Supporting Information

for

ENDOR/HYSCORE Studies of the Common Intermediate Trapped During Nitrogenase Reduction of N_2H_2, CH_3N_2H, and N_2H_4 Support an Alternating Reaction Pathway for N_2 Reduction

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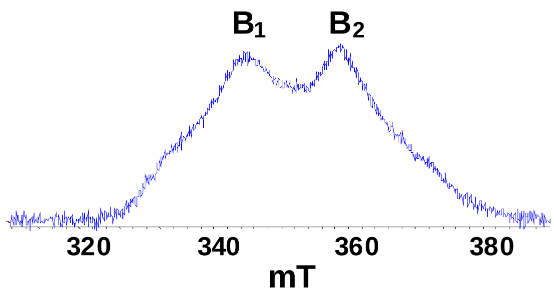

Fig S1. Two-pulse field-sweep ESE spectra of intermediate ***I*** (*l*(N_2H_4)) recorded with τ = 200 ns. Microwave frequency is 9.702 GHz.

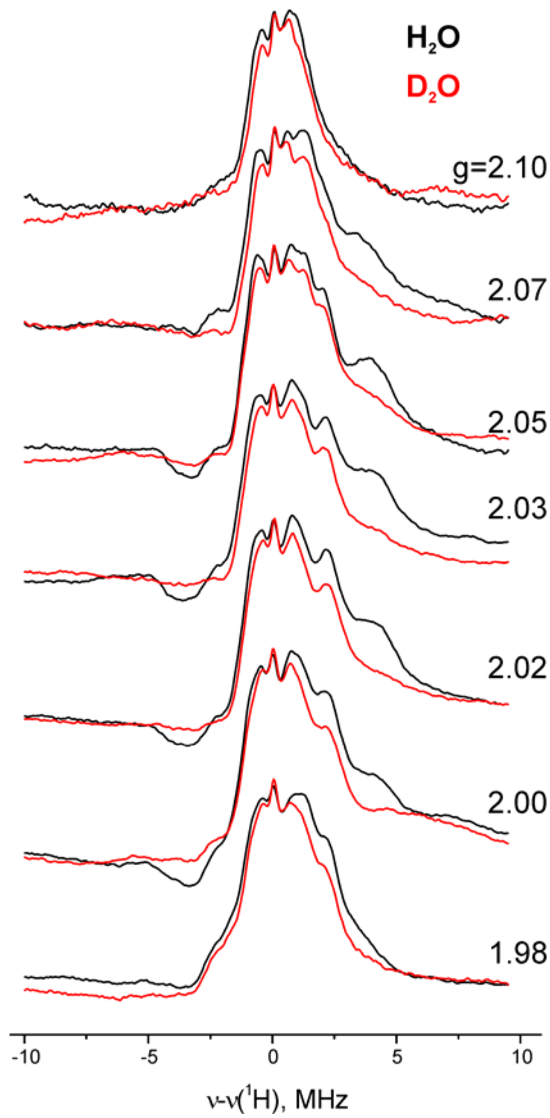

Fig S2. Field dependence of 35 GHz CW [1]H ENDOR spectra of ***I*** (*l*(N_2H_4)) obtained for samples prepared in H_2O (black) and D_2O (red) buffers. *Conditions:* microwave frequency, 35.002 GHz (H_2O), 35.096 GHz (D_2O); modulation amplitude, 2.5 G; time constant, 64 ms; bandwidth of RF broadened to 100 kHz; RF sweep speed, 1 MHz/s, 50-100 scans; temperature, 2 K.

1D Four-Pulse ESEEM

Additional information about the exchangeable proton(s) was obtained from 1D four-pulse ESEEM spectra (**Fig S3**). These spectra contain lines in the region of the double proton Larmor frequency ($2\nu_H$ ~ 28-29 MHz) that are sum-combination harmonics ($\nu_\alpha + \nu_\beta$) of two basic frequencies ν_α and ν_β. These harmonics are not created in HYSCORE experiments, and the approach is particularly useful for the resolution of protons with different anisotropic couplings.[35]

The four-pulse ESEEM spectrum of the ***I*** intermediate recorded at the low-field edge of the spectrum (329 mT, g = 2.106) contains two well-resolved lines in the region of the proton $2\nu_H$, as shown in **Fig S3A.** The most intense line represents the contribution of weakly coupled protons from the protein environment with $(\nu_\alpha + \nu_\beta) \cong 2\nu_H$. The spectrum also reveals a peak of lower intensity shifted from $2\nu_H$ to higher frequencies by ~ 0.9 MHz. This feature is greatly diminished in the spectrum of the sample prepared in D_2O, indicating that it contains a major contribution from the exchangeable proton(s) seen in ENDOR and as cross-peaks **2** (with larger deviation from antidiagonal) in the HYSCORE spectra. The resolved shifted peak was observed in the narrow interval up to field 332 mT (g = 2.0876). In orientation-selected spectra the shift depends on the part of the spectra excited by microwave pulses. The resolved shifted line was observed at the low-field edge of the EPR spectrum when the magnetic field is directed along g_1 axis, i.e. the single-crystal like conditions. In this case the shift of the sum combination line is described by the relation,[35, 36]

$$\Delta = 9/4(T^2/\nu_H) \sin^2\theta \cos^2\theta \qquad \textbf{(S1)}$$

in which θ is the angle between the unique axes of g-tensor and hyperfine tensors in axial approximation. For $\Delta = 0.9$ MHz and $T = 4.6$ MHz obtained from HYSCORE spectra analysis, **eq S1** gives an estimate that $\sin^2\theta \sim \cos^2\theta \sim 1/2$. This result indicates that either the orientation-selection at X band is not effective enough even near the edge of the spectrum or that θ is about 45^o; the latter agrees with the ENDOR measurements.

At the higher fields, **Fig S3B**, the sum combination peak at $2\nu_H$ is accompanied by unresolved shoulder that extends up to 0.8-0.9 MHz. This behavior is consistent with increase of intensity and width of the cross-peaks **1** (along the diagonal) in HYSCORE spectra.

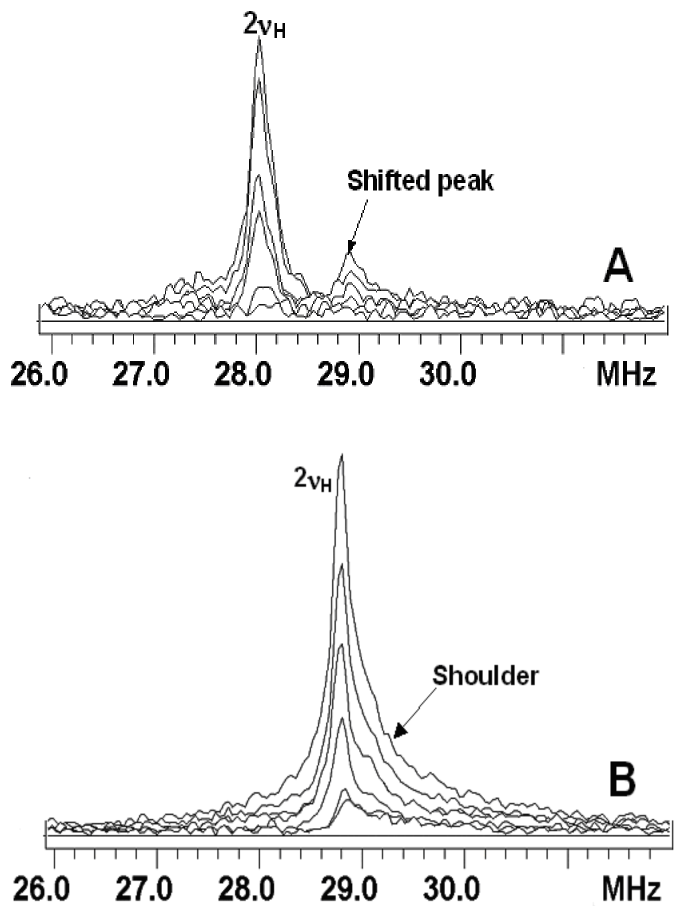

Figure S3. Stacked presentations of sets of four-pulse ESEEM spectra of *l*(N_2H_4) intermediate The spectra show the modulus of the Fourier transform along the time (T) axis for different times between first and second pulses, τ. The initial time τ is 100 ns in the farthest trace, and was increased by 12 ns in successive traces. Microwave frequency and magnetic field were 9.6995 GHz and 329.0 mT (g=2.106) (A), 9.7004 GHz and 338.0 mT (g=2.05) (B).

Analysis of ^{1}H and ^{15}N HYSCORE spectra.

The contour lineshape in the powder 2D spectrum from I=1/2 nuclei such as ^{1}H and ^{15}N (Zeeman frequency, ν_I) for axial hyperfine interactions is described (Dikanov, S.A. and Bowman, M.K. (1995) *J. Magn. Reson. A* **116**, 125-128) by equation:

$$\nu_1^2 = Q\nu_2^2 + G \qquad \textbf{(S2)}$$

where $$Q = \frac{\mathrm{T} + 2a - 4\nu_\mathrm{I}}{\mathrm{T} + 2a + 4\nu_\mathrm{I}} \quad \text{and} \quad G = 2\nu_\mathrm{I}\left(\frac{4\nu_\mathrm{I}^2 - a^2 + 2\mathrm{T}^2 - a\mathrm{T}}{\mathrm{T} + 2a + 4\nu_\mathrm{I}}\right)$$

For each cross-peak contour, the frequency values along the ridge can be plotted as ν_1^2 versus ν_2^2, transforming the contour lineshape into a straight line segment whose slope and intercept are proportional to *Q* and *G*, respectively. These values can then be used to obtain two possible solutions of isotropic (*a*) and anisotropic (T) couplings with the same value of $|2a+\mathrm{T}|$ and interchanged $A_\perp=|a-\mathrm{T}|$ and $A_{||}=|a+2\mathrm{T}|$.

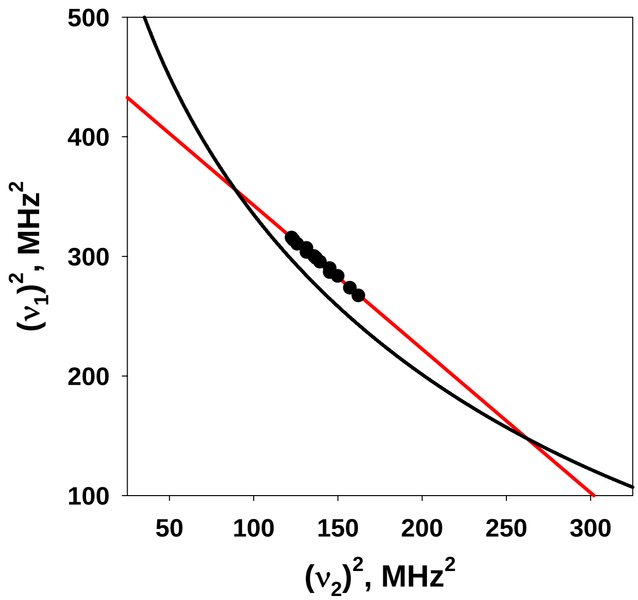

Figure S4. ^{1}H HYSCORE. The example of the cross-peaks **2** from the spectrum in **Fig S3A**. The coordinates ν_1 and ν_2 of arbitrary points along the ridge of cross-peaks **2** were plotted as sets of values for ν_1^2 versus ν_2^2. The points have been fitted by linear regression (*red line*) to give the slope Q= -1.20 (0.03) and intercept G=462.8 (3.8) MHz^2. **Eq S1** with these coefficients define two solutions: T=4.6 MHz and *a*=-4.9 MHz; T=4.6 MHz and *a*=0.3 MHz (signs are relative). In addition, the curve $|\nu_1 + \nu_2| = 2\nu_I$ (using ν_I =14.136 MHz corresponding to the proton Zeeman frequency in the field 332 mT) is plotted in **Fig S4** to explain the nature of the two solutions determined by Eq.(1). The points at which the curve crosses each extrapolated straight line correspond to the nuclear frequencies ν_α and ν_β at canonical orientations. For an axial hyperfine tensor, there are two possible assignments of the parallel or perpendicular orientations and consequently, two sets of hyperfine tensors, one for each assignment. This approach gives hyperfine couplings *a* and *T* identical to those determined from the slope and intercept.

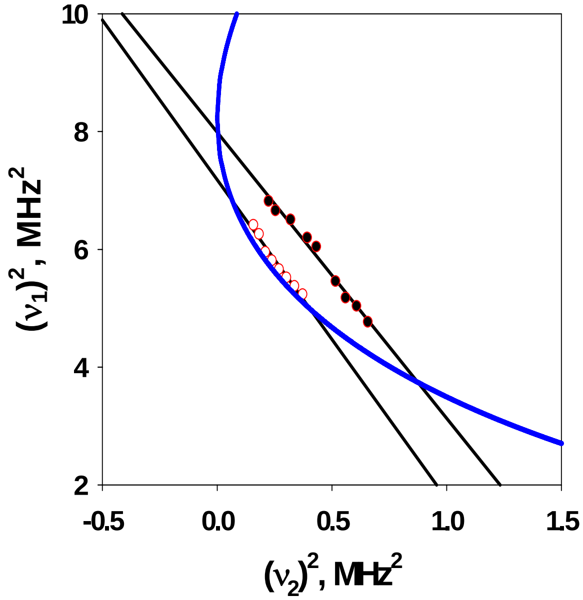

Figure S5. [1]H HYSCORE. The ($(\nu_1)^2$ vs. $(\nu_2)^2$) plot for two arcs forming the ^{15}N cross-feature seen in **Figure 8c**.

For the nonaxial hyperfine tensor, the cross-peak contour lineshape from single nitrogen in powder sample is a triangle with the corners located at the $|\nu_1+\nu_2|=2\nu_I$ line (*blue line* in the figure) (Dikanov, S. A.; Tyryshkin, A. M.; Bowman, M. K. *J. Magn. Reson.* **2000**, *144*, 228-242). The coordinates of the corners determine the principal values of the hyperfine tensor A_i (i=1,2,3) via the relations $[(\nu_I + A_i/2)^2, (\nu_I - A_i/2)^2]$, $A_i = a + T_i$. However, the spectrum in Figure 9C is orientation-selected one where only limited part of orientations forms the lineshape. It suggests that the observed arcs only part of the total lineshape, which would be observed in hypothetical case of complete excitation of all orientations. Theoretical prediction suggests that linear regressions of these arcs should give the estimate of two crossing points with the $|\nu_1+\nu_2|=2\nu_I$ line for each arc. If two resolved arcs forming a cross-feature are resulted from non-axial tensor then two straight lines in coordinates ($(\nu_1)^2$ vs. $(\nu_2)^2$) corresponding to these arcs should cross on $|\nu_1+\nu_2|=2\nu_I$ curve (or at least near this curve in the analysis of real spectrum). However, the regression lines of the points from two arcs are crossing somewhere far away from the $|\nu_1+\nu_2|=2\nu_I$ line indicating that this cross-feature is not produced by single nitrogen with nonaxial hyperfine tensor. If suggest that two arcs are produced by different nitrogens then their regression parameters Q=-4.859 (0.22), G=7.991 (0.1) MHz^2 and Q= -5.41 (0.387), G=7.18 (0.1) MHz^2 define two possibilities for each tensor in axial approximation, respectively: *T*=0.6 MHz and *a*=-2.2 MHz , *T*=0 .6 MHz and *a*=1.6 MHz; and T=0.25 MHz and *a*=-2.1 MHz and T=0.25 MHz and *a*=-1.85 MHz.