fig. 1 is 40 gauss.

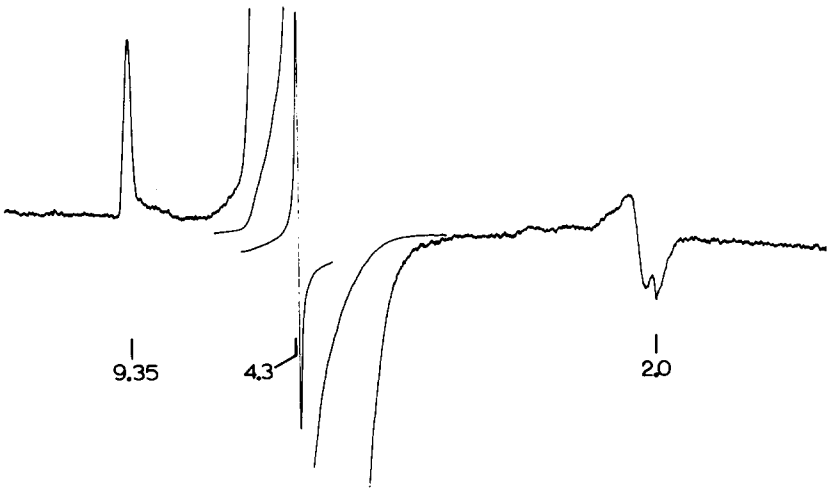

Fig. 1. EPR spectrum of polycrystalline protocatechuate 3,4-dioxygenase, packed in its mother liquor. Conditions: T = 12°K; microwave frequency, 9.196 GHz; microwave power, 3 mW; modulation amplitude, 10 gauss; sweep rate, 1000 gauss/min; time constant, 0.3 s; receiver gain, 3200 in the low field region and 320 and 32 in the g = 4.3 region, respectively. Abscissa: magnetic field strength, increasing to the right; ordinate: an arbitrary linear function of the first derivative of microwave absorption with respect to the field. For the estimation of g-values the frequency and field readings from the E-9 bridge were used. The feature in the g = 2 region is due to 0.1 mole Cu^{2+} per mole enzyme according to comparisons of this signal with that of a standard copper-EDTA solution. We believe the copper is an adventitious contaminant.

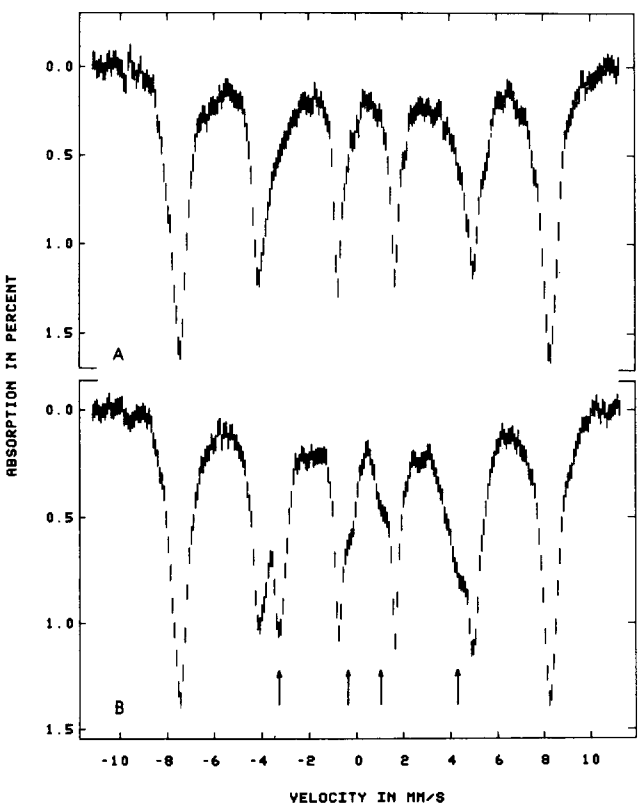

Fig. 2. Mössbauer spectra of ^{57}Fe-enriched protocatechuate 3,4-dioxygenase taken at 1.5°K (A) and 4.2°K (B), respectively. The spectra were taken in a field of 600 gauss applied parallel to the Mössbauer radiation. The absorption lines of the middle Kramers doublet are indicated by arrows.

suring the temperature dependence of the resonance at $g = 4.3$ between 1.5° K and 40° K Blumberg and Peisach determined the zero-field splitting parameter $D = 1.5$ cm^{-1}. The Mössbauer data presented below are totally consistent with the zero-field splitting parameters reported in [5].

Low temperature Mössbauer spectra of ^{57}Fe-enriched protocatechuate 3,4-dioxygenase (~ 50μg ^{57}Fe/cm^2) taken in parallel applied fields of 600 gauss are shown in Fig. 2; the spectra were taken at 1.5° K and 4.2° K, respectively. The spectra can readily be understood by using the parameters D and λ determined by Blumberg and Peisach. The middle doublet is located 7.75° K above the ground doublet, i.e. at 1.5° K only the latter is populated (99.4%). Since the ground doublet has extremely anisotropic g-values the effective A-tensor associated with this doublet is also very anisotropic; the magnetic hyperfine field is along the y-direction for practically all molecular orientations. Such a situation gives rise to a Mössbauer spectrum with six fairly sharp absorption lines. From the total magnetic splitting we can readily compute $H_{sat} = (-525 \pm 15)$ kG; here we have used Eqn. 6 and the fact that the low-field resonance occurs at $g_y = 9.35$.

The EPR data place the middle doublet at 7.75° K above the ground state; accordingly at 4.2° K this state is populated to 13.3%. The Mössbauer spectrum taken at 4.2° K is shown in Fig. 2B. Since the middle doublet is magnetically rather isotropic it gives rise to a magnetic Mössbauer spectrum consisting of four lines in parallel applied field (the nuclear $\Delta m = 0$ transitions are strongly suppressed in parallel field). Moreover, since its g-values are only half as large as g_y of the ground state, Eqn. 6 predicts a corresponding reduction of the total magnetic splitting. The four absorption lines of the middle doublet are indicated by the arrows in Fig. 2B. By subtracting the 1.5° K spectrum from the 4.2° K data in such a way that the ground state Mössbauer spectrum is completely removed, the population of the ground doublet was found to be (88 ± 5)% which is, within the experimental uncertainties, in agreement with the EPR data of Blumberg and Peisach.

The electronic relaxation rate of the native enzyme at 200° K is still slow compared to the nuclear precession frequency and a quadrupole doublet is not observed. We have obtained, however, some limited information about the electric field gradient tensor from the low-temperature data. The hyperfine field associated with the ground Kramers doublet is directed along the y-direction and the Mössbauer spectrum therefore measures the component of the field gradient which is along the hyperfine field, V_{yy}. The 1.5° K spectrum does not exhibit any quadrupolar perturbation, i.e. $V_{yy} \simeq 0$. We have taken also spectra in the temperature range from 15° K to 25° K. At this temperature a third spectrum, due to the upper Kramers doublet, is observed. Eqns. 2 and 6 predict the hyperfine field of this doublet to be in the z-direction; hence it is sensitive to V_{zz}. The complexity of the data (three superimposed spectra) does not presently allow a reliable data decomposition. The data, however, show clearly that $V_{zz} > 0$.

2. Protocatechuate 3,4-dioxygenase reduced with dithionite

The Mössbauer spectrum of dithionite reduced protocatechuate 3,4-dioxygenase taken at 4.2° K consists of a symmetric quadrupole doublet with reason-

ably sharp lines (0.33 mm/s full width). the quadrupole splitting, ΔE_Q = (3.13 ± 0.02) mm/s, and the isomeric shift, δ = (1.21 ± 0.02) mm/s (relative to Fe metal), unambiguously establish the material to be in the high-spin ferrous state. ΔE_Q is independent of temperature (measured up to 200° K). We have just started a systematic investigation of the reduced enzyme using applied magnetic fields up to 60 kG. Such measurements yield information about the zero-field splitting, the magnetic hyperfine interaction, and details about the electric field gradient tensor [9,12]. This enzyme yields well resolved high-field spectra which are rich in information. Analysis of these data is presently in progress.

3. The ternary complex: protocatechuate 3,4-dioxygenase-3,4-dihydroxyphenylpropionate-O_2

Fujisawa and Hayaishi [4] have reported optical and kinetic data for a fairly long-lived oxygenated complex using the slow substrate 3,4-dihydroxyphenylpropionate. They showed that this complex, at 24° C, decays with a half-life of about 30 s and that this decay parallels product formation. Since such a complex is of great interest in understanding the catalytic mechanism, we have studied it with EPR and Mössbauer spectroscopy. EPR spectra taken at 12° K are displayed in Fig. 3. The upper trace shows a spectrum of the oxygenated complex frozen after steady state conditions had been achieved.

The most prominent features in Fig. 3A are strong resonances at g = 6.7 and g = 5.3 *. Such resonances can result from a high-spin ferric ion in an environment of almost axial symmetry (λ = 0.03 in Eqn. 2; they result commonly from the ground Kramers doublet of high-spin ferric heme proteins, for which $D >$ [5]). Fig. 4 shows a Mössbauer spectrum of an oxygenated sample prepared under the same conditions as the EPR sample for which the upper trace in Fig. 3 was obtained. The spectrum in Fig. 4 was taken at 4.2° K in a magnetic field of 600 gauss applied parallel to the Mössbauer radiation. The intensities of the six prominent lines did not change when the field was applied perpendicular to the Mössbauer radiation. This observation implies that this spectrum results from a Kramers doublet for which $g_x \simeq g_y << g_z$; such doublets yield either no EPR signal (if $g_x = g_y = 0$) or only extremely weak signals. On the other hand, a Kramers doublet with g-values at 6.7 and 5.3 has to yield a Mössbauer spectrum with intensities depending quite sensitively on the direction of the applied field (see for instance [8,9]). Thus the EPR and Mössbauer spectra result from two different Kramers doublets. These observations can be reconciled if we assume a high-spin ferric system with a negative zero-field splitting, i.e. $D < 0$ in Eqn. 2.

Fig. 5 shows an energy level diagram of the high-spin ferric system for $D < 0$ and λ = 0.03. We have labeled each doublet with the quantum numbers perti-

* Resonances around g = 9 and g = 4.3 seen in Fig. 3A reflect material not attributable to the ternary complex discussed here. We have noticed that all preparations we have examined yield this remnant absorption; we consider it likely that it reflects iron bound to non-specific, non-catalytic sites. Up to 17 more iron atoms can be bound by the enzyme; its catalytic activity, however, is increased only by 25% [15]. The additional iron atoms yield EPR absorption around g = 4.3 and g = 9.0. Notice that these minority species (about 20% of the iron present) are also observed in the Mössbauer spectrum shown in Fig. 5 (especially around −3.2 and +4.2 mm/s). For the preparations discussed in this paper we consistently obtained 7 ± 0.2 irons/molecule.

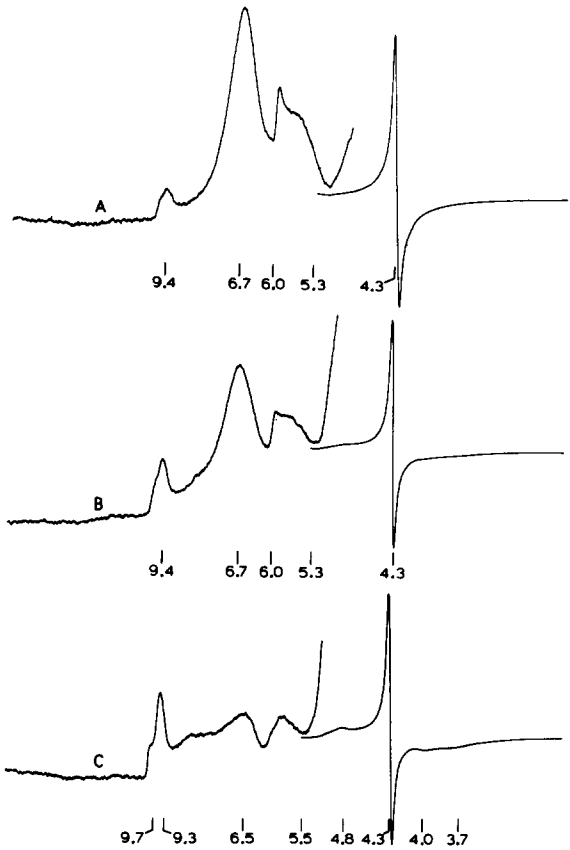

Fig. 3. EPR spectra of the ternary complex of protocatechuate 3,4-dioxygenase with 3,4-dihydroxyphenylpropionate and O_2. Conditions: $T = 12.3°K$; 9.196 GHz, 1 mW; modulation amplitude, 10 gauss; sweep rate, 1000 gauss per min; receiver gain, 3200 in the low-field region, 160 in the $g = 4.3$ region in (A) and 63 in (B) and (C). The magnetic field increases linearly to the right and selected values of the frequency-to-field ratio (g-value) are given on the abscissa. A. The sample was frozen after steady-state conditions had been achieved. B. The sample was frozen after the ternary complex had decayed for one half-life (4 min) as monitored by optical spectroscopy. C. The sample was frozen after the ternary complex had decayed for four half-lives.

nent to axial symmetry, i.e. $\lambda = 0$. For $\lambda = 0.03$ the doublets are still essentially pure $\pm 1/2$, $\pm 3/2$, and $\pm 5/2$ states. Likewise the energy separations are practically (to within 0.1%) $4D$ and $2D$, respectively. Our data can be explained on the basis of the diagram in Fig. 5, using $D \simeq -2\ \mathrm{cm}^{-1}$.

At 4.2° K practically only the $\pm 5/2$ doublet is populated. This doublet is EPR-silent and should give rise to a six-line Mössbauer spectrum with intensities of 3 : 2 : 1 : 1 : 2 : 3. These intensities have to be independent of the orientation of an applied magnetic field, which is indeed observed.

The $\pm 1/2$ doublet is predicted to give rise to intense resonances at $g = 6.7$ and $g = 5.3$. If the observed resonances originate indeed from a excited state, their temperature dependence can be described by the function

$$I(D,T) \propto \frac{1}{T} \frac{\exp(6D/kT)}{1 + \exp(4D/kT) + \exp(6D/kT)} \cdot \tag{8}$$

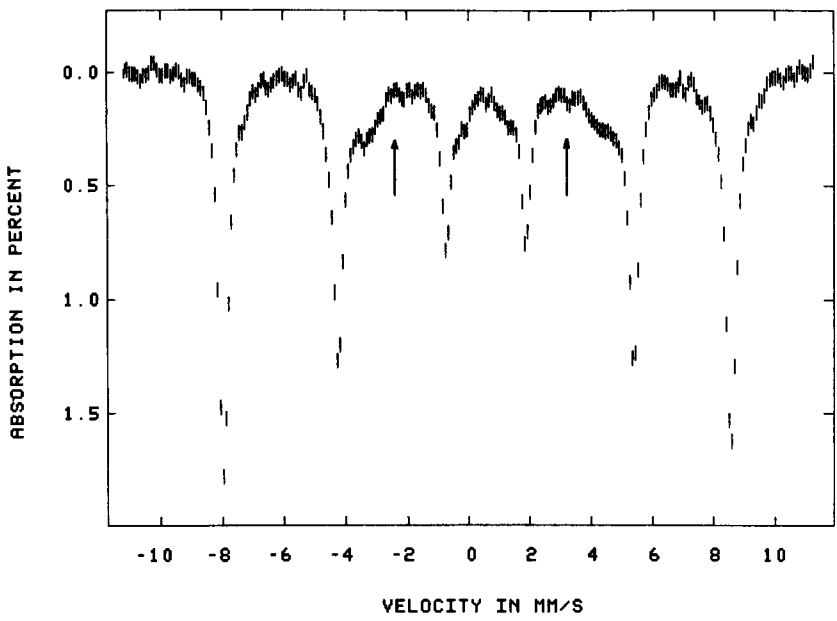

Fig. 4. Mössbauer spectrum of the ternary complex of protocatechuate 3,4-dioxygenase with 3,4-dihydrophenylpropionate and O_2 taken at 4.2°K in a 600 gauss parallel field. The prominent six-line pattern results from the ground Kramers doublet of the ternary complex. The residual absorption (20%) is due to species which give rise to EPR signals at $g \simeq 9$ and $g = 4.3$. The arrows indicate where the nuclear $\Delta m = 0$ transitions of the Mössbauer spectrum associated with the $\pm 3/2$ Kramers doublet should occur. Computer simulations indicate that the $\pm 3/2$ doublet is less than 6% populated at 4.2°K.

The $1/T$ dependence describes the population difference within the upper doublet; the exponential factor results from the Boltzmann distribution of the three doublets (Note that $D < 0$). Plotted as a function of temperature, the function $I(D,T)$ has a maximum at $T = 4.17D$ if D is measured in units of temperature. We have measured the area under the $g = 6.7$ resonance as a function of temperature (Fig. 6). The error bars indicated reflect essentially the uncertainties associated with establishing a reliable baseline under the $g = 6.7$ signal. The solid line in Fig. 6 was computed from Eqn. 8 using $D = -1.8$ cm^{-1}. Due to the large

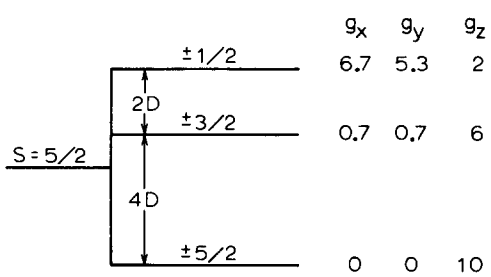

Fig. 5. Energy level diagram of an $S = 5/2$ system according to Eqn. 2 with $D < 0$, $\lambda = 0.03$, and $g_0 = 2.0$. The effective g-values computed from Eqn. 2 are given for each doublet.

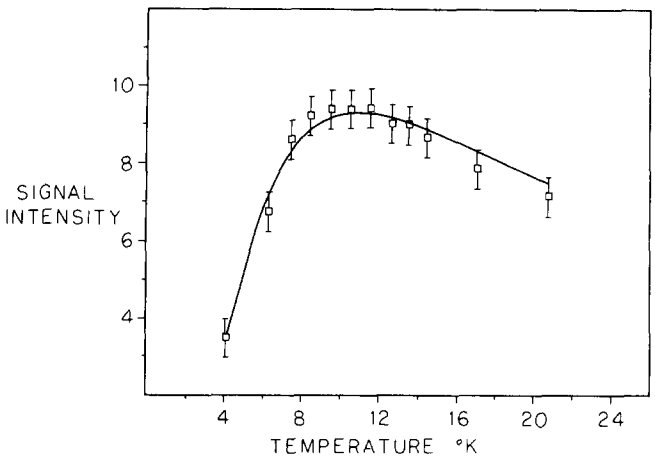

Fig. 6. The area (amplitude times width at half height) under the $g = 6.7$ resonance is plotted as a function of temperature. A representative spectrum is shown in Fig. 3A. The solid line was genearated from Eqn. 8 using $D = -1.8\ \text{cm}^{-1}$.

uncertainties the zero-field splitting parameter D cannot be restricted to better than $\pm 0.4\ \text{cm}^{-1}$. The margins of error, however, can be reduced if the Mössbauer data are taken into account.

We now discuss the Mössbauer spectrum shown in Fig. 4 in more detail. From the overall magnetic splitting we obtain the saturation field, $H_{sat} = -513 \pm 7$) kG. Note that the spacing between the two rightmost lines is somewhat narrower than that between the two leftmost lines. This means that the component of the electric field gradient tensor along the hyperfine field (which is along the z-direction since the dominant g-value of the ground doublet is $g_z = 10$) is negative; $eQV_{zz} = -0.5$ mm/s. Furthermore the isomeric shift is evaluated to be $\delta = (0.44 \pm 0.08)$ mm/s; this value is in a range typically found for high-spin ferric compounds.

The $\pm 3/2$ doublet is predicted to have resonances at $g_x = g_y = 0.7$ and $g_z = 6$. The EPR spectrum shown in Fig. 3A shows a fairly sharp resonance at $g \simeq 6$. We have used the procedure described by Aasa and Vänngård to estimate the intensity of the $g = 6$ resonance relative to the $g = 6.7$ resonance [16]; within the uncertainties the resonance at $g = 6$ has an intensity compatible with assigning it to the $\pm 3/2$ doublet. Within the framework of our spin Hamiltonian the Mössbauer spectrum associated with this doublet can be computed accurately from the parameters obtained from the ground doublet spectrum. This spectrum will have a six-line pattern like that for the ground doublet, the magnetic splitting scaled down, however, by the factor 6/10. Most conspicuously, the middle doublet should yield two peaks (the nuclear $\Delta m = 0$ lines) at +3.3 and −2.4 mm/s (indicated by arrows in Fig. 4). The absence of any discernible lines in the spectrum shown in Fig. 4 at these velocities suggests (a judgement based on computer simulations) that the middle doublet is populated less than 6% at 4.2°K. This observation implies that $|D| > 1.8\ \text{cm}^{-1}$; taken together, the

EPR and Mössbauer data suggest $D = (-2 \pm 0.2)\ cm^{-1}$ *.

To establish that these signals do indeed arise from the oxygenated intermediate, the time course of the $g = 6.7$ resonance must parallel the decay of the complex as observed by optical spectroscopy. Fig. 3 shows three representative EPR spectra taken under steady state conditions (A), after one half-life (4 min at 6° C, B) and after four half-lives (C), respectively. These spectra show that the decay of the $g = 6.7$ resonance correlates with the decay of the oxy complex. (Note that the signal at $g = 6$ follows the decay kinetics also.) Since the reaction mixture was O_2 limited and substrate binding is rapid [4], the EPR spectra shown in Fig. 3C should reflect an enzyme-substrate complex. Several distinct species are evident in this spectrum. Two species give rise to absorption around $g = 4.3$ (note the weak by broad side bands) and at $g = 9.3$ and $g = 9.7$). Another species gives rise to an almost continuous absorption from $g = 9$ to g 5. A spectrum similar to that in Fig. 3C is observed after anaerobic incubation of native enzyme with 3,4-dihydroxyphenylpropionate **.

We have also investigated complexes of the enzyme with the product, β-carboxy-*cis*,*cis*-muconate, and product analogues, such as glutarate and adipate. In no case do we observe EPR signals around $g = 6$. Thus, the resonance at $g = 6.7$, which arises from a high-spin ferric system with a large and negative zero-field splitting, is associated not with an enzyme-product complex, but with a ternary enzyme-substrate-oxygen complex.

Discussion

A knowledge of the charge and spin states of the iron, its ligand environment, and the modulating effects of substrates and inhibitors on these properties is critical for understanding the catalytic mechanism for protocatechuate 3,4-dioxygenase. Our EPR and Mössbauer investigation has yielded some parameters of the stable states of this enzyme and shown them to be unique among previously known biological iron complexes.

The zero-field splitting parameters for protocatechuate 3,4-dioxygenase and rubredoxin are remarkably similar. The saturation field obtained from the Mössbauer data for ferric enzyme (−525 kG), however, is in striking contrast to that found for rubredoxin (−370 kG). Therefore, Mössbauer spectroscopy shows that protocatechuate 3,4-dioxygenase has a ligand environment unlike rubre-

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* We have attempted to get more information about the middle doublet by taking a Mössbauer spectrum at 12° K. We have observed increasing absorption at velocities where the lines of the ±3/2 doublet should appear. However, resonance absorption of iron atoms not associated with the ternary complex also increases at this temperature due to the increased population of the doublet associated with the $g = 4.3$ signal and, hence, obscures the spectrum due to the ±3/2 doublet of the ternary complex. Furthermore, the electronic spin relaxation time of the iron in the ternary complex becomes shorter at 12° K, resulting in broadening of the absorption lines. This prevents us from decomposing the spectrum taken at 12° K by subtracting the absorption lines of the ±5/2 doublet.

** The Mössbauer spectrum of the enzyme-substrate complex reflects the same complexity as the EPR spectrum in Fig. 4C. The features around $g = 6.5$ and 5.5 appear to result from the ground Kramers doublet of a species with a positive zero-field splitting parameter D. Similar resonances have been reported for a complex of protocatechuate 3,4-dioxygenase with homocaffeic acid [5]. Presently, we do not understand why enzyme-substrate complexes exhibit a variety of species. Despite the complexities, the Mössbauer spectra clearly show that all the iron atoms remain in a high-spin ferric state.

doxin. Although chemical evidence for a cysteine residue near the iron site has been reported for the enzyme [15], the observed saturation field of −525 kG suggests that this residue is not coordinated to the iron. Saturation field data on high-spin ferric complexes having one sulfur coordination, such as cytochrome P-450 [17], Fe(PPIXDME)($SC_6H_4NO_2$) [18], Fe(Ph_2[16]N_4)(SCH_2Ph) [19], Fe(salen)(pyrr-dtc) [20], all reflect fields of approx. −450 kG. An octahedral oxygen coordination is also unlikely, since FeO_6 complexes have much smaller zero-field splitting parameters and somewhat larger saturation fields (see Oosterhuis [10] for a compilation of data).

The Mössbauer data obtained for the reduced protein further substantiates the dissimilarity of protocatechuate 3,4-dioxygenase with rubredoxin. The characteristic isomer shift for reduced rubredoxin (δ = 0.68 mm/s [11,12]) reflects its more covalent, tetrasulfur environment while that of protocatechuate 3,4-dioxygenase (δ = 1.21 mm/s) suggests a more ionic, oxygen-nitrogen type environment. (In contrast to other charge and spin states the isomeric shift of high-spin ferrous compounds is quite sensitive to the nature of the ligand environment [12]). Interestingly, the isomer shift of the reduced enzyme is quite close to that of deoxyhemerythrin (δ = 1.15 mm/s, ΔE_Q = 2.85 mm/s, [21]) suggesting that the irons in both proteins may reside in similar environments. X-ray diffraction studies of myohemerythrin [22] and methemerythrin [23] have been reported; both investigations implicate histidine and tyrosine residues in the iron coordination. This similarity between reduced protocatechuate 3,4-dioxygenase and deoxyhemerythrin may help explain the optical spectrum of the native enzyme.

Protocatechuate 3,4-dioxygenase exhibits an intense absorption near 460 nm ($\epsilon \simeq 3$ $mM^{-1} \cdot cm^{-1}$/Fe); this probably arises from a ligand-to-metal charge transfer transition since metal *d-d* transitions have much smaller extinction coefficients ($\epsilon \simeq 50$ $\mu M^{-1} \cdot cm^{-1}$). With cysteine very likely excluded as a ligand, the most probable candidate for the charge transfer band is tyrosine. Transferrin, an iron transport protein, exhibits an optical maximum at 470 nm (ϵ = 2.2 $mM^{-1} \cdot cm^{-1}$/Fe). Resonance Raman investigations on this protein showed enhancement of phenolate vibrational modes upon laser excitation within its visible absorption band [25], implicating tyrosine coordination to the iron. A resonance Raman study on this enzyme is in progress.

Absorbance spectra for oxygenated intermediates of protocatechuate 3,4-dioxygenase have been reported by Fujisawa et al. [4]. While the nature of such complexes has been extensively investigated for heme proteins, very little work has been published for such intermediates in dioxygenase reactions. Here we have characterized the ternary complex of native enzyme with 3,4-dihydroxyphenylpropionate and molecular oxygen by EPR and Mössbauer spectroscopy. Our investigation reveals a high-spin ferric complex whose electronic structure gives rise to a large and negative zero-field splitting. To our knowledge, such parameters have not been observed for any other iron protein. Although we do not yet fully understand the spectroscopic data obtained for enzyme-substrate complexes, it is quite clear that these complexes are characterized by large and positive zero-field splitting parameters. Thus oxygen binding causes a dramatic change in the electronic environment of the iron. This, however, does not necessarily imply that the oxygen molecule is directly coordinated to the

iron atom. The mode of oxygen binding in this ternary complex clearly needs further elucidation. Besides Mössbauer and EPR studies using different substrates, resonance Raman and electron nuclear double resonance experiments may give valuable clues to the nature of this catalytically important intermediate. Whatever the details of the reaction mechanism our spectroscopic investigations show that the electronic structure of the iron is affected by both substrate and subsequent oxygen binding; in all identified intermediates, however, the iron retains its high spin ferric character.

The iron environment in protocatechuate 3,4-dioxygenase is as yet unidentified though our data limits the possible ligands. The spectroscopic parameters obtained here differ in many respects from those found for other iron proteins and suggest a new mode of iron coordination in biological systems. Our data provides, therefore, a set of specific spectroscopic parameters that must be reflected by postulated dioxygenase model compounds.

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References

1 Fujisawa, H. and Hayaishi, O. (1968) J. Biol. Chem. 243, 2673—2681
2 Fujisawa, H., Uyeda, M., Kojima, T., Nozaki, M. and Hayaishi, O. (1972) J. Biol. Chem. 247, 4414—4421
3 Lipscomb, J., Howard, J., Lorsbach, T. and Wood, J. (1976) Fed. Proc. 35, 1536
4 Fujisawa, H., Hiromi, K., Uyeda, M., Okuno, S., Nozaki, M. and Hayaishi, O. (1972) J. Biol. Chem. 247, 4422—4428
5 Blumberg, W.E. and Peisach, J. (1973) Ann. N.Y. Acad. Sci. 222, 539—560
6 Tsai, R., Yu, C.A., Gunsalus, I.C., Peisach, J., Blumberg, W.E., Orme-Johnson, W.H. and Beinert, H. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 1157—1163
7 Wickman, H.H., Klein, M.P. and Shirley, D.A. (1966) Phys. Rev. 152, 345—357
8 Lang, G., Aasa, R., Garbett, K. and Williams, R.J.P. (1971) J. Chem. Phys. 55, 4539—4548
9 Münck, E. and Champion, P.M. (1974) J. Phys. (Paris) Colloque C6, Suppl. 12, 33—46
10 Oosterhuis, W.T. (1974) Struct. Bonding 20, 59—99
11 Rao, K.K., Evans, M.C.W., Cammack, R., Hall, D.O., Thompson, C.L., Jackson, P.J. and Jonhson, C.E. (1972) Biochem. J. 129, 1063—1070
12 Debrunner, P.G., Münck, E., Que, Jr., L. and Schulz, C.E. (1976) in Iron-Sulfur Proteins (Lovenberg, W., ed.), Vol. 3, pp. 381—417, Academic Press, New York
13 Lane, R.W., Ibers, J.A., Frankel, R.B. and Holm, R.H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2868—2872

14 Orme-Johnson, W.H. and Sands, R.H. (1973) in Iron-Sulfur Proteins (Lovenberg, W., ed.), Vol. 2, pp. 195—238, Academic Press, New York
15 Fujiwara, M. and Nozaki, M. (1973) Biochim. Biophys. Acta 327, 306—312
16 Aasa, R. and Vänngård, T. (1975) J. Magn. Res. 19, 308—315
17 Sharrock, M., Münck, E., Debrunner, P.G., Marshall, V., Lipscomb, J.D. and Gunsalus, I.C. (1973) Biochemistry 12, 258—264
18 Koch, S., Tang, S.C., Holm, R.H., Frankel, R.B. and Ibers, J.A. (1975) J. Am. Chem. Soc. 97, 916—918
19 Koch, S., Holm, R.H. and Frankel, R.B. (1975) J. Am. Chem. Soc. 97, 6714—6723
20 Petridis, D., Simopoulos, A. and Kostikas, A. (1971) Phys. Rev. Lett. 27, 1171—1173
21 Okamura, M.Y. and Klotz, I.M. (1973) in Inorganic Biochemistry (Eichhorn, G.L. ed.), pp. 320—343, Elsevier, Amsterdam
22 Hendrickson, W.A., Klippenstein, G.L. and Ward, K.B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2160—2164
23 Stenkamp, R.E., Sieker, L.C. and Jensen, L.H. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 349—351
24 Gaber, B.P., Miskowski, V. and Spiro, T.G. (1974) J. Am. Chem. Soc. 96, 6868—6873