MODEL SYSTEM STUDIES OF AXIAL LIGATION IN THE OXIDIZED REACTION STATES OF CYTOCHROME P-450 ENZYMES

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I. INTRODUCTION

Cytochrome P-450 enzymes have been isolated from bacteria, microsomes, and mitochondria and catalyze the insertion of one atom of dioxygen into substrate while the other is reduced to water. The biological and many of the physical properties of these enzymes are summarized elsewhere (Hill et al., 1970a; Ullrich, 1972; Gunsalus et al., 1973; Tomaszewski et al., 1974). Substantial clarification of the nature of P-450 dependent oxygenase reactions has been afforded by isolation of the soluble cytochrome (P-450_{cam}) from *Pseudomonas putida* grown on camphor. *In vitro* assembly of the of the enzyme system, which also includes an electron transfer chain comprised of a reductase and the 2Fe-2S protein putidaredoxin, has led to deduction of reaction sequence (1) (Tyson et al., 1972; Gunsalus et al., 1973). In this scheme S = substrate, ox = Fe(III), red = Fe(II), and s-red is the one-electron reduction product of

the Fe(II)·O₂ form. More recently microsomal P-450 cytochromes have been purified and enzyme systems assembled (Imai and Sato, 1974; van der Hoeven and Coon, 1974; Haugen et al., 1975; Guengerich

et al., 1975). While scheme (1) appears to apply to these systems as well, the details of substrate binding, oxygen activation, and the hydroxylation step remain to be established. This matter is exacerbated by the lack of structural information for the heme active site. While all P-450 enzymes are b-type (protoporphyrin IX) cytochromes, the axial ligand(s) to iron, on which structural, electronic, and reactivity properties are significantly dependent in both natural and synthetic Fe(II,III) porphyrin systems, have not been securely identified in any of the five reaction states. Considerable speculation has centered on cysteinate sulfur (Cys-S) ligation, a possibility first suggested in the mid-1960's (Mason et al., 1965; Murikami and Mason, 1967) and subsequently supported by the findings that thiol addition to met-Mb and Hb and hemin chloride (usually in the presence of nitrogenous bases) afforded epr g-values close to those of the low-spin ($S=1/2$) ox-P-450 state (Jefcoate and Gaylor, 1969; Bayer et al., 1969; Röder and Bayer, 1969). The substrate-bound state ox-P-450·S of at least the P. putida enzyme is predominantly high-spin ($S=5/2$) (Peterson, 1971; Tsai et al., 1970; Sharrock et al., 1973).

The possibility of axial sulfur ligation in one or more of the enzyme reaction states has proven difficult to assess in the absence of known properties of fully characterized sulfur-bound iron porphyrins. Indeed, the $[\text{Fe(III)N}_4\text{SR}]$ coordination unit, potentially unstable to intramolecular electron transfer, has only recently been obtained in isolable porphyrin (Koch et al., 1975c; Collman et al., 1975; Ogoshi et al., 1975; Tang et al., 1976) and other tetraazamacrocyclic complexes (Koch et al., 1975ab). In this investigation we have attempted to develop certain empirical criteria for identification of ligands L and L' in porphyrin complexes containing the high-spin $[\text{Fe(III)N}_4\text{L}]$ and low-spin $[\text{Fe(III)N}_4\text{LL}']$ coordination units, in which L and L' are O-, S-, and N-donor ligands intended to simulate binding by protein side-chains. Two basic assumptions underlie this approach: (i) high-spin ox-P-450·S and low-spin ox-P-450 states are effectively five- and six-coordinate, respectively, there being no exceptions to these spin state-structure correlations in natural and synthetic porphyrin systems (Hoard, 1971, 1975); and (ii) at nominal parity of axial ligand(s) properties of synthetic porphyrins and enzyme sites will be sufficiently similar to allow deduction of axial ligation modes in the latter.

II. RESULTS AND DISCUSSION

A. Synthetic Fe(III) Porphyrins

Five-coordinate complexes of protoporphyrin IX dimethyl ester

dianion (PPIXDME) were obtained by cleavage of the μ -oxo dimer with HL and six-coordinate species were formed *in situ* by reaction with ligands L', as shown in reactions (2) and (3). Five-coordinate

thiolate complexes sufficiently stable for isolation were obtained only from arylthiols; alkylthiols caused reduction to Fe(II). Fe-(PPIXDME)L species served as precursors to six-coordinate species containing a variety of L/L' axial ligand combinations. If the initial cleavage was performed in the presence of excess L', it was possible in some cases to trap at low temperatures six-coordinate complexes with L = alkylthiolate. Low-spin complexes containing acetylcysteinate-N-methylamide (S-Cys(Ac)NHMe) were obtained in this way. The following ligands were used as simulators of protein side-chain coordination: Cys, ArS⁻, MeNH(Ac)Cys-S⁻; Met, tetrahydrothiophene (THT); His, N-methylimidazole (N-MeIm); Tyr, ArO⁻; Asp, Glu, OAc⁻; Ser, Thr, RO⁻; Lys, Arg, RNH₂; Asn, Gln, dimethylformamide (DMF). Stable five-coordinate complexes were isolated with L=p-ClC₆H₄S, p-O₂NC₆H₄S, p-O₂NC₆H₄O, OEt, and OAc. Analogous complexes were obtained in the octaethylporphyrinate (OEP) series.

Amino acid analyses of two P-450 enzymes (Tsai *et al.*, 1971; Dus *et al.*, 1974) reveal the presence of all residues potentially capable of coordinating to metal ions. Not all of the large number of possible enzyme ligation modes could be tested in synthetic five- and six-coordinate Fe(III) porphyrins owing to the failure to isolate or generate in solution all desired species. Magnetic and spectroscopic features of accessible complexes were determined and, together with available data for the synthetically inaccessible ligation modes in met-Hb and Mb (His-Fe) and cytochromes c (Met-Fe-His), were compared to corresponding properties determined for ox-P-450·S and ox-P-450. Such comparisons are listed in Table I, to which reference should be made in the following discussion, and are limited to those synthetic species which most closely approach the enzyme properties. Full details concerning preparation of compounds, ligation modes examined, and necessary theory, as well as the complete body of physical data, are given elsewhere (Tang *et al.*, 1976).

B. High-Spin Forms

All Fe(PPIXDME)L complexes are high-spin with magnetic moments near the theoretical spin-only value of 5.92 BM for the d⁵ configuration. In non-coordinating solvents these complexes exhibit diagnostic high-spin absorption spectra (Caughey *et al.*, 1973; Smith and Williams, 1970). Of various species with anionic sulfur and

TABLE I
COMPARISON OF PHYSICAL PROPERTIES OF SYNTHETIC Fe(III) PORPHYRIN THIOLATES
AND OXIDIZED CYTOCHROME P-450 REACTION STATES

High-Spin Forms				
<u>Magnetism and Epr Data</u>	μ (BM)	g-values ^o		
Fe(PPIXDME)(SC ₆ H ₄ NO ₂)	5.90 ^a	---		
Fe(PPIXDME)(SC ₆ H ₄ Cl)	5.87 ^a	7.2, 4.8, 1.9		
ox-P-450 _{cam} •S	5.2 ^b	8.0, 4.0, 1.8 ^c		
microsomal ox-P-450•S	--	8.1, 3.7, 1.7 ^d		
<u>R. japonicum</u> ox-P-450•S	--	7.9, 3.8, 1.8 ^e		
<u>Electronic Spectra</u>	$\lambda_{\max}$, nm (cmM)			
	α	β	γ (Soret)	
Fe(PPIXDME)(SC ₆ H ₄ NO ₂)	646(4.8)	540(sh,10.4), 517(11.2)	391(86.8)	
Fe(PPIXDME)(SC ₆ H ₄ Cl)	643(4.4)	571(10.4), 533(11.5)	396(83.3)	
ox-P-450 _{cam} •S ^f	646(4.5)	540(sh,10), ~520	391(87)	
microsomal ox-P-450•S ^g	647	540(sh), 517	394	
<u>Mössbauer Data</u> ^h	δ (mm/sec) ⁱ	ΔE_Q (mm/sec)	H _{hf} ^o (kOe)	D(cm ⁻¹)
Fe(PPIXDME)(SC ₆ H ₄ NO ₂) ^j	0.33	0.76	-476±10	10≤D≤12
(CH ₂ Cl ₂ soln.)	0.32	0.88	--	--
ox-P-450 _{cam} •S ^k	0.35	0.79	-448±10	3.8 ^c

TABLE I (Cont'd.)

<u>Electronic Spectra</u>	<u>Low-Spin Forms</u>		
	$\lambda_{\max}$, nm (ϵ_{mM})		
	α	β	γ (Soret)
Fe(PPIXDME)(SC ₆ H ₄ NO ₂)(2-MeTHF) ^l	566(1.1)	535(1.0)	418(14.9)
Fe(PPIXDME)(SC ₆ H ₄ Cl)(2-MeTHF) ^l	566(1.0)	536(1.0)	422(14.2)
Fe(PPIXDME)(SC ₆ H ₄ NO ₂)(DMF) ^l	567(1.1)	533(1.0)	420(14.1)
ox-P-450 _{cam} ^f	571(10.5)	535(10)	417(105)
microsomal ox-P-450 ^m	568(14.2)	534(13.6)	417(124)
mitochondrial ox-P-450 ⁿ	567	532	416
<u>Epr Data</u>	<u>Ligand(L')</u>		<u>g-values^o</u>
Fe(PPIXDME)(SC ₆ H ₄ NO ₂)	N-MeImP	2.42	2.26 1.91
	n-PrNH ₂	2.48	2.26 1.90
	THTq	2.40	2.28 1.92
	PhSH ^s	2.43	2.28 1.92
	DMF ^r	2.46	2.28 1.90
Fe(PPIXDME)(S-Cys(Ac)NHMe)	N-MeImP	2.37	2.24 1.94
	n-PrNH ₂	2.40	2.23 1.93
	THTq	2.35	2.24 1.94
	DMF ^r	2.37	2.24 1.95
ox-P-450 _{cam} ^c	--	2.45	2.26 1.91
microsomal ox-P-450 ^t	--	2.41	2.25 1.91
mitochondrial ox-P-450 ^u	--	2.42	2.26 1.91

a295°K. ^b94-253°; Peterson, 1971. ^cTsai et al., 1970. ^dPeisach and Blumberg, 1970. ^ePeisach et al., 1972. ^fGunsalus et al., 1973. ^gEstabrook et al., 1973. ^hValues at 4.2°K unless otherwise noted. ⁱRelative to Fe metal. ^jSolid state. ^k200°K, Sharrock et al., 1973. ^l77°K, values in parentheses are the absorbance ratios A/A _{β} . ^mvan der Hoeven and Coon, 1974. ⁿMitani and Horie, 1969. ^o~90°K. PN-methylimidazole. ^qTetrahydrothiophene. ^rN,N-dimethylformamide. ^sLow-spin form is minority component. ^tMiyake et al., 1969. ^uCheng and Harding, 1973.

oxygen ligands and with His(Im) coordination (met-Hb, Mb, Antonini and Brunori, 1971), only thiolate complexes showed a high degree of correspondence with the substrate-bound reaction state in terms of α and γ band positions and intensities. Further comparisons are afforded by MCD spectra, a conspicuous feature of which for ox-P-450_{cam}•S is substantial negative ellipticity in the Soret region (Dolinger *et al.*, 1974; Vickery *et al.*, 1975). Among all synthetic porphyrin complexes examined this feature is observed only with thiolate species; all others exhibit positive ellipticity of their Soret bands (Dawson *et al.*, 1976).

The epr spectra of all ox-P-450•S states are characterized by large rhombic splittings manifested by three g-values, two of which are symmetrically split about $g = 6$ and the other is < 2 . For strict axial symmetry $g_{\perp} = 6$ and $g_{\parallel} = 2$. From the observed g-values the spin Hamiltonian parameter ratio $E/D = 0.083-0.092$, corresponding to degrees of rhombicity of 25-28% (Peisach and Blumberg, 1971). The epr spectra of Fe(PPIXDME)(SC₆H₄Cl) and Fe(OEP)(SPh) reveal substantial rhombic splittings with $E/D \approx 0.05$ or 15% rhombicity. In contrast, complexes with O- and N-donor ligands have axial or near-axial symmetry, with the largest splitting observed for Fe(PPIXDME)-(OEt) ($E/D = 0.02$). For Mbs and normal intact Hbs the degree of rhombicity is usually $\leq 3\%$ (Peisach and Blumberg, 1971; Peisach *et al.*, 1969; Kotani and Watari, 1971). Consequently, substantial rhombicity in the present group of enzymes and synthetic complexes appears to correlate with thiolate ligation.

The Mössbauer spectral parameters of isomer shift (δ) and quadrupole splitting (ΔE_Q) are typical of high-spin Fe(III) porphyrins and are in good agreement with results for ox-P-450_{cam}•S. However, these parameters are rather insensitive to the nature of axial ligands (Tang *et al.*, 1976; Maricondi *et al.*, 1972; Torrens *et al.*, 1972) and do not serve to identify enzyme ligation. Considerably more useful in this respect is the quantity H_{hf}^o , the saturation magnetic field at the ⁵⁷Fe nucleus, which is expected to be sensitive to changes in axial ligation. This has proven to be the case. Values of H_{hf}^o for synthetic porphyrins were evaluated from low magnetization and Mössbauer measurements in large applied magnetic fields and the preceding epr data. Experimental methods and necessary theory are available elsewhere (Tang *et al.*, 1976). For typical Fe(III) salts, met-Mb (Lang, 1970), and oxygen-ligated porphyrins $-H_{hf}^o \geq 500$ kOe, substantially larger than the value of -448 kOe for ox-P-450_{cam}•S. Only in the thiolate complex Fe(PPIXDME)-(SC₆H₄NO₂), for which $-H_{hf}^o = 476 \pm 10$ kOe, is the enzyme value reasonably closely approached.

C. Low-Spin Forms

Combination of reactions (2) and (3) has led to the formation

of six-coordinate low-spin Fe(III) porphyrin complexes in solution. Attempts to isolate thiolate species proved unsuccessful. Epr monitoring of reaction mixtures with a variety of ligands L' has shown that, after the initial low temperature quench, further thaw-quench cycles were accompanied by rapid decrease in the intensity of low-spin Fe(III) signals with no other resonances detectable at 80-95°K. This behavior is interpreted as arising from the redox reaction (4). Because of the instability of six-coordinate species and in some cases the lack of complexation by excess L', the scope of physical

studies of these complexes is less extensive than for five-coordinate complexes.

Satisfactory electronic spectra of Fe(PPIXDME)LL' complexes were obtained only in glass media at ~77°K and only with certain combinations of L and L'. Spectral data for 2-MeTHF and DMF adducts are those of low-spin species (Smith and Williams, 1970) and are in fairly good agreement with results for the ox-P-450 state. Further spectral examination has revealed that insufficient differences exist between the N-MeIm/N-MeIm and SC₆H₄NO₂/DMF cases to permit secure ligation criteria to be established. However, epr results (vide infra) eliminate the first mode of ligation. Because of the inability to survey a wide range of Fe(PPIXDME)LL' species, it can be noted only that spectral similarities are consistent with, but do not require, thiolate coordination in the resting enzyme state.

Prior to this study ample evidence has been presented, and usefully quantitated in "truth" diagrams (Peisach and Blumberg, 1971; Peisach et al., 1973a), that g-values of the rhombic epr spectra of low-spin ferrihemes are sensitive to the nature of axial ligands. Using both PPIXDME and OEP complexes the spectra of species containing some 25 L/L' ligand combinations were examined. Together with data for cytochromes b and c and Fe(PPIXDME)(N-MeIm)₂⁺, this information indicated that in the ox-P-450 state one ligand is cysteinate and the other corresponds to the biological counterparts of ligands L' in Table I, which in combination with L = ArS produced satisfactory agreement with the g-values of the enzymes.

D. Conclusions

Noting the modes of axial ligation which could not be directly tested with synthetic porphyrin complexes and the limitations of such complexes as models for biological heme coordination, matters which are dealt with more fully elsewhere (Tang et al., 1976), the following principal conclusions are drawn: (i) On the basis of

collective comparative results from electronic absorption, MCD, epr, and magnetically perturbed Mössbauer spectra, the most probable axial ligation mode in the high-spin ox-P-450·S state is Cys-S-Fe. (ii) From comparative epr and, marginally, electronic absorption spectra, the Cys-S-Fe coordination in ox-P-450·S is retained in the resting ox-P-450 state with axial ligation Cys-S-Fe-L'; no choice can be made among the possibilities L' = His, Lys(Arg), Cys-SH, Met, Asn(Gln), and H₂O. All but the last have been directly tested. From these conclusions the simplified representation of substrate binding, reaction (5), follows for those cases in which a spin-state change occurs. It is consistent with certain explicit earlier proposals concerning one or both reaction states (Hill *et al.*, 1970ab; Peisach *et al.*, 1973b) except that a weakly bound sixth ligand is

not included in the high-spin state. Removal of L' upon substrate binding with retention of thiolate coordination conflicts with an earlier proposal of sulfur ligand displacement (Tsai *et al.*, 1970), but is in accord with another proposal (Estabrook *et al.*, 1973) that L' = His is detached from the coordination sphere. More recently it has been suggested that in ox-P-450 L' = H₂O, which is displaced when substrate is bound (Griffin and Peterson, 1975). We have been unable to detect H₂O binding to Fe(III) porphyrin thiolates by epr measurements. If 2-MeTHF is considered a rough simulator of axial H₂O coordination, spectral data for Fe(PPIXDME)-(SC₆H₄NO₂)(2-MeTHF) widen the choice of L' ligands to include H₂O. In assigning the ligation modes above, the resemblance between certain electronic features of the oxidized P-450 reaction states and chloroperoxidase is noted (Chiang *et al.*, 1975). In the latter the two cysteinyl residues are described as existing as a disulfide in both the Fe(III) and Fe(II) forms of the enzyme and thus are unavailable as thiolate ligands. Possibly in at least this one case thiolate coordination may be a sufficient but not necessary condition for development of properties similar to those of oxidized P-450 reaction states. However, this situation is not consistent with

the collective body of data for model Fe(III) PPIXDME complexes presented here and elsewhere (Tang *et al.*, 1976).

E. Structure of Fe(PPIXDME)(SC₆H₄NO₂)

In order to define in detail the stereochemistry of a presumably representative Fe(III) porphyrin thiolate and thus provide the

Figure 1. Overall molecular structure of Fe(PPIXDME)(SC₆H₄NO₂).

Figure 2. Coordination sphere of Fe(PPIXDME)(SC₆H₄NO₂).

first structural model for the active site of the ox-P-450·S reaction state, the molecular structure of Fe(PPIXDME)(SC₆H₄NO₂) was determined by X-ray diffraction. A view of the entire molecule is presented in Figure 1 and the coordination sphere geometry with bonded distances is given in Figure 2. The Fe(III)N₄S coordination unit has the pyramidal arrangement found in all high-spin Fe(III) porphyrins but with marginally significant differences in Fe-N distances, which average to 2.064 Å. The Fe atom lies 0.448 Å out of the mean porphyrin plane and 0.434 Å out of the N₄ plane toward the axial sulfur. The porphyrin ring is "domed" toward the metal by 0.014 Å. The average Ct-N distance is 2.017 Å, although definition of the N₄ center (Ct) is somewhat arbitrary in the absence of axial symmetry. These values agree closely with representative parameters for high-spin Fe(III) porphyrins of Fe-N = 2.065 Å, Ct...N = 2.015 Å, and Ct...Fe = Fe...N₄ plane = 0.45 Å given by Hoard (1971, 1975). The Fe-S distance is at the long end of the 2.21-2.31 Å range for non-porphyrin high-spin Fe(III) thiolate complexes (Tang *et al.*, 1976). The locus of the phenyl ring (Figure 1) is apparently dictated by steric effects and, with the marginally different Fe-N distances, degrades the Fe site symmetry in the coordination sphere from axial to rhombic. This situation may contribute to the substantial rhombic splittings observed in the epr spectra.

Provided the conclusion that Fe-S-Cys ligation occurs in the oxidized enzymes is correct, the utility of well-defined synthetic Fe(III) porphyrin thiolate complexes may extend beyond providing electronic and structural models for active sites. It has recently been shown that P-450 enzymes can function as peroxidases (Hrycay *et al.*, 1975b), that certain organic hydroperoxides can replace NADH and O₂ in enzymic substrate hydroxylation (Rahmitula and O'Brien, 1975), and that hydroperoxide, NaClO₂, NaIO₄, or H₂O₂ support substrate hydroxylation with the oxidized enzyme in the absence of molecular oxygen (Hrycay *et al.*, 1975a). The latter finding implies that the three step process from ox-P-450·S to s-red-P-450·S·O₂ in the reaction sequence (1) can be "short-circuited" by supplying to the oxidized enzyme a reagent which in combination with Fe(III) results in the same oxidation level as the s-red state before hydroxylation. A formalistic scheme is indicated in reaction (6), where

the ferryl (FeO) entity may be the active hydroxylating agent and conceivably could be formed directly with chlorite or periodate. Employment of such reagents in combination with synthetic Fe(III) porphyrin thiolates and potential substrate could afford useful model hydroxylation systems for mechanistic studies, which would be more easily manipulated than others requiring formation and reduction

of $\text{Fe(II)} \cdot \text{O}_2$ species prior to hydroxylation. Aromatic hydroxylation has been accomplished in model systems composed of hemin chloride, aqueous base, excess thiol, O_2 , and substrate aniline (Sakurai and Ogawa, 1975). In this case presumably thiolate reduces Fe(III) to Fe(II) , and subsequent reactions may proceed in a manner similar to scheme (1).

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