The high-potential flavin and heme of nitric oxide synthase are not magnetically linked: implications for electron transfer

Jason M Perry[1], Namdoo Moon[2], Yunde Zhao[3], W Richard Dunham[2] and Michael A Marletta[1,3]

Background: The homodimeric nitric oxide synthase (NOS) catalyzes conversion of L-arginine to L-citrulline and nitric oxide. Each subunit contains two flavins and one protoporphyrin IX heme. A key component of the reaction is the transfer of electrons from the flavins to the heme. The NOS gene encodes two domains linked by a short helix containing a calmodulin-recognition sequence. The reductase domain binds the flavin cofactors, while the oxygenase domain binds heme and L-arginine and additionally mediates the dimerization of the NOS subunits. We investigated the origin of the unusual magnetic properties (rapid-spin relaxation) of an air-stable free radical localized to a reductase domain flavin cofactor.

Results: We characterized the air-stable flavin in wild-type NOS, both in the presence and absence of calcium and calmodulin, the imidazole-bound heme complex of wild-type NOS, the NOS Cys415→Ala mutant, and the isolated reductase domain. All preparations of NOS had the same flavin electron-spin relaxation behavior. No half-field transitions or temperature-dependent changes in the linewidth of the radical spin signal were detected.

Conclusions: These data suggest that the observed relaxation enhancement of the NOS flavin radical is caused by the environment provided by the reductase domain. No magnetic interaction between the heme and flavin cofactors was detected, suggesting that the flavin and heme centers are probably separated by more than 15 Å.

Addresses: Howard Hughes Medical Institute, [1]Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, [2]Biophysics Research Division and [3]Department of Biological Chemistry, School of Medicine, University of Michigan, Ann Arbor, Michigan 48109-1065, USA.

Correspondence: Michael A Marletta
E-mail: marle@umich.edu

Key words: electron paramagnetic resonance, electron transfer, flavin, heme

Received: **11 March 1998**
Revisions requested: **7 April 1998**
Revisions received: **22 April 1998**
Accepted: **29 April 1998**

Published: **11 June 1998**

Chemistry & Biology July 1998, **5**:355–364
http://biomednet.com/elecref/1074552100500355

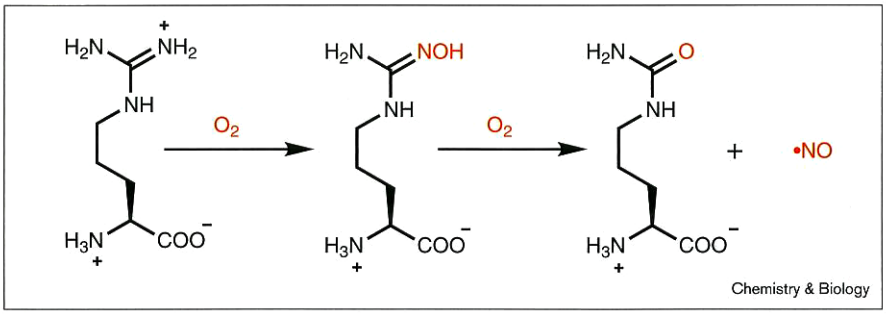
Introduction

Nitric oxide synthase (NOS; EC 1.14.13.39) catalyzes the conversion of L-arginine to nitric oxide (NO) and L-citrulline at the expense of NADPH and molecular oxygen (Figure 1). The physiological role of NO has been a subject of intense investigation: NO has been shown to be a signal transduction agent that acts through stimulation of soluble guanylate cyclase, a component of the host response to infection, and a participant in other processes such as synaptogenesis, neuronal development, and apoptosis [1]. The particular activity that NO mediates seems to be a function of the tissue type and cellular location in which it is synthesized. Despite the growing body of information regarding the actions of NO, important questions about the molecular mechanism of the NOS-catalyzed reaction remain unanswered.

Two general classes of NOS isoforms have been characterized [2]. The constitutive isoforms, which are best represented by neuronal NOS (nNOS) and endothelial NOS (eNOS), are regulated by a Ca^{2+}-dependent interaction with calmodulin that permits NADPH-dependent heme reduction. The cytokine-induced NOS isoforms, such as the one found in macrophages, are generated as a product of dynamic gene regulation and copurify with calmodulin as a tightly bound subunit [3,4]. All of the isoforms isolated to date are catalytically competent only as homodimers, and each subunit has been shown to contain stoichiometric amounts of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), (6R)-5, 6, 7, 8-tetrahydro-L-biopterin (H_4B) and protoporphyrin IX heme [5–11]. The protein-donated ligand to the axial position of the NOS heme is a cysteine thiolate, and thus it displays electronic absorption and electron paramagmetic resonance (EPR) properties similar to those observed with substrate-bound cytochrome P450, including the signature ferrous–CO complex with a λ_{max} at approximately 445 nm [8,12].

Each NOS subunit is composed of two separate domains that are linked to each other by the region of the enzyme that contains a calmodulin-recognition sequence, and, upon proteolytic digestion of this region, the independent domains can be isolated [13]. The amino-terminal heme domain contains the putative active site of the enzyme. The amino-terminal domain mediates dimerization of NOS subunits and binds heme, H_4B and L-arginine. The carboxy-terminal reductase domain displays significant sequence

Figure 1



The reaction catalyzed by NOS. L-Arginine is converted to NO and L-citrulline.

homology to cytochrome P450 reductase and likewise contains one equivalent each of FAD and FMN. The striking functional resemblance between the reductase domain of NOS and P450 reductase is the ability to stabilize a neutral flavin-semiquinone radical, shown in P450 reductase (and presumed in NOS) to be localized to FMN [14]. The catalytic cycle of NOS is believed to be fueled by the calmodulin-dependent sequential transfer of NADPH-derived electrons from the reductase domain to the active site, where heme reduction has been observed. Whether there is a direct electron-transfer event between a reduced flavin and the heme or if other functional groups participate in an electronic relay to bridge the two centers is not yet clear; the former, however, has been the putative model based on analogies drawn to the cytochrome P450/cytochrome P450 reductase system and previous investigations of the magnetic properties of the flavin-semiquinone radical [7].

The flavin-semiquinone radical of NOS has an intrinsic spin-relaxation rate that is somewhat faster than would be expected for an isolated free radical, which has prompted a model of a dipolar spin–spin interaction between the flavin radical and the high-spin heme which could result in a relaxation enhancement of the radical [7]. In this report, we have applied progressive microwave power saturation EPR to several preparations of recombinant rat nNOS in order to locate the origin of the observed magnetic properties characteristic to the flavin radical spin. Our findings demonstrate that all of the components necessary for the relaxation behavior of the flavin semiquinone radical are contained within the reductase domain of NOS, and indicate that the distance between the flavin radical and the active-site heme is probably at least 15 Å. This distance is likely to be too great to allow direct electron transfer between the flavin and heme centers and suggests the participation of other protein components in the electron-transfer process.

Results

Relationship between heme spin state and flavin-radical relaxation

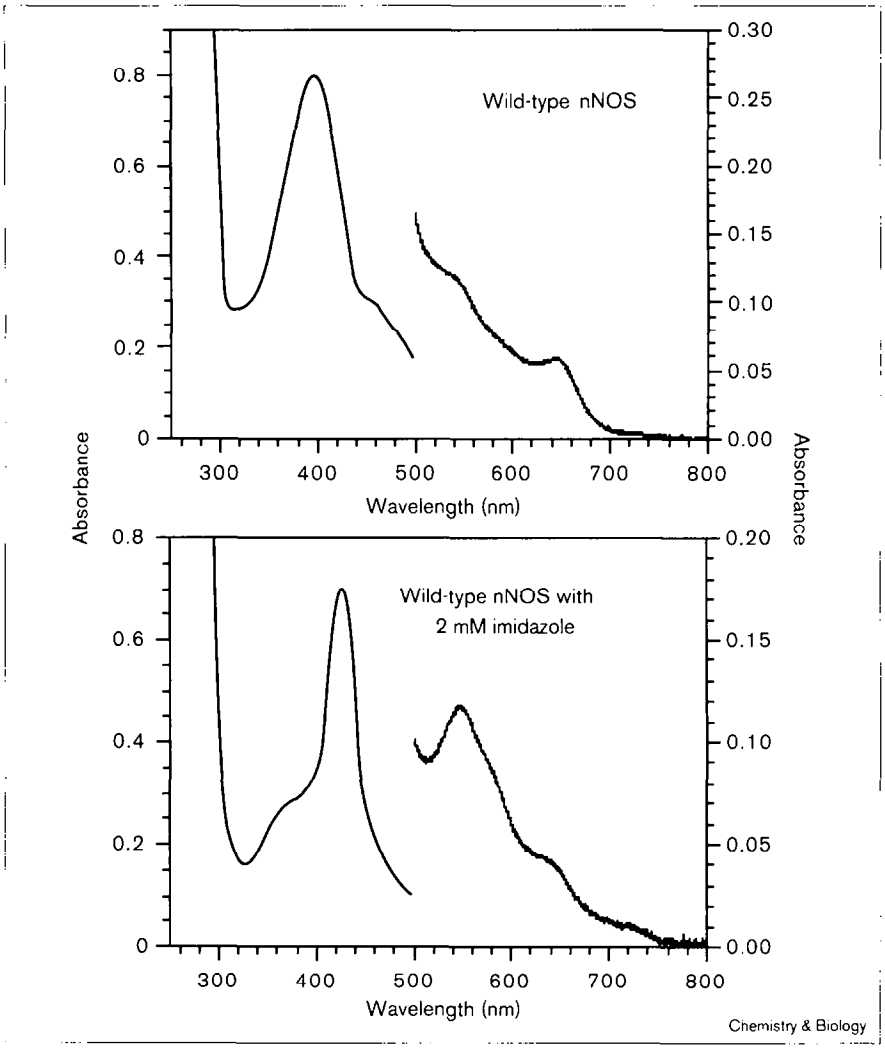
As expected from previous results, the NOS heme was observed to be predominantly high-spin ferric (spin = 5/2) following the purification procedure [8], and it is with this species of heme that the flavin-semiquinone radical has been reported to engage in a dipolar spin–spin interaction [15]. In the presence of 2 mM imidazole, the heme can be quantitatively converted to the low-spin ferric state (spin 1/2), by coordinating imidazole as a distal ligand [6]. Electronic absorption spectra of these heme species are shown in (Figure 2). The prominent features of the wild-type nNOS optical spectrum include a rather broad Soret band centered at 396 nm, a β/α band centered at 544 nm, and a Fe(III)→porphyrin charge-transfer band centered at 646 nm. The bound flavins also contribute to the spectrum by adding maxima at 454 nm and 480 nm (oxidized flavin species) and by adding to the absorbance between 550 and 700 nm (neutral flavin semiquinone). Flavin transitions at 380 nm and 355 nm are not observed because of the spectrally dominating heme chromophore. The imidazole-bound wild-type nNOS exhibits a sharp Soret band with a maximum at 427 nm, β/α transitions between 510 nm and 590 nm and a δ band with a maximum at approximately 365 nm. The flavin absorbance is largely masked by the heme, but the shoulder at 380 nm and that between 450 nm and 500 nm probably represent oxidized flavin; the absorbance from 620 nm to 700 nm is the semiquinone.

Field-swept EPR spectra confirm the electronic nature of the two heme species (Figure 3). The wild-type high-spin heme has transitions at $g_x = 7.68$, $g_y = 4.13$, and $g_z = 1.82$, showing a high degree of rhombic distortion similar to that observed in other cytochrome P450 enzymes [16]. Transitions at $g_x = 2.44$, $g_y = 2.28$, and $g_z = 1.88$ result from a minor amount of low-spin heme that is consistently observed in wild-type nNOS preparations. The imidazole-bound low-spin heme transitions appear at $g_x = 2.57$, $g_y = 2.28$, and $g_z = 1.81$. Both spectra display an isotropic free-radical signal centered at $g = 2.003$ with a linewidth of approximately 20 G, which arises from the neutral flavin-semiquinone radical.

We used progressive microwave power saturation to determine if the flavin-radical spin could engage in an electron- spin interaction with either population of heme.

Figure 2

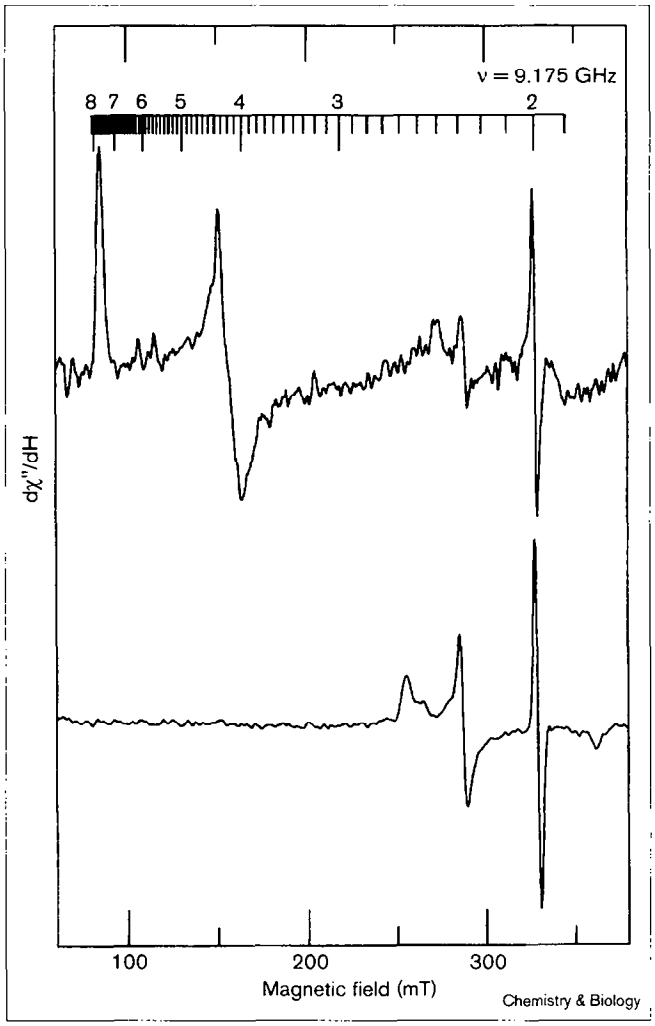
Electronic absorption spectra of nNOS immediately following purification and after exposure to 2 mM imidazole. The scale on the left refers to the heme Soret region and the scale on the right refers to the long-wavelength visible region. Heme Soret absorbance maxima occur at 396 and 427 nm respectively; other features arise as described in the text.



This technique can be applied to calibrate spin-relaxation rates by measuring the peak-to-trough height of a signal as a function of the incident microwave power at a constant temperature [17]. All power saturation data presented in this report were collected at 25 K. The relaxation properties of the flavin-semiquinone radical show no dependence on the spin state of the heme, which suggests that there is no dipolar interaction between the two spins (Figure 4). Both heme species relax faster than the flavin radical and would be expected to dominate the relaxation behavior of the semiquinone if the spins were relaxing together through dipolar or exchange interactions. The high-spin heme should relax faster than the low-spin species because of the complexity of its ground state, however; this allows for a more efficient interchange of energy quanta (i.e., coupling of the spin and phonon systems). For a high-spin ferric ion, there is an ^6S ground term that is partitioned into three doublets by high-order perturbations dependent on the ligand field and spin–orbit interactions; there will therefore be some relatively low lying excited states (within the phonon continuum) available to participate in the relaxation process that allows the phonon system to efficiently interchange quanta with the spin system [16]. Also, there is no change in the linewidth of the flavin radical upon conversion of the heme from spin 5/2 to spin 1/2. Dipolar magnetic interactions often induce spectral broadening that would be particularly pronounced if the monitored species has a T_1 that is significantly longer than that of the perturbing species, and this would be the case when considering the relaxation of a flavin radical compared to the relaxation of a high-spin iron [17].

In addition to these observations, no half-field spin interaction signals were detected between the flavin radical and either heme species at an incident microwave power of 200 mW (data not shown); this transition is almost always forbidden when considering an interaction between a radical and a spin 5/2 iron, however. We also examined the

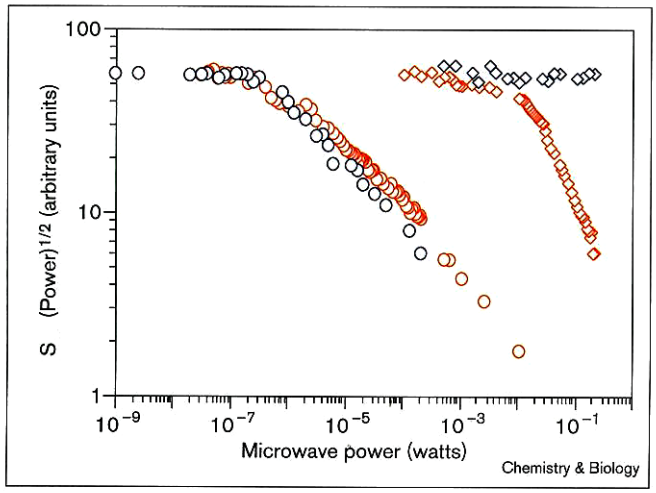
Figure 3



Field-swept EPR spectra of wild-type nNOS immediately following purification (upper trace) and wild-type nNOS in the presence of 2 mM imidazole (lower trace). EPR parameters: modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; temperature 25 K; microwave frequency, 9.175 GHz. The upper spectrum was collected with an incident microwave power of 20 mW, and the lower spectrum was collected with an incident microwave power of 10 mW. A buffer blank was subtracted from each spectrum to eliminate resonance cavity signals. The y-axis is the derivative of the magnetic susceptibility with respect to the magnetic field strength. The integral of this derivative is the EPR intensity. The scale bar reflects the effective g value at the given magnetic field and is nonlinear because it is proportional to the inverse of the field strength.

imidazole-bound nNOS flavin radical linewidth as a function of temperature and observed no additional broadening (data not shown). Cooling from 25 K to 12 K would be expected to dampen some vibrational modes that could mask a spin–spin interaction between a rapidly relaxing system and a radical, allowing the interaction to be observed as a change in linewidth with the concomitant appearance of a half-field transition. In accord with this, no half-field signals were detected at 12 K; this experiment

Figure 4



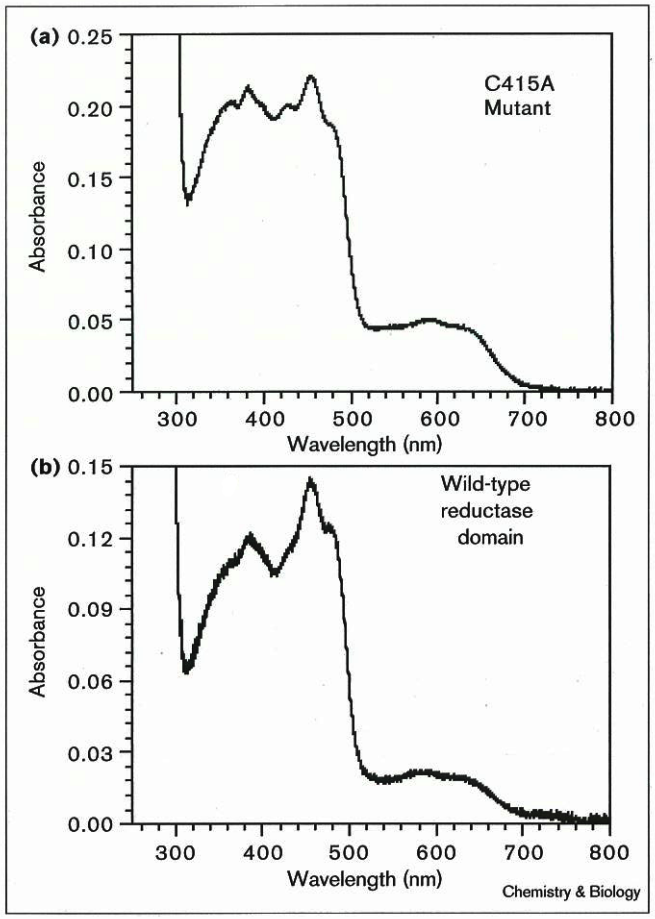
Progressive microwave power saturation EPR of wild-type nNOS and wild-type nNOS in the presence of 2 mM imidazole. Blue symbols represent nNOS immediately following purification (circles are the flavin radical, diamonds are the high-spin heme) and red symbols represent nNOS + 2 mM imidazole (circles are the flavin radical, diamonds are the low-spin heme). S′ is the peak-to-trough amplitude of the flavin radical or the g_y of the low-spin heme or the peak height of the g_x of the high-spin heme. EPR parameters were identical to those used to collect the field-swept spectral data shown in Figure 3.

was also performed at 6 K, and, likewise, no change in linewidth of the flavin radical signal was observed. Similar power saturation results have been demonstrated by Tsai *et al.* [18] on a recent EPR investigation of the eNOS.

Relationship between heme occupancy and flavin radical relaxation

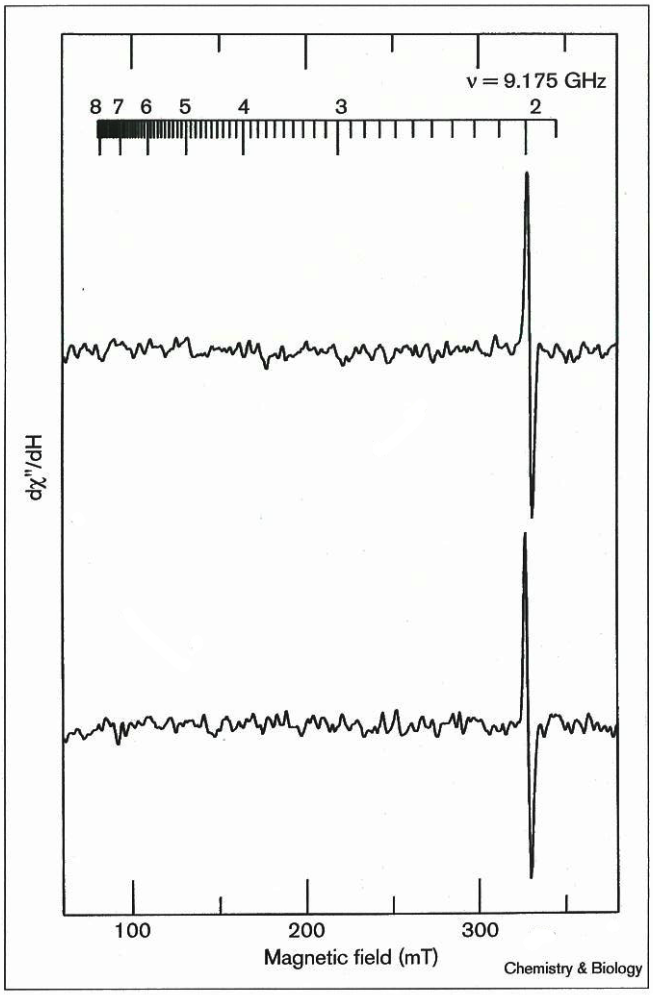
To circumvent the potential complication of a similar net effect on the flavin radical relaxation properties brought about by the different heme spin states, we wished to assess the radical relaxation behavior in the absence of heme. This was accomplished by using two heme-free NOS polypeptides: the Cys415→Ala (C415A) point mutant (heme-ligand mutant) and the reductase domain, which was generated by proteolysis of the wild-type nNOS. Electronic absorption spectra (Figure 5) of these species demonstrate the absence of bound heme, which is corroborated by field-swept EPR (Figure 6). The optical spectra of the C415A mutant and the reductase domain are not identical; both, however, demonstrate the full complement of transitions imparted to the nNOS spectrum by the flavin prosthetic groups. The semiquinone absorbance is most prevalent in the visible region of these spectra from 530–700 nm. The other major transition associated with the semiquinone redox species is centered at 355 nm and 357 nm in the C415A mutant and the reductase domain, respectively. Spectral maxima, which represent the optical contribution from the fully oxidized population of flavin, appear at 385, 454, and 480 nm in both

Figure 5



Electronic absorption spectra of the C415A point mutant and the reductase domain of wild-type nNOS. Both spectra were collected immediately following the purification procedure and illustrate the absorbance contributions of the oxidized flavin and the neutral flavin semiquinone chromophores.

Figure 6



Field-swept EPR spectra of the C415A point mutant (upper trace) and the reductase domain (lower trace) of nNOS. Both spectra were collected with an incident microwave power of 2 mW; other EPR parameters were identical to those used to generate the field-swept spectra of the wild-type nNOS shown in Figure 3.

preparations. The reason for the optical discrepancy between the two preparations is unclear; it could, however, be accounted for by a low extinction charge transfer band derived from the heme domain. The EPR spectra of the C415A mutant and the reductase domain are identical, and each contains the 20 G semiquinone radical as the only observable paramagnetic center. When the power saturation experiment was applied to these preparations, the relaxation behavior of the semiquinone radical in each was determined to be identical to that observed in the full length, wild-type preparations of nNOS (Figure 7).

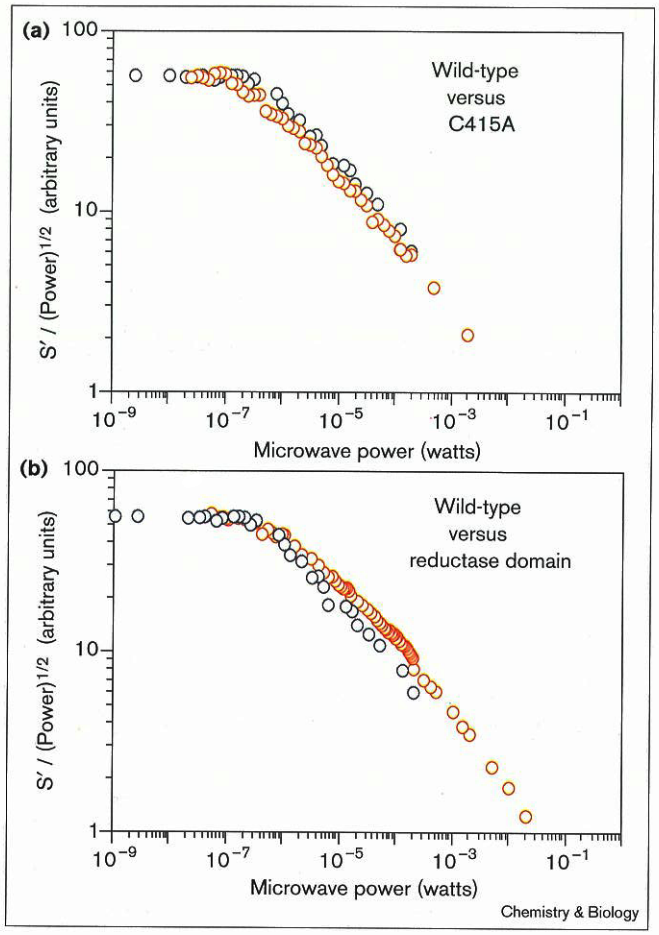
Effect of Ca^{+2}/calmodulin binding on flavin radical relaxation

Because the binding of the Ca^{+2}/calmodulin complex is known to trigger NADPH-supported electron transfer from the high potential flavin to the heme of nNOS, we investigated whether the addition of the Ca^{2+}/calmodulin complex would alter the magnetic properties of the flavin radical. As in all other preparations studied, power saturation of the nNOS flavin radical in the presence of the Ca^{+2}/calmodulin complex demonstrates no perturbation of the flavin radical relaxation properties from those measured on nNOS immediately following purification (Figure 8).

Discussion

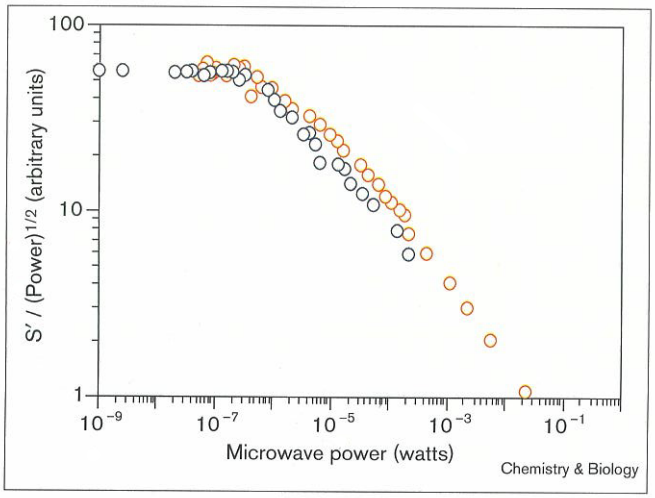
EPR studies on the air-stable neutral flavin semiquinone radical of nNOS have been carried out as part of our ongoing investigation of the overall reaction mechanism that results in the conversion of L-arginine to L-citrulline and NO. At least some part of the overall conversion is driven by NADPH-dependent heme reduction, which is presumably mediated by electron-pair splitting on the isoalloxazine rings of the two-flavin system contained in

Figure 7



Progressive microwave power saturation of the flavin-semiquinone radical observed in the C415A point mutant and the reductase domain of nNOS. Blue circles represent the saturation behavior of the flavin radical of wild-type nNOS in both plots, and red circles represent the saturation behavior of the flavin radical in the **(a)** C415A point mutant and **(b)** the reductase domain. S′ is the measured peak-to-trough amplitude of the flavin radical; EPR parameters were identical to those used to collect the field-swept spectral data shown in Figure 3.

Figure 8

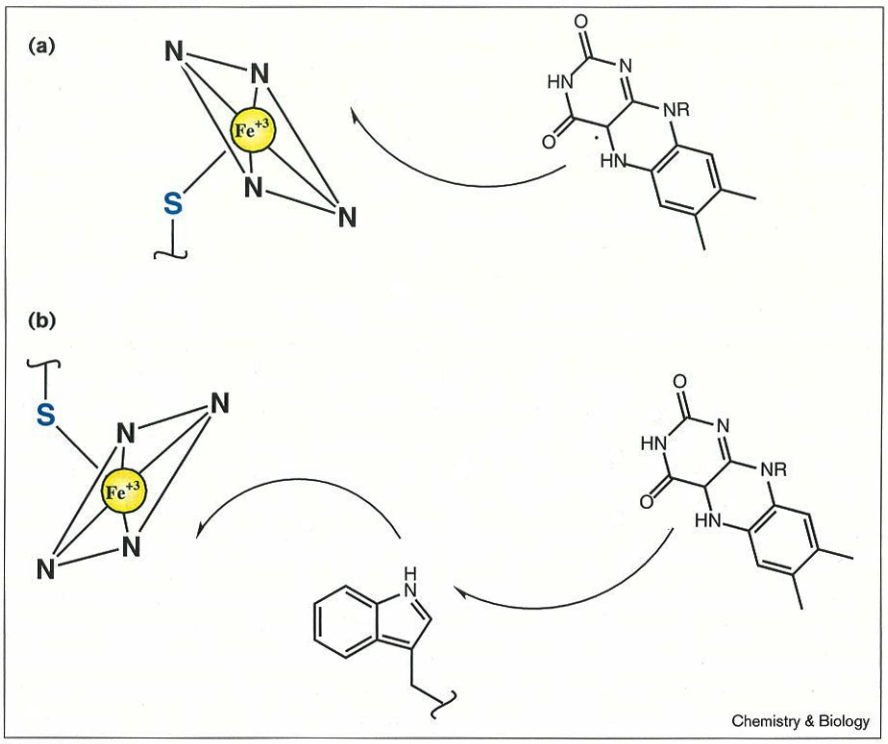


Comparison of the flavin radical saturation behavior in the presence and absence of calmodulin. Blue circles represent the saturation behavior of the flavin radical of wild-type nNOS as it is isolated, and red circles represent the saturation behavior of the flavin radical (12 μM nNOS) in the presence of 2 mM $CaCl_2$, and 36 μM calmodulin. S′ is the measured peak-to-trough amplitude of the flavin radical; EPR parameters were identical to those used to collect the field-swept spectral data shown in Figure 3.

the reductase domain of NOS. Therefore, an assessment of any observable interactions between the flavin and heme cofactors is integral to gaining a better understanding of the architecture of the active site as well as the mechanism by which electron transfer and, ultimately, catalysis proceeds. It had previously been speculated that the flavin radical and the high-spin heme were close enough to engage in a dipolar spin–spin interaction, which was based on an EPR-visible flavin radical signal at an incident microwave power of 10 mW [7]. This hypothesis was supported by a recent power saturation EPR experiment in which the relaxation properties of the resting wild-type nNOS were compared to those of a heme-depleted sample [15]. It was observed that the flavin-radical spin of the heme-depleted sample relaxed more slowly than that of the intact wild-type enzyme. In addition, data from a saturation–recovery experiment on the flavin radical were fit to a non-single exponential, and similar studies on the Y_D radical of manganese-depleted photosystem II indicated that this type of curve could be generated by a dipolar-spin interaction [19]. Surprisingly, however, neither the substrate L-arginine [15] nor the addition of Ca^{+2}/calmodulin [18] (Figure 8) (which triggers NADPH-dependent heme reduction) had any significant effect on the relaxation properties of the NOS flavin radical. The lack of an effect by Ca^{+2}/calmodulin is particularly interesting because it apparently rules out the rather simple model that calmodulin binding serves as a switch that reorients the flavin and heme cofactors to permit efficient electron transfer between them.

Our data contrasts with the previous observations regarding the relaxation behavior of the flavin-semiquinone radical. First, we observed no change in flavin relaxation on conversion of the heme from high (S = 5/2) to low (S = 1/2) spin. The high-spin heme has five unpaired electrons and a magnetic moment approximately 3.5 times that of the low-spin heme, which has only one unpaired electron. The altered magnetic properties of the heme center should affect the relaxation properties of the flavin radical if the two spins have a dipolar interaction; in fact, if the interaction is strong, the spins should relax together. If the low-spin heme were to bring about a similar net

Figure 9



Models of cofactor positioning and electron transfer in NOS. **(a)** An earlier model was based on speculation on the relationship between the high-potential flavin and heme cofactors of NOS. A magnetic linkage between these two cofactors in the resting or Ca^{+2}/calmodulin-activated state of the enzyme would indicate that they are in close proximity to each other, and that there is probably a direct electron-transfer event between them. **(b)** Based on the current data, we present the possibility of an alternative heme-reduction pathway that invokes the participation of other amino-acid residues in the electron-transfer process (for example, tryptophan residues as shown in the figure), and permits a greater distance between the flavin and heme cofactors ($\geq$ 15 Å).

enhancement of radical relaxation as the high-spin heme, so that progressive microwave power saturation of the radical spin could not distinguish between the interactions (as could be the case if the low-spin iron were moved closer to the radical center or into a geometry more favorable for spin diffusion), it should generate a half-field resonance. We were unable to detect any half-field interaction signals between the flavin radical and the low-spin heme at either 25 K or 12 K. These data suggested that there might not be any magnetic interaction between the flavin and heme cofactors.

To examine whether the flavin relaxation properties were dependent on any other unspecified interaction with the heme domain, we generated two heme-free NOS polypeptides for comparison: the C415A point mutant and the independent reductase domain. Both of these preparations were able to maintain the air-stable semiquinone radical, and the spin-relaxation properties of these radicals were indistinguishable from each other and from the wild-type nNOS. This has led us to the conclusion that any components necessary for enhancing the relaxation of the flavin radical are contained within the reductase domain, and are most likely to be the direct effects of local polypeptide composition and conformation.

The reason for the discrepancy between the current results and the previously reported observations is unclear; it could, however, reflect differences in sample preparation. At the time when the earlier studies were initiated, the *Escherichia coli* expression system of nNOS was not readily available and the authors had to rely on the relatively harsh procedure of prolonged dialysis against 2 M urea to generate the heme-free apoNOS that was used for comparison with the intact wild-type enzyme [20]. This urea dialysis preparation was not able to stabilize the semiquinone radical, and the sample used for the power saturation experiment had to be pre-reduced immediately prior to freezing; hence, the environment of observed radical signal might not have been homogeneous [15]. If the structure of the reductase domain were perturbed during the sample preparation, and structural integrity is required for the flavin-radical relaxation enhancement, one might expect to observe a more slowly relaxing radical species that more closely resembles an isolated free radical.

Relaxation enhancements of a protein-associated radical greater than what would be expected for an isolated free radical are not unprecedented. $P^{1/2}$ is the microwave power needed for half-saturation of an EPR signal and is a convenient comparative measure of spin-relaxation rates. The $P^{1/2}$ value reported for the NOS flavin radical ranges between 1 and 50 μW depending on the protein source and amount of dissolved oxygen in the sample. Therefore, although there is an intense semiquinone signal visible up to 20 mW, the spin is heavily saturated. The $P^{1/2}$ of the FMN semiquinone radical of flavodoxin was measured to be 200 μW at 170 K, which indicates a significant relaxation

enhancement over an isolated free radical [21]. As a caveat, this value cannot provide a direct comparison between the NOS flavin radical and that of flavodoxin because relaxation properties are temperature-dependent and the process is accelerated at a higher temperature as more relaxation pathways become accessible. This trend is particularly apparent when considering the EPR properties of high-spin species, such as the wild-type nNOS heme, for which it is necessary that the sample be cooled to 25 K or lower to observe the associated resonance transitions.

Local magnetic fields induced by unpaired electron spins that are in a fixed orientation because of the protein matrix should have distance-dependent interactions with each other. There are two major electron-spin interactions to consider, dipole–dipole and exchange. The exchange component is of electrostatic origin and at distances of less than 5 Å, or when bridging atoms can participate, it usually dominates and gives rise to a coupled-spin system such as that observed between heme a_3 and the mononuclear copper center (CuB) in cytochrome c oxidase. Dipolar interactions, however, are the result of overlapping magnetic fields and operate through a significantly greater range, usually up to ~15 Å [22]. Because the flavin radical of nNOS seems to have no dipolar interactions with the active-site heme (even in the presence of Ca^{+2} and calmodulin), we infer that the distance between the two cofactors is probably greater than 15 Å. This is not surprising in light of the proposed domain structure of NOS; it is in contrast, however, with the current model which places the high-potential flavin in spatial proximity to the high-spin heme, a juxtaposition that would presumably permit a direct electron transfer (Figure 9a). Although a direct electron transfer between these two cofactors cannot be ruled out by the absence of a magnetic interaction, the presumed minimal distance between the flavin radical and the heme suggests that other functional groups, perhaps aromatic amino acids, could be involved in the process. The NOS heme domain contains ten tryptophans that are completely conserved in the 30 genes that have been sequenced. Any of these tryptophans could potentially serve to facilitate long-range electron transfer. Consistent with this, the recently solved crystal structure of the NOS heme domain reveals that two of these tryptophans are positioned adjacent to the heme, one on each side of the heme plane [23]. Figure 9b depicts a potential route of electron transfer that is consistent with both the crystal structure and the data presented in this report. Alternatively, the process could be mediated by electron tunneling along the peptide backbone, which is an increasingly attractive model of electron transfer in other known electron-transfer proteins such as azurin and cytochrome c [24].

In conclusion, we judge the relaxation behavior of the flavin-semiquinone radical of nNOS to be a product of its environment within the reductase domain and is probably not a result of an electron-spin interaction with the active-site heme, or any other component of the heme domain. These observations are in contrast to the current model which contends that efficient electron-spin relaxation of the flavin radical is a consequence of an interaction with the active-site heme. In addition, our results indicate that the high-potential flavin of NOS is probably not incorporated into the active site; rather, the flavin donates electrons to the heme from a more remote location.

Significance

Nitric oxide synthase (NOS) is a homodimeric enzyme that catalyzes the NADPH-dependent conversion of L-arginine to L-citrulline and nitric oxide. Each subunit contains one equivalent each of the two flavin cofactors FAD and FMN, as well as one equivalent of a protoporphyrin IX heme. The NOS gene encodes two domains, a reductase domain that binds the flavin cofactors and NADPH and an oxygenase domain that binds the heme and L-arginine. A short helix containing a calmodulin-recognition sequence is believed to link these two domains. The catalytic mechanism of NOS is thought to be driven by the calmodulin-dependent sequential transfer of NADPH-derived electrons from the reductase domain to the active site where the heme is reduced. It is not yet clear whether there is a direct electron transfer between a reduced flavin and the heme or if other protein components participate in an electronic relay to bridge the two centers. The pre-existing model, based on similarities between NOS and cytochrome P450 reductase enzymes, favors a direct electron-transfer process. Our demonstration that two paramagnetic species of redox-active cofactors (high-potential flavin-semiquinone radical and ferric heme) localized to separate domains of nitric oxide synthase (NOS) have no magnetic interaction with each other contradicts the previously described model of electron transfer. The former model placed the high potential flavin of the reductase domain of NOS in close proximity to the active site of the oxygenase (heme) domain and implied that a direct electron-transfer event occurred between flavin and heme. We have proposed a novel and testable model of the electron transfer between these two cofactors that recruits the redox capability of conserved amino-acid residues. This model is permissive of the long-range electron transfer that is implied by our results, which indicate that the distance separating the high-potential flavin and heme of NOS is at least 15 Å.

Materials and methods

Materials

2′, 5′-ADP-Sepharose, calmodulin-Sepharose 4B, and the HiLoad 26/60 Superdex-200 gel filtration column were obtained from Pharmacia. (6R) -5, 6, 7, 8-tetrahydro-L-biopterin was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland), and 10 mM stock vials of this reagent were prepared in 50 mM Hepes buffer pH 7.4 containing 100 mM dithiothreitol (DTT). *E. coli* DH5α competent cells, T4 DNA ligase, and IPTG were from Gibco-BRL. All necessary restriction enzymes, ampicillin, and the Expand High Fidelity PCR kit were purchased from Boehringer Mannheim. The original cDNA clone for rat

neuronal NOS contained in the vector Bluescript (SK^-) was a gift from Dr Solomon H. Snyder (Johns Hopkins University), whereas the expression plasmid used, pCWori, was generously provided by Dr Michael R. Waterman (Vanderbilt University). All materials and reagents not described above were obtained from Sigma.

Cloning and mutagenesis

The pCWnNOS expression vector was constructed using the protocol developed by Gerber and Ortiz de Montellano [25]. The C415A mutant was generated using the overlap extension PCR method [26]. The external primers used for this purpose were (5′-GGCTCCAAGGCCCACATGGAC-3′) and (5′-CACTTCCAGCACCAGCTCTGG-3′). The mutagenic primers were (5′-ACGCCTCTCGAGCTGTGGGCAGGA-3′) and (5′-TCCTGCCCACAGCTCGAGAGGCGT-3′). The resulting PCR fragment was subcloned into pCWnNOS that had been pretreated with *Sac*I, and the orientation of the inserted fragment was assessed by screening transformants with a mixture of *Apa*I and *Nar*I. Mutations and fidelity of the PCR reactions were confirmed by DNA sequencing. All primer synthesis and DNA sequencing was performed by the University of Michigan Biomedical Research Core Facility.

pCWnNOS expression

An overnight culture of DH5α–pCWnNOS was used to inoculate four 4 l flasks each containing 1 l of modified 2xYT media and 50 mg ampicillin. Casein N-Z plus enzymatic hydrolysate was used in lieu of the tryptone recommended for the YT media. These cultures were grown at 37°C until the OD_{600} was 0.6, at which time the temperature in the incubator was equilibrated to 22°C. Upon cooling of the cultures, pCWnNOS expression was induced by adding IPTG to a final concentration of 1 mM. After induction, the cultures were allowed to grow for an additional 18 h at 22°C before being harvested by low-speed centrifugation. The expression scheme used for the C415A mutant was identical to that used to express the wild-type nNOS.

Purification

The cell pellets generated from 4 l of culture were resuspended in 50 ml of sonication buffer (100 mM Hepes pH 7.4, 2 mM $CaCl_2$, 35% glycerol, 1 μg/ml antipain, and 1 μM benzamidine) and lysed in the presence of 100 μM H_4B and 1 mM DTT. The cell debris was pelleted by ultracentrifugation at 100,000 × g for 1.5 h and the resulting supernatant was applied to a 30 mL calmodulin–Sepharose column that had been pre-equilibrated with 2 bed volumes of buffer A (100 mM Hepes pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$, and 10% glycerol). After loading, the column was washed with 250 ml of buffer A containing 10 μM H_4B and 0.1 mM DTT. The bound protein was eluted from the calmodulin–Sepharose column with buffer B (100 mM Hepes pH 7.4, 150 mM NaCl, 10 μM H_4B, and 0.1 mM DTT) containing 10 mM EGTA and passed directly onto a 10 ml 2′, 5′-ADP–Sepharose column that had been pre-equilibrated with 2 bed volumes of buffer B. This column was washed with 100 ml of buffer B and subsequently with 100 mL of buffer B supplemented with 300 mM NaCl. The bound protein was eluted with 50 ml of buffer B containing 10 mM NADPH and concentrated to 10 mL using ultrafiltration. The protein concentrate was subjected to gel filtration on a Superdex–200 column pre-equilibrated with buffer C (100 mM Hepes pH 7.4, 400 mM NaCl, and 10% glycerol), and fractions containing NOS, which migrates as a single band at 160 kDa on a Coomassie-stained SDS–PAGE, were pooled. Samples were concentrated and desalted, if necessary, and stored at −80°C until use. This purification procedure typically yields between 3 and 5 mg of pure nNOS per litre of culture with a specific activity ranging from 480–640 nmol min^{-1} mg^{-1} as determined by monitoring the conversion of ferrous-oxy hemoglobin to methemoglobin at 37°C [27]. The purification procedure for the C415A mutant was identical to that of the wild-type NOS with the exception that it elutes from the gel filtration column at a significantly longer retention time, consistent with it being monomeric. The reductase domain was obtained as a by-product of the nNOS overproduction system and copurified with the full-length protein until the gel filtration step where it was separated to greater than 90% purity, as judged by Coomassie-stained SDS–PAGE. The amino terminus of the reductase domain was sequenced using Edman degradation at the University of Michigan core facility; the readable sequence was LAEAVKFS (using single-letter amino-acid code), which corresponds to amino acid residues 734–741 of the rat nNOS. Imidazole-bound wild-type nNOS was prepared by concentrating the sample in buffer B containing 2 mM imidazole. Protein concentrations were determined using the Bradford microassay procedure with bovine serum albumen (BSA) as the standard.

Electronic absorption characterization

All optical spectra were recorded on a Cary 3E spectrophotometer at a constant temperature of 25°C, maintained by a circulating water bath. A buffer blank was subtracted from each spectrum.

Electron paramagnetic resonance characterization

Measurements were made using a Varian Century E-Line X-band EPR Spectrometer. Protein samples used in the EPR studies were 50–100 μM, and all spectra reported here were collected at 25 K. The final concentration of glycerol in all EPR samples ranges between 20 and 30% (v/v). The precise concentration is unknown because protein samples were concentrated by water evaporation under vacuum immediately prior to freezing; this technique also limits the amount of dissolved oxygen in the sample. Progressive microwave power saturation data were fitted to the following equation:

$$y = \text{peak amplitude} \times \text{gain}_{(r)} / \text{gain}_{(s)} \times (\text{power}_{(s)} / \text{power}_{(r)})^{1/2} \quad (1)$$

where r and s represent the reference point and the recorded spectrum used to generate data points respectively.

Acknowledgements

We wish to thank members of the Marletta laboratory for their critical comments on this manuscript, and the National Institutes of Health for support of this research (CA50414; M.A.M.), (GM32785; W.R.D.). M.A.M. is an Investigator at the Howard Hughes Medical Institute.

References

1. Jaffrey, S.R. & Snyder, S.H. (1996). PIN: protein inhibitor of neuronal NOS. *Science* **274,** 774-776.
2. Marletta, M.A. (1994). Nitric oxide synthase: aspects concerning structure and catalysis. *Cell* **78,** 927-930.
3. Cho, H.J., Xie, Q.-W., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D. & Nathan, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J. Exp. Med.* **176,** 599-604.
4. Stevens-Truss, R. & Marletta, M.A. (1995). Interaction of calmodulin with the inducible murine macrophage nitric oxide synthase. *Biochemistry* **34,** 15638-15645.
5. Marletta, M.A. (1993). Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268,** 12231-12234.
6. McMillan, K., Bredt, D.S., Hirsch, D.J., Snyder, S.H., Clark, J.E. & Masters, B.S.S. (1992) Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl Acad. Sci. USA* **89,** 11141-11145.
7. Stuehr, D.J. & Ikeda-Saito, M. (1992). Spectral characterization of brain and macrophage nitric oxide synthases. *J. Biol. Chem.* **267,** 20547-20550.
8. White, K.A. & Marletta, M.A. (1992). Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* **31,** 6627-6631.
9. Hevel, J.M., White, K.A. & Marletta, M.A. (1992). Purification of the inducible murine macrophage nitric oxide synthase: identification as a flavoprotein and detection of enzyme-bound tetrahydrobiopterin. In *The Biology of Nitric Oxide.* (Moncada, S., Marletta, M.A., Hibbs Jr, J.B. & Higgs, E.A., eds), pp. 19-21, Portland Press, London.
10. Stuehr, D.J., Cho, H.J., Kwon, N.S., Weise, M.F. & Nathan, C.F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl Acad. Sci. USA* **88,** 7773-7777.
11. Hevel, J.M. & Marletta, M.A. (1992). Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity. *Biochemistry* **31,** 7160-7165.
12. Richards, M.K. & Marletta, M.A. (1994) Characterization of neuronal nitric oxide synthase and a C415H mutant, purified from a baculovirus overexpression system. *Biochemistry* **33,** 14723-14732.

13. Sheta, E.A., McMillan, K. & Masters, B.S.S. (1994). Evidence for a biodomain structure of constitutive cerebellar nitric oxide synthase. *J. Biol. Chem.* **269,** 15147-15153.
14. Vermilion, J.L. & Coon, M.J. (1978). Identification of the high and low potential flavins of liver microsomal NADPH-cytochrome P-450 reductase. *J. Biol. Chem.* **253,** 8812-8819.
15. Galli, C., MacArthur, R., Abu-Soud, H.M., Clark, P., Stuehr, D.J. & Brudvig, G.W. (1996). EPR Spectroscopic characterization of neuronal NO synthase. *Biochemistry* **35,** 2804-2810.
16. Herrick, R.C. & Stapleton, H.J. (1976). Determination of the zero-field splitting of Fe^{3+} in. cytochrome $P\text{-}450^{cam}$ from electron spin-lattice relaxation rates. *J. Chem. Phys.* **65,** 4786-4790.
17. Hales, B.J. (1993). Intrinsic and extrinsic paramagnets as probes of metal clusters. *Methods Enzymol.* **227,** 384-395.
18. Tsai, A.-L., Berka, V., Chen, P.-F. & Palmer, G. (1996). Characterization of endothelial nitric-oxide synthase and its reaction with ligand by electron paramagnetic resonance spectroscopy. *J. Biol. Chem.* **271,** 32563-32571.
19. Koulougliotis, D., Tang, X.-S., Diner, B.A. & Brudvig, G.W. (1995). Spectroscopic evidence for the symmetric location of tyrosines D and Z in photosystem II. *Biochemistry* **34,** 2850-2856.
20. Abu-Soud, H.M., Yoho, L.L. & Stuehr, D.J. (1994). Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. *J. Biol. Chem.* **269,** 32047-32050.
21. Ohnishi, T., King, T.E., Salerno, J.C., Blum, H., Bowyer, J.R. & Maida, T. (1981). Thermodynamic and electron paramagnetic resonance characterization of flavin in succinate dehydrogenase. *J. Biol. Chem.* **256,** 5577-5582.
22. Cammack, R. & Cooper, C.E. (1993). Electron paramagnetic resonance spectroscopy of iron complexes and iron-containing proteins. *Methods Enzymol.* **227,** 353-384.
23. Crane, B.R., *et al.*. & Tainer, J.A. (1997). The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. *Science* **278,** 425-431.
24. Langen, R., Chang, I.J., Germanas, J.P., Richards, J.H., Winkler, J.R. & Gray, H.B. (1995). Electron tunneling in protein: coupling through a beta strand. *Science* **268,** 1733-1735.
25. Gerber, N.C. & Ortiz de Montellano, P.R. (1995). Neuronal nitric oxide synthase. *J. Biol. Chem.* **270,** 17791-17796.
26. Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. & Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77,** 51-59.
27. Hevel, J.M. & Marletta, M.A. (1994). Assays for NO synthase. *Methods Enzymol.* **233,** 250-258.

Because *Chemistry & Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published via the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cmb – for further information, see the explanation on the contents pages.