Characterization of Endothelial Nitric-oxide Synthase and Its Reaction with Ligand by Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance was used to characterize the heme structure of resting endothelial nitric-oxide synthase (eNOS), eNOS devoid of its myristoylation site (G2A mutant), and their heme complexes formed with 16 different ligands. Resting eNOS and the G2A mutant have a mixture of low spin and high spin P450-heme with widely different relaxation behavior and a stable flavin semiquinone radical identified by EPR as a neutral radical. This flavin radical showed efficient electron spin relaxation as a consequence of the dipolar interaction with the heme center; 

P1/2 is independent of Ca2+-calmodulin and tetrahydrobiopterin. Seven of the 16 ligands led to the formation of low spin heme complexes. In order of increasing rhombicity they characterize the heme structure of resting endothelial nitric-oxide synthase (eNOS). These isoforms share ~60% overall homology in their protein sequences, and their reaction mechanisms are also very similar (1, 2). All three isozymes contain heme, FAD, and FMN and require the participation of Ca2+-calmodulin and tetrahydrobiopterin for enzyme catalysis (1–4). Characterization of NOS by different biophysical methods including UV-Vis (5–7), electron paramagnetic resonance (EPR) (7–9), resonance Raman (10–12), and magnetic circular dichroism spectroscopy (13) in the last few years has provided insights into the structure-function relationship and the oxidation-reduction behavior of each catalytic component. EPR is superior to UV-Vis electronic spectroscopy in providing information about heme symmetry, identifying heme ligands, and understanding the electronic structure of the heme iron. The much higher sensitivity of EPR to changes in heme symmetry than other spectroscopic methods proves to be a useful tool in delineating the structural perturbation caused by different axial ligands (14).

In contrast, 4-ethylpyridine, 4-methylpyrimidine, acetylguanidine, ethylguanidine, 2-aminohiozazole, 2-amino-4,5-dimethyliothiazole, 1-histidine, and 7-nitroindazol resulted in high spin heme complexes of eNOS. A re-definition of the P and O zones is proposed. As eNOS and chloroperoxidase lie closer of chloroperoxidase. Are-definition of the P and O zones on the “truth diagram” originally derived by Blumberg and Peisach (1971) in Probes and Structure and Function of Macromolecules and Membranes (Chance, B., Yonetani, T., and Mildvan, A. S., eds) Vol. 2, pp. 215–229, Academic Press, New York) and had significant overlap with complexes of chloroperoxidase. In this P-redefinition, the P and O zones are implied that the distal heme environment in eNOS resembles chloroperoxidase more than P450cam.

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The free radical nitric-oxide serves as an important messenger in a wide variety of physiological and pathophysiological processes. Biosynthesis of this simple gaseous molecule is catalyzed by three different isoforms of nitric-oxide synthase (NOS). These isoforms share ~60% overall homology in their protein sequences, and their reaction mechanisms are also very similar (1, 2). All three isozymes contain heme, FAD, and FMN and require the participation of Ca2+-calmodulin and tetrahydrobiopterin for enzyme catalysis (1–4). Characterization of NOS by different biophysical methods including UV-Vis (5–7), electron paramagnetic resonance (EPR) (7–9), resonance Raman (10–12), and magnetic circular dichroism spectroscopy (13) in the last few years has provided insights into the structure-function relationship and the oxidation-reduction behavior of each catalytic component. EPR is superior to UV-Vis electronic spectroscopy in providing information about heme symmetry, identifying heme ligands, and understanding the electronic structure of the heme iron. The much higher sensitivity of EPR to changes in heme symmetry than other spectroscopic methods proves to be a useful tool in delineating the structural perturbation caused by different axial ligands (14).

Electro NOS complexes fell in a region between the P and O zones on the “truth diagram” originally derived by Blumberg and Peisach (1971) in Probes and Structure and Function of Macromolecules and Membranes (Chance, B., Yonetani, T., and Mildvan, A. S., eds) Vol. 2, pp. 215–229, Academic Press, New York) and had significant overlap with complexes of chloroperoxidase. In this P-redefinition, the P and O zones are implied that the distal heme environment in eNOS resembles chloroperoxidase more than P450cam.

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**EXPERIMENTAL PROCEDURES**

| Compound   | Description                                      |
|------------|--------------------------------------------------|
| Imidazole  | 2-methylimidazole, 2-hydroxylimidazole, pyridine, 4-ethylypyridine, pyrimidine and 4-methylpyrimidine, thiazole, 2-aminohiozol |

1. The abbreviations used are: NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate. G2A mutant, mutant with the glycine replaced by an alanine at residue number 2; CaM, calmodulin.

2. P.-F. Chen, A.-L Tsai, V. Berka, and R. K. Wu, manuscript in review.
zole, 2-amino-4,5-dimethylthiazole, ethylglycine, and acetylglu- 
dine are from Aldrich. Potassium cyanide, l-arginine, l-lysine and 
l-histidine, NADPH, 7-nitroindazole, adenosine 2’,3’-monophosphate, 
CHAPS, and calmodulin were purchased from Sigma. L-Citrulline 
was obtained from ALEXIS Biochemicals, San Diego, and l-(2,3,4,5’-
H)arginine was obtained from Amersham Corp. (specific activity, 77 Ci/mmol). Recombinant human endothelial nitric-oxide synthase (eNOS) was 
prepared using a baculovirus expression system as described previously 
(15, 16). The cell pellets were suspended in buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 20 mM CHAPS, 10% glycerol, 1 μM antipain, 1 μM leu-
peptin, 1 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride) and soni-
cated three times for 10 s each. The 100,000 × g supernatant was 
directly applied to a 2’,3’-ADP-Sepharose column (1.5 × 2 cm) pre-
equilibrated with buffer A. The column was washed with 25 ml of buffer 
A plus 0.5 M NaCl, followed with 10 ml of buffer A and then eluted with 
buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 5 mM CHAPS, 10% glycerol) plus 30 mM adenosine 2’,3’-
monophosphate. The eluate was concentrated by Centriprep-100 (Ami-
con) at 500 × g and then applied to a 10-DG column (Bio-Rad). The yield 
was about 10–20 mg of purified eNOS from 3 × 10⁸ Spodoptera frugi-
perda cells. Soluble endothelial nitric-oxide synthase was prepared 
by specific replacement of the second amino acid, glycine, to alanine 
through oligonucleotide-directed mutagenesis described elsewhere.² 
Expression and purification of this mutant eNOS were very similar to 
the wild-type enzyme but without detergent solubilization, and the 
enzyme was purified directly from the soluble fraction after the step of 
sonication. Details of the characterization of both wild-type and G2A 
mutant eNOS were presented elsewhere.² Activity of eNOS was measured as the conversion of tritium-labeled 
L-arginine to L-citrulline as described previously (15, 16). A 3-min 
incubation of the reaction mixture at 37 °C before quenching was ap-
plied to maintain a linear production of L-citrulline.

EPR was recorded mainly at liquid helium temperature and occa-
sionally at liquid nitrogen temperature on a Varian E-6 spectrometer 
with an Air Product liquid helium transfer line (17). Calibration of 
magnetic field was done by using either a,α’-diphenyl-β-picrylhydrazyl 
standard (g = 2.0037) or the stable flavin semiquinone for the high field 
region, and sperm whale myoglobin (g = 2.0050) for the low field signal 
(18). A Hewlett-Packard HP5342 microwave frequency counter was 
used to record the frequency. Data obtained from progressive power 
saturation were fitted to

\[ \log(S/P^{1/2}) = -b/2 \log(P_{12} + P) + b/2 \log(P_{10}) + \log(K) \]  

where \( P_{10} \) is the power to achieve half-saturation of the signal; \( b \) is set 
to 1 for inhomogeneous broadening as found for most hemeprotein 
states, and \( K \) is a proportionality factor (19).

In the case of low spin heme, the \( g_{\text{max}} (g_e) \) was either measured 
directly, or calculated from (20, 21)

\[ g_z^2 + g_x^2 + g_y^2 - g_{\text{ex}} g_z - g_{\text{ey}} g_z + g_{\text{ex}} g_y + 4g_z - 4g_y - 4g_z = 0 \]  

(Eq. 2)

(rather than \( g_z^2 + g_x^2 + g_y^2 = 16 \) because low spin P450 always exhibits 
a \( g_{\text{max}} \) Value smaller than 3). The parameters, \( V \) and \( \Delta \), the rhombic and 
axial ligand field terms, were then calculated in units of \( \lambda \) (the 
spin-orbit coupling constant) using the equations derived by Taylor (22):

\[ V\lambda = g_e(g_x + g_y) + g_z(g_z - g_x - g_y) \]  

(Eq. 3)

\[ \Delta \lambda = g_z(g_x + g_y) + g_x(g_y - g_z) - V2\lambda \]  

(Eq. 4)

using an axis systems defined by Blumberg and Peisach (23). For the 
high spin heme, \( g_z \) and \( g_x \) were measured directly, and \( E \) and \( D \) were 
calculated using Equation 5. \( g_z \) was measured directly when observable, 
otherwise it was calculated using Equation 6 and the previously deter-
mined values of \( E \) and \( D \). The ratio of rhombic (E) and axial (D) ligand 
field components is \( <0.1 \) for NO (9) and most other P450s (14, 24).

\[ g_{z} = 3g_e \pm 24E/D = 168E/(9D^2) \]  

(Eq. 5)

\[ g_x = g_z - 304E/(9D^2) \]  

(Eq. 6)

where \( g_z = 2.0 \).

² P. F. Chen, A.-L. Tsai, V. Berka, and K. K. Wu, manuscript in review.
**TABLE I**

| Ligand                   | Ligand Structure | Spin | $g_x^1$ | $g_y^1$ | $g_z^1$ | %Rhombicity | $\Delta^4$ $\lambda$ | V$^4$ $\lambda$ |
|--------------------------|------------------|------|---------|---------|---------|-------------|-----------------------|----------------|
| None                     | ---              | 5/2  | 7.67    | 4.34    | 1.84    | 20.8        |                       |                |
| None                     | ---              | 1/2  | 1.87    | 2.30    | 2.45    | 33.1        | 5.22                  | 1.73           |
| Imidazole                |                  | 1/2  | 1.75    | 2.30    | 2.70    | 62.7        | 4.0                   | 2.50           |
| 4-Methyl imidazolene     |                  | 1/2  | 1.81    | 2.28    | 2.66    | 64.8        | 4.52                  | 2.92           |
| Pyridine                 |                  | 1/2  | 1.85    | 2.30    | 2.50    | 39.7        | 4.92                  | 1.95           |
| 4-Ethyl pyridine         |                  | 5/2  | 7.72    | 4.19    | 1.82    | 22.0        |                       |                |
| Pyrimidine               |                  | 1/2  | 1.86    | 2.29    | 2.48    | 52.0        | 4.70                  | 2.44           |
| 4-Methyl pyrimidine      |                  | 5/2  | 7.70    | 4.24    | 1.84    | 21.7        |                       |                |
| Thiazole                 |                  | 1/2  | 1.84    | 2.31    | 2.53    | 43.1        | 4.77                  | 2.06           |
| 2-Amino thiazole         |                  | 5/2  | 7.79    | 4.23    | 1.82    | 22.2        |                       |                |
| 2-Amino-4,5-dimethyl-    |                  | 5/2  | 7.83    | 4.06    | 1.81    | 23.6        |                       |                |
| indazole                 |                  |      |         |         |         |             |                       |                |
| 7-nitro indazole         |                  | 5/2  | 7.70    | 4.18    | 1.84    | 22.0        |                       |                |
| Acetylguanidine          |                  | 5/2  | 7.80    | 4.17    | 1.81    | 22.3        |                       |                |
| Ethylguanidine           |                  | 5/2  | 7.78    | 4.20    | 1.83    | 22.1        |                       |                |
| Cyanide                  | NaCN             | 1/2  | 1.78    | 2.33    | 2.60    | 44.5        | 4.12                  | 1.83           |
| L-arginine               |                  | 5/2  | 7.85    | 4.33    | 1.84    | 20.2        |                       |                |
| L-lysine                 |                  | 1/2  | 1.82    | 2.28    | 2.58    | 54.9        | 4.66                  | 2.56           |
| L-histidine              |                  | 5/2  | 7.71    | 4.19    | 1.81    | 22.0        |                       |                |

1 Values in square brackets are calculated from Equation 1.
2 Values in square brackets are calculated from Equation 6.
3 Values for the high spin heme was calculated as $6.25 \times (g_x - g_y)$, and values for the low spin heme was calculated as $100 \times V\Delta$.
4 Calculated according to Equation 3 in units of $\lambda$, the spin-orbital coupling constant.
5 Calculated according to Equation 4 in units of $\lambda$ also.
spin. Low spin heme signals have a much smaller amplitude than high spin signals, a consequence of the derivative representation of the absorption envelopes of the two heme species (9, 14).

The eNOS sample isolated from the G2A mutant cells exhibits an EPR with high spin heme as the dominant species. Approximately 60% of the total heme estimated from the amplitude of either $g_\parallel$ or $g_\perp$ signal was present as high spin. The $g_\parallel$ component is easily identified at 1.84, indistinguishable from that of the l-arginine-treated sample (Fig. 1, bottom panel). The EPR results corroborate the optical data that show the Soret peak is more blue-shifted for the resting G2A mutant than that of the wild-type eNOS.5

The relaxation behavior of the high spin and low spin heme EPR are quite different. The low spin heme showed inhomogeneous saturation with an average $P_{1/2}$ of 0.3 mW (Fig. 2B and Table II). In contrast, the high spin heme EPR did not show any saturation even at 200 mW, the maximal output of our klystron with power leveler enabled. This difference in relaxation rate enabled a clean discrimination between the $g_{\text{min}}$ originating from the high spin or low spin heme centers by selective saturation of one heme species. For example, the spectrum recorded at 0.2 mW gave mainly the low spin heme signals (Fig. 3, spectrum a) with only a small contribution from the high spin heme.

EPR of the Stable Flavin Radical and Its Interaction with Heme Centers—A prominent radical signal was observed in all isolated eNOS samples (Fig. 1) and was attributed to a flavin semiquinone radical (7–9). This EPR signal is isotropic and centered at $g = 2.004$ with an overall line width of 20 G, indicative of a neutral flavin semiquinone radical (Fig. 2A, inset) (26). Double integration of this radical revealed that it represented about 15–20% of the total FMN, the flavin species proposed to generate the stable radical. A power saturation study gave a $P_{1/2}$ of 50 μW. To evaluate the effect of heme spin states on the relaxation behavior of this flavin radical, eNOS was also prepared as the imidazole derivative containing pure low spin heme and also as the l-arginine-bound form, a pure high spin hemoprotein, for this study. These reagents did not change the EPR spectral characteristics of the flavin radical. As shown in Fig. 2 and Table II, the $P_{1/2}$ of the flavin radical in the imidazole eNOS (Fig. 2A) and l-arginine-bound eNOS (Fig. 2C) were the same as that of the resting eNOS (Fig. 2B). Addition of calcium plus calmodulin to either of these three samples did not change the $P_{1/2}$ of the flavin radical and produced only a marginal change of the high spin or low spin heme centers (Fig. 2 and Table II). The presence and absence of tetrahydrobiopterin (data not shown) did not have any effect on $P_{1/2}$ either, indicating the relaxation rate of the flavin radical is not influenced by the substrate binding or binding of either Ca$^{2+}$–calmodulin or tetrahydrobiopterin.

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**Table II**

| Samples                  | Heme species | Flavin semiquinone |
|--------------------------|--------------|--------------------|
| eNOS                     | Low spin     | Flavin semiquinone |
| eNOS + CaM               | 240          | NS$^d$             |
| eNOS + Im$^c$            | 490          | NS$^d$             |
| eNOS + l-Arg$^c$         | 520          | 50                 |
| eNOS + l-Arg + CaM       | 620          | 54                 |
| eNOS + l-Arg + CaM       | 490          | 49                 |

$^a$ Not saturable in the range of our microwave power, 0–200 mW.
$^b$ Concentrations of calmodulin and calcium were 10 μM and 2 mm, respectively.
$^c$ Imidazole (Im) was present as 6 mm.
$^d$ l-Arginine was present as 0.2 mm.
(Fig. 3 and Table I). The EPR spectra of these low spin heme complexes are more anisotropic than is the less anisotropic component of the resting enzyme. The \( g_{\text{max}} \) values vary from 2.48 to 2.70 compared with 2.42 found in the resting eNOS, and the \( g_{\text{min}} \) values decrease from 1.86 to 1.75 compared with 1.90 found for the resting enzyme. With the exception of the two imidazole ligands, each low spin heme complex exhibited a unique set of \( g \) values with the cyanide and thiazole complexes displaying much broader \( g_{\text{max}} \) and \( g_{\text{min}} \) signals. A minimum of two low spin heme species are present in the imidazole complex, as characterized by two discrete sets of \( g \) values, 2.70, 2.30, 1.75, and 2.57, 2.30, 1.83 (Fig. 6). The splitting of \( g = 2.57 \) signal is not reproducible from batch to batch and is best treated as one single species but raises the possibility of the existence of more than two low spin heme complexes in this case. The cognate 4-methylimidazole complex showed very broad signals for \( g_{\text{max}} \) and \( g_{\text{min}} \) making it difficult to assign accurate \( g \) values for either of them.

Axial (\( \Delta \)) and rhombic (\( V \)) ligand field parameters were calculated from the \( g \) values according to Equations 3 and 4 (Table I). The resting enzyme, which has two low spin components, gave two axial components of 4.8 and 5.7 and two rhombic component of 1.8 and 2.9. The other seven low spin heme complexes have values of \( \Delta \) ranging from 4.0 to 5.1 and values of \( V \) parameters ranging from 1.8 to 2.5. The rhombicity, calculated as the \( 100 \times V/\Delta \), of these seven low spin complexes fell in the range between 40 and 65%, relatively higher than those of the wild-type low spin heme species, 32 and 49%.

The ligands that led to the formation of high spin heme complexes were 4-ethylpyridine, 4-methylpyrimidine, acetylguanidine, ethylguanidine, 2-aminothiazole, 2-amino-4,5-dimethylthiazole, L-histidine, and 7-nitroindazole. The important features of the spectra are shown in Figs. 4 and 5 and are summarized in Table I. Compared with the low spin heme complexes, the changes appear to be smaller in extent and manifested primarily as a subtle shift in all three \( g \) values following reaction with different ligands. The \( g_{\text{max}} \) varied between 7.70 and 7.83, \( g_{\text{mid}} \) varied between 4.06 and 4.20, and \( g_{\text{min}} \) ranged from 1.81 to 1.84. However, each high spin complex was significantly different to the resting enzyme in two aspects. The first is a major change in heme spin state composition, with these heme complexes essentially present as pure high spin in contrast to a mixture of high and low spin heme found for the resting eNOS. The second difference is that the rhombicity of all these high spin complexes is larger than that of the resting enzyme as recognized by the larger separation between \( g_{\text{max}} \) and \( g_{\text{mid}} \) (Fig. 4 and Table I).

The degree of perturbation appears to be related to the
FIG. 5. Shift of the EPR signal of high spin eNOS derivatives from that of resting eNOS. The low field signal shown in Fig. 4 was enlarged to show the change in line width and magnetic field position. The spectra follow the same sequence as shown in Fig. 4, with additional two spectra i and j as the eNOS treated with 200 μM L-arginine and resting eNOS, respectively.

The intensity of the flavin radical is ~20% of the concentration of FMN. This amount of EPR-detectable radical exhibited no correlation with the concentration of the high spin heme (data not shown). On the other hand, EPR line shape and relaxation behavior of the flavin radical remained the same for the pure low spin imidazole eNOS complex as well as the pure high spin L-arginine-bound eNOS (Fig. 2 and Table II). Although the high spin heme, with five unpaired electrons, is more magnetic than the low spin heme, the latter might exhibit a more potent intrinsic spin-lattice relaxation rate. This might bring about a dipolar interaction between the low spin heme with the flavin radical comparable with that of the high spin heme center. Incidentally, the same P1 value was obtained by fitting the data of power saturation to Equation 1 using either one or two components in the relaxation process (data not shown). All these analyses led to the conclusion that it is not the heme spin state but most likely the heme redox potential that dictates the amount of radical formed.

The dipolar interaction between the flavin semiquinone and the high spin heme of resting eNOS results in a shift in g value of similar extent. The low spin center relaxes via Raman relaxation of the aminothiazole complex clearly showing heterogeneity in the EPR signal generated by acetylguanidine and 7-nitroindazole. The high spin state of the EPR signal upon arginine binding. The high spin heme is characterized by the high frequency modulation of the orbital magnetism (41). The relaxation of high spin heme is dominated by coupling to nearby Kramer's doublets, an Orbach process (42). For P450-type high spin hemes these lie at ~7 cm−1 (42). The fast relaxation of the high spin heme presumably accounts for the substantial flavin semiquinone radical signal at liquid helium temperature even at incident microwave powers as high as 10–20 mW (Fig. 1 and Refs. 7–9). As inferred from a comparison of the line widths of flavin radicals in 16 different flavoproteins (28), the peak-to-trough separation of 20 G of the semiquinone EPR signal indicates a neutral rather than an anionic radical. Neutral flavin radicals typically have a 19 G EPR line width, in contrast to the 15 G for the anionic flavin radical; this extra width arises from the additional H at N5 in neutral flavin and can be almost eliminated by increasing the pH; using D2O buffer (26). The optical spectrum of both the wild-type and G2A mutant eNOS shows clear peaks at 500–650-nm region consistent with a blue neutral flavin chromophore rather than a red anion flavin radical (25).

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EPR of Endothelial Nitric-oxide Synthase

RESULTS

Among the 37 ligands tested for their low spin EPR effects, we found that only the imidazole ring, thiazole, or 2-aminothiazole could cause the formation of low spin EPR spectra for eNOS, whereas the nonimidazole ligands tested resulted in high spin EPR spectra. These results were obtained for thiazole and 2-aminothiazole or L-arginine versus L-lysine (Figs. 3–5). The optical data and the EPR results are in good agreement and thus confirm the contrasting spectral behavior exhibited by a family of ligands with common functional moieties.

We conclude that those ligands that cause type II spectral changes and yield low spin EPR spectra are directly ligated to the heme iron. On the other hand, those ligands that result in type I spectral changes and high spin EPR are not directly coordinated to heme but rather cause a heme structural perturbation by one or more indirect mechanisms such as puckering or doming of the heme porphyrin, change of bond length between heme iron and the sulfur atom, or change of the dihedral angle between Fe-S and the N$_7$N$_8$ pyrrole axis. Presumably these ligands, either because they are too bulky or because of unfavorable steric factors, are unable to bind to the iron. It also appears that these indirect perturbations lead to a displacement of the original 6th heme ligand of eNOS.

Dawson, Sono, and their colleagues (31, 32) have made an extensive study on the effect of ligands on the heme spin state of P450$_{cam}$ and found more than 20 different oxygen, nitrogen, and sulfur donors that led to the formation of low spin heme complexes. The proximal thiolate has an abnormally strong crystal field, and other than a few halide anions most other ligands produce a low spin 6-coordinate complex (31, 33). This also explains the smaller dispersion of g values caused by different ligands in P450 heme complexes (31) than in those hemeproteins that have histidine as the proximal ligand. Precautions must thus be taken in the assignment of the unknown 6th heme ligand of P450 complexes based solely on the similarities in EPR g values. One example of this difficulty in assignment is that the axial ligand at the distal position of both low spin P450$_{cam}$ and chloroperoxidase, each with a proximal thiolate ligand, is water (34, 35), rather than the nitrogen or carboxylate ligands proposed earlier (30, 36).

With the exception of cyanide, the seven ligands that induce the formation of low spin heme complex are all nitrogen donors. Thiazole is potentially ambiguous, but "nitrogen on" rather than "sulfur on" is supported by the observation that no hyperporphyrin spectrum is observed for this complex (data not shown). The heterogeneous EPR of both imidazole ligands might be the result of two different chemical forms of the same ligand, i.e. a neutral imidazole versus imidazolate anion. The latter should give an EPR signal with less anisotropy, due to its stronger ligand field. The same situation occurs with the methylimidazole complex of eNOS. The very broad low field and high field EPR signals prohibited the accurate location of $g_{\text{max}}$ and $g_{\text{min}}$ (Fig. 3). Table I also reveals a big difference in $g_{\text{min}}$ between that observed and that calculated using $g_{\text{imid}}$ and $g_{\text{max}}$ and Equation 2. The proposal that EPR heterogeneity is a consequence of the distal ligand present as two different protonated forms is not supported by the EPR changes induced by pH. With both imidazole and 4-methylimidazole, a decrease of sample pH from 8.0 to 6.9 led to an increase of the high field $g_{\text{max}}$ at the expense of the low field component (Fig. 6), a change opposite to that expected from simple arguments involving the ligand field strength of the distal ligand. One possible explanation is that the two signals represent two orientations of the imidazole ring relative to the heme plane and that lowering the pH eliminates a H bond that stabilizes one of the two orientations.

The Truth Diagram originated by Blumberg and Peisach (23, 24) correlates the electronic effect of the axial ligands, represented by $\Delta$, with the heme rhombicity, expressed as the ratio of $V\Delta$, for various heme complexes. This diagram provides some empirical guidance for the assignment of axial ligands.
and has been widely used (8, 14, 23, 33, 37). Although a number of pitfalls have been found in the use of this correlation diagram, and it should not be used indiscriminately as the only method for ligand assignment (38), many successful examples warrant its continued application.

The correlation diagram constructed from the rhombic and tetragonal field strength according to Blumberg and Peisach (23) is shown in Fig. 7. The four domains that are about the same in their heme rhombicity are zone C (cytochrome c, with methionine and histidine ligands), zone B (cytochrome b, with two histidine or imidazole ligands), zone H (hemoglobin histidine, with histidine/imidazole or histidine/azide ligands) and zone O (hemoglobin hydroxide, with histidine and hydroxide ligands). Complexes in these four fields all have histidine as the proximal ligand, thus their heme geometry is relatively similar, defined by the four pyrrole nitrogen ligands and the imidazole ligand of the histidine. Ligation of the sixth ligand modulates the tetragonal field effect with the more electronegative ligand showing the stronger influence (14). Compounds falling in the P zone having a proximal thiolate ligand and appear to exhibit a different geometry from compounds in the other four domains.

For simplicity, we selected P450 and chloroperoxidase and their derivatives containing mainly a nitrogen donor at the sixth position for direct comparison with our low spin eNOS complexes. To provide a contrast with these thiolate-ligated heme proteins, we also included a number of hemeproteins containing non-thiolate proximal ligands but with a nitrogen ligand at the sixth position. The seven eNOS low spin heme complexes (two from the imidazole complexes) in this study clustered in a distinct region between the P and O zones of low spin heme centers as originally defined by Blumberg and Peisach (23) (dashed line in Fig. 7). There is substantial overlap between the heme derivative of chloroperoxidase and eNOS and also, to a lesser extent, between eNOS and P450cam. The region defined by these three enzymes is clearly separated from the other hemeproteins in this presentation (Fig. 7). Comparison of Fig. 7 with the original description of Blumberg and Peisach (23) reveals two major differences. There is now a significant overlap between the C and B zones and an expansion of zone P with a corresponding shrinking of zone O. This latter modification was due to substantial overlap among the three thiolate-ligated heme proteins, P450, chloroperoxidase, and NOS in Fig. 7, and is substantiated by the extensive comparisons between P450 and chloroperoxidase of Sono and Dawson (30–32).

As pointed out previously, thiolate is a sufficiently dominating ligand that attenuates the effect of the distal ligand. One example, shown in Fig. 7, is that the cyanide derivative of all three thiolate-ligated hemeproteins falls nicely in the P zone. In contrast, cyanide significantly changes the location of b- and c-type hemeproteins in the truth diagram, because cyanide dominates when there are N or O donors at the proximal position (Fig. 7, CN− zone). A similar logic can be applied to the significant overlap between the C and B zones. In this case, the proximal imidazole becomes the decisive ligand and significantly outweighs the influence of methionine, thus attenuating differences in electronic effects exerted by His/Met and His/His ligand pairs for some of the c- and b-type cytochromes (Ref. 38 and Fig. 7).

The x-ray crystallographic data of P450cam (39) and chloroperoxidase (35) reveal the differences of the heme environment at the distal ligand pocket. P450cam and other P450 proteins have a very hydrophilic pocket for the distal heme ligand (39); by contrast chloroperoxidase resembles other peroxides with much more polar surroundings in the distal pocket (35). The similarity in tetragonal field between eNOS and chloroperoxidase contrasted with P450cam suggests that the eNOS distal ligand has a more hydrophilic environment than regular P450. This is not unexpected considering that it must interact with the relatively polar substrates, L-arginine or N-hydroxyarginine during catalysis.

In summary, these EPR studies are in strong support of our earlier optical characterization in defining changes of heme spin states and heme geometry produced by different heme and amino acid ligands. Some ligands result in low spin heme complexes and change the heme structure by directly coordinating with the heme iron. Other ligands yield high spin complexes presumably by perturbing the heme geometry by indirect mechanisms and by excluding the original distal heme ligand of the resting enzyme. The structural perturbation induced by the high spin ligands, although apparently subtle, does seem to depend on the chemical structure of the ligands. The flavin radical of eNOS is a neutral semiquinone radical. The high stability of the flavin semiquinone detected by EPR is the consequence of efficient dipolar interaction with a heme center, either the low spin or high spin heme. Calcium plus...
calmodulin did not change the saturation of the flavin radical, indicating that calmodulin binding might not bring about a significant change of the distance between the reductase and the oxygenase domains. The distal heme ligand of eNOS has a significant change of the distance between the reductase and the oxygenase domains. This is undoubtedly dictated by its more polar environment than P450 and more closely resembles the oxygenase domains. The distal heme ligand of eNOS has a significant change of the distance between the reductase and the oxygenase domains. This is undoubtedly dictated by its more polar environment than P450 and more closely resembles the oxygenase domains.

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