Spectral Characterization of Diarylpropane Oxygenase, a Novel Peroxide-dependent, Lignin-degrading Heme Enzyme*

(Received for publication, October 12, 1984)

Laura A. Andersson, V. Renganathan, Andrew A. Chiu, Thomas M. Loehr†, and Michael H. Gold
From the Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, Beaverton, Oregon 97006

Diarylpropane oxygenase, an $H_2O_2$-dependent lignin-degrading enzyme from the basidiomycete fungus *Phanerochaete chrysosporium*, catalyzes the oxygenation of various lignin model compounds with incorporation of a single atom of dioxygen ($O_2$). Diarylpropane oxygenase is also capable of oxidizing some alcohols to aldehydes and/or ketones. This enzyme ($M_r = 41,000$) contains a single iron protoporphyrin IX prosthetic group. Previous studies revealed that the Soret maximum of the ferrous-CO complex of diarylpropane oxygenase is at ~420 nm, as in ferrous-CO myoglobin (Mb), and not like the ~450 nm absorption of the CO complex of the ubiquitous heme monooxygenase, cytochrome P-450. This spectral difference between two functionally similar heme enzymes is of interest. To elucidate the structural requirements for heme iron-based oxygenation reactions, we have compared the electronic absorption, EPR, and resonance Raman (RR) spectra of diarylpropane oxygenase with those of other heme proteins and enzymes of known axial ligation.

The absorption spectra of native (ferric), cyano, and ferrous diarylpropane oxygenase closely resemble those of the analogous myoglobin complexes. The EPR g values of native diarylpropane oxygenase, 5.83 and 1.89, also agree well with those of aquometMb. The RR spectra of native, cyano, and ferrous diarylpropane oxygenase have their spin- and oxidation-state marker bands at frequencies analogous to those of aquometMb and indicate a high-spin, hexacoordinate ferric iron. The RR spectra of ferrous diarylpropane oxygenase have frequencies analogous to those of deoxy-Mb that suggest a high-spin, pentacoordinate Fe(II) in the reduced form. The RR spectra of both ferric and ferrous diarylpropane oxygenase are less similar to those of horseradish peroxidase, catalase, or cytochrome C peroxidase and are clearly distinct from those of P-450. These observations suggest that the fifth ligand to the heme iron of diarylpropane oxygenase is a neutral histidine and that the iron environment must resemble that of the oxygen transport protein, myoglobin, rather than that of the peroxidases, catalase, or P-450. Given the functional similarity between diarylpropane oxygenase and P-450, this work implies that the mechanism of oxygen insertion for the two systems is different.

Lignin is a complex, optically inactive, heterogeneous, and random biopolymer (1) that comprises 20–30% of woody plants. Thus, its catabolism and utilization as a renewable resource are of great interest. A variety of white rot basidiomycetous fungi are capable of catabolizing lignin (2) in an apparently nonspecific process (3–7) which has been shown by both chemical and physiological studies to be oxidative (8–13). Recent studies have revealed that *Phanerochaete chrysosporium*, a white rot basidiomycete, produces an extracellular, $H_2O_2$-dependent, ligninolytic enzyme when cultured under aerobic conditions (14, 15). Enzyme activity is absent both from nonligninolytic cultures and from cultures of a nonligninolytic mutant of this organism (14, 16, 17).

Diarylpropane oxygenase has recently been purified to electrophoretic homogeneity (18–20) and shown to be a protein of $M_r = 41,000$ with a single iron protoporphyrin IX prosthetic group (19, 20). Diarylpropane oxygenase catalyzes $H_2O_2$-dependent oxygenation as well as oxidation of a variety of lignin model compounds (14, 15, 18–20). For example, it catalyzes the $\alpha,\beta$ cleavage of 1-(3',4'-diethoxyphenyl)-1,3-dihydroxy-2-(4"-methoxyphenyl)propane (I) during which a single atom of oxygen from $O_2$ is specifically incorporated into the $\alpha$ position of the product 1-(4'-methoxyphenyl)-1,2-dihydroxyethane (III; Scheme I) (16, 18, 19).

Diarylpropane oxygenase also catalyzes hydroxylation of the olefinic substrate 1-(4'-ethoxy-3'-methoxyphenyl)-2-propene (IV), during which a single atom of oxygen from $O_2$ is specifically incorporated into the $\beta$ position of the product 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol (V; Scheme II) (16). By reference to these examples, diarylpropane oxy-

---

*This work was supported by the National Science Foundation Grant DMB 8311441 to M. H. G., the Crown Zellerbach Company (M. H. G.), the Medical Research Foundation of Oregon (T. M. L.), and the National Institutes of Health Grant GM 19865 and GM 34468 to T. M. L. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, 19600 N. W. Von Neumann Drive, Beaverton, OR 97006.

††Incorporation of $^18O$ into the $\beta$ position of diol (V) has recently been confirmed with highly purified preparations of diarylpropane oxygenase: K. Miki, V. Renganathan, and M. H. Gold, manuscript in preparation.
Spectral Characterization of Diarylpropane Oxygenase

**EXPERIMENTAL PROCEDURES**

Diarylpropane oxygenase was isolated and purified from the extracellular medium of agitated, aerobically cultured *P. chrysogenum*, as described previously (20). Electrophoretically homogeneous diarylpropane oxygenase (20) was concentrated to ~200 μM in 20 mM sodium succinate buffer, pH 4.50, 100 mM NaCl. Sperm whale myoglobin (Sigma Type II) was made to ~300 μM in H2O, dialyzed against ferricyanide to ensure full oxidation, and exchanged into 20 mM sodium phosphate buffer, pH 7.0, 100 mM NaCl. All of the proteins studied herein were compared at their respective pH optima.

Resonance Raman spectroscopic studies were performed on diarylpropane oxygenase samples from two different protein preparations. No differences could be observed in the RR spectra of the two samples. Complexes of diarylpropane oxygenase and Mb with CN- and N2 were generated by addition of concentrated stock solutions to a final ligand concentration of 15 and 30 mM, respectively. The ferrous form of the enzyme was prepared under rigorously anaerobic conditions, as described previously (20). Integrity of enzyme samples was confirmed by absorption spectroscopy immediately prior to and following RR spectroscopy. In no case was evidence of laser-induced degradation observed.

Resonance Raman spectra of diarylpropane oxygenase and Mb were obtained on samples maintained at ~2 °C in melting point capillaries using a back-scattering geometry from a sample Dewar. Excitation was provided by Spectra-Physics ion lasers (164-05 Ar, 164-01 Kr). The Raman spectrophotometer and computer interface have been reported previously (27).

**RESULTS**

The optical absorption band positions of ferric, ferrous, and liganded forms of diarylpropane oxygenase, adapted from Gold *et al.* (20), are compared to those of a variety of other heme systems in Table I. The EPR spectrum of native diarylpropane oxygenase at 4 K is shown in Fig. 1. EPR g values for ferric diarylpropane oxygenase and its cyanide derivative are shown in Table II, along with *g* values for other heme proteins. The *g* values for the native oxygenase at 5.83 and 1.99 are characteristic of an axial high-spin (*S* = 5/2) ferric state, whereas the *g* values for the cyano complex at 3.18 and 2.13 are indicative of low-spin (*S* = 1/2) hexacoordinate ferric heme. The *g* values determined for aquometMb (this work) are in good agreement with the literature (Table II; Ref. 35).

High-frequency (>1000 cm⁻¹) resonance Raman spectra of native (ferric), reduced (ferrous), and CN⁻-ligated diarylpropane oxygenase obtained with 406.7- and 488.0-nm excitation lines are shown in Figs. 2-4, along with RR spectra of the analogous Mb complexes (Figs. 2 and 4). Key vibrational bands (spin- and oxidation-state markers (40)) for diarylpropane oxygenase and for heme systems of known axial ligation are compared in Table III. The vibrational bands of diarylpropane oxygenase are listed and assigned in Table IV, by comparison with the previously assigned RR bands for the analogous myoglobin complexes. RR oxidation- and spin-state frequencies for the native oxygenase at 1612, 1558, 1479, and

---

**Scheme II. Olefin hydroxylation by DAPOX, diarylpropane oxygenase.**

**Scheme III. Veratryl alcohol oxidation by DAPOX, diarylpropane oxygenase.**

---

2 The N-dealkylating enzyme, secondary amine monooxygenase from *Pseudomonas aeruginosa*, has also been suggested to be a heme monooxygenase (23, 24). In addition, the enzyme-substrate complex of heme oxygenase, the system that degrades hemin to biliverdin, is spectrally similar to myoglobin and also appears to function as an oxygenase (25).

3 Under nonphysiological conditions, hemoglobin will hydroxylate aniline, in a reaction that is inhibited by catalase (26).

4 The abbreviations used are: EPR, electron paramagnetic resonance; RR, resonance Raman; Mb, myoglobin; HRP, horseradish peroxidase; mW, milliwatt; Hb, hemoglobin.

---

5 A study of the low-frequency (<1000 cm⁻¹) RR spectra of diarylpropane oxygenase and its complexes is in progress: L. A. Anderson, V. Renganathan, T. M. Loehr, and M. H. Gold, manuscript in preparation.
Electronic absorption spectral maxima of diarylpropane oxygenase (DAPOX) and other heme proteins

| System                  | nm    | Ref. |
|-------------------------|-------|------|
| Ferric, high spin       |       |      |
| Native DAPOX, pH 4.5    | 407   | 500  |
| AquometMb, pH 7.0       | 409.5 | 505  |
| Catalase                | 405   | 505  |
| HRP, pH 6.0             | 403   | 500  |
| Cytochrome P-450-cam*   | 391   | 500  |
| Ferric, low spin        |       |      |
| Cytochrome b_s          | 360   | 425  |
| CN^-DAPOX               | 360   | 423  |
| CN^-metMb               | 359   | 423  |
| CN^-HRP                 | 422   | 580* |
| CN^-P-450               | 439   |      |
| N_2^-DAPOX              | 418   | 540  |
| N_2^-metMb              | 420   | 540  |
| N_2^-HRP                | 416   | 534  |
| N_2^-P-450              | 427   |      |
| Ferrous CO              |       |      |
| DAPOX                   | 435   | 556  |
| deoxy-Mb                | 434   | 556  |
| HRP                     | 437   | 556  |
| P-450                   | 411   | 540  |
| Ferrous CO              |       |      |
| DAPOX                   | 420   | 535  |
| Mb                      | 343   | 423  |
| HRP                     | 423   | 541  |
| P-450                   | 368   | 446  |

* P-450-cam, the camphor hydroxylating P-450 from Pseudomonas putida.
* Shoulder.

TABLE II

| System                  | g Values | Ref. |
|-------------------------|----------|------|
| High-spin ferric        |          |      |
| Native DAPOX            | 5.83     | 1.99 |
| AquometMb               | 5.85     | 1.99 |
| HRP                     | 5.9      | 2.0  |
| Catalase                | 6.3      | 5.4  |
| P-450                   | 6.4      | 5.6  |
| Low-spin ferric         |          |      |
| CN^-DAPOX               | 3.18     | 2.13 |
| CN^-metMb               | 3.45     | 1.88 |
| CN^-HRP                 | 3.05     | 1.2  |
| CN^-catalase            | 2.84     | 2.25 |
| CN^-CCP                 | 3.0      | 2.1  |
| CN^-P-450               | 2.54     | 2.28 |
| CN^-CFO                 | 2.70     | 2.30 |

EPR spectroscopy can provide information on both the spin state and the symmetry of the metal center. For example, among a series of high-spin ferric heme proteins that include aquometMb, hemoglobin (Hb), HRP, cytochrome c peroxidase, and catalase, EPR spectra of the oxygen carriers were shown to possess a more axial or tetragonal character as compared with a more rhombic symmetry for the peroxidases and catalase (46), suggesting that the protein environments of the hemes in the peroxidases and catalase are dissimilar from those of Hb and Mb (47). Resonance Raman spectroscopy generally provides detailed structural information for heme protein prosthetic groups and porphyrin complexes (41, 44, 48–51). Recently, Desbois et al. (43) suggested that the RR spectral properties of a variety of oxygen carriers are different from those of a variety of peroxidases (all having an iron protoporphyrin IX prosthetic group), further indicating that the two spectrally distinct heme structures (or environments) reflect their different biological functions.

**Electrical Absorption Spectroscopy**

The absorption spectrum of native diarylpropane oxygenase (DAPOX) (Table I) has features typical of a high-spin ferric heme system. For example, its Soret maximum (407 nm) is within 2.5 nm of that of high-spin hexacoordinate aquometMb and catalase and within 4 nm of that of high-spin pentacoordinate acid HRP; however, the diarylpropane oxygenase Soret band is significantly red-shifted (>1000 cm⁻¹) from that of ferric cytochrome P-450 (391 nm). The oxygenase absorption at 500 nm and the high-spin marker band at 632 nm are similar within a narrow 350-cm⁻¹ spread in all five of these heme proteins; this agreement supports the high-spin ferric assignment of native diarylpropane oxygenase (20). Furthermore, the finding that both pentacoordinate HRP and P-450 have their high-spin marker bands at ≥641 nm, whereas those of hexacoordinate aquometMb and diarylpropane oxygenase are at ~633 nm, suggests that the native oxygenase may also be hexacoordinate with a loosely bound sixth ligand, such as H₂O, as found in aquometMb. Contrary to a previous suggestion (19), the ferric diarylpropane oxygenase absorption spectral pattern is distinct from that of low-spin, hexacoordinate cytochrome b₅.

The absorption maxima of the cyano and azido adducts of the oxygenase are virtually identical to those of the corresponding low-spin, hexacoordinate metMb derivatives, but do
show some distinction from the spectra of the low-spin, hexacoordinate $N_2$-HRP, CN$^-$-HRP, and CN$^-$-P-450 complexes (Table I). Unlike catalase, diarylpropane oxygenase can be reduced; the ferrous enzyme has its Soret and visible bands at 435 and 556 nm, respectively, in good agreement with the analogous bands of both high-spin, pentacoordinate deoxy-h4b and ferrous HRP. However, only a poor match is observed between these spectral features and those of ferrous P-450. The bands of the ferrous-CO complex of diarylpropane oxygenase are at 420, 535, and 568 nm and agree fairly well with those of the ferrous-CO complexes of both Mb and HRP, but again are distinctly different from those of ferrous-CO P-450. These observations strongly suggest that the fifth ligand to the heme iron of diarylpropane oxygenase is likely to be a histidine or histidinate as found in Mb and HRP, respectively, but not a cysteinate anion as found in cytochrome P-450.

**Electron Paramagnetic Resonance Spectroscopy**

The EPR $g$ values of native diarylpropane oxygenase, 5.83 and 1.99 (Fig. 1), are essentially identical to those of high-

![Fig. 2. Resonance Raman spectra of native diarylpropane oxygenase (DAPOX) and aquometmyoglobin. A, ferric DAPOX, pH 4.50. Conditions: 406.7-nm excitation; laser power, 30 mW at sample Dewar; slitwidth, 5 cm$^{-1}$; scanning speed 2 cm$^{-1}$/s with repetitive scanning; sample temperature $-2^\circ$C. B, aquometMb, pH 7.0; 406.7-nm excitation. Other conditions as for A. C, ferric DAPOX, pH 4.50; 488.0-nm excitation. Laser power, 75 mW at sample Dewar; other conditions as for A. D, aquometMb, pH 7.0; 488.0-nm excitation; laser power, 75 mW at sample Dewar; other conditions as for A.](image1)

![Fig. 3. Resonance Raman spectra of dithionite-reduced ferrous diarylpropane oxygenase (DAPOX). A, 406.7-nm excitation; laser power, 30 mW at sample Dewar; other conditions as in Fig. 1. B, 488.0-nm excitation; laser power, 75 mW at sample Dewar; other conditions as in Fig. 1.](image2)

![Fig. 4. Resonance Raman spectra of the cyanide complexes of diarylpropane oxygenase (DAPOX) and metmyoglobin. A, CN$^-$-DAPOX, 406.7-nm excitation; laser power, 25 mW at sample Dewar; other conditions as in Fig. 1. B, CN$^-$-DAPOX; 488.0-nm excitation; laser power, 75 mW at sample Dewar; other conditions as in Fig. 1. C, CN$^-$-metMb; 488.0-nm excitation; laser power, 75 mW at sample Dewar; other conditions as in Fig. 1.](image3)
Spectral Characterization of Diarylpropane Oxygenase

Comparison of key resonance Raman bands for diarylpropane oxygenase (DAPOX) and other heme systems

The abbreviations used are: DAPOX, diarylpropane oxygenase; Me₂SO, dimethyl sulfoxide; PP, iron protoporphyrin IX; P-450-cam, the camphor hydroxylating P-450 from P. putida; Im, imidazole; 2-MeIm, 2-methylimidazole. (p), polarized; (dp), depolarized; (ap), anomalously polarized.

| System                  | 5th Excitation | 6th Excitation |
|-------------------------|----------------|----------------|
| High-spin Fe(III)       |                |                |
| DAPOX                  |                |                |
| AquometMb               |                |                |
| F₃-metMb                |                |                |
| (Me₂SO)₂PP             |                |                |
| Cl⁻·PP                 |                |                |
| HRP                    |                |                |
| Catalase               |                |                |
| P-450-cam              |                |                |
| Low-spin Fe(III)        |                |                |
| CN⁻·DAPOX              |                |                |
| CN⁻·metMb              |                |                |
| CN⁻·HRP                |                |                |
| N₂·DAPOX               |                |                |
| N₂·metMb               |                |                |
| Im₂PP                  |                |                |
| High-spin Fe(II)        |                |                |
| DAPOX                  |                |                |
| Deoxy-Mb                |                |                |
| HRP                    |                |                |
| P-450-cam              |                |                |
| 2-MeIm·PP              |                |                |
| Low-spin Fe(II)         |                |                |
| Im₂PP                  |                |                |
| Cytb                   |                |                |

### TABLE IV

High-frequency resonance Raman spectral features of diarylpropane oxygenase (DAPOX) and myoglobin

>1300 cm⁻¹; 406.7- and 488.0-nm excitation.

| Assignments* | Ferric | Ferrous |
|--------------|--------|---------|
|              | DAPOX | H₂O-metMb | CN⁻·DAPOX | CN⁻·metMb | N₂·DAPOX | N₂·metMb | DAPOX | Mb⁺ |
| v(C=O)       | 1612  | 1620      | 1620      | 1619      | 1624      | 1621      | 1626  | 1618 |
| v(C=O)       | 1581  | 1582      | 1588      | 1590      | 1560      | 1584      | 1603  | 1607 |
| v(C=O)       | 1577  | 1577      | 1577      | 1579      | 1578      | 1580      | 1554  | 1552 |
| v(C=O)       | 1558  | 1562      | 1584      | 1583      | 1581      | 1583      | 1566  | 1568 |
| v(C=O)       | 1514  | 1512      | 1512      | 1512      | 1512      | 1512      | 1512  | 1512 |
| v(C=O)       | 1479  | 1483      | 1501      | 1506      | 1504      | 1502      | 1470  | 1472 |
| v(C=O)       | 1453  | 1446      | 1471      | 1470      | 1481      | 1477      | 1451  | 1452 |
| v(C=O)       | 1425  | 1425      | 1430      | 1429      | 1425      | 1425      | 1426  | 1426 |
| v(C=O)       | 1402  | 1402      | 1399      | 1399      | 1399      | 1399      | 1399  | 1399 |
| v(C=O)       | 1372  | 1369      | 1372      | 1374      | 1374      | 1375      | 1357  | 1357 |
| v(C=O)       | 1337  | 1335      | 1340      | 1339      | 1343      | 1342      | 1333  | 1338 |
| v(C=O)       | 1311  | 1318      | 1315      | 1315      | 1315      | 1315      | 1313  | 1304 |
| v(C=O)       | 1306  | 1306      | 1305      | 1306      | 1310      | 1310      | 1309  | 1303 |

* Adapted from Ref. 41.
^ This work.
₁ ¹肩, shoulder.

spin, hexacoordinate aquometMb and confirm the high-spin ferric assignment (Table II). Both of their EPR spectra are quite distinct from those of high-spin pentacoordinate ferric catalase, HRP, cytochrome c peroxidase, and P-450 (Table II). As mentioned above, a comparison of the g values of catalase and the two peroxidases with those of the O₂-binding proteins, Mb and Hb, led to suggestions that the heme environments differed between the two functionally distinct types
of heme proteins (47). Thus, the great similarity between the EPR \( g \) values of native diarylpropylene oxygenase and metMb is an indicator of similar active-site environments in the two systems. The \( g \) values for the CN\(^-\) complex of the oxygenase (Table II) are in the range observed for other low-spin, hexacoordinated cyano-heme complexes.

**Resonance Raman Spectroscopy**

**Native Diarylpropylene Oxygenase**—Resonance Raman spectra of the native enzyme obtained in the high-frequency region (Fig. 2) are also indicative of a high-spin ferric heme. The spin- and oxidation-state marker bands (40) of native diarylpropylene oxygenase \( \left( v_{10}, \ 1612; v_{19}, \ 1558; v_2, \ 1479; \right) \) and \( v_4, \ 1372 \text{ cm}^{-1} \) are at frequencies expected for high-spin ferric hemes and are within \( \pm 4 \text{ cm}^{-1} \) of the analogous RR bands of high-spin ferric aquometMb (Tables III and IV). A comparison of these marker bands with those of other high-spin Fe(III) porphyrin systems further supports a hexacoordinate structure of the oxygenase heme that agrees with the electronic absorption spectral evidence. For example, \( v_3 \) in the oxygenase spectra \( (1479 \text{ cm}^{-1}) \) is within \( 1-4 \text{ cm}^{-1} \) of \( v_3 \) in the spectra of hexacoordinate bis(dimethylsulfoxide)-Fe(III)(protoporphyrin IX complex \( (1480 \text{ cm}^{-1}) \) (41) and hexacoordinate F\(^-\)-metMb \( (1482 \text{ cm}^{-1}) \) (41). In contrast, pentacoordinate high-spin ferric systems such as chloro-Fe(III)-protoporphyrin IX (41), HRP \( (40), \) catalase \( (40) \), and P-450 \( (40) \) all have their \( v_4 \) at \( \sim 1488-1500 \text{ cm}^{-1} \), some \( 9-21 \text{ cm}^{-1} \) above the diarylpropylene oxygenase frequency. The spin-state band, \( v_{19} \), for the oxygenase \( (1558 \text{ cm}^{-1}) \) is also within the narrow range \( (1557-1569 \text{ cm}^{-1}) \) seen for the hexacoordinate heme systems, whereas \( v_{10} \) ranges from \( \sim 1567 \) to \( 1576 \text{ cm}^{-1} \) for pentacoordinate ferric heme systems. Overall, the general RR characteristics of native diarylpropylene oxygenase and, by inference, the active-site environment are very similar to those of aquometMb, but unlike those of cytochrome P-450, HRP, or catalase. These observations again preclude a cysteinate ligand for the oxygenase (as found in P-450) and argue against a tyrosinate fifth ligand (as found in catalase) or a histidinate/strongly hydrogen-bonded histidine \( (52, \ 53) \) ligand (as found in HRP). Resonance Raman spectroscopy has been a particularly useful technique for the identification of tyrosinate coordination in both heme and non-heme iron proteins. A set of four characteristic peaks appears at approximately 1600, 1500, 1275, and 1170 \text{ cm}^{-1} \) that are enhanced by excitation within the tyrosinate \( \rightarrow \) Fe(III) charge transfer band \( (64, \ 55) \). The unequivocal absence of any such features in the RR spectra of diarylpropylene oxygenase almost certainly rules out tyrosinate as a possible axial ligand to the heme group of this enzyme.

**Ligated Diarylpropylene Oxygenase**—The complexes of the ferric diarylpropylene oxygenase with CN\(^-\) and N\(_7\) have RR spectra typical of low-spin, hexacoordinate ferric heme systems (Fig. 4; Tables III and IV). For example, \( v_{10} \) is shifted from \( 1612 \text{ cm}^{-1} \) in the native form to \( 1639 \text{ cm}^{-1} \) in the spectra of CN\(^-\) and N\(_7\) addsucts; a similar shift is seen when aquometMb is converted to the cyan and azido forms. The frequencies assigned to \( v_{10} \) and \( v_2 \) are also shifted in the spectra of the low-spin CN\(^-\) and N\(_7\) oxygenase species, from \( 1588 \) and \( 1479 \text{ cm}^{-1} \) to \( \sim 1577 \) and \( 1500 \text{ cm}^{-1} \), respectively. These spectral shifts concur with those observed for the conversion of high-spin Fe(II) to low-spin Fe(III) heme complexes. The most notable difference in the spectra of the cyan complexes of diarylpropylene oxygenase and metMb is that the 1639- \text{ cm}^{-1} \) feature \( (v_{10}) \) of the former is markedly less intense than the analogous feature in the CN\(^-\)-metMb spectrum. Whereas RR peak positions are a property of the electronic ground state of a given molecule, RR peak intensities are related to the excited electronic state of the molecule (56). Thus, the decreased intensity of \( v_{10} \) in the CN\(^-\)-oxygenase RR spectrum may indicate a different excited state for this species than for CN\(^-\)-metMb. It is unlikely that the weak \( v_2 \) feature is due to incomplete formation of the low-spin complex, since the other spin-state marker bands \( (v_10 \) and \( v_2 \) \) are in the expected frequency range and have more typical intensity patterns. As was seen with native diarylpropylene oxygenase, the RR spectral properties of its CN\(^-\) and N\(_7\) derivatives most closely resemble those of the analogous metMb complexes and are again less similar to those of CN\(^-\)-HRP. This is especially evident for \( v_{19} \), observed at \( 1577 \) and \( 1579 \text{ cm}^{-1} \) for the oxygenase and metMb-cyano complexes, respectively, but at \( 1590 \text{ cm}^{-1} \) for CN\(^-\)-HRP. These findings again suggest that neutral histidine is a most likely candidate for the fifth ligand of diarylpropylene oxygenase.

**Ferrous Diarylpropylene Oxygenase**—The RR spectra of the reduced form of the enzyme (Fig. 3) are indicative of a high-spin pentacoordinate ferrous iron. The oxidation-state marker, \( v_4 \), is at \( 1357 \text{ cm}^{-1} \), a frequency typical of normal Fe(II) heme systems, rather than the low-frequency \( 1346 \text{ cm}^{-1} \) of ferrous cytochrome P-450 (Table III). Since the position of \( v_4 \) in RR spectra of P-450 has been correlated with the electronic properties of its cysteinate ligand (57), we conclude that the axial ligand of ferrous diarylpropylene oxygenase is not a cysteine sulfur. The spin-state markers of the reduced oxygenase, \( v_{10} \) \( (1603 \text{ cm}^{-1}) \), \( v_2 \) \( (1554 \text{ cm}^{-1}) \), and \( v_3 \) \( (1470 \text{ cm}^{-1}) \), are at frequencies expected for high-spin, pentacoordinate ferrous heme systems and differ notably from the analogous bands of low-spin, hexacoordinate bis(imidazole)Fe(II)protoporphyrin IX or cytochrome \( b_6 \).

In general, the RR band positions of ferrous diarylpropylene oxygenase are similar to those of both deoxy-Mb and ferrous HRP. An interesting exception is observed for the band assigned as \( v(C=C): 1625 \text{ cm}^{-1} \) for the oxygenase and 1627 cm\(^{-1} \) for HRP, but 1618 cm\(^{-1} \) for deoxy-Mb. Desbois et al. (43) recently reported that the functionally distinct \( O_2 \)-binding proteins and peroxidases were also distinct in their RR spectra. These authors noted that the position of the vinyl \( C=C \) stretching mode of the ferrous proteins could distinguish between the two functional systems, being found at 1618-1621 cm\(^{-1} \) for oxygen carriers and at 1625-1627 cm\(^{-1} \) for peroxidases. They proposed that this spectral difference arises from an increase in electron density on the vinyl carbons of the protoporphyrin IX prosthetic group in the peroxidases, relative to that in the \( O_2 \)-binding systems (43). However, this raises a question as to whether an increased electron density on the vinyl groups of peroxidases (and possibly diarylpropylene oxygenase) serves a functional role. DiNello and Dolphin (58) demonstrated that apo-HRP reconstituted with deuterohemion (iron protoporphyrin IX with hydrogen atoms in place of the two vinyl substituents) still retains considerable peroxidase activity (26% of native HRP activity) and that the key substituents for retention of peroxidase activity were the propionic acid side chains.

Application of these observations to the RR spectra of ferrous diarylpropylene oxygenase indicates an electronic structural similarity to ferrous HRP and the peroxidases, at least with respect to the vinyl substituents on the common iron protoporphyrin IX prosthetic group. Extension of these ideas to the high-spin ferrous systems (Table III) also places diarylpropylene oxygenase among the peroxidases; its \( v(C=C) \) at 1627 cm\(^{-1} \) is somewhat closer to the vinyl frequency of HRP (1632 cm\(^{-1} \)) than to that of aquometMb (1620 cm\(^{-1} \)). Our previous observations, however, showed that electronic ab-
Spectral Characterization of Diarylpropane Oxygenase

It appears that the hormone coordination sphere of diarylpropane oxygenase, including axial ligation and active-side environment, is closest to that of Mb, whereas the vinyl substituents of the enzyme prothetic group might have increased electron density proposed for the peroxidases. Since diarylpropane oxygenase functions both as a peroxidase and as an oxygenase but apparently not as an oxygen-carrier, it is then not surprising that this enzyme does not readily fit into any one particular structural category. The diverse chemistry of the enzyme may well be determined by the presence of specific functional domains, as originally conceived by Mason (59, 60).

Structural and Mechanistic Implications

Based upon the results of the three complementary spectroscopic techniques, few, if any, similarities were observed between the properties of H$_2$O$_2$-dependent diarylpropane oxygenase and cytochrome P-450, despite previous speculations (61). In addition, the oxygenase is spectrally and, consequently, structurally distinct from both catalase and HRP. The significance of these findings is 2-fold: first, they essentially eliminate the possibility of either a cysteinyl or tyrosinate axial ligand to the heme iron of diarylpropane oxygenase; second, they raise questions as to the general mechanism of heme monoxygenase reactions.

The original definition of a monoxygenase is an enzyme which catalyzes the introduction of a single atom of dioxygen into a substrate with concomitant reduction of the second oxygen atom to water (Scheme IV; 21, 26).

\[
\text{RH} + \frac{1}{2} \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{R}^+\text{OH} + \text{H}_2\text{O}^8
\]

**SCHEME IV**

As recently as 1978, cytochrome P-450 was described as the only heme protein known to be normally capable of monoxygenase reactions (22) and under typical conditions, is not H$_2$O$_2$-dependent. The observation that diarylpropane oxygenase can catalyze two types of H$_2$O$_2$-dependent reactions, the monoxygenase of model compounds with concomitant insertion of one atom of molecular oxygen into the product, and the oxidation of model lignin compounds, renders this heme enzyme of particular interest. Reactions catalyzed by diarylpropane oxygenase include olefinic hydroxylations (14, 16), as well as aliphatic dial cleavage and C$_2$ oxidations (14-16, 19, 20).

It is commonly assumed that the special coordination sphere and environment of the porphyrin iron are directly related to P-450 monoxygenase functions (22, 63). P-450 has been convincingly demonstrated to have a cysteinyl anion as the fifth ligand to the heme iron in its ferrous, ferrous- and ferrous-CO states. This ligand creates an unusually electron-rich environment at the heme iron center (33, 66) which is believed to be responsible both for the spectral properties of P-450 and for its ability to activate molecular oxygen for insertion into organic molecules. In contrast, respiratory proteins such as myoglobin have histidine as the fifth ligand to the heme iron, whereas horseradish peroxidase has a histidine anion or strongly hydrogen-bonded histidine as its ligand (52, 53). Thus, the observation by Gold et al. (20) that the ferrous-CO complex of diarylpropane oxygenase has its Soret maximum at ~420 nm, a wavelength typical of Mb, Hb, and HRP, but distinct from the ~450 nm Soret maximum of ferrous-CO P-450 (22) led us to seek further understanding of the enzyme's oxygenation and oxidation reactions.

Our results, indicating that the oxygenase has a neutral histidine as a fifth ligand, suggest that the mechanisms of oxygen activation and insertion are different for diarylpropane oxygenase and cytochrome P-450. This may be supported by previous studies of lignin degradation (67) which indicate that photosensitizing riboflavin is able to catalyze lignin model compound oxidations similar to those carried out by both the fungus and by the purified enzyme. In addition, O$_2$ is incorporated during the photosensitizing riboflavin reaction (68) in a monoxygenase-like manner. Photosensitizing riboflavin appears to cleave the diarylpropane in a Type I hydrogen abstraction reaction where the radical produced may react subsequently with the ground state oxygen (67).

This mechanism is analogous to that which has been proposed for prostaglandin cyclooxygenase, a heme enzyme which also catalyzes both H$_2$O$_2$-dependent oxidations and insertion reactions (69). In one of the proposed mechanistic pathways for prostaglandin cyclooxygenase, a carbon-centered radical is produced, and this radical reacts with molecular oxygen to form a peroxy radical. The latter intermediate ultimately yields the final product via a chain reaction (69). Enzymatic substrate activation followed by reaction of the activated substrate with O$_2$ has also been postulated for the mechanism of protocatechuate oxygenase (70, 71). This mechanism has been supported by several spectroscopic studies (72, 73) which indicate that O$_2$ may not bind directly to the Fe(III) in the enzyme-active site.

The fact that H$_2$O$_2$-dependent diarylpropane oxygenase reacts with H$_2$O$_2$ to form a stable peroxy-adduct (19) suggests that the substrate is oxidized after binding to this adduct. O$_2$ may then react with the enzyme-organic substrate complex to form the aldehyde (II) and diol (III) products. Electron absorption and RR studies of the peroxy-adduct of ferric diarylpropane oxygenase show that it is an Fe(IV) complex, similar to compound II of horseradish peroxidase (32). Despite its H$_2$O$_2$ dependency, the spectral properties of the oxygenase are generally dissimilar to those of horseradish peroxidase, with the exception of the RR frequencies for the vinyl substituents, and are more closely related to those of Mb. Ferrous diarylpropane oxygenase also forms a stable O$_2$ adduct, which is not thought to play a role in the catalytic mechanism. Additional spectroscopic and mechanistic studies are in progress to better define the novel enzymatic capabilities of diarylpropane oxygenase and to correlate these properties with those of other heme proteins and enzymes.

REFERENCES

1. Sarkar, K. V. (1971) in *Lignins: Occurrence, Formation, Structure and Reactions* (Sarkar, K. V., and Ludwig, C. H., eds) pp. 95-195, Wiley-Interscience, New York
2. Crawford, R. L. (1981) *Lignin Biodegradation and Transformation*, Wiley-Interscience, New York
3. Lundquist, K., Kirk, T. K., and Connors, W. J. (1977) *Arch. Microbiol.* 112, 291-296
4. Crawford, R. L., Robinson, L. E., and Foster, R. D. (1981) *Appl. Environ. Microbiol.* 41, 1112-1116
5. Enoki, A., Goldab, G. P., and Gold, M. H. (1981) *Arch. Microbiol.* 129, 141-145
6. Enoki, A., and Gold, M. H. (1982) *Arch. Microbiol.* 132, 123-130
7. Nakatsubo, F., Kirk, T. K., Shimada, M., and Higuchi, T. (1981)

*V. Renganathan, K. Miki, and M. H. Gold, manuscript submitted.*

*L. A. Anderson, V. Renganathan, T. M. Loehr, and M. H. Gold, unpublished results.*

---

*See Refs. 24, 64, and 65 for extensive discussions of the evidence for the P-450 coordination environment.*
