respectively. These assignments are of course reversed in (*R*)-I-*d*.

The present results show that the configuration of an *intact* stereogenic methyl group,[21] CHDT (so-called "chiral" methyl group[22]), can be determined directly by tritium NMR.[23-27] The diastereomer, (1-*S*,7-*S*)-I-*d*-*t*, is calculated to have a tritium chemical shift at room temperature that is about 15 ppb greater (i.e., less shielded) than that of the other diastereomer, (1-*S*,7-*R*)-I-*d*-*t*. With proton and deuterium decoupling, a mixture of these diastereomers should then give sharp lines separated by 4.8 ± 0.3 Hz at 320 MHz, thus allowing an easy and accurate integration of the two signals on available instrumentation.

Acknowledgment. The NMR spectrometer was purchased through funds provided in part by the National Science Foundation and the National Institutes of Health. We thank the Research Corp. for support.

(21) Mislow, K.; Siegel, J. *J. Am. Chem. Soc.* **1984**, *106*, 3319-3328.
(22) Floss, H. G.; Tsai, M.-D. In *Advances in Enzymology and Related Areas of Molecular Biology*; Meister, A., Ed.; Wiley: New York, 1979; Vol. 50, pp 253-302. Floss, H. G.; Tsai, M.-D.; Woodward, R. W. *Top. Stereochem.* **1984**, *15*, 215-320. Floss, H. G. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986. Frenzel, T.; Beale, J. M.; Kobayashi, M.; Zenk, M. H.; Floss, H. G. *J. Am. Chem. Soc.* **1988**, *110*, 7878-7880.
(23) Altman, L. J.; Han, C. Y.; Bertolino, A.; Handy, G.; Laungani, D.; Muller, W.; Schwartz, S.; Shanker, D.; de Wolff, W. H.; Yang, F. *J. Am. Chem. Soc.* **1978**, *100*, 3235-3237).
(24) For tritium NMR, see: Bloxsidge, J. P.; Elvidge, J. A. *Prog. NMR Spectrosc.* **1983**, *16*, 99-113. Evans, J. A.; Warrell, D. C.; Elvidge, J. A.; Jones, J. R. *Handbook of Tritium NMR Spectroscopy and Applications*; Wiley: New York, 1985. Tritium NMR has been used to determine the configurations of chemical products (e.g., R*CDTX and R*CHTX, where R* is a chiral group, or RR'C=CDT and RR'C=CHT) derived, usually enzymatically, from a compound containing a stereogenic methyl group, XCHDT; if the stereochemistry of the reaction is known, the configuration of XCHDT is established indirectly, whereas if the configuration of XCHDT is known, the stereochemistry of the reaction can be established.[22,23]
(25) It has been thought[22] that such an NMR procedure would require finding conditions where the rotation of the methyl group is slow on the NMR chemical shift time scale,[2] but such conditions are neither necessary nor indeed desirable.
(26) Excess (*S*)-2-methylpiperidine should react[8] with CHDTX, where X is a leaving group; a route to such a CHDTX compound from chiral acetic acid is known.[21]
(27) Investigations of the chemical shift differences in the CHDT groups of diastereomers of I-*d*-*t* and in the CH_2D groups of chiral molecules other than I are planned.

Structure and Properties of a Pterin-Containing Ternary Copper(II) Complex, $[Cu(bpy)(PC)(H_2O)]\cdot 3H_2O$ (bpy = 2,2'-Bipyridine; PC = Pterin-6-carboxylate). Implications for the Active-Site Copper-Cofactor Bonding in *Chromobacterium violaceum* Phenylalanine Hydroxylase

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Received September 14, 1988

Phenylalanine hydroxylase and other aromatic amino acid hydroxylases are metalloenzymes that introduce a hydroxyl group into the side-chain aromatic rings in the presence of the pterin cofactor biopterin (**1a**).[2,3] While the hydroxylases from mammalian and pseudomonas species require iron for their activity, the phenylalanine hydroxylase from *Chromobacterium violaceum* involves 1 mol of type 2 copper per mol of enzyme in place of iron,[4,5] and a reduced pterin ring has been inferred to coordinate to copper through the nitrogen atom from electron spin resonance (ESR) spectroscopic studies by Benkovic and his collaborators.[6] Their electron spin echo studies reported very recently indicated that two imidazole groups from the enzyme are equatorially bound to copper.[7] In the course of the studies on Cu(II)-folic acid interactions, we found that folic acid (FA, **1b**), also a pterin

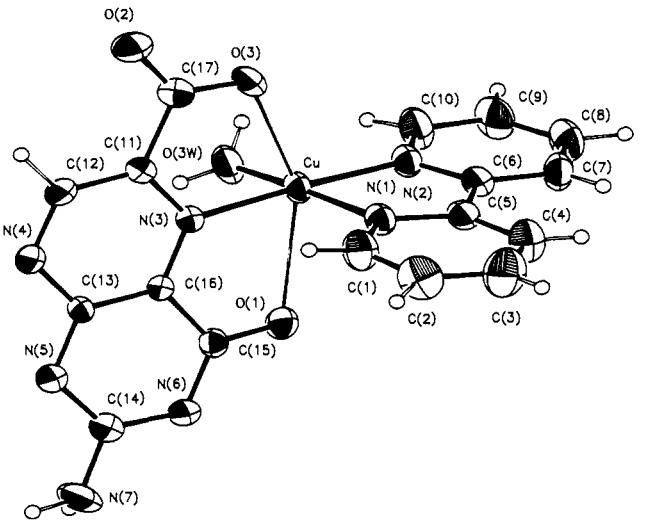
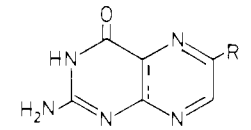


Figure 1. ORTEP view of $[Cu(bpy)(PC)(H_2O)]$ (**2**) showing 50% thermal ellipsoids. Selected bond lengths (Å) and angles (deg) are as follows: Cu-N(1) = 2.005 (3), Cu-N(2) = 1.993 (3), Cu-N(3) = 2.013 (3), Cu-O(1) = 2.499 (3), Cu-O(3) = 2.391 (3), Cu-O(3W) = 1.972 (3); N(1)-Cu-N(2) = 81.1 (1), N(1)-Cu-N(3) = 97.0 (1), N(1)-Cu-O(1) = 83.0 (1), N(1)-Cu-O(3) = 94.7 (1), N(1)-Cu-O(3W) = 171.1 (2), N(2)-Cu-N(3) = 177.1 (1), N(2)-Cu-O(1) = 104.4 (1), N(2)-Cu-O(3) = 108.0 (1), N(2)-Cu-O(3W) = 93.0 (1), N(3)-Cu-O(1) = 73.1 (1), N(3)-Cu-O(3) = 74.3 (1), N(3)-Cu-O(3W) = 88.5 (1), O(1)-Cu-O(3) = 146.7 (1), O(1)-Cu-O(3W) = 92.0 (1), O(3)-Cu-O(3W) = 93.6 (1).



1a; R = $-CH(OH)CH(OH)CH_3$

1b; R = $-CH_2NH-C_6H_4-CONHCH(COOH)CH_2CH_2COOH$

1c; R = $-COOH$

cofactor, suffers oxidative cleavage at the side chain by $Cu(bpy)^{2+}$ (bpy = 2,2'-bipyridine) at pH > 10 under aerobic conditions to give a ternary copper(II) complex involving bpy and pterin-6-carboxylate (PC, **1c**), Cu(bpy)(PC).[8] Since the same oxidation was observed for $Cu(phen)^{2+}$ (phen = 1,10-phenanthroline) but not for $Cu(en)^{2+}$ (en = ethylenediamine), electronic and steric

(1) (a) Kanazawa University. (b) Institute for Molecular Science. (c) Nagoya University.

(2) *Folates and Pterins*; Blakley, R. L.; Benkovic, S. J., Eds.; John Wiley & Sons: New York, 1985; Vol. 2.
(3) Dix, T. A.; Benkovic, S. J. *Acc. Chem. Res.* **1988**, *21*, 101-107.
(4) Nakata, H.; Yamauchi, T.; Fujisawa, H. *J. Biol. Chem.* **1979**, *254*, 1829-1833.
(5) Pember, S. O.; Villafranca, J. J.; Benkovic, S. J. *Biochemistry* **1986**, *25*, 6611-6619.
(6) Pember, S. O.; Benkovic, S. J.; Villafranca, J. J.; Pasenkiewicz-Gierula, M.; Antholine, W. E. *Biochemistry* **1987**, *26*, 4477-4483.
(7) McCracken, J.; Pember, S. O.; Benkovic, S. J.; Villafranca, J. J.; Miller, R. J.; Peisach, J. *J. Am. Chem. Soc.* **1988**, *110*, 1069-1074.
(8) Kohzuma, T.; Odani, A.; Morita, Y.; Takani, M.; Yamauchi, O. *Inorg. Chem.* **1988**, *27*, 3854-3858.

0002-7863/89/1511-3431$01.50/0

effects due to coordination of two aromatic nitrogens of bpy or phen seemed to be essential for the redox activities of copper–pterin systems, which is in accord with the conclusion for the copper site of the natural system.[6,7]

We here report the molecular structure and ESR spectral properties of the first known pterin-containing ternary Cu(II) complex, $[Cu(bpy)(PC)(H_2O)]\cdot 3H_2O$ (**2**), having a square-planar structure with weak apical bonds suggestive of the copper environment of the enzyme.

The green complex **2** was prepared as reported previously[8] from $Cu(NO_3)_2$, bpy, and **1b** at pH > 10, and crystals of **2** suitable for X-ray diffraction studies were obtained by recrystallization from water.[9] The molecular structure of **2** shown in Figure 1 reveals that the Cu(II) ion is basically in a square-planar geometry with two nitrogens of bpy, one nitrogen of PC, and one water oxygen in the plane and with two oxygens of the pterin moiety occupying remote axial positions. Although the Cu(II)···O(1) and Cu(II)···O(3) (numbered according to Figure 1) distances, 2.499 (3) and 2.391 (3) Å, are within the range of axial Cu–O bond lengths,[10] axial coordination of O(1) is very weak, and the C(15)–O(1) bond (1.238 (5) Å) may better be regarded as a double bond, because it is shorter than usual single bonds and appears to be similar to a bond observed for 6-methyl-7,8-dihydropterin hydrochloride and (6*R*)-pentaacetyl-5,6,7,8-tetrahydro-L-neopterin (1.24 Å).[11] Considering that the C(15)–C(16) (1.471 (6) Å), N(5)–C(14) (1.361 (6) Å), and N(3)–C(11) (1.343 (5) Å) bond lengths are longer than the corresponding values, 1.402, 1.333, and 1.294 Å, respectively, for 6-methyl-7,8-dihydropterin,[11a] we may conclude that a proton has dissociated from the N(6)H group of the pterin ring with a negative charge left in the ring. Coordination of bpy and PC molecules at right angles is probably attributed to the steric hindrance arising from the large planar rings. The two axial bonds may further stabilize the structure. In this connection xanthopterin in a molybdenum complex has been shown to be coordinated through its nitrogen and oxygen atoms by X-ray crystal structure analysis.[12,13] The square-pyramidal structure inferred for the ternary complex, Cu(bpy)(FA),[8] is related to **2** in that the bpy and pterin rings are perpendicular to each other.

The ESR spectra measured for Cu(DA)(PC) (DA = bpy or phen) at neutral pH were of axial symmetry with almost identical spin Hamiltonian parameters, $g_\parallel$ = 2.268 (2.269), $g_\perp$ = 2.061 (2.061), and $|A_\parallel|$ = 16.9 (16.7) mT for DA = bpy (phen), and seven nitrogen superhyperfine structures were detected in the perpendicular region (Figure 2a). The spectral parameters are reminiscent of those reported for a dihydropterin adduct of the phenylalanine hydroxylase ($g_\parallel$ = 2.27; $|A_\parallel| = 166 \times 10^{-4}$ cm^{-1} (15.7 mT)).[6] Addition of 1 equiv of imidazole (Im) to the above solutions gave ESR spectra ($g_\parallel$ = 2.245 (2.246), $g_\perp$ = 2.051 (2.053), and $|A_\parallel|$ = 18.1 (17.9) mT for DA = bpy (phen)) with nine nitrogen superhyperfine structures in both parallel and perpendicular regions (Figure 2b), from which we see that Im displaces the coordinated water molecule to form a 4N chromophore, Cu(bpy)(PC)(Im), with an increased ligand field. The electronic absorption spectrum of **2** at pH 6.8 has a peak at 676 nm, which shifts to 650 nm upon addition of Im. This is consistent with the change from a 3N to a 4N chromophore.[14]

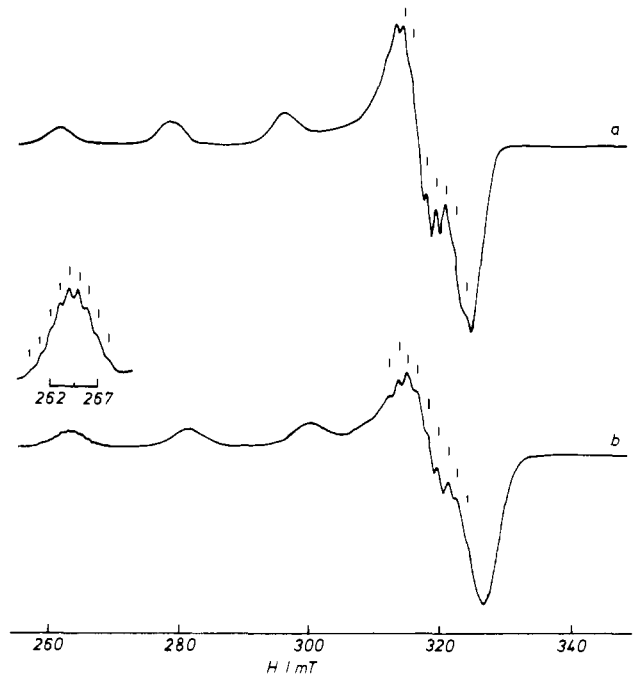


Figure 2. ESR spectra of Cu(bpy)(PC) (pH 7.2) (a) and Cu(bpy)(PC) containing 1 equiv of imidazole (pH 7.0) (b) at 77 K. Conditions: microwave frequency (GHz), 9.126 (a) and 9.133 (b); microwave power (mW), 2 (a and b); modulation (mT), 0.63 (a and b).

The molecular structure and the ESR spectra demonstrate some important aspects probably characteristic of the active site type 2 Cu(II) of *Chromobacaterium violaceum* phenylalanine hydroxylase,[6,7] i.e., (a) coordination of two pyridine nitrogens[7] which have been found to be necessary for $Cu(bpy)^{2+}$-catalyzed oxidation of folic acid,[8] (b) Cu(II)-cofactor bonding through the pterin nitrogen atom,[6] and (c) presence of a readily displaceable water molecule in an equatorial position which could be the binding site for molecular oxygen or a substrate. The negative charge left after deprotonation of the pterin ring is probably delocalized within the ring.[15] Interestingly McCracken et al.[16] have identified by electron spin echo studies on amine oxidase, which requires pyrrolloquinolinequinone (PQQ), two magnetically distinct imidazoles, and an equatorially bound water which is displaced by anions such as cyanide and azide. The present findings lend strong support to the recent conclusions on the phenylalanine hydroxylase Cu(II) site with two imidazoles, a pterin cofactor bound through the ring nitrogen, and a displaceable water and further suggest that the coordination structure of **2** may be common to other type 2 copper sites such as in amine oxidase requiring PQQ[16] whose steric requirement[17] is similar to that of PC.

Further studies on spectral and redox properties of related systems are under way.

(9) Crystal data: $C_{17}H_{19}N_7O_7Cu$, M = 496.94, monoclinic, space group $P2_1/n$, a = 7.046 (1) Å, b = 26.459 (3) Å, c = 11.001 (2) Å, β = 102.90 (1)°, V = 2002.5 Å^3, Z = 4, D_c = 1.648 g cm^{-3}, μ(MoKα) = 11.45 cm^{-1}, crystal dimensions 0.20 × 0.20 × 0.15 mm^3. Intensity data in the range $2\theta < 60°$ were collected by the ω–2θ scan technique using a Rigaku AFC-5R automated diffractometer. The structure was solved by a heavy-atom method and was refined by least-squares techniques. The final R and R_w values were 0.040 and 0.044, respectively, for 2394 unique reflections with $|F_o| \geq 3\sigma|F_o|$. Full details are provided in the Supplementary Material.

(10) Procter, I. M.; Hathaway, B. J.; Nicholls, P. *J. Chem. Soc. A* **1968**, 1678–1684.

(11) (a) Bieri, J. H. *Helv. Chim. Acta* **1977**, *60*, 2303–2308. (b) Antoulas, S.; Prewo, R.; Bieri, J. H.; Viscontini, M. *Helv. Chim. Acta* **1986**, *69*, 210–220.

(12) Burgmayer, S. J. N.; Stiefel, E. I. *J. Am. Chem. Soc.* **1986**, *108*, 8310–8311.

(13) During revision of the manuscript the crystal structure analysis of the cobalt(II) complexes of 2-(ethylthio)-4-hydroxypteridine was reported (Burgmayer, S. J. N.; Stiefel, E. I. *Inorg. Chem.* **1988**, *27*, 4059–4065).

(14) The agreement between the structures of **2** in the solid state and in solution is supported by the similarity between the reflectance spectrum (λ_{max}, 668 and 381 nm) and the absorption spectrum in aqueous solution (λ_{max}, 676 and 375 nm) and between the EXAFS data for the solid and the aqueous solution (unpublished results).

(15) Several resonance and tautomeric forms are possible with the deprotonated PC. Experimental and theoretical approaches to the stability of dihydropterins have been reported, e.g.: (a) Eberlein, G.; Bruice, T. C.; Lazarus, R. A.; Henrie, R.; Benkovic, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 7916–7924. (b) Gready, J. E. *Ibid.* **1985**, *107*, 6689–6695.

(16) McCracken, J.; Peisach, J.; Dooley, D. M. *J. Am. Chem. Soc.* **1987**, *109*, 4064–4072.

(17) Suzuki, S.; Sakurai, T.; Itoh, S.; Ohshiro, Y. *Inorg. Chem.* **1988**, *27*, 591–592.

Acknowledgment. We thank Takahiro Saito for assistance with the experiment. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan, to which our thanks are due.

Supplementary Material Available: Listings of interatomic distances and angles, positional parameters and isotropic temperature factors for non-hydrogen atoms, positional parameters for hydrogen atoms, and anisotropic temperature factors for non-hydrogen atoms (5 pages). Ordering information is given on any current masthead page.

Elementary Electronic Excitations and the Mechanism of Cytochrome P450

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Received November 4, 1988

The class of heme proteins known as cytochromes P450[1] are involved in the cleavage of molecular oxygen and the stereospecific insertion of a single atom of oxygen into a variety of substrates.[2] A specific cytochrome $P450_{cam}$, isolated from *pseudomonas putida*, has been used in a variety of physical–chemical studies[3] in order to elucidate some of the key structure–function relationships common to the broad class of P450 enzymes. In particular, an isotopically sensitive vibrational mode has been observed at 351 cm^{-1} with resonance Raman spectroscopy and assigned to Fe–S stretching involving cysteine.[4] Recent studies of the resonance Raman excitation profile (REP) of this mode have resolved unusual and unexpected S → Fe charge-transfer electronic structure to the blue of the Soret transition.[5] In the present communication, we consider these results in the context of the fundamental steps involved in the mono-oxygenase reaction cycle. (For a recent review see Dawson.[6]) We suggest that these steps are related to specific electronic excitations of the sulfur, iron, porphyrin, and oxygen orbitals.

The samples of cytochrome $P450_{cam}$ used in the Raman studies were prepared as discussed previously.[5,7] Figure 1 shows the REP's of the 1488-cm^{-1} high-spin marker band of the heme and the 351-cm^{-1} Fe–S axial ligand mode of oxidized, substrate-bound $P450_{cam}$. Absorption spectra are also shown for solution (thin solid line) and for *z*-polarized single crystals[8] (thick solid line). Notice the extreme blue shift and structure in the Fe–S REP (solid triangles). We expect the REP's of high-frequency modes to peak to the blue of low-frequency modes when both modes are coupled to the same electronic transitions. Thus, it is quite certain that the 351- and 1488-cm^{-1} modes are coupling to different electronic excitations and that the Fe–S mode is activated by *z*-polarized charge-transfer transitions, one of which is clearly observed in the single-crystal absorption at 323 nm. The other *z*-polarized transition, at 360 nm, was apparently missed in the single-crystal analysis due to its proximity to the Soret band and the difficulty

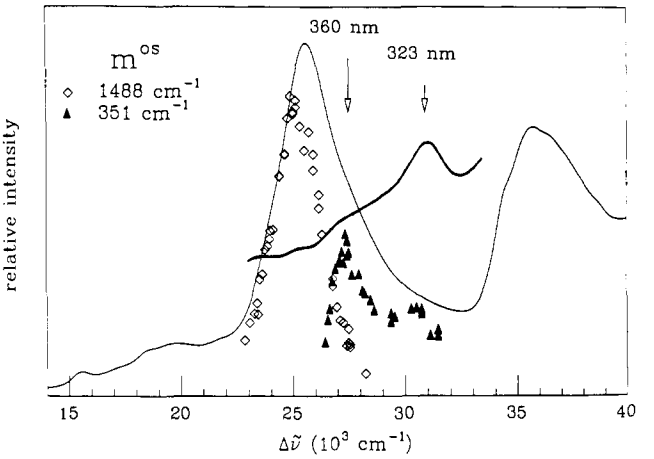
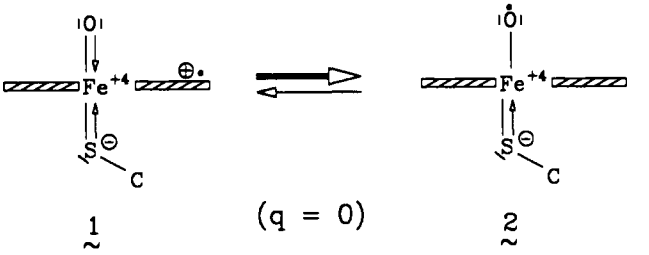
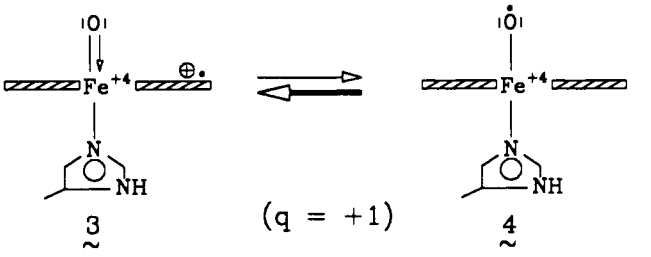


Figure 1. The Raman excitation profiles and absorption spectra of $P450_{cam}$. The REP of the Fe–S mode is shown as solid triangles. The thin solid line is the solution absorption spectrum. The thick solid line is the *z*-polarized single crystal absorption (from ref 8).

Scheme I



Scheme II



of subtracting the in-plane Soret transition moment from the *z*-polarized spectrum (two hemes/unit cell with different orientation). The small inflection at 360 nm is the residual of this transition.

We believe that the two charge-transfer transitions in the high-spin complex involve S → Fe excitations arising from sp^2-hybridized sulfur orbitals[4] that increase the π overlap with the 4-fold symmetric heme while still accounting for the anisotropic esr *g* values.[3] The $S(sp^2) \rightarrow Fe(d\pi)$ excitations are quenched in the low-spin complex, possibly due to filling of the $d\pi$ orbitals when the iron moves into the heme plane (and the *xy* orbital is increased in energy). In this respect, substrate-mediated spin-state equilibria can act as a "switch" to control the S → Fe electron-donation properties of the system.[4]

The sulfur → iron π-electron donation appears to have important mechanistic consequences not only in the dioxygen cleavage step but also in the final hydroxyl insertion step. The high-valent iron porphyrin intermediates that play a key role in the mechanism of both peroxidases and cytochromes P450 have generally been characterized as iron–oxo π cation complexes (Fe^{IV} $S = 1$, porphyrin $S = {}^1/_2$),[9,10] although recent calculations[11] indicate the

(1) *Cytochrome P450*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986.
(2) White, R.; Coon, M. J. *Annu. Rev. Biochem.* **1980**, *49*, 315.
(3) (a) Gunsalus, I. C.; Wagner, G.; Debrunner, P. In *Microsomes, Drug Oxidations and Chemical Carcinogenesis*; Coon, M. J., Ed.; Academic: New York, 1980; p 233. (b) Dawson, J.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255.
(4) Champion, P. M.; Stallard, B.; Wagner, G.; Gunsalus, I. C. *J. Am. Chem. Soc.* **1982**, *104*, 5469.
(5) Bangcharoenpaurpong, O.; Champion, P. M.; Martinis, S.; Sligar, S. *J. Chem. Phys.* **1987**, *87*, 4273.
(6) Dawson, J. *Science* **1988**, *240*, 433.
(7) Gunsalus, I. C.; Wagner, G. *Methods Enzymol.* **1978**, *52*, 166.
(8) Hanson, L. K.; Sligar, S.; Gunsalus, I. C. *Croat. Chem. Acta* **1977**, *49*, 237.
(9) (a) Lowe, G.; Kert, C.; Hjelmeland, L.; Kirchner, R. *J. Am. Chem. Soc.* **1977**, *99*, 3534. (b) Lowe, G.; Herman, Z. *J. Am. Chem. Soc.* **1980**, *102*, 6173.
(10) Groves, J.; Haushalter, R.; Nakamura, M.; Nemo, T.; Evans, B. *J. Am. Chem. Soc.* **1981**, *103*, 2884.

0002-7863/89/1511-3433$01.50/0