**17O-Water and Cyanide Ligation by the Active Site Iron of Protocatechuate 3,4-Dioxygenase**

EVIDENCE FOR DISPLACEABLE LIGANDS IN THE NATIVE ENZYME AND IN COMPLEXES WITH INHIBITORS OR TRANSITION STATE ANALOGS*

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Hyperfine broadening is observable in the EPR spectrum of *Brevibacterium fuscum* protocatechuate 3,4-dioxygenase after lyophilization and rehydration in 17O-enriched water, demonstrating H2O ligation to the active site iron. Lack of detectable broadening in the sharp features of the spectra of three substrate complexes suggests that H2O is displaced by substrate. Water is bound in the monodentate complex with the competitive inhibitor 3-hydroxybenzoate which binds directly to the iron showing that two iron ligation sites can be occupied by nonprotein ligands. Ketonized substrate analogs which mimic a proposed transition state of the reaction cycle, 2-hydroxyisonicotinic acid N-oxide (2-OH INO) and 6-hydroxyx nicotinic acid N-oxide (6-OH NNO), have H2O bound in their final, bleached enzyme complexes, suggesting that these complexes are also monodentate. In contrast, a transient, initial complex of 6-OH NNO which is spectrally similar to the substrate complex, apparently does not have H2O bound.

Cyanide binding occurs in two steps. The active site Fe**+** of the initial, rapidly formed, violet complex is high spin while that of the second, slowly formed, green complex is low spin; a unique state for mononuclear non-heme iron enzymes. The data suggest that the Fe-CN**−** and Fe-(CN)**−**2 complexes form sequentially. CN**−** binds to enzyme complexes with 2-OH INO and 6-OH NNO in one step to yield high spin Fe**+** species. In contrast, preformed substrate complexes prevent CN**−** binding. CN**−** binding eliminates the broadening due to 17O-water in the EPR spectra of both native enzyme and the enzyme-ketonized analog complexes. A model is proposed in which H2O is displaced by bidentate binding of the substrate but can potentially rebind after a subsequent substrate ketonization. The proximity of the vacatable H2O-binding site of the iron to the site of oxygen insertion suggests, however, that this site may serve to stabilize an oxygenated intermediate during the reaction cycle.

Protocatechuate 3,4-dioxygenase (EC 1.13.1.3) is a non-heme iron microbial enzyme which is central to the degradative pathways for many aromatic compounds (1). Molecular O2 is cleaved in the catalyzed reaction and both atoms of oxygen are incorporated into PCA resulting in aromatic ring fission. The open chain product, β-carboxy-cis,cis-muconic acid, is further metabolized to succinate and acetyl-CoA by Pseudomonads (2). The iron-binding site, which appears to be part of the enzyme active site, has been extensively characterized by EPR (3-5), Mössbauer (4, 5), resonance Raman (5, 6), optical (8), CD (9), and magnetic CD (10) spectroscopies. The techniques show that protocatechuate 3,4-dioxgenase and related intradiol dioxygenases possess a new class of iron center which is characterized by tyrosinate ligation in one or more coordination positions (4, 6). Tyrosinate to iron charge transfer interactions give rise to the red-brown color of the enzymes. EPR and Mössbauer spectra show that the iron of the native enzymes is high spin ferric in an electronic environment of low symmetry (4). The dioxygenase iron centers can be distinguished from rubredoxin-type iron centers, which are spectroscopically similar, by the degree to which the electronic environment of the former centers is alterable by substrates, inhibitors, and other iron ligands. Complexes have been formed with protocatechuate 3,4-dioxygenase which span the theoretical range of electronic symmetry (E/D = 0.01 to 0.33) with both positive and negative zero field splitting (11). Resonance Raman spectroscopy shows that the tyrosine ligation persists in substrate and inhibitor complexes (7). The other Fe ligands are unknown although cysteine ligation is made unlikely by the fact that *Brevibacterium fuscum* protocatechuate 3,4-dioxygenase shows spectroscopic features very similar to other protocatechuate 3,4-dioxygenases but is devoid of cysteine (13). This supports an earlier interpretation of Mössbauer spectra which suggested that the magnitude of the internal saturation field of the iron is larger than expected for cysteinylic ligation (4). Preliminary extended x-ray absorption fine structure (21) and model studies (14) are consistent with only nitrogen- and oxygen-type ligands.

In the first paper of this series (13), we described the preparation and characterization of *B. fuscum* protocatechuate 3,4-dioxygenase which has much sharper spectral features than other intradiol dioxygenases. Recently, we have reported that the sharp features of the EPR spectrum are all broadened when the enzyme is transferred to 17O-enriched water, directly demonstrating water ligation to iron (11). In the same preliminary report (11), we showed that CN**−** can complex with the iron to produce a unique low spin non-heme...
iron species. In the present study, we have investigated water ligation in protocatechuate 3,4-dioxygenase complexes with substrates, substrate analogs, and putative transition state analogs which mimic intermediates in the proposed reaction cycle (8). We observe no broadening in the EPR spectra of substrate complexes, suggesting that water is displaced. In contrast, water is bound in the complexes with most mono- dentate inhibitors and the transition state analogs, showing that they form a distinctly different type of complex and that at least the two sites in the Fe coordination can be occupied by external ligands or water. This conclusion is supported by the observation that CN\textsuperscript{-} binds in two successive steps apparently forming the monocyanato- and dicyano-Fe\textsuperscript{III} complexes. CN\textsuperscript{-} also appears to displace the water from the native enzyme and all of its complexes with inhibitors, suggesting that the water-binding site is accessible to small molecules and may be used mechanistically to bind \( O_2 \) or, more likely, to stabilize an oxygeneous intermediate during turnover.

**MATERIALS AND METHODS**

Samples of water enriched to 52.7 atom % \( ^{18}O \) were obtained from Mound Laboratory and KOR. 3,4-Dihydroxyphenylacetic acid and 3,4-dihydroxyphenylpropionic acid (Aldrich) were decolorized with charcoal and recrystallized from ethylacetoacetate-chloroform. 2-OH NNO and 6-OH NNO were prepared according to the procedures described in (12). \( F. fuscum \) protocatechuate 3,4-dioxygenase was purified as described previously (13). All other chemicals were reagent grade and water was deionized and then glass distilled. The ligand coordination sphere were analyzed using the spin Hamiltonian:

\[
\hat{H} = \hat{H}_{\text{Isot}} + \hat{H}_\text{zero field} + \hat{H}_\text{hyperfine}
\]

\[
\hat{H} = \hat{\alpha}_S \cdot \hat{g}_S \cdot \hat{D}(S_z, \frac{3}{2}) \cdot (S(S + 1)) + E/D(S_z, \frac{3}{2}) + I_{\mu\nu} \cdot A_{\mu\nu} \cdot \hat{S} + I_{\nu\mu} \cdot A_{\nu\mu} \cdot \hat{S}
\]

where \( D \) and \( E/D \) are zero field-splitting parameters, \( A_{\mu\nu} \) is the transferred hyperfine coupling tensor of the \( ^{19}O \)-ligand, and the other parameters have their usual definitions. The hyperfine terms \( I_{\mu\nu} \cdot A_{\mu\nu} \cdot \hat{S} \) and \( I_{\nu\mu} \cdot A_{\nu\mu} \cdot \hat{S} \) describing interaction of the spin with the nuclear spin of the iron and the nuclear spin of the ligand, respectively. Since the nuclear spin of \( ^{35}Fe \) is 0, the first term has no effect on the observed spectrum. The second term results in split EPR resonances due to the \( I = \frac{1}{2} \) nuclear spin of \( ^{13}O \); however, the splitting is not resolved in the spectra reported here, and the effect is observed as a broadening of the signal. The term \( E/D \) is a measure of the departure of the electronic environment of the iron from axial symmetry. A perfectly axial center gives an \( E/D \) value of 0 while a maximally rhombic center gives a value of 256 (25). The \( E/D \) value can be determined directly from the \( g \) values. The quadrupole moment of the \( ^{35}O \) ligand does not affect the EPR spectrum unless it becomes significant relative to the Zeeman interaction. In this case, second order effects can result in an unsymmetrical shift in the hyperfine splitting pattern giving an apparent change in the \( g \) values (18).

**RESULTS**

**Native Enzyme**—The protocatechuate 3,4-dioxygenase isolated from \( F. fuscum \) is stable to lyophilization, unlike the isofunctional enzymes from Pseudomonas. Thus, it is possible to efficiently change solvents without increase in line width or other indications of loss of homogeneity. In addition, the EPR features of the iron in this enzyme are extremely sharp (Fig. 1), which is essential for the observation of hyperfine broadening from a ligand nucleus having a small gyromagnetic ratio. For the 52.7 atom % enriched \( ^{18}O \)-water used in these experiments, the broadening was 4-pica-0.2 to 0.6 milliTesla. As shown in Fig. 1, the EPR resonances of protocatechuate 3,4-dioxygenase in \( ^{18}O \)-enriched water are broader than those of enzyme in \( ^{16}O \)-water. This result is reproducible and does not depend on the source of the enriched water. The low field \( g \) value is broadened from 1.4 to 1.6 milliTeslas and shifted slightly from \( g = 9.67 \) to 9.65. The feature from the middle Kramer’s level around \( g = 4.28 \) is also broadened, but part of the broadening can be attributed to separation of the three \( g \) values due to the apparent decrease in \( E/D \) value. In a similar experiment using \( O_{enriched} \) water (98% enrichment), no shift or broadening could be detected. Water is bound to the iron in the native enzyme at both \( pH \)7.0 and 9.5, the low and high ends of the \( pH \) activity profile for the enzyme, yielding EPR spectra with the same \( g \) values but slightly different line widths. The results are summarized in Table I.

**Substrate Complexes**—The anaerobic complexes of PCA with all known protocatechuate 3,4-dioxygenases yield EPR spectra devoid of features sufficiently sharp to detect broadening by \( ^{18}O \)-water. However, the spectra from anaerobic complexes of \( F. fuscum \) protocatechuate 3,4-dioxygenase with other catecholic substrates generally contain sharp features. The complex with catechol exhibits an EPR spectrum similar to that of native enzyme with equally sharp features. In \( ^{18}O \)-water solution, there is no observable broadening of the EPR spectrum (Fig. 1). Similar results have been obtained with two other diphenolic substrates, 3,4-dihydroxyphenylacetate and 3,4-dihydroxyphenylpropionate. The absence of broadening in the EPR spectra of these complexes could result from either displacement of water from the iron or fortuitous alignment of the hyperfine coupling field axes with the principal axis system of the \( g \) tensor. In several nonsubstrate ligand complexes, however, differential hyperfine broadening was observed in two or three of the EPR features representing different principal axes of the \( g \) tensor (see Table I and text below). Thus, at least in these cases, the hyperfine and magnetic frames were not aligned.

**Inhibitor Complexes**—The complex of protocatechuate 3,4-dioxygenase with 4-hydroxybenzoate, a substrate analog lacking the 3-hydroxy group, contains water as an iron ligand (Table I). The halogenated derivatives in which the 3-position is substituted with fluorine or chlorine have also been studied. The optical spectra of these complexes is similar in shape and intensity to those of competent substrates, but blue shifted. Broadening of the sharp EPR spectral features in the presence of \( ^{18}O \)-water demonstrates that water is an Fe ligand in the complex with 3-fluoro-4-hydroxybenzoate, but no broadening was detected in the EPR spectrum of the complex with 3-chloro-4-hydroxybenzoate. Broadening was also not detected in the EPR spectrum of the 3-hydroxybenzoate complex with enzyme in \( ^{18}O \)-water.

**Transition State Analogs**—We have previously reported that protocatechuate 3,4-dioxygenases from \( F. fuscum, Pseudomonas aeruginosa, \) and \( Pseudomonas putida \) all react with the tautomizerable substrate analogs, 2-OH INO and 6-OH NNO, forming extremely strong complexes (11, 12). We have recently found that coumenate, another heterocyclic substrate analog, is also very tightly bound by protocatechuate 3,4-dioxygenases. The optical spectra or the putative transition state analog complexes are distinguished by a shift of the...
absorbance out of the visible range resulting in a bleaching of the native red color. The sharp features of the EPR spectra of the complex with 2-OH INO are shown in Fig. 1, and the results are summarized in Table I. Broadening by ¹⁷O-enriched water ligand is observed in these each of these complexes. The bleached complexes with 2-OH INO and 6-OH NNO are formed via a short-lived intermediate (12). When this intermediate in the reaction with 6-OH NNO was formed at low temperature and frozen for EPR measurements, samples prepared in ¹⁶O- and ¹⁷O-water solutions have the same EPR line widths.

Cyanide Complexes—Cyanide rapidly forms a complex with native protocatechuate 3,4-dioxygenase at neutral pH, changing the color of the enzyme to an intense violet (Fig. 2). The EPR spectrum of the complex (Fig. 3) shows that it is high spin with a decreased E/D value reflecting a shift to a more axial electronic symmetry, E/D = 0.090. This early high spin complex is converted in a cyanide-dependent, pseudo-first order process to a low spin (S = ½) iron complex (Fig. 3) which, to our knowledge, the first observed in a nonheme, non-spin-coupled protein iron center. When analyzed in the manner suggested by Blumberg and Peisach (24), the EPR spectrum of the low spin form yields values of $\Delta/\lambda = 5.2$ and $V/\Delta = 0.80$ where $\Delta$ and $V$ are crystal field parameters and $\lambda$ is the spin orbit coupling energy. The values show that the ligands contribute a relatively large degree of charge to the iron which is consistent with dicyano-Fe ligation. The complex displays a green color due to the large red shift in the spectrum (Fig. 2). The low spin conversion is relatively slow, occurring over several minutes. As can be seen from the repetitive scans shown in Fig. 2, an isosbestic point exists between the spectra of initial and final complexes, but not between those of the native enzyme and the final complex. Thus, it seems likely that the final complex cannot form directly from the native enzyme. The cyanide is bound very tightly in the final complex, but it can be removed by prolonged dialysis with recovery of the spectrum of the native enzyme.

Cyanide does not change any of the spectral characteristics of the anaerobic PCA complex with enzyme even after a 12-h incubation at 23 °C, nor does PCA apparently bind to the low spin cyanide complex. However, if PCA is added to the initial high spin cyanide complex, a new complex is formed which exhibits the unusual EPR spectrum shown in Fig. 3. Cyanide also binds to the enzyme complexes with the putative transition state analogs, 2-OH INO and 6-OH NNO (Fig. 3).
**TABLE I**

| Ligand                        | g value*       | Assignment      | Line width$^b$ | Water ligation |
|--------------------------------|----------------|-----------------|---------------|---------------|
| None, pH 7.0                  | 9.67 (9.65)    | GS$^S$, $g_x$   | 1.4           | 1.6           | +             |
| None, pH 9.5                  | 6.77 (9.65)    | MS, $g_y$, $g_z$| 1.6           | 1.9           | +             |
| Substrates                    |                |                 |               |               |               |
| Catechol, pH 7.0              | 9.69 (9.70)    | GS$, g_x$       | 1.38          | 1.35          | -             |
| 3,4-(OH)$_2$-phenylacetate, pH 7.0 | 9.5            | US, $g_y$       | 1.5           | 1.5           | -             |
| 3,4-(OH)$_2$-phenylacetate, pH 9.5 | 9.63 (9.63)    | GS$, g_x$       | 2.08          | 2.05          | -             |
| 3,4-(OH)$_2$-phenylpropionate | 9.28           | MS, $g_x$, $g_z$| 1.9           | 1.9           | -             |
| Inhibitors                    |                |                 |               |               |               |
| 3-OH-benzoic acid             | 9.7            | GS$^4$, $g_z$   | 1.55          | 1.55          | -             |
| 4-OH-benzoic acid             | 4.75           | MS, $g_y$       | 4.8           | 4.8           | -             |
| 3-F-4-OH-benzoic acid         | 9.29           | GS$, g_y$       | 1.92          | 2.08          | +             |
| 3-Cl-4-OH-benzoic acid        | 5.2            | MS, $g_y$       | 3.4           | 4.1           | +             |
| Tropolone                     | 9.79 (9.79)    | GS$^4$, $g_z$   | 1.5           | 1.5           | -             |
| Coumarate                     | 9.4            | US, $g_y$       | 2.4           | 2.4           | -             |
| Carboxylate ligand Terephthalate | 9.78          | GS$^4$, $g_z$   | 1.3           | 1.3           | -             |
| CN$^-$ complexes              |                |                 |               |               |               |
| CN$^-$, 1st complex           | 7.83 (7.84)    | GS$, g_x$       | 5.55          | 5.6           | -             |
| CN$^-$, final complex         | 5.68 (5.68)    | MS, $g_y$       | 4.9           | 4.9           | -             |
| 2-OH INO + CN$^-$             | 2.43           | LS, $g_y$       | 5.6           | 5.6           | -             |
| 6-OH NNO + CN$^-$             | 2.21           | LS, $g_y$       | 2.2           | 2.2           | -             |
| Addition of hydrogen peroxide rather than cyanide also produced new complexes which fail to show hyperfine broadening when the EPR spectrum is measured in $^{17}O$-water, but the line widths are too large for the absence of broadening to be unequivocal.

**DISCUSSION**

We have used transferred hyperfine interactions between $^{17}O$-enriched water and active site high spin ferric ion to provide the first definitive evidence for water ligation to the active site iron of a protocatechuate 3,4-dioxygenase. The data show that water is also present in a number of inhibitor complexes, and indicate that it is absent in competent substrate complexes. Ligand hyperfine structure can only be observed in an EPR spectrum when the spin system undergoing resonance extends onto neighboring atoms. It is highly selective, demonstrating that there is chemical bonding between the atoms (15). Distant nuclei do not interact strongly enough to be detected. Nuclei having no nuclear spin, I = 0,
hyperfine broadening at pH 9.5.

The observed differential broadening of the $g_z = 9.67$ and $g_{xy} = 4.28$ features of the EPR spectrum indicates anisotropy in the hyperfine interactions. In low symmetry (lower than rhombic) which can be expected in metal-binding sites such as these, the principal axes for Zeeman and hyperfine interactions will not generally coincide. The hyperfine interaction will be different along each principal axis of $g$ and the full splitting may not be observed along any of the $g$ axes. Thus, it is possible to identify water as a ligand from broadening of any of the EPR features, but the absence of broadening of a single observed feature is an equivocal indication of the absence of water in the complex. Failure to observe broadening in two or three $g$ values is much stronger evidence that water is not bound because it is unlikely that the hyperfine coupling will be weak along all of the $g$ tensor axes.

**Substrate Complexes**—Resonance Raman studies show that substrates are directly coordinated to the iron through at least one of the hydroxyl groups (6, 7); thus, it is reasonable that they displace at least one ligand from the native iron complex when they bind. The absence of broadening in the EPR spectrum of the anaerobic alternate substrate complexes suggests that one such displaced ligand is water, but the data do not allow us to determine if more than one ligand is displaced. In order to investigate this further, we have studied the complexes with inhibitors lacking one of the hydroxyl groups.

**Inhibitor Complexes**—The inhibitor 4-hydroxybenzoate and derivatives bearing fluorine, or chlorine in the 3-position, are bound to protocatechuate 3,4-dioxygenase in complexes exhibiting a long wavelength optical transition characteristic of a substrate complex, but they are not oxygenated. The complexes with the parent compound and the 3-fluoro derivative have water as an iron ligand while water is apparently absent from the complex with the 3-chloro derivative. Resonance Raman spectroscopy has been used to demonstrate that each of these inhibitors is bound directly to the iron through the 4-hydroxyl function in complexes with *P. aeruginosa* protocatechuate 3,4-dioxygenase (20). Similar measurements have not been made with our enzyme, but all other spectra of these complexes are very similar. Thus, we suspect that the relatively bulky chlorine displaces water by steric interference, suggesting that one water is bound in the Fe ligand site near the 3-position of the inhibitor as illustrated below (Illustration 1).

Together these data demonstrate that there are two sites in the iron coordination which can be occupied by water or exogenous ligands. In the native enzyme, one coordination site is occupied by water, but there are several alternatives for the second coordination site. For example, this site might be occupied by a second water ($a$) or an amino acid side chain from the protein ($b$). Alternatively, the iron may be pentacoordinate ($c$) (Illustration 2).

Thus far, we have not found a method to clearly distinguish between these possibilities; however, several aspects of the iron coordination are suggested by the studies of $^{17}$O-water binding in conjunction with other inhibitors and cyanide.

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**FIG. 2. Optical spectra of the CN$^-$ binding reaction.** 70 µM enzyme was mixed with 300 mM CN$^-$ in 50 mM MOPS buffer, pH 7.0 at 23°C. The CN$^-$ was added from a stock solution of 2 mM NaCN neutralized with HCl in the same buffer. The spectra shown were scanned at 40-s intervals.

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such as $^{16}$O, the isotope of largest natural abundance, or $^{18}$O, the residual isotope in $^{18}$O-enriched samples, have no effect on the line width, although they may shift the resonance position by a heavy atom effect well known in NMR. Nuclei with $|I| > 1/2$ may also produce spectral shifts via quadrupole interactions. Ligand hyperfine interactions involving $^{17}$O have previously been used to identify oxygen atoms in the composition of paramagnetic species of laccase (16) and bleomycin (17). For species in which the electron magnetic moment resides on oxygen (as in superoxide or other oxygen radicals), the hyperfine splitting is typically 5 mTels or more (16). When the radical is centered on another atom, iron in this case, the splitting is much smaller, often less than 1 mTels. In complementary NMR experiments reported by Lauffer et al. (19), model complexes for the iron coordination of intradiol dioxygenases were shown to exhibit contact-shifted resonances corresponding to the hyperfine splitting observed in an EPR spectrum. Similar contact-shifted resonances are also observed in the NMR spectra of catechol 1,2-dioxygenase complex with catechol (26). This provides an independent demonstration of the considerable spin density delocalization in these centers. This delocalization of spin density may be one of the catalytic functions of the ferric ion in the intradiol phenoloxidase dioxygenases, providing a mechanism for circumventing the spin conservation barrier introduced by the triplet ground state of $O_2$.

**Native Enzyme**—The presence of water as an iron ligand in the resting enzyme is demonstrated by the broadened EPR absorption derivative features observed in $^{17}$O-water solution. The slight change observed in the $g$ values of the EPR features of the complex could, in principle, be due to either the heavy atom effect or quadrupolar interactions as described above, but the absence of any detectable shift in $^{18}$O-enriched water indicates that it probably due to the latter. We were unable to determine the ionization state of the bound water, but a change in ionization between pH 7.0 and 9.5 is unlikely since the $g$ values of the EPR spectra are essentially identical. However, an increase in the net charge on the water oxygen may be reflected in the observed increase in the $^{17}$O-water
One approach was to test whether water is also bound in the complex with the isomer of 4-hydroxybenzoate, 3-hydroxybenzoate. Resonance Raman spectroscopy failed to show evidence for direct iron ligation by this competitive inhibitor in the complex with *P. aeruginosa* protocatechuate 3,4-dioxygenase (20); again, the optical spectrum of this complex is nearly identical with that of the inhibitor complexed to our enzyme. Despite this lack of direct evidence for coordination, the EPR and optical spectra show that the iron site is signif-

**FIG. 3.** EPR spectra of enzyme-CN⁻ complexes. A, approximately 150 µM enzyme was mixed with 300 mM CN⁻ in 50 mM MOPS buffer at pH 7.0 and frozen immediately to trap the initial CN⁻ complex. B, a sample prepared as in A was allowed to stand at room temperature for 60 min, and then excess CN⁻ was removed by gel filtration chromatography prior to freezing. C, 1 mM PCA was added anaerobically to a sample prepared as in A. D, 300 mM CN⁻ was added to approximately 150 µM enzyme plus 1 mM 2-OH INO and allowed to stand for 5 min at room temperature before freezing. E, as in D except the inhibitor is 6-OH NNO. Instrumental conditions were: power = 0.2 milliwatt; modulation frequency = 100 kHz; modulation amplitude = 1 milliTesla (mT); microwave frequency = 9.220 GHz; sweep rate = 100 milliTeslas/min; center field = 210 milliTeslas; temperature = 4.5 K.

**ILLUSTRATION 2**
transition state analogs differs in several important respects. Including 2-OH INO and 6-OH NNO as transition state analog site. The water binding in enzyme complexes with displaced by a ligand which can occupy at most one coordination sites of the iron coordination. Thus, if the iron in native complexes employed for the resonance Raman experiments. In either case, water is probably not present in any of the replaceable sites of the iron coordination. Thus, if the iron in native enzyme has two water ligands, they are both apparently displaced by a ligand which can occupy at most one coordination site. The water binding in enzyme complexes with transition state analogs differs in several important respects.

Transition State Analogs—We describe a group of inhibitors including 2-OH INO and 6-OH NNO as transition state analogs due to their high affinity for the enzyme and the spectral similarity of their enzyme complex to early bleached intermediates of the reaction cycle (11, 12). Both inhibitors tend to assume a ketonized configuration in solution which we have proposed as the reactive configuration of the bound substrate (5). The optical and EPR spectra of the isomeric analog complexes are similar but not identical, and the kinetics of their complex formation is quite different (12), showing that they form distinct complexes. Although no direct evidence for iron ligation by these inhibitors exists, the dramatic changes in optical, EPR, and Mössbauer spectra which accompany complex formation clearly suggest that such a bond is formed. Since water is also bound to iron in both enzyme complexes, it is likely that each inhibitor is coordinated exclusively through one group, presumably the N-O hydroxymate oxygen. Moreover, water binding in the isomeric analog complexes implies that water remains bound when the inhibitor is coordinated through either of two ligand positions of the iron. Thus, in contrast to the monohydroxybenzoate complexes, there appear to be two different binding sites for water in the transition state analog complexes.

In view of the large differences between the spectral properties of the transition state analog complexes and those of substrate or the monohydroxybenzoate complexes, it is not unexpected that the iron ligation shows some major differences. It is possible, for example, that 3-hydroxybenzoate and 3-chloro-4-hydroxybenzoate exclude water from the two iron sites by coordinating to one and sterically blocking the other. If this is the case, then the binding orientation of the analog complexes must be quite different since they contain equally bulky carbonyl groups in the positions which should sterically hinder the approach of water.

Cyanide Complexes—Cyanide is observed to reversibly form two successive complexes with native protocatechuic acid dioxygenase; a high spin form appears rapidly and is then converted slowly to a low spin form in a cyanide-dependent reaction. This strongly supports the proposal for two iron coordination sites which can be occupied by exogenous ligands. The EPR features of these complexes are relatively broad, but they show no evidence for further broadening in $^{17}$O-enriched water. Thus, it appears that the binding of the first CN$^-$ is sufficient to displace water from the iron. This implies that there is only one water bound in the native enzyme. The slow conversion to what is apparently the dicyano low spin complex suggests that a major change in the iron site must occur prior to binding. It is possible, for example, that an amino acid side chain must dissociate from the iron or that the iron site must adjust its structure to stabilize a 6 rather than 5 coordinate iron (Illustration 3).

When CN$^-$ binds to the preformed enzyme complexes with 2-OH INO or 6-OH NNO, water is displaced and the resulting high spin complexes have different EPR spectral features than either of the CN$^-$ complexes with the native enzyme. The inhibitor prevents formation of a low spin CN$^-$ complex, presumably by occupying the second binding site. As shown above, the order of addition of CN$^-$ and inhibitor do not affect the final complex, but this is not true for addition of substrate and CN$^-$. The optical and EPR spectra of a preformed substrate complex are not altered by CN$^-$, but PCA added to the high spin CN$^-$ complex (presumably with one CN$^-$ bound) causes a new complex to form which is distinct from either

![Illustration 3](https://example.com/illustration3.png)
the substrate or CN\textsuperscript{−} derivatives. These results, taken together with spectral data in the absence of CN\textsuperscript{−}, suggest that the substrate and transition state analog complexes are fundamentally different with respect to their geometry and susceptibility to reaction with potential iron ligands.

**Bideterminate Substrate Complex**—One appealing explanation for the observed difference between the substrate and transition state analog complexes is that in the former case the iron may be chelated. Bideterminate complexes of iron and catechols are formed exclusively in solution and would be the expected form in the enzyme if both hydroxyl groups of the substrate have access to the iron; that this is the case is suggested by the apparent proximity of the 3-chloro group of 3-chloro-4-hydroxybenzoic acid to the water-binding site, indicating that the 3-hydroxyl group of the substrates may have the same access. Such coordination would account for the displacement of water and the exclusion of CN\textsuperscript{−} from the active site.

We have observed that the binding of the transition state analogs is a multistep process (12) ending with the formation of the tightly bound, bleached complex which has water as an iron ligand. The earlier intermediates have spectra similar to that of the substrate complex and have much higher dissociation constants than the final complexes. In the case of 6-OH NNO, the decay of one early complex is slow enough to allow trapping for EPR measurements, and, as in the substrate complexes, shows no broadening. These observations can also be accounted for in terms of an initial bideterminate binding of the transition state analog which displaces all bound water from the iron. A subsequent change in the conformation of either the inhibitor, the enzyme, or both could lead to monodeterminate binding of the ketonized inhibitor and rebinding of water. This is consistent with the observation that the inhibitors immediately displace CN\textsuperscript{−} from the enzyme when added to the high spin CN\textsuperscript{−} complex. Cyanide rebinding occurs slower than in the absence of inhibitor but much faster than formation of the low spin (presumably dicyano) complex with native enzyme.

**Model for the Initial Steps of the Reaction**—We have proposed (5) a model for the reaction of intradiol dioxygenases in which the substrate is bound initially as a monodeterminate ligand promoting substrate ketonization and development of a center of high electron density at the 4-carbon. This site could then be attacked directly by O\textsubscript{2}. The results presented here suggest that ketonization, while still important for the reaction, does not occur in the initial ES complex. If it did, then we would expect to find water bound in this complex as it is in the ketonized analog complexes. The properties of the ketonized analog complexes do suggest, however, that they mimic some stage of the reaction cycle. Thus, it is possible that ketonization occurs coincident with or after the initial interaction with O\textsubscript{2}. If this is the case, then the vacant water-binding site in the iron ligand sphere adjacent to the ketonized substrate-binding site could be used to stabilize the developing oxy intermediate as shown in Scheme 1. Such a role is also suggested by the ability of the transition state analog complexes to bind hydrogen peroxide in the water and CN\textsuperscript{−} binding site (12). This model requires that there be a substantial rotation of the substrate relative to the iron during the reaction because both the 3-hydroxyl group of the substrate and a peroxy intermediate are proposed to bind at the same iron coordination site at different stages of the reaction; the 4-carbon is proposed to become tetrahedral so that oxygen must extend either above or below the distorted plane of the ring, while the 3-hydroxyl is in the plane of the ring prior to ketonization. Such rotation would require either considerable flexibility in the substrate-binding site or a major change in the enzyme configuration during turnover. We have shown in the first paper of this series (13) that the protocatechuate 3,4-dioxygenase-PCA complex is exceptionally heterogeneous especially in comparison to the native enzyme and the transition state analog complexes. Thus, it is very likely that the substrate has more than one binding orientation. The proposed rotation would account for the accessibility of the iron to small molecules in the ketonized analog complexes as well as the relatively slow conversion rate from the initial, substrate-like analog complex to the final, water-binding complex.

It is clear from the work presented here that PCA in the absence of O\textsubscript{2} does not spontaneously assume the configuration and binding orientation of the ketonized analogs despite the large increase in stability of this type of complex. It is possible that this could be accounted for solely on the basis of the propensity of the species to ketonize. For example, there might be a hydrophobic pocket in the active site which could better accommodate the carbonyl group of the ketonized form than the hydroxyl group of the phenolic form. Alternatively, the site might be specific for the "buckled" form of the ketonized substrate which would occur if the 4-carbon became tetrahedral. It is unlikely that ketonization alone would cause a significant distortion in the planarity of the ring due to the possibilities for electron delocalization; however, formation of a fourth bond to the 4-carbon would force this position to become tetrahedral. In the case of the substrate, this fourth bond is proposed in Scheme 1 to derive from O\textsubscript{2}; thus, the bleached, high affinity intermediate would not be stable until after O\textsubscript{2} attacks. In the case of the ketonized N-oxide analogs, the fourth bond may be provided as a hydrogen bond from the water proposed here to occupy the adjacent iron coordination site oxygen (Illustration 4).

In the later steps of the reaction cycle proposed in Scheme
1, the O-O bond would be broken by a variation of the Baeyer-Villiger reaction as previously proposed (5), except that the noninserted oxygen might be retained in the iron coordination as shown. This oxygen would be incorporated during the breakdown of the anhydride intermediate as required by the stoichiometry of oxygen incorporation. The resulting chelated dicarboxylic acid product would be consistent with the observed red shift in the optical spectrum of the last intermediate of the reaction cycle (23). Such a reaction scheme would also account for the stoichiometry of oxygen incorporation, the slow (rate limiting) release of product, and the ability of the enzyme to selectively release one of the atoms from oxygen during the pyrone-forming side reactions of the enzyme (22).

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