are observed in the expected ratio for incorporation from solvent of no ^{18}O, one ^{18}O, and two ^{18}O atoms. (Scheme II, Figure 2) The first-formed exocyclic product is ethylene phosphate (mono-^{18}O labeled from one water molecule), which under our conditions rapidly further hydrolyzes to 2-hydroxyethyl phosphate (incorporating a second water molecule giving some di-^{18}O-labeled product.)

These results confirm that *no* oxygen exchange from solvent occurs during the course of the reaction or with starting material or products and that there is 100% P–O cleavage for all products at pH 2–15 (other results not reported). In addition, most significantly we have found no evidence under any conditions for formation of a hexacoordinate intermediate.

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Registry No. Ethyl ethylene phosphate, 823-31-4; ethyl 2-hydroxyethyl phosphate, 5178-07-4.

EXAFS and Raman Evidence for Histidine Binding at the Active Site of Protocatechuate 3,4-Dioxygenase

R. H. Felton,* W. L. Barrow,[1] S. W. May,* A. L. Sowell, and S. Goel

School of Chemistry, Georgia Institute of Technology Atlanta, Georgia 30332

G. Bunker and E. A. Stern*

Department of Physics, University of Washington Seattle, Washington 98195

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The coordination environment at the active site iron of the the catechol-cleaving dioxygenases has been examined by a number of spectroscopic probes. One of these enzymes, protocatechuate 3,4-dioxygenase (PCD), which catalyzes the intradiol cleavage of protocatechuic acid to β-carboxy-*cis,cis*-muconic acid, has been isolated from a number of microbial genera,[2-6] with the crystalline enzyme from *Pseudomonas aeruginosa* being most extensively studied.[7-10] Previous resonance Raman studies[9,11] provided evidence that the ferric ion is coordinated by two tyrosines; however, the identity of other iron ligands is not firmly established. Based upon EPR[12] and sulfhydryl titration,[13] suggestions were advanced that sulfur binding was possible, although the original proposal of tetrahedral sulfur ligation is no longer viable. Instead, histidyl coordination was proposed due to the similarity between reduced PCD and deoxyhemerythrin Mössbauer spectra.[14]

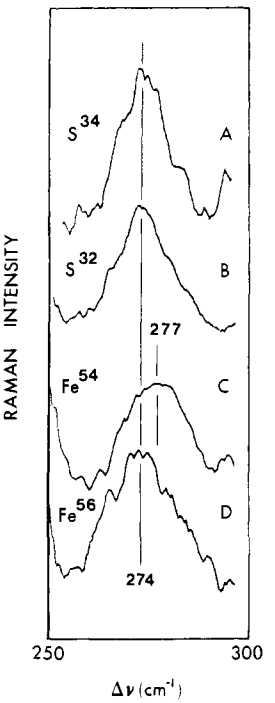

Figure 1. Resonance Raman spectra of native and labeled PCD: (A) $^{56}Fe^{34}S$; (B) $^{56}Fe^{32}S$; (C) $^{54}Fe^{32}S$; (D) $^{56}Fe^{32}S$. Spectra A, B and C, D, are measured from samples in a partitioned, rotating cell. Laser wavelength is 514.5 nm; power is 500 mW.

A Raman peak at 274 cm^{-1} in the native enzyme disappears when the enzyme is reversibly inhibited by 3-chloro-4-hydroxybenzoate (3-ClHB); yet, Raman peaks assigned to tyrosines suffer no concomitant intensity reduction.[9] This observation suggests that displacement or modification of nontyrosine groups is being monitored. Although the 274-cm^{-1} frequency is below that of ca. 350 cm^{-1} found for ν(Fe–S) in iron–sulfur proteins[15-17] and cytochrome $P\text{-}450_{cam}$,[18,19] a frequency shift to lower energies is conceivable in a highly ionic environment provided by tyrosinate coordination. Characterization of this vibration, at least in terms of sulfur participation, becomes feasible by a study of the ^{34}S isotope shift. On the other hand, a mode at 267 cm^{-1} is identified as ν(Cu–ImH) (ImH = imidazole) in the UV resonance Raman study of oxyhemocyanin,[20] and imidazole ligation in PCD becomes an attractive alternative assignment of the 274-cm^{-1} peak, if a $^{54}Fe/^{56}Fe$ isotope shift is observed with no accompanying sulfur isotope shift.

A complementary probe of local geometry about a metal ion is provided by extended X-ray absorption fine structure (EXAFS). It is now appreciated that coordinated histidine is characterized by a prominent peak in the Fourier transform of EXAFS data.[21,22] Consequently, combined Raman and EXAFS studies further delineate the active-site structure of PCD.

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PCD was isolated from strain ATCC 23975 as described previously.[8,23] Crystalline PCD labeled with ^{34}S was obtained from the same bacteria by using $H_2{}^{34}SO_4$ (90%) as the sole sulfur source.[24] Apo-PCD was prepared according to established procedures,[13] and the ^{54}Fe-labeled PCD was reconstituted from apo-PCD with 97.6% labeled ferrous ammonium sulfate.[25] To increase the accuracy of the small spectral shifts anticipated, we collected Raman spectra in a partitioned, rotating cell that is a modification of the apparatus described by Kiefer.[26] By placement of labeled and unlabeled enzyme solutions in separate compartments, scattered Raman intensity from the two samples was collected in the same run and monochromator positioning errors and source fluctuations were minimized. Otherwise, experimental protocol was that described previously.[9] EXAFS spectra were collected in the fluorescence mode[21,27] on 250 μM (2 mM Fe) samples of PCD and PCD·3-ClHB and as absorption spectra on solid standards. Specific activities of samples measured following laser and synchrotron irradiation confirmed sample integrity during the runs.

Figure 1 presents $^{32}S/^{34}S$ and $^{54}Fe/^{56}Fe$ Raman data. The 274-cm^{-1} peak rides on the Rayleigh shoulder, and fluorescence background counts are high. Spectra are shown after linear background subtraction and data smoothing. Irrespective of the slope of the background subtraction, we find no measurable shift in the experiments using labeled sulfur enzyme, while a +3-cm^{-1} shift is observed for ^{54}Fe-labeled sample. For a pure Fe–S stretch, a 5-cm^{-1} shift is anticipated with ^{34}S, and it is evident that this vibration cannot be so assigned. Since scattering intensity in the 350-cm^{-1} region is weak and exhibits no discernible isotope shift, we conclude that Raman data provide no support for iron–sulfur binding.

In contrast, a 3-cm^{-1} shift is found and is predicted for ν(Fe–ImH). A band at 292 cm^{-1} in azidomethemerythrin is assigned to ImH,[28] and Walters and Spiro[29] have assigned a shoulder at 270 cm^{-1} to ImH in oxymyoglobin. The comparison is relevant for the oxygenated ferrous porphyrins are known to have frequencies similar to those found in ferric porphyrins.[18] Furthermore, these authors report a value of 203 cm^{-1} for the symmetric stretch ν(ImH–Fe–ImH) in low-spin ferric protoporphyrinbis(imidazole). The datum predicts ν(Fe–ImH) in ferric complexes should be at 300 cm^{-1} for either one ImH bound to ferric or a cis arrangement of imidazoles in a ferric complex as found in azidomethemerythrin.[30] Consequently, we assign the 274-cm^{-1} peak to either structure in PCD.

The presence of imidazole (histidyl) ligation in PCD is confirmed by the EXAFS transforms shown in Figure 2. The major peak at 1.5 Å in Figure 2 is in all cases assigned to nearest neighbor scattering, and the prominent peak at 3.3 Å in Figure 2, parts a, c, and d, is the backscattering peak arising from N_2 and C_4 of the imidazole, the third shell of atoms in ImH.[31] Its prominence is thought to arise from shadowing of N_2 and C_4 by N_1 caused by the nearly collinear arrangement of Fe–N_1–N_2 or Fe–N_1–C_4.[21]

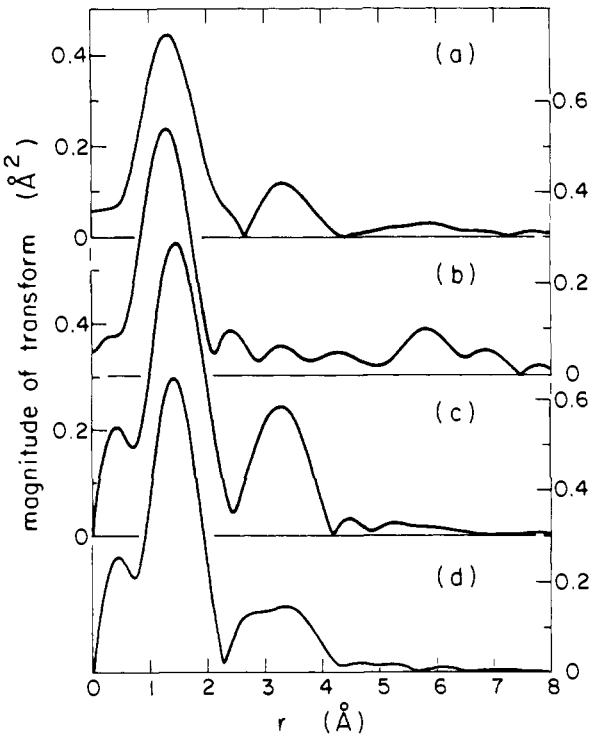

Figure 2. Fourier transforms over the range $3.0 \leq k \leq 8.2$ Å^{-1}. The window edges were tapered to reduce cutoff effects: (a) PCD; (b) PCD·3-ClHB; (c) $Cu(ImH)_4(NO_3)_2$; (d) $Cu(ImH)_2$(2,4,6-trichlorophenolato)$_2$.

The enhancement requires in addition a rigid arrangement of atoms as in the five-membered ImH ring to overcome thermal quenching of the third shell backscattering amplitude. Concern that the phenyl ring of tyrosine could cause or interfere with the 3.3-Å peak is vitiated, for we find no such signature in the EXAFS transform of tripotassium tris(catecholato)iron(III), and the trace in Figure 2d of the transform from EXAFS data of bis(imidazolato)bis(2,4,6-trichlorophenolato)copper(II)[33] displays the characteristic maximum of ImH.

Quantitative analysis of the enzyme data yields the number of histidines bound to the Fe in the native and inhibited forms. Standard analysis methods were used: background subtraction, normalization, interpolation to k space, and Fourier analysis.[34] The nominal energy threshold was set to the absorption maximum. The data range of k was taken to be between 3.0 and 8.2 Å^{-1}; tapered windows and k^2 weighting were used. Comparison of native PCD to deoxyhemerythrin spectra[35] by the ratio method[34] directly shows the presence of 1.7 ± 0.3 histidine ligands in the native form. Hemerythrin is known to have 2.5 histidine ligands per Fe.[30] Furthermore, direct comparison of the transforms of the native and inhibited forms and the difference spectrum between the two show that 1.0 ± 0.3 histidines remain bound in the inhibited enzyme. This is confirmed by ratio method analysis. The 1 standard deviation error is obtained by analyzing partial sums of the data and their difference spectra and comparing the results.

In the PCD·3-ClHB complex, the 274-cm^{-1} Raman peak is not observed,[36] and the reduced EXAFS amplitude can be interpreted

(23) The ATCC has reclassified this strain as *Pseudomonas putida*, although it is still referred to in the literature by its original identification as *P. aeruginosa*.

(24) The growth medium contained 3 g/L trisodium citrate, 3 g/L $(H_4N)_2HPO_4$, 1.2 g/L K_2HPO_4, 5 g/L 4-hydroxybenzoic acid, 0.35 g/L NaCl, 0.072 g/L $FeCl_2{\cdot}4H_2O$, 0.17 g/L $MgCl_2{\cdot}6H_2O$, and 0.126 g/L H_2SO_4 (95% solution enriched with 90% ^{34}S).

(25) Specific activities: ^{34}S-labeled PCD, 55 units/mg; apo-PCD, 0.51 units/mg; ^{54}Fe-labeled PCD, 56 units/mg.

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(36) The inability to detect the remaining bound ImH in the Raman spectrum of PCD-3-ClHB could be caused, in part, by diminished resonance enhancement. The peak absorption maximum shifts from 450 nm in PCD to 420 nm in the inhibited complex and thus further from the 514.5-nm laser source. Attempts to examine the low-frequency Raman spectrum with 457.9-nm irradiation are unsucessful due to increased fluorescence background.

as indicating either (a) ImH remains bound but the Fe–N bond is forced out of the ImH plane or (b) the number of bound histidines is reduced from two in the native enzyme to one in the inhibitor–enzyme complex. We favor (b) since previous binding studies[8,10] of 3-ClHB and its fluoro analogue, 3-FHB, demonstrated that prior ionization of the relatively acidic *p*-hydroxyl in these inhibitors was not responsible for high potency. Instead, it was suggested that a base, likely a histidine, was present at the active site to provide for removal of even relatively nonacidic *p*-hydroxyl protons (such as those found in all known PCD substrates), as the hydroxyl nears the coordination sphere of iron. When considered together with the fact that 3-ClHB forms an Fe–O bond with PCD iron,[9] these conclusions rationalize the displacement of one histidine ligand in the enzyme–inhibitor complex. In the enzyme–substrate complex, approach of the *o*-dihydroxyphenyl group accompanied by displacement of the two cis histidines and consequent conversion of the hydroxy functionalities to the oxyanion form provides an attractive picture for the eventual storage of substrate as a chelating ligand.[9] The replacement of a backbone amino acid ligand by an inhibitor (and likely substrate) is noteworthy, since metalloenzymes usually possess a vacancy or labile water in the first coordination shell so as to accommodate the binding molecule.

Analysis of EXAFS data in terms of first-shell composition is currently under investigation.

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A Reactive Three-Metal Carbide Cluster Mimic, $[Fe_3(CO)_9(CCO)]^{2-}$

J. W. Kolis,† E. M. Holt,*‡ M. Drezdzon,† K. H. Whitmire,† and D. F. Shriver*†

Departments of Chemistry, Northwestern University Evanston, Illinois 60201 and Oklahoma State University Stillwater, Oklahoma 74078

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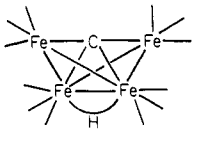
In view of the importance of surface carbides in the heterogeneous catalytic reduction of CO, there is considerable interest in the reactivity of the carbide ligand in molecular metal cluster compounds.[1-4] Recently, molecular cluster compounds containing four metal atoms (e.g., **1**) have been shown to be reactive at the



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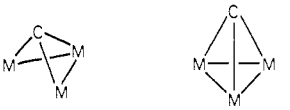
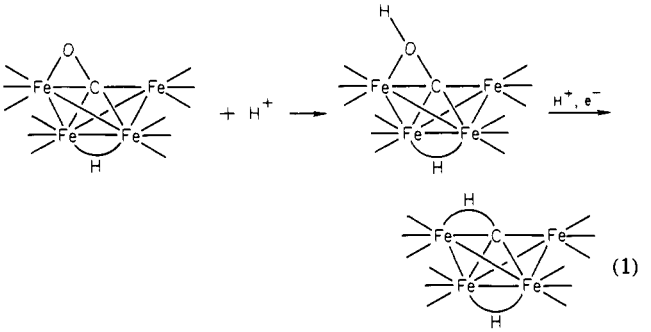
carbide atom,[5-7] which contrasts with the inertness at the carbide in previously studied five-metal and higher carbide molecules. This trend in reactivity has been correlated with the degree of exposure of the C in the Fe_4 butterfly carbides[4-6] and with the relative proximity to the HOMO–LUMO gap of molecular orbitals having high C-orbital coefficients.[8] These same considerations lead one to expect high reactivity at a carbide coordinated to an array of three metal atoms, **2a,b**.[8] In this communication we report the



2a **2b**

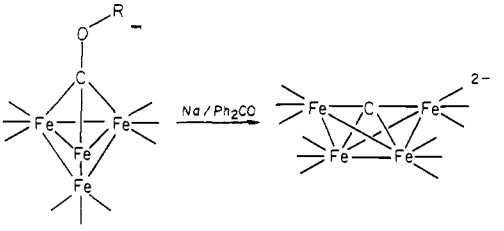
first evidence for an isolable three-metal system having carbide-like reactivity.

The synthetic approach to an Fe_3 carbide is based on our recent observation that CO can be converted to a carbide or methyne in a strong acid medium.[9,10] The proposed key reactions, eq 1, are the formation of a good O-containing leaving group via protonation and the reduction of this species by a second metal cluster.[9,10]



(1)

In analogy with these reactions, we explored the reduction of metal cluster carbonyl compounds in which a variety of oxophilic groups are attached to the carbonyl oxygen, eq 2. Success in the

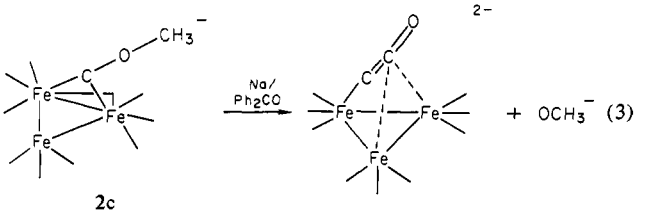


(2)

R = acetyl, CH_3

preparation of the known cluster $[Fe_4(CO)_{12}C]^{2-}$ has led us to extend this technique to the Fe_3 system.[11]

In a typical preparation, 0.3 g of $[PPN]_2[Fe_3(CO)_{11}]$ was O-methylated or O-acetylated as previously described,[12] and this was reduced over a period of 4 h by benzophenoneketyl (1,2-dimethoxyethane or THF, containing 0.3 g of Na and 0.1 g of benzophenone), eq 3. The solution was filtered to remove excess



(3)

2c

† Northwestern University.
‡ Oklahoma State University.

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