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RAMAN SPECTRUM OF PROTOCATECHUATE DIOXYGENASE FROM *PSEUDOMONAS PUTIDA* NEW LOW FREQUENCY BANDS

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Summary The Raman spectrum (441.6 nm excitation) of protocatechuate 3,4-dioxygenase (PCD) from *Pseudomonas putida* shows resonance enhanced bands at 1605, 1504, 1270, 858, and 830 cm^{-1} which are due to the p-hydroxyphenyl group of tyrosine coordinated to iron. In addition, we observe strong resonance enhanced bands at 592 and 524 cm^{-1} and weak (presumably iron-ligand)vibrations at 465, 423, and 371 cm^{-1}. Recent publications of the Raman spectrum of PCD from *Pseudomonas aeruginosa* (Tatsuno et al, *J. Am. Chem. Soc. 100,* 4614-4615 (1978) and Keyes et al, *Biochem. Biophys. Res. Comm. 83,* 941-945 (1978) using 488 and 514 nm excitation did not report these bands. Our 441.6 nm excitation Raman spectrum of human serum transferrin, another metalloprotein with an iron-tyrosine linkage, does not show the 592 and 524 cm^{-1} bands and has only two very weak bands at about 423 and 364 cm^{-1}. We discuss several interpretations of these data.

Nonheme iron dioxygenases are essential enzymes for the degradation of aromatic compounds by soil bacteria (1). One of these enzymes is protocatechuate dioxygenase (PCD) which catalyzes the intradiol addition of dioxygen to protocatechuate (3,4-dihydroxybenzoate) forming β-carboxy-*cis,cis* muconic acid. This enzyme has been isolated from several sources and studied extensively (for reviews see ref. 2 and 3). PCD from *P. aeruginosa* has been studied most extensively. It is composed of 32 subunits and is of the molecular form $8(\alpha_2\beta_2Fe)$ where α and β are protein subunits of molecular weights 22,500 and 25,000 (4). Although the ferric iron in the active site of this enzyme is characterized by a g = 4.3 high spin EPR signal (5), the metal ligands have not been positively identified. Whereas EPR data suggest tetrahedrally coordinated cysteine sulfur groups (5), Mössbauer data suggest coordination to oxygen or nitrogen (6). Very recently resonance Raman spectra of PCD from *P. aeruginosa* were reported by Tatsuno et al, (7) (488 nm excitation) and by Keyes et al, (8) (514.5 nm excitation) showing that tyrosine is an iron ligand.

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We report here resonance Raman spectra (441.6 nm excitation) of a new PCD isolated from *Pseudomonas putida*. This enzyme has the molecular form 4(αβFe) (manuscript in preparation), in contrast to the form $8(\alpha_2\beta_2Fe)$ of PCD from *P. aeruginosa*. In addition to resonance enhanced Raman bands of tyrosine, we observe strong bands at 524 and 592 cm^{-1} and weak ones at 370, 423, and 465 cm^{-1} not previously reported for the spectrum of the *P. aeruginosa* enzyme. Since resonance Raman spectra recorded with different excitation frequencies may not be identical, we also report the resonance Raman spectrum of human serum transferrin, another metalloprotein with a ferric iron-tyrosine linkage and similar spectral properties to PCD. While the Raman bands due to tyrosine are very similar in the two proteins, the absence of the strong low-frequency bands in the transferrin spectrum suggests the involvement of an additional ligand in the PCD chromophore which is not present in transferrin.

MATERIALS AND METHODS

PCD, isolated from *Pseudomonas putida* grown on phthalate as the sole carbon source, was shown to be homogeneous by polyacrylamide gel electrophoresis with or without SDS and by sedimentation equilibrium analysis. The purification procedure and properties of PCD from *P. putida* (the bacterial strain was generously donated by Douglas Ribbons of the University of Miami) will be reported in detail elsewhere. In contrast to PCD from *Pseudomonas aeruginosa*, our preparation of PCD shows a lower molecular weight (190,000 vs 700,000) and a different iron content [4 moles of Fe/mole of enzyme vs 7-8 moles of Fe/mole of enzyme in *P. aeruginosa* PCD (4,6)]. Nevertheless, the optical absorption, CD, and EPR spectra of *P. putida* PCD are similar to those of *P. aeruginosa*, implying that the active sites of these two enzymes are also similar.

Samples of PCD for Raman studies were concentrated by ultrafiltration to 0.9 mM in iron and centrifuged prior to use. Buffer for the PCD was 0.01 M tris-Cl, pH 8.1 containing 0.1 M KCl. Reduced PCD was prepared by adding a few crystals of dithionite to the same PCD solution under an argon atmosphere in the sample cuvette.

Human serum transferrin was a gift of Dr. Philip Aisen. Its spectrum was recorded in 0.01 M tris-Cl, pH 8.0 at a concentration of 0.8 mM in iron.

Raman spectra were recorded with the 441.6 nm output of a He-Cd laser (RCA model LD 2186) with the beam transmitted through the samples which were contained in standard 5 x 10 mm cuvettes. The spectrometer and photon counting system are described elsewhere (9). Nominal laser output power was 25 mW (16×10^6 mW/cm^2 at the focus in the samples). The scan rate was 1 cm^{-1}/sec and the spectral slit width was 5 cm^{-1}. The spectrometer optics were optimized on the water band at 3300 cm^{-1}. All samples were maintained at 4°C.

RESULTS AND DISCUSSION

The Raman spectra for oxidized and reduced PCD are shown in traces A and B respectively of Figure 1. Trace C is the spectrum of human serum transferrin. The visible absorption spectrum of the oxidized PCD is shown in the inset in the upper left panel of Figure 1 with the laser wavelength indicated by the arrow. Since the laser wavelength is within the optical absorption

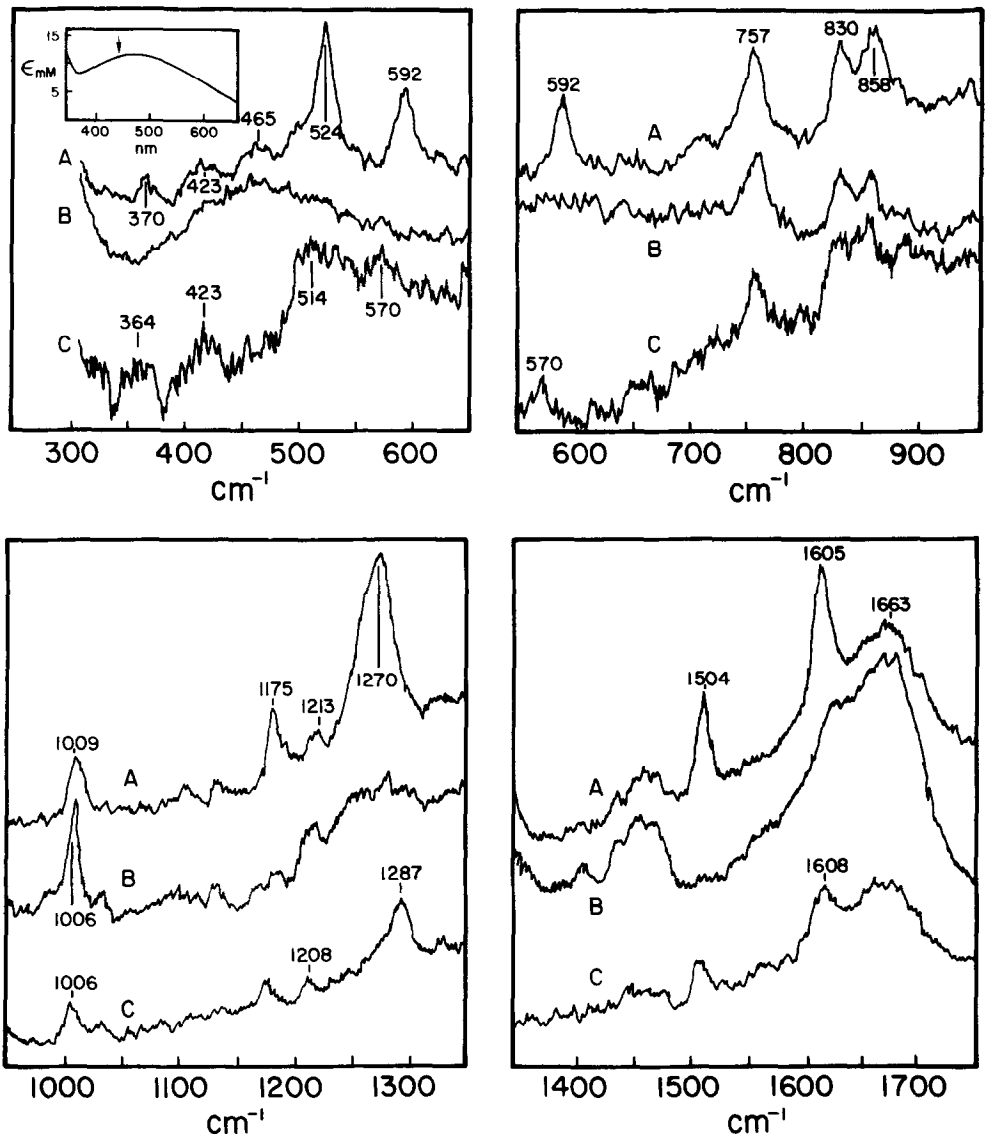

Figure 1. Raman spectra of: (A) oxidized protocatechuate 3,4-dioxygenase (PCD); (B) the same PCD sample as in (A) after reduction with dithionite; (C) human serum transferrin. The spectra displayed in the upper two panels are the sum of 8 scans; those in the lower two panels are the sum of 4 scans. All spectra of PCD are displayed with the same output amplifier time constant and gain. The spectrum of transferrin in the lower panels is recorded at the same gain as that for PCD. Spectrum C in the upper left and upper right panels are shown at 2.5 fold and 1.7 fold higher gain. The spectra shown were traced from the original data. No attempt was made to subtract the slight fluorescence background of unknown origin which causes the sloping baseline. The inset in the upper left panel shows the position of the laser excitation (arrow) in the visible absorption spectrum of PCD (extinction is expressed for the holoenzyme).

band, we expect the Raman spectrum of the oxidized enzyme to show resonance enhanced bands corresponding to vibrations of the chromophore comprised of the iron and its ligands. The resonance enhanced bands correspond to ligand internal normal modes (perhaps modified by bonding to the metal), to ligand-metal vibrations, or to chromophore normal modes which are a mixture of ligand internal vibrations and ligand-metal vibrations. The spectrum of the reduced PCD was recorded on the same sample with identical instrumental parameters as used for the spectrum of the oxidized enzyme. The reduced enzyme has no absorption band near 441.6 nm; therefore its Raman spectrum cannot have resonance enhanced bands. The spectrum of the apoenzyme (not shown) was indistinguishable from that of the reduced protein.

In addition to resonance enhanced bands, the Raman spectrum of the oxidized enzyme has bands which are not resonance enhanced. These usually correspond to amino acids not part of the chromophore. These nonresonance bands occur in the spectra of both oxidized and reduced enzyme. The absolute intensity of the non-resonance enhanced bands in the oxidized enzyme spectrum should be less than those in the reduced enzyme spectrum, because both the exciting and scattered light are absorbed by the chromophore. The bands which occur only in the spectrum of the oxidized enzyme correspond to resonance enhanced vibrations of the chromophore. Traces A and B of Figure 1 clearly establish chromophore vibrations at 370, 423, 465, 524, 592, 1175, 1270, 1504, and 1605 cm^{-1}.

Careful consideration of the rest of the spectrum shows that several other bands are also resonance enhanced. The band near 1009 cm^{-1} in the spectrum of the oxidized enzyme consists mainly of a strong nonresonant band at 1006 cm^{-1}, as shown in the reduced enzyme spectrum. A small resonant band at about 1010 cm^{-1} causes both the slight difference between the frequencies of the peaks in the oxidized and reduced protein spectra and the asymmetry in the band of the oxidized protein spectrum. The 1006 cm^{-1} agrees well in frequency with that of a strong phenylalanine mode at 1004 cm^{-1} (10) and serves as an approximate relative intensity reference for other bands in the spectra (11). Thus the ratio of the intensity of the 757, 830, and 858 cm^{-1} bands to that of the 1006 cm^{-1} band is about 2 in the spectrum of oxidized enzyme and slightly less than 1 in the spectrum of reduced protein. These ratios imply that at least 50% of the intensity in the bands at 757, 830, and 858 cm^{-1} in the spectrum of oxidized protein is resonance enhanced.

The broad band centered at about 460 cm^{-1} for the reduced enzyme is a nonresonant water mode (12). The other broad band centered about 1660 cm^{-1} consists of both water and protein amide bands. The band due to water occurs at 1631 cm^{-1}, and this frequency may also be used as an approximate relative intensity reference for the other bands. Again, the conclusion is reached that

about half of the intensity of scattering at 757, 830 and 858 cm^{-1} is due to resonance enhancement.

The bands at 1605, 1270, 1170, 858, and 830 cm^{-1} agree well with the frequencies of tyrosine p-hydroxyphenyl vibrations (10) regardless of the phenolic ionization state. These PCD bands also correspond, with minor frequency differences, to those observed for transferrin (see trace C in Figure 1 and references 11,13-15) and for PCD from *P. aeruginosa* (7,8). While the band at 1504 cm^{-1} does not correspond as closely to the observed free tyrosine vibration at 1488 cm^{-1} (10), it does occur in all of the metalloproteins mentioned above and has been ascribed to tyrosine (15). Recent work by Tomimatsu et al. (11) has confirmed this assignment. Thus at least one iron ligand in *P. putida* PCD is tyrosine.

The comparison of the oxidized PCD spectrum with that of transferrin in the low frequency range is striking. The transferrin spectrum does not show the strong bands at 524 and 592 cm^{-1}, even at 2.5 fold higher gain. Because these two bands are so intense in the PCD spectrum we expect them to be associated with vibrations of the conjugated part of an aromatic amino acid. Although tyrosine shows weak bands at 526 and 574 cm^{-1}, these are not due to the p-hydroxyphenyl group and therefore should not give rise to such strongly enhanced bands. The only other aromatic amino acid vibrations with frequencies near these two PCD bands are tryptophan indole vibrations (10) at 535 (solid), 541 (pH 1.0), and 546 (pH 12); and 575 (solid), 576 (pH 1.0) and 577 (pH 12). The prominent band at 757 cm^{-1} also corresponds to a tryptophan indole vibration. Thus assigning the 524 and 592 cm^{-1} bands to resonance enhanced tryptophan indole bands is consistent with the 50% resonance enhancement of the tryptophan band at 757 cm^{-1}. The slight resonance enhanced intensity near 1010 cm^{-1} in the spectrum of the oxidized enzyme also agrees well with another tryptophan indole vibration (10).

However, tryptophan is not known to be a metal ligand in other iron proteins and is not expected to be a good ligand from a chemical standpoint. The possibility that the 524 and 592 cm^{-1} bands are purely metal-ligand vibrations seems unlikely since known metal ligand vibrations occur below 400 cm^{-1} (16,17). A possible alternative is that these two bands arise from chromophore normal modes which are mixtures of metal-ligand and parahydroxyphenyl vibrations.

The bands at 371, 423, and 465 cm^{-1} are in the frequency range of pure metal-ligand vibrations (17). Although tyrosine also has weak bands in this general frequency range, they are not due to the p-hydroxyphenyl group and are not expected to be resonance enhanced. Assignment of these low frequency

bands to metal-ligand vibrations is reasonable, but metal isotope substitution experiments are needed for verification to prove this assignment.

Thus in spite of the many spectral similarities between PCD and transferrin, these low frequency Raman data show that the chromophores have different structures, and possibly different ligands. Further resonance Raman experiments should prove helpful in elucidating this structure.

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