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Modulation of Substrate Binding to Naphthalene 1,2-Dioxygenase by Rieske Cluster Reduction/Oxidation

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The naphthalene 1,2-dioxygenase system (NDOS) from *Pseudomonas* sp. NCIB 9816-4 is comprised of three protein components and catalyzes the NAD(P)H and O_2-dependent *cis*-dihydroxylation of naphthalene to yield *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene.[1−3] X-ray crystallographic studies show that the active site of NDOS lies within the oxygenase component (NDO) and contains both a Rieske-type [2Fe2S] cluster and a catalytically active mononuclear iron site.[4] Three pairs of these metal sites are juxtaposed across the α−α-subunit boundaries in the $(\alpha\beta)_3$ enzyme. Further structural studies of NDO reveal that the alternative substrate indole binds close to the mononuclear iron.[5] Because both metal centers become reduced in the X-ray beam, all structural data are of the fully reduced enzyme (denoted ferro-NDO^R).[6]

Recently, we established that O_2 reactivity at the mononuclear iron site is gated by both naphthalene binding *and* Rieske cluster reduction, implying that there is some structural reorganization that occurs during these processes.[7] As a result, a reactive form of O_2 is generated only when all of the required elements for catalysis (i.e., naphthalene and 2 reducing equiv) are present in the active site. The O_2 surrogate NO is similarly gated by substrate binding in the case of ferro-NDO^R, but unlike O_2, it will also bind to NDO in which only the mononuclear iron is reduced (denoted ferro-NDO) in the absence of substrate. In each case, the EPR silent $S = 2$ mononuclear ferrous center is converted to an EPR active $S = {}^3/_2$ center which provides both a probe for the active site and a model for the oxygen complex.[7]

To understand the "gating" phenomena that underlie the unique dihydroxylation reaction of NDO, we have employed Q-band pulsed deuteron ENDOR spectroscopy to obtain the first precise information about the position of naphthalene bound in the active site with an oxygen analogue coordinated to the mononuclear iron. These disclose a novel conformational coupling between the position of substrate and the redox state of the Rieske cluster.

Substrate-free $S = {}^3/_2$ NO-ferro-NDO[8] exhibits apparent *g* values of $\mathbf{g} = [4.18, 3.88, 2.01]$,[7] where g_3 corresponds to the unique axis of the zero-field splitting tensor, which should lie essentially along the Fe−N(O) bond. Binding of the substrate, naphthalene, causes a minimal change in *g*-values, to $\mathbf{g} = [4.21, 3.84, 2.01]$, and a sharpening of the spectrum. Reduction of the Rieske center (NO-ferro-NDO^R) introduces a signal from its [2Fe, 2S]$^{1+}$ state, but it leaves the signal from the non-heme-Fe center largely unchanged.

The Mims single-crystal-like ^{2}H-ENDOR spectra collected at g_1 for NO-ferro-NDO-d_8-naphthalene show the four-line pattern expected for a single interacting deuteron (Figure 1).[9] The spectrum

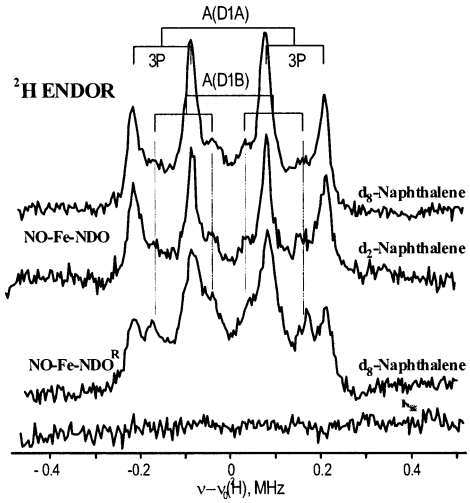

Figure 1. Q-band Mims ^{2}H-ENDOR spectra at g_1 for NO-ferro-NDO and NO-ferro-NDO^R bound with deuterated naphthalenes as indicated, with the spectrum for d_8-naphthalene as the control. Conditions: microwave frequency ≈ 34.76 GHz, microwave pulse length ($\pi/2$) = 52 ns, τ = 500 ns, repetition rate = 100 Hz, spectral resolution = 256 points, samplings of each point = 50, scans ≅ 20; RF pulse width, $T = 60$ μs.

is characterized by two splittings, 131 and 297 kHz, one of which corresponds to the hyperfine coupling, *A*, and the other to the quadrupole interaction, 3*P*. As the maximum quadrupole splitting for a ring deuteron is only $3P^{\circ}_{max} = 266$ kHz,[10] the splittings must be assigned as $3P = 131$ kHz; $A = 297$ kHz (Figure 1). The sharpness of the ^{2}H quartet further requires that *both* the quadrupole and the hyperfine couplings must be near extremal values. From the ratio $3P/3P^{\circ}_{max} \approx {}^1/_2$, one can then calculate that the C−D bond must lie roughly perpendicular to the g_1 axis. The quartet pattern observed for NO-ferro-NDO-[d_8-naphthalene] is preserved in the g_1 Mims ENDOR spectra from NO-ferro-NDO with bound dideutero-naphthalene(D1,D4) (Figure 1), and so this pattern must arise from a single D1 deuteron.

As the substrate cannot covalently bond to the metal center, the deuteron hyperfine coupling must arise from the through-space dipolar interaction between the electron spin and this substrate deuteron. The *intrinsic* hyperfine coupling in the g_1 spectrum of this $S = {}^3/_2$ center has magnitude $A_{int} = (g_e/g_1)A$. For a point-dipole interaction, the requirement that the coupling is near an extremum requires that $A_{int} = T$ or $2T$, where $T = \rho_{Fe}g_e\beta_e g_D\beta_D/r^3_{Fe-D}$.[3] Here ρ_{Fe} is the electron spin density on Fe, g_e, and β_e, g_D and β_D have their usual meanings, and r_{Fe-D} is the Fe−D1 distance. For ρ_{Fe} close to unity, $A = 297$ kHz yields $r_{Fe-D} \approx 4.4$ Å for an Fe−D1 vector roughly perpendicular to g_1 ($A_{int} \approx T$), quite compatible with the

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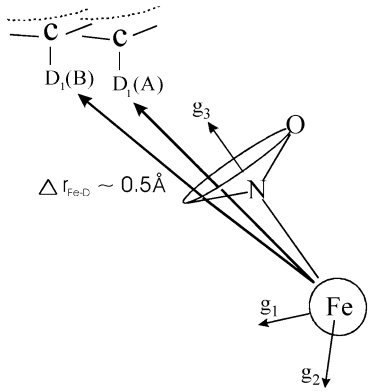

Figure 2. Orientation of D1 for naphthalene relative to the NO−Fe fragment in the "A" and "B" binding configurations: $r_{Fe-D} \approx 4.4$ Å for Fe−D1(A); $r_{Fe-D} \approx 5$ Å for Fe−D1(B).

distance found for indole bound in the substrate pocket;[5] preliminary analysis of 2D patterns of ENDOR spectra collected across the EPR envelope indicates that r_{Fe-D} points roughly along the g_3 axis (Fe−N(O) bond) (Figure 2) and confirms the value for the distance.[11]

The spectrum of the d_8-naphthalene adduct shows weaker features that comprise a four-line pattern from a second deuteron, D(B), Figure 1. These features also persist with dideutero-naphthalene, which shows that they do not represent a signal from the other deuterons (D2/D8) of d_8-naphthalene. Instead, we must assign D(B) to D1 of a minor conformation of bound naphthalene. The "D1(B)" pattern exhibits essentially the same quadrupole splitting as D1 of the majority ("D1(A)"), $3P(B) = 127$ kHz, but the hyperfine coupling is smaller, $A(B) = 200$ kHz. The smaller coupling for D1(B) suggests a greater Fe−D1(B) distance, with the ratio of $r_{Fe-D(B)}/r_{Fe-D(A)} \approx (297 \text{ kHz}/200 \text{ kHz})^{1/3} \approx 1.14$, or $r_{Fe-D(B)} \approx 5$ Å for $r_{Fe-D(B)}$ perpendicular to g_1 (Figure 2).

Figure 1 displays absolute ^{2}H ENDOR spectra of d_8-naphthalene collected at g_1 for NO-ferro-NDO^R-[d_8-naphthalene] as well as NO-ferro-NDO-[d_8-naphthalene]. Both spectra show the four-line patterns of D1(A) and D1(B), but the intensity of the D1(B) pattern is greatly enhanced in the NDO^R spectrum, indicating the occurrence of an allosteric shift from A to B binding geometries/sites upon reduction of the Rieske center.[12] The total intensities of the NDO and NDO^R spectra are roughly the same, which indicates that reduction does not shift naphthalene from the A,B sites to a third, more distant site.

The X-ray crystal structure of the indole complex of NDO[5] shows the closest proton to be about 4.1 Å away from the Fe^{2+}, comparable to the value we have estimated here for D1 of naphthalene. Together, the current spectroscopic and past crystallographic studies provide views of the ferrous mononuclear center of the substrate bound enzyme with and without NO bound as an O_2 surrogate. They indicate that there is ample room for binding dioxygen to the Fe of a substrate adduct without major rearrangement of the catalytic site. The current study shows that the terminal atom of the dioxygen could attack the reactive atoms of the aromatic ring, but it does not rule out mechanisms in which prior O−O bond cleavage occurs.

Although substrate-bound ferro-NDO possesses a mononuclear iron site that could, in principle, react with O_2 to yield a reactive oxygen species, it is not observed to do so at a high rate unless triggered by reduction of the Rieske cluster. The quaternary structure of NDO brings the Rieske cluster of one α-subunit within 12 Å of the mononuclear iron in the adjacent α-subunit with a single Asp residue forming a bridge between the ligands of each center. Thus, it is reasonable that structural changes caused by reduction of the Rieske cluster might be transmitted to the mononuclear iron center and, conversely, that substrate binding could influence the Rieske center.

The current results show for the first time that the redox state of the Rieske center modulates the separation of the substrate and the catalytic non-heme iron. It is possible that the shift to the distal (B) site upon cluster reduction is required for O_2 to gain access to the iron, accounting for the observed lack of O_2 interaction with the enzyme when the Rieske center is oxidized. Note, however, that during catalysis the Rieske cluster becomes reoxidized as it transfers an electron to the oxygen-bound mononuclear center. Thus, the substrate may shift to the proximal (A) position during conversion from the O_2-ferro-NDO^R-naphthalene to [O_2-ferro]$^-$-NDO-naphthalene state critical to catalysis. Consequently, the shift in substrate position disclosed here may hold the key to both oxygen gating and oxygen reactivity by Rieske aromatic dioxygenases. Further studies will refine the ENDOR-derived information about substrate binding, both for naphthalene and for other substrates.

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References

(1) Jeffrey, A. M.; Yeh, H. J. C.; Jerina, D. M.; Patel, T. R.; Davey, J. F.; Gibson, D. T. *Biochemistry* **1975**, *14*, 575−584.
(2) Gibson, D. T.; Parales, R. E. *Curr. Opin. Biotechnol.* **2000**, *11*, 236−243.
(3) Ensley, B. D.; Gibson, D. T. *J. Bacteriol.* **1983**, *155*, 505−511.
(4) Kauppi, B.; Lee, K.; Carredano, E.; Parales, R. E.; Gibson, D. T.; Eklund, H.; Ramaswamy, S. *Structure (London)* **1998**, *6*, 571−586.
(5) Carredano, E.; Karlsson, A.; Kauppi, B.; Choudhury, D.; Parales, R. E.; Parales, J. V.; Lee, K.; Gibson, D. T.; Eklund, H.; Ramaswamy, S. *J. Mol. Biol.* **2000**, *296*, 701−712.
(6) Karlsson, A.; Parales, J. V.; Parales, R. E.; Gibson, D. T.; Eklund, H.; Ramaswamy, S. *J. Inorg. Biochem.* **2000**, *78*, 83−87.
(7) Wolfe, M. D.; Parales, J. V.; Gibson, D. T.; Lipscomb, J. D. *J. Biol. Chem.* **2001**, *276*, 1945−1953.
(8) NO-ferro-NDO-[substrate] samples were prepared as described previously.[7] 100 mM MES pH 6.8, 10% glycerol. The NO-ferro-NDO samples thus prepared contain an oxidized Rieske center. NO-ferro-NDO^R was prepared by anaerobic reduction with a 5-fold excess of sodium dithionite and 100 μM methyl viologen. Deuterated naphthalenes were from Isotec Inc.
(9) Mims[13] Q-band pulsed ENDOR spectra were recorded at 2 K.[14] The ENDOR pattern for a deuteron ($I = 1$) exhibits paired features that, to first order, are given by $\nu(\pm, m) = |\nu_n \pm A/2 + (2m - 1)3P/2|$, where $m = 1, 0$; ν_n is the nuclear Larmor frequency, and A and $3P$ are the hyperfine and quadrupole coupling constants.
(10) Barnes, R. G.; Bloom, J. W. *J. Chem. Phys.* **1972**, *57*, 3082−3086.
(11) *J. Am. Chem. Soc.*, submitted.
(12) We cannot rule out incomplete reduction.
(13) Mims, W. B. *Proc. R. Soc. London* **1965**, *283*, 452−457.
(14) Davoust, C. E.; Doan, P. E.; Hoffman, B. M. *J. Magn. Reson.* **1996**, *119*, 38−44.

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