ENDOR Studies of L-Arginine and N^G-Hydroxy-L-Arginine Bound to All Three Holo-Nitric Oxide Synthase Isozymes

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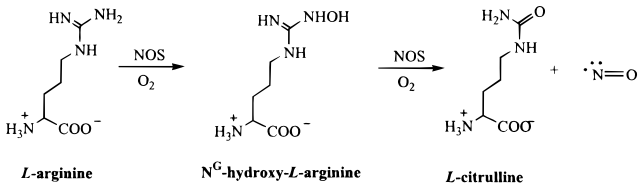
Nitric oxide plays diverse roles in cellular signaling, and improper regulation of NO production has been implicated in a wide array of diseases. The pathologies associated with a breakdown in the regulation of NO production can be partitioned into three major classes, each associated with a distinct isoform of nitric oxide synthase (NOS).[1] The endothelial isozyme (eNOS) is involved in regulation of vascular smooth muscle tone and is constitutive.[2−4] Neuronal NOS (nNOS), also constitutive, is involved in neurotransmission[5] and has been associated with stroke damage[6] and Alzheimer's disease.[7] The third isoform, inducible NOS (iNOS), is synthesized in an immune response[8] and has been implicated in inflammation.[9] All three utilize FAD, FMN, tetrahydrobiopterin, and a P450-type heme as cofactors in the two-step, five-electron oxidation of L-arginine (L-Arg) to L-citrulline and NO via the intermediate N^G-hydroxy-L-arginine (NOHA) (Scheme 1). All three isozymes have been shown to contain zinc with a stoichiometry of 1 Zn:2 hemes at the heme domain dimer interface.[10−12] The reactivities of eNOS and nNOS are regulated by the binding of Ca^{2+}-calmodulin, while iNOS binds Ca^{2+}-calmodulin with such affinity that calmodulin is bound at very low Ca^{2+} levels. Substrate binding and oxygen activation occur at the P450-type heme site.[13−15]

Given the variety of important physiological processes that utilize NO and the importance of overproduction or underproduction of NO by the three different isoforms in a range of pathophysiological processes, any attempt to regulate NO production through NOS inhibition must consider isoform selectivity.[15,16] Generally, inhibitor design is based on substrate structure and assumptions of binding orientations. However, for NOS, these efforts have been conducted without full structural or spectroscopic comparison of the binding of *either* substrate. A series of EPR studies[17−19] showed that the high-spin NOS ferriheme is sensitive to the presence and the identity of substrates, and that the three isoforms behave somewhat similarly, although classification of behavior with substrates and analogues is possible. A resonance Raman (rR) study[20] of the three NOS isoforms with bound L-arginine intriguingly showed that the Fe−C and C−O stretching modes of CO-ferrous nNOS were shifted to lower frequency from those of e- and iNOS. X-ray structural studies have been reported for the e- and iNOS heme domains,[10−12,21,22] and are progressing for the nNOS heme domain.[23] These demonstrate strikingly similar active sites with highly homologous amino acid compositions and overall folds. However, *no* structural studies have been reported on L-Arg binding to the *fully functional holoenzymes*, and we are aware of *no* studies, structural or spectroscopic, addressing isoform selectivity of the second catalytic step, the conversion of NOHA to L-citrulline and NO (Scheme 1). We present here ^{15}N pulsed Q-band electron−nuclear double resonance (ENDOR) studies of L-Arg and NOHA, bound to e-, i-, and nNOS holoenzymes, thereby providing the first step in assessing the binding geometries of both natural substrates within the three holoenzyme isoforms.

Scheme 1



An earlier 35 GHz pulsed ^{15}N ENDOR study[24] of $^{15}N^G$-L-arginine bound to holo-nNOS used spectra taken near $g_2 = 4.19$ to derive a location of the reactive guanidino nitrogen of the first substrate, which was subsequently confirmed by X-ray crystallography of the nNOS oxygenase domain[23] with bound L-arginine. To initiate the present work, ^{15}N ENDOR data was collected at additional fields across the EPR envelope (Figure S1, Supporting Information), and this has allowed us to refine the geometric parameters for $^{15}N^G$-L-arginine binding: Fe−N distance, $r_{FeN} = 4.1 \pm 0.05$ Å; angle between the Fe−N vector and the normal to the heme plane, $\theta = 10°-15°$, with the N lying over a line roughly between heme-pyrrole nitrogens, as illustrated in the **top left** of Figure 1. We then studied binding of L-Arg to the other two holo-NOS isoforms.[25−27] Presented in the **bottom left** of

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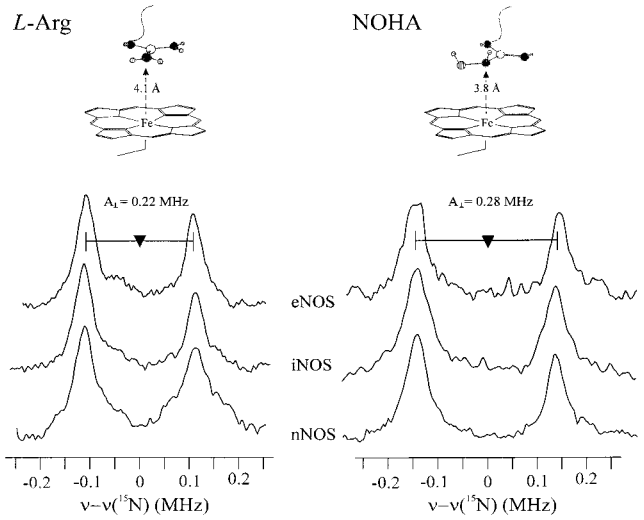

Figure 1. (Top) Depictions of the current level of ENDOR-derived models of $^{15}N^{G}$-L-arginine (L-arg, left) and $^{15}N^{G}$-hydroxy-L-arginine (NOHA, right) binding near to the high spin ferriheme of nNOS. In the models, substrate nitrogens are shown in black, nearby substrate protons are shown in gray, carbon atoms in white and oxygen atoms hatched. The dashed line is included to guide the reader's eye to the pertinent Fe−N interaction. (Bottom) 35 GHz Mims pulsed-ENDOR spectra at g_2 of $^{15}N^{G}$-L-arg (left) and ^{15}NOHA (right) bound to eNOS ($g_2 = 4.18$),[19] iNOS (4.13)[17] and nNOS (4.19).[24] *Conditions:* $T = 2$ K, $\nu_{MW} = 34.7$ GHz, MW pulse lengths = 40 ns, $\tau = 800$ ns, RF pulse length = 60 μs, repetition rate = 200 Hz. Each spectrum consists of 256 points, with each point an average of 4000−6000 transients.

Figure 1 are 35 GHz Mims pulsed-ENDOR spectra for $^{15}N^{G}$-L-arg (left) bound to e-, i-, and nNOS, taken at the peak of the high spin ferriheme EPR signal ($g_2 = 4.18$ (eNOS),[19] 4.13 (iNOS),[17] and 4.19 (nNOS)[24]). With $^{15}N^{G}$-L-arg bound, all three isoforms show a ^{15}N doublet whose splitting of $A \approx 0.22$ MHz is determined by the Fe−N^{G} distance.[24] The splitting and, thus, this distance, is negligibly different in the three holoenzymes. The small, but reproducible increase (∼5%) of the doublet splitting in the iNOS data translates into an r_{FeN} in iNOS that is smaller by only 0.06 Å. This relative difference in distance between isoforms is reliably within the precision of the measurement, although it is below the absolute accuracy of the distances themselves. Such precision in a distance difference is not attainable crystallographically for light atoms in the vicinity of a transition ion.

Closer inspection of the spectra shows subtle differences in line shapes. In particular, the pronounced shoulders on the outside of the central doublet in the nNOS spectrum (Figure 1, **left, bottom**) are conspicuously reduced in the eNOS and iNOS spectra. The occurrence of these shoulders for nNOS can only be attributed to a small difference in the offset of N^{G} from the heme normal (<10°) relative to the other two isoforms. These results may speak to the interpretation of the isoform-dependent resonance Raman spectra seen previously,[20] and could represent the influence of the >300 residues at the N-terminus of nNOS that are not present in eNOS or iNOS. The rR effects were interpretable *either* in terms of a greater shielding of the charge on the guanidino nitrogen when bound to nNOS, *or* of an arginine-CO distance that is ∼1 Å greater in nNOS. As the latter is ruled out here, a small difference in substrate positioning in nNOS, *without* a significant difference in distance, might be responsible.

An earlier ENDOR study of the second substrate, NOHA,[28] singly labeled with ^{15}N at the hydroxylated nitrogen, showed that the reactive nitrogen is slightly closer to the Fe and more nearly 'overhead' ($r_{FeN} = 3.8$ Å, ∼10° off the heme normal) than the corresponding nitrogen of L-arg. The combination of ^{15}N, ^{1,2}H ENDOR and X-ray diffraction results,[23] further allowed us to conclude that NOHA binds as the hydroxylamine-like tautomer, as summarized pictorially in the **top right** of Figure 1 (see Note Added in Proof). Examination of the ^{15}N ENDOR data for NOHA bound to the three holoenzyme isoforms, shown in the **lower right** panel of Figure 1, demonstrates that for all three holoenzymes the doublet splitting is, $A \approx 0.28$ MHz. The precision of this measurement requires that r_{FeN} is the same for all three isoforms, to within 0.02 Å; the overall accuracy is such that $r_{FeN} = 3.8 \pm 0.05$ Å for all three isoforms. None of the spectra shows evidence for shoulders with significant intensity outside the central doublet, indicating that in each case the ^{15}N is very close to the heme normal. In short, there is no meaningful isoform variation in the placement of the $^{15}N^{G}$ of NOHA relative to the heme.

The present ENDOR study presents the first direct comparison of substrate binding geometries for all three NOS isoform *holoenzymes*. Although subtle differences are noted, it appears that the binding geometries of *both* L-arg and NOHA, as manifest in the positions of the nitrogen atom that undergoes oxidation in each, are essentially the same in e-, i-, and nNOS. This helps to explain why selective inhibitor design based on a comparison of oxygenase domain crystal structures has proven ineffective.

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Note Added in Proof. A recent low-resolution (2.6 Å) crystal structure of the iNOS oxygenase domain complexed with NOHA wholly confirms the ENDOR-derived structure of NOHA, although this point was suppressed in the discussion (Crane, B. R.; Arvai, A. S.; Ghosh, S.; Getsoff, E. D.; Stuehr, D. J.; Tainer, J. A. *Biochemistry* **2000**, *39*, 4608−4621.

Supporting Information Available: One Figure showing field-dependent ^{15}N ENDOR data, and representative simulations, from $g_1 = 7.6$, past g_2, to $g = 3.5$ for L-Arg bound to nNOS.

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(25) Endothelial and inducible NOS were isolated according to published procedures.[26,27] Complexes with ^{15}N-labeled substrates were prepared for ENDOR spectroscopy as described previously.[24,28] Final sample concentrations were 0.4−0.6 mM in heme, 0.2−0.3 mM in functional NOS homodimer.

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