# Substrate Binding-Induced Changes in the EPR Spectra of the Ferrous Nitric Oxide Complexes of Neuronal Nitric Oxide Synthase†

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ABSTRACT: A versatile diatomic physiological messenger, nitric oxide (NO), is biosynthesized by a group of flavo-heme enzymes, the nitric oxide synthases. We have examined the active site of the neuronal isoform by EPR spectroscopy of the ferrous nitric oxide complex. The nitric oxide complex of the substrate-free enzyme exhibits a cytochrome P450-type EPR spectrum typical of a hexacoordinate NO−heme complex with a non-nitrogenous proximal axial heme ligand. The NO complex of the substrate-free enzyme is rather unstable and spontaneously converts to a cytochrome P420 type pentacoordinate denatured form. Binding of L-arginine (L-Arg) enhances the stability of the hexacoordinate NO form. The EPR spectrum of the NO adduct of the enzyme−substrate complex has an increased *g*-anisotropy and well-resolved hyperfine couplings due to the $^{14}N$ of nitric oxide. Significant perturbations in the NO EPR spectrum were observed upon $N^{\omega}$-monomethyl-L-Arg and $N^{\omega}$-hydroxy-L-Arg binding. The perturbations in the EPR spectrum indicate that L-Arg and its derivatives bind on the distal site of the heme in very close proximity to the bound NO to cause alterations in the heme−NO coordination structure. Interactions between the bound NO and the substrate or its analogues appear to affect the Fe−NO geometry, resulting in the observed spectral changes. We infer that analogous interactions with oxygen might be involved in the hydroxylation events during enzyme catalysis of nitric oxide synthase.

The free radical NO[1] serves as a physiological vasodilator, neurotransmitter, and cytostatic agent (Nathan & Hibbs, 1991; Marletta, 1993; Masters, 1994; Bredt & Snyder, 1994; Griffith & Stuehr, 1995). NO is produced by isoenzymes termed NOS which catalyze the NADPH-dependent conversion of L-Arg to NO and L-citrulline, with $N^{\omega}$-hydroxy-L-Arg being formed as an intermediate (Klatt et al., 1993). Neuronal NOS is a homodimer that contains one molecule of heme, FAD, FMN, and $H_4$biopterin per subunit (Marletta, 1993; Masters, 1994; Bredt & Snyder, 1994; Griffith & Stuehr, 1995). The consensus binding sites for NADPH, FAD, and FMN all reside in the carboxyl-terminal half of NOS, whose sequences are highly homologous to the flavoprotein NADPH−cytochrome P450 reductase, that also contains FAD and FMN (Bredt et al., 1991). The N-terminal domain contains the heme, substrate (L-Arg), and $H_4$biopterin binding sites (McMillan & Masters, 1995). The flavins mediate electron transfer from NADPH to the heme group. In neuronal NOS, this intramolecular electron transfer is facilitated by the binding of $Ca^{2+}$-calmodulin and substrate L-Arg (Abu-Soud & Stuehr, 1993; Matsuoka et al., 1994).

The heme group of NOS has recently been studied by EPR, MCD, and resonance Raman spectroscopies (Stuehr & Ikeda-Saito, 1992; Wang et al., 1993, 1994; Sono et al., 1995; Salerno et al., 1995, 1996a,b). The results of these spectroscopic studies have consistently shown that the axial ligand of the heme iron is a thiolate as in the cytochrome P450 enzymes, indicating that cytochrome P450-type oxygen activation and catalysis (Dawson & Sono, 1987) are likely to take place at the heme site in NOS. L-Arg binding to NOS alters the electronic structure of the ferric heme iron and reduces association rates and affinity constants of exogenous ligands, such as cyanide and carbon monoxide (Matsuoka et al., 1994; Salerno et al., 1995; McMillan & Masters, 1993). On the basis of these observations, L-Arg has been considered to bind in the distal heme pocket close to the sixth coordination position of the heme iron (Matsuoka et al., 1994; Salerno et al., 1995; Berka et al., 1996) as observed in cytochrome P450 enzymes (Sligar & Murray, 1987; Raag & Poulos, 1991).

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[1] Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; Tris, tris(hydroxymethyl)aminomethane; Arg, arginine; cytochrome $P450_{LM}$, cytochrome P450 of livers of phenobarbital-treated rat; cytochrome $P450_{SCC}$, cytochrome P450 of bovine adrenal cortex; cytochrome $P450_{CAM}$, cytochrome P450 from *Pseudomonas putida*; mT, millitesla.

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NO binds as an axial ligand of the heme iron of many hemoproteins including NOS (Sono et al., 1995; Wang et al., 1994). Previous spectroscopic studies on the NOS−NO complex have been limited to optical absorption, MCD, and resonance Raman studies. EPR spectroscopy of ferrous NO complexes of hemes and hemoproteins directly detects an unpaired electron spin residing on the heme−NO system, thus serving as a sensitive probe for the nature of the heme iron ligand *trans* to the bound NO and the ligand coordination geometry (Yonetani et al., 1972; Chien & Dickinson, 1977; Hori et al., 1981; Waleh et al., 1989). The major objective of this work was to detect effects of substrate binding upon the NOS heme center by EPR spectroscopy of its ferrous NO complex. In cytochrome $P450_{SCC}$, substrate binding significantly altered the ferrous NO EPR spectral properties (Tsubaki et al., 1987b). In cytochromes $P450_{CAM}$ and $P450_{SCC}$, substrate binding appears to enhance the stability of the hexacoordinate ferrous NO complexes (O'Keeffe et al., 1978; Tsubaki et al., 1987b). If the stereochemical positioning of the substrate binding site and the heme group in NOS is similar to that of cytochrome P450 enzymes as previously proposed (Matsuoka et al., 1994; Berka et al., 1996), then we would expect that L-Arg binding should enhance the stability of the hexacoordinate NO complex and alter the EPR spectral properties of the ferrous NO complex. Furthermore, experiments on the $N^{\omega}$-hydroxy-L-Arg- and $N^{\omega}$-methyl-L-Arg-bound NOS are expected to provide information on the enzyme interactions with the intermediate substrate and mechanism-based inhibitors, respectively. In light of the recent report on the formation of the inhibitory ferrous NOS−NO complex with self-generated NO during catalysis (Wang et al., 1994), we think it also important to characterize the NOS−NO complex by EPR.

We have studied the EPR spectroscopic properties of the ferrous NO complexes of neuronal NOS. Our results show that the reaction of the ferrous NOS with NO forms a hexacoordinate NO species with partial but inevitable formation of a pentacoordinate P420-type NO complex in the absence of L-Arg. In the presence of L-Arg, we found that the NO complex of neuronal NOS is very stable and shows an EPR spectrum slightly different from that in the absence of L-Arg. A significant change in the NO EPR spectrum was observed upon $N^{\omega}$-monomethyl-L-Arg and $N^{\omega}$-hydroxy-L-Arg binding. This is the first report on the EPR spectral properties of the NOS−NO complexes, and provides clear evidence for the changes in the heme−NO system induced by substrate binding.

## EXPERIMENTAL PROCEDURES

Rat neuronal NOS was purified from stably transfected kidney 293 cells as described previously (McMillan et al., 1992). The protein samples were in 0.05 M Tris buffer, pH 7.5, containing 10% glycerol. $N^{\omega}$-Monomethyl-L-Arg and $N^{\omega}$-hydroxy-L-Arg were purchased from Alexis, and L-Arg was obtained from Sigma.

NO complexes were prepared by the reaction of dithionite-reduced enzyme or CO complex of reduced enzyme with NO gas. The NOS solution was placed in a test tube with a screw top fitted for a rubber septum. The enzyme solution was deoxygenated by repeated evacuation and filling with nitrogen, and then the test tube was filled with CO gas to 1.2 atm. Sodium dithionite was dissolved in anaerobic 0.05 M Tris buffer, pH 7.5, in a test tube with a screw top fitted with a rubber septum. Sodium dithionite was added to a final concentration of 10 mM to the NOS solution in the anaerobic test tube through a gas-tight syringe. The enzyme was kept on ice for at least 30 min to ensure complete reduction (Sono et al., 1995). The reduced NOS was transferred to an EPR sample tube which was previously flushed with nitrogen gas through a rubber septum, and then NO gas, washed by water, was introduced into the EPR sample tube. After gentle mixing and approximately 5 min incubation on ice, the sample was flash-frozen at 200 K. The sample tube at 200 K was evacuated and then stored at 77 K until use. Usually the measurements were carried out within 30 min of NO complex formation, but an overnight storage at 77 K did not cause detectable alterations in the spectrum. Formation of a pentacoordinate NO species, the P420-type NO complex, was achieved by aerobic mixing of NO gas and sodium dithionite with the NOS solution or prolonged incubation with NO gas at room temperature (O'Keeffe et al., 1978; Tsubaki et al., 1987b). In both cases, the same EPR spectrum was observed.

EPR spectra were recorded by a Bruker ESP-300 spectrometer operating at 9.45 GHz with a field modulation of 0.2 mT at 100 kHz. Measurements were carried out at 20 or 30 K using an Oxford liquid helium flow cryostat (ESR-300). Microwave frequency was monitored by a frequency counter (HP-5350), and an NMR gauss meter (Bruker ER-035M) was used to determine the magnetic flux density.

## RESULTS

Upon reaction with NO, the dithionite-reduced ferrous form of neuronal NOS yields an EPR spectrum as shown in spectrum A in Figure 1. Spectrum B in Figure 1 is the EPR spectrum of the NO complex of NOS generated by the reaction of NO with the reduced enzyme in the absence of CO. In the presence of air or upon prolonged incubation at room temperature, the same type of spectrum was obtained. The spectrum, with sharp triplet splittings at $g_2$ with a hyperfine coupling constant of 1.66 mT, is very similar to those of the NO complexes of cytochromes $P420_{LM}$, $P420_{CAM}$, and $P420_{SCC}$ (O'Keeffe et al., 1978; Tsubaki et al., 1987b). Spectra of this type are known to arise from pentacoordinate ferrous heme−NO complexes (Kon, 1968). The iron proximal ligand bond is either broken or severely distorted in the NO complex of the P420-type NO complex. At first sight, the spectrum of the NOS−NO complex in the absence of substrate (Figure 1, spectrum A) could be regarded as a hexacoordinate NO−heme complex with a non-nitrogenous axial ligand *trans* to the bound NO. The spectrum, however, shows an absorption with a weak amplitude below 0.322 T and a slightly deformed triplet hyperfine signal. Spectrum A of Figure 1 seems to have a P420-type pentacoordinate NO EPR signal overlapping on a hexacoordinate NO EPR spectrum. Subtraction of the pentacoordinate spectrum B from spectrum A yields spectrum C. In spectrum C, besides a major component with $g_1 = 2.080$, $g_2 = 2.005$, and $g_3 = 1.970$, a minor component is present at $g \sim 2.03$ (marked by the asterisk). Similar minor components at $g \sim 2.03$ were reported for several ferrous NO hemoproteins including cytochromes $P450_{LM}$ and $P450_{SCC}$ (O'Keeffe et al., 1978; Tsubaki et al., 1987b); they have been attributed to a minor species which has an Fe−NO geometry different from that

Table 1: EPR Parameters for the NO Complexes

| complex | principal $g$-values $g_1$ | $g_2$ | $g_3$ | $A_2$, hyperfine coupling constant at $g_2$ (mT) | reference |
|---|---|---|---|---|---|
| NOS | 2.080 | 2.005 | 1.970 | 2.03 | this work |
| NOS-L-Arg | 2.086 | 2.005 | 1.970 | 2.08 | this work |
| NOS−NHA | 2.077 | 2.021 | 1.990 | 1.94 | this work |
| NOS−NMA | 2.070 | 2.021 | 1.990 | 2.18 | this work |
| NOS−P420 | | 2.009 | | 1.66 | this work |
| $P450_{CAM}$ | 2.073 | 2.009 | 1.976 | 1.92 | O'Keeffe et al. (1978) |
| $P450_{SCC}$ | 2.071 | 2.001 | 1.962 | 2.20 | Tsubaki et al. (1987b) |
| $P420_{SCC}$ | | 2.007 | | 1.67 | Tsubaki et al. (1987b) |

[a] NHA and NMA are $N^{\omega}$-hydroxy-L-Arg and $N^{\omega}$-monomethyl-L-Arg, respectively.

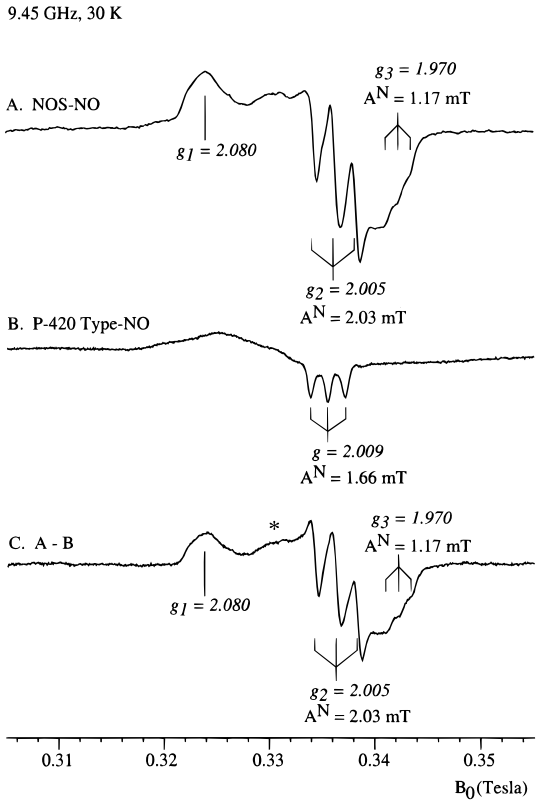


FIGURE 1: EPR spectra of the reaction product of ferrous NOS with NO (spectrum A), and the P420-type NO complex of NOS (spectrum B). Spectrum C is a difference spectrum, spectrum A − spectrum B. Measurements were carried out as described under Experimental Procedures.

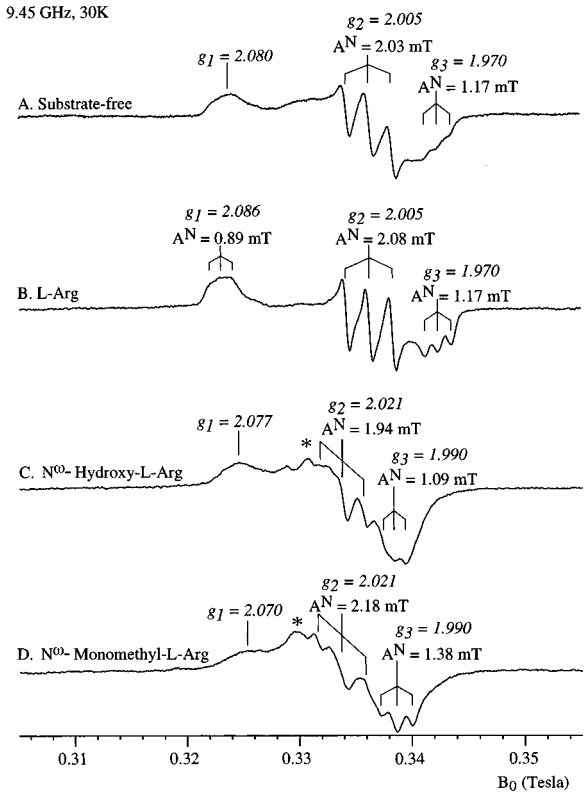


FIGURE 2: EPR spectra of the ferrous NO complex of substrate-free NOS (spectrum A, same as spectrum C of Figure 1), compared to those of L-Arg-bound NOS (spectrum B), $N^{\omega}$-hydroxy-L-Arg-bound NOS (spectrum C), and $N^{\omega}$-monomethyl-L-Arg-bound NOS (spectrum D). Conditions are described under Experimental Procedures.

in the major species (Tsubaki et al., 1987b). The amount of pentacoordinate NO species in spectrum A is roughly 20%.

In our repeated experiments using the same neuronal NOS preparation, the amount of pentacoordinate species formed varied between 20 and 40%. The fraction of pentacoordinate species significantly increased when NO gas was added to ferrous neuronal NOS in the absence of CO. On the basis of these observations, we conclude that the P420-type pentacoordinate NO complex is a denatured form of the NO complex, and we regard the hexacoordinate species (the main component of spectrum C) as the NO complex of neuronal NOS in the absence of substrate. The EPR spectrum of the NO complex of ferrous neuronal NOS is rhombic with $g_1 = 2.08$, $g_2 = 2.005$, and $g_3 = 1.97$. Triplet hyperfine couplings due to the $I = 1$ of $^{14}NO$ are seen at the $g_2$ and $g_3$ components. The spectrum is similar to those of the NO complexes of ferrous cytochrome P450 enzymes reported by O'Keeffe et al. (1978) and Tsubaki et al. (1987b), as compared in Table 1. The absence of superhyperfine couplings associated with the $g_2$ component is consistent with the axial cysteine ligation to the heme iron *trans* to the bound NO.

Figure 2 exhibits the EPR spectra of the NO complexes of neuronal NOS recorded in the presence of L-Arg (spectrum B), $N^{\omega}$-hydroxy-L-Arg (spectrum C), and $N^{\omega}$-monomethyl-L-Arg (spectrum D). The spectrum of substrate-free NOS (spectrum A, same as spectrum C of Figure 1) is also included for comparison. In the presence of L-Arg or its derivatives, no significant contribution of the P420-type NO EPR was detected in the spectrum. The EPR spectrum of the NO complex in the presence of L-Arg is clearly different from that in its absence. The spectrum in the presence of L-Arg is more rhombic: the $g_1$ value shifts to 2.086 without appreciable changes in the $g_2$ and $g_3$ values. In addition, the hyperfine couplings at the $g_1$ and $g_3$ components in the L-Arg-bound spectrum are better resolved. It is apparent that the electronic structure of the Fe−NO system in the NO-bound ferrous NOS is affected by L-Arg binding.

More significant changes are seen in the EPR spectra of the ferrous NO complexes in the presence of $N^{\omega}$-hydroxy-L-Arg and $N^{\omega}$-monomethyl-L-Arg (spectra C and D). The spectra are less anisotropic than those of the substrate-free and the L-Arg-bound NO complexes, with a distinct change in the $g_3$ value from 1.97 to 1.99. Complicated $g_2$ features and the appearance of a pronounced absorption around $g = 2.03$ (signals marked with an asterisk) are indications of the presence of multiple species. This situation makes assignment of the $g_2$-value and its associated hyperfine coupling difficult.

## DISCUSSION

In the absence of L-Arg, the reaction of ferrous neuronal NOS with NO forms a mixture of the hexa- and pentacoordinate NO species. The pentacoordinate species has also been observed in NO reactions with substrate-free cytochrome P450 enzymes. O'Keeffe et al. (1978) reported that NO binding to ferrous cytochrome $P450_{LM}$ induced the denaturation of the enzyme, resulting in the formation of a pentacoordinate P420 NO species, and that a predominantly hexacoordinate ferrous NO EPR spectrum could be obtained only when the mixture of NO and the ferrous enzyme was freeze-quenched in 15 ms after mixing. Tsubaki et al. (1987b) reported that the reaction of ferrous cytochrome $P450_{SCC}$ with NO yielded a mostly hexacoordinate NO complex with a small amount of a pentacoordinate species even in the absence of substrate. The NO complex of neuronal NOS appears to be much more stable than that of cytochrome $P450_{LM}$, and as stable as the cytochrome $P450_{SCC}$−NO complex. L-Arg binding enhances the stability of the hexacoordinate NO species of neuronal NOS. Similar observations have been reported for the cytochrome P450 enzymes (O'Keeffe et al., 1978; Tsubaki et al., 1987b). Therefore, substrate-enhanced stability of hexacoordinate NO complexes appears to be a common characteristic of cytochrome P450-type hemoproteins.

The EPR spectral changes induced by the binding of L-Arg and its derivatives indicate that the electronic state of the Fe−NO system is altered upon binding of L-Arg and its analogues. The most straightforward interpretation of the NO EPR spectral change is a direct interaction between the bound NO and L-Arg and its derivatives. It is possible, however, that the change in the EPR spectrum reflects the binding of L-Arg and its derivatives at a distant site which interacts with the heme ligand binding site through structural changes of the enzyme molecule. Substrates and mechanism-based inhibitors are expected to bind very close to the sixth coordination position of the heme iron where oxygen activation takes place, as seen in cytochrome P450 enzymes (Raag & Poulos, 1991). Therefore, the substrate is expected to bind in the distal pocket sufficiently close to the sixth position of the heme iron where both $O_2$ and NO bind. The possibility of a distant substrate binding site also seems very remote when considering the opposite effects induced by L-Arg and $N^{\omega}$-hydroxy-L-Arg which are known to bind at the same site (McMillan & Masters, 1993; Griffith & Stuehr, 1995). L-Arg binding increases the *g*-anisotropy while the *g*-anisotropy is reduced by $N^{\omega}$-hydroxy-L-Arg binding. Furthermore, EPR spectral changes similar to what we have observed for the NOS−NO complex were reported for cytochrome $P450_{SCC}$ (Tsubaki et al., 1987b), where direct interaction of substrates with the heme-bound ligand was manifested by resonance Raman scattering (Tsubaki et al., 1987a). We conclude that L-Arg, $N^{\omega}$-hydroxy-L-Arg, and $N^{\omega}$-monomethyl-L-Arg are most likely to interact directly with the coordinated NO in the ferrous NOS−NO complex. This supports the proposal that the L-Arg and the heme ligand binding sites are in close proximity as deduced from ligand binding studies by Matsuoka et al. (1994) and Berka et al. (1996), and is also consistent with the L-Arg binding-induced shift in the Fe−NO stretching mode of the NO complex of ferrous NOS by Wang et al. (1994), who have also suggested that L-Arg directly interacts with the coordinated NO.

Previous studies of ferrous heme−NO complexes have shown that the NO EPR spectral properties are sensitive to the changes in the Fe−NO geometry (Chien, 1969; Chien & Dickinson, 1977; Morse & Chan, 1980; Hori et al., 1981; Waleh et al., 1989).[2] The EPR spectral changes induced by the binding of L-Arg and its derivatives represent alterations of the Fe−NO geometry by substrate and inhibitor binding. In ferrous heme−NO complexes, bound NO assumes a bent, end-on Fe−N−O geometry (Scheidt et al., 1977; Deatherage & Moffat, 1979). The characteristic bent Fe−NO geometry (Figure 3) is highly likely in the NOS−NO complex, as the EPR spectrum of the NOS−NO complex is very similar to those of the NO complexes of myoglobin, hemoglobin, and cytochrome P450 enzymes in which the bent, end-on Fe−NO geometry has been established (Deatherage & Moffat, 1979; Chien & Dickinson, 1977; Hori et al., 1991; Hu & Kincaid, 1991). The increase in the *g*-value anisotropy upon L-Arg binding indicates an increase in the spin-delocalization into the iron d-orbitals. According to calculations by Waleh et al. (1989), the electron spin is distributed more in the iron $d\pi$ orbital than in the $dz^2$ orbital, indicating that changes in the $d\pi-2p\pi^*$ overlap have a more pronounced effect on the *g*-value anisotropy than those in the $dz^2-2p\pi^*$ overlap. The increase in the *g*-value anisotropy could be caused by an increase in the $d\pi-2p\pi^*$ overlap that could be attained in the less bent Fe−NO geometry. On the basis of this consideration, we propose that L-Arg binding increases the $d\pi-2p\pi^*$ overlap by slightly increasing the Fe−N−O bond angle as schematically illustrated in Figure 3 (A and B). The L-Arg binding to NOS appears to increase the $\pi$-back bonding of the Fe−NO bond, as manifested in the change of the Fe−NO stretching mode from 536 $cm^{-1}$ to 549 $cm^{-1}$ (Wang et al., 1994). An increase in the $\pi$-back bonding is attained for the less-bent Fe−NO geometry (Chien &

[2] *g*-Value anisotropy in a ferrous heme−NO EPR spectrum reflects the unpaired electron distribution over the metal and the axial ligand orbitals through the Fe−NO bond. Upon formation of the Fe−NO bond, the unpaired electron originally residing on the $2p\pi^*$ of NO delocalizes into the iron d-orbitals mainly through the direct overlap of the NO $2p\pi^*$ orbital with the iron $d\pi$ and $dz^2$ orbitals (Enemark & Feltham, 1974). This results in a much larger *g*-value anisotropy in the ferrous NO−heme complexes than that of free radical NO due to the large spin−orbit coupling of the iron d-orbitals (Kon & Kataoka, 1969; Hoffman et al., 1974; Loew, 1983). In the linear Fe−N−O geometry of heme−nitrosyl complexes, maximum overlap between the NO $2p\pi^*$ and iron $d\pi$ orbitals is attained, whereas no direct overlap exists between the $2p\pi^*$ and the iron $dz^2$ orbitals because of their orthogonality. Bending of the Fe−N−O unit will reduce the $2p\pi^*-d\pi$ overlap, and the $2p\pi^*$ orbital will overlap with the $dz^2$ orbital. The extent of orbital admixing alters the d-orbital spin population depending on the Fe−NO geometry, and, hence, the changes in the *g*-value anisotropy in the ferrous NO EPR spectrum of hemoproteins could be considered due to changes in the Fe−NO geometry. By a different approach, Walech et al. (1989) also demonstrated that *g*-value anisotropy is highly sensitive to minor changes in the Fe−NO geometry.

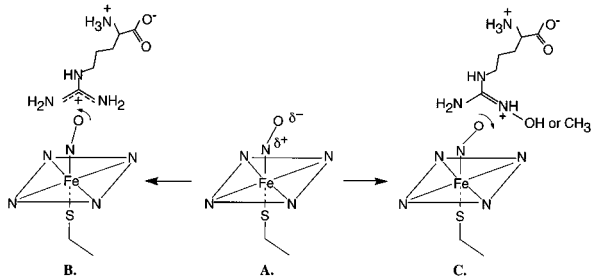


FIGURE 3: Schematic diagram of the proposed Fe−N−O unit for substrate-free NOS (A), L-Arg-bound NOS (B), and $N^{\omega}$-hydroxy-L-Arg- or $N^{\omega}$-monomethyl-L-Arg-bound NOS (C).

Dickinson, 1977; Hu & Kincaid, 1991; Ray et al., 1995); thus, the resonance Raman results appear to be consistent with a less bent Fe−NO geometry in the L-Arg- bound NOS−NO complex. The L-Arg binding could alter the Fe−NO geometry possibly through either hydrogen bonding, polar interaction, or steric hindrance. It is tempting to consider that the L-Arg binding changes the Fe−NO geometry by an attractive electrostatic interaction between the partly-anionic oxygen atom of the NO and the positive charge on the protonated guanidino group of the bound L-Arg.

The hyperfine splitting of the NO EPR spectrum is notably sharpened upon binding of L-Arg. In the substrate-free enzyme, a significant range of orientations could be available to the Fe−NO unit. The existence of multiple orientations introduces a distribution in *g*-anisotropy and hyperfine coupling, leading to line broadening. Binding of L-Arg in the distal pocket of the NOS−NO complex will restrict the range of orientations available to NO because of interactions between NO and bound L-Arg. With the restricted range of orientations (possibly with a single orientation) of the Fe−NO unit, the line broadening is reduced, and a well-resolved hyperfine splitting is observed in the L-Arg-bound enzyme.

When $N^{\omega}$-hydroxy-L-Arg and $N^{\omega}$-monomethyl-L-Arg bind to the NO complex of NOS, the *g*-value anisotropy is reduced. As discussed above and in footnote 2, the reduction of the *g*-value anisotropy would be an indication of the reduced overlap of the NO $2p\pi^*$ and the iron $d\pi$ orbitals that could be attained by a more sharply bent Fe−NO structure than in the substrate-free enzyme (Figure 3C). The difference in the *g*-value anisotropy between the nitrosyl heme in the $N^{\omega}$-monomethyl-L-Arg-bound NOS and that in the $N^{\omega}$-hydroxy-L-Arg-bound NOS is relatively small, suggesting that the both L-Arg derivatives impose a similar effect on the Fe−NO system in spite of the fact that $N^{\omega}$-monomethyl-L-Arg has a nonpolar substituent whereas $N^{\omega}$-hydroxy-L-Arg has a polar side chain. One of the possible factors for the altered Fe−NO geometry is the steric pressure exerted by the hydroxyl and methyl groups of the substituted L-Arg by forcing the Fe−NO unit to adopt a further bent Fe−NO geometry. These two L-Arg derivatives, which are isosteric, bind at the same site as L-Arg (McMillan & Masters, 1993; Griffith & Stuehr, 1995), and the positions of their hydroxyl and methyl groups relative to the heme are expected to be similar. Such a scenario appears to be consistent with similar EPR spectra of both L-Arg derivative-bound neuronal NOS−NO complexes. It is also possible that the electrostatic interaction between the L-Arg derivatives and the bound NO plays a role in defining the further bent Fe−NO geometry, since the electron-donating hydroxyl or methyl group stabilizes the limiting structure (Figure 3C) which has the localized positive charge on the substituted amino nitrogen. An electrostatic interaction between the localized positive charge and the partial negative charge on the oxygen of NO would compel the Fe−NO bond to have a more sharply bent structure in these systems than in substrate-free NOS. The proposed further bent Fe−NO structure might have some relevance to NOS enzyme catalysis. The NOS-catalyzed conversion of $N^{\omega}$-hydroxy-L-Arg to citrulline has been considered as a hydrogen atom abstraction reaction by the ferric peroxide or peroxo species, $Fe^{III}-OOH$, or $Fe^{III}-O-O^{-}$ (Marletta, 1993; Korth et al., 1994). The more sharply bent structure of the Fe−O−O bond in the $N^{\omega}$-hydroxy-L-Arg-bound NOS would facilitate hydrogen atom abstraction from $N^{\omega}$-hydroxy-L-Arg by placing the terminal oxygen atom of Fe−O−O close to the substituted amino nitrogen.

In conclusion, the results of this study have revealed that (1) the heme moiety of NO-bound NOS is fundamentally the same as that in cytochrome P450, (2) NO-bound NOS is relatively unstable and is readily converted to a P420-type denatured form upon complex formation, (3) substrate binding stabilizes hexacoordinate NO complex formation and, in addition, is responsible for distinctive changes in *g*-anisotropy, (4) changes in *g*-anisotropy among substrate-free and substrate-bound NOS−NO complexes suggest direct interaction between the bound NO and the guanidino group of the arginines, and (5) the differences in *g*-anisotropy among L-Arg-bound NOS−NO complex and L-Arg derivative-bound NOS−NO complexes suggest that the coordination geometry of heme-bound NO is regulated differently in these complexes. Changes in *g*-anisotropy can be explained in terms of the differences in spin-delocalization from the bound NO to the heme iron. These differences originate from the coordination geometry of the NO relative to heme, which is regulated by the bound L-Arg and its derivatives. The regulation of coordination geometry of exogenous NO to NOS by the bound substrates is almost certainly reflected in the interaction of the ferrous heme−$O_2$ adduct with the substrates, L-Arg and $N^{\omega}$-hydroxy-L-Arg; the position of these substrates in the binding site and the resulting orientation of the bound $O_2$ might specify the hydroxylation events in the catalytic cycle.

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