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A RESONANCE RAMAN STUDY OF SUBSTRATE AND INHIBITOR BINDING TO PROTOCATECHUATE-3,4-DIOXYGENASE

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SUMMARY: Resonance Raman spectra were obtained for complexes of protocatechuate-3,4-dioxygenase with substrate and hydroxybenzoate inhibitors. The data establish metal coordination by these bound species and demonstrate further that tyrosine ligation, present in the resting enzyme, is not altered in the complexes. For the inhibitors, 3-chloro-4-hydroxybenzoate and 3-fluoro-4-hydroxybenzoate, the data are interpreted as indicating iron ligation by the phenolate functionality. For the substrate, 3,4-dihydroxyphenylproprionate, chelation via the *o*-dihydroxy grouping is proposed. In all three complexes tyrosine ligands present in the resting enzyme are not displaced. The inhibitor scattering intensity was utilized as an internal standard to estimate that two tyrosines are coordinated to the iron at the active site.

INTRODUCTION: The coordination environment of the metal atom in non-heme iron oxygenases and the effect of substrate and inhibitor binding on this environment are subjects of current interest. Protocatechuate-3,4-dioxygenase (PCD) is an oxygenase of this type which catalyzes the intradiol oxygenation of 3,4-dihydroxybenzoate (protocatechuate) to β-carboxy-*cis*,*cis*-muconic acid (1). Recently, two groups presented resonance Raman evidence for tyrosine coordination of the ferric ion in resting PCD but were unable to obtain spectral information regarding iron coordination changes upon substrate binding (2,3). We now report resonance Raman spectra of PCD complexes with substrate and hydroxybenzoate inhibitors that establish metal coordination by these exogenous compounds and demonstrate further that tyrosine ligation is not altered in the complexes. Additionally, the present Raman spectra are

[≠]Alfred P. Sloan Foundation Fellow, 1977-1979.

Abbreviations: Protocatechuate-3,4-dioxygenase [protocatechuate: oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.3], PCD; 3,4-dihydroxyphenylpropionate, DHPP; 3-chloro-4-hydroxybenzoate, 3-ClHB; 3-fluoro-4-hydroxybenzoate, 3-FHB.

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of sufficient quality to allow examination of changes in the low-frequency Stokes scattering upon substrate or inhibitor binding.

EXPERIMENTAL SECTION:

Raman spectra were obtained with CW excitation as described previously (4). Excitation at 514.5 nm was provided by a Coherent Radiation Model CR-5 argon ion laser and at 647.1 nm by a Model 500K Krypton ion laser. Samples were contained in a rotating cell with 200-300 mW power on the sample. Spectral resolution was 6 cm^{-1} at 514.5 nm.

All commercial materials were the highest grades obtainable. 3-chloro-4-hydroxybenzoic acid (3-ClHB), obtained from Aldrich, was recrystallized from water and dried *in vacuo* prior to use. 3-fluoro-4-hydroxybenzoic acid (3-FHB) was synthesized, as previously described (5). Protocatechuic acid from various commercial sources was decolorized with charcoal and recrystallized from water before use in enzyme assays. 3,4-dihydroxyphenylpropionic acid (DHPP) from Aldrich was treated with charcoal in ethyl acetate and recrystallized by addition of chloroform.

PCD was isolated from 4-hydroxybenzoate induced cells of *Pseudomonas aeruginosa* as described previously (5). The isolated enzyme exhibited normal spectral properties and a specific activity of 65-75 units/mg. Fresh samples of 90 μM PCD (~ 0.7 mM in Fe^{3+}) in 50 mM tris-chloride at pH 7.5 were placed in a rotating cell and were irradiated at 514.5 nm for several hours to reduce fluorescence. Enzyme assay and optical spectroscopy following illumination verified that pre-illumination caused no detectable alteration in the properties of PCD (specific activity before irradiation, 74; after,69).

Enzyme inhibitor complexes were obtained by the addition of 5 mM 3-FHB (K_I = 0.6 μM) or 3-ClHB (K_I = 4 μM) to PCD in 50 mM Tris-chloride, pH 7.5, to assure saturation of the binding site by these competitive inhibitors (5). Apo-PCD was prepared according to Fujiwara and Nozaki (6). The DHPP complex with PCD was prepared by adding 10 fold excess of DHPP to PCD in 50 mM tris-chloride under a nitrogen atmosphere. Markedly increased fluorescence was noted in the enzyme-substrate complex, which was not attributable to unbound substrate. Upon exposure to air, both the Raman peaks of the substrate and the fluorescence disappeared. Ferric DHPP complexes were prepared under N_2 by dissolving ferric nitrate and DHPP at the desired ratio and adjusting the pH to 10 for the tris complex and to 3.8 for the monocatecholato complex (7). Poor scattering at 514.5 nm for the latter complex at 25 mM concentration necessitated the use of the Kr^+ line. Even then large slit widths precluded recording the Raman spectrum near the Rayleigh line.

RESULTS AND DISCUSSION: Figure 1 presents resonance Raman spectra of native PCD, its complexes with the competitive inhibitors, 3-FHB and 3-ClHB, in both H_2O and D_2O, and the Raman spectrum of the apo-enzyme. The spectrum of resting PCD exhibits the features observed by Tatsuno et al.(2) and Keyes et al. (3). These are enhanced lines at 1608, 1508, 1264, and 1177 cm^{-1} attributable, upon comparison to transferrin and model complexes (8,9) to scattering by tyrosine modes upon excitation into the charge-transfer state.

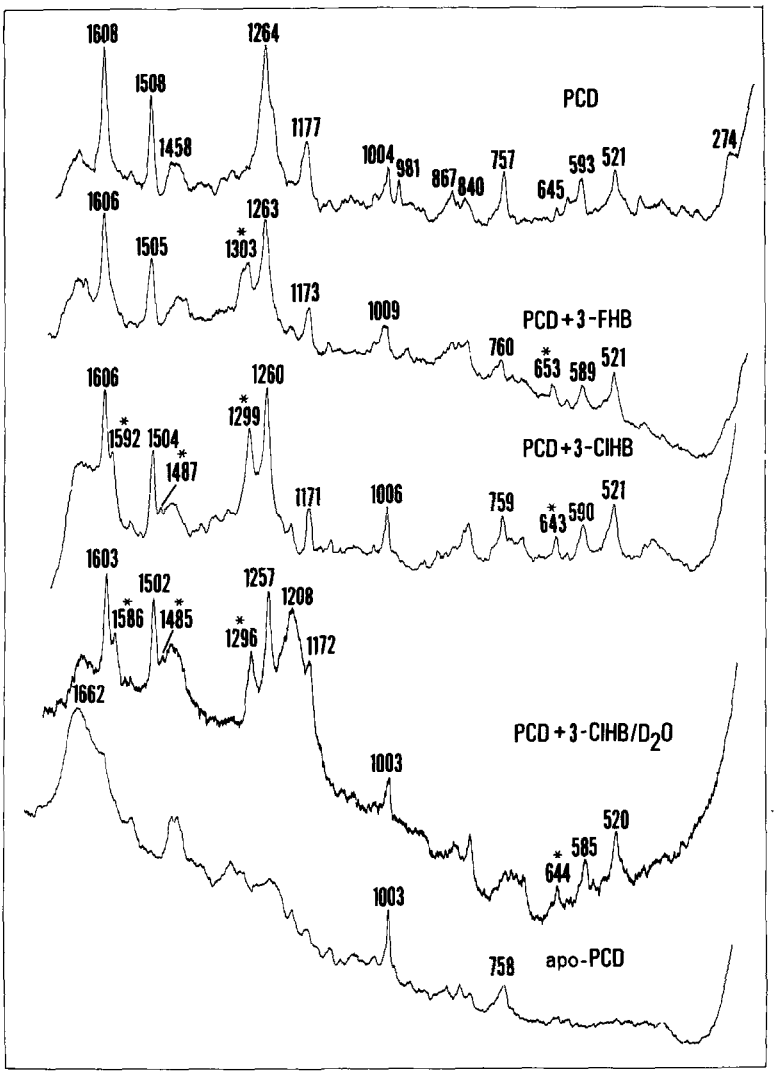

Figure 1: Resonance Raman spectra of protocatechuate-3,4-dioxygenase, inhibited complexes with 3-fluoro-4-hydroxybenzoate and 3-chloro-4-hydroxybenzoate, and the Raman spectrum of apoenzyme. Spectra are excited at 514.5 nm. Asterisked features are assigned to enhanced scattering of inhibitor modes.

Non-resonant peaks are assigned as 1662 (H_2O), 1458 (δ-CH_2), ~ 1208 (D_2O) and 1004 cm^{-1} (phenylalanine). Weak enhancement is possible for the 757 cm^{-1} peak (9) due to overlapping of an enhanced tyrosine mode with a tryptophan vibration. In comparison with other resonance Raman studies of PCD, the low-frequency region is fluorescent-free. This difference is possibly a result

of the higher purity of the present enzyme preparation. In the low-frequency region, prominent peaks at 593 and 521 cm^{-1} differ in position and intensity from those reported at 507 and 360-370 cm^{-1} for human serum transferrin iron chelate (9). The vibrations may correspond, respectively, to an in-plane deformation mode of tyrosine (10) and a mode containing some Fe-O stretching character (11). Alternatively, scattering may be caused by the two totally symmetric vibrations, derived from iron-oxygen stretches and present in low-symmetry octahedral complexes (12).

In Figure 1, the asterisked features are assigned to resonance enhanced ring vibrations of 3-FHB (1303 cm^{-1}) and 3-ClHB (1592, 1487, 1299 cm^{-1} in H_2O and 1586, 1485 and 1296 cm^{-1} in D_2O), which correlate with increased C-O stretching contribution to the potential energy distribution of the mode as determined by normal coordinate analyses. The increased intensity at 644 or 653 cm^{-1} (*cf* native PCD) is consistently reproducible and is assigned to a new vibration of the inhibitor-iron complex. We have examined non-resonant spectra of 3-ClHB and 3-FHB in various states of ionization, as well as resonance Raman spectra of ferric complexes of *o*-chlorophenol, *o*-fluorophenol, and the methyl ester of 3-ClHB. There is excellent agreement between the frequencies of the new bands in the enzyme-inhibitor complexes and various model compounds.

It is apparent from the conservation of tyrosine scattering intensity upon inhibitor binding that tyrosine ligands are not displaced by the structurally similar phenolate functionality of the inhibitors. Some minor changes are observed in relative intensities in the 500-650 cm^{-1} region, which is consistent with replacement of some non-tyrosine ligand of iron by the inhibitor. Also, the 274 cm^{-1} feature (Fe-S?) in native PCD is lost upon binding. Since the Raman spectrum of PCD·3-ClHB shows an approximate 2:1 ratio of tyrosine to inhibitor mode scattering intensity for corresponding vibrations, we believe that there are two tyrosines ligated to iron. These data together with the blue shift of the absorption maximum from 450 nm

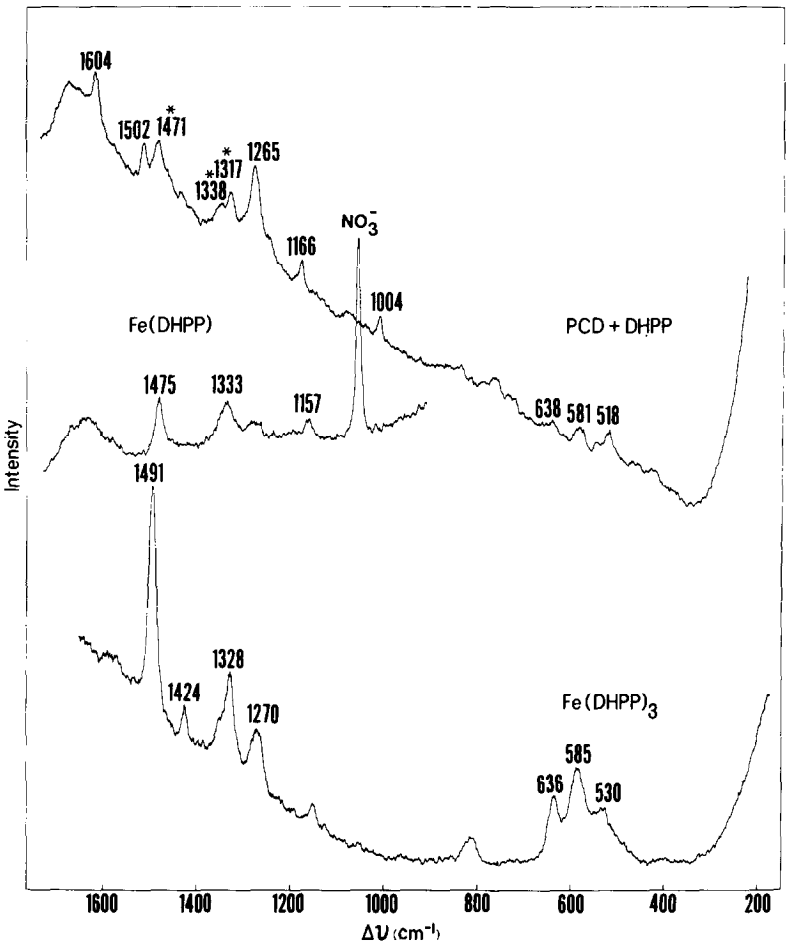

Figure 2: Resonance Raman spectrum of the enzyme-substrate complex of protocatechuate-3,4-dioxygenase with 3,4-dihydroxyphenylproprionate and spectra of model complexes. Excitation of Fe(DHPP) is at 641.7 nm. Other conditions and symbols are as indicated in Figure 1.

in PCD to 420 nm in the inhibited complexes (5) are compelling evidence for direct ligation of hydroxybenzoates.[#]

Figure 2 presents the Raman spectrum of the PCD complex with the substrate DHPP obtained under anaerobic conditions. To provide evidence that

[#]Relative scattering intensities of the ring modes of inhibitor and enzyme do not vary appreciably with excitation frequency. In this instance, but not generally, the inhibitor scattering intensity can be employed as an internal standard to estimate the relative number of electronically analogous tyrosines. Also, the blue shift of the visible maximum by ca. 1600 cm^{-1} is consistent with phenolate complexation (13).

vibrations at 1471, 1338, and 1317 cm^{-1} are associated with substrate, we have included spectra of the mono and tris catecholato iron complexes with DHPP, which show the largest scattering for those vibrations noted in the enzyme-substrate complex.

The slow substrate, DHPP, is ligated to ferric iron in a manner that provides a spectrum similar to those of the model complexes and enteriobactin Fe(III) complexes (10). Additionally, the optical absorption spectrum of the enzyme-substrate complex exhibits marked absorption in the 500-750 nm region (1) and is consistent with the optical absorption spectrum of Fe(DHPP) with the ligand as a chelating agent (7). We propose that substrate binding involves chelation of the iron via the *o*-dihydroxy grouping, since all known PCD substrates contain relatively non-acidic hydroxyls (pK_a 9.5-10). In sharp contrast, 3-FHB and 3-ClHB contain relatively acidic *p*-hydroxyl functionalities (pK_a 7.6-8.0), and phenolate binding to iron is the dominant binding mode. In support of this hypothesis, we have obtained kinetic data for the binding of a series of 3-substituted-4-hydroxybenzoates and find that their K_I values correlate directly with steric substituent constants, if it is assumed that the phenolate ionic form of the inhibitor binds to PCD [R. S. Phillips, unpublished observations].

While chelation may represent the predominant mode of binding in the enzyme-substrate complex, mechanistic considerations suggest that during the catalytic process one of the catechol oxygens swings away to provide for ketonization of the substrate and possible oxygen binding at the metal. This picture could explain why certain catechol derivatives (5) bind well to PCD but are inert to oxygenation.

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