Binding of Intermediate, Product, and Substrate Analogs to Neuronal Nitric Oxide Synthase: Ferriheme is Sensitive to Ligand-Specific Effects in the L-Arginine Binding Site†

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ABSTRACT: The electron paramagnetic resonance spectra of purified neuronal nitric oxide synthase indicates that the binding of ligands to the arginine site perturbs the environment of the high-spin ferriheme in a highly ligand-specific manner. Four categories of high-spin complex can be distinguished; all are five-coordinate, and all retain the axial thiolate ligand, but they differ in their ligation geometries. These spectroscopic species reveal distinct local conformations which can be stabilized individually by the binding of L-arginine, N^{ω}-hydroxy-L-arginine, N^{ω}-methyl-L-arginine, and N^{ω}-nitro-L-arginine. Other arginine analog inhibitors stabilize one or more of these states, revealing patterns based on the nature of substituents at the terminal amino group.

Enzymatically generated nitric oxide is an important molecular messenger in the control of smooth muscle tone (Palmer *et al*., 1987) and in neuronal communication (Garthwaite *et al*., 1988). Endothelial and neuronal nitric oxide synthases, the constitutive isoforms of the enzyme, generate NO as a signal molecule under the control of calcium/calmodulin (Bredt & Snyder, 1990; Pollack *et al*., 1991). The inducible isoform of the enzyme (iNOS) is synthesized in response to an immune challenge (Hauschildt *et al*., 1990; Knowles *et al*., 1990; McCall *et al*., 1989; Curran *et al*., 1989) and produces NO at cytotoxic levels even at low intracellular calcium levels. Overproduction of NO by iNOS is a problem in both chronic (diabetes, arthritis) and acute (toxic shock syndrome) conditions with an autoimmune component (Cho *et al*.,1992).

Nitric oxide synthases (NOS) catalyze the formation of NO from arginine in two sequential monooxygenation reactions (Marletta *et al*., 1988; Bromberg & Pick, 1989; Iyengar *et al*., 1987; Palmer *et al*., 1988). *N*-Hydroxyarginine and citrulline are the products of these reactions, which consume a total of 1.5 mol of NADPH/mol of NO formed.

The enzymes contain heme (McMillan *et al*., 1992; White *et al*., 1992; Stuehr & Ikeda-Saito, 1992), tetrahydrobiopterin (Mayer *et al*., 1991), and flavin mononucleotide and flavin adenine dinucleotide (FMN and FAD) (McMillan *et al*., 1992; Mayer *et al*., 1991; Bredt *et al*., 1991, 1992; Xie *et al*., 1992; Lyons *et al*., 1992; Janssens *et al*., 1992; Lamas *et al*., 1992). The heme appears to be the site of oxygen activation for both monoxygenation reactions, while the C-terminal halves of the enzymes contain regions highly homologous to the FMN- and FAD-binding domains of NADPH−cytochrome P450 reductase (Bredt *et al*., 1991). The N- and C-terminal halves of the neuronal isoform (nNOS) have been independently expressed; the N-terminal half contains the heme-binding region and the biopterin and arginine binding sites (McMillan & Masters, 1993, 1995; Nishimura *et al*., 1995).

Inhibition of NO synthase by arginine analogs was reported by Hibbs *et al.* (1987) and investigations aimed at producing clinically useful NOS inhibitors have concentrated on ligands which bind to the arginine site. We recently reported spectroscopic evidence of perturbation of the heme site on binding of arginine or the inhibitors thiocitrulline and homothiocitrulline (Salerno *et al*., 1995); arginine and thiocitrulline had previously been shown to bind tightly and competitively to nNOS (Frey *et al*., 1994). In the following communication we show that the intermediate *N*-hydroxyarginine and several arginine analog inhibitors of nNOS cause characteristic changes in the electron paramagnetic resonance spectra of nNOS ferriheme attributable to distortions of the ligand geometry of the heme iron.

MATERIALS AND METHODS

Enzyme Purification. Rat nNOS was purified from stably transfected kidney 293 cells as described previously (McMillan & Masters, 1993).

Sample Preparation and Spectroscopy. Enzyme samples were prepared as described previously (Salerno *et al.*, 1995) and concentrations were determined on the basis of heme concentration. Samples for spectroscopy were prepared by addition of concentrated solutions of ligand to purified enzyme; in all cases, the volume change caused by ligand addition was <2%. Samples (0.25 mL) for electron paramagnetic resonance (epr) measurements were frozen in quartz epr tubes in a 6:1 isopentane−cyclohexane mixture cooled with liquid nitrogen and stored in liquid nitrogen. Spectra were recorded using a Bruker 300D EPR spectrometer and an Oxford liquid helium cryostat. Spectra were acquired at 9.445 GHz, with instrument parameters as noted in the figure legends. Analysis of epr spectra was performed using standard methods as described previously (Salerno *et al.*, 1995).

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Several methods of quantitation were used. We estimated the relative proportions of high- and low-spin states by simulating the spectra and comparing the areas under the g_{max} feature with the results of simulation at 1:1 stoichiometry. This was checked by converting the enzyme to high-spin completely, *i.e.*, with N^{ω}-methyl-L-arginine, and assuming that all the ferriheme was present as either the detectable high-spin or low-spin features; this implies that the decrease in low-spin concentration is equal to the increase in high-spin concentration. Reasonable agreement was obtained. The relative concentrations of high-spin forms were estimated by comparison of the areas under the g_{max} feature with a small intensity factor correction as previously described (Salerno *et al.*, 1995).

Materials. All chemicals used for purification were obtained from Sigma Chemical Co. N^{ω}-Methyl-L-arginine (NMA), 7-nitroindazole (7-NI), and N^{δ}-iminoethyl-L-ornithine were purchased from Alexis Corp. (San Diego, CA), and N^{ω}-nitro-L-arginine (NNA) was from Research Biochemicals International (Natick, ME).

RESULTS

Binding of N^{ω}-Hydroxy-L-arginine to NOS. The majority of the ferriheme of nNOS as isolated is in a high-spin state with significant rhombic distortion resembling that observed in other high-spin species with a thiolate axial ligand. Previously, we showed that arginine binding to nNOS converted the remaining low-spin ferriheme to a high-spin form and that, in the presence of saturating L-arginine, essentially all the ferriheme was represented by a single high-spin species with slightly smaller rhombicity than the original high-spin species (Salerno *et al.*, 1995).

Figure 1, line A, shows the electron paramagnetic resonance spectrum of nNOS at 10 K as isolated. As previously reported, the majority species is a high-spin ferriheme with $g_x = 7.65$, $g_y = 4.04$, and $g = 1.8$. The low-spin state contributes features at $g_x = 1.9$, $g_y = 2.28$, and $g_z = 2.43$, while the free radical signal at $g = 2.0$ is contributed by flavosemiquinone. A signal at $g = 4.3$ from high-spin rhombic Fe^{3+} represents a small amount of non-heme iron; it is intense because the middle Kramers' doublet is almost magnetically isotropic and has high transition probability. The broad signal on the low-field side of $g = 2$ is due to a cavity contaminant.

As shown in line B, N^{ω}-hydroxy-L-arginine (NHA) binding converts the enzyme almost quantitatively to high-spin and leads to a sharpening of the high-spin epr spectrum. A small remnant of broad low-spin features can be detected at higher temperatures (>20 K; data not shown). The **g** tensor of the new high-spin species is similar to that of the enzyme as isolated; $g_x = 7.67$, $g_y = 3.98$, and $g_z = 1.79$, and the differences are difficult to detect over such a broad scan range.

Figure 1, line C shows the epr spectrum of nNOS holoenzyme incubated with the inhibitor, N^{ω}-amino-L-arginine. The epr spectrum of the N^{ω}-amino-L-arginine complex is strikingly similar to that of the NHA complex (Figure 1B), with $g_x = 7.67$, $g_y = 3.98$, and $g_z = 1.79$. Broad signals near $g = 10$ and $g = 3.5$ are due to liquid oxygen present in minute cracks in the frozen sample. As in the case of *N*-hydroxyarginine binding, *N*-aminoarginine binding results in epr spectral changes that are more subtle than those observed when arginine or thiocitrulline binds.

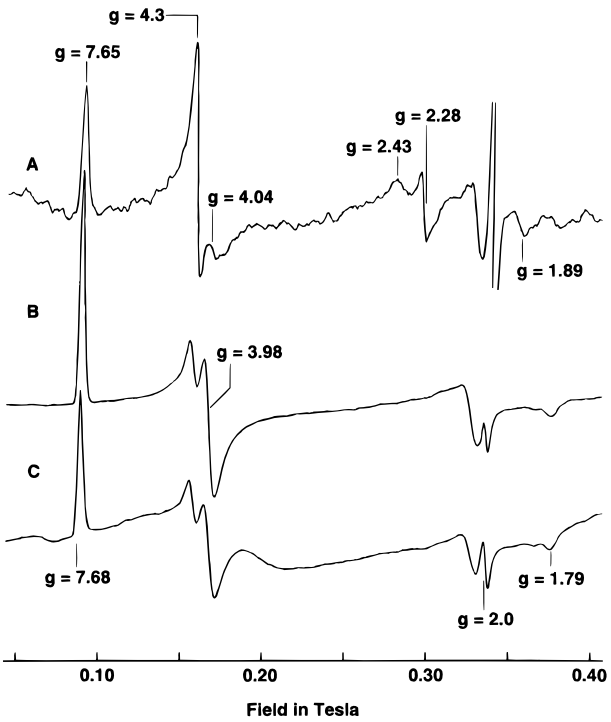

FIGURE 1: Effects of *N*-hydroxy-L-arginine and *N*-amino-L-arginine on the electron paramagnetic resonance spectrum of nNOS ferriheme. Line A, epr spectrum of purified nNOS as isolated. Line B, epr spectrum of nNOS after addition of 100 μM *N*-hydroxy-L-arginine. Line C, epr spectrum of nNOS after addition of the inhibitor *N*-amino-L-arginine. Enzyme concentrations were 20 μM as measured by ferriheme CO binding spectra. Experimental conditions were as follows: sample temperature, 10 K; microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 14.3 Gauss; scan width, 0.4 T, center field, 0.21 T.

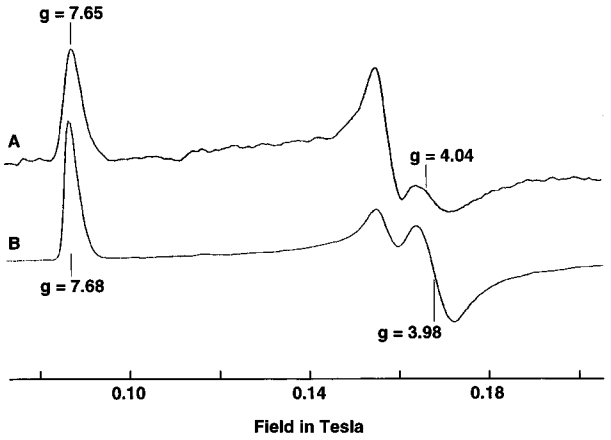

FIGURE 2: Effect of *N*-hydroxy-L-arginine binding on the electron paramagnetic resonance spectrum of high-spin nNOS ferriheme in the $g_x - g_y$ region. Line A, epr spectrum of purified nNOS as isolated; line B, epr spectrum of nNOS after addition of 100 μM *N*-hydroxy-L-arginine. Sample temperature, 7 K; other conditions were as in Figure 1.

In Figure 2, lines A and B compare the high-spin epr signals at 7 K in the $g_x - g_y$ region of nNOS as isolated and incubated with NHA, respectively. The small shifts in the positions of the lines are more obvious because of the expanded scale. It is clear that NHA binding converts nearly all the heme to a single, well-defined high-spin state with a rhombicity slightly greater than that of the high-spin ferriheme in nNOS as isolated.

Binding of N^{ω}-Methyl-L-arginine. *N*-Methyl-L-arginine (NMA) is a potent inhibitor of all NOS isoforms. It binds tightly but reversibly to the enzyme as isolated, but in the

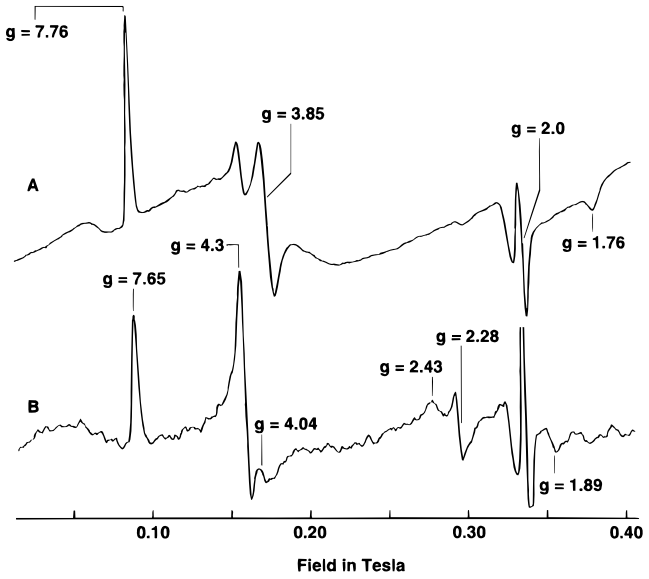

FIGURE 3: Effect of *N*-methyl-L-arginine binding on the epr spectrum of nNOS. Line A: epr spectrum at 10 K of nNOS incubated with NMA; line B: epr spectrum of nNOS as isolated. Conditions were as in Figure 1.

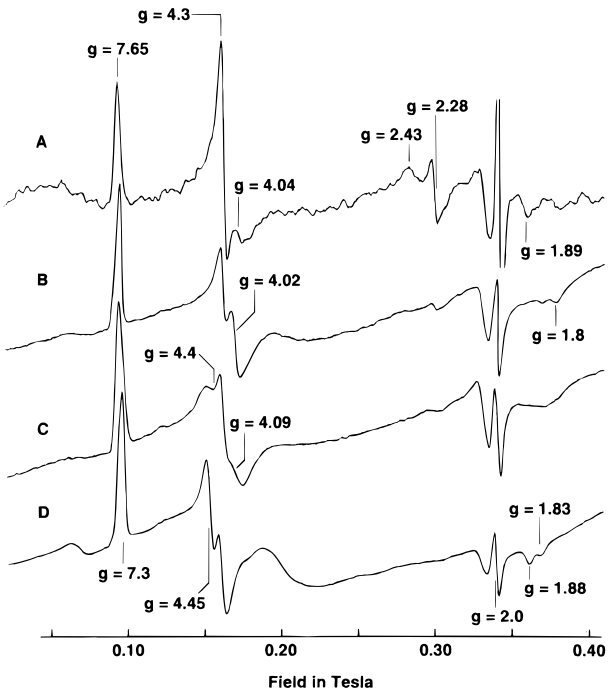

FIGURE 4: Effects on the nNOS ferriheme electron paramagnetic resonance spectra of the binding of the inhibitors *N*-iminoethyl-L-ornithine, 7-nitroindazole, and *N*-nitro-L-arginine. Line A, epr spectrum of nNOS as isolated at 10 K. Line B, epr spectrum of nNOS after addition of *N*-iminoethyl-L-ornithine. Line C, epr spectrum of nNOS in the presence of 7-nitroindazole. Line D, epr spectrum of the *N*-nitro-L-arginine complex. Conditions were as in Figure 1.

presence of NADPH it can be metabolized to form an irreversible inhibitor−enzyme complex. In Figure 3, the effect of NMA binding on the epr spectrum of nNOS is illustrated. Trace A shows the epr spectrum at 10 K of nNOS incubated with NMA; for comparison, trace B shows the spectrum of the enzyme as isolated. NMA caused a shift in the spin state equilibrium of the enzyme toward the high-spin state; almost all the enzyme is represented by a new high-spin species with $g_x = 7.76$, $g_y = 3.85$, and $g_z = 1.76$. This represents a significant increase in rhombicity relative to high-spin ferriheme in enzyme as isolated. A small signal in Figure 3A from much less rhombic ferriheme is visible between $g = 5$ and $g = 6$. This species represents only about 1% of the total ferriheme.

Binding of Arginine-like Inhibitors. Figure 4 illustrates the effects on the high-spin ferriheme ligation geometry of N^{δ}-iminoethyl-L-ornithine, 7-nitroindazole (7-NI) and N^{ω}-nitro-L-arginine (NNA), three nNOS inhibitors which are competitive with respect to L-arginine. Trace A shows the epr spectrum of nNOS as isolated at 10 K. As shown in trace B, binding of *N*-iminoethyl-L-ornithine to nNOS produces a majority high-spin state with $g_x = 7.59$, $g_y = 4.02$, and $g_z = 1.8$; this shift is similar to that produced by arginine binding but slightly smaller in magnitude.

In trace C of Figure 4, the epr spectrum of nNOS in the presence of 7-NI at 10 K indicates a conversion of most of the low-spin species to high-spin forms. The single peak at $g = 7.51$ is much broader than the corresponding high-spin features of the other inhibitor complexes of nNOS. The g_y region reveals the presence of two high-spin species of comparable intensity. The g_x feature is clearly the sum of two poorly resolved signals corresponding to the better resolved features at g_y; the g_x and g_z values for the two species can be estimated from the spectra using the relationship between the **g** tensor and the axial and rhombic zero field splitting parameters *D* and *E*. The principal values of the **g** tensors are approximately $g_x = 7.55$, $g_y = 4.09$, and $g_z = 1.81$ and $g_x = 7.3$, $g_y = 4.4$, and $g_z = 1.83$. The former species closely resembles the arginine complex, while the latter species is similar to the minority species observed in the presence of thiocitrulline and homothiocitrulline. The small signal near $g = 6$ from axial ferriheme represents less than 1% of the total heme.

Binding of N^{ω}-Nitro-L-arginine. N^{ω}-Nitro-L-arginine (NNA) is a reversible inhibitor of all NOS isoforms which has slow on and off rate constants for tight binding. Trace D of Figure 4 shows the epr spectrum of the NNA complex. The g_x and g_y regions are dominated by a single high-spin species, but at g_z there is a stronger signal at $g = 1.88$ and a weaker signal at 1.83. The majority species has $g_x = 7.3$, $g_y = 4.45$, and $g_z = 1.88$, but a significant minority species may be present with $g_x = 7.5$, $g_y = 4.1$, and $g_z = 1.83$. The g_x features are not resolved, and the g_y feature of the second species falls on top of the $g = 4.3$ signal of low-symmetry high-spin ferric iron.

Spin State Equilibria and Zero Field Splitting. Data presented here and previously (Salerno *et al.*, 1995) indicate that the ferriheme of nNOS is predominantly high-spin as isolated and in the presence of L-arginine analogs. The spin state equilibria of nNOS as isolated and nNOS saturated with L-arginine or NHA appear similar at low temperature (10−25 K) and at room temperature (∼293 K). In these cases, the high-spin form predominates at all temperatures. Arginine-saturated enzyme, in particular, is essentially 100% high-spin by epr criteria, in keeping with the type I spectral change elicited by arginine at room temperature.[1] As we previously pointed out, in the presence of thiocitrulline, a slight temperature dependence in the spin state is observed between ambient temperature and 25 K.

No evidence of temperature dependence in the spin state equilibrium could be detected at temperatures between 7 and 23 K. The temperature dependence of the high-spin ferriheme epr spectra in nNOS as isolated or in the presence of L-arginine, NHA, or L-thiocitrulline could be accounted for

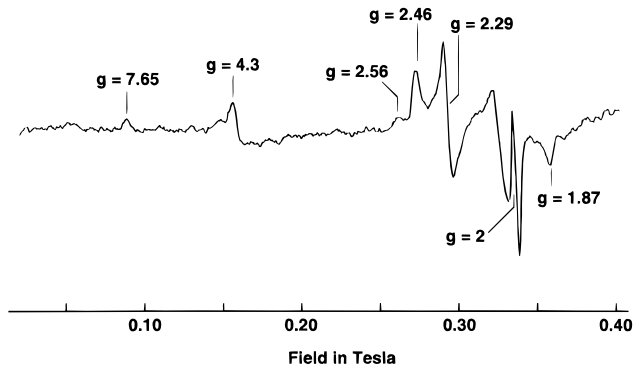

FIGURE 5: Electron paramagnetic resonance spectrum at 20 K of nNOS in the presence of 10 mM citrulline. Other conditions were as in Figure 1.

by the Curie law dependence arising from the population ratio within the lowest Kramers' doublet and an additional factor representing the Boltzmann equilibrium of the three $S = {}^5/_2$ Kramers' doublets, assuming a value of the axial zero field splitting parameter $D \sim 3.8$ cm^{-1} [see Salerno *et al*. (1995)]. This is smaller than the value of D expected for a ferriheme with histidyl ligation, but is reasonable for a ferriheme with a thiolate axial ligand, as indicated by other evidence. A more detailed set of experiments, requiring measurements over a wide range of precisely determined temperatures, will be required to produce an accurate measurement of D.

L-Citrulline Complex. L-Citrulline is a product of the NO-generating reaction catalyzed by NOS. Although citrulline has been reported to be a poor inhibitor of the enzyme, this merely reflects a high K_d compared to substrate. Figure 5 shows the electron paramagnetic resonance spectrum at 20 K of nNOS in the presence of 10 mM citrulline. The spectra indicate a significant shift in the spin state equilibrium toward the low-spin states; the proportion of low-spin ferriheme is 2−4 times greater than in nNOS as isolated. Approximately half the ferriheme is in the low-spin state.

The high-spin species in the presence of citrulline resembles the original high-spin state, and it is not clear that the enzyme is saturated with citrulline under these conditions. It is possible that citrulline is also bound to the high-spin species but that citrulline is incapable of perturbing the heme ligation geometry without serving as a sixth ligand.

Two new low-spin forms can be observed; the majority low-spin form has $g_x = 1.87$, $g_y = 2.29$, and $g_z = 2.46$. A minor species with $g_z = 2.56$ is also visible, and a remnant of the original low-spin species may be present as a shoulder to the left of the $g_z = 2.46$ feature. It is clear that the thiolate ligand has been retained in the low-spin states as well as in the high-spin state. Comparison with appropriate model complexes (Sono & Dawson, 1982) suggests that the new low-spin species observed in the presence of high citrulline concentrations probably represent six-coordinate ferrihemes in which the carbonyl oxygen ($g_z = 2.46$) or the ϵ-amino nitrogen ($g_z = 2.56$) of citrulline serve as the sixth ligand of the heme.

Table 1: High-Spin Complexes of nNOS Holoenzyme[g]

ligand	g_x	g_y	g_z	E/D
group I: NMA-like				
N-methylarginine	7.76	3.85	1.76	0.087
thiocitrulline 1[a]	(7.76)	3.83	∼1.75	0.087
homothiocitrulline maj.[b]	7.75	3.9	∼1.75	0.087
group II: NHA-like				
thiocitrulline 2[a]	(7.68)	3.98	1.79	0.081
N-aminoarginine	7.68	3.98	1.79	0.081
N-OH-arginine	7.68	3.98	1.79	0.081
as isolated[c]	7.65	4.04	1.8	0.079
group III: arginine-like				
N-iminoethylornithine	7.59	4.02	1.8	0.078
arginine[c]	7.56	4.09	1.805	0.075
7-nitroindazole 1[d]	(7.55)	4.09	(1.81)	0.075
group IV: NNA-like				
homothiocitrulline min.[e]	7.33	4.46	1.86	0.063
7-nitroindazole 2[d]	(7.3)	4.4	(1.86)	0.061
N-nitroarginine[f]	7.26	4.43	1.88	0.059

[a] High-spin species present at comparable concentrations (Salerno *et al*., 1995). [b] Majority species (Salerno *et al*., 1995). [c] (Salerno *et al*., 1995). [d] High-spin species present at comparable concentrations. [e] Minority species; similar species observed in the presence of thiocitrulline and in the enzyme as isolated at very low concentrations are not listed in the table (Salerno *et al*., 1995). [f] Majority species; a second slightly less rhombic species which may also be present is not shown in the table (see text). [g] Entries show principal g values and zero field splitting parameters of the most important high-spin species observed in electron paramagnetic resonance experiments with nNOS. The species listed represent the majority of the ferriheme present unless otherwise noted. Values in parentheses were resolved by computation (e.g., because two species were overlapped).

DISCUSSION

The results presented here and in the previous communication (Salerno *et al*., 1995) demonstrate not only that binding of substrate (L-arginine), intermediate (N^{ω}-hydroxy-L-arginine), product (L-citrulline), and L-arginine analog inhibitors perturbs the spin state equilibrium of nNOS ferriheme but also that the ligation geometry of the ferriheme is altered, producing ligand-specific high and low-spin species. This constitutes a spectroscopic fingerprint for occupation of the substrate binding site. The sensitivity of electron paramagnetic resonance spectra to substitutions on the arginine side chain provides a powerful probe of interactions between the oxygen-binding position on the heme and the arginine-binding site on the polypeptide.

The characteristics of the high-spin nNOS ferriheme species observed in these experiments are summarized in Table 1. The high-spin nNOS ferriheme species observed have been separated into four groups, as discussed below; while the classification of a few of the species is slightly ambiguous, the existence of clusters of similar species is at this point obvious. The ability of several different arginine analogs to produce species with virtually identical epr spectra suggests that much of the effect is produced by the stabilization of local conformational states by ligand binding; this could produce a tendency to cluster around preexisting conformational energy minima. Small changes within each group could be caused by substituent-specific modifications of these favored conformations.

The differences between groups I−III are comparable to the differences between P450 isoforms and represent changes

[1] The constant high-spin/low-spin ratio observed in the enzyme as isolated over a wide temperature range suggests that the spin state equilibrium is frozen in at a fairly high temperature, perhaps even during freezing of the solution. It would be remarkable if the temperature dependence of the free energy difference between the states exactly matched the kT energy denominator in the Boltzmann distribution, and examples of temperature dependent spin state equilibria are well-known [e.g., Tamura (1971)]. Since spin state changes are coupled to processes (i.e., conformational changes or changes in ligand orientation) with strongly temperature-dependent kinetics, however, the spin state distribution can sometimes be effectively frozen in by a relatively small temperature decrease.

in ligation geometry rather than axial ligand replacement. It is likely that the axial thiolate ligand has also been retained in the group IV; although the rhombicity is smaller than that observed in other heme proteins with thiolate axial ligands, similar spectra have been obtained for ferriheme model complexes with aryl thiolate axial ligation.

Group I: N^{ω}-Methyl-L-arginine. The first group consists of the NMA complex, the more rhombic of the two major L-thiocitrulline species, and the majority species formed by L-homothiocitrulline. The differences in the *g* values of the first two complexes are of the same order as the measurement error resulting from overlapped signals. The L-homothiocitrulline complex is also similar but is slightly less rhombic ($E/D \sim 0.085$ vs $E/D \sim 0.087$).

These are the three most rhombic high-spin nNOS species so far detected, and they represent a substantial change in ligation geometry from the enzyme as isolated ($E/D \sim 0.079$). Binding of these arginine analogs results in a heme ligation geometry outside the range available to nNOS ferriheme in the enzyme as isolated. The thiolate ligand is not displaced, however; in fact, these species are the nNOS species which most resemble P450 ferriheme spectroscopically.

Group II: N-Hydroxy-L-arginine. The second group consists of the second thiocitrulline complex, the *N*-amino-L-arginine complex, the NHA complex, and the high-spin form of nNOS as isolated. The first three species are virtually indistinguishable and exhibit a small increase in rhombicity relative to the enzyme as isolated ($E/D \sim 0.081$ vs $E/D \sim 0.079$). We note that the intermediate (NHA) binds tightly with minimal disruption of the heme ligation geometry.

The epr spectra of these species are *g* strain-broadened; the principal contribution to line width is likely to be a distribution in *E*/*D* reflecting the range of heme ligation geometries conformationally accessible at ambient temperature and frozen in as the temperature was lowered. Binding of NHA or *N*-amino-L-arginine produces a single high-spin species with a narrow line width. The difference between the line positions within this group is less than the line widths. This suggests that these ligands restrict the portion of the conformational space available to the enzyme in the vicinity of the active site but do not force the ligation geometry of the ferriheme outside of the range available to it in the absence of ligand.

Group III: L-*Arginine.* This group consists of the *N*-imino-L-ornithine complex, the arginine complex, and the more rhombic of the two species observed in the presence of 7-nitroindazole. These species are characterized by moderate decreases in the rhombicity of the ferriheme compared to nNOS as isolated. Inclusion of N^{ω}-imino-L-ornithine in this group rather than in group II is somewhat arbitrary, since the complex it forms with nNOS lies midway between the arginine and NHA complexes in rhombicity. It was included in group III because the resulting rhombicity is lower than that of the enzyme as isolated and because g_x, which in this case can be measured more accurately than g_y, is close to g_x of the arginine complex. It is possible that the estimate given here for *E*/*D* (0.078) is slightly high because of the difficulty in estimating g_y and that *E*/*D* is actually 0.076 or 0.077.

The group III species are characterized by a change in the heme ligand geometry opposite that seen with the complexes in the first two groups. Arginine binding induces a larger change, in terms of *E*/*D*, than NHA binding. Like NHA, arginine binding produces a sharpened epr spectrum, suggesting that the region of conformational space accessible to the heme pocket is restricted by ligand binding.

Group IV: N^{ω}-Nitro-L-arginine. The species in group IV include the NNA complex, the minority homothiocitrulline high-spin species, and the less rhombic of the two species observed in the presence of 7-nitroindazole. Similar species are observed as minority components under other conditions (e.g., in the presence of thiocitrulline and as isolated). They are characterized by a large decrease in rhombicity in comparison to the enzyme as isolated; in the case of the NNA complex, *E*/*D* decreases from 0.079 to 0.059.

The reason for this large change in rhombicity is unclear. Rhombicity in ferric porphyrinoid model thiolates is sensitive to the character of the thiolate (e.g., to the acidity of the group), but geometric changes imposed by the polypeptide are likely to be more important in heme proteins. Interactions with the porphyrin periphery are unlikely to affect *E*/*D*, but stretching or bending of the sulfur−iron bond could produce the changes observed.

Epr Spectra, Ligand Field Geometry, and Arginine Analogs. The features of the high-spin nNOS ferriheme epr spectrum at $g \sim 7.6$, 4, and 1.8 are associated with transitions within the lowest Kramers' doublet of the $S = {}^5/_2$ sextet of high-spin ferric heme. They are derived from molecules in which the orientation of the Zeeman magnetic field lies along the *x* and *y* directions in the plane of the heme; the z direction (normal to the heme) gives rise to a transition near $g = 1.8$. As long as intermixing of the Kramers' doublets by Zeeman terms ($\sim$0.3 cm^{-1}) is negligible,[2] the spectra are controlled by the ratio *E*/*D*, where *E* is the rhombic zero field splitting parameter [see Salerno et al. (1995) and references therein]. Terms in *E* mix the three $S = {}^5/_2$ Kramers' doublets, splitting g_x and g_y about the axial value of 6 to first-order and lowering both g_z and $(g_x + g_y)/2$ as a second-order effect. The primary role of *E*/*D* in determining the epr spectra allows reasonable estimates of individual g_x values to be made when features in this region are not resolved (*e.g.*, with 7-nitroindazole).

Both *E* and *D* are determined by ligand-induced splittings of the iron d orbitals (Kotani, 1969), which, in general, are a function of both the identity of the axial ligands and the geometry of the heme site. All the high-spin nNOS epr spectra fall within the range of *E*/*D* characteristic of enzymes and model complexes with thiolate ligands; the identity of the axial ligands is the same in all the high-spin complexes of nNOS produced by binding of substrate and substrate analogs which we have examined.

Changes in *E*/*D* observed here are controlled through E by changes in the relative energy of *the d_{xz} and d_{yz}* orbitals. Rhombic character is in general dependent on the axial ligand, which can split the d_{xz} and d_{yz} orbitals by π interactions. Our estimates of *E*/*D* were obtained by exact diagonalization of the matrix of the $S = {}^5/_2$ spin Hamiltonian $D\{S_z^2 - {}^1/_3[S(S+1)]\} + E(S_x^2 - S_y^2)$. The spectral changes observed after the addition of arginine are caused by a decrease in *E*/*D* from about 0.079 to 0.075. Since *D* is probably about 3.8 cm^{-1}, this corresponds to a change in *E* from about 0.30 cm^{-1} to about 0.285 cm^{-1}.

As we pointed out previously, the changes in *E*/*D* observed in the high-spin complexes are not indicative of direct

[2] The effect of the Zeeman terms lowers the average value of g_x and g_y slightly in these complexes, from about 6 to 5.8 or 5.9.

binding to the heme iron by arginine analogs. This would produce low-spin complexes of the type seen with imidazole or in a fraction of the enzyme molecules in the presence of citrulline or thiocitrulline. The changes in *E*/*D* reflect distortions in the ligand field geometry which are likely to be mediated by local conformational changes transmitted to the iron orbitals by distortions in the porphyrin and by polypeptide-imposed strain on the metal−thiolate interaction trans to the arginine binding site. Direct steric interaction between the porphyrin and substrate analogs cannot be ruled out but would not account for the observed specificity of the changes for substituent groups.

Shape of the Binding Site and Modes of Binding. Arginine and NHA, the substrate and intermediate of nNOS, both bind tightly to the substrate binding site. Both produce essentially a single high-spin species with narrow lines, suggesting that each ligand binds in a single well-defined orientation and that binding restricts the conformational flexibility of both the enzyme and the active site. Since NHA produces a very small distortion of the heme site, the shape of the binding site as isolated may closely resemble the intermediate. The larger distortion in heme ligation geometry produced by arginine further suggests that both arginine and the substrate binding site experience some strain associated with arginine binding.

N-methyl-L-arginine (NMA) produces a larger distortion of the heme environment as measured by *E*/*D*. NMA still binds tightly to nNOS and, like the substrate and intermediate, produces a single species characteristic of a unique binding mode and restricted conformational flexibility. The greater distortion it induces may be in part a steric consequence of the methyl group but it also depends on the hydrophobic nature of the substituent, since other substituents at this position have far different consequences.

N-Nitro-L-arginine (NNA) is intriguing because of the large decrease in *E*/*D* it produces without causing the loss of the axial ligand. It is likely that the appearance of this species is due to a significant conformational rearrangement of the binding site−heme pocket region. Such a rearrangement would explain why NNA has slow on and off constants associated with its tight binding mode. It may be possible to trap intermediates in *N*-nitro-L-arginine binding even without rapid freezing equipment.

It seems likely that much more information about the substrate binding sites of the NOS isoforms will be made available through further inhibitor studies. It may be productive to consider inhibitors producing group I type as NMA-like. In the same way, *N*-amino-L-arginine can be considered an NHA-like inhibitor, and *N*-iminoethyl-L-ornithine might be considered an arginine-like inhibitor. *N*-amino-L-arginine is a reasonable steric match for NHA; NMA can be differentiated from these by the hydrophobicity of the substituent group. *N*-Iminoethyl-L-ornithine is a reasonable steric match for arginine; the difference between these complexes can be attributed to the greater hydrophobicity of the methyl group of *N*-iminoethyl-L-ornithine vs the second ϵ-amino group of arginine.

Thiocitrulline, homothiocitrulline, and 7-nitroindazole are examples of inhibitors which bind in multiple modes. As we have previously pointed out, the multiple species produced by thiocitrulline could result from alternative ligand orientations or structures, or from alternative conformational adjustments induced in the binding site. These explanations are not mutually exclusive. It now appears that the two major thiocitrulline species represent an NMA-like binding mode and an NHA-like binding mode. The thiol group may be about equally well accommodated in conformations favored by the methyl and hydroxyl substituents.

Homothiocitrulline and 7-nitroindazole appear to be bound, respectively, in NMA-like and arginine-like modes but also to stabilize some of the NNA-like state. Homothiocitrulline, NNA, and 7-nitroindazole all represent steric challenges to the binding site, because of the extra methyl group, the relatively bulky nitro substituent, and the large fused ring system, respectively. It would be of considerable interest to examine the time dependence of the appearance of these multiple forms of nNOS inhibitor complexes.

The methods applied here suggest a description of the substrate binding site which will be refined by the extension of these studies to inhibitors with different substitutions. It remains to be seen whether all NOS isoforms will follow the pattern observed here, or whether a different series of states will be revealed when data from those systems become available.

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