Elucidation of the Coordination Chemistry of the Enzyme–Substrate Complex of Catechol 1,2-Dioxygenase by NMR Spectroscopy

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Abstract: The coordination chemistry of the active site iron in catechol 1,2-dioxyygenase (CTD) from *Pseudomonas putida* (ATCC 23974) has been probed with NMR spectroscopy. The high-spin ferric center gives rise to paramagnetically shifted resonances in the range of 105 to −67 ppm, assigned to protons on the iron ligands. Observed endogenous ligand resonances are assigned to β-CH_2 protons of tyrosines and histidines. Exogenous ligand resonances are also observed, particularly those of methyl protons on substrates and inhibitors. The methyl resonance of the CTD–4-methylcatechol complex is found at 105 ppm. Comparisons of the 105-ppm shift with corresponding ones in models and in CTD–methylphenol and other CTD–substrate complexes indicate that 4-methylcatechol coordinates to the iron via O1 alone and that both catecholate protons are likely to be dissociated. The implications of the observations are discussed in terms of the proposed substrate activation mechanism.

Catechol 1,2-dioxyygenase (CTD) is a non-heme iron enzyme that catalyzes the oxidative cleavage of catechols to *cis,cis*-muconic acids.[1] The enzyme serves as part of nature's mechanism for degrading aromatic molecules in the biosphere. Many spectroscopic techniques have been applied to the study of the non-heme iron site[2-8] and have provided insights into the coordination chemistry of the iron center. The metal center is coordinated by two tyrosines, which results in the stabilization of the ferric oxidation state relative to the ferrous and a lowered $Fe^{III/II}$ redox potential.[9] Stong reductants such as dithionite are required to reduce the ferric center. The coordination of substrate to the metal[2a,3b] introduces yet a third phenolate to the coordination environment and should further lower the $Fe^{III}/^{II}$ potential. As a result, the iron center appears not to be reduced during the catalytic cycle.[4,8] The absence of a ferrous center in the cycle would seem to preclude the participation of an O_2 complex, so a novel substrate activation mechanim involving a monodentate catecholate has been proposed for the oxidative cleavage catalyzed by this enzyme.[10]

The presence of a high-spin ferric center in a metalloprotein can result in the observation of paramagnetically shifted resonances.[11-15] Such shifts have been used effectively in the study of heme proteins to elucidate structures of the active sites.[11] Studies of non-heme iron proteins in the past have been limited to iron–sulfur proteins[12] and have only recently been expanded to include hemerythrin,[13] ribonucleotide reductase,[14] and uteroferrin.[15] NMR studies of CTD and its various complexes have been undertaken to provide further insight into the coordination chemistry of the metal center; a preliminary report on the enzyme–substrate complexes has been published.[16] Two aspects of the iron(III) coordination chemistry can be gleaned from the data—the identification of active site ligands and an understanding of the mode of substrate binding. Our observations support the proposed substrate activation mechanism, and we detail our evidence for the mechanism in this report.

Experimental Section

Catechol 1,2-dioxygenase was purified from *Pseudomonas putida* (ATCC 23974, also *Ps. arvilla* C-1) cells following the procedure of Roe et al.[6] Catechol, 4-methylcatechol, 3-methylcatechol, and pyrogallol were obtained from Aldrich and sublimed before use. *p*-Cresol, 3,5-dimethylphenol, 2,3-dimethylphenol, thiophenol, and *p*-thiocresol were purchased from Aldrich and used without further purification. 5-Methylpyrogallol was synthesized by the $LiAlH_4/AlCl_3$ reduction of 3,4,5-trimethoxybenzoyl chloride,[17] followed by deprotection with BBr_3.[18] NMR ($CDCl_3$): 2.1 (s, 3 H), 5.0 (br), 6.2(s, 2 H). 3,6-Dimethylcatechol was prepared by the Mannich condensation of catechol with formaldehyde and morpholine, followed by hydrogenation over 10% Pd/C at 60 psi H_2.[19] NMR ($CDCl_3$): 2.25 (s, 6 H), 5.7 (br s, 2 H), 6.48 (s, 2

Table I. NMR Chemical Shifts (ppm) of Exogenous Resonances of CTD–X and Fe(salen)X Complexes

X	resonance	CTD–X[a]	Fe(salen)X[b]
4-methylcatechol	4-CH_3	105	109 (O1 isomer, monodentate) −27 (O2 isomer, monodentate) 47 (chelated)
5-methylpyrogallol	5-CH_3	91	
3,6-dimethylcatechol	3-CH_3	n.o.	
	6-CH_3	−26	
4-methylphenol	4-CH_3	88	110
3,5-dimethylphenol	3,5-CH_3	−22	−31
2,3-dimethylphenol	2-CH_3	n.o.	83
	3-CH_3	−24	−32
p-thiocresol	4-CH_3	85	95
thiophenol	4-H	−67	−79

[a] This work: obtained in 50 mM potassium phosphate, pH 7.5, in D_2O at 30 °C. [b] Reference 21.

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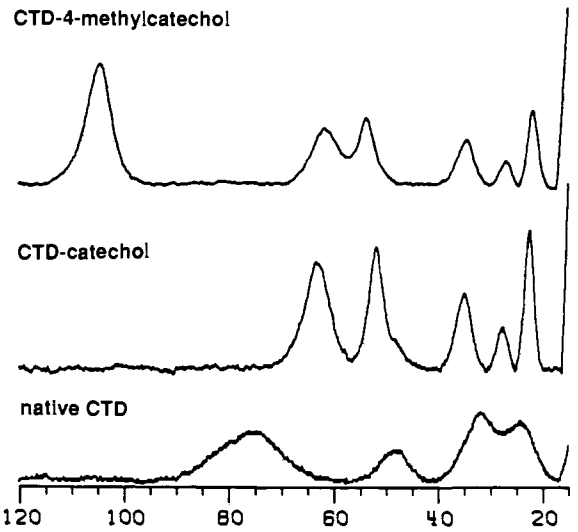

Figure 1. ^1H NMR spectra of native catechol and 4-methylcatechol complexes. Conditions: [CTD], ca. 2 mM in 50 mM potassium phosphate buffer pH 7.5 (uncorrected) in D_2O; [catechol], 10 mM; [4-methylcatechol], 10 mM.

Table II. NMR Chemical Shifts of Endogenous Ligands of CTD–X Complexes

CTD–X complexes[a]	δ (ppm)				
native CTD	75	48	32	24	
CTD–X, where X =					
catechol	63	52	35	27	22
4-methylcatechol	62	53	34	26	21
3-methylcatechol	68	57	38	29	21
3,6-methylcatechol	63	53	37	27	20
pyrogallol	70	56	36	29	22
5-methylpyrogallol	67	61	35	28	21
4-methylphenol	74	66	34	26	
3,5-dimethylphenol	73	62	33	26	
2,3-dimethylphenol	70		33		
thiophenol	83	80	40	27	
p-thiocresol		80	35	25	

[a] This work: obtained in 50 mM potassium phosphate, pH 7.5, in D_2O at 30 °C.

H). 4-Methyl-d_3-catechol was synthesized by a similar procedure with $LiAlD_4/AlCl_3$ and 3,4-dimethoxybenzoyl chloride. Ring-deuteriated catechols were obtained by dissolving the desired catechol in D_2O and a half equivalent of KO-*t*-Bu and heating the solution in a sealed tube at 140 °C. This reaction is conveniently run in an NMR tube, allowing periodic monitoring of the extent of deuteriation. The reaction was terminated when NMR integrated showed >90% deuteriation of the ring protons (ca. 4 h).

NMR spectra of the CTD complexes were obtained from samples in 50 mM potassium phosphate buffer pH 7.5 in D_2O. CTD stored in 50 mM Tris–OAc buffer (pH 8.5 at 4 °C) was transferred into the D_2O buffer with a Pharmacia PD-10 column. The resulting CTD solution was then concentrated to ca. 2 mM with a Minicon (Amicon) concentrator. Enough substrate or inhibitor was added to the concentrated CTD solution in an NMR tube to achieve saturation of the active sites. The procedures were performed under a N_2 atmosphere for the CTD–substrate complexes.

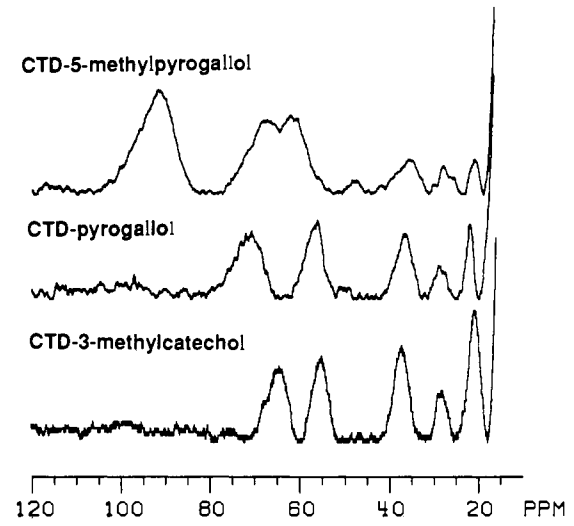

Figure 2. ^1H NMR spectra of CTD complexes with 3-methylcatechol, pyrogallol, and 5-methylpyrogallol. Conditions: as in Figure 1; [3-methylcatechol], 10 mM; [pyrogallol], 14 mM; [5-methylpyrogallol], 11 mM.

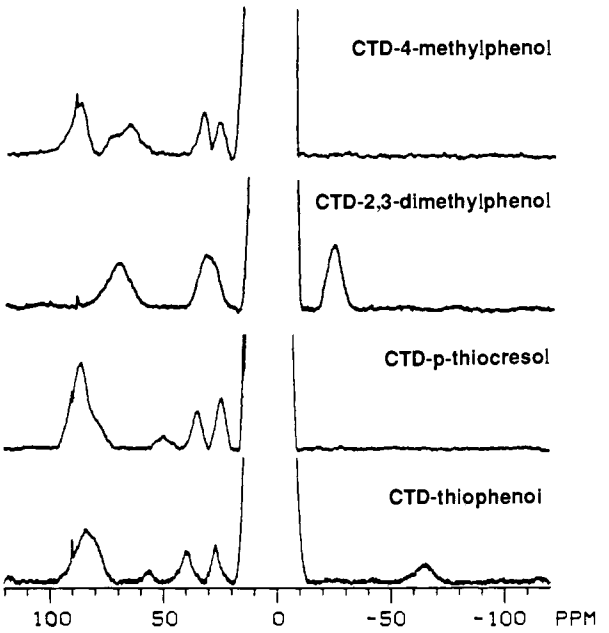

Figure 3. ^1H NMR spectra of CTD complexes with 4-methylphenol, 2,3-dimethylphenol, thiophenol, *p*-thiocresol. Conditions: as in Figure 1; [4-methylphenol], 20 mM; [2,3-dimethylphenol], 40 mM; [thiophenol], 3 mM; [*p*-thiocresol], 3 mM.

^1H NMR spectra were obtained on a Nicolet NT-300 FT NMR spectrometer operating in the quadrature detection mode (^1H frequency, 300 MHz). Suppression of solvent and diamagnetic protein resonances was accomplished by employing a presaturation pulse[15] (30 ms, 30–50 dB) or a modified DEFT pulse sequence[20] (τ = 30 ms). Between 30000 and 40000 transients were accumulated for the CTD complexes over a 50-kHz bandwidth. The spectra contained 8K data points and the signal-to-noise ratio was improved by apodization of the free induction decay, which introduced a 100–150-Hz line broadening.

Results and Discussion

NMR spectra of CTD and several of its complexes have been obtained and are illustrated in Figure 1–3. Resonances for exogenous ligands are listed in Table I and they have been shown to be in slow exchange with free ligand. Resonances for en-

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dogenous ligands are tabulated in Table II. For non-heme iron complexes, the dominant component of the isotropic shift is the contact contribution, because of the near-spherical symmetry of the high-spin Fe(III) ground state and the small zero-field splittings associated with such complexes.[21] The dominance of the contact shift allows the assignment of resonances to particular protons by comparison with the shifts of model complexes. The contact shift, which is proportional to A, the electron–nuclear hyperfine splitting constant, arises from the delocalization of spin density onto the ligand orbitals from the paramagnetic metal center. When delocalized via a σ mechanism, the unpaired spin density gives rise to downfield shifts which attenuate rapidly, usually by an order of magnitude with every intervening bond between the proton and the metal center. When delocalized with a π mechanism, the unpaired spin density gives rise to shifts which alternate in sign and do not attenuate despite increased distance from the paramagnetic center.

The Mode of Substrate Binding. The NMR spectrum of native CTD is characterized by broad ill-defined features at 75, 48, 32, and 24 ppm, downfield of DSS (Figure 1). The addition of catechol to native CTD produces significant changes at the active site as monitored by NMR spectroscopy (Figure 1). The NMR spectrum of the ES complex exhibits sharper peaks at 63, 52, 35, 27, and 22 ppm. No additional solvent exchangeable peaks are observed in H_2O. The significant sharpening of the spectra of the ES complexes probably results from the differences in the zero-field splittings of the high-spin ferric center in the complexes. The D values for native CTD and the CTD–catechol complex have been determined by Mössbauer spectroscopy to be 0.4–0.7 and 2 cm^{-1}, respectively.[4c] Larger zero-field splittings, i.e., larger D values, result in more efficient electronic relaxation and sharper NMR lines.[22]

Deuteriation of the catechol produces no change in the number of position of the resonances demonstrating that all observed features arise from endogenous ligands. Likewise, the deuterium NMR spectrum of the CTD–catechol-d_4 complex does not exhibit any resonances observed in the proton NMR spectrum of the CTD–catechol complex.[16] A comparison of the NMR spectra of the CTD–catechol and CTD–4-methylcatechol complexes (Figure 1) shows a new feature at 105 ppm. This resonance, assigned to the methyl group of the catechol by methyl deuteriation,[16] demonstrates that catechol coordinates to the metal center. the positions and line widths of the endogenous resonances change very little in the 4-methylcatechol complex, suggesting that the addition of the methyl group does not significantly alter the active site relative to the CTD–catechol complex.

The interpretation of the methyl shift in the ^1H NMR spectrum of the CTD–4-methylcatechol complex is based on comparisons with the spectra of model complexes and CTD–phenol complexes (Table I and Figure 2). The Fe(salen) complexes are particularly suitable models because the active site has been shown to consist of two tyrosine ligands, a water molecule which is displaced by substrate or inhibitor binding and possibly two histidine ligands.[1-3,5,7] Salen provides two phenolates and two imine nitrogens in a tetradentate framework to simulate the proposed active site.[23] When X is a methyl-substituted phenolate in Fe(salen)X complexes, the methyl resonances are found at 83, −31, and 110 ppm for *o*-, *m*-, and *p*-methylphenolates, respectively, as expected for a π mechanism for spin delocalization.[23]

The transferability of these observations to those of the protein resonances is demonstrated by the results on the CTD–phenol complexes (Table I and Figure 2). A comparison of the 4-methylphenol and the 3,5-dimethylphenol complexes shows that the methyl resonance for the former must be assigned to the feature at 88 ppm, while that for the latter can be associated with the −22 ppm feature. These values exhibit the same positional dependence for the sign of the isotropic shift as the corresponding Fe(salen) complexes, but their magnitudes are somewhat smaller than those observed for the model complexes. This decrease probably reflects the difference between the salicyaldimine and the tyrosine phenolates.[9] The more electron-donating tyrosine ligands would render the iron center less Lewis acidic, thus blue-shifting the charge-transfer band of the exogenous phenol and decreasing the isotropic shift. These effects have been observed in the Fe(salen)X series.[9]

The CTD–*o*-cresol complex does not exhibit a methyl resonance, while the CTD–2,3-dimethylphenol complex exhibits the *m*-methyl peak at −24 ppm, but the *o*-methyl resonance is not observed. The endogenous ligand resonances observed for these two complexes also differ from those of the 4-methylphenol and 3,5-dimethylphenol complexes, suggesting that the presence of an ortho methyl group substantially affects the enzyme active site.

The binding of 4-methylcatechol to the active site iron could occur via O1 or O2 (i.e., a monodentate catecholate) or O1 and O2 (i.e., a chelated catecholate). Fe(salen)4-MecatH, a monodentate catecholate complex, has been shown to exist as both O1 and O2 isomers on the basis of methyl shifts that match those of corresponding methyl phenolate complexes (Table I); [Fe(salen)4-Mecat]$^-$, the chelated form, exhibits a methyl resonance at 47 ppm.[23] The observation of the methyl resonance of the CTD–4-methylcatechol complex at 105 ppm thus demonstrates that this substrate is coordinated to the iron center via only the O1 oxygen.[16]

Other substrate complexes studied corroborate the monodentate coordination of substrate to the iron center. The 5-methylpyrogallol complex has its methyl resonance at 88 ppm, indicating that substrate binding to the iron is via O2 alone. The 3-methylcatechol complex does not exhibit a methyl resonance, perhaps because of the proximity of the methyl group to the iron center. This would be the case for substrate coordination via O2; O1 coordination, on the other hand, would be expected to show a methyl resonance near −25 ppm. The 3,6-dimethylcatechol complex confirms our interpretation of the 3-methylcatechol data. The twofold symmetry of this substrate would be broken upon coordination to the metal center via either oxygen, giving rise to an *o*- and a *m*-methyl group. The *o*-methyl is not observed, and the *m*-methyl resonance is found at −26 ppm. These observations thus support monodentate coordination of the substrate in the proposed mechanism. The preference for a particular catecholate oxygen is presumably dictated by steric factors in the active site.

A comparison of the methyl shifts of the 4-methylcatechol, 5-methylpyrogallol, and 4-methylphenol complexes provides further mechanistic insight. The methyl resonance of the 4-methylcatechol complex is significantly more downfield shifted than those of the other two complexes. The methyl shifts of Fe(salen)4MecatH and Fe(salen)OC_6H_4-4-Me are essentially identical,[23] indicating that the substitution of H with OH does not significantly perturb the delocalization of unpaired spin density onto the methyl group in these complexes. The larger methyl shift of the CTD–4-methylcatechol complex relative to the corresponding phenol shows that more unpaired spin density is delocalized onto the 4-methylcatechol. We have demonstrated that the ligand-to-metal charge-transfer transition is responsible for spin delocalization in these systems.[9] A larger shift corresponds to a lower energy charge transfer band. The decrease in energy would result if the monodentate catecholate would lose both its OH protons, thereby raising the catecholate ligand orbital energies and narrowing the ligand–metal energy gap. The dissociation of both protons as a requirement for substrate activation is consistent with observations made on model complexes which exhibit catechol cleavage reactivity.[24]

The similarity of the methyl shifts of the CTD–5-methylpyrogallol and the CTD–4-methylphenol complexes suggests that

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the pyrogallol O1–H and O3–H protons are not significantly dissociated in the active site. The introduction of a third OH group would increase the pK_a of the second OH, rendering it more difficult for an active site base to pull off this proton. On the basis of foregoing discussion, one might expect that the 5-methylpyrogallol complex may be less activated for O_2 attack. This expectation is borne out by comparing the stopped flow rates of the ES + O_2 step for various substrates. In a comparison of such rates for 4-methylcatechol, catechol, and 4-fluorocatechol, we have shown that the more electron donating substituent enhances the rate of oxygenation.[8b] Pyrogallol is expected to be more electron-rich than 4-methylcatechol; however, the O_2 reaction is an order of magnitude slower.[25] These observations thus suggest that the dissociation of the second catecholate proton enhances the tendency of the substrate to react with O_2.

Assignments for Endogenous Ligand Resonances. The chemical shifts of the resonances arising from the endogenous ligands of the CTD complexes are summarized in Table II. A perusal of the table indicates that the resonances arising from endogenous ligands can be grouped into two sets. The two more downfield-shifted resonances comprise one set. The isotropic shifts of these features are quite sensitive to the nature of the exogenous ligand and vary from 52 to 83 ppm downfield. The separation between the two peaks also varies, ranging from 11 ppm in the catechol complexes to essentially zero in the CTD–2,3-dimethylphenol complex. The second group of resonances, at ca. 35 and 26 ppm, is relatively insensitive to the nature of the exogenous ligand. This difference in sensitivity to exogenous ligand binding aids in the peak assignments.

A second factor to take into account in assigning the endogenous ligand resonances is that some of the expected resonances are not observed. For example, none of the ring protons of the exogenous ligands in the catechol and phenol complexes are observed, though the isotropic shifts for these absent features are expected in the 100 to −100 ppm range.[9,23] The methyl groups meta and para to the coordinated oxygen are observed, while the *o*-methyl group is not. In contrast to the phenol complex, the para proton of the thiophenol in the CTD–thiophenol complex is observed at −67 ppm. The ortho and meta protons, expected at ca. −70 and 70 ppm, respectively, are not evident. Replacement of thiophenol and *p*-thiocresol results in the disappearance of the −67-ppm and the appearance of the CH_3 resonance at 88 ppm, thus confirming the *p*-H assignment. The ortho and meta protons and the coordinated *p*-thiocresol are also not observed. These observations indicate that the observability of a particular resonance depends on two factors: the proximity of the proton to the ferric center and the coordination environment of the metal center.

The paramagnetic line width of a particular resonance in a non-heme iron protein is determined by three components—a dipolar term (T_{2D}), a Fermi contact term (T_{2F}), and a Curie spin relaxation term (T_{2C});[26] i.e., $T_2^{-1} \approx T_{2D}^{-1} + T_{2F}^{-1} + T_{2C}^{-1}$ where $T_{2D}^{-1} = f(\tau_c/r^6)$, $T_{2F}^{-1} = f(\tau_s)$, $T_{2C}^{-1} = f(\tau_R/r^6)$, and $\tau_c^{-1} = \tau_R^{-1} + \tau_s^{-1}$. r is the metal–proton distance, τ_R is the rotational correlation time, and τ_s is the electronic spin–lattice relaxation time. For the endogenous resonances of CTD, τ_c^{-1} is probably dominated by τ_s^{-1} [ca. 10^9–10^{10} s^{-1} for high spin Fe(III) in non-heme systems[26,27]], since τ_R^{-1} is estimated to be ca. 10^8 s^{-1} for a protein of 63 000 D.[28] For the methyl resonances on the exogenous ligands, methyl group rotation may also contribute to τ_c.

The dipolar and Curie terms have r^{-6} dependences such that protons in closer proximity to the metal center have larger line widths; for the CTD complexes, some resonances are so broad that they are not observed. The minimum distance at which a proton may have an observable line width, however, depends on the exogenous ligand. This reflects the importance of τ_s in determining T_2^{-1}, since τ_s depends on the coordination environment of the iron center. It has been demonstrated in high-spin ferric porphyrin complexes that τ_s is inversely proportional to the square of the zero-field splitting parameter, D; larger D values yield shorter τ_s values and sharper NMR lines.[22] Of the CTD complexes, the catechol complexes have the sharpest peaks and the native enzyme the broadest, with thiophenol and phenol complexes intermediate. The zero-field splittings for these complexes, as determined by Mössbauer spectroscopy,[4c] follow this trend, with the catechol complex having the largest D value (2 cm^{-1}) and the native enzyme the lowest (0.4–0.7 cm^{-1}).

Our observations on the CTD complexes suggest that a particular proton must have an Fe–H distance of ca. >6 Å to be observed. None of the catechol protons are observed in the CTD–catechol complex. The longest Fe–H distance is ca. 6.5 Å, based on the crystal structure of Fe(saloph)catH;[29] on the other hand, the methyl group in the 4-methylcatechol complex with an estimated distance of >7 Å is observed. The *o*-methyl resonance in the CTD–2,3-dimethylphenol complex is also not observed, while the *m*-methyl peak is. On the basis of the crystal structure of $[Fe(salen)]_2hq$,[29] the two methyl groups have estimated distances of 5.0 ± 0.5 and 6.5 ± 0.3 Å, respectively. Interestingly, the *p*-H of the thiophenol complex is observed. The Fe–H distance is estimated to be ca. 6.4 Å based on the crystal strucure of Fe(saloph)SPh.[25]

Thus, the amino acid ligands to be associated with the endogenous ligand resonances must have Fe–H distances greater than 6 Å, in order to be observable. Furthermore, these protons must have effective spin delocalization mechanisms that would give rise to the observed isotropic shifts. Table III lists the chemical shifts observed for synthetic complexes which model high-spin ferric–amino acid interactions. Given these structures, the possible candidates narrow down to protons on Cys, His, and Tyr.

The α-CH of Cys has an isotropic shift in the appropriate range, though its Fe–H distance is somewhat shorter than 6 Å. However, cysteine ligation to the ferric center has been shown to be unlikely by Mössbauer studies.[4] Furthermore, the related enzyme, protocatechuate 3,4-dioxygenase (PCD) from *Brevibacterium fuscum*,[4b] does not contain any cysteine in its primary structure, despite strong spectral similarities with CTD and the PCD from *Pseudomonas aeruginosa*.

The β-CH_2's of Tyr have the appropriate Fe–H distance to be observed. Because of the phenolate π-delocalization mechanism these resonances are expected in the 50–80 ppm range (Table III). There are two such features in the CTD complexes. These resonances are sensitive to the nature of the exogenous ligand; this sensitivity is consistent with their assignment to the Tyr β-CH_2 protons. The exogenous ligand alters the Lewis acidity of the iron center, which in turn affects the phenolate-to-metal charge-transfer band and the extent of spin delocalization. We have shown in model complexes that the shifts observed in phenolate complexes can be correlated to the energy of the LMCT band.[9] The higher the energy of the LMCT band is, the smaller the shifts are. In the model studies, thiophenol, phenol, and catechol are ranked in increasing order of ligand strength, rendering the ferric center progressively less Lewis acidic. The shifts observed on the invariant salen ligand decreases in this order. This decreasing order is also observed for the CTD–thiophenol, –phenol, and –catechol complexes.

The two features observed may arise from the β-CH_2's of two different tyrosines in the active site or from diastereotopic CH_2's

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Table III. NMR Chemical Shifts (ppm at 300 K) of Model Complexes for High-Spin Iron(III)–Amino Acid Interactions[a]

amino acid	model complex	chemical shift	ref
aspartate (or glutamate)	$Fe(salen)O_2CCH_3$	CH_3: 144	30
	$[Fe_2(HXTA)(O_2CCH_3)_2]^-$	CH_3: 89 (122)[b]	31
	$[Fe_2(HXTA)(O_2CCH_2CH_3)_2]^-$	CH_2: 83, 67 (113, 91)[b]	32
histidine	$Fe(salen)(4\text{-}CH_3\text{-}Im)_2^+$[c]	CH_3: 15 (20)[b] N–H: 103 2,5-H: n.o. CH_3: 19	15
	$Fe(salen)(Im)_2^+$[c]	N–H: 103 2,5-H: n.o. 4-H: 78	15
	$Fe(salpyr)_2(4\text{-}CH_3\text{-}Im)_2^+$[c]	N–H: 97 2,5-H: n.o. CH_3: 20	15
	$Fe(salhis)_2^+$[c]	N–H: 103 2-H: n.o. 4-H: 81 5-CH_2: −2, −21	15
tyrosine	$Fe(salen)OPh\text{-}4\text{-}CH_3$	*o*-H: −101 *m*-H: 89 *p*-CH_3: 110	23
	Fe(salen)tyrOMe	*m*-H: 87 β-CH_2: 73, 53	15
	Fe(salen)Z-tyr-PNB	*m*-H: 86 β-CH_2: 65, 59	33
	Fe(salen)Ac-tyrOMe	*m*-H: 85 β-CH_2: 61, 58	15
serine, threonine	Fe(TPP)O-*t*-Bu	CH_3: 30	32
cysteine	$[Fe_2S_2(S_2\text{-}o\text{-}xyl)_2]^{2-}$	CH_2: 40 (500–600)[d]	34

[a] Abbreviations used: salen, *N,N'*-ethylenebis(salicylideneamine); HXTA, 2-hydroxy-5-methyl-1,3-xylylenediamine-*N,N,N',N'*-tetraacetate; salpyr, 3-(*o*-hydroxyphenyl)-5-methylpyrazole; salhis, 4-[2′-(salicylideneamino)ethyl]-imidazole; Im, imidazole; Z-tyr-PNB, *N*-α-carbobenzyloxytyrosine *p*-nitrobenzyl ester; $(HS)_2$-*o*-xyl, *o*-xylene-α,α′-dithiol; TPP, *meso*-tetraphenylporphin. [b] Estimated shift after correction for $J = -10$ cm^{-1}. [c] Coordinated N designated as N-1. [d] Estimated shift after correction for $J = -150$ cm^{-1}.

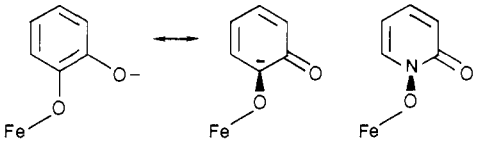
of similar tyrosines. In our model studies (Table III), the diastereotopic β-CH_2 protons exhibit shift differences of 3–20 ppm, depending on the orientation of the CH_2 protons relative to the p-orbitals of the phenol π system. The shift difference will be minimized when the C-1′ p orbital bisects the H–C–H angle in a Newman projection.[35] An examination of tyrosines in protein structures indicates that tyrosines typically adopt a conformation that minimizes the diastereotopic difference.[36] We favor the first alternative because of the resonance Raman evidence for two different tyrosines.[2b,3e] The Raman excitation profiles have been interpreted as indicating a square-pyramidal geometry for the iron center with an apical and a basal tyrosine.[9] The apical tyrosine would have the shorter Fe–O bond and the lower energy LMCT band and, consequently, the more downfield shifted β-CH_2 resonance. The differences in the tyrosine β-CH_2 shifts in the CTD complexes would thus reflect the extent to which the apical vs. basal distinction is maintained from complex to complex.

The remaining features in the 20–40 ppm range are assigned to β-CH_2's of histidines. The assignment is consistent with the insensitivity of the observed shifts to changes in the exogenous ligands and the magnitude of the shifts assigned to histidine β-CH_2's in other high-spin ferric proteins. In cytochrome *c′* from *Rhodopseudomonas palustris*, the β-CH_2 protons have been assigned to features at 35 and 36 ppm.[37] The presence of histidine in the CTD active site is also suggested by EXAFS studies of the related protocatechuate 3,4-dioxygenase.[5]

Summary. The observation of paramagnetically shifted features in the NMR spectra of CTD and its complexes has allowed us to gain insight into the coordination chemistry of the active site iron. Because of the dominance of the contact shift in high-spin ferric complexes and extensive studies on related model systems, endogenous ligand resonances can be assigned to tyrosine and histidine β-CH_2 protons. Other protons on these amino acid ligands are too broad to be observed due to their proximity to the metal center. The NMR identification of these ligands is consistent with Raman evidence for tyrosine[2,3] and EXAFS evidence for histidine.[5]

More significantly, these studies have provided a greater understanding of how substrate interacts with the iron center and how substrate activation may occur. The position of the methyl resonance of 4-methylcatechol bound to CTD clearly demonstrates that this substrate is coordinated in a monodentate fashion (via O1) and that the proton on the uncoordinated oxygen (O2) is partially, if not wholly, dissociated in the active site. This iron-bound catecholate dianion is proposed to be the species that reacts with O_2.

The observation on the CTD–4-methylcatechol complex contrasts with that on the corresponding PCD complex. On the basis of the methyl shift, we concluded that the substrate is chelated in the PCD case.[16] It is not clear why there is a difference in the way catechol is coordinated to the metal center in CTD and PCD. Several lines of evidence suggest the monodentate catecholate as the species which reacts with O_2. Model studies indicate that a chelated catecholate is a poor reductant for O_2.[24,38] Steady-state kinetic studies on PCD show that *p*-hydroxybenzoates are much better inhibitors of enzyme activity than the corresponding meta isomers.[10,39] Furthermore, resonance Raman studies on the PCD–hydroxybenzoate complexes demonstrate the coordination of the parahydroxy group but not the meta.[3e] Lastly, investigations of PCD substrate analogues designed to mimic the ketonized tautomeric form of the substrate, i.e.



bind tightly and irreversibly; EPR studies on these complexes indicate that these 2-hydroxypyridine *N*-oxides occupy only one coordination site on the metal.[40]

We speculate that the presence of the carboxylate in the native substrate of PCD, i.e., 3,4-dihydroxybenzoate, provides the enzyme with a handle to render the chelated substrate monodentate during the reaction with O_2. The absence of such a handle for the CTD substrates may have required the evolution of an alternative strategy. Perhaps the presumed active site residue in CTD which accepts the proton from the uncoordinated catechol oxygen also serves to keep the catecholate monodentate via an ion pairing interaction.

We conceive the reaction of the ES complex with O_2 as an attack of O_2 on the substrate, which would be activated by coordination to the iron and the loss of both its protons. The catecholate-to-iron(III) charge-transfer interaction delocalizes unpaired spin density onto the substrate, as indicated by the contact shifts observed. The acquired radical character on the catechol could then overcome the singlet–triplet barrier for reaction with dioxygen and thus facilitate the oxygenation reaction.

Acknowledgment. This work was supported by the National Institutes of Health (GM-33162). J.B.L. is an N.I.H. predoctoral trainee (GM-07323). L.Q. is grateful for an Alfred P. Sloan Research Fellowship (1982–1986) and an N.I.H Research Career Development Award (1982–1987).

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