Kinetic and Mössbauer Studies on the Mechanism of Protocatechuic Acid 4,5-Oxygenase†

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ABSTRACT: Protocatechuic acid 4,5-oxygenase (PCA-4,5-oxygenase) has been purified from cell extracts of *Pseudomonas testosteroni* grown with *p*-hydroxybenzoic acid as sole carbon and energy source. Substrate specificity studies with catechols indicate that the carboxylate group and the hydroxyl groups are essential for cleavage of the aromatic ring. Substitution at C_5 of the benzene ring is allowed because gallic acid ($K_m = 7.35 \times 10^{-5}$ M) and 5-methoxygallic acid ($K_m = 12.5 \times 10^{-5}$ M) function as alternative substrates to protocatechuic acid ($K_m = 4.6 \times 10^{-5}$ M). PCA-4,5-oxygenase is inhibited competitively by substrate analogs protocatechualdehyde ($K_I = 9.6 \times 10^{-4}$ M) and 4-nitrocatechol ($K_I = 5.6 \times 10^{-4}$ M). Competitive inhibition is observed with sulfhydryl inhibitors. PCA-4,5-oxygenase contains four iron atoms and two free sulfhydryl groups per molecule (mol wt 140,000). The coordination complex formed between PCA-4,5-oxygenase apoenzyme and iron is quite unstable, and the iron is easily removed from the enzyme. Removal of the iron causes a total loss of catalytic activity. Restoration of enzyme activity can be accomplished by preincubating PCA-4,5-oxygenase apoenzyme with ferrous ions in the presence of cysteine. Mössbauer studies on $^{57}Fe^{2+}$-reconstituted PCA-4,5-oxygenase show that all four iron atoms occupy similar but not identical sites. Two assignments for the charge and spin states are compatible with the Mössbauer parameters. (a) The iron atoms are in a low-spin ferrous state. (b) The enzyme has two active sites, each containing two antiferromagnetically coupled high-spin ferric ions. Kinetic and chemical evidence favor the latter interpretation.

Upon incubation of PCA-4,5-oxygenase[1] with the substrates protocatechuic acid, gallic acid, and 5-methoxygallic acid, respectively, the irons become high-spin ferric ($S = 5/2$). This change at the active site is independent of the presence of oxygen, but absolutely dependent on the addition of each respective catechol substrate. The enzyme–substrate complexes show an electron spin resonance (esr) signal at $g = 4.3$. This signal disappears when the enzyme has converted the substrate to product. Preincubation of PCA-4,5-oxygenase with the competitive inhibitors protocatechualdehyde or 4-nitrocatechol also gives an esr signal at $g = 4.3$. These data indicate that the two inhibitors form a complex with high-spin Fe^{3+} at the active site. For the PCA-4,5-oxygenase–protocatechualdehyde complex, Mössbauer data show that all four iron atoms are high-spin ferric and equivalent. The internal magnetic field $H_{int} = -(551 \pm 15)$ kG shows that the bonding to high-spin ferric is highly ionic. The zero-field-splitting tensor which describes the splitting of the ^6S ground state is characterized by $E/D \approx 1/3$ and $D = (0.7 \pm 0.3)$ cm^{-1}. Reduction of the protocatechualdehyde–PCA-4,5-oxygenase complex with sodium borohydride (T_4) gives significant covalent incorporation of tritium into the enzyme–inhibitor complex. This tritium incorporation is dependent on the presence of protocatechualdehyde and suggests the formation of an enamine derivative from the reduction of the Schiff's base formed by a reaction of the aldehyde functional group with an ϵ-amino group of lysine.

Although the properties of PCA[1]-3,4-oxygenase have been documented in detail by Hayaishi (1966), little progress has been made until recently on the purification and properties of PCA-4,5-oxygenase. Dagley and Patel (1957) first demonstrated that cleavage of protocatechuic acid occurred adjacent to the two hydroxyl groups to give a semialdehyde product. Subsequently Dagley *et al.* (1960), Ribbons and Evans (1962), and Dagley *et al.* (1964) worked out the details of this reaction by proving that 4,5 fission of the benzene ring occurred to give α-hydroxy-γ-carboxy-*cis,cis*-muconic semialdehyde as the ring fission product. The cyclization of α-hydroxy-γ-carboxy-*cis,cis*-muconic semialdehyde with ammonium ions to give 2,4-lutidinic acid as product helped to establish the position of ring cleavage (Scheme I). Dagley and Patel (1957) showed that Fe^{2+} was required as the sole cofactor for PCA-4,5-oxygenase. Wheelis *et al.* (1967) reported that all attempts to purify PCA-4,5-oxygenase proved unsuccessful because of the extreme instability of this enzyme. However, in 1968 Dagley *et al.* purified the enzyme to homogeneity (mol wt 140,000). PCA-4,5-oxygenase was found to be unstable upon dilution, but this inactivation was shown to be partially prevented by L-cysteine (Dagley *et al.*, 1968) and completely prevented by the inclusion of 5 mM L-cysteine and 10% glycerol in buffer solutions for the enzyme (Ganguli *et al.*, 1970). This communication deals with further studies on the specificity and mechanism of PCA-4,5-oxygenase with special emphasis on the role of iron in this dioxygenase reaction.

Materials and Methods

Culture Methods and Purification of PCA-4,5-Oxygenase. *Pseudomonas testosteroni* was grown with *p*-hydroxybenzoic acid as sole carbon and energy source on the medium described by Dagley *et al.* (1968). Cell extracts were prepared by subjecting a suspension of cells (1 g wet weight of cells/2.0 ml of 0.05 M KH_2PO_4 buffer at pH 7.3) to the maximum output of a Branson sonic probe for 5 min at 0°. PCA-4,5-oxygenase was purified to constant specific activity by the pro-

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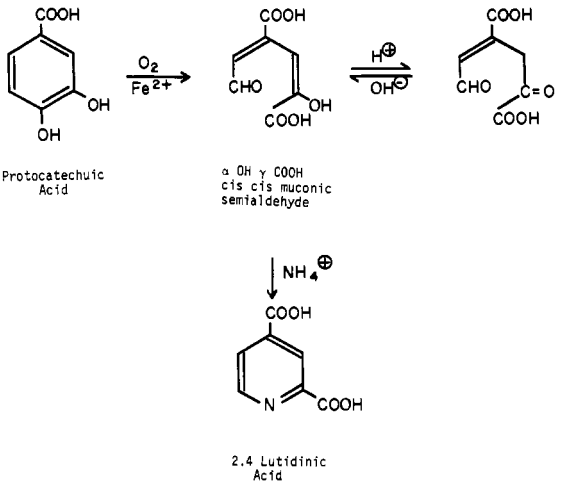
[1] Abbreviations used are: PCA, protocatechuic acid; TPTZ, 2,4,6-tripyridyl-*s*-triazine.

SCHEME I



cedure outlined by Dagley *et al.* (1968). The addition of 5 mM L-cysteine and 10% glycerol to 0.05 M KH_2PO_4 buffer (pH 7.3) protects the enzyme during purification. Therefore a Sephadex G-200 column (52 × 2.5 cm) was used routinely as a final step to ensure homogeneity of the enzyme. (Sedimentation coefficient at 20° = 2.5×10^{-13} sec, and the diffusion coefficient for 8.0 mg of protein = 3.4×10^{-7} cm^2 sec^{-1}.) Assuming a partial specific volume of 0.73, then the molecular weight of PCA-4,5-oxygenase was confirmed to be 140,000.

Enzyme Assays. PCA-4,5-oxygenase was assayed by measuring the rate of oxygen uptake with a YSI Clark oxygen electrode assembly on a Gilson Model K oxygraph. The reaction vessel was jacketed to maintain a constant temperature of 30°. The reaction volume for each assay was 1.5 ml.

Determination of the Total Iron Content of PCA-4,5-Oxygenase. A modification of the method of Fischer and Price (1964) was used to determine the number of atoms of iron present in PCA-4,5-oxygenase. Glass-distilled water was distilled twice more and a calibration graph was prepared for iron using ferrous ammonium sulfate·$6H_2O$ as a standard. This calibration involves the formation of a chromophore with 2,4,6-tripyridyl-*s*-triazine (TPTZ). The iron complex formed with Fe^{2+} has a λ_{max} at 593 nm with an extinction coefficient of ϵ_{593} = 22,600 $mole^{-1}$ cm^{-1}. Different concentrations of pure PCA-4,5-oxygenase (from 3 to 25 nmoles of Fe^{2+}) were diluted to 0.4 ml with twice-distilled water, and 0.05 ml of 8 N HCl was added to each solution. The solution was allowed to stand for 10 min with occasional agitation, then 0.05 ml of 80% trichloroacetic acid was added and the precipitated protein was removed by centrifugation. The clear supernatant (0.4 ml) was transferred to an iron-free glass tube and 0.1 ml of 75% ammonium acetate and 0.04 ml of 10% hydroxylamine hydrochloride (an iron-free solution obtained from G. F. Smith Chemical Co.) was added with mixing. Then 0.04 ml of 4.0 mM TPTZ was added with mixing and the solution was allowed to stand for 10 min before reading the absorbance at 593 nm. For PCA-4,5-oxygenase 3.8–4.2 g-atoms of iron/molecule of enzyme was routinely determined.

Reconstitution of PCA-4,5-Oxygenase Apoenzyme with $^{57}Fe^{2+}$. Pure PCA-4,5-oxygenase was taken and 85% of the iron was removed from the protein by passing a 1.5-ml solution of enzyme (40 mg/ml) down a Chelex-100 column (Bio-Rad analytical grade chelating resin 200–400 mesh). The resulting apoenzyme was eluted from the column with 0.05 M KH_2PO_4 buffer (pH 7.3). The eluate was concentrated to 1.75 ml in an Amicon ultrafiltration cell fitted with a PM_{10} membrane. The resulting 1.75 ml of apoenzyme was passed down a second Chelex-100 column (1.5 × 15 cm) and concentrated to a volume of 1.5 ml. This procedure removed 85% of the total iron from PCA-4,5-oxygenase. $^{57}Fe^{2+}$ was incorporated into PCA-4,5-oxygenase apoenzyme by preincubating a tenfold excess of 90% enriched $^{57}Fe^{2+}$ (40.8 μatoms/ml) for 24 hr, with 40 mg of apoenzyme in a total volume of 1.15 ml of 0.05 M KH_2PO_4 buffer which contained 10% glycerol and 5 mM L-cysteine at pH 7.3. This reconstituted enzyme was passed down a Sephadex G-25 column (1.8 × 25 cm). The eluate from the Sephadex G-25 column was concentrated to 50 mg/ml and the total iron concentration in this preparation was determined as 4.05 g-atoms of iron/molecule of enzyme.

Preparation of PCA-4,5-Oxygenase for Esr Experiments. Homogeneous PCA-4,5-oxygenase (72 mg in 3.0 ml) was passed down a Sephadex G-25 column (1.5 × 16 cm) to remove any extraneous iron. The column was generated with 0.05 M KH_2PO_4 buffer which contained 10% glycerol and 5 mM L-cysteine (pH 7.3), and the enzyme was eluted from the column with the same buffer. The eluted protein was concentrated to 66 mg/ml for the esr experiments. The iron content of this protein was determined at 3.9 g-atoms of iron/molecule.

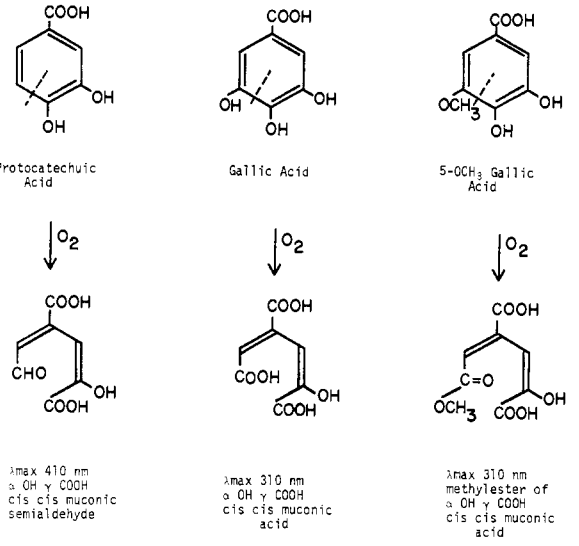
Determination of Free Sulfhydryl Groups on PCA-4,5-Oxygenase. The number of free sulfhydryl groups present in PCA-4,5-oxygenase was determined by reacting an excess of [1-^{14}C]iodoacetamide (58.3 mCi/mole) with different aliquots of enzyme. This enzyme preparation was purified without L-cysteine added to buffer systems. Enzyme was passed down a Sephadex G-25 column (25 × 1.5 cm) to remove excess [1-^{14}C]iodoacetamide, then 50-μl samples of enzyme were counted in a scintillation fluid for aqueous samples described by Wood *et al.* (1965). The scintillation counting was done on a Beckman LS-133 liquid scintillation system.

Spectroscopic Methods. Uv–visible spectra of derivatives were recorded with a Beckman DB-G spectrophotometer. Molar extinction coefficients were measured with a Carl Zeiss PMQ II spectrophotometer. Electron spin resonance studies at 77°K were performed on a Varian V-4502 electron paramagnetic resonance spectrometer operating in the X-band.

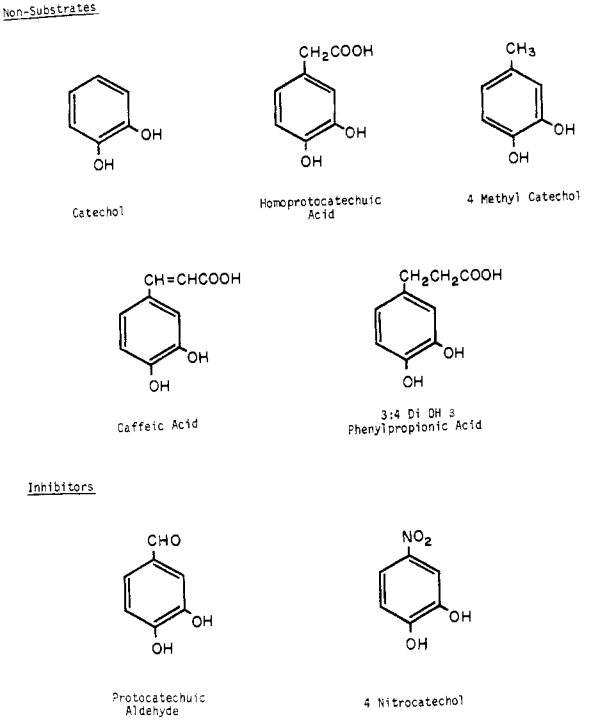
The Mössbauer spectrometer was of the constant acceleration type. A 30-mCi source of ^{57}Co in copper was used which gave a minimum observable line width (full width at half-maximum) of 0.22 mm/sec. The system was calibrated with a metallic iron absorber; the isomeric shifts are listed with respect to this standard. A Janis variable-temperature cryostat was used for the measurements. The samples were inserted from the top and γ rays passed horizontally through the samples *via* two pairs of mylar windows. A pair of coils were mounted around the tail section to produce a moderate parallel magnetic field at the sample.

For measurements at helium temperatures a transverse magnetic field of some hundred gauss was produced by sandwiching the sample container (nylon) between two niobium washers; these can be energized at higher temperatures by a permanent magnet mounted around the tail section of the cryostat. As the temperature is lowered the niobium rings become superconducting trapping the magnetic flux when the permanent magnet is removed.

SCHEME II: Substrates and Position of Ring Cleavage by PCA-4,5-oxygenase.



SCHEME III: Specificity of PCA-4,5-oxygenase.



The ^{57}Fe-enriched protein was prepared as described earlier. The sample for the native PCA-4,5-oxygenase contained about 20 mg of protein in 0.3 ml, and the sample for the protocatechualdehyde–inhibitor complex contained about 40 mg of protein (~30 μg of ^{57}Fe/cm^2) in 0.8 ml.

Materials

Synthesis of 5-Methoxygallic Acid. 5-Methoxygallic acid was synthesized by the method of Jurd (1959). The product was characterized by mp 220° and by CH analysis. *Anal.* Calcd for $C_8H_8O_5$: C, 52.5; H, 4.40 Found: C, 50.22; H, 4.67. Protocatechuic acid and protocatechualdehyde were obtained from J. T. Baker Chemical Co. Protocatechualdehyde was recrystallized from toluene. 4-Nitrocatechol, caffeic acid, and *p*-chloromercuribenzoic acid were obtained from the Aldrich Chemical Co. Homoprotocatechuic acid, 4-methylcatechol, and methylgallate were obtained from K & K Laboratories. 3,4-Dihyxroxy-β-phenylpropionic acid was obtained from G. F. Smith Chemical Co. [1-^{14}C]Iodoacetamide (58.3 Ci/mole) was obtained from Amersham-Searle, and sodium borotritide (25 mCi/mole) was obtained from New England Nuclear Corp.

Results

PCA-4,5-Oxygenase Substrate Specificity. When it was discovered that gallic acid and 5-methoxygallic acid function as substrates with PCA-4,5-oxygenase, then it was of interest to determine whether cleavage of the benzene nucleus still takes place at the 4,5 position with these substrates. Adachi *et al.* (1964) reported on the spectral properties of α-hydroxy-*cis,cis*-muconic acid showing that the λ_{max} for this compound was 295 nm (log ϵ 4.06) at pH 7.0 shifting to 350 nm (log ϵ 4.43) at pH 12.4. This spectral shift is due to base promoted ionization of the enol tautomer of α-hydroxy-*cis,cis*-muconic acid. The ring fission products formed when PCA-4,5-oxygenase cleaves either gallic acid or 5-methoxygallic acid both gave a λ_{max} at 310 nm (log ϵ 4.00) at pH 12.4. Base-catalyzed ionization of the enol product formed by cleavage of 5-methoxygallic acid by PCA-4,5-oxygenase could only occur if the position of cleavage of the benzene nucleus was 4,5. Furthermore, Dagley *et al.* (1960) showed that the enol form of α-hydroxy-γ-carboxy-*cis,cis*-muconic semialdehyde (λ_{max} 410 nm) would rapidly cyclize with NH_4^+ to give 2,4-lutidinic acid as product (λ_{max} 272 nm). No cyclization reaction occurred with NH_4^+ with the products formed from gallic acid cleavage by the enzyme. A summary of the position of cleavage of the three substrates is presented in Scheme II. A thorough chemical characterization of the reaction product from gallic acid cleavage has been worked out recently by Tack *et al.* (1972). K_m values of 4.6×10^{-5}, 7.35×10^{-5}, and 12.5×10^{-5} M were obtained for protocatechuic acid, gallic acid, and 5-methoxygallic acid, respectively. The substrate analogs catechol, 4-methylcatechol, homoprotocatechuic acid, caffeic acid, and 3,4-dihydroxy-β-phenylpropionic acid were tested with PCA-4,5-oxygenase and shown to function neither as substrates nor inhibitors (Scheme III). Dagley *et al.* (1968) tested vanillic acid as a substrate and showed no activity indicating that the hydroxyl group cannot be replaced by a methoxyl group at C_3. Therefore, hydroxyl groups must occupy C_3 and C_4 of the benzene ring for the enzyme to function. Also, this study shows an absolute requirement for the presence of a carboxylate substituent at C_1 of the aromatic ring; even the presence of carboxymethyl or carboxyethyl substituents do not allow correct binding to the enzyme active site for either catalysis or inhibition.

Catechol Inhibitors of PCA-4,5-Oxygenase. Protocatechualdehyde and 4-nitrocatechol are good competitive inhibitors of PCA-4,5-oxygenase (Figure 1), giving K_I values of 9.6×10^{-4} and 5.6×10^{-4} M, respectively. For protocatechualdehyde use can be made of the aldehyde functional group to determine whether an interaction with an ϵ-amino group of lysine can occur at the active site. Equal aliquots of PCA-4,5-oxygenase were taken (10.5 mg of protein) and to one aliquot 1.0 ml of protocatechualdehyde (10 mM) was added to give a total volume of 2.0 ml in 0.05 M KH_2PO_4 buffer

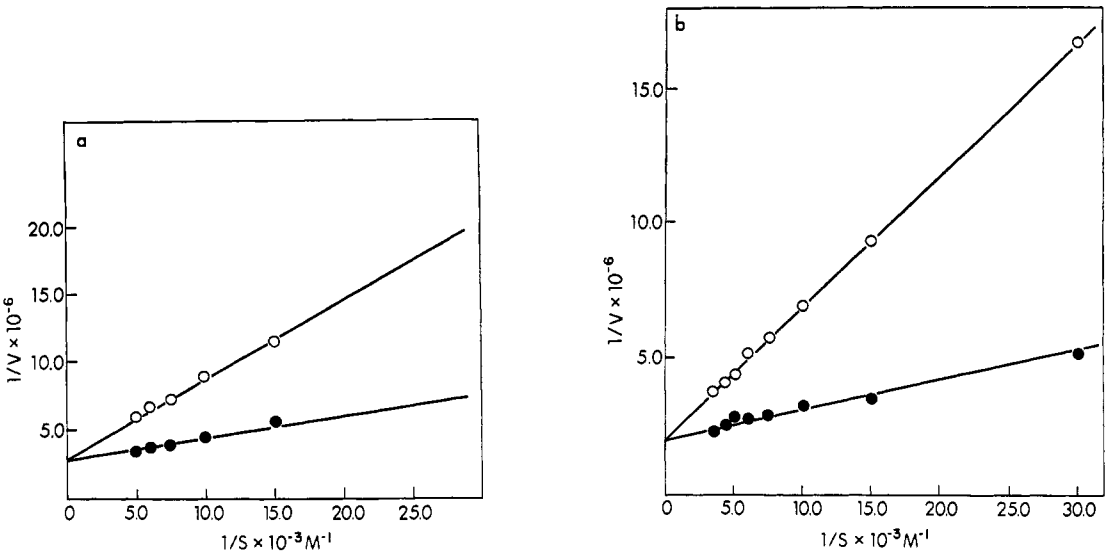


FIGURE 1: Competitive inhibition of PCA-4,5-oxygenase with substrate analogs. (a) Protocatechualdehyde and (b) 4-nitrocatechol.

(pH 7.3) containing 10% glycerol and 5 mM L-cysteine. The second aliquot was diluted with the same buffer to 2.0 mg, except that no protocatechualdehyde was added. After equilibration, 2.0 mg of sodium borohydride (T_4) (25 Ci/mole) was added to both the reaction and the control, and reduction was allowed to proceed for 24 hr at 0°. The two reduced samples were dialyzed for 48 hr against 3.5 l. of 0.05 M KH_2PO_4 buffer (pH 7.3) containing 10% gycerol and 5 mM L-cysteine. The buffer was changed every 8 hr. After this treatment 50-μl samples of the PCA-4,5-oxygenase–protocatechualdehyde complex and the PCA-4,5-oxygenase control were taken and counted for the covalent incorporation of tritium. From this experiment an average of 5.6×10^{-8} mole of tritium was covalently incorporated into 4.6×10^{-8} mole of PCA-4,5-oxygenase–protocatechualdehyde complex. For the control reactions 0.92×10^{-8} mole of tritium was incorporated into 4.6×10^{-8} mole of PCA-4,5-oxygenase. This experiment suggests that protocatechualdehyde forms a Schiff base with an ϵ-amino group of lysine allowing sodium borotritide reduction of this complex to give a enamine derivative with covalent incorporation of tritium.

Involvement of Sulfhydryl Groups at the Active Site of PCA-4,5-Oxygenase. *p*-Chloromercuribenzoic acid is an excellent competitive inhibitor of PCA-4,5-oxygenase. The implication that free sulfhydryl groups are present at the active site of PCA-4,5-oxygenase was confirmed and quantitated by a reaction with [^{14}C]iodoacetamide. Preincubation of PCA-4,5-oxygenase with iodoacetamide completely inhibits enzyme activity. To determine the number of free sulfhydryl groups in the enzyme 0.25 ml of [^{14}C]iodoacetamide (58.3 Ci/mole) was dissolved in 1.0 ml of water and 0.5 ml of this reagent was reacted with 1.0 ml of native homogeneous PCA-4,5-oxygenase (6.9 mg/ml). After equilibration, preincubation, and determination of total loss of enzyme activity, the enzyme–inhibitor complex was passed down a Sephadex G-25 column (25 × 1.5 cm) to remove the excess [^{14}C]iodoacetamide. The specific activity of the resulting ^{14}C-labeled iodoacetamide–PCA-4,5-oxygenase complex was determined and it was found that 2.7×10^{-8} mole of [^{14}C]iodoacetamide was incorporated into 1.3×10^{-8} mole of enzyme. This experiment was repeated with several different enzyme preparations and also with the protocatechualdehyde–PCA-4,5-oxygenase complex. Based on these experiments homogeneous PCA-4,5-oxygenase was shown to contain 1.8–2.2 free sulfhydryl groups/enzyme molecule.

Electron Spin Resonance Studies with PCA-4,5-Oxygenase. When PCA-4,5-oxygenase reacts with protocatechuic acid, gallic acid, or 5-methoxygallic acid, the esr can be used to demonstrate that each catechol substrate generates a typical high-spin Fe^{3+} signal at $g = 4.3$. (Figure 2). The appearance of this esr signal is dependent on the presence of the respective catechol substrate, and independent of the presence of molecular oxygen. When each of the three substrates tested are converted to corresponding reaction products, the $g = 4.3$ signal disappears. These data show that each substrate

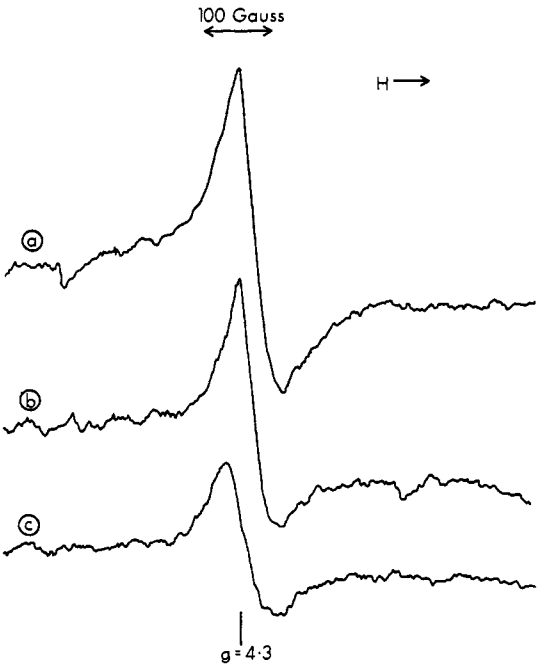


FIGURE 2: Electron spin resonance spectra of PCA-4,5-oxygenase in the presence of excess substrate. (a) Protocatechuic acid, (b) gallic acid, and (c) 5-methoxygallic acid. Spectra were recorded at 77° K.

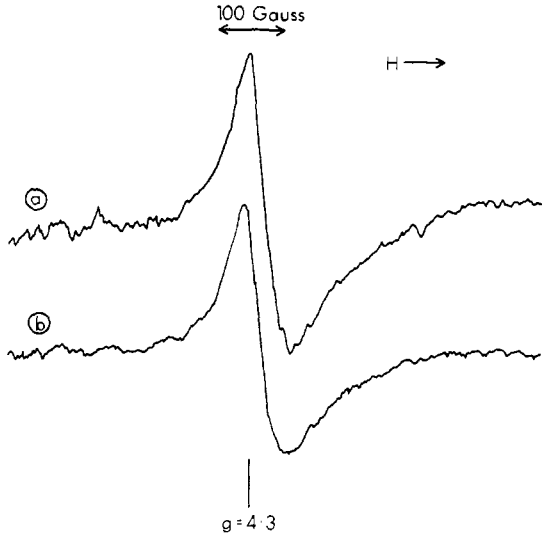


FIGURE 3: Electron spin resonance spectra of PCA-4,5-oxygenase in the presence of inhibitors. (a) Protocatechualdehyde and (b) 4-nitrocatechol. Spectra were recorded at 77° K.

reacts with the iron at the active site of PCA-4,5-oxygenase to generate a paramagnetic high-spin Fe^{3+} coordination complex.

Examination of the formation of the enzyme–inhibitor complexes formed with protocatechualdehyde and 4-nitrocatechol by esr shows that each inhibitor forms a high-spin Fe^{3+} complex with PCA-4,5-oxygenase (Figure 3). The formation of this high-spin Fe^{3+} complex with the inhibitors is dependent on the presence of each inhibitor, but independent of the presence of oxygen. The formation of high-spin Fe^{3+} PCA-4,5-oxygenase–inhibitor complexes lends support to the kinetic data which shows that both protocatechualdehyde and 4-nitrocatechol are competitive for protocatechuic acid. Furthermore, with the PCA-4,5-oxygenase–inhibitor complexes the $g = 4.3$ signal increases to a maximum intensity on addition of either protocatechualdehyde or 4-nitrocatechol, whereas with the three substrates, the signal increases in intensity until the respective substrate is depleted, at which time the $g = 4.3$ signal disappears.

Native Enzyme. Mössbauer spectra of the native protein were taken at 4.2°K and 100°K. They consist of a pair of quadrupole split lines of equal intensities with a separation of about 0.6 mm/sec. At 4.2°K velocities up to ±7 mm/sec were used to look for additional absorption lines, but none were found. The spectrum taken at 100°K is shown in Figure 4. Application of a magnetic field of 150 G had no effect on the spectra. The quadrupole splitting ΔE_Q and the isomeric shift δ (except for a second-order Doppler shift) are independent of temperature. The observed isomeric shift, the magnitude of ΔE_Q and its temperature independence exclude a high-spin ferrous or a low-spin ferric assignment for the four iron atoms.

The observed isomeric shift and quadrupole splitting do not rule out the possibility that the iron atoms are high-spin ferric ($S = 5/2$), though an unusually fast spin relaxation would be required at 4.2°K to average out the magnetic hyperfine interactions. To check on this possibility, the magnetic susceptibility[2] of unenriched native enzyme was measured between 1.5°K and 4.2°K. The sample showed some paramagnetism, but even if all the paramagnetism is attributed to iron in a high-spin ferric state, not more than 10% of the enzyme's total iron content can be high-spin ferric ($S = 5/2$).

When the sample used for the susceptibility measurements was checked with esr spectroscopy, a weak high-spin ferric signal at $g = 4.3$ and a weak signal typical of iron in a low-spin ferric state were observed. A quantitative estimate revealed that less than 10% of the iron in the sample contributed to the esr absorption, which is in agreement with the susceptibility measurements. The appearance of a high-spin ferric signal in fact is not surprising since the purified native enzyme still contains traces of the product, α-hydroxy-γ-carboxy-*cis,cis*-muconic semialdehyde, a weak competitive inhibitor which can complex with the iron to form a high-spin ferric complex. The nature of the low-spin ferric signal is not clear. It is, however, easily saturated at 40°K. This implies that the relaxation time at 4.2°K is long enough that magnetic splitting should be observed in the Mössbauer spectrum. It is, therefore, very likely that the low-spin ferric signal represents a small iron impurity not removed in the purification procedure. We conclude that a high-spin ferric ($S = 5/2$) assignment must be excluded. Thus, it appears that the irons are in a diamagnetic complex.

The absorption lines of the Mössbauer spectrum in Figure 4 have a width (full width at half-maximum) of about 0.45 mm/sec, much larger than the widths of 0.28–0.30 mm/sec which we have observed in other proteins with iron in a diamagnetic complex. The probable explanation for the broader lines is that the four iron atoms give rise to slightly different quadrupole spectra. The solid line in Figure 4 is the result of a least-squares fit to the data assuming two quadrupole doublets of equal intensities.[3] The line width of about 0.35 mm/sec necessary to fit the data is still a little bit large, but the computer fits are satisfactory. The four lines fitted to the 100°K data may be assigned in two ways (the isomeric shift δ is referred to iron metal):

	Site A	Site B
Case 1	$\Delta E_Q = 0.80$ mm/sec	$\Delta E_Q = 0.45$ mm/sec
	$\delta = 0.49$ mm/sec	$\delta = 0.52$ mm/sec
Case 2	$\Delta E_Q = 0.64$ mm/sec	$\Delta E_Q = 0.63$ mm/sec
	$\delta = 0.42$ mm/sec	$\delta = 0.60$ mm/sec

On the basis of these data alone, no choice can be made between the two sets of parameters. It is clear, however, that the data have to be explained as a superposition of at least two slightly different doublets. This could mean that the iron atoms occupy structurally inequivalent sites.

As discussed above, the susceptibility data eliminate a high-spin ferric ($S = 5/2$) assignment for the iron atoms. Thus, it appears from the Mössbauer data that the irons are low-spin ferrous ($S = 0$). However, this is not the only assignment compatible with the Mössbauer and susceptibility data. In recent years, many proteins have been studied with active sites involving spin-coupled pairs (Dunham *et al.*, 1972; Münck *et al.*, 1972; Dawson *et al.*, 1972). In these proteins two high-spin ferric atoms are antiferromagnetically coupled

[2] We are indebted to Dr. Tom Moss, Thomas J. Watson Research Center, Yorktown Heights, New York, N. Y., for conducting the magnetic susceptibility experiments reported in this manuscript.

[3] It is in principle justified to fit eight lines with equal intensities to the data (four sites each giving rise to a quadrupole doublet), but this is not a very meaningful procedure, since at least 18 free parameters are involved.

to give a diamagnetic ground state. A similar assignment for PCA-4,5-oxygenase would agree with the susceptibility data and would explain why no magnetic hyperfine interactions are observed in the Mössbauer spectra.

Protocatechualdehyde–PCA-4,5-Oxygenase Complex. The esr experiments show a signal at $g = 4.3$ when PCA-4,5-oxygenase is incubated with protocatechualdehyde. Some important questions can be answered by investigating the inhibitor complex with Mössbauer spectroscopy. Are all four iron sites affected by incubation with protocatechualdehyde? If so, are the four sites equivalent?

Mössbauer spectra taken at 4.2°K in parallel and transverse magnetic fields are shown in Figures 5 and 6. For both spectra about 10^7 counts/channel was accumulated with a running time of about 75 hr for each spectrum. The spectra show a complex hyperfine pattern typical for iron in a high-spin ferric state. Attempts to measure the quadrupole splitting, ΔE_Q, at 230°K failed; the spin relaxation is still too long for the quadrupole splitting to be observed.

Even without a detailed analysis of the spectra, it is obvious that all four iron atoms are affected by inhibitor binding. The quadrupole pattern observed in the native enzyme has completely disappeared. (Since the sample used to study the inhibitor complex contained about twice as much protein as the sample for the native enzyme, one iron site left unconverted upon incubation with protocatechualdehyde should give a quadrupole doublet with at least 0.75% resonance absorption). In the following discussion we will show that all four irons are high-spin ferric and equivalent.

A spin Hamiltonian sufficient to describe the Mössbauer spectra of the high-spin ferric ion ($S = {}^5/_2$) in an applied magnetic field $\mathbf{H}$ is

$$\mathcal{H} = 2\beta\mathbf{H}\cdot\mathbf{S} + D\left[S_z^2 - \frac{1}{3}S(S+1) + \frac{E}{D}(S_x^2 - S_y^2)\right] + A_0\mathbf{S}\cdot\mathbf{I} + H_Q \quad (1)$$

The first two terms, the electronic Zeeman interaction and the zero-field splitting, depend only on electronic parameters. Since the high-spin ferric iron has an almost spherical subshell, we consider only the Fermi contact term for the magnetic hyperfine interaction, $A_0\mathbf{S}\cdot\mathbf{I}$. For the nuclear excited state H_Q describes the interaction of the nuclear quadrupole moment Q with the electric field gradient tensor

$$H_Q = \frac{eQV_{zz}}{4}\left[I_z^2 - \frac{5}{4} + \frac{1}{3}\eta(I_x^2 - I_y^2)\right] \quad (2)$$

where $\eta = (V_{xx} - V_{yy})/V_{zz}$ is the asymmetry parameter. In the absence of magnetic interaction, only the quadrupole splitting

$$\Delta E_Q = \frac{eQV_{zz}}{2}\sqrt{1 + \frac{1}{3}\eta^2}$$

is observed. For the measurements described here (applied fields of a few hundred gauss), the nuclear Zeeman term can be omitted.

Aasa (1970) and Blumberg (1967) have shown that the $g = 4.3$ esr signal arises if $\lambda = E/D \simeq 1/3$ and $D \gg \beta H$. From the width of the esr signal of the PCA–inhibitor complex (Figure 3), we can conclude that $0.315 < \lambda < 0.333$. Since the effective g values practically do not change for this range of λ values, we choose $\lambda = 1/3$ for our further discussion.

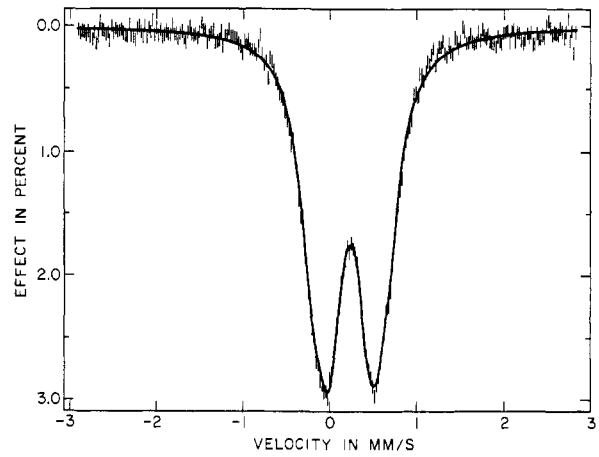


FIGURE 4: Mössbauer spectrum of PCA-4,5-oxygenase taken at 110°K. The solid line is the result of a least-squares fit assuming four lines of equal intensity (*i.e.*, two sites).

For $\lambda = 1/3$ the spin sextet is split into three equally spaced Kramer's doublets, separated by an energy $\Delta = 3.538D$ (Wickman *et al.*, 1965). For applied magnetic fields $\mathbf{H}$ such that $A_0 \ll \beta H \ll D$, it is convenient to treat each doublet separately using a spin Hamiltonian with an effective spin $S' = {}^1/_2$. For the nth doublet ($n = 1, 2, 3$) we get:

$$\mathcal{H}^{(n)} = E_n + \beta\mathbf{H}\cdot\mathbf{g}^{(n)}\cdot\mathbf{S}' + S'\cdot\mathbf{A}^{(n)}\cdot\mathbf{I} + H_Q \quad (3)$$

with $E_1 = 0, E_2 = \Delta, E_3 = 2\Delta$. In the framework of the effective spin Hamiltonian, and with the above-mentioned restrictions, the corresponding components of $\mathbf{g}$ and $\mathbf{A}$ are proportional, *i.e.*, $A_x/g_x = A_y/g_y = A_z/g_z$ for each doublet. For long electronic relaxation times (long compared to the nuclear precession time), each doublet gives rise to a distinct Mössbauer spectrum weighted with a Boltzmann factor $\exp(-E_n/kT)$.

The effective g values for the Kramers doublets can be calculated by diagonalizing the 6×6 electronic Hamiltonian. For $\lambda = 1/3$ the middle doublet gives rise to an isotropic value $g = 4.29$. For $D > 0$ (E can always be chosen as $E > 0$), the ground doublet has g values of 0.8, 9.7, and 0.6 in the x, y, and z directions; the upper doublet has similar behavior except that $g_z = 9.7$ is now the largest component. For $D < 0$ the lower and upper doublets are interchanged.

For a detailed discussion of the Mössbauer spectra arising from high-spin ferric irons with $E/D = 1/3$ the reader is referred to the literature (Lang *et al.*, 1971).

We now interpret the data. In Figures 5 and 6 we see two spectra superimposed. The one with the wide magnetic splitting (lines 1–6) arises from the doublets with the strong magnetic anisotropy. These absorption lines belong essentially to the ground doublet; due to a small Boltzmann factor the contribution of the upper doublet is small. A second spectrum (lines 1′–6′) arises from the $g = 4.3$ doublet. It is easily recognized in transverse field where the $\Delta m = 0$ lines, forbidden in parallel field, appear at about +2.3 and −2.0 mm per sec.

The solid lines in Figures 5 and 6 are the results of computer calculations.[4] Within the framework of our Hamiltonian and the mentioned approximations the results are quite satisfactory. The absorption line at −0.2 mm/sec (less than 5%

[4] The general features of the computer program used in this work have been described by Münck *et al.* (1972).

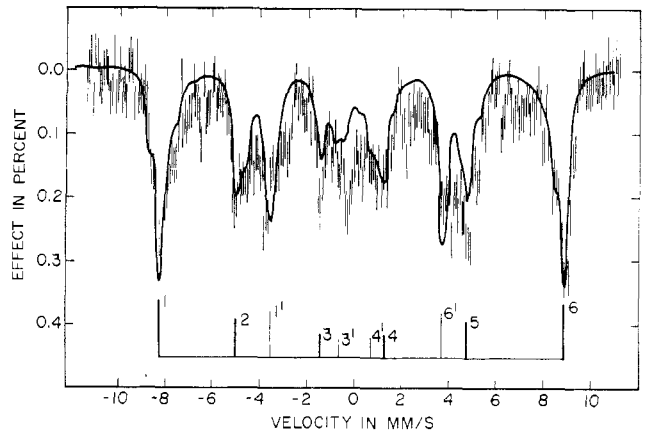


FIGURE 5: Mössbauer spectrum of the PCA-4,5-oxygenase–protocatechualdehyde complex taken at 4.2°K in a magnetic field of 150 G applied parallel to the observed γ rays. In Figures 5 and 6 the lines marked 1–6 arise from the upper and lower Kramer's doublets. The lines 1′–6′ belong to the isotropic ($g = 4.3$) middle doublet. For this doublet the nuclear $\Delta M = 0$ lines (2′ and 5′) are forbidden in a parallel field. The solid line in Figure 5 is a computer simulation using the hyperfine parameters quoted in the text and $D = 0.7$ cm^{-1}.

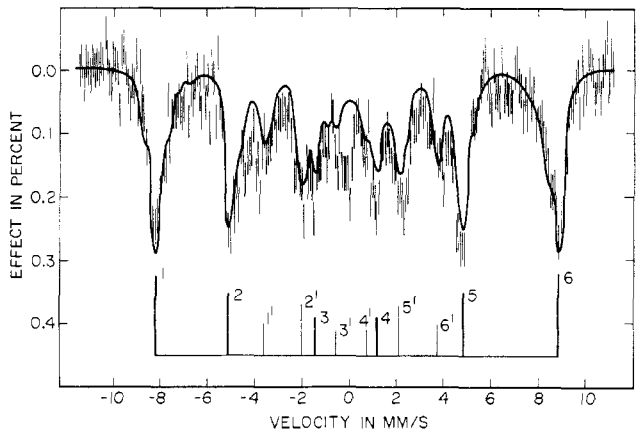


FIGURE 6: Mössbauer spectrum of the PCA-4,5-oxygenase–protocatechualdehyde complex taken at 4.2°K in a 150-G magnetic field applied perpendicular to the γ rays. The solid line is a computer simulation using the same parameters as in Figure 5.

of the total absorption) can be partly accounted for by Fe impurities in the beryllium window of the proportional counter. The best results were obtained for the following hyperfine parameters:[5] $H_{int} = -5A_0/(2g_g\beta_n) = -(551 \pm 15)$ *kG*, $|\Delta E_Q| = 0.55$ mm/sec, $\eta = 1$ and $\delta = 0.5$ mm/sec (with respect to iron metal). The large value found for the internal magnetic field shows that the bonding of the iron is highly ionic. The line width (full width at half-maximum) used to calculate the curves was $\Gamma = 0.35$ mm/sec. The curves do not require additional line broadening due to inequivalencies in the iron sites; hence we can conclude that the four high-spin ferric atoms are equivalent.

The zero-field splitting parameter D was varied to achieve the correct Boltzmann distribution among the three Kramers doublets. The simulations are somewhat insensitive to D, but upper and lower limits could be obtained. The spectrum taken in parallel magnetic field favors a slightly larger value of D than the transverse field data. By comparing curves computed for different values of D, we estimate $|D| = (0.7 \pm 0.3)$ cm^{-1}. The parameters for the PCA-4,5-oxygenase–protocatechualdehyde complex are close to those found for Fe·EDTA 5- and 6-dentate complexes (Lang *et al.*, 1971).

Discussion

PCA-4,5-oxygenase differs quite markedly from PCA-3,4-oxygenase. PCA-3,4-oxygenase contains 7 atoms of Fe^{3+}/molecule (mol wt 700,000) and 12 free sulfhydryl groups which apparently are not involved in catalysis. PCA-4,5-oxygenase contains four iron atoms per molecule (mol wt 140,000) and two sulfhydryl groups which are involved in catalysis. For PCA-4,5-oxygenase–substrate complexes and PCA-4,5-oxygenase–inhibitor complexes we observe high-spin Fe^{3+} iron. Substrate specificity studies show an absolute requirement for the carboxylate group on C_1, and the two hydroxyl groups on C_3 and C_4. Substitutions at C_5 are allowed since both gallic acid and 5-methoxygallic acid function as alternative substrates to PCA. Reduction of the protocatechualdehyde–PCA-4,5-oxygenase complex with sodium borohydride (T_4) gives significant covalent incorporation of tritium into the enzyme. Stoichiometry for tritium incorporation is not to be expected since the reduction of protocatechualdehyde to the corresponding alcohol together with a possible reduction of Fe^{3+} to Fe^{2+} constitute competing reactions to the reduction of the Schiff's base formed between protocatechualdehyde and an ϵ-amino group of lysine in the enzyme. These competing reactions are certainly significant since the reduction of the totally inactivated protocatechualdehyde–PCA-4,5-oxygenase complex with sodium borohydride always gives partial restoration of enzyme activity. In the tritium-labeling experiments which we reported, we find the incorporation of approximately one tritium per enzyme molecule.

Difficulties are encountered in determining the number of substrate or inhibitor molecules which bind to PCA-4,5-oxygenase because equilibrium dialysis experiments cause loss of iron from the enzyme. The instability of the complex with iron has proven useful in some respects because the iron in the enzyme is easily replaced by $^{57}Fe^{2+}$ for Mössbauer studies. Recently we have shown that Co^{2+}, Ni^{2+}, and Cu^{2+} form complexes at the iron binding site, but substitution with these other metal yields inactive enzyme.

As discussed above, the Mössbauer and susceptibility data on native PCA-4,5-oxygenase allow two assignments for the charge and spin states of the iron atoms. On the basis of these measurements we cannot distinguish between irons in a low-spin ferrous state and spin-coupled ($S_{tot} = 0$) high-spin ferric ions. However, chemical and kinetic data give evidence in favor of spin-coupled pairs. (a) The irons can

[5] The value for ΔE_Q was not measured directly. The quoted value is based on the following arguments. The magnetic hyperfine tensors of the upper and lower doublets are extremely anisotropic. Since the sign of D is unknown, the direction of the largest component of $\tilde{\mathbf{A}}$ for the ground doublet is not known. This component is A_y if $D > 0$ or A_z if $D < 0$. The quadrupole splitting is small compared to the magnetic hyperfine interaction. Therefore, the Mössbauer spectrum of the ground doublet is sensitive only to the component of the electric field gradient along the largest component of $\tilde{\mathbf{A}}^{(1)}$. The computer calculations give the best results if this component is positive and has a value $eQV_{ii} = +0.95$ mm/sec. Recently, Lang *et al.* (1971) have evaluated Mössbauer spectra of Fe·EDTA complexes. They obtained good results by assuming that the components of the electric field gradient tensor and the zero-field-splitting tensor are proportional, *i.e.*, $\eta = 3(E/D) = 1$. With the same assumptions it follows that $|E_Q| = 0.55$ mm/sec for the PCA-4,5-oxygenase complex. Furthermore, $\Delta E_Q > 0$ if $D < 0$ or $\Delta E_Q < 0$ if $D > 0$.

easily be removed from the enzyme. This fact is difficult to reconcile with a low-spin ferrous assignment. (b) The native enzyme is colorless. Low-spin ferrous complexes require strong ligands and are generally colored. (c) Coordination complexes formed between catechols and Fe^{2+} are less stable than those formed with Fe^{3+}. (d) The enzyme has four iron atoms and only two free sulfhydryl groups are found to be involved at the active sites. This could mean that the enzyme has two active sites, each containing two iron atoms. If spin-coupling occurs in PCA-4,5-oxygenase, the atoms must be high-spin ferric. Spin-coupled pairs are not so unusual and have been found in ferredoxins (Dunham *et al.*, 1972; Münck *et al.*, 1972) and in hemerythrin (Dawson *et al.*, 1972).

The kinetic and chemical data obtained so far give rather indirect evidence for spin-coupled pairs and do not allow a conclusive assignment to be made. Samples free from paramagnetic impurities are now in preparation for further magnetic susceptibility experiments. The existence of higher magnetic states which become populated as the temperature is increased could give definite evidence for antiferromagnetic coupling in PCA-4,5-oxygenase. Furthermore, spin-coupled high-spin ferric irons may allow the observation of weak d–d transitions which are spin forbidden for uncoupled irons. Optical absorption experiments designed to observe weak absorption bands in the near-infrared region could yield further information on the likelihood of spin-coupled Fe^{3+} pairs.

The analysis of the Mössbauer spectra of the PCA-4,5-oxygenase–protocatechualdehyde complex shows that the four irons are high-spin ferric and equivalent. We believe that this complex is representative of the enzyme–substrate complex (which also shows the esr signal at $g = 4.3$) formed during catalysis. If the spin-coupling hypothesis turns out to be correct, the Mössbauer data on the enzyme–inhibitor complex imply that the spin coupling is broken when the enzyme–inhibitor (substrate) complex is formed, and that the valence state of the irons does not change upon inhibitor (substrate) binding.

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