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RAMAN SPECTRAL EVIDENCE FOR TYROSINE COORDINATION OF IRON IN PROTOCATECHUATE 3,4-DIOXYGENASE

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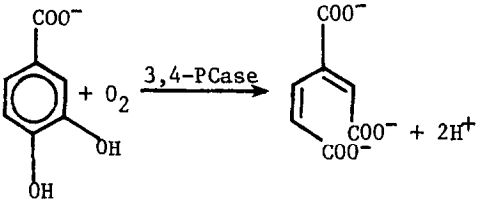
SUMMARY: The Raman spectrum of protocatechuate 3,4-dioxygenase [EC 1.13.11.3] shows four principal resonance-enhanced peaks at 1602, 1503, 1263 and 1171 cm^{-1} with 514.5 nm laser excitation. These frequencies are associated with ring-mode vibrations of one or more tyrosinate residues coordinated with the Fe(III) at the active site. These data provide the first direct evidence for the identity of a permanent iron ligand in this enzyme. The great similarity in the resonance Raman spectrum of protocatechuate 3,4-dioxygenase with those of iron-transferrins suggests the existence of a class of proteins characterized by Fe(III)-tyrosinate coordination.

INTRODUCTION: Protocatechuate 3,4-dioxygenase≠ is an aromatic ring-cleaving enzyme which converts 3,4-dihydroxybenzoate (protocatechuate) to β-carboxy-*cis,cis* muconate by incorporation of both atoms of an oxygen molecule:



The phenolytic oxygenation reaction was demonstrated by Hayaishi and coworkers over 20 years ago (1). The enzyme, 3,4-PCase, is a non-heme iron protein which has received much attention in recent years (2). The enzyme is easily obtained in pure form via crystallization; a popular procedure involves the harvesting

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≠ Protocatechuate 3,4-dioxygenase [protocatechuate:oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.3] is abbreviated as 3,4-PCase.

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of hydroxybenzoate induced cells of *Pseudomonas aeruginosa* and subsequent isolation of the enzyme (3). While crystals of this enzyme are readily obtainable, no crystallographic structure determination of the protein or the iron coordination site has yet been published. However, EPR and Mössbauer spectroscopic studies indicate that the iron atom remains high-spin and trivalent throughout the catalytic cycle of the enzyme. Que et al. (4) have recently reviewed much of the available physical data on this enzyme and proposed a mechanism of action.

In view of the similarity of the optical spectrum of 3,4-PCase to that of various transferrins ($\lambda_{max} \sim 450$ nm), it has been suggested by analogy that tyrosinate may be a ligand of iron and that the optical spectrum also arises from tyr$\longrightarrow$Fe(III) charge transfer (5). In this communication, we wish to report that a Raman spectral study of 3,4-PCase clearly establishes the presence of tyrosinate as an iron ligand, based on the observation of resonance-enhanced vibrational modes of the tyrosine ring.

EXPERIMENTAL SECTION:

Pseudomonas aeruginosa (ATCC 23975) were grown at 26°C in a medium containing p-hydroxybenzoate as inducer and primary carbon source in vigorously agitated 2L flasks. The medium contained 3.0 g p-hydroxybenzoic acid, 0.1 g yeast extract, 3.0 g $(NH_4)_2HPO_4$, 1.2 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 1.2 g NaCl and 0.1 g Fe $SO_4 \cdot 7H_2O$ per liter of solution and was adjusted to pH 7 with 5N NaOH.

To monitor growth of cells an aliquot of the culture solution was centrifuged and the concentration of p-hydroxybenzoate in the supernatant determined from its uv-spectrum. When approximately 5% p-hydroxybenzoate remained ($\sim$ 1½ days), an additional 3.0 g p-hydroxybenzoic acid (dissolved in 10 mL and adjusted to pH7 with NaOH) was added per liter of culture medium; growth was continued until the level of inducer dropped to 10% ($\sim$ ½ day).

Cells were harvested by continuous-flow centrifugation, yielding 5 g of wet cells per liter. Purification and crystallization of 3,4-PCase followed the procedure of Fujisawa and Hayaishi (3) with the omission of the protamine sulfate precipitation step and the use of Tris-Cl buffer (50 mM, pH 8.5) throughout.

Raman samples were prepared by dissolving the enzyme in a minimum volume of Tris-Cl buffer (pH 8.5) and concentrating them by ultrafiltration (Millipore Pellicon membrane, 100K MW cut-off) to $\sim$ 12% by weight (1.4 mM Fe). Laser power incident at the sample was measured at 200 mW. The sample temperature was maintained at 0°C throughout the spectral studies and no decomposition was apparent visually or as determined from constant specific activities of $\sim$ 20 units/mg enzyme (3) before and after the Raman experiments.

Spectra were recorded on a modified Jarrell-Ash 25-300 Raman spectrophotometer equipped with an RKB, Inc. digital grating drive, an ITT FW 130 photomultiplier, a Computer Automation 24K minicomputer and peripherals. The laser is a Coherent Radiation CR-04 argon ion source.

RESULTS AND DISCUSSION: Figure 1 shows the Raman spectrum (100-1900 cm^{-1}) of the resting enzyme, protocatechuate 3,4-dioxygenase, maintained at $\sim$ 0°C in an aqueous Tris-Cl buffer solution. This spectrum is a superposition of several resonant and nonresonant Raman peaks as well as a sharp fluorescence band, shown here at $\sim$ 365 cm^{-1}. We have observed four principal resonance-enhanced Raman peaks in this enzyme at 1602, 1503, 1263 and 1171 cm^{-1}. Several, generally weaker, nonresonant peaks are attributable to the solvent (e.g., $\nu_2(H_2O)$ at 1657 cm^{-1}) or to the protein itself (e.g., C-H deformation at 1447 cm^{-1}, phenylalanine at 1003 cm^{-1} and tryptophan at 757 cm^{-1}) (6). The peak at 981 cm^{-1} arises from the $\nu_1(A_1)$ vibration of sulfate ion, added as an internal standard. The strong feature at 365 cm^{-1} (514.5 nm excitation) was observed to "shift" to $\sim$ 1068 cm^{-1} with 496.5 nm excitation and, thus, indicates that it is at constant energy, corresponding to emission at 524 nm. This feature is most probably a fluorescence band ascribable either to the enzyme itself or to enzyme-bound substances not removed by the isolation procedure. We have observed, for example, that 3,4-dihydroxyphenylpropionic acid, a slow-substrate, also shows a strong band at this frequency upon 514.5 nm excitation. Alternatively, this fluorescence band may be associated with an unidentified impurity.

The four principal resonance Raman peaks in 3,4-PCase (Figure 1 and Table I) most likely arise from ring mode vibrations of one or more Fe(III)-coordinated tyrosinate residues as permanent iron ligands. A nearly identical set of four peaks has been observed in the resonance Raman spectra of Fe(III)-transferrins (Table I). Interestingly, a very similar resonance Raman spectrum has also been observed for human lactoferrin (7). The assignment of these four peaks to Fe(III)-tyrosinate vibrations is based on Raman studies of model metal phenolate complexes (8-10) and the identification of tyrosine as an iron ligand in transferrin by spectrophotometric titrations and chemical modifications (11). It appears, therefore, that Fe(III)-tyrosinate proteins can be considered a distinct class of non-heme iron proteins and that this class of pro-

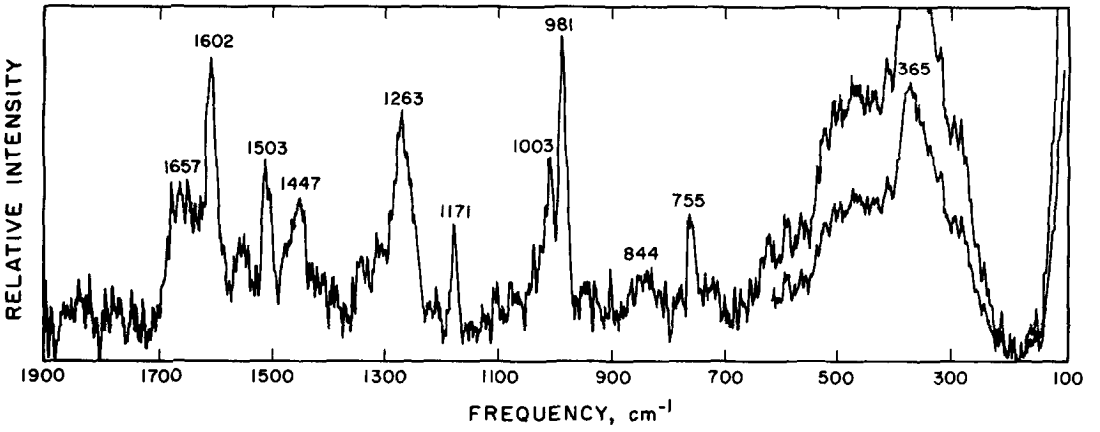

Figure 1: Raman spectrum of protocatechuate 3,4-dioxygenase (0°C, Tris-Cl buffer solution) obtained in a backscattering geometry from a glass capillary with 514.5 nm (200 mW) excitation. This spectrum is the accumulation of 12 repetitive scans where each scan was obtained with 8.0 cm^{-1} slits at a rate of 1.0 cm^{-1}/s and a digitizing increment of 1.0 cm^{-1}. A very strong background fluorescence under the entire spectral range has been subtracted using our available computer graphics.

TABLE I: Resonance Raman Peaks Attributed to Iron(III)-Coordinated Tyrosine in Iron(III)-Transferrins and in Protocatechuate 3,4-Dioxygenase.

Protein	Principal Resonance Raman Peaks, cm^{-1}				Ref.
Ovotransferrin/HCO_3^-	1605	1504	1270	1170	(8)
Serum Transferrin/HCO_3^-	1613	1508	1288	1174	(9)
Ovotransferrin/$C_2O_4^{2-}$	1604	1505	1264	1175	(10)
3,4-PCase	1602	1503	1263	1171	this work

teins is readily characterized as such by resonance Raman spectroscopy.

The present finding that protocatechuate 3,4-dioxygenase is an Fe(III)-tyrosinate protein represents the first definitive identification of an iron ligand in this enzyme and indicates that the metal is coordinated to one or more tyrosine residues at the active site of this protein.

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