Comparative Effects of Substrates and Pterin Cofactor on the Heme Midpoint Potential in Inducible and Neuronal Nitric Oxide Synthases

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Abstract: The nitric oxide synthases (NOS) are heme-containing enzymes responsible for catalyzing the five-electron oxidation of a guanidino nitrogen of L-arginine to produce the free radical nitric oxide. The binding sites of the heme group, as well as of the L-arginine substrate and tetrahydrobiopterin cofactor, are located within the oxygenase domain of the NOS enzymes. Reduction of the heme is the first committed step in catalysis, as this allows for binding and activation of molecular oxygen, followed by oxidative attack on the L-arginine substrate. As with heme groups in other enzymes, the electronic properties of the NOS heme are modified by substrate and cofactor binding in its vicinity. Here we present the first quantitative thermodynamic data of the NOS heme with the determination of the heme midpoint reduction potentials for the neuronal NOS and inducible NOS oxygenase domains. In the absence of L-arginine and tetrahydrobiopterin, the midpoint potential of the inducible NOS oxygenase heme iron is over 100 mV lower than that of the neuronal NOS oxygenase heme iron. Binding of the substrate alone, cofactor alone, or both combined with the inducible NOS oxygenase increases the heme iron reduction potential by 112, 52, and 84 mV, respectively. On the basis of these data, we calculate that the binding affinities of L-arginine and tetrahydrobiopterin increase by about 80-fold and 8-fold, respectively, for the reduced heme iron form of the enzyme. These data support interactive binding of L-arginine and tetrahydrobiopterin in proximity to the inducible NOS heme group, as observed in the crystal structure of this enzyme. In contrast, addition of L-arginine, tetrahydrobiopterin, or both to neuronal NOS oxygenase do not markedly change its heme iron midpoint potential, with observed shifts of +19, −18, and −10 mV, respectively. These data explain the contrasting reactivities between the two NOS isoforms regarding their different NADPH consumption rates and capacity to support heme iron reduction and are indicative of the regulatory mechanisms that each enzyme employs toward electron transfer. We also examine the effects of three substrate-based inhibitors of NOS on the heme iron midpoint potentials. Among these inhibitors, *S*-ethylisothiourea decreased the heme potentials of tetrahydrobiopterin-bound inducible NOS and neuronal NOS by 27 and 24 mV, respectively, *N*-nitro-L-arginine methyl ester lowered both potentials below −460 mV, and aminoguanidine slightly increased both potentials. This work suggests the following: (1) A thermodynamic block of reductase-catalyzed heme reduction exists in inducible NOS but not in neuronal NOS in the absence of substrate and tetrahydrobiopterin. This reveals distinct heme environments for the two isoforms. (2) Heme iron reduction thermodynamics in inducible NOS are improved by tetrahydrobiopterin and L-arginine, implying that this isoform is uniquely configured to respond to substrate and pterin control. (3) Some, but not all, inhibitors that reduce electron flux through NOS act by affecting the thermodynamics of heme iron reduction.

Introduction

Over the past decade, the free radical nitric oxide (NO)[1] has come under intensive scientific investigation.[2] This simple diatomic molecule has an amazing range of physiologic effects, including neurotransmission, vasodilation, cytotoxicity, and involvement in almost all pathologic states. NO is produced in the body by the nitric oxide synthase (NOS) enzymes,[3] a family of three isozymes that catalyze the five-electron oxidation of L-arginine to L-citrulline and NO. The three NOS isoforms can be distinguished according to their initial cellular identifications (neuronal NOS; endothelial NOS; inducible NOS in cytokine-treated cells), primary sequence, function of the evolved NO product (nNOS, neurotransmission; eNOS, maintenance of vascular tone; iNOS, killing foreign cells), and modes of enzyme activity (nNOS, Ca^{2+}-dependent and high activity; eNOS, Ca^{2+}-dependent and low activity; iNOS, Ca^{2+}-independent and high activity).

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(1) Abbreviations used: NO, nitric oxide; NOS, NO synthase; iNOS, inucible NOS; nNOS, neuronal NOS; H4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; SEITU, *S*-ethylisothiourea; L-NAME, *N*-nitro-L-arginine methyl ester.

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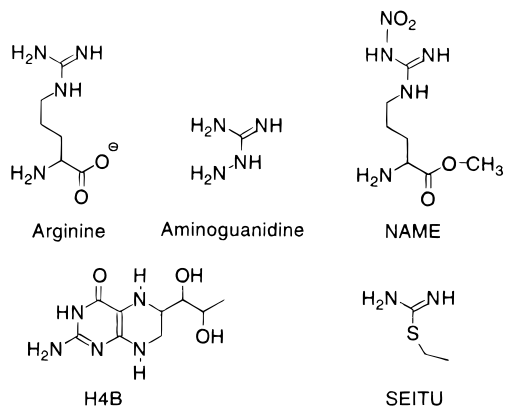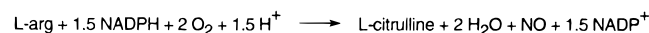
Heme Midpoint Potential in Nitric Oxide Synthases

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Scheme 1

$$\text{L-arg} + 1.5\ \text{NADPH} + 2\ \text{O}_2 + 1.5\ \text{H}^+ \longrightarrow \text{L-citrulline} + 2\ \text{H}_2\text{O} + \text{NO} + 1.5\ \text{NADP}^+$$

Arginine   Aminoguanidine   NAME

H4B   SEITU

Figure 1. Structures of compounds used in this study.

Figure 2. Proposed mechanism for oxygen activation during NO synthesis. Sequential reduction of ferric NOS heme (Fe^{3+}—S) by two NADPH-derived electrons (e^-) enables generation of an iron—oxo species (Fe^{4+}=O) that is envisioned to react with bound L-arginine to form N^ω-hydroxy-L-arginine (Arg-NOH) as an enzyme-bound intermediate. Subsequent one-electron reduction of ferric NOS enables formation of the iron—dioxy species (Fe^{3+}O$_2$)$^-$ that reacts with Arg-NOH, forming NO and L-citrulline as products (from refs 3, 4, and 9a).

Despite these differences, the three NOS isoforms display many structural and mechanistic similarities. Each NOS utilizes L-arginine, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and O$_2$ as substrates and catalyzes a reaction in which 1.5 equiv of NADPH is consumed for every 1 equiv of NO produced.[4] The balanced reaction is shown in Scheme 1. The NOS enzymes are dimeric and have a bidomain structure, with binding sites for flavin adenine dinucleotide, flavin mononucleotide, and NADPH located in the reductase domain (by analogy with the cytochrome P450 reductases). The reductase domain also has a binding site for calmodulin, which is tightly bound in the case of iNOS but transiently bound in nNOS and eNOS dependent on the free Ca^{2+} concentration. The flavins accept electrons from NADPH[5] and, in the calmodulin-bound state, transfer them one at a time to a heme group located in the NOS oxygenase domain.[6] Heme binding to NOS is via cysteine thiolate ligation, suggesting that it functions as in the cytochromes P450. The NOS oxygenase domain also binds the L-arginine substrate and tetrahydrobiopterin (H4B) cofactor in the vicinity of the heme, thus modulating its electronic and spectral properties.[7] The structures of arginine and H4B are shown in Figure 1. The function of H4B in the mechanism of NOS is as yet not clear, but this cofactor is known to facilitate assembly of NOS subunits into the active dimeric structure[7f,8]

and modify the reactivity of heme—oxygen complexes.[7f,9] In the absence of arginine and H4B, the heme group is predominantly in a low-spin state with a Soret maximum at 415—420 nm. Addition of either L-arginine or H4B favors the high-spin state with a shift in the Soret absorbance to about 400 nm. When both arginine and H4B are bound, the heme group exists almost completely in the high-spin state with a Soret maximum at 395 nm. Studies have shown that a similar shift to the high-spin state occurs for cytochrome P450 heme groups upon substrate binding and is associated with increased reduction potential of the heme.[10,11]

Reduction of the heme to its ferrous state is the first committed step toward NO synthesis, leading to O$_2$ binding and activation, and eventual L-arginine oxidation. The proposed mechanistic cycle for NO synthesis catalyzed by NOS is shown in Figure 2. Controlling heme reduction is a central feature for regulating NOS activity.[6,7,12] In contrast to most cytochromes P450, NOS heme reduction has been observed in the absence of substrate, leading to uncoupled electron transfer and the production of the toxic superoxide radical anion or hydrogen peroxide.[7f,13—15] For example, with calmodulin bound, neuronal NOS displays a significant NADPH consumption rate in the absence of both arginine and H4B, generating large amounts of superoxide, although its heme is in the low-spin state.[14] This appears contrary to the current thinking that the low-spin state should have the lowest reduction potential and thus limited

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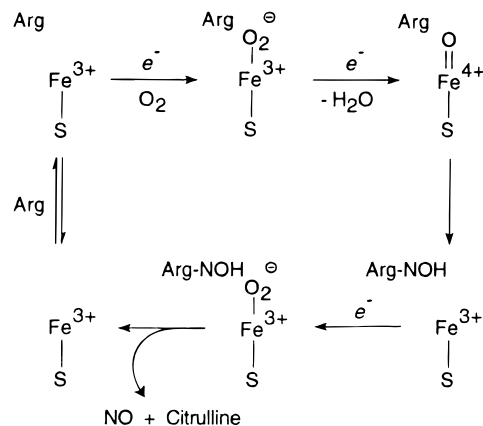
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electron transfer. Several NOS inhibitors are known to decrease NADPH consumption,[7c,f,12,16] possibly by interfering with heme reduction. With the preparation of nNOS and iNOS oxygenase domains that are totally free of arginine and of H4B, we were able to determine and compare the midpoint redox potentials of the heme moieties in the absence and presence of substrate and cofactor and to examine the effects of the substrate-based inhibitors, aminoguanidine, *S*-ethylisothiourea (SEITU), and *N*-nitro-L-arginine methyl ester (L-NAME) (structures shown in Figure 1) on the reducibility of each NOS's heme group.

Experimental Methods

Dimeric arginine- and H4B-free nNOS and iNOS oxygenase domains (amino acid residues 1−720 for neuronal NOS and 1−498 for inducible NOS) were prepared according to previously published methods.[17]

Spectroelectrochemical titrations were performed at 25 °C in 0.1 M potassium phosphate, pH 7.0, with 125 mM NaCl. The glass spectroelectrochemical cell and electrodes are as previously described,[18] and reduction−oxidation titrations were performed using a Radiometer PGP201 potentiostat/galvanostat. The reference electrode was calibrated using a 5 mM solution of ferricyanide/ferrocyanide in 0.1 M potassium phosphate, pH 7.0, at 25 °C (E_m = +425 mV). The NOS oxygenase domain (6−8 μM) was dissolved in the phosphate buffer in the absence or presence of 2.5 mM L-arginine, 25 μM H4B, or the substrate analogue NOS inhibitors 2 mM aminoguanidine, 40 μM SEITU, and 2 mM L-NAME. The concentrations of these species were chosen such that their binding to the enzyme reached saturation, based on the following binding constants to the oxidized heme form of the enzymes: for inducible NOS, $k_{D,L-arginine}$ = 2.3 μM,[4a] $k_{D,H4B}$ = 600 nM in the absence of L-arginine,[7f] $k_{D,H4B}$ = 110 nm in the presence of L-arginine,[7f] $k_{i,aminoguanidine}$ = 16 μM,[16] $k_{i,SEITU}$ = 5 nM;[19] for neuronal NOS, $k_{D,L-arginine}$ = 1.5 μM,[20] $k_{D,H4B}$ = 250 nM in the absence of L-arginine,[21] $k_{D,H4B}$ = 37 nM in the presence of L-arginine,[21] $k_{i,SEITU}$ = 30 nM.[19] Redox mediators (Sigma, St. Louis, MO) with midpoint potentials in the range of the heme potential were also added to the spectroelectrochemical cell. These were 50−150 μM anthraquinone-2-sulfonate (E_m = −225 mV), 15−40 μM phenosafranin (E_m = −252 mV), 20−40 μM neutral red (E_m = −325 mV), 20−50 μM benzylviologen (E_m = −350 mV), and 50 μM methylviologen (E_m = −440 mV).[22] All other chemicals were purchased from sources as previously published.[7f]

The contents of the spectroelectrochemical cell were made anaerobic by alternating cycles of evacuation and refilling with argon. The cell was maintained under positive argon pressure throughout the titration. Spectra were recorded on a Hitachi 3110 spectrophotometer. The one-electron midpoint potentials of the NOS hemes were determined from the absorbance difference at 440 nm minus 390 nm following the subtraction of absorbance contributions from the mediators at each point in the titrations. Using these data and the corresponding measured potentials (vs SHE) the midpoint potential of the half-reaction can be determined using the Nernst equation:

$$E = E_m + 2.303(RT/nF) \log([\text{oxidized}]/[\text{reduced}]) \quad (1)$$

where E is the measured equilibrium potential at each titration point, R is the gas constant (8.314 J/mol·K), T is the experimental temperature in Kelvin, n is the number of electrons in the half-reaction, F is the Faraday constant (96 485 C/mol), and ([oxidized]/[reduced]) is the ratio of oxidized to reduced species determined spectrometrically. From eq 1, a plot of E vs log([oxidized]/[reduced]) gives a slope of 0.059/n (at 25 °C) and a y intercept of the half-reaction midpoint potential, E_m, in volts. The reported values are derived from data of at least three replicate experiments (one replicate done as an oxidative titration) with a standard error in the midpoint potential of less than 10 mV. The potential values are reported versus the standard hydrogen electrode. The experimental method was verified by determining the one-electron midpoint potential of camphor-containing cytochrome P450$_{cam}$ to be −185 mV, which is in agreement with previously published values.[10b]

Results and Discussion

The data for the redox titrations of inducible NOS oxygenase heme along with the best-fit Nernst plots are shown in Figure 3A. These data are summarized in Table 1, along with the heme Soret absorbance maxima and steady-state NADPH oxidase activities (at 37 °C) of the full-length enzymes. In the absence of L-arginine and H4B, the inducible NOS oxygenase heme has a midpoint potential of E_m = −347 mV. This is as low or lower than any midpoint potential reported for a substrate-free cytochrome P450 heme (e.g. E_m = −300 mV for cytochrome P450$_{cam}$[10]) and may reflect the hydrophilic or polar environment of the inducible NOS heme in this state.[11,23,24] Not surprisingly, in the absence of substrate and cofactor, the inducible NOS heme is mostly low-spin (λ_{Soret} = 420 nm) and cannot be reduced by NADPH via the reductase domain,[7f] and full-length inducible NOS exhibits a very low heme-independent NADPH oxidase activity.[7f] Addition of L-arginine or H4B to inducible NOS is known to shift the heme Soret toward high-spin, allow for heme reduction by NADPH and moderately increase the steady-state NADPH consumption rate.[7f,13] It is then predicted that L-arginine and H4B would increase the inducible NOS heme reduction potential. As shown in Figure 3A and Table 1, the addition of H4B alone increases the inducible NOS oxygenase heme E_m to −295 mV, while L-arginine alone increases the E_m to −235 mV. This disparity indicates differences in the effects of L-arginine and H4B binding on the inducible NOS heme environment and in their relative affinities for the reduced versus oxidized form of the enzyme. From the binding constants known for L-arginine (k_D = 2.3 μM)[4a] and H4B (k_D = 0.6 μM)[7f] binding to the oxidized heme form of inducible NOS and the heme reduction potentials under different binding conditions, we are able to calculate apparent k_D values for L-arginine and H4B binding to the reduced heme form of the enzyme according to the following equation:

$$k_{D,red} = 10^{-[(E_m(\text{bound}) - E_m(\text{free}))nF/(2.303RT)]}k_{D,ox} \quad (2)$$

For inducible NOS, the $k_{D,red}$ (L-arginine) = 0.03 μM and $k_{D,red}$ (H4B) = 0.08 μM, meaning that the binding affinities for

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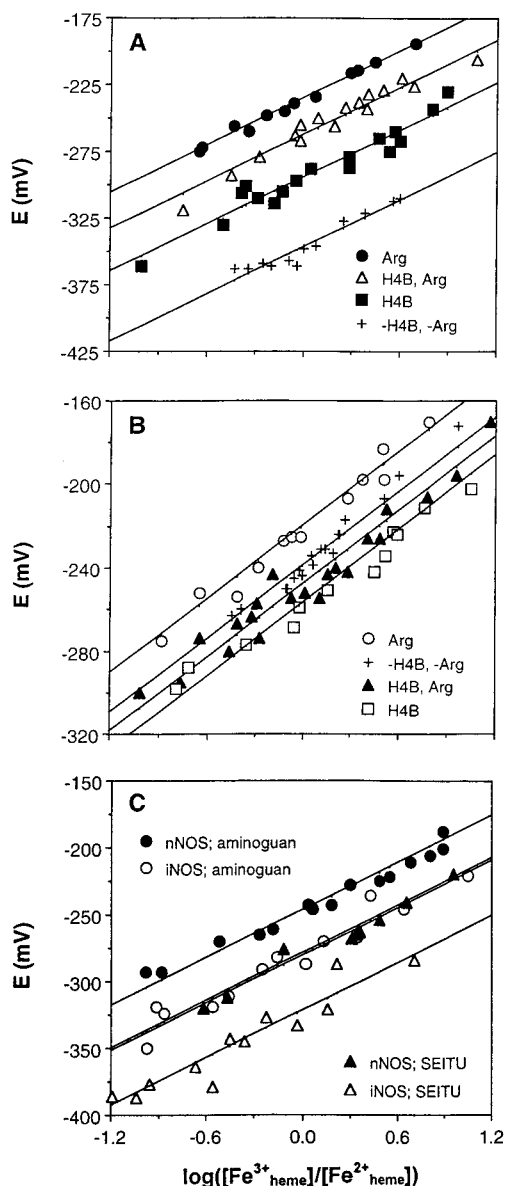
Figure 3. Potentiometric redox titrations of NOS oxygenase domain heme groups and effects of arginine substrate, H4B cofactor, and substrate binding analogues. The data are presented as the measured potential (*E*) vs the logarithmic ratio of ferric/ferrous heme. Panel A is for inducible NOS in the absence and presence of arginine and/or H4B. Panel B is for neuronal NOS in the absence and presence of arginine and/or H4B. Panel C shows the effects of aminoguanidine and SEITU on the redox titrations of both isoforms. The solid lines represent the theoretical one-electron Nernst plots under each condition.

L-arginine and H4B increase by approximately 80-fold and 8-fold, respectively, for the ferrous heme form of inducible NOS. This is consistent with the proposed reaction mechanism since the ferrous heme enzyme reacts with O_2, and increased affinity of this enzyme form for L-arginine and H4B would ensure productive reduction of O_2. Moreover, it indicates that inducible NOS enzyme activity is thermodynamically regulated by substrate and cofactor binding. A summary of the experimentally determined and calculated thermodynamic constants for L-arginine and H4B binding to inducible NOS is presented as a partial thermodynamic cube diagram in Figure 4A. Adding both L-arginine and H4B to inducible NOS slmost completely shifts the heme spin state to high-spin ($\lambda_{Soret} = 396$ nm) and causes a large increase in NADPH oxidation as L-arginine is metabolized to NO.[7f,13] However, under this condition, the

inducible NOS oxygenase heme has $E_m = -263$ mV, which is between the heme midpoint potentials observed with L-arginine or H4B alone. This indicates a nonadditive effect of L-arginine and H4B on the heme reduction potential and further alludes to cooperative binding between the substrate and cofactor within the oxygenase domain of inducible NOS wherein the binding of one species perturbs the binding site of the other.[9] These redox data are supported by the recent crystal structure of the inducible NOS oxygenase,[24] indicating that the pterin cofactor plays a fundamental role in controlling subunit interaction and heme active site formation. The structure shows that H4B binds to two elements involved in L-arginine binding, a heme propionate group and an α-helix element,[24] to participate in the creation of the extensive hydrogen bonding network that facilitates substrate binding.

Next, three substrate analogues that inhibit full-length inducible NOS NADPH oxidase activity were tested for their effects on the heme midpoint potential (Figure 3C). In the presence of H4B, the substrate-based inhibitors aminoguanidine, SEITU, and L-NAME all decrease NADPH consumption by inducible NOS to near the basal, heme-independent level (Table 1) even though they stabilize the heme in a high-spin state. Under these same conditions, aminoguanidine allows for heme reduction by NADPH (Table 1) and is believed to be a mechanism-based inhibitor of NOS.[16] SEITU does not allow the full-length inducible NOS heme to be reduced by NADPH, and L-NAME even inhibits heme reduction by sodium dithionite. Consistent with these findings, the inducible NOS oxygenase domain heme midpoint potentials were −279 and −322 mV with aminoguanidine and SEITU, respectively. The midpoint potential of the heme could not be determined in the presence of L-NAME, suggesting that the electronegative nitro group of L-NAME greatly destabilizes the ferrous heme state.

Figure 3B shows the redox titration data for neuronal NOS oxygenase domain heme with accompanying one-electron Nernst plots. These data are also summarized in Table 1. In the absence of substrate or H4B, the neuronal NOS heme midpoint potential was −239 mV. This value is 100 mV higher than for inducible NOS under identical conditions, despite the fact that the heme group of both NOS are predominantly low-spin. We have observed that the heme in full-length calmodulin-bound L-arginine- and H4B-free neuronal NOS is reduced by NADPH, and the enzyme has significant NADPH oxidase activity (Table 1), consistent with the high heme reduction potential for neuronal NOS under these conditions. Remarkably, the addition of L-arginine and H4B to the neuronal NOS oxygenase does not significantly change the heme midpoint potential ($E_m = -248$ mV) even though they shift the heme almost completely to high-spin. Separately, arginine or H4B caused slightly more significant changes in the neuronal NOS heme midpoint potential. L-Arginine increased the neuronal NOS oxygenase heme E_m to −220 mV, whereas H4B alone actually decreased the midpoint potential to −257 mV. From these experimentally determined reduction potentials for neuronal NOS and the known binding constants for L-arginine and H4B binding to the oxidized from of neuronal NOS of 2 and 0.25 μM, respectively, we have calculated the binding constants for these species to the reduced heme form of the enzyme using eq 2. For L-arginine, the $k_{D,red}$ decreases by one-half to 1 μM, whereas for H4B, the $k_{D,red}$ increases to 0.5 μM. The experimentally determined and calculated thermodynamic constants for L-arginine and H4B binding to neuronal NOS are summarized in Figure 4B. These data indicate a lack of significant preference for L-arginine and H4B binding to either the oxidized

Table 1

enzyme	condition	λ_{Soret} (nm)	NADPH oxidase[7f,12a] (nmol/(min·mg of enzyme))	heme reduction by NADPH[a]	E_m (mV)	$k_{D,ox}$ (μM)[b]	$k_{D,red}$ (μM)[c]
	−H4B, −arg	420	61 ± 5	no	−347		
	−H4B, +arg	399	165 ± 1	yes	−235	2.3	0.03
	+H4B, −arg	402	245 ± 1	yes	−295	0.6	0.08
iNOS	+H4B, +arg	396	1270 ± 140	yes	−263		
	+H4B, +aminoguan	400	76 ± 3	yes	−279		
	+H4B, +SEITU	398	127 ± 15	no	−322		
	+H4B, +L-NAME	400	54 ± 1	no	<−460[27]		
	−H4B, −arg	415	540 ± 90	yes	−239		
	−H4B, +arg	398	360 ± 10	yes	−220	2.0	1.0
	+H4B, −arg	401	1450 ±30	yes	−257	0.25	0.50
nNOS	+H4B, +arg	394	880 ± 10	yes	−248		
	+H4B, +aminoguan	400	104 ± 9	yes	−247		
	+H4B, +SEITU	397	650 ± 5	yes	−281		
	+H4B, +L-NAME	398	115 ± 5	no	<−460[27]		

[a] Determined in the absence of CO. [b] Experimental values, referenced in text. [c] Calculated according to eq 2.
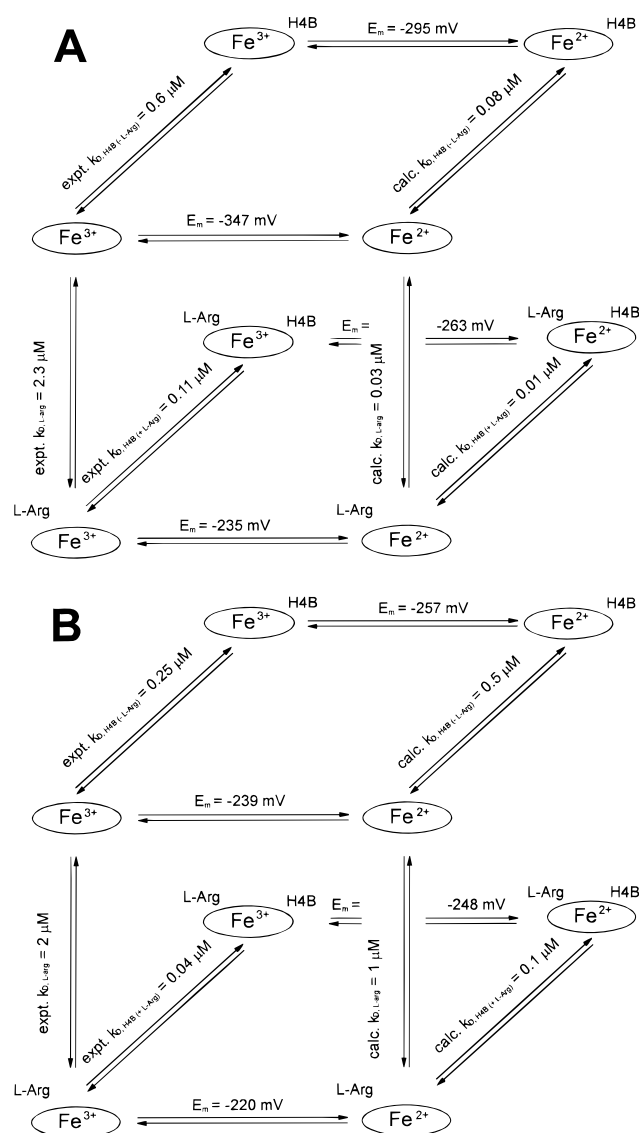

Figure 4. Thermodynamic cube diagrams for (A) inducible NOS and (B) neuronal NOS. Equilibrium midpoint potentials for the respective heme groups under various conditions of L-arginine and tetrahydrobiopterin (H4B) binding are presented, as well as the binding constants for the substrate and cofactor to the oxidized heme and reduced heme forms of the enzymes.

or reduced heme form of neuronal NOS, implying that electron transfer to the heme group of neuronal NOS and hence the

reactivity of this enzyme are not regulated by substrate or cofactor binding. Instead, we propose that heme reduction and neuronal NOS activity are primarily controlled by the surrounding Ca^{2+} levels through the transient binding of calmodulin.[6,13]

Regarding the effects of substrate analogues on the electronic properties of neuronal NOS (Figure 3C and Table 1), the addition of aminoguanidine to H4B-containing neuronal NOS oxygenase stabilized the high-spin form of the heme and allowed for heme reduction by NADPH, similar to the results observed with inducible NOS. Also, under these conditions, the neuronal NOS heme has a midpoint potential of −247 mV, which is identical to that of neuronal NOS oxygenase containing H4B and L-arginine and is consistent with aminoguanidine being a mechanism-based inhibitor of NOS.[16] By comparison, SEITU addition to H4B-bound neuronal NOS also maintains a high-spin heme but the heme reduction potential decreases to −281 mV. However, this midpoint potential is sufficiently high such that reduction of the heme by NADPH via the reductase domain flavins is thermodynamically allowed and a relatively high steady-state rate of NADPH oxidation by SEITU-bound neuronal NOS is observed (Table 1). As with inducible NOS, the addition of L-NAME to neuronal NOS decreased the oxygenase heme midpoint potential to a level that could not be determined. This again shows that L-NAME binds to and stabilizes the oxidized heme form of the enzyme, thus providing a thermodynamic barrier to heme reduction.

The data presented here must ultimately be interpreted in the context of whether the NOS oxygenase domain heme is thermodynamically poised to accept an electron from the terminal flavin of the reductase domain. If this is so, then the subsequent steps leading to O_2 binding, activation, and substrate oxidation may proceed. The reduction potentials of the NOS reductase domain flavins are as yet unknown, however the considerable homology between the three NOS reductase domains and the enzyme cytochrome P450 reductase[25] may allow for the use of the flavin reduction potentials of the latter enzymes as an estimate of the NOS reductase domain flavin potentials. The reduction potential of the three-electron reduced state of cytochrome P450 reductase was determined to be −290 mV.[26] Therefore, we surmise that NOS heme reduction should

(25) (a) Bredt, D. S.; Hwang, P. M.; Glatt, C. E.; Lowenstein, C.; Reed, R. R.; Snyder, S. H. *Nature* **1991**, *351*, 714−718. (b) Xie, Q.-W.; Cho, H. J.; Calaycay, J.; Mumford, R. A.; Swiderek, K. M.; Lee, T. D.; Ding, A.; Troso, T.; Nathan, C. F. *Science* **1992**, *256*, 225−228.

(26) (a) Iyanagi, T.; Makino, N.; Mason, H. S. *Biochemistry* **1974**, *13*, 1701−1710. (b) We have determined by reductive titration of the nNOS reductase domain that NADPH reduces the flavins to the three-electron reduced state (see ref 5).

occur under any condition in which the heme E_m is higher than this approximate value. From our results (Table 1), we see that this indeed is the case. Only for inducible NOS without L-arginine or H4B ($E_m = -347$ mV), inducible NOS containing SEITU ($E_m = -322$ mV), or both isoforms containing L-NAME ($E_m < -460$ mV[27]) was no heme reduction by NADPH observed. Similar to its effect with inducible NOS, SEITU binding to H4B-bound nNOS oxygenase lowers the heme midpoint potential from -257 to -281 mV, but the neuronal NOS heme remains poised to accept an electron from the reductase domain. Therefore, SEITU is only an effective inhibitor of inducible NOS heme reduction. Because uncoupled NOS heme reduction generates the toxic superoxide radical anion, the rational design of selective NOS inhibitors must take into consideration their effects on the redox properties of each NOS isozyme.

Another concept that these data address is the correlation between the heme iron spin state and its reduction potential. Regulation of the iron redox potential within a hemeprotein has been ascribed to the polarity of the heme environment,[23a,b] structural and bonding interactions at the metal center,[23c,d] the solvent exposure of the heme,[23b] electrostatic effects at the redox active site,[23e,f] the degree of hydration at the heme active site,[11] and ligand field effects.[10,23c] In cytochrome P450cam,[10b] there is a clear correlation between the equilibrium spin state of the ferric heme and its redox potential. Our data show that such a correlation does not exist for the NOS. First, the substrate- and H4B-free neuronal NOS has a predominantly low-spin heme and yet has a higher heme reduction potential than several examples of inhibitor-bound neuronal NOS whose heme is mostly high-spin. Indeed, binding SEITU to either H4B-bound NOS isozyme maintains or enhances the high-spin character of the heme, but results in a decrease in heme reduction potential. To explain this we must invoke other possible factors that regulate hemeprotein redox potentials. For example, in the substrate- and H4B-free neuronal NOS, the heme may have a strong-field sixth ligand (stabilizes low spin), but this ligand is very apolar or the heme is in a very insulated environment that favors the ferrous heme state. In both NOS isozymes, binding H4B and/or L-arginine favors the five-coordinate high-spin heme and these compounds bind as strong or stronger to the reduced heme over the oxidized heme form of the NOS enzymes, thus promoting heme reduction. With SEITU or L-NAME, the five-coordinate high-spin heme iron is still preferred, but their electron-rich functional groups may be directed toward the heme[24b] such that they bind with greater affinity to the ferric heme state of the enzyme effectively preventing reduction of the heme.

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(27) This value was chosen since sodium dithionite ($E_m = -460$ mV) does not reduce the L-NAME-containing NOS heme groups.