Location of Guanidino Nitrogen of L-Arginine Substrate Bound to Neuronal Nitric Oxide Synthase (nNOS): Determination by Q-band Pulsed ENDOR Spectroscopy

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Nitric oxide synthase (NOS) catalyzes the formation of NO from L-arginine according to eq 1.[1−5]

$$\text{L-arginine} \xrightarrow[O_2]{\text{NADPH}} \underset{\text{arginine}}{\text{N}^{\omega}\text{-hydroxy}} \xrightarrow[O_2]{\text{NADPH}} \text{citrulline} + \text{NO} \quad (1)$$

Central to all proposed chemical mechanisms for this reaction is the close proximity of the reactive guanidino nitrogen of substrate to the heme iron. However, optical difference spectroscopy performed on neuronal NOS (nNOS) in the Masters[6] laboratory and on inducible NOS (iNOS) in the Marletta[7] laboratory, followed by EPR studies on nNOS[8−10] and examination of binding to a module derived from nNOS,[11] showed that the substrate is not a ligand to the ferriheme iron of NOS, which remains five-coordinate, but binds in close proximity to it.[8−10,12] The recently published crystal structures of monomeric iNOS oxygenase domain contain imidazole as sixth ligand to the ferriheme, with either a second imidazole or the inhibitor aminoguanidine bound nearby.[13] Thus, the nature of L-arginine binding remains to be established. The guanidino nitrogen (N_g) of enzyme-bound L-arginine in the vicinity of the high-spin heme iron must experience a dipolar hyperfine interaction that depends on its distance to the Fe. In the present study, ^{15}N Q-band Mims pulsed ENDOR[14−17] spectroscopy has been used to measure this interaction for L-arginine isotopically labeled at the terminal guanidino nitrogens ($^{15}N_g$). This has enabled us to establish the distance of this moiety from the heme iron and its position relative to the heme normal.[18]

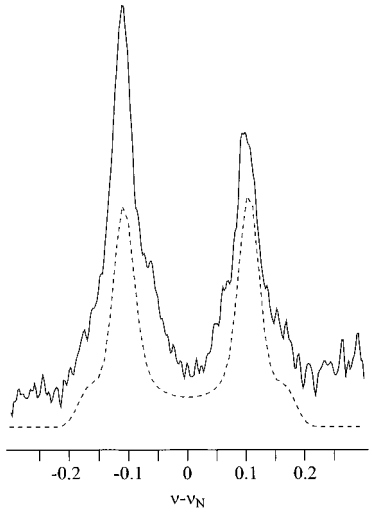

Figure 1. The 35 GHz Mims pulsed ENDOR of [$^{15}N_g$]-L-arginine bound to nNOS (solid line) at $g_2 = 4.19$. Conditions: $T = 2$ K, $\nu_{MW} = 34.72$ GHz, MW pulse lengths = 40 ns, $t = 800$ ns, RF pulse length = 40 ms, repetition rate = 200 Hz. Each spectrum consists of 256 points, with each point an average of 6000 transients. The dashed line represents a simulation based on a distance from N_g to Fe of 4.05 Å (**T** = [−0.115, −0.115, 0.23] MHz and $A_{iso} = 0.015$ MHz), with Euler angles for the Fe−N_g vector relative to the **g** frame of $\theta = 10°$, $\varphi = 15°$. The unequal peak intensities is common in Q-band pulsed ENDOR but cannot be modeled at present.

The five-coordinate ferriheme of substrate-bound nNOS is high-spin (hs) and its effective **g** tensor has principal values of **g** = [g_1, g_2, g_3] = [7.56, 4.19, 1.81], where g_3 lies approximately normal to the heme plane.[8−10] CW ^{1}H Q-band ENDOR[20] measurements have been performed on L-arginine-bound nNOS prepared in H_2O and D_2O buffers. Spectra taken at g_1 show several nonexchangeable doublets with hyperfine couplings of $A_H \leq 3$ MHz.[21] The absence of more strongly coupled exchangeable protons (data not shown), such as are seen in studies of water-bound hemes ($A_H > 6$ MHz),[23] is consistent with the previous conclusion that the heme is five-coordinate and that there is no solvent-derived sixth ligand.[9,12]

Q-band CW and Mims pulsed ENDOR experiments with natural-abundance (^{14}N) L-Arg disclose strong responses from the pyrrole ^{14}N ligands but fail to show any resonances attributable to substrate. However, Mims pulsed ENDOR spectra of [$^{15}N_g$]-L-Arg bound to nNOS show well-resolved signals from the guanidino ^{15}N. Figure 1 presents a spectrum of enzyme with ^{15}N-labeled substrate where the field is set to g_2.[24] It shows a remarkably sharp doublet (line widths ≈35 kHz) centered at the

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(18) These experiments were performed with nNOS purified from an *E. coli* strain in which a plasmid containing the nNOS cDNA was coexpressed with a plasmid encoding the *E. coli* chaperonins groEL and groES, as described previously.[19] After elution from a 2′,5′-ADP−Sepharose 4B column (Pharmacia), nNOS containing fractions were pooled (nNOS concentration = 10 μM, based on heme content) and incubated with 2 mM L-arginine or [$^{15}N_g$]-L-arginine and 50 mM tetrahydrobiopterin (BH_4) for 20 min on ice. The incubation was followed by repeated concentration and dilution using Centriprep concentrators (50 kDa cutoff) while maintaining constant concentrations of [$^{15}N_g$]-L-arginine and BH_4. Samples in D_2O were prepared by a 10:1 dilution of [$^{15}N_g$]-L-arginine and BH_4 bound nNOS in D_2O buffer, incubation for ca. 24 h and reconcentration. Final sample concentrations (ca. 60 μL of 0.48 mM nNOS) were attained using Millipore Ultrafree-MC microconcentrators (30 kDa cutoff, <10 μL dead volume).

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^{15}N Larmor frequency and split by a hyperfine interaction of $A = 0.22$ MHz; the individual peaks of the doublet bear shoulders, with the outer shoulders having a breadth of ~0.3 MHz.[21] This signal is absent for samples that contain natural-abundance L-Arg, confirming that it arises from $^{15}N_g$ of the labeled substrate. The mere observation of this signal confirms that the reactive guanidino nitrogen of the substrate is indeed close to the heme iron. The simplicity of the spectrum requires either that the two N_g are equidistant from Fe or, more probably, that the arginine is oriented such that the second N_g is too far away to be observed.

Simulations of a set of spectra taken over a range of fields near g_2 further show that the hyperfine coupling to $^{15}N_g$ is determined by the dipolar interaction with the hs ferric ion.[25] The intrinsic dipolar interaction between the $S = {}^5/_2$ ferric ion and $^{15}N_g$ has axial symmetry, $\mathbf{T} = [-T, -T, 2T]$, where the unique direction corresponds to the Fe−$^{15}N_g$ vector and the magnitude of the perpendicular component is given by eq 2.

$$T = \rho_{Fe} g_e g_N \beta_e \beta_N / r^3 \qquad (2)$$

Here, g_N and g_e are the nuclear and electronic g factors, respectively, β_e and β_N are the electronic and nuclear magnetons, r is the Fe−N_g distance, and ρ_{Fe} is the fraction of the electron spin on the iron, which we here take to be $\rho_{Fe} \approx 0.9$. The strong **g** anisotropy of the nNOS ferriheme modifies the intrinsic hyperfine interaction.[14] For example if the Fe−N_g vector were parallel to g_3, the effective coupling would be rhombic, with components, $\mathbf{T}' = [T_1', T_2', T_3'] = [-(g_1/g_e)T, -(g_2/g_e)T, 2(g_3/g_e)T]$ (noncoaxiality of **g** and **T** modifies this result). The EPR intensity at g_2 for a frozen solution comes not only from those molecules where the g_2 axis happens to lie along the field direction but also from all orientations of the heme relative to the external field such that the orientation-dependent g factor equals g_2.[14] Hence, in general, a spectrum taken near g_2 will exhibit a range of dipolar splittings. However, simulations show that no matter what the actual orientation of the Fe−N_g vector, the doublet splitting in ENDOR spectra such as that of Figure 1 depends only on the Fe−N_g distance (it is roughly equal to T_2'), while the shapes are fixed by the orientation of the Fe−N_g vector relative to the heme. As an illustration, Figure 1 includes a simulation calculated using precise formulas for the effective interaction,[14,15] with Fe−N_g distance, $r_{Fe-N} = 4.05$ Å, and orientation parameters given in the figure legend.[26] The value for r_{Fe-N} has error bounds of less than ±0.15 Å, based on the uncertainties in the observed hyperfine coupling and in ρ_{Fe}.

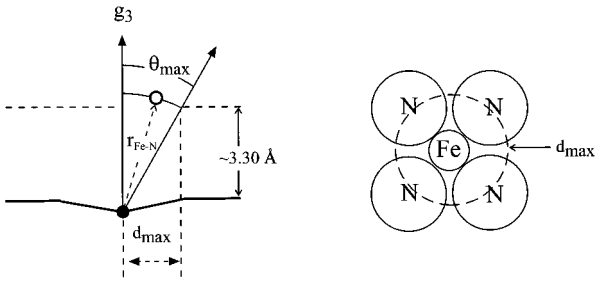

Figure 2. Position of N_g relative to the heme active site of nNOS. (Left) Side view. The ENDOR results place N_g at a distance, r_{Fe-N}, of 4.05 Å, on the portion of the sphere near the heme normal (g_3) delimited by $\theta_{max} = 27°$, with a maximum displacement from the heme normal of $d_{max} = 1.9$ Å. (Right) Top view. The dashed circle represents d_{max}, showing that the guanidino nitrogen must lie above the inner FeN_4 core of the heme.

The constraints imposed by the van der Waals thickness of the heme (~3.3 Å) and van der Waals radius of N_g (~1.6−1.7 Å) limit the closest approach of the guanidino nitrogen to the heme plane to ~3.30 Å (Figure 2). Taking this into account, the experimentally determined value of r_{Fe-N} restricts the angle between the Fe−N_g vector and the g_3 axis to $\theta_{max} \leq 27°$ when Fe is out-of-plane toward the proximal ligand by ~0.4 Å, as expected[27] (Figure 2, left). As a result, the guanidino nitrogen can be displaced laterally from the heme normal by no more than $d_{max} \approx 1.9$ Å, which means that it must be located above the inner FeN_4 core of the heme (Figure 2, right).[28] The shape of the shoulders on the central doublet show that N_g does *not* lie directly above Fe along the g_3 axis; the outer shoulders can be reproduced only for angles of, $10 < \theta < 20°$, which correspond to $d \approx 1-2$ Å. Analysis of the hyperfine interactions to the pyrrole ^{14}N, coupled with full field-dependent ENDOR data for $^{15}N_g$, will give more precise information about the displacement of N_g relative to the FeN_4 heme core.[29]

These measurements provide the first direct demonstration of the proximity of the reactive guanidino nitrogen of the L-arginine substrate to the nNOS heme iron. The best interpretation of the data is that N_g is held at a distance from the heme iron of $r_{Fe-N} \approx 4$ Å above the FeN_4 core and displaced slightly from the heme normal ($d \approx 1-2$ Å). Thus, as for P450cam[27] and unlike P450BM-3,[30] nNOS holds the substrate target atom, in this case the guanidino nitrogen, in a *very* well-ordered position near to Fe, favorably placed for subsequent hydroxylation by an iron-bound oxygenic species. Perhaps of even greater importance, this study has demonstrated that Q-band pulsed ENDOR is able to resolve extremely small dipolar couplings to a *noncovalently bound* substrate. This remarkable ability will be of critical use in a broad range of applications.

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(21) ENDOR spectra were recorded at 35 GHz in either CW[20] or pulsed[22] mode with instruments of local design. The ENDOR response from a weakly coupled nucleus with $I = {}^1/_2$ (e.g., ^{1}H, ^{15}N) is made up of doublets centered at the nuclear Larmor frequency, ν_n, and split by the hyperfine coupling, A.

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(24) The value of g_2 given here is slightly different from that previously reported and is based on the fact that ENDOR simulations predict that the maximal intensity of the $^{15}N_g$ signal near g_2 must occur exactly at g_2. The maximum ENDOR intensity (normalized to the percent ENDOR effect by dividing the data by the echo intensity) was observed at $g = 4.19$.

(25) The simulations show that any isotropic contribution must be negligibly small (≤15 kHz).

(26) Allowing the arrangement of substrate with respect to the heme to place the second guanidino group at its maximum distance from Fe would place the second N_g at a distance of ca. 6−7 Å. This distance would predict a hyperfine coupling of ≤0.05 MHz, a value unlikely to be observable in a Mims pulsed ENDOR experiment.

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