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PROTOCATECHUATE 3,4-DIOXYGENASE. RESONANCE RAMAN STUDIES OF THE OXYGENATED INTERMEDIATE

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SUMMARY: Resonance Raman spectra of protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* have been investigated during the reaction of the enzyme with substrate and oxygen. It is found that the spectrum of the turned-over enzyme is indistinguishable from that of the resting enzyme in the absence of substrate, and is characterized by resonance-enhanced tyrosinate ring vibrational modes at 1263 and 1174 cm^{-1}. In the ternary ESO_2 complex, however, the tyrosinate vibrational modes are shifted to 1252 and 1165 cm^{-1}, respectively. There is no evidence for any dioxygen vibrations in the spectra of ESO_2 complexes prepared with $^{16}O_2$, $^{18}O_2$, and $^{16}O^{18}O$ in the region between 1300 and 200 cm^{-1}. The results of this resonance Raman study are interpreted to indicate that molecular oxygen is attached only to the substrate (but not iron) in the stable intermediate, and that the concomitant rearrangement at C4 of the substrate induces a substantial change in geometry of the tyrosine residues associated with the iron complex. Furthermore, the optical spectrum of the ESO_2 complex (λ_{max} = 520 nm) is dominated by tyrosinate → Fe(III) charge transfer and contains little or no peroxide → Fe(III) charge transfer. These results invalidate the previously advanced analogy in spectral properties between this enzyme and the respiratory protein, oxyhemerythrin.

INTRODUCTION: Protocatechuate 3,4-dioxygenase (3,4-PCase)#, an intradiol aromatic ring cleaving enzyme, has a molecular weight of 700,000 and an $8(\alpha_2\beta_2Fe)$ structure [1]. Electron paramagnetic resonance (EPR) and Mössbauer spectroscopic studies on the resting enzyme (E), enzyme-substrate complex (ES), and enzyme-substrate-oxygen ternary complex (ESO_2) have shown that the metal ion occurs as high spin Fe(III) in each of these species [2,3]. These studies clearly indica-

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

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ted changes in the electronic environment of the iron upon formation of the ternary complex and led to the proposal that the iron in ESO_2 may be coordinated to peroxide since the absorption maximum at 520 nm is similar to that of oxyhemerythrin. Recent resonance Raman (RR) investigations of E [4-6] have shown that the metal is coordinated to tyrosinate groups. Subsequent studies on ES and enzyme-inhibitor complexes [7,8] have revealed new Raman frequencies due to iron-bound substrate or inhibitor, but no further information on the structure of the iron center. We have now investigated the ESO_2 ternary complex by RR spectroscopy and find that a significant structural rearrangement occurs in the iron-tyrosinate environment upon reaction of the ES complex with molecular oxygen. However, we were unable to obtain any spectral evidence for the presence of dioxygen in the metal-ligand chromophore.

MATERIALS AND METHODS: Protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* was prepared as described previously [5] and had a specific activity >40 units/mg enzyme. The enzyme was dissolved at a concentration of ∿120 mg/mL in 0.05 M Tris-Cl (pH 8.5). For preparation of the ternary complex [9], the enzyme was stirred in the presence of O_2 for 24 hrs. at 4°C. The enzyme solution was transferred to a capillary tube and the reaction initiated by addition of a small volume of a deoxygenated substrate, 3,4-dihydroxyphenylpropionate (pH 8.1). The contents were mixed with a syringe and frozen in liquid N_2 within 15 s of the addition of substrate. The resulting trapped intermediate could be maintained at liquid N_2 temperature for several days with little or no decay as judged by its Raman spectrum. However, prolonged laser irradiation led to a slow degradation of the ESO_2 complex, permitting observation of the decay process. Ternary complexes were also made with isotopically-enriched dioxygen (Miles Laboratories): $^{18}O_2$ (99 atom-% ^{18}O) and $^{16}O^{18}O$ (58 atom-% ^{18}O). Raman spectra were recorded on a computerized Jarrell-Ash spectrophotometer as reported elsewhere [10]. The presence of a fluorescent background and the desire for spectra of high signal-to-noise ratio necessitated the accumulation of approximately 300 scans per sample, each collected at a rate of 4.0 cm^{-1}/s with 8 cm^{-1} slits and a digitizing increment of 0.6 cm^{-1}. Spectra of the resting enzyme (λ_{max} = 465 nm) were obtained by excitation at 514.5 nm (∿200 mW) while spectra of the ES complex (λ_{max} = 480 nm) and the ternary complex (λ_{max} = 520 nm) were obtained by excitation at 530.9 nm (∿50 mW at the sample).

RESULTS: The Raman spectrum of 3,4-PCase after the enzymatic reaction is allowed to go to completion is shown in the bottom trace of Figure 1. This spectrum is indistinguishable from that of the resting enzyme [4-6] and no spectral features due to products could be discerned. The 1300-700 cm^{-1} spectrum of the enzyme (resting or turned-over) is characterized by resonance-enhanced Raman peaks at 1263 and 1174 cm^{-1} due to tyrosinate ligand and at 755 cm^{-1} due to tyrosine or

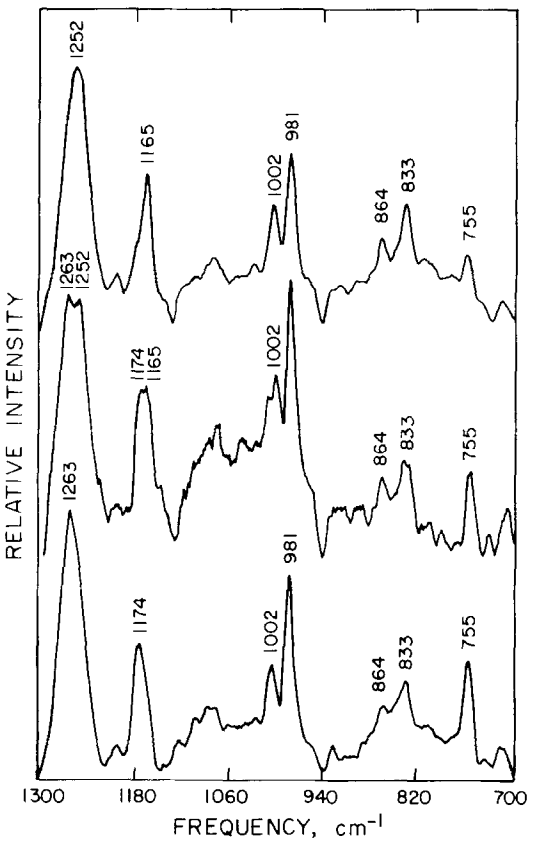


Figure 1. Raman spectra of protocatechuate 3,4-dioxygenase in frozen solution after reaction with substrate (3,4-dihydroxyphenylpropionate) and oxygen. Upper spectrum: reaction with $^{18}O_2$ (2 atm) to yield ∿75% ternary complex, ESO_2. Middle spectrum: reaction with $^{16}O_2$ (1 atm) to yield ∿50% ternary complex. Lower spectrum: reaction with $^{16}O_2$ followed by complete decay of ternary ESO_2 complex into products at -185°C. Excitation at 530.9 nm (∿50 mW at the sample). Data collection parameters are given in the text.

tryptophan [6]. In addition, there are non-enhanced peaks at 1002 cm^{-1} from protein phenylalanines and 981 cm^{-1} from sulfate ion present as an internal standard, while the less intense peaks at 864 and 833 cm^{-1} are most likely protein tyrosine modes [11].

In agreement with previous work [4,7], we have found that reaction of the resting enzyme with substrate alone produces no significant changes in the resonance-enhanced vibrational modes of the iron protein. However, formation of the ternary ESO_2 complex results in marked changes in the resonance-enhanced spectral

lines attributed to tyrosinate and tyrosine/tryptophan (Fig. 1, upper trace). The tyrosinate vibrational modes at 1252 and 1165 cm^{-1} in ESO_2 are ~10 cm^{-1} lower in frequency than the corresponding modes in the resting or turned-over enzyme. The shifts in the vibrational frequencies are illustrated by the middle spectrum of Fig. 1 from a sample in which approximately half of the enzyme is in the ESO_2 form. In this spectrum it is clearly seen that the two tyrosinate bands are both split into doublets, have greater bandwidths, and have lower band intensities relative to the 981 cm^{-1} standard than in either the top or bottom spectrum in Fig. 1. The tyrosine/tryptophan vibration at 755 cm^{-1} disappears entirely upon complete formation of the ESO_2 complex according to a difference spectrum of ESO_2 and E. The remaining intensity at 755 cm^{-1} in the upper spectrum in Fig. 1 is due to ~25% unreacted enzyme.

Ternary complexes of 3,4-PCase were prepared with three different isotopic species of dioxygen in order to locate Raman spectral features due to metal-bound peroxide or superoxide. Previous spectroscopic studies of metalloproteins containing bound dioxygen have revealed peroxide ion vibrations between 740 and 850 cm^{-1} [12-14], superoxide vibration at 1107 cm^{-1} [15], and metal-oxygen vibrations between 500 and 570 cm^{-1} [13,16]. Close examination of the Raman spectra of the three ternary complexes of 3,4-PCase failed to reveal any features between 1300 and 200 cm^{-1} which are not also present in the spectra of the resting or turned-over enzyme and which shift to appropriately lower frequency upon substitution by the heavier oxygen isotope.

DISCUSSION: Resonance Raman excitation within the 465 nm visible absorption band of 3,4-PCase has established the presence of tyrosinate as an iron ligand [4-7] and the assignment of the visible absorption to tyrosinate → Fe(III) charge transfer (CT) as in other iron-tyrosinate proteins [5,17]. The shift in the apparent absorption maximum for tyrosinate → Fe(III) CT from 465 nm to 480 nm upon anaerobic reaction of E with S [9] is accompanied by the appearance of new substrate peaks at 1471, 1338, 1317, and 1269 cm^{-1} in the RR spectra [7,8], but without a change in the frequencies of the tyrosinate vibrational modes. These results

provide direct evidence for substrate coordination to Fe(III) and suggest that the change in the optical absorption spectrum of ES arises from the contribution of a substrate → Fe(III) CT transition which enhances the RR modes of S, particularly with longer wavelength excitation [8].

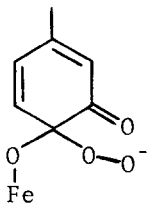
Conversion of ES to ESO_2 is accompanied by marked changes in both the electronic and the vibrational spectra of the iron-tyrosinate moiety. The visible absorption maximum at 520 nm in ESO_2 [9] can still be assigned to tyrosinate → Fe(III) CT on the basis of resonance-enhanced tyrosinate ring vibrations, although these vibrations have decreased $\sim$10 cm^{-1} in frequency. It is likely that these changes reflect structural alterations at the iron center in ESO_2 since the valence of the iron remains constant [2] and the electronic effects of binding substrate to iron are by themselves not sufficient to perturb the tyrosinate vibrational frequencies in ES.

The resonance-enhanced vibrational mode at 755 cm^{-1} in the Raman spectrum of 3,4-PCase could be assigned to either tyrosine or tryptophan [6]. Although the unlikelihood of tryptophan as an iron ligand favors the assignment of this peak to a tyrosine mode, fluorescence measurements [18] have located a tryptophan near the iron center. The disappearance of the 755 cm^{-1} peak upon formation of ESO_2 also favors a tryptophan assignment since the tyrosinate ring modes at 1252 and 1165 cm^{-1} do not decrease in intensity. The enhancement of the 755 cm^{-1} vibration in E and ES could be explained by a ring stacking interaction [19] with the iron-tyrosinate chromophore without requiring the direct involvement of tryptophan as an iron ligand. A disruption of the tryptophan-tyrosine interaction resulting from the distortion of the iron-tyrosinate coordination geometry in ESO_2 would account for the loss of the 755 cm^{-1} peak in ESO_2.

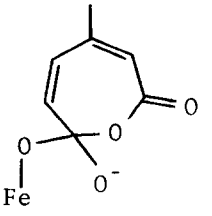
Recently it has been proposed [3] that the ESO_2 complex of 3,4-PCase contains a ferric-peroxide moiety on the basis of the similarity of its 520 nm absorption to the intense O_2^{2-} → Fe(III) CT transitions of oxyhemerythrin, $\varepsilon_{500\ nm}$ = 1150 $M^{-1}cm^{-1}$/Fe [20]; $[Fe(III)(EDTA)O_2]^-$, $\varepsilon_{520\ nm}$ = 530 $M^{-1}cm^{-1}$/Fe [21]; and the 13-L-hydroperoxolinoleic acid complex of lipoxygenase-1, $\varepsilon_{570\ nm}$ = 1000 $M^{-1}cm^{-1}$/

Fe [22]. If an iron-peroxide were present in the ESO_2 complex of 3,4-PCase, it would account for a considerable portion of the 520 nm absorption band ($\varepsilon_{520\ nm}$ = 2800 $M^{-1}cm^{-1}$/Fe [9]) and should give rise to a resonance-enhanced peroxide vibrational mode. Since no vibration attributable to a bound dioxygen moiety was observed in the 1300 - 700 cm^{-1} region of the RR spectrum of ESO_2, it is unlikely that the iron is coordinated to peroxide or superoxide in the ESO_2 complex.

Although the present experiments yielded no direct information on the nature of the enzyme-bound intermediate in ESO_2, the appearance of a characteristic RR spectrum supports the view that ESO_2 is a distinct reaction intermediate. Presumably, it represents a structure in which the substrate has already reacted with dioxygen to form either a peroxide intermediate (such as I) or a more advanced intermediate such as the anhydride, II, proposed by Hamilton [23]. An intermediate



(I)



(II)

such as structure II is favored by recent studies on the denaturation of the ESO_2 complex [24] since it would be more likely to decay spontaneously to the β-carboxyethylmuconic acid product than would an intermediate analogous to structure I. This question might be resolved if RR spectral lines due to the bound intermediate could be detected, possibly by investigating a broader range of frequencies and the use of several excitation wavelengths. For either structure I or II, the reaction of oxygen at C4 of the substrate would have converted this carbon from planar in ES to tetrahedral in ESO_2. Assuming that the substrate is

firmly held in position by the enzyme, the rearrangement of substituents at C4 would very likely result in changes in the iron-tyrosinate geometry and, thereby, explain the decreased vibrational frequencies in the RR spectrum of ESO_2 [25].

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REFERENCES:

1. Yoshida, R., Hori, K., Fujiwara, M., Saeki, Y., Kagamiyama, H., and Nozaki, M. (1976) Biochemistry 15, 4048-4053.
2. Que, L., Jr., Lipscomb, J.D., Zimmermann, R., Münck, E., Orme-Johnson, N.R., and Orme-Johnson, W.H. (1976) Biochim. Biophys. Acta 452, 320-334.
3. Que, L., Jr., Lipscomb, J.D., Münck, E., and Wood, J.M. (1977) Biochim. Biophys. Acta 485, 60-74.
4. Tatsuno, Y., Saeki, Y., Iwaki, M., Yagi, T., Nozaki, M., Kitagawa, T., and Otsuka, S. (1978) J. Am. Chem. Soc. 100, 4614-4615.
5. Keyes, W.E., Loehr, T.M., Taylor, M.L. (1978) Biochem. Biophys. Res. Commun. 83, 941-945.
6. Bull, C., Ballou, D.P., and Salmeen, I. (1979) Biochem. Biophys. Res. Commun. 87, 836-841.
7. Felton, R.H., Cheung, L.D., Phillips, R.S., and May, S. (1978) Biochem. Biophys. Res. Commun. 85, 844-850.
8. Que, L., Jr., and Heistand, R.H., II, (1979) J. Am. Chem. Soc. 101, 2219-2221.
9. Fujisawa, H., Hiromi, K., Uyeda, M., Okuno, S., Nozaki, M., and Hayaishi, O. (1972) J. Biol. Chem. 247, 4422-4428.
10. Loehr, T.M., Keyes, W.E., and Pincus, P.A. (1979) Anal. Biochem. 96, in press.
11. Lord, R.C., and Yu, N.T. (1970) J. Mol. Biol. 50, 509-524.
12. Freedman, T.B., Loehr, J.S., and Loehr, T.M. (1976) J. Am. Chem. Soc. 98, 2809-2815.
13. Dunn, J.B.R., Shriver, D.F., and Klotz, I.M. (1973) Proc. Nat. Acad. Sci. USA 70, 2582-2584.
14. Eickman, N.C., Solomon, E.I., Larrabee, J.A., Spiro, T.G., and Lerch, K. (1978) J. Am. Chem. Soc. 100, 6529-6531.
15. Barlow, C.H., Maxwell, J.C., Wallace, W.J., and Caughey, W.S. (1973) Biochem. Biophys. Res. Commun. 55, 91-95.
16. Brunner, H. (1974) Naturwissenschaften 61, 129.
17. Gaber, B.P., Miskowski, V., and Spiro, T.G. (1974) J. Am. Chem. Soc. 96, 6868-6873.
18. Hou, C.T. (1978) Bioinorg. Chem. 8, 237-243.
19. Sigel, H., and Naumann, C.F. (1976) J. Am. Chem. Soc. 98, 730-739.

20. Dunn, J.B.R., Addison, A.W., Bruce, R.E., Loehr, J.S., and Loehr, T.M. (1977) Biochemistry 16, 1743-1749.

21. Walling, C., Kurz, M. Schugar, H.J. (1970) Inorg. Chem. 9, 931-937.

22. DeGroot, J.J.M.C., Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., and Egmond, M.R. (1975) FEBS Letters 56, 50-54.

23. Hamilton, G. (1974) in Hayaishi, O., ed., Molecular Mechanisms of Oxygen Activation, Academic Press, New York, 405-451.

24. Nakata, H., Yamauchi, T., and Fujisawa, H. (1978) Biochim. Biophys. Acta 527, 171-181.

25. Loehr, T.M., Keyes, W.E., and Loehr, J.S. in King, T.E., Mason, H.S., and Morrison, M., eds., Oxidases and Related Redox Systems, submitted for the Third International Symposium, Albany New York, 1979.