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EPR Studies on the Photo-Induced Intermediates of Ferric NO Complexes of Rat Neuronal Nitric Oxide Synthase Trapped at Low Temperature[1]

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Rat neuronal nitric oxide synthase (nNOS) was expressed in *Escherichia coli* and purified. Although the nitric oxide (NO) complex of the ferric heme was EPR-silent, photo-illumination at 5 K to the NO complex of the ferric nNOS in the substrate-free form produced a new high spin EPR signal similar to that of the ferric heme of N^{ω}-nitro-L-arginine-bound nNOS, suggesting that the photo-dissociated NO might move away from the heme. Low photo-dissociability of NO in this complex indicated less restricted movement of the dissociated NO in the distal region of the heme, which might result in the rapid rebinding of the NO to the ferric heme at 5 K. In the presence of substrate L-arginine, derivatives, or product L-citrulline, the photo-products from the ferric NO complexes exhibited large novel EPR signals with a spin-coupled interaction between the ferric heme (S=5/2) and the photolyzed NO (S=1/2), suggesting a stereochemically restricted interaction between the photo-dissociated NO and the guanidino- or the ureido-group of the substrate analogues at the distal heme region of nNOS. The photo-product from the NO complex produced from citrulline-bound nNOS might be the same intermediate species as that formed in the last step of the catalytic cycle.

Key words: EPR, hemoprotein, nitric oxide, neuronal nitric oxide synthase, photo-products.

Nitric oxide (NO) is an important messenger molecule in the control of vascular tone, platelet function, and neurotransmission, and is produced with L-citrulline from L-arginine by catalysis of three isoforms of nitric oxide synthase (NOS) with the consumption of two molecules of molecular oxygen and 3/2 molecules of NADPH (*1-7*). NOS consists of two domains: the N-terminal oxygenase domain contains binding sites for heme, tetrahydrobiopterin (H_4B), and L-arginine and the C-terminal domain has the sites for FAD, FMN, and NADPH, in which the amino acid sequence of the C-terminal domain is homologous to NADPH-cytochrome P450 reductase, except that a calmodulin binding site is located between the two domains (*8-10*). The three isoforms of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), show significant homology in their primary amino acid sequences except for additional N-terminal sequences in nNOS (*11, 12*). Recently, the molecular structures of the oxygenase domains of iNOS and eNOS with and without the substrate have been reported (*12-14*). Substrate L-arginine and its analogues have been shown to bind to the distal region above the sixth coordination position of the heme.

In one cycle of the NOS reaction, two monooxygenase reactions occur; the first produces N^{ω}-hydroxyl-L-arginine from L-arginine and the second forms L-citrulline and NO (*15, 16*). P450 type oxygen activation takes place at the heme of the NOS in which the proximal axial ligand is a cysteine thiolate as that in the cytochrome P450 (*17-20*). The coordination structure of the NOS heme is very similar to that of cytochrome P450 and the mechanism of oxygen activation is considered to be similar. It has been reported that the NO produced by NOS itself acts as a feedback inhibitor of the NOS reaction during normal catalytic reaction (*21-23*). NO is known to bind to both the ferric and ferrous heme irons of many hemoproteins including NOS, and induces characteristic visible light absorption spectra (*23, 24*). Characteristic EPR spectra have been reported for the ferrous NO complexes of cytochrome P450 and nNOS (*25, 26*). Since NO-bound ferric heme complexes are diamagnetic due to spin-pairing of S=5/2 (Fe^{3+}) and S=1/2 (NO), we can not obtain information from the EPR. Upon

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Abbreviations: NO, nitric oxide; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; EPR, electron paramagnetic resonance; Arg, L-arginine; OH-Arg, N^{ω}-hydroxy-L-arginine; NO_2-Arg, N^{ω}-nitro-L-arginine; Cit, L-citrulline; H_4B, (6*R,S*)-5,6,7,8-tetrahydro-L-biopterin; CHAPSO, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate; SF, substrate free; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CaM, calmodulin.

photolysis of NO-bound ferric heme complexes with visible light at low temperature, however, the photo-induced intermediates trapped in the distal heme cavity show novel EPR spectra that provide information about the steric crowding of the distal heme cavity (*27-31*). If the dissociated NO is trapped at a location close to the ferric heme, a spin-coupled pair, $S=5/2$ (Fe^{3+}) and $S=1/2$ (NO), leads to a widespread EPR absorption together with a zero-field absorption. When the dissociated NO is moved farther away from the ferric heme, the photo-product might exhibit a new broad high spin signal due to the magnetic dipolar interaction between the dissociated NO and the ferric heme.

In this study, we report the EPR spectra of photo-induced intermediates of ferric NO complexes of nNOS trapped at low temperature. On the basis of the molecular structures of iNOS, we will discuss the modes of interaction of the heme iron and the photo-dissociated NO molecule in the distal heme region of nNOS.

MATERIALS AND METHODS

Materials—Yeast extract and bactotryptone were obtained from Difco Laboratory (Detroit, MI, USA). Calmodulin-agarose, δ-aminolevulinic acid, and N^{ω}-nitro-L-arginine were from Sigma (St. Louis, MO, USA), DEAE-Sephacel and 2′,5′-ADP-Sepharose 4B from Pharmacia Biotech, β-D-thiogalactoside, L-arginine, phenylmethylsulfonyl fluoride (PMSF), and L-citrulline from Nacalai Tesque (Kyoto), 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) from DOJIN Laboratories (Kumamoto), (6*R,S*)-5,6,7,8-tetrahydro-L-biopterin (H_4B) from Schircks Laboratories (Jona, Switzerland), N^{ω}-hydroxy-L-arginine acetate from Cayman Chemical (Ann Arbor, MI, USA). *Escherichia coli* BL21 competent cells were from Novagen (Madison, WI, USA). L-[1,2,3,5-^{3}H]Arginine monohydrochloride was purchased from Amersham Life Science (Amersham, England). Rat calmodulin was purified from BL21 transfected with rat calmodulin cDNA which was kindly provided by Drs. Hayashi and Taniguchi of Institute for Comprehensive Medical Science, Fujita Health University, Aichi (*32*).

Protein Expression and Purification—The plasmid-containing rat nNOS cDNA in pBluescript SK(−) was a kind gift from Dr. Snyder of Johns Hopkins Medical School, Baltimore, USA (*33*). pCWori was a gift from Dr. Dahlquist of Oregon University, Oregon, USA (*34*). The construction of pCWnNOS and protein expression were carried out according to the method of Roman *et al.* (*35*). Briefly, *Escherichia coli* BL21 transfected with pCWnNOS were cultivated in 500 ml Sakaguchi-flasks with 300 ml of Terrific Broth containing 50 μg/ml ampicillin, 0.4% (v/v) glycerol, 0.5 mM β-D-thiogalactoside, 450 μM δ-aminolevulinic acid, and 3 μM riboflavin. The flasks were kept at 25°C with constant shaking at 180 rpm in the dark for 40 h. The final cultivated solution of *E. coli* contained about 500 nmol of nNOS per liter, estimated from the dithionite-reduced CO difference spectrum using the difference absorption coefficient (444-490 nm) = 91 $mM^{-1}\cdot cm^{-1}$ (*36*).

The *E. coli* paste from 1,500 ml of culture was suspended in 130 ml of buffer B) 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.1 mM EGTA , 1 μM FAD, 1 μM FMN, 5 μM H_4B, 100 μM L-arginine (L-Arg), and 10% (v/v) glycerol) containing 1 mM PMSF. The cell walls were destroyed by lysozyme treatment and ultrasonication. The nNOS was loaded onto a DEAE-Sephacel column (2.5×35 cm) and eluted from the column with a linear gradient to 300 mM NaCl. Fractions showing a CO-difference spectrum were collected and loaded onto a 2′,5′-ADP-Sepharose column (1.5×15 cm), that was washed sequentially with 0.5 M NaCl and 0.3% CHAPSO using the FPLC system (Pharmacia). nNOS was eluted with 10 mM NADPH and was loaded directly onto a Calmodulin-agarose column (1.5×15 cm) with the FPLC system. The column was washed with 0.3 M NaCl and eluted with 2.5 mM EGTA and 0.5 M NaCl. The fractions showing a red color were collected and concentrated to 200 μM with a 100 kDa-Microsep (Gelman Science). The purification took three days and the yield was about 20%. The purified nNOS showed one protein band in SDS-PAGE after silver staining; the activity was 130 nmol citrulline formed per minute per nmol of nNOS at 25°C. The activity was measured with ^{3}H-L-arginine as the substrate in the presence of 10 μM H_4B, 2 μM FAD, 2 μM FMN, 3 mM $CaCl_2$, and 5 μM calmodulin (CaM); the product ^{3}H-L-citrulline was separated by HPLC (JASCO, Tokyo) with a reverse-phase column (0.75×15 cm, RP-18, Kanto Chemicals,Tokyo) using a mixture (1:4, v/v) of 2-propanol and an aqueous solution containing 25 mM phosphoric acid, 25 mM sodium dihydrogenphosphate, and 18.5 mM SDS (*37*).

EPR Measurements and Photo-Illumination—One hundred microliters of 200 or 20 μM nNOS in 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 10% (v/v) glycerol was put into an EPR sample tube 4 mm in diameter. L-Arginine, N^{ω}-hydroxy-L-arginine, L-citrulline, or N^{ω}-nitro-L-arginine were mixed with the purified nNOS for EPR measurements to a final concentration of 400 μM. The samples of substrate-free nNOS and the complexes with L-arginine, N^{ω}-hydroxy-L-arginine, N^{ω}-nitro-L-arginine, and L-citrulline are hereafter referred to as SF-nNOS, Arg-nNOS, OH-Arg-nNOS, NO_2-Arg-nNOS, and Cit-nNOS, respectively. The NO complex of nNOS in the oxidized state was prepared according to the method described previously (*30*). The complete complex formation of the ferric heme with NO was confirmed by the disappearance of the ferric EPR signals at 5 K. EPR measurements were carried out at the X-band microwave frequency (9.23 GHz) using a Varian E-12 spectrometer, operated with the 100 kHz magnetic field modulation. An Oxford flow cryostat (ESR-900) was used for measurements at cryogenic temperature. Photo-illumination was achieved by focusing white light from an unfiltered tungsten lamp (50 W) onto the samples through the slit of the EPR cavity in the front panel for 15 min while the samples were immersed in the cryostat. The microwave frequency was calibrated with a microwave frequency counter (Takeda Riken, Model TR5212). The magnetic field strength was determined by nuclear magnetic resonance of water protons (*30, 31*). Accuracy of the values in this study was approximately ±0.005 for low spin signals and ±0.01 for high spin signals.

RESULTS

EPR Spectra of Ferric Heme in nNOS—The EPR spectra

of the ferric heme of nNOS in substrate-free and substrate- or analogue-bound forms were measured at 15 K and 5 K. SF-nNOS and Cit-nNOS exhibited low spin signals dominantly at 15 K (Fig. 1) The ferric low spin signals could hardly be detected in the other nNOS samples at 15 K. From the signal intensities of the high spin signals, about 40% of the absorptions in SF-nNOS and 20% in Cit-nNOS were attributable to the high spin forms. The high spin EPR spectra were essentially the same as those reported previously (*17, 38*). Upon the addition of NO_2-Arg to SF-nNOS, two distinct high spin signals were observed (Fig. 1e). The high spin EPR signals of SF-nNOS and NO_2-Arg-nNOS were expanded around the in-plane (g_1-g_2) region as illustrated in Fig. 2, a and c, respectively. The in-plane g-anisotropy of the high spin species of NO_2-Arg-nNOS decreased markedly from that of SF-nNOS. The g values of the EPR signals are summarized in Table I. The signal intensity of the semiquinone radical at $g_e = 2.003$ in Fig. 1 was very small compared with those reported previously from other laboratories (*17*). This signal increased upon the addition of NADPH to the samples (spectrum not shown). In our preparation, we mixed L-Arg in buffer B for stabilization and used NADPH for the elution from the 2′,5′-ADP-Sepharose column, a procedure that might produce product citrulline during the elution. The nNOS from the 2′,5′-ADP-Sepharose column was adsorbed on a Calmodulin-agarose column and washed extensively to remove free flavins and amino acids as described in "MATERIALS AND METHODS." The semiquinone radical might be oxidized during the washing process.

Photo-Products from Ferric Heme-NO Complexes of nNOS—Upon binding of NO at the sixth coordination

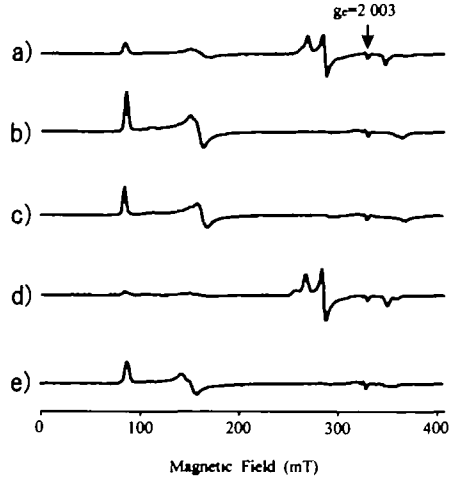

Fig. 1. **EPR spectra of ferric nNOS (20 μM) in the presence of various substrate analogues.** Spectra a, b, c, d, and e were measured at 15 K for substrate-free nNOS and L-arginine, N^{ω}-hydroxy-L-arginine, L-citrulline, and N^{ω}-nitro-L-arginine bound nNOS, respectively. All measurements were carried out at the same sensitivity with an incident microwave power of 10 milliwatts using a field modulation of 1 millitesla at 100 kHz. The details are described in "MATERIALS AND METHODS."

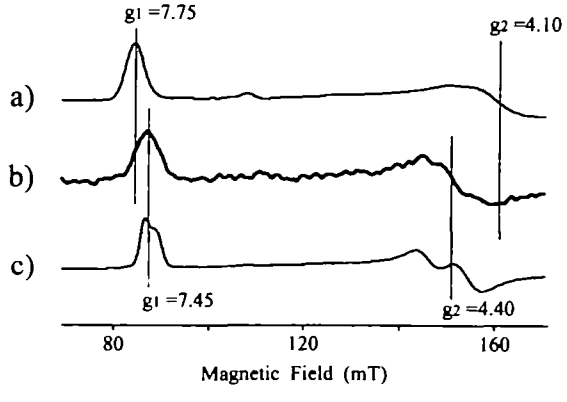

Fig. 2. **Comparison of the high spin EPR spectrum of the photo-product from the ferric NO complex of substrate-free nNOS (b) with those of ferric substrate-free (a) and N^{ω}-nitro-L-arginine bound nNOS (c).**

TABLE I. **The g-values of ferric high and low spin hemes in nNOS.**

Sample	Spin state[a]	g_1	g_2	g_3
SF-nNOS[b]	H	7.75	4.10	1.80
	L	2.450	2.300	1.899
		2.50	(2.30)	1.875
Arg-nNOS[c]	H	7.71	4.16	1.817
OH-Arg-nNOS[c]	H	7.80	4.05	1.79
Cit-nNOS[c]	H	7.80	4.05	1.79
	L	2.469	2.309	1.885
		2.550	(2.30)	1.837
NO_2-Arg-nNOS[c]	H	7.54	4.30	1.84
		7.37	4.49	1.89
Photoproduct from NO-nNOS in SF	H	7.48	4.34	–

The ferric high and low spin EPR spectra were measured at 5 K and 15 K, respectively. [a]H and L denote ferric high and low spin states, respectively. [b]SF represents the substrate-free form of nNOS. [c]EPR spectra of Arg-, OH-Arg-, Cit-, and NO_2-Arg-bound nNOS were obtained in the presence of 400 μM L-arginine, N^{ω}-hydroxy-L-arginine, L-citrulline, and N^{ω}-nitro-L-arginine, respectively. Details are described in "MATERIALS AND METHODS."

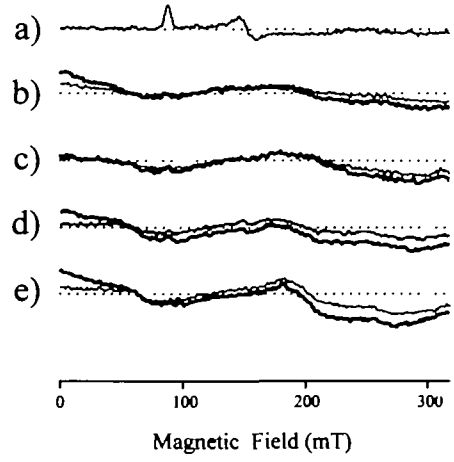

Fig. 3. **EPR difference spectra of the photo-products from NO complexes of ferric nNOS (200 μM) in various states.** The difference spectra were obtained by subtracting the spectra before visible light illumination from those after illumination at 5 K (fine lines) and 3.6 K (bold lines). The EPR spectra were measured at the same sensitivity at 5 K after photo-illumination. Spectra a, b, c, d, and e are those of photo-products from ferric NO complexes of nNOS in the substrate-free, and L-arginine-, N^{ω}-hydroxy-L-arginine-, L-citrulline-, and N^{ω}-nitro-L-arginine-bound forms, respectively. Dotted lines are base lines.

position of the heme, the NOS isozymes in both the oxidized and reduced forms show a sharp peak around 440 nm in visible light absorption spectrum (*24*). NO-complexes of hemoproteins in the reduced form show typical EPR spectra that reflect the surroundings of the NO in the distal heme moiety of the hemoprotein (*26, 39*). Although NO complexes of the oxidized hemoproteins are EPR inactive due to a strongly spin-coupled system caused by one electron being donated to the ferric heme from NO, new EPR spectra could be observed after visible light illumination of the NO complexes of ferric heme in various forms of nNOS at 5 K and 3.6 K as shown in Fig. 3. Reversion of the photo-products to the initial strong spin-coupled EPR-inactive species was achieved after the temperature of the photolyzed sample was raised to 100 K or more for a few minutes. No EPR signals were observed at 5 K for these annealed samples (*30*). Photo-illumination of the annealed samples at 5 K produced the same spectra as those observed at the first illumination. This indicates that the photo-dissociated NO neither reacted chemically with molecules in the distal region nor stepped out of the protein interior at 100 K. The lower the temperature (3.6 K) of the photolysis induced, the greater the EPR signal intensities, indicating that the photo-induced intermediate might be trapped more at lower temperature (Fig. 3).

The EPR spectrum of the photo-product of the NO complex of ferric SF-nNOS exhibited a typical high spin spectrum of NOS as shown in Fig. 3a. The signal intensity of the photo-product was very low. The content of the EPR active species in the photo-illuminated NO complex of the ferric SF-nNOS, which corresponds to photo-dissociability, was estimated to be roughly about 7% of the total ferric hemes. Low photo-dissociability has been reported also for ferric NO-complexes of P450cam and P450scc in substrate-free forms, which were explained by the rapid rebinding of the photo-dissociated NO to the ferric heme even at 5 K (*30, 31*). The high spin EPR signals of the photo-product from the ferric NO complex of SF-nNOS were expanded around the in-plane (g_1-g_2) region in order to clarify the details of the in-plane g-anisotropy and linewidth (Fig. 2b). The g values of the high spin signals in the photo-product of NO complex of SF-nNOS were 7.45 and 4.40, which were clearly different from those of the high spin ferric heme of SF-nNOS (Fig. 2a), but very similar to those of NO_2-Arg-nNOS without NO (Fig. 2c). In addition, the linewidths of the photo-product of NO-SF-nNOS were slightly broader than those of the high spin ferric heme of SF-nNOS.

In the presence of L-arginine and its analogues, the photo-products from the ferric NO complexes of nNOS exhibited novel broad and widespread EPR absorptions from zero magnetic field to about 400 millitesla as shown in Fig. 3. These peculiar EPR absorptions indicate that the electronic spin of the photolyzed NO molecule ($S=1/2$) might spin-couple with the high spin heme iron ($S=5/2$) in these samples. The EPR spectra of these photo-products were very similar to each other. Photo-product from the ferric NO complex of OH-Arg-nNOS exhibited an EPR spectrum that was somewhat different in the zero field region from the others. Although the areas of EPR absorption in Fig. 3, b–e, were difficult to estimate because of their peculiar broad line shapes, it is clear that the amounts of EPR active photo-induced intermediate species were 5–10 times more than the amount of the high spin species observed in the photo-product from the ferric NO complex of SF-nNOS.

DISCUSSION

In cytochrome P450cam, low spin EPR was observed in the substrate-free form where a water molecule exist at the sixth coordination position of the ferric heme. On the other hand, a dominant high spin EPR with a minor low spin EPR was observed in the substrate-bound form, which has no water molecule at the sixth coordination position (*40–42*). Very recently, the molecular structures of the oxygenase domain of iNOS and eNOS with and without substrate have been reported (*13, 14*). The molecular structure of the substrate-free oxyganase domain shows two water molecules in the distal heme moiety: one water molecule bonds to a substrate-binding residue (glutamic acid-371 in iNOS) and the other resides 0.42 nm above the heme iron as shown in Fig. 4a (*13*). There are no water molecules above the ferric heme in the molecular structure of the substrate analogue-bound oxygenase domain (*13*). The configuration of the ferric heme of cytochrome P450 is very similar to that of NOS and the conversion of the high to low spin EPR of NOS could be attributed to the presence of a water molecule at the sixth position. The substrate-free iNOS

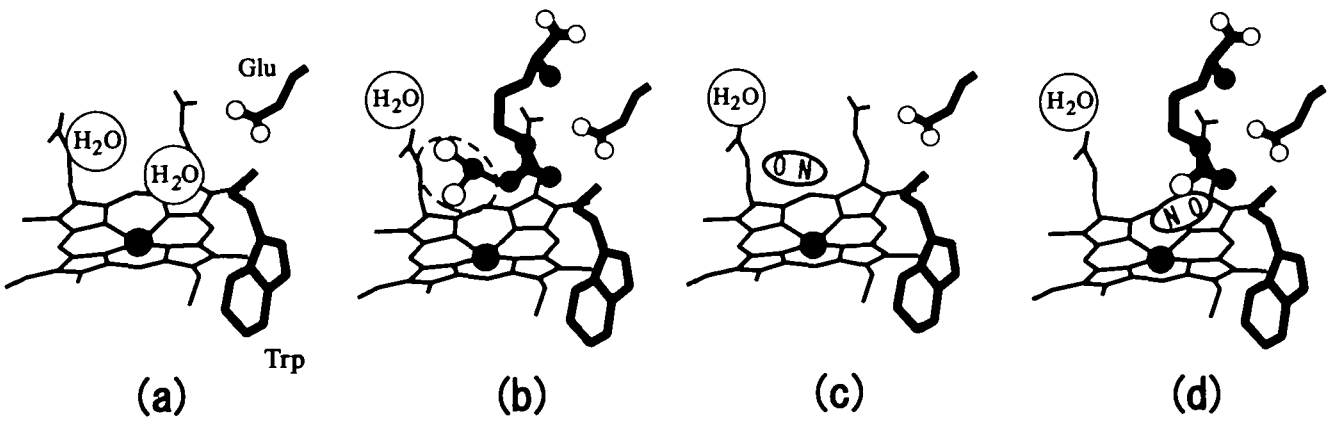

Fig. 4. **Schematically illustrated structures of the distal region of the ferric hemes in SF-nNOS (a), N^{ω}-nitro-L-arginine-bound nNOS (b), and the photo-products of NO complexes of ferric SF-nNOS (c) and ferric Cit-nNOS (d).** These figures are drawn according to the molecular structure of the oxygenase domain of iNOS in Ref. *13*.

showed dominant low spin EPR at 20 K and substrate-bound iNOS showed major high spin EPR (*43*). In our present study on nNOS, low spin EPR signals were observed dominantly in the substrate-free and Cit-bound forms. About 60% of the heme in SF-nNOS was a low spin species, in which one water molecule might coordinate as the axial ligand. In Cit-nNOS, the sixth coordination position of the heme is probably occupied by a water molecule that might be interacting with an electronegative carboxyl oxygen of the ureido group. The minor high spin species in SF-nNOS and Cit-nNOS might indicate that the water molecule has swung out of the coordination position of the hemes.

The g_1-g_2 value of the high spin signals was markedly decreased upon NO_2-Arg binding to SF-nNOS, indicating a decrease in the rhombicity of the high spin heme (Fig. 2). There are two distinct high spin species in NO_2-Arg-nNOS, indicating that two different NO_2-Arg conformations exist in this complex. The large anionic NO_2 group might push the water molecule out of the distal heme site, which could cause a rotation of the NO_2-group around the N^{ω}-NO_2 bond axis. The anionic charge of an NO_2-group near the sixth coordination position of the heme (Fig. 4b) might be attributable to the lower rhombicity of the heme. Salerno *et al.* classified ligands for ferric nNOS into several groups from the rhombicity of the high spin EPR, but could not explain the origin of the rhombicity (*44*).

Although the ferric heme-NO complexes are EPR-silent due to spin-pairing, the photo-induced intermediates trapped in the distal heme cavity at low temperature have EPR spectra showing the interaction between the photo-dissociated NO ($S=1/2$) and the ferric high spin heme ($S=5/2$) (*28-31*). The photo-product of the ferric NO complex of SF-nNOS exhibits only high spin signals. The EPR signals are slightly broader than those of the ferric heme indicating a magnetic dipolar interaction between the dissociated NO and the ferric heme iron (Fig. 2b). The EPR spectrum is quite similar to that of the ferric NO_2-Arg-nNOS. The similar rhombicity suggests that the photo-dissociated NO might be trapped in the distal cavity of SF-nNOS at a position similar to where the NO_2 group in NO_2-Arg-nNOS locates, and might produce an electrostatic field in the distal heme region similar to that of the NO_2 group of the NO_2-Arg (Fig. 4c). Furthermore, the extremely low photo-dissociability for the ferric NO complex of SF-nNOS means that the photo-dissociated NO can not be trapped stably in the distal heme pocket, and that most of the photo-dissociated NO rebinds to the heme iron and forms the original strongly spin-coupled EPR-inactive species immediately after photolysis even at low temperature.

In the presence of L-arginine and its derivatives, the photoproducts of the ferric-NO complexes of nNOS exhibit a unique widespread EPR spectra indicating the spin-coupled interaction. The overall spectral patterns in Fig. 3, b-e, are indistinguishable from each other except for a slightly different zero field absorption in the OH-Arg-nNOS complex. These spectra resemble those of the photo-products from the ferric-NO complexes of P450scc in the presence of 20(*S*)-hydroxycholesterol and from NO-ferric myoglobin (*29, 30*). The spectra in Fig. 3, b-e, indicate that the photolyzed NO might not be so far from the heme iron and are trapped at similar positions near the heme iron. The photo-dissociated NO from the NO-ferric heme of nNOS complexed with the substrate or the analogues might be trapped stably by the sterically restricted guanidino-groups. The interaction between NO in the sixth coordination position in ferrous nNOS and the guanidino groups of substrate analogues has been shown by Migita *et al.* (*26*).

The EPR spectra of the photo-induced intermediate species in Fig. 3, b-e, were attributed to the superimposition of spectra from several molecular species. The existence of almost no zero field absorption in the photo-products from ferric-NO complex of OH-Arg-nNOS could be explained by the disappearance of some intermediates with special configurations having had quite strong spin-exchange interactions between the NO and Fe^{3+}. It is plausible that OH-Arg might have a special configuration with respect to the ferric heme because it is the product from L-arginine and the substrate for the formation of L-citrulline, and it might perturb the interaction of the photo-dissociated NO with ferric heme. Very recently, an ENDOR study of OH-Arg bound nNOS showed that the OH group was held just above the ferric heme (*45*).

During the reaction cycle, a significant amount of NO has been reported to bind to the sixth coordination position of the ferrous heme iron (*21-23*). The photo-dissociated NO from the ferric NO complex of Cit-nNOS in this study might be trapped by the ureido group of the citrulline, at least at 5 K (Fig. 4d). The results of the present EPR analysis at cryogenic temperature might not be applicable to the catalytic reaction at physiological temperature. The ferrous-NO complex of nNOS in the reaction cycle can be explained, however, by trapping in the ureido group of the citrulline. The molecular species obtained by the photo-illumination of the ferric heme-NO complex of Cit-nNOS might be formed during the last step of the NOS reaction cycle, in which the NO-ferric heme is reduced to the ferrous NO-complex by electron transfer from the reductase domain of nNOS.

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