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Electron Paramagnetic Resonance Detectable States of Cytochrome P-450$_{cam}$†

John D. Lipscomb*

ABSTRACT: Cytochrome P-450$_{cam}$ is a low-spin Fe^{3+} hemoprotein (g = 2.45, 2.26, and 1.91) which is made 60% high spin (g = 7.85, 3.97, and 1.78) at 12 K by the addition of 1 mol of substrate per mol of enzyme. Low-temperature EPR spectra show that the low-spin fraction of substrate-bound P-450$_{cam}$ contains two magnetic species. The majority species has an unusual EPR spectrum (g = 2.42, 2.24, and 1.97) which cannot be simulated by using the range of crystal field parameters known for other heme proteins. The minority species has the same g values as substrate-free enzyme. Both low-spin species show Curie law temperature dependence below 50 K and have similar saturation behavior. Above 50 K the g = 2.42, 2.24, and 1.97 species rapidly loses signal intensity. The distribution of low-spin species is pH dependent (apparent pK_a = 6.2) with the g = 2.42, 2.24, and 1.97 magnetic species favored at high pH. The substrate binding stoichiometry and the equilibria observed in the low-spin fraction suggest that there are not multiple protein forms of cytochrome P-450$_{cam}$. Putidaredoxin and other effector molecules which specifically catalyze hydroxylation convert either the high-spin or the g = 2.42, 2.24, and 1.97 low-spin species to another new magnetic species (g = 2.47, 2.26, and 1.91). This species is only seen in the presence of substrate, and its stability reflects the catalytic potency of the effector molecule. The EPR and UV–visible spectra of cytochrome P-420 depend upon the manner in which the P-420 is generated. Incubation with acetone or reaction with *N*-ethylmaleimide or diethyl pyrocarbonate generates P-420 with different spectral characteristics. Through identification of active-site amino acids by chemical modification and comparison with porphyrin model complexes, the range of ligands likely to participate in each of the EPR detectable species is assigned. Mechanisms of interconversion of these species and their bearing on catalysis are discussed.

Cytochrome P-450$_{cam}$ (cytochrome m)[1] is the central enzyme of a three-component mixed-function oxidase which converts the 5-methylene group of camphor to the 5-exo alcohol (Katagiri et al., 1968). Dramatic changes in the magnetic resonance spectra of the heme iron accompany each step in the catalytic cycle (Gunsalus et al., 1973). In particular, Mössbauer spectroscopy has been valuable in detailing the electronic properties in each of the semistable ferric and ferrous intermediates of the cycle (Sharrock et al., 1973, 1976), and EPR spectroscopy of the ferric intermediates has demonstrated the sensitivity of the iron environment to substrates. At 12 K the native cytochrome m (m^o) is low spin ($S = {}^1/_2$) with EPR g values at 2.45, 2.26, and 1.91, but addition of substrate (m^{os}) is accompanied by a 60% conversion to a rhombically distorted high-spin ($S = {}^5/_2$) form with g = 7.85, 3.97, and 1.78 (Tsai et al., 1970). Substrate binding also causes a 26-nm blue shift in the optical Soret band which has been associated with the EPR detectable high-spin transition. Quantitation of the cytochrome absorption at 25 °C in the Soret region implies that only 6% of the substrate-bound cytochrome is in the low-spin form (Sligar, 1976). The low-spin fraction increases with decreasing temperature according to the usual Arrhenius relationship to a maximum of 40% at ~200 K. Below this temperature, Mössbauer spectroscopy shows that the Arrhenius dependence is not observed and there is only a small change in the high-/low-spin distribution. (Sharrock et al., 1973). At temperatures above 100 K, the EPR spectrum of the low-spin m^{os} magnetic species is identical with that observed for m^o; however, at lower temperatures an anomalous signal at g = 1.97 is observed (Tsai et al., 1970). This signal was postulated to arise from either an excited state or a contaminant. We present evidence in this report which shows that the signal represents in fact a new low-spin form of cytochrome m.

† This work was initiated in the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801, and completed in the Department of Biochemistry, Medical School, University of Minnesota, Minneapolis, Minnesota 55455. *Received January 14, 1980.* The work was supported by National Institutes of Health Grants AM 00562, GM 21161, and GM 24689. The EPR spectrometer at the University of Minnesota was purchased in part through grants from the Minnesota Medical Foundation and the Graduate School.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; NEM, *N*-ethylmaleimide; DEP, diethyl pyrocarbonate; cytochrome P-450$_{cam}$ is shortened to cytochrome m or m (for monoxygenase) (Sligar, 1976) to facilitate presentation of its various redox and substrate-bound states.

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The semistable intermediates of the cytochrome m reaction cycle have been clearly defined, and the dynamics of their interconversion have been studied in some detail (Tyson et al., 1972; Peterson, 1971). Substrate binding occurs with multiple changes in the redox potential, reactivity, and stability of the cytochrome (Lipscomb et al., 1976). Electron transfer to the cytochrome occurs in two independent steps after substrate binding. The first step, a ferric-to-ferrous transition ($m^{os} \rightarrow m^{rs}$), allows formation of an oxygenated complex ($m_{O_2}^{rs}$). The second step occurs only in the presence of an effector molecule which is required to elicit hydroxylation (Tyson et al., 1972). Putidaredoxin (PD) serves both the first electron donor and the effector roles, but several other proteins and small molecules have been found to fulfill one function or the other (Lipscomb et al., 1976). Physical (Sligar et al., 1974) and kinetic (Lipscomb et al., 1976) studies indicate that mixed-function oxidation occurs in an $m_{O_2}^{rs}$–PD complex. It is shown in this report that PD binding by cytochrome results in a new low-spin magnetic species in the m^{os} EPR spectrum, suggesting that the protein–protein interaction directly changes the cytochrome active site.

Comparatively little is known about the details of active-site structure. Evidence for a heme iron axial mercaptide ligand has accumulated from studies by EPR spectroscopy (Mason et al., 1965; Blumberg & Peisach, 1971), magnetic circular dichroism (Dawson et al., 1976), single-crystal optical spectroscopy (Hanson et al., 1976), and inorganic model complexes (Chang & Dolphin, 1975; Collman et al., 1975; Tang et al., 1976). This ligand is thought to be fixed during the reaction cycle because each semistable intermediate shows evidence of sulfur ligation with the possible exception of the oxygenated form for which the data cannot be uniquely interpreted (Hanson et al., 1976). The second axial ligand is unknown and may exchange with solvent, substrate, or another ligand from the protein to account for spin-state changes observed during the reaction cycle. We show here that the changes in the cytochrome m EPR spectrum induced by substrate and ligand binding are compatible with a model of this sort which proposes one fixed and one exchangeable ligand. The identities of the exchangeable ligands cannot be directly ascertained from these studies. However, the spectral properties of the EPR detectable species can be compared with those observed in model complexes to identify the most likely set of ligands for each species. Chemical modifications of active-site amino acid residues reported here and in previous studies (Yu & Gunsalus, 1974; Lipscomb et al., 1978) are also useful in determining the feasibility of proposed ligand arrangements.

Cytochrome P-420 is a class of inactive cytochrome m molecules which form when the cytochrome is denatured or chemically modified by sulfhydryl reagents (Yu & Gunsalus, 1974). The position of the Soret band at ~420 nm for the reduced cytochrome P-420–CO complex is typical of heme proteins lacking a cysteinyl axial ligand. Thus, the formation of cytochrome P-420 from cytochrome m is thought to involve the displacement of the fixed thiolate ligand. In this report, we compare some of the various forms of cytochrome P-420 which can be distinguished by EPR or optical spectroscopy and conclude that they can also arise through processes such as configurational changes and titration of noncysteinyl active-site residues which sometimes leave the fixed thiolate ligand in place in the ferric state.

Materials and Methods

Cytochrome m and PD were purified from *Pseudomonas putida* strain PpG786 (ATCC 29607) grown on camphor as the sole carbon source as previously described (Yu et al., 1974; Gunsalus & Wagner, 1978). The ^{57}Fe-labeled cytochrome m was prepared by growth of the organism on enriched media (Sharrock et al., 1973). The enzyme concentrations were estimated from the published optical extinction coefficients and activities from the utilization of NADH by a reconstituted system containing putidaredoxin reductase, putidaredoxin, camphor, and the cytochrome m (Gunsalus & Wagner, 1978). Cytochrome P-420 was estimated from the absorbances at 420 and 450 nm after degassing, reduction with sodium dithionite, and bubbling with carbon monoxide (Yu & Gunsalus, 1974). Cytochrome m is prepared and stored in the presence of camphor. When required, camphor was removed by exclusion chromatography at 4 °C on a Bio-Rad Bio-Gel P-10 column equilibrated in 50 mM Tris-HCl buffer at pH 8 followed by dialysis vs. 50 mM K^+PO_4 buffer at pH 7.1.

The two carboxy-terminal residues of PD were removed by carboxypeptidase A treatment to produce des-Trp-Gln-PD (Sligar et al., 1974). *Peptococcus aerogenes* rubredoxin (Rd) was the generous gift of Dr. N. Stombaugh. Apo-Rd was prepared by the method Bachmayer et al. (1968).

Lipoic acid was purchased from Sigma Chemical Co. and reduced with sodium borohydride to dihydrolipoic acid [Lip-$(SH)_2$] according to Gunsalus et al. (1956), and its concentration was estimated by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959). 5-*exo*-Hydroxycamphor was obtained from Dr. I. C. Gunsalus. Diethyl pyrocarbonate was obtained from Sigma Chemical Co., and the other chemicals were reagent grade. Buffer indicates 50 mM K^+PO_4, pH 7.1, prepared by mixing 50 mM solutions of K_2HPO_4 and KH_2PO_4. Camphor was quantitated by vapor-phase chromatography on a 3% QF-1 resin according to Lipscomb et al. (1976).

EPR spectra were recorded on a Varian E-9 spectrometer fitted with an Air Products flow helium Dewar at the School of Chemical Sciences, University of Illinois, or a Varian E-109 spectrometer equipped with a 30-dB microwave power attenuator and fitted with an Oxford Instruments ESR-10 helium Dewar at the Department of Biochemistry, Medical School, University of Minnesota. All measurements were made with a microwave frequency of ~9.2 GHz and a modulation frequency of 100 kHz. Ferric myoglobin fluoride and α,α-diphenyl-β-dipicrylhydrazyl were used as standards to calibrate the measurements of g values in the $g = 6$ and $g = 2$ regions, respectively. Temperature was measured by using a gold–iron–chromel thermocouple or the resistance of a calibrated carbon resistor. Overlapping spectra in the $g = 2.4$ region were separated by using a Du Pont Instruments curve revolver or by curve-fitting procedures. These procedures used the g value and the shape of the isolated peaks in this region obtained from spectra of m^o ($g = 2.45$) and m^{os} at pH 8, 2.5 K ($g = 2.42$). These spectra are greater than 95% due to a single species. The spin distribution was quantiated by using the method of Aasa & Vänngård (1975) and the phenylimidazole complex of m^o as a standard. Samples were frozen by immersion in liquid nitrogen.

Cytochrome m was modified with diethyl pyrocarbonate (DEP) by reacting 14.5 μM m^o or m^{os} with 1 mM DEP at 20 °C for 6 h in 5 mM K^+PO_4, pH 6.2, and removing excess reagent by exclusion chromatography on Bio-Rad Bio-Gel P-10 equilibrated in 50 mM K^+PO_4 at pH 7.1. Prior to EPR measurements, the modified protein was concentrated on an Amicon PM-10 ultrafilter.

Results

EPR Detectable Species in Cytochrome m–Substrate Complex. EPR spectra of ferric cytochrome m in the low-spin substrate-free and mixed-spin substrate-bound forms are shown

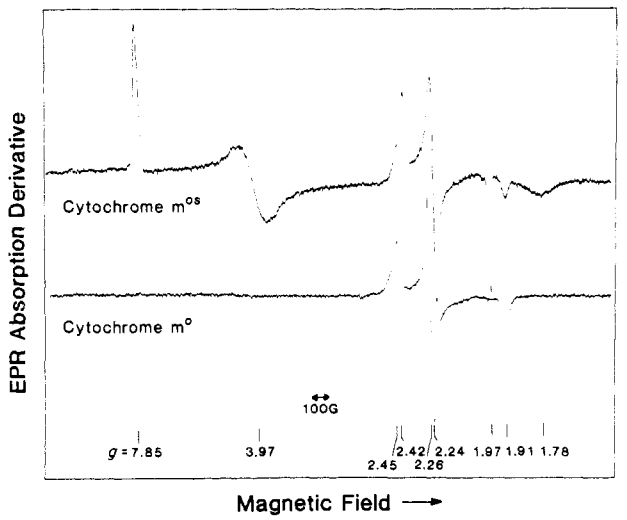

FIGURE 1: EPR spectra of m° and m^{os}. Spectra of 2 mM m^{os} (top) or 640 μM m° (bottom) were measured in 50 mM K^+PO_4 buffer, pH 7.1, S = camphor, 3.5 mM. Measurement parameters were as follows: T = 12 K, modulation amplitude = 12.5 G, modulation frequency = 100 kHz, microwave power = 0.5 mW, time constant = 0.3 s, receiver gain = 500, scan rate = 500 G/min.

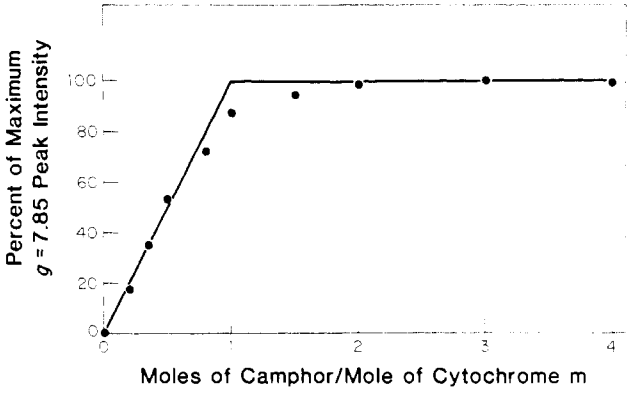

FIGURE 2: Titration of m° with D-camphor. 300 μM m° solutions were prepared with the camphor concentrations in the proportions shown in 100 mM K^+PO_4 buffer, pH 7.1. The signal intensity of each sample was measured at g = 7.85 under the instrumental parameters detailed in Figure 1.

in Figure 1. The origin of the spin mixture was sought by the titration of 300 μM, substrate-free m° with camphor while observing the increase in the high-spin signal intensity. The substrate binding constant is less than 5 μM (Gunsalus et al., 1973) so a sharp break in the titration curve is expected at the high cytochrome and substrate concentrations used in the experiment. Figure 2 shows that the signal maximizes when 1 ± 0.05 substrate molecule is added per heme. Thus, the spin distribution appears not to arise from a mixture of camphor binding and nonbinding species but rather from either a spin equilibrium or the presence of a species which can bind substrate but does not undergo a spin change.

Comparison of the low-spin regions of the m° and m^{os} EPR spectra in Figure 1 shows that the signal at g = 2.45 in m° appears as a shoulder on a larger signal at g = 2.42 in m^{os}. Also, a prominent signal at g = 1.97 appears in the m^{os} spectrum. The narrow line width of the g = 1.97 signal allows hyperfine splitting in ^{57}Fe-enriched cytochrome m^{os} to be easily observed (Figure 3), showing that the signal arises from iron. Figure 4 shows that the low-spin signals can be assigned to two different magnetic species on the basis of their temperature dependence. One species has the same g values as observed in m°, g = 2.45, 2.26, and 1.91.[2] The second magnetic species,

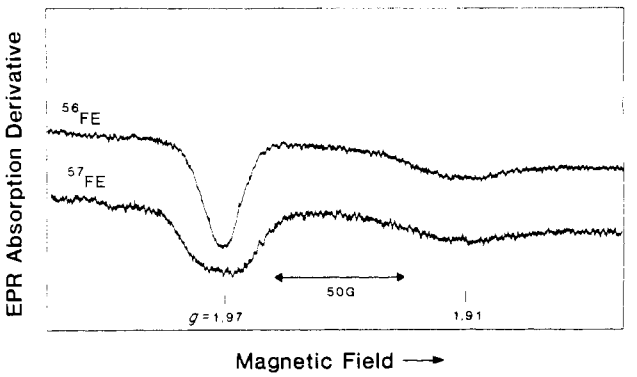

FIGURE 3: Hyperfine broadening of the ^{57}Fe-m^{os} g = 1.97 signal of 400 μM m^{os} in buffer plus 1.2 mM camphor. ^{57}Fe enrichment was ~88%. Measurement conditions were as in Figure 1 except the modulation amplitude was 8 G and the gain was 8 × 10^3.

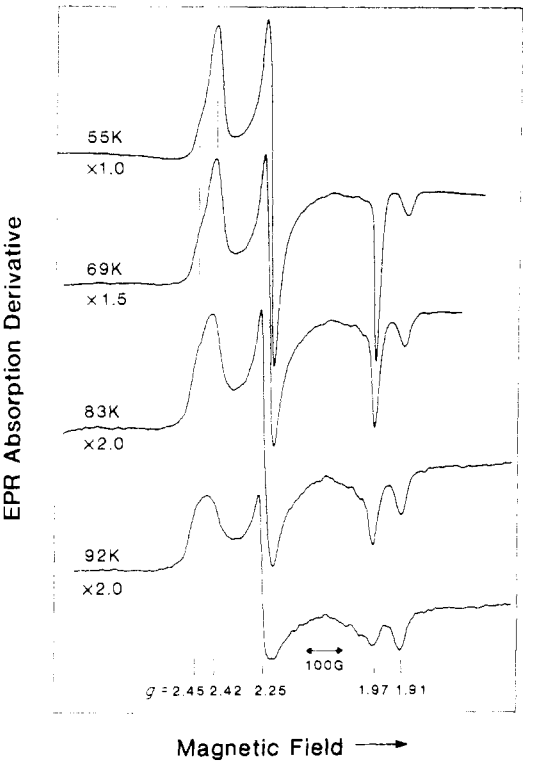

FIGURE 4: Temperature effects on m^{os} EPR spectra of 640 μM m^{os} plus 5 mM camphor measured in buffer at the temperature shown. Instrumental conditions were as follows: modulation frequency = 100 kHz, microwave power = 20 mW, time constant = 0.5 s, receiver gain for the 55 K spectrum = 4 × 10^3, gains for the other spectra are indicated on the figure.

g = 2.42, 2.24, and 1.97, is unique to the substrate-complexed enzyme.

Cytochrome m–Product Complex. The synthetically prepared 5-*exo*-hydroxycamphor complex with m° also has at least two EPR detectable low-spin species at 12 K. The majority species shown in Figure 5 is a new low-spin form at g = 2.48,

[2] Peterson (1971) showed that potassium or another small divalent cation is necessary for substrate binding. We can estimate from optical and EPR titrations that the K_D for K^+ is 14.5 mM. Consequently, in 50 mM K^+PO_4 buffer, pH 7.1 (70 mM K^+), a small fraction of the cytochrome may not have camphor bound and thus may give the native low-spin EPR spectrum. The intensity of the g = 1.91 signal is decreased 10% in 500 mM K^+PO_4 at pH 7.1. High buffer concentrations were not used to avoid changes in protein characteristics observed in other studies.

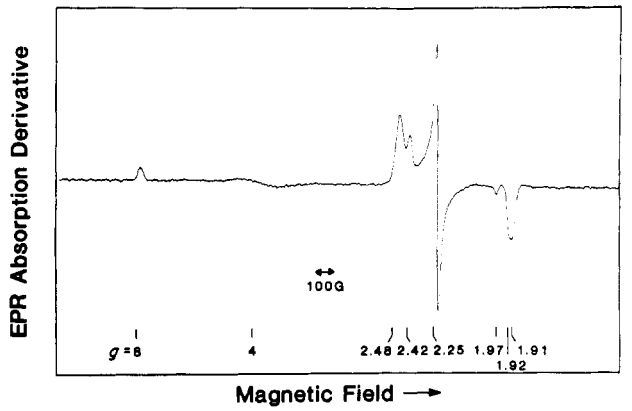

FIGURE 5: EPR spectra of m°-product complex. 640 μM m° in solution with 2 mM 5-*exo*-hydroxycamphor in buffer. Instrumental parameters were as described in Figure 1. Gain = 2.5 × 10^3.

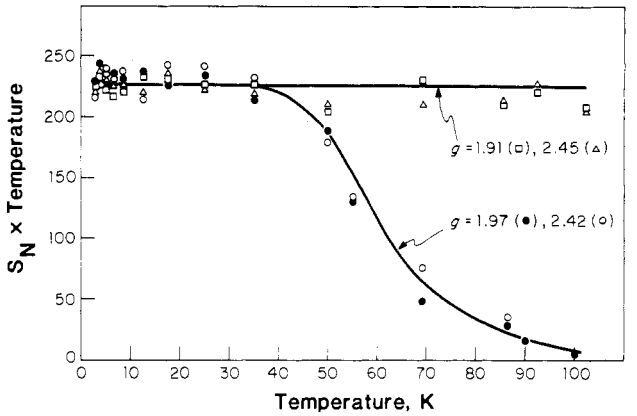

FIGURE 6: Temperature dependence of the low-spin m^{os} signals. Spectra such as those shown in Figure 4 were measured at the temperatures shown. The signals at g = 2.45 and 2.42 were deconvoluted by the procedures described under Materials and Methods. The intensities of the signals were measured and normalized to a power of 0.2 μW and the following gain values: g = 2.45, gain = 5.7 × 10^5; g = 2.42, gain = 5.7 × 10^4; g = 1.97, gain = 4 × 10^4; g = 1.91, gain = 4 × 10^5. Nonsaturating power levels were chosen at each temperature. Other measurement parameters were as given in Figure 4.

2.24, and 1.90. There is also a minority species with g values at 2.42, 2.24, and 1.91. The small amount of g = 1.97 and high-spin species also formed may be due to low levels of camphor in the 5-*exo*-hydroxycamphor preparation although none is detected by gas chromatography or biological assay. In contrast to the camphor complex, the two new low-spin forms in this complex show a very similar temperature dependence between 2.9 and 100 K. The g = 1.97 species formed in the product complex disappears at high temperature as observed in the substrate complex.

Temperature Dependence of the m^{os} Magnetic Species. The normalized amplitudes of high- and low-field signals from m^{os} are shown in Figure 6. At temperatures below 50 K all of the signals show the expected Curie law temperature dependence. The signals at g = 1.91 and 2.45 continue to show this dependence beyond 100 K. The signals at g = 1.97 and 2.42, however, lose amplitude above 50 K more rapidly than expected from a Curie law dependence. This loss in signal amplitude is accompanied by a slight broadening of the g = 1.97 signal and a curvature in the base line on either side of the signal. These effects could arise from many causes including power saturation, fast relaxation, heterogeneity of the iron ligation sphere which gives rise to the g = 1.97 species, or exchange of the g = 1.97 species with a high-spin or EPR

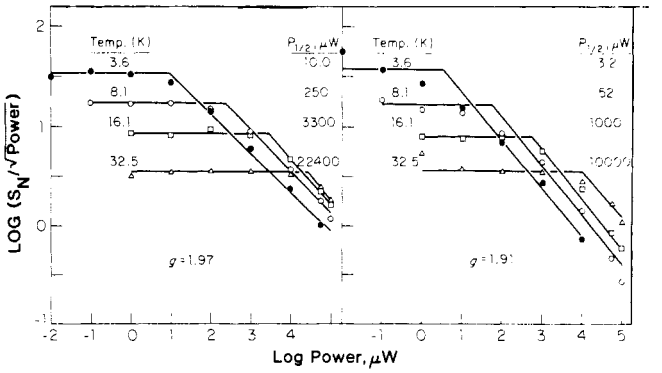

FIGURE 7: Saturation of low-spin m^{os} signals. The signal intensity of the g = 1.97 (left) and g = 1.91 (right) bands of 640 μM m^{os} was measured as a function of microwave power normalized to a gain of 1000 (g = 1.97) or 10000 (g = 1.91). The half-saturation power ($P_{1/2}$) is given by the sharp break in the curves. Other measurement parameters are given in Figure 4.

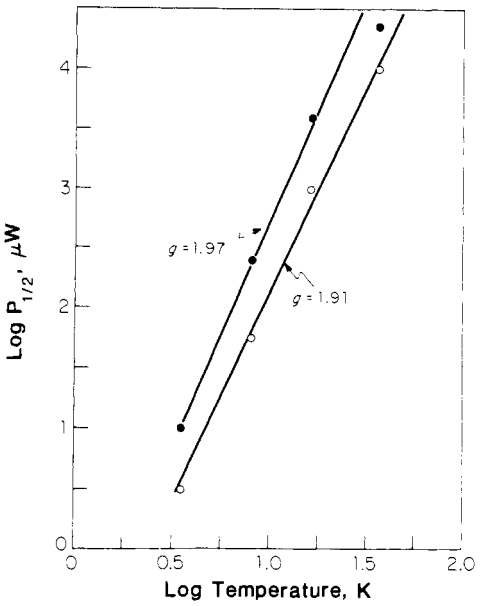

FIGURE 8: Temperature dependence of half-saturation power. Log half-saturation powers from Figure 7 are plotted vs. log temperature for the m^{os} low-spin bands shown.

silent species. The Curie law dependence of the g = 1.91 species through the temperature range in which the g = 1.97 signal amplitude is lost shows that it is not in equilibrium with the g = 1.97 species at these temperatures. The saturation behavior of the g = 1.97 species is compared in Figure 7, and the log of the half-saturation values is plotted as a function of log temperature in Figure 8. The slope of this plot is indicative of the relaxation mechanism (Beinert & Orme-Johnson, 1967). Clearly, the two low-spin species have similar power saturation curves, suggesting that they relax via the same mechanism and with slightly different relaxation times. The power saturation curves for m° were measured and found to be identical with those of the g = 1.91 species. Herrick & Stapleton (1976) have studied the relaxation of m° by the technique of pulse saturation recovery. They found that the species has an unusually long spin–lattice relaxation time and relaxes via a Raman process with T^7 rather than T^9 dependence at low temperature. Figure 8 shows that saturation occurs at higher power levels in the g = 1.97 species than in the g = 1.91 species. Since the g = 1.91 species has a broader line width than the g = 1.97 species, it appears that the line width is determined by microheterogeneity and not the re-

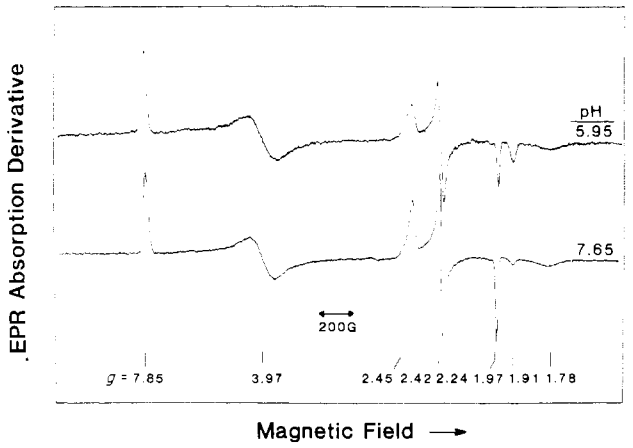

FIGURE 9: pH effects on the distribution of m^{os} species. 640 μM m^{os} was dialyzed vs. K^+PO_4 buffer of ionic strength equal to 50 mM buffer at 25 °C at pH 7.1 plus 1.2 mM camphor at the pH values shown. The same effect is seen in buffers with an ionic strength equivalent to 300 mM K^+PO_4 buffer at pH 7.1 plus camphor. Measurement conditions are described in Figure 1 with gain = 2×10^3.

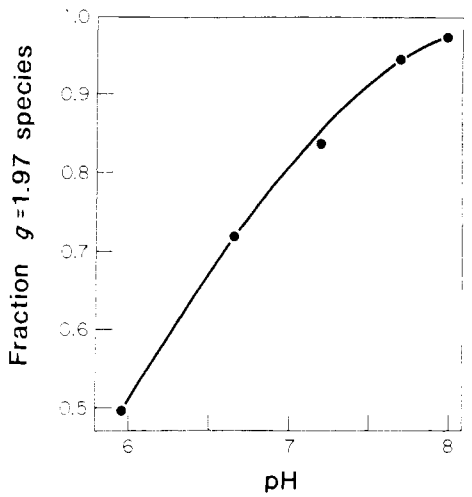

FIGURE 10: pH dependence of the $g = 1.97$ species. The fraction of low-spin m^{os} present as the $g = 1.97$ species is estimated from the signal intensity at $g = 1.97$ as a function of pH. The solid line is the 50–95% portion of a theoretical titration curve for a single proton dissociation from a group with a $pK_a = 6.2$. The inverse of this curve can be used to fit the data taken at $g = 1.91$ for the $g = 1.91$ species.

laxation time. It seems unlikely, then, that the $g = 1.97$ species is decreasing in amplitude due to relaxation or saturation under the conditions of the measurements.

pH-Induced m^{os} Species Equilibrium. Although interconversion of the $g = 1.97$ and 1.91 species does not occur on variation of temperature, it can be effected by varying the pH in solution prior to freezing. EPR spectra of m^{os} frozen at pH 5.95 and 7.65 are shown in Figure 9. There is a shift from the $g = 1.91$ species to the $g = 1.97$ species with increasing pH clearly reflected in both the low- and high-field signals while the high-spin signal intensity does not change appreciably. The fractional change in the $g = 1.97$ signal intensity is plotted as a function of pH in Figure 10 and appears to follow a typical ionization curve for a single ionizing group. The variation of pH in buffer systems with temperature is well-known. The pH of phosphate buffer increases ~0.2 pH unit between room temperature and 0 °C (Douzou, 1975). Variation of pH in the frozen state is less well understood (Williams-Smith et al., 1977), and no attempt was made to correct for these changes. The apparent pK_a for the process

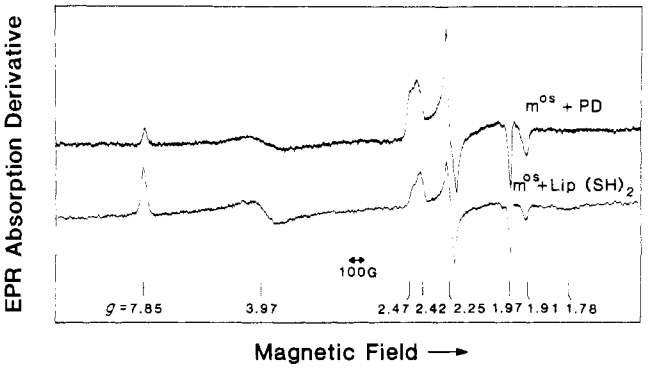

FIGURE 11: Effector complexes of m^{os}. 80 μM PD (top) or 6 mM $Lip(SH)_2$ (bottom) were complexed with 80 μM m^{os} in 50 mM K^+PO_4 buffer, pH 7.1, plus 1.2 mM camphor. $T = 12$ K. Measurement conditions were as in Figure 1; gain = 1×10^4.

depicted in Figure 10 based on the pH values at freezing is 6.2. The constant signal intensity at $g = 7.85$ throughout the titration suggests that the effect does not result from changes in the substrate or potassium ion binding constant. The interconversion of the low-spin magnetic species suggests that they do not arise from two different classes of cytochrome m.

EPR Detectable m^{os}–Effector Molecule Complexes. The complex between effector molecules and m^{os} causes conversion of either the high-spin or the $g = 1.97$ species to another new magnetic low-spin species, $g = 2.47$, 2.26, and 1.91. Although the exact nature of the change depends upon the effector, it differs from small molecule iron ligand binding in that the low-spin species formed always has the same g values and there is no effect in the absence of camphor. The high-spin and low-spin conversions of cytochrome m–effector complexes are illustrated in Figure 11. PD causes a shift away from high spin, giving rise to a pronounced low-field shoulder on the $g = 2.42$ signal and an increase in the $g = 1.91$ signal, but the $g = 1.97$ signal suffers only a slight decrease in intensity. Dihydrolipoic acid [$Lip(SH)_2$], a small molecule effector (Lipscomb et al., 1976), causes very little change in the high-spin spectrum but promotes a decrease in intensity of the $g = 1.97$ signal with the advent of a shoulder at $g = 2.47$.

Treatment of PD with carboxypeptidase A produces a homogeneous species missing the glutamine and tryptophan residues from the carboxy terminal (des-Trp-Gln-PD) (Sligar et al., 1974). The modified PD forms a much weaker complex with m^{os} but still serves as an effector protein for mixed-function oxidation. Saturating des-Trp-Gln-PD causes ~15% less conversion of the high-spin form than PD but is otherwise similar in effect. Aporubredoxin causes an effect similar to that of $Lip(SH)_2$.

The affinity of m^{os} for an effector molecule can be estimated by the change in signal amplitude at $g = 7.85$ or 1.97 as a function of effector concentration. Equal concentrations of PD and m^{os} cause the maximum change in the $g = 7.85$ signal even at 80 μM, the lowest protein concentration measured. Thus, the K_D for the PD–m^{os} interaction must be less than 10 μM, in agreement with the $K_D = 3$ μM measured from fluorescence quenching (Sligar et al., 1974) of fluorescein-labeled cytochrome m. The concentration at which PD reaches one-half of its maximum effectiveness in promoting hydroxylated product formation has been termed $K_{1/2}$ and equals 0.16 μM. The $K_{1/2}$ is a complex parameter composed of a combination of rate and binding constants previously defined (Lipscomb et al., 1976). Values of K_D and $K_{1/2}$ for the effector molecules are compared in Table I. It is apparent that changes in the binding constant are reflected in the concen-

Table I: Relationship of Effector Binding and Activity

effector	K_D, EPR	K_D	$K_{1/2}$[a]
PD	<10 μM	3 μM[b]	0.16 μM
des-Trp-Gln-PD	100 μM	150 μM[b]	10 μM
apo-Rd	40 μM		4.6 μM
Lip(SH)$_2$	3 mM	1.4 mM[c]	0.5 mM

[a] Concentration required to obtain half the maximum yield of 5-*exo*-hydroxycamphor when combined with 30 μM $m_{O_2}^{rs}$ at 20 °C (see text). [b] From fluorescence quenching. See Sligar et al. (1974). [c] From kinetic analysis. See Lipscomb et al. (1976).

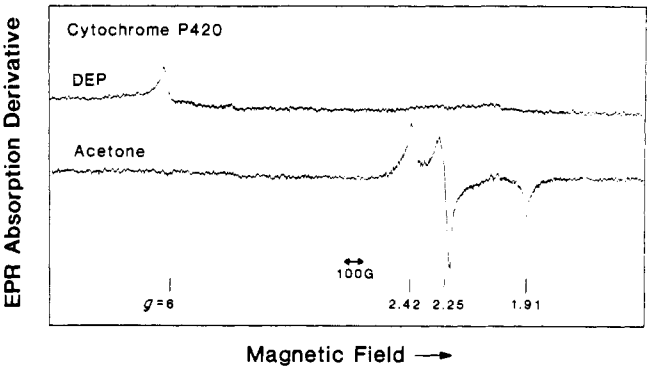

FIGURE 12: Cytochrome P-420 high- and low-spin forms. (Top) Approximately 400 μM m° after modification with DEP and concentration. Camphor causes no shift in this spectrum. (Bottom) 400 μM m^{os} incubated for 1 h with 20% acetone at 25 °C in 50 mM K^+PO_4, pH 7.1, plus 600 μM camphor. Measurement conditions were as described in Figure 1 with microwave power = 0.25 mW and gain = 2×10^3.

tration required for efficient hydroxylation.

Cytochrome m–Ligand Complexes. The observed *g* values for the EPR spectra of some m°–small molecule ligand complexes, as well as the Soret absorption maxima and K_D values, are compared in Table II. All molecules tested produced low-spin spectra with *g* values typical of thiolate heme ligation. Molecules such as propanethiol and cyanide produce more than one low-spin magnetic species. This may reflect different binding geometries or binding of both protonated and unprotonated species.

The phenylimidazole series shows marked spectral differences which are probably due to a combination of steric factors and changes in electron density on the binding nitrogen. Several ligands are capable of causing the optical red shift in the ferrous Soret band to the 450-nm region typical of the carbon monoxide complex.

In general, based on the nephelauxetic series determined by Peisach et al. (1973), the low-field *g* values of the ligand complexes of m° listed in Table II are in the predicted order. Cyanide, for instance, has the smallest charge contribution and the largest low-field *g* values of the ligands measured. The binding constants measured by optical titration are, however, in nearly the reverse order expected from the analogous constants for heme proteins such as hemoglobin (Hill et al., 1970; Antonini & Brunori, 1971). Effects of this type have been attributed to the probable presence of a negatively charged amino acid residue in the heme pocket which would decrease the affinity of halides, cyanide, and azide (Hill et al., 1970; Smith & Williams, 1970). Lipscomb et al. (1978) reported the presence of at least three cysteinyl groups which are partially protected by substrate from alkylation in the m^{os} complex. The titration of one of these by NEM is pH independent, suggesting that it is maintained in the ionized form by a nearby base. Such a group might be responsible for the decreased affinity of charged ligands. The affinity of pyridine, neutral imidazole, and other uncharged ligands should be unaffected, however, suggesting that the steric factors and charge contribution of the fixed ligand may also be the determining factors in complex formation.

EPR Detectable Species of Cytochrome P-420. Cytochrome P-420 can be generated in a variety of ways, including treatment with detergents, acetone, and sulfhydryl reagents (Yu & Gunsalus, 1974; Lipscomb et al., 1978). The optical and EPR characteristics of some forms of P-420 were determined and are summarized in Table III. Clearly, these forms have some fundamental differences. For instance, addition of 25% v/v acetone to m^{os} for 1 h produces a low-spin P-420 with *g* values typical of a thiol-liganded heme (Figure 12). On the other hand, sulfhydryl reagents such as NEM, which presumably modify all cysteine residues, generate P-420 with an identical reduced CO spectrum. P-420 generated by treatment of m^{os} with either acetone or NEM has an optical Soret maxima near 417 nm, similar to that of m° and other low-spin *b*-type heme proteins. At low temperature, however, EPR spectra show that the acetone-generated form is low spin while the NEM-treated material is high spin, suggesting that it undergoes a spin transition. The P-420 which forms when m° or m^{os} is reacted with DEP has not been previously reported and differs from both types of P-420 discussed above in that the optical Soret maximum is near 380 nm, suggesting that it is high spin. The EPR spectrum shows that the species is high spin at low temperature. The nearly axial symmetry indicated by the *g* value at 6 (Figure 12) is quite different from that observed in high-spin m^{os}. The DEP reaction was studied in more detail to determine whether amino acids in the active site were being directly affected and whether there is a direct correlation between modification and P-420 generation. The

Table II: Properties of Cytochrome m–Ligand Complexes

ligand	g_1[a]	g_2[a]	g_3[a]	λ_{max}^{ox} (nm)[b]	λ_{max}^{rd} (nm)[b]	K_D^{ox} (mM)[c]
azide	2.6/2.52	2.23	1.82/1.87	418	410	100
cyanide	2.60	2.28	1.82	439	430	4
imidazole	2.56	2.27	1.87	418	410	0.4
1-phenylimidazole	2.47	2.26	1.90	423	444	0.0001
2-phenylimidazole	2.41	2.25	1.91	417	414	0.007
4-phenylimidazole	2.5/2.45	2.25	1.89	423	417	0.04
dimethyl sulfide	2.47	2.26	1.89	424	446	8.5
thioxane	2.43	2.26	1.92	418	445	0.8
propanethiol	2.41/2.34	2.24	1.92/1.94	418	412	3.4
pyridine	2.48	2.26	1.88	421	444	0.02

[a] Measured at 79 K. [b] Conditions: 50 mM K^+PO_4, pH 7.1, 20 °C, reduction by sodium dithionite. [c] Determined by optical titration at 7.1, 20 °C.

Table III: Optical and Magnetic Characteristics of Cytochrome P-420

causative reagent[a]	soret max (nm)[b]	g values[c]
acetone	417	2.46, 2.25, 1.91
NEM	419	6, (6), (2)
DEP	380	6, (6), (2)

[a] See text for conditions. [b] Measured at 25 °C in buffer. [c] Measured at 10 K under instrumental conditions stated in Figure 1. Parentheses indicate probable values.

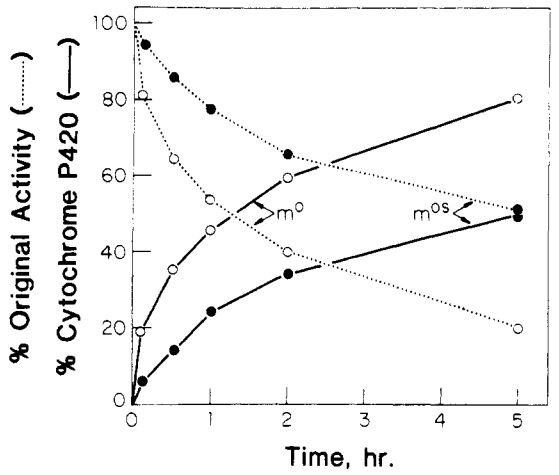

FIGURE 13: Time dependence of DEP–P-420 formation. 14.5 μM m° with (●) or without (○) 250 μM camphor was incubated with 1.0 mM DEP equilibrated at 20 °C. Activity and P-420 formation were estimated as described under Materials and Methods.

formation of ethoxyformylated cytochrome m by the procedure described under Materials and Methods results in the concurrent shift of the ferric Soret absorption band, the shift of the ferrous–CO spectrum to 420 nm, and the loss of catalytic activity (Figure 13). Both NADH oxidation and hydroxylation of camphor are completely inhibited. Camphor has a protective effect as seen in Figure 13, suggesting that the titratable group is near the camphor binding site in the active center. DEP also reacts readily with NEM-generated P-420 to yield a form with the same optical and EPR spectra as P-420 generated by DEP alone. Conversely, the same number of sulfhydryl groups react with NEM in DEP-generated P-420 as in m°. These results suggest that an amino acid other than cysteine is modified.

Discussion

The EPR detectable species of cytochrome m and their exchange are summarized in Figure 14. Under some conditions all of these magnetic species exist simultaneously, testifying to the delicately balanced nature of the heme axial ligand structure. The fraction of any one species is dependent on experimental conditions, strongly implying that the species do not represent cytochrome m molecules which differ in their fundamental protein composition. In the presence of substrate, three species are found in either temperature- or pH-controlled equilibria. Under the model for the ligand structure of cytochrome P-450 cited earlier, the species are proposed to reflect changes in the position, electronic configuration, or identity of the variable heme ligand.

In contrast, Ebel et al. (1977) have described buffer-dependent line shape changes in m°. These changes are probably due to minor alterations in conformation or active-site polarity and do not represent major changes in the ligand structure.

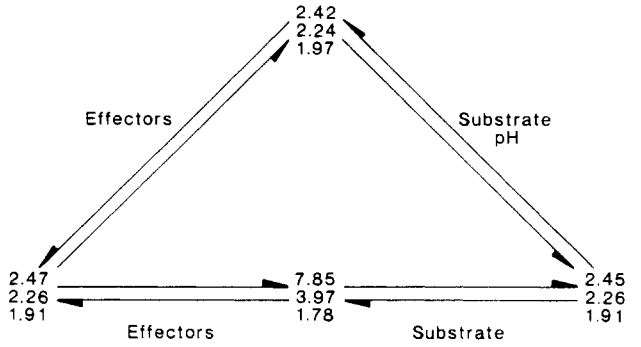

FIGURE 14: Interconversion of EPR detectable states of cytochrome m.

Table IV: EPR g Values for $g = 1.97$ Species Model Complexes

modeled protein ligand	model complex[a]	model ligand[a]	g values	ref
Cys	Fe(PPIX)(*n*-BuS⁻)	*n*-BuSH	2.32, 2.23, 1.96	*b*
Cys	Fe(TPP)(PhS⁻)	PhSH	2.40, 2.25, 1.97	*c*
Lys	Fe(TPP)(PhS⁻)	$MeNH_2$	2.38, 2.22, 1.96	*c*
Asn, Gln	Fe(PPIXDME)(CH_2PhS^-)	DMF	2.36, 2.24, 1.95	*d*
camphor	Fe(TPP)(PhS⁻)	camphor	2.37, 2.27, 1.94	*c*
?	$g = 1.97$ species	?	2.42, 2.24, 1.97	*e*

[a] Abbreviations: PPIX, protoporphyrin IX; TPP, tetraphenylporphyrin; PPIXDME, protoporphyrin IX dimethyl ester; *n*-BuSH, *n*-butylthiol; PhSH, phenylthiol; $MeNH_2$, methylamine; DMF, dimethylformamide. [b] Ullrich et al., 1977. [c] Collman et al., 1975. [d] Tang et al., 1976. [e] This work.

The prominent signal at $g = 1.97$ in the EPR spectrum of m^{os} has never been assigned, although it was observed in the first low-temperature spectra of the protein (Tsai et al., 1970). At that time it was posulated to be an impurity or an excited state. Both of these suggestions are unlikely since the signal is specifically elicited by camphor, occurs in the same proportion in crystalline preparations of cytochrome m, and continues to increase in signal intensity with T^{-1} to the lowest temperature measured (2.5 K). In this paper, we have used the unusual temperature dependence of the signal above 50 K to associate it with strong signals of $g = 2.42$ and 2.24 which together represent the majority low-spin magnetic species at low temperature. This association has not previously been made because traditional crystal field theory calculations (Griffith, 1957; Blumberg & Peisach, 1971; Bohan, 1977) show that the g value set 2.42, 2.24, and 1.97 is not allowed without a large excursion from the parameter range encountered in virtually all other heme complexes. Other magnetic species observed in EPR spectra of cytochrome m complexes fit well within the known parameter set. The data set can be fit by assuming an improbably large orbital reduction factor or by dropping the requirement that the wave functions are normalized. These assumptions may be reasonable if the excited states of the $g = 1.97$ species are much closer in energy to the ground state than usually encountered in low-spin heme iron. This sort of approach was used by Sharrock (1978) to obtain a good simulation of the spectrum. The occurrence of the $g = 1.97$ species spectrum suggests that the current formulation of the crystal field theory as applied to heme centers is not valid in all cases. Appropriate corrections such as those discussed above are being investigated.

Despite the difficulties encountered in determining a parameter set to allow theoretical interpretation of the $g = 1.97$ species, several model complexes have been synthesized which closely approximate the observed g values. Some of these are listed in Table IV. One thiolate ligand is required to obtain even an approximate fit. EPR spectra of model complexes with imidazole nitrogen, thioether, or phenolic oxygen ligands in addition to the thiolate do not resemble the $g = 1.97$ species (Tang et al., 1976). We cannot eliminate any of the ligands represented by the complexes in Table IV. Camphor has not been postulated as a possible iron ligand in the past because the carbonyl function would be expected to form a rather weak bond. Nevertheless, the neutral oxygens of camphor and DMF do bind in model complexes and give g values approaching those of the $g = 1.97$ species. Since this species is observed only when camphor is bound, substrate must be considered as a possible ligand. The camphor binding site in cytochrome m is thought to be near the heme in the active-site pocket because it is competitively displaced by phenylimidazoles and other known iron ligands. Recent ENDOR results (LoBrutto et al., 1980) favor pentacoordinate iron in the high-spin m^{os} fraction. Thus, the two new magnetic species resulting from substrate binding may represent the direct iron–camphor ligation ($g = 1.97$ species) and a distal camphor form (high spin) in which camphor blocks access to the iron by other potential ligands but remains, itself, unliganded.

Data are also available which suggest that the $g = 1.97$ species represents thiol–thiolate ligation of the heme iron. The model complex phenylthiolate–tetraphenylporphyrin–phenylthiol (Collman et al., 1975; Wickman et al., 1977) cited in Table IV has the g values which best fit the observed values for the $g = 1.97$ species. We have recently reported evidence which shows that free cysteine is available in the active site (Lipscomb et al., 1978). Titration of substrate-maskable thiol groups causes loss of the high-spin and $g = 1.97$ species in the m° EPR spectrum. Reduction of the cytochrome by putidaredoxin is inhibited and substrate is bound with lower affinity, but modified $m_{O_2}^{rs}$ remains catalytically competent. The data suggest that there are at least two and probably three cysteine residues near the heme on the substrate side. It could be postulated that in the substrate-complexed enzyme one of these sulfhydryl groups is brought into position to bind directly to the heme iron. As in the case of camphor ligation, the thiol bond would probably be rather weak and equilibration with a thiol-off (high-spin) form is reasonable.

No experiments have been reported which specifically implicate involvement of lysine, asparagine, or glutamine in the function or spectral properties of cytochrome m.

The axial ligands of the $g = 1.91$ species are not determined by the work reported here, although the g values strongly indicate one ligand to be thiolate. The species has the same g values and power saturation behavior as the low-spin species in m°, suggesting that they are the same or very similar. All techniques thus far applied show that m° has one thiolate ligand. The identity of the sixth ligand has been approached by several techniques. Chevion et al. (1977) have presented convincing evidence that the sixth ligand is imidazole based on the EPR-derived crystal field parameters of model complexes and various cytochrome P-450 enzymes. However, ^{1}H NMR relaxation results (Griffin & Peterson, 1975; Philson et al., 1979) argue against imidazole in favor of a ligand such as water. The data of Philson et al. (1979) limit the possible ligands to water or an amino acid with an exchangeable proton which has a pK_a above 8.4 and is located within 2.9 Å of the iron binding atom (Tyr, Ser, Thr, Asn, Gln, Lys, or Arg). The exchangeable histidine proton is ~4.8 Å from the iron in a heme complex. These results also argue against cysteine as a sixth ligand because the pK_a of the proton on sulfur when liganded to heme is thought to be lower than that of cysteine in solution, therefore dissociable at neutral pH or below. Also, we have shown that all the active-site cysteines can be titrated with only slight changes in the EPR and optical spectra of m° (Lipscomb et al., 1978). Model complexes synthesized with thiolate–imidazole ligation (Tang et al., 1976) exactly reproduce the g values observed in the $g = 1.91$ species. However, other complexes designed to model lysine, asparagine, and glutamine ligation give similar g values. The g values of model complexes for water, serine, and threonine ligation in thiolate–iron complexes have not been reported. The $g = 1.91$ species is converted to an axial high-spin magnetic species by DEP. Under the conditions used for the DEP reaction, histidine is usually the amino acid modified (Burstein et al., 1974). This, coupled with the observed pK_a value of 6.2 for the interconversion of $g = 1.91$ and 1.97 species, supports the postulate that histidine may be the variable ligand or is important for the configuration of the active center. DEP is sufficiently nonspecific, however, that the modified amino acid or acids must be directly identified before its mode of action can be evaluated.

Two general explanations for the distribution and interconversion of cytochrome m EPR detectable species are indicated by the data presented here. One possibility arises from the fact that models for ligands such as lysine, asparagine, and glutamine can be synthesized which resemble either the $g = 1.97$ and/or the $g = 1.91$ species depending upon the type of porphyrin and thiolate ligand chosen (Tang et al., 1976). Thus, it is possible that all the low-spin species have the same axial ligand but camphor binding causes large changes in the electronic environment of the porphyrin or the ligands. A second, and we feel more likely, possibility is that the protein can exist in two conformations. In solution one conformation has high-spin iron and a high affinity for camphor while the other configuration has low-spin iron and a lower (but not zero) affinity for camphor. The low-spin conformation with camphor bound is stable with either of two different axial heme ligands ($g = 1.97$ and 1.91 species) in a pH-dependent distribution. In a model of this sort, the high-/low-spin equilibrium would not reflect the pH-dependent distribution of low-spin forms if the magnitude of the free energy for ligand exchange is small compared with the $-\Delta G$ for conversion from one conformation to the other. The high-spin fraction is, in fact, observed to be pH independent. Effector molecules might change the EPR spectra of either the high-spin or the low-spin form depending upon which effector–m^{os} complex is more stable. Effectors are observed to preferentially shift either the high-spin or $g = 1.97$ species. Extensive thermodynamic and kinetic evidence for interconvertible high-/low-spin forms of cytochrome m in solution has been reported by Sligar (1976), but the methods used in the study could not have resolved different low-spin forms. The biological advantage appears to be that the high-spin form has a high redox potential. Thus, reducing equivalents are directed toward the form which most strongly binds substrate. In the absence of camphor, the low-spin form similar to the $g = 1.91$ species is more stable. The camphor binding energy apparently shifts the equilibrium toward the high-spin form and perhaps toward the $g = 1.97$ species in solution.

The central role of camphor in the regulation of the cytochrome m system is quite clear. The $g = 1.97$ and high-spin species are seen only in the presence of camphor. The mod-

ulation of the heme environment by complexation of effector molecules appears to be mediated by camphor. There is no change in the EPR spectrum caused by effectors when the product is substituted for camphor. Thus, it seems likely that the effector molecules do not themselves interfere with the heme ligation and their proximity to the heme when complexed to cytochrome m cannot be directly ascertained from these studies. The correlation between effector binding affinity and catalytic potency strongly supports the model based on kinetic data (Lipscomb et al., 1976) which proposes that an active complex between an effector and cytochrome m is critical to mixed-function oxidation.

The data presented in Figures 4 and 6 show that the g = 1.97 species loses signal intensity with increasing temperature much faster than would be expected for a species which is neither saturating nor has an unusually fast relaxation rate. The g = 1.97 species could be exchanging with the high-spin form which is difficult to quantitate above 13 K because population of low-lying Kramer levels and rapid relaxation cause loss of signal intensity and broadening. Earlier Mössbauer results (Sharrock et al., 1973) suggest that this is not the case. The high-spin fraction changes from 55% at 4.2 K to 70% at 200 K while the g = 1.97 species disappears almost entirely over this temperature range. It is possible that the g = 1.97 species has a low-lying excited state which, due to relaxation or other constraints, is EPR silent in the temperature range in which it is populated. The depopulation of the ground state depends upon the energy separating the two states as well as entropy contributions from multiplicity changes and other modified degrees of freedom within the molecule (Iizuka & Kotani, 1969). This does not readily allow prediction of the energy splitting between the ground state and a proposed excited state. However, the lack of any conversion below 50 K followed by a rather sharp and total conversion above 50 K does not fit well with this model. The most likely explanation is the introduction of small molecular motions above 50 K which are sufficient to make g = 1.97 species heterogeneous. This motion may be a precursor to the low- to high-spin transition which has been shown to occur between 200 K and room temperature (Sligar, 1976).

The nature of cytochrome P-420 has received a great deal of speculation (Jefcoate & Gaylor, 1969; Peisach et al., 1973; Chevion et al., 1977). The term clearly encompasses a wide variety of species with quite fundamental differences unified by the absorption wavelength of the ferrous–CO complex. The three forms of P-420 described in this paper are probably the result of three independent denaturation processes: titration of the fixed axial thiol group by sulfhydryl reagents, a protein conformational change caused by acetone which leaves the characteristic EPR spectrum and thiol ligand intact, and titration of a group or groups other than thiol by DEP. These results as well as the EPR spectra of undenatured cytochrome m reported here emphasize the fact that there are many elements which contribute to an active P-450 reaction center. These elements must be considered when proposing mechanisms for reactions within the active site and when designing synthetic model complexes.

Acknowledgments

The author thanks Dr. I. C. Gunsalus for supplying materials and proteins as well as advice and encouragement during this investigation. Many useful discussions were held with Drs. E. Munck and P. Debrunner. Some of the initial studies reported here were suggested by Dr. W. H. Orme-Johnson. Expert technical assistance was provided by M. J. Namtvedt and R. Salo.

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Binding of Iron from Nitrilotriacetate Analogues by Human Transferrin†

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ABSTRACT: At neutral pH, human transferrin firmly binds iron at two specific iron-binding sites which lose affinity for metal under moderately acidic or alkaline conditions. Ferric nitrilotriacetate (FeNTA), at neutral pH, will efficiently provide iron to transferrin but in a sequential, rather than random, fashion. It initially directs the metal to the transferrin iron-binding site located closest to the protein's C terminus, while other commonly employed iron reagents, for reasons which are not yet clear, provide more iron to the alternate site. We studied the transferrin iron-donating characteristics of two analogues of FeNTA, where one and two of the chelator's acetic acid ligand groups were hydroxyethyl ligands. Although there is little difference in the iron-binding strengths of these chelators, there were marked differences in the rate of iron exchange to transferrin as well as differences in apportionment of metal between sites. Apportionment was studied by measuring the acidic dissociation (pH 5.8) of fractionally ^{59}Fe-labeled diferric transferrin prepared by sequential binding of chelated ^{59}Fe and ^{56}Fe isotopes and by 6 M urea–polyacrylamide gel electrophoresis of partially iron-saturated transferrin solutions that were prepared by using these chelates. Replacement of one NTA ligand had a small effect upon site allocation of iron but enhanced the exchange rate. Substitution of two alcoholic ligands resulted in an iron chelate which provided iron nearly randomly to both sites, but it was sluggishly reactive. Between pH 9 and 10, iron dissociates from transferrin but, in contrast to behavior under acidic conditions where the site closer to the N terminus loses iron affinity at a more neutral pH than the C-terminal site, iron was randomly released from both sites.

Ferric nitrilotriacetate (FeNTA) will furnish its iron completely and efficiently to transferrin's specific iron-binding sites. The chelate does not hydrolyze or form a ternary complex with the protein when bicarbonate is present and circumvents problems encountered when iron salts are used to adjust iron saturation levels or provide a radioiron label to transferrin (Bates & Schlabach, 1973; Workman et al., 1975).

As a reagent, however, FeNTA is not entirely faultless. When less than saturating quantities of FeNTA are added to human apotransferrin, the iron is not equally apportioned to each site but instead is predominately bound to one transferrin iron-binding site (Zapolski & Princiotto, 1977a; Harris, 1977a; Aisen et al., 1978; Van Eijk et al., 1978). The site is located closest to the protein's C terminus (Evans & Williams, 1978) and, among several iron compounds that have been studied in this respect, transferrin binding of FeNTA iron was atypical; all the other compounds provided more iron to the site closest to the N terminus.

The present study was undertaken to determine what effect a slight modification of NTA's iron ligand structure would have upon the characteristics of iron donation to transferrin. Two readily available NTA homologues which form stable and well characterized ferric chelates are Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] and *N*-(2-hydroxyethyl)iminodiacetic acid (Toren & Koltoff, 1955; Anderegg & Schwarzenbach, 1955; Chaberek & Martell, 1959). We compared the iron-donating characteristics of these chelates to the well-known iron reagents FeNTA and iron citrate. Transferrin loses affinity for iron at high pH as well as low pH (Laurell, 1952). We studied transferrin dissociation with respect to the behavior

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0006-2960/80/0419-3599$01.00/0