Studies of the Oxygen Binding Site of Cytochrome P-450

NITRIC OXIDE AS A SPIN-LABEL PROBE*

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Pseudomonas putida and rat liver microsomal cytochromes P-450 form both ferric- and ferrous-NO complexes. The ferric-NO complexes are stable and do not possess ESR spectra in the temperature range 5-300 K which suggests that the unpaired electron of the bound NO is spin-paired with the single unpaired d electron of the heme iron. The ferrous-NO complex of the bacterial cytochrome is quite stable; however, a portion of the comparable complex of the microsomal enzyme rapidly denatures to a five-coordinate P-420-NO species. The percentage of the ferrous-NO complex of the microsomal enzyme which becomes five-coordinate appears to be dependent upon the amount of high spin cytochrome present in the original ferric enzyme since it is less in the presence of exogenous Type I substrates. The addition of the substrate, d-camphor, to the bacterial cytochrome P-450 does not affect the optical absorbance or electron spin resonance (ESR) spectra of either the ferric- or ferrous-NO complexes. The optical absorbance difference spectrum of the ferric-NO versus ferric liver microsomal cytochrome P-450 is dependent upon the percentage of the enzyme in the high (substrate-bound) versus low spin (substrate-free) state. The Soret absorbance band maxima of the ferric- and ferrous-NO complexes of these cytochromes P-450 are shifted to longer wavelengths than those of the corresponding complexes of horseradish peroxidase. The ESR spectra of the ferrous-NO complexes of cytochromes P-450 reveal rhombic symmetry with a triplet hyperfine interaction in the g2 signal. Unlike horseradish peroxidase, there is no nine-line superhyperfine structure for the g2 signal. These data have been interpreted as follows: 1) the unique structure of the active site of cytochrome P-450 which accounts for the long wavelength Soret absorbance band maximum of the ferrous-CO complex is presumably also present in both the ferric- and ferrous-NO complexes; 2) the NO complexes of P-420 prepared from these cytochromes P-450 are identical by ESR spectroscopy, as well as being similar to the corresponding complex of denatured hemoglobin in which the bond between the heme iron and the axial ligand bound trans to NO is either severely distorted or completely broken; 3) the absence of superhyperfine splitting in the g2 ESR signal of the ferrous-NO complex of cytochrome P-450 can be considered consistent with the proposal of a mercaptide sulfur at the axial ligand bound trans to the nitric oxide (dioxygen) binding site or with a bonding scheme in which there exists a strong interaction between the heme iron and NO and weaker bonding between the trans axial ligand and the heme iron. Given the latter possibility, nitrogen (histidine ?) cannot be excluded as the trans axial ligand.

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Nitric oxide has been used as both an electronic and a paramagnetic probe of the chemical and physical properties of a variety of hemoproteins. Hemoglobin (Hb) and myoglobin (Mb) do not form stable ferric-NO complexes (1, 2). The ferric-NO complexes of horseradish peroxidase and cytochrome c peroxidase, on the other hand, are stable spin-paired diamagnetic complexes (3). Since NO contains one unpaired electron it is a unique spin-label ligand and the ferrous-NO complex of a number of hemoproteins has been used as a model of diamagnetic dioxygen and carbon monoxide complexes. ESR spectral studies have indicated that the paramagnetic HbNO species has rhombic symmetry with the unpaired electron at least partially transferred to the heme iron and the nitrogen of the histidine which is the trans axial ligand (1, 2, 4). The ESR spectrum of HbNO is very sensitive to the conformational state of the complex (1, 2, 5-10). Extensive studies with horseradish peroxidase and cytochrome c peroxidase have revealed that their ferrous-NO complexes have an

1 The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; α, β, γ, isolated subunits of human adult and fetal hemoglobins; SDS, sodium dodecyl sulfate; Fe(III)(NO)(1-MeIm), nitrosyl α, β, γ, δ-tetraphenylporphinato(1-methylimidazole)iron(II); Fe(III)(NO), nitrosyl α, β, γ, δ-tetraphenylporphinato iron(II).
cytochrome P-450 nitric oxide complexes

even more dramatically distorted field than the comparable complexes of Hb and Mb. The $g_z$ ESR signal of the ferrous-NO complexes of horseradish peroxidase and cytochrome c peroxidase has readily observable superhyperfine splitting, which has been used as evidence for electron-spin, nuclear-spin interaction of the unpaired electron of the heme-bound NO with the nuclear spin of the trans axial ligand which is presumed to be a nitrogen atom (1-4). The ferrous-NO complexes of catalase and lactoperoxidase lack this superhyperfine splitting in the $g_z$ signal, a result which was interpreted to indicate either fast electron spin relaxation or the absence of a nitrogen at the proximal site (3). Recent studies with abnormal hemoglobins (11), subunits (12, 13) and $\alpha \beta$ hybrids of human Hb (12), and tryptophan dioxygenase (14) have revealed that the superhyperfine splitting of the $g_z$ ESR signal of ferrous-NO complexes is dependent on the conformation of the protein and thus the interaction of the axial ligands with the heme iron. The suggestion was made that alterations in the bonding of the NO and trans axial ligand to the heme iron will control whether or not superhyperfine splitting is observed in the $g_z$ signal (13).

In order to elucidate the mechanism of action of the hemeprotein monooxygenase cytochrome P-450, it is necessary to have a detailed description of its active site. Preliminary reports have indicated that nitric oxide combines with the soluble cytochrome P-450$_{cam}$ from Pseudomonas putida (15, 16) and the membrane-bound cytochrome P-450$_{cam}$ in liver microsomes (16) in both their ferric and ferrous oxidation states. In this study we have further characterized the chemical and physical properties of these NO complexes with respect to one another in the presence or absence of substrate and in the native versus denatured (P-420) states. Furthermore, the ferrous-NO complexes were utilized as electronic and paramagnetic probes of the dioxygen binding site, specifically as models of the diamagnetic carbon monoxide and dioxogen complexes which give rise to the red-shifted 450 nm and more typical 418 nm Soret absorbance band maxima, respectively.

Materials and Methods

Nitric oxide was purchased from the Linde Division, Union Carbide Corp. (14NO) and from Merck and Co. (14NO). Nitric oxide (14NO) was passed through NaOH pellets before use to remove any possible contamination by higher oxides of nitrogen. Horseradish peroxidase (type II) and metmyoglobin (whale skeletal muscle, type II) were purchased from Sigma Chemical Co. Sodium hexobritallate was obtained from Winthrop Laboratories as a gift, 5α-androstane-3,17-dione from Sigma Chemical Co., and d-camphor from Baker Chemical Co. All other reagents were of the highest quality commercially available.

Cytochrome P-450$_{cam}$ was purified in the camphor-bound form from P. putida (ATCC 17453) mutants d-camphor using a modification (17) of the original procedures (18, 19). Substrate (d-camphor) was removed by column chromatography using Sephadex G-10 as previously described (17). The purity of cytochrome P-450$_{cam}$ was evaluated using the ratio of absorbance at 392 nm to that at 280 nm. A ratio of >1.2 indicated that the protein was at least 80% pure (17, 18). The concentration of cytochrome P-450$_{cam}$ was determined from the optical absorbance spectrum of the ferric camphor-bound form ($e$ = 104 mm$^{-1}$ cm$^{-1}$ at 392 nm) or the ferrous-carbonyl form ($e$ = 120 mm$^{-1}$ cm$^{-1}$ at 446 nm) (17). Unless otherwise noted all optical absorbance and ESR spectra of the camphor-bound enzyme were recorded in 0.05 M potassium phosphate, pH 7.4, containing 0.1 M KC1 and 1 mM d-camphor (Buffer A) and those of the camphor-free enzyme in 0.05 M morpholinopropanesulfonic acid neutralized with Tris base to pH 7.0 (Buffer B).

Liver microsomes were prepared as previously described (20) from male albino rats (Sprague Dawley strain, Charles River Co., 290 to 350 g) that received a daily intraperitoneal injection of phenobarbital in saline (0.8% NaCl solution) 80 mg/kg for 4 days. The microsomal pellet was resuspended by homogenization in a small volume of 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA (Buffer C). Protein was determined by the biuret method according to the method of Gornall et al. (21). Cytochrome P-450$_{cam}$ content was determined from the ferric carbon monoxide minus ferrous optical absorbance difference spectrum (Buffer C), using $\Delta A$ = 91 mm$^{-1}$ cm$^{-1}$ for 450 nm minus 480 nm (22).

Cytochrome P-450$_{cam}$ was denatured to P-420 by adjusting the pH of a sample of the ferric camphor-bound enzyme (200 $\mu$M in Buffer A) to pH 11 with 5 M KOH (23). Liver microsomal P-420 (50 mg of proteins/ml in Buffer C) was prepared by the addition of sodium deoxycholate (0.5% final concentration) (24) or potassium thiocya-

Optical absorbance spectra were recorded at room temperature with either a Cary model 14R (Varian Instrument Co.) or an Aminco model DW-2 dual wavelength/split spectrophotometer (American Instrument Co.). Electron spin resonance spectra were recorded with a Varian model E-4 spectrometer equipped with a variable temperature attachment. Unless otherwise indicated all ESR spectra were recorded using the following instrumental parameters: temperature, -170°C; power, 10 milliwatts; modulation amplitude, 1.0 G. A PDP II minicomputer (Digital Equipment Corp.) was utilized to collect and enhance the signal to noise ratio of some of the ESR spectra and to obtain the second derivative spectra. A freeze-quench device, which was a modification of the design of Ballou and Palmer (25), was used to prepare short reaction time ESR samples. The samples for freeze-

ESR spectroscopy were prepared in buffer deoxygenated with argon.

Results

Cytochrome P-450$_{cam}$—Addition of nitric oxide to either the camphor-bound (high spin) or the camphor-free (low spin) ferric cytochrome P-450$_{cam}$ results in the formation of a single, spectrally distinct species (Fig. 1). This ferric-NO complex is characterized by intense $\alpha$ and $\beta$ absorbance band maxima which are similar to those previously reported for the ferric-NO complex of horseradish peroxidase with the exception that the intensities are reversed (Table I). The Soret absorbance band maximum is significantly red-shifted relative to that for the ferric-NO complex of horseradish peroxi-

![Fig. 1. Optical absorbance spectra of ferric cytochrome P-450$_{cam}$ (5 $\mu$M); the high spin d-camphor(K+) complex (---) in Buffer A (see "Materials and Methods"); the low spin camphor-free form (-- - -) in Buffer B; the NO complex of either species (---).](http://www.jbc.org/)

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dase and it has a lower extinction coefficient. The ferric-NO complex of cytochrome P-450, is quite stable in the absence of oxygen (several hours at 25°C, pH 7 to 8). The original ferric enzymes can be recovered when the NO is removed by evacuation demonstrating the reversible nature of this interaction (data not shown). The ESR spectra of the high spin (camphor-bound) or low spin (camphor-free) ferric forms of the cytochrome disappear upon formation of the ferric-NO complex, and no new ESR signals are observed in the temperature range from 5-300 K. This type of complex has been represented as a spin-paired system in which the unpaired electron of the hemin iron is coupled to the unpaired electron of the bound NO molecule (3).

The ferrous-NO complex formed by reduction of the ferric-NO complex or addition of NO to the ferrous or ferrous-NO complex of the cytochrome is also a spectrally distinct species (Fig. 2). This complex of the bacterial enzyme is also stable in the absence of oxygen. The ferrous-NO complex, like the ferrous-CO complex (18), is spectrally identical in the presence or absence of camphor. Additionally, as has been observed in the case of the ferrous-CO complexes of cytochrome P-450, (18, 29) and chloroperoxidase (30), the Soret absorbance band maxima of their ferrous-NO complexes are dramatically red-shifted, they have smaller extinction coefficients, and only a single absorbance band maximum is observed in the visible portion of their spectra relative to other hemeproteins investigated (Table I).

The second derivative ESR spectra of the ferrous-NO complexes of horseradish peroxidase is also shown in Fig. 3; however, the z absorption resonance in the case of horseradish peroxidase is changed from a triplet of triplets to a doublet of triplets upon replacement of 14NO with 15NO (3).

The second derivative ESR spectra of the ferrous-NO and 15NO complexes of cytochrome P-450, and horseradish peroxidase are shown in Fig. 4. As already noted the axial z absorption center resonance is transformed from a triplet to a doublet with cytochrome P-450, and from a triplet of triplets to a doublet of triplets in the case of horseradish peroxidase when 14NO is exchanged for 15NO. These second derivative spectra also allow resolution of the hyperfine structure of the g, and g, resonances both of which are sensitive to replacement of 14NO with 15NO (3). The number of apparent lines in the g, and g, signals changes from three to two for cytochrome P-450, and from four to three in the case of horseradish peroxidase (Fig. 4).

Cytochrome P-450, (Liver microsomal cytochrome P-450 also forms a ferric-NO complex either in the presence or absence of exogenous Type I substrates. The optical absorbance difference spectrum of the ferric NO complex of cyto-

### Table I

<table>
<thead>
<tr>
<th>Complex</th>
<th>( \lambda_{\text{max}} ) nm (( e_{\text{max}} \text{ cm}^{-1} )</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric P-450, NO</td>
<td>430 (103) 541 (15.0), 571 (12.3)</td>
<td>This work</td>
</tr>
<tr>
<td>Ferric P-450, NO (2 camphor)</td>
<td>419 (144) 533 (13.0), 568 (16.0)</td>
<td>3</td>
</tr>
<tr>
<td>Ferric HRP NO</td>
<td>438 (75.7) 557 (12.8)</td>
<td>This work</td>
</tr>
<tr>
<td>Ferric P-450, NO (2 camphor)</td>
<td>421 (116) 542 (11.5), 570 (10.5)</td>
<td>3</td>
</tr>
<tr>
<td>Ferric HRP NO</td>
<td>442 (85) 558 (14), 589 (sh)</td>
<td>27</td>
</tr>
<tr>
<td>Ferric HbNO</td>
<td>416.7 (99) 544, 573</td>
<td>28</td>
</tr>
<tr>
<td>Ferric MbNO</td>
<td>415.2 (129) 542, 571</td>
<td>28</td>
</tr>
</tbody>
</table>

* The abbreviations used are: cytochrome P-450, (P-450,); horseradish peroxidase (HRP); chloroperoxidase (CPO); hemoglobin (Hb); inositol hexaphosphate (IHP); myoglobin (Mb).

**Fig. 2.** Optical absorbance spectra of ferrous cytochrome P-450, (5 \( \mu \)M): the ferrous (-----), ferrous-CO (-----), and ferrous-NO (----) forms of either the d-camphor (K') complex in Buffer A or the camphor-free form in Buffer B.

**Fig. 3.** ESR spectra of 14NO (upper) and 15NO (lower) complexes (500 \( \mu \)M) of ferrous cytochrome P-450, (A, B) (camphor-bound or camphor-free) and horseradish peroxidase (C, D).
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Cytchrome P-450 versus the ferric enzyme in the presence of a typical Type I substrate is shown in Fig. 5. For comparison purposes, the optical absorbance difference spectrum of the ferric-NO complex of cytochrome P-450$_{cam}$ versus the ferric enzyme in the presence of camphor is shown in Fig. 6. Note that the ratio of the intensities of the $\alpha$ and $\beta$ bands is reversed in the ferric-NO complex of the liver microsomal enzyme (Fig. 5) relative to that seen in the bacterial enzyme (Fig. 6). Therefore, it can be suggested that not all of the cytochrome P-450$_{cam}$ molecules have substrate bound (see the ESR spectral results below). As was observed with the bacterial enzyme, the ESR signals of both the high and low spin forms of cytochrome P-450$_{cam}$ disappear upon formation of their respective ferric-NO complexes, and no new signals are observed in the 5-300 K temperature range. Also this ferric-NO complex is quite stable at room temperature in the pH range from 7 to 8 as long as it remains anaerobic.

**Table II**  
**Electron spin resonance parameters of nitric oxide complexes of cytochrome P-450$_{cam}$**

<table>
<thead>
<tr>
<th>Complex</th>
<th>Principal $g$ value</th>
<th>Hyperfine coupling constant ($G_s$ (axial))</th>
<th>No. of lines</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450$_{cam}$-NO (camphor)</td>
<td>2.073</td>
<td>2.009</td>
<td>1.976</td>
<td>19.2</td>
</tr>
<tr>
<td>P-450$_{cam}$-NO</td>
<td>2.08</td>
<td>2.004</td>
<td>1.976</td>
<td>20</td>
</tr>
<tr>
<td>P-450$<em>{cam}$-NO$</em>+$ (substrate)</td>
<td>2.006</td>
<td>2.008</td>
<td>1.976</td>
<td>20</td>
</tr>
<tr>
<td>HRP-14NO</td>
<td>2.076</td>
<td>2.008</td>
<td>1.987</td>
<td>20.8</td>
</tr>
<tr>
<td>CPO-14NO</td>
<td>2.082</td>
<td>2.004</td>
<td>1.975</td>
<td>3</td>
</tr>
<tr>
<td>TDO-14NO</td>
<td>2.084</td>
<td>2.009</td>
<td>1.988</td>
<td>16.5</td>
</tr>
<tr>
<td>LPO-14NO</td>
<td>2.088</td>
<td>2.004</td>
<td>1.982</td>
<td>20</td>
</tr>
<tr>
<td>LPO-14NO</td>
<td>2.070</td>
<td>2.004</td>
<td>1.958</td>
<td>16</td>
</tr>
<tr>
<td>Catalase-14NO$_+$</td>
<td>2.050</td>
<td>2.003</td>
<td>1.970</td>
<td>21</td>
</tr>
<tr>
<td>P-450$_{cam}$-NO (camphor)</td>
<td>2.074</td>
<td>2.009</td>
<td>1.976</td>
<td>27.4</td>
</tr>
<tr>
<td>HRP-15NO</td>
<td>2.076</td>
<td>2.008</td>
<td>1.966</td>
<td>30</td>
</tr>
<tr>
<td>P-420$_{cam}$-NO</td>
<td>2.072</td>
<td>2.015</td>
<td>2.072</td>
<td>22</td>
</tr>
<tr>
<td>P-420$<em>{cam}$-NO$</em>+$</td>
<td>2.072</td>
<td>2.015</td>
<td>2.072</td>
<td>16</td>
</tr>
<tr>
<td>P-420$<em>{cam}$-NO$</em>+$</td>
<td>2.072</td>
<td>2.015</td>
<td>2.074</td>
<td>16</td>
</tr>
<tr>
<td>P-420$<em>{cam}$-NO$</em>+$</td>
<td>2.072</td>
<td>2.015</td>
<td>2.074</td>
<td>16</td>
</tr>
</tbody>
</table>

$^a$ Contains also a small signal at 2.03 to 2.05.

$^b$ Abbreviations not already listed in Table I: cytochrome P-450$_{cam}$ (P-450$_{cam}$); tryptophan dioxygenase (TDO); lactoperoxidase (LPO).

Reduction of the ferric-NO complex of cytochrome P-450$_{cam}$ with dithionite, or the addition of NO to the ferrous form, yields another spectrally distinct species. This ferrous-NO complex is less stable than the corresponding complex of the bacterial enzyme; however, the presence of a Type I substrate aids significantly in maintaining its native conformation. For example, the optical absorbance difference spectrum of the ferrous-NO complex versus ferrous cytochrome P-450$_{cam}$ in the presence of androstanedione, shown in Fig. 5, is similar to that of the equivalent spectrum of the bacterial enzyme shown in Fig. 6, suggesting that most of the ferrous NO complex of cytochrome P-450$_{cam}$ remains native within the time required to record the spectrum (1 to 2 min). Nonetheless, changes in the optical absorbance spectrum do occur as a function of time and have been shown by ESR spectroscopy to correspond to denaturation of the ferrous NO complex to a five-coordinate P-420 ferrous-NO species (16).

The ESR spectrum of the ferrous-NO complex of cytochrome P-450$_{cam}$ prepared by rapidly mixing the dithionite reduced enzyme with NO-saturated buffer and freeze-quenching within 15 ms (see "Materials and Methods") is illustrated in Fig. 4. Second derivative ESR spectra of the 14NO (upper) and 15NO (lower) complexes (500 pM) of ferrous cytochrome P-450$_{cam}$ (A, B) and horseradish peroxidase (C, D).

**Fig. 4.** Second derivative ESR spectra of the 14NO (upper) and 15NO (lower) complexes (500 pM) of ferrous cytochrome P-450$_{cam}$ (A, B) and horseradish peroxidase (C, D).

**Fig. 5.** Optical absorbance difference spectra of the NO complexes of cytochrome P-450$_{cam}$ (5 μM) in Buffer C containing 0.1 mM 5α-androstane-3,17-dione: ferric-NO versus ferric (—); ferrous NO versus ferrous (—).
FIG. 6. Optical absorbance difference spectra of the NO complexes of cytochrome P-450$_{cam}$ (5 µM) in Buffer A: ferric-NO versus ferric (---); ferrous-NO versus ferrous (--).
excess sodium dithionite since similar data are obtained when
the liver microsomal enzyme is enzymatically reduced with
NADPH. The fact that the ferrous-NO complex of cytochrome
P-450$_{LM}$ is unstable would seem to indicate a relatively "weak"
bonding interaction between the heme iron and the axial
ligand trans to the NO. A slight perturbation of the ligand
field at the heme iron results in the formation of a more
strongly bonded NO versus the bonding to the trans ligand
furnished by the protein moiety. This propensity to denature
may be important with respect to identifying the trans axial
ligand itself.

Using the intensity of the $g_z$ ESR signal of the native
ferrous-NO complex of cytochrome P-450$_{a}$ as a measure of the
native conformation (Fig. 8) it is possible to estimate the
amount of denatured (P-420) form present in each of the
samples frozen at 2 min. From a measure of the ratio of the
intensity of the respective $g_z$ signals of the sample quenched
at 15 ms versus the two samples frozen after 2 min, it is
determined that the sample with hexoxygenal present con-
tains 22% denatured complex while that without exogenous
substrate contains 56%. The difference (34%) corresponds to
the extent of low spin to high spin conversion measured at
room temperature by optical absorbance difference spectro-
copy upon addition of a typical type 1 substrate to a micro-
somal suspension.

P-420—The ESR spectra of the P-420-NO complex arising
from denaturation of the ferrous-NO complex of cytochrome
P-450$_{LM}$ is identical (Fig. 9) to that of the NO complex of P-420
prepared by treating the liver microsomal enzyme with deox-
ycholate (24) or potassium thiocyanate (25) or of P-420 formed
by alkali denaturation of the bacterial cytochrome (23). These
spectra are very similar to that of the NO complex of SDS-
denatured hemoglobin (1, 31). Several lines of evidence indi-
cate that in the SDS-denatured HbNO complex the heme iron
has become five-coordinate. Both proton magnetic resonance
and ESR spectral studies of heme-NO complexes compared
with HbNO and SDS-denatured HbNO (2, 31) have shown that
when HbNO is denatured the trans axial ligand (histi-
dine) to the heme-bound NO is removed or the bond severely
distorted. It is interesting to note that the addition of an
allosteric effector to (8, 9) or acid-modification of (5-7) HbNO
causes the heme iron of two of the subunits to become five-
coordinate. The ESR spectra of the P-420-NO complexes
closely resemble that of the SDS-denatured HbNO species
suggesting a similar structural interpretation in the case of
cytochrome P-450.

**DISCUSSION**

A comparison of the optical absorbance difference spectra
obtained for the ferric- and ferrous-NO complexes of cyto-
chromes P-450$_{LM}$ and P-450$_{a}$ (Figs. 5 and 6) suggest that
their respective hemein and heme electronic environments are
quite similar. The ESR spectra of their respective ferrous-NO
complexes also indicate a nearly identical magnetic environ-
ment at the dioxygen binding site of these cytochromes P-450.

Thus, conclusions obtained from comparisons of the chemical
and physical properties of the soluble bacterial cytochrome P-
450 with those of other hemeproteins are also applicable to the
membrane-bound liver microsomal cytochrome P-450.

The existence of superhyperfine splitting in the $g_z$ ESR
signal of ferrous-NO complexes of several hemeproteins in-
cluding Hb (2, 32), Mb (33), horseradish peroxidase and
cytochrome c peroxidase (3) has been used as evidence for the
presence of a nitrogen-containing ligand bound trans to the
heme-bound NO. This triplet of triplets $g_z$ signal is believed to
arise from electron spin-nuclear spin interaction of the un-
paired electron of the bound NO with a nitrogen atom of the
ligand bound trans to it. In the case of Hb and Mb, where an
imidazole moiety of a histidine residue is known to serve as
the axial ligand, the observed coupling constant for the
superhyperfine interaction is 6 to 7 G (2, 3, 32, 33). A similar
coupling has been observed in the signal of the ferrous-NO complexes of
catalase or lactoperoxidase which suggested that, for these
hemeproteins, electron spin relaxation was very rapid or that
a ligand not possessing nuclear spin was bound trans to the
heme-bound NO (3). However, the absence of superhyperfine
splitting in the $g_z$ ESR signal in both of these hemeproteins,
as well as in cytochromes P-450 and chloroperoxidase, may be
the result of other phenomena.

Studies with isolated $\alpha$, $\beta$, and $\gamma$ subunits of hemoglobin as
well as $\alpha\beta$ hybrids have indicated that even when the trans
axial ligand is known to be a histidine nitrogen atom, super-
hyperfine splitting is not always observed (11, 12). The pres-
ence or absence of superhyperfine splitting in the $g_z$ signal of
the ESR spectra of the NO complexes of $\alpha$ and $\beta$ subunits, as
well as of two mutant hemoglobins (Hb Zurich and Hb M
Iwate) was shown to be conformationally dependent (11). Two
types of spectrally distinct NO complexes were proposed to
result from changes in the bonding between the trans axial
ligand, histidine, and the NO to the heme iron (11). Thus the
type II ESR spectrum is characterized by the presence of the
superhyperfine splitting in the $g_z$ signal due to a strong $\sigma$
bond between the proximal histidine and the heme iron such
that electron spin-nuclear spin interaction occurs. The type I
ESR spectrum, on the other hand, shows smaller hyperfine
coupling in the $g_z$ signal, the absence of any superhyperfine
interaction, and the presence of an observable hyperfine
interaction in the $g_z$ signal. These factors are taken in the
case of type I to indicate a weakened bonding interaction
between the trans nitrogen atom and the heme iron with a
concomitant strengthening of the iron NO bonding (11). A
similar proposal has been offered to explain the results previ-
ously observed for the ferrous-NO complex of tryptophan
dioxygenase (14). In the absence of its substrate, L-tryptophan,
a three-line hyperfine splitting was observed in the $g_z$ signal,
while in its presence a nine-line pattern was seen indicative of
superhyperfine splitting of the ESR signal of the bound NO by
a trans axial nitrogen atom (histidine ?). It was suggested that
the substrate induced a conformational change in the
enzyme which further strengthened the bond between the
heme iron and the fifth ligand, which presumably is nitrogen
since a nine-line $g_z$ signal was observed (14). Thus the three
line $g_z$ signal observed for both the cytochromes P-450 and
chloroperoxidase (Table II) may be an indication of weakened
proximal ligand to heme iron bonding with concomitantly
stronger NO to heme iron bonding. This bonding pattern
would be similar to that designated as type I by Trittelvita et
al. (11).

Another possibility for the absence of superhyperfine split-
ing of the $g_z$ signal in the ferrous-NO complex of cytochromes
P-450, as well as chloroperoxidase, lactoperoxidase, and cata-
lase, is also related to the bonding interactions among the

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results.
NO, heme iron, and proximal ligand. As previously indicated the electron spin-nuclear spin coupling, believed to be responsible for the superhyperfine splitting of the \( g \) \( z \) ESR signal, is favored when the unpaired electron of the NO is localized primarily in a molecular orbital appropriate for mixing with the \( d_{x^2-y^2} \) orbital of the heme iron (1, 11, 34). Support for this idea has come from studies with horseradish peroxidase (3).

Replacement of \(^{14}\)NO with \(^{15}\)NO was observed to change the shape of the \( g \) \( x \) and \( g \) \( y \) signals of the ferrous-NO complexes; however poor spectral resolution precluded the direct determination of coupling constants. Spectral simulations were interpreted to indicate that the large splitting observed in these signals came primarily from interaction with the porphyrin nitrogens (3). We have observed that the second derivative ESR spectra of both cytochromes P-450 and horseradish peroxidase exhibit changes in the splitting of their \( g \) \( x \) and \( g \) \( y \) signals when \(^{14}\)NO is replaced with \(^{15}\)NO (Fig. 4), which may indicate that significant electron spin density is also delocalized onto the porphyrin nitrogens. Consequently, we suggest that the metal-nitric oxide bonding may be complex enough so as to require further perturbations of the molecular orbital diagrams (11, 34) which have been presented. For example, the origin of the differences in the number of lines observed in the \( g \) \( x \) and \( g \) \( y \) signals between these two heme-proteins does not seem obvious.

As indicated in Figs. 3 and 4 the ESR spectra of the ferrous-NO complexes of cytochromes P-450_<sub>cam</sub> and P-450_<sub>LM</sub> resemble that of the ferrous-NO complex of horseradish peroxidase with the exception that superhyperfine splitting of the \( g \) \( z \) signal is observed. Assuming that the absence of splitting is not due to fast relaxation of the electron spin, then one obvious possibility is that the axial ligand bound \( \text{trans} \) to the heme-bound NO does not have nuclear spin, e.g. sulfur. This possibility is supported by the recent report of Stern and Peisach (35) in which a model compound for the ferrous-NO complex of cytochrome P-450_<sub>cam</sub> was presented. These authors were able to generate an optical absorbance spectrum having wavelength maxima near those of the enzyme from a complex presumed to have a mercaptide axial ligand \( \text{trans} \) to a heme-bound NO. These data would then support the proposal that the unique electronic spectrum of the ferrous-NO complex of cytochrome P-450 may be the result of a \( \text{trans} \) mercaptide ligand presumably interacting with a porphyrin molecular orbital to produce the 450 nm Soret band (36). However, the lack of superhyperfine splitting in the \( g \) \( z \) signal cannot be considered positive evidence for the proximal ligand not having nuclear spin since, as noted above, even when histidine is thought to be the proximal ligand the splitting is not always observed (11, 12). Therefore it is our contention that the identity of the axial ligand at position 5 in the ferrous-NO complex of cytochrome P-450 remains unknown.

Based upon the results obtained in our studies we would like to propose the scheme shown in Fig. 10 to represent the bonding between the iron and nitric oxide in the ferri- and ferrous forms of cytochromes P-450 and P-420. In this model the spin-paired ferric-NO complex (A) is indicated to have linear bonding between the iron and NO to maximize the \( d_{x^2-y^2} \) \( \pi \) backbonding. The dotted lines represent \( \pi \) bonding and X is the \( \text{trans} \) axial (proximal) ligand at position 5. In the ferrous-NO complex (B), however, the bonding between the heme iron and the \( \text{trans} \) axial ligand is judged to be weaker as indicated by the dashed line between X and iron. Consequently, the NO to heme iron bonding is stronger as indicated by the heavy solid and dotted lines between iron and NO.

Support for this conclusion comes from the fact that at least a portion of the liver microsomal cytochrome P-450 is easily denatured to a five-coordinate P-420-NO complex (C) in which the bonding between the heme iron and NO is even stronger than in the ferrous-NO complex (B). It is also interesting to note that while the binding of substrate appears to stabilize the native conformation of the ferrous-NO complex, it does not alter its ESR spectrum, i.e. no conformationally dependent change occurs in the \( g \) \( z \) signal. The indicated bonding of the Fe-NO in the cytochrome P-450 ferrous (B) and P-420-NO (C) complexes is based upon crystallographic data obtained from Fe(TPP)(NO)(1-Me-Im) and Fe(TPP)(NO)<sub>2</sub> complexes (37, 38). It is important to note in the case of the ferrous-NO complex (B) that the Fe—N—O bond angle is very sensitive both to the strength of the bonding between the \( \text{trans} \) axial ligand and the heme iron as well as to the steric environment about the Fe—NO bond. For example a nearly linear bond angle (165-170°) has been recently reported in the case of the \( \beta \)-nitrile-\( \text{trans} \) histidines of hemoglobin Kansas (10) relative to the 142° angle reported for Fe(TPP)(NO)(1-Me-Im) (37). If in fact the proximal ligand bonds only weakly to the heme iron prohibit-
suggesting that the protons are probably on an atom within the inner coordination sphere of the hemin iron. Furthermore, it was reasoned that the most likely candidate for this ligand was in fact water. A more extensive investigation has subsequently confirmed these data (40). Thus contrary to recent reports (41, 42), the replaceable sixth ligand of low spin substrate free cytochrome P-450 (A) is not an imidazole moiety. The current study has shown that the optical absorbance spectrum of the ferric-NO complex of cytochrome P-450 (D) is the same whether the hemin iron was initially low spin (A) (water as the presumed replaceable ligand) or high spin (B) (pentacoordinate). This is shown in Reactions 1 and 2 of Fig. 11 in which the fifth ligand of the heme iron is the same. The Mossbauers spectral (43) and magnetic susceptibility (44) studies of ferrous cytochrome P-450 have shown that the heme iron of the ferrous form (C) is high spin (pentacoordinate) in either the presence or absence of the substrate d-camphor. The addition of O2, CO, or NO to ferrous cytochrome P-450 results in the formation of low spin ferrous forms (E, F), and it is reasonable to assume that these ligands attach the vacant position 6 of the heme iron. The optical absorbance and ESR spectra of the ferrous-NO complex of cytochrome P-450 (in the presence or absence of camphor) are the same whether it is formed by the addition of NO to the ferrous enzyme (Reaction 4) or by reduction of the ferric NO complex (Reaction 3). It has already been shown that CO and O2 are directly competitive with each other for binding to the heme iron (19) and now NO can be added to this group. These results may be reasonably interpreted to indicate that the fifth ligand of cytochrome P-450, represented by X in Fig. 11, does not change during Reactions 3 to 6. Therefore, the recent proposal (42) that O2 and CO bind on opposite sides of the heme iron to account for the "unusual" optical absorbance spectrum of the CO complex and "normal" spectrum of the O2 complex seems quite unlikely.

REFERENCES
Studies of the oxygen binding site of cytochrome P-450. Nitric oxide as a spin-label probe.
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