Supporting Information

A Conformational Equilibrium in the Nitrogenase MoFe Protein with an α-V70I Amino Acid Substitution Illuminates the Mechanism of H_2 Formation

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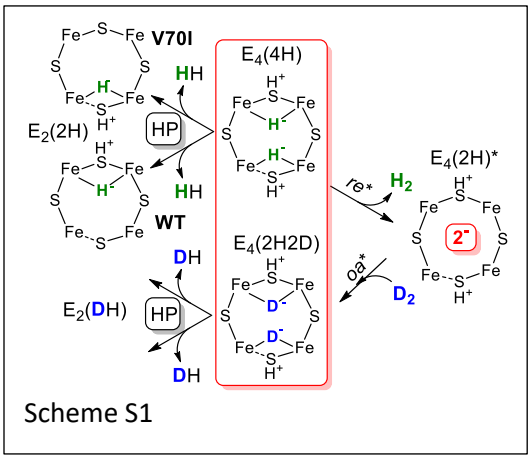
Table S1. Detailed information of EPR and ENDOR samples with different MoFe protein variants studied in the present work.[a]

E_n state/Intermediate	MoFe protein concentration (μM)	Fe protein concentration (μM)	Substrate	pH/pD	Data presented in
WT E_0	50	—	H^+ (1 atm Ar)	7.0	Figure 4 and 6
α-V70I E_0	50	—	H^+ (1 atm Ar)	7.0	Figure 4 and 6
^{95}Mo-WT E_0	150	—	H^+ (1 atm Ar)	7.3	Figure 4
^{95}Mo-α-V70I E_0	150	—	H^+ (1 atm Ar)	7.3	Figure 4
α-V70I E_0 and E_2 (RFQ)[b]	80	800	H^+ (1 atm Ar)	7.4	Figure 5
WT E_0[c]	50	0.5	H^+ (1 atm Ar)	7.0	Figure 6
α-V70I E_0[c]	50	0.5	H^+ (1 atm Ar)	7.0	Figure 6
α-V70I E_4(4H) and E_4(2H)*	150	120	D^+ (1 atm Ar)	7.1	Figure 10
α-V70I E_4	60	40	H^+/D^+ (1 atm Ar/D_2/H_2)	7.3	Figure S1

[a] Unless otherwise stated, all samples were prepared in a *ca.* 200 mM MOPS buffer, pH as specified in the table, with a MgATP regeneration system having final concentrations of *ca.* 13-15 mM ATP, 15-20 mM $MgCl_2$, ca. 20-25 mM phosphocreatine, 2.0 mg/mL BSA, 0.3 mg/mL creatine phosphokinase and ca. 40 mM sodium dithionite. For turnover samples with the presence of Fe protein, all reaction mixtures were incubated at room temperature for about 20-25 sec followed by rapid freezing in a pentane slurry and stored in liquid nitrogen before measurement. [b] The rapid freeze quenching samples were prepared as described earlier.[1,2] [c] The low flux ([MoFeP]:[FeP] = 100:1) turnover samples were made after being incubated at room temperature for different times as indicated in Figure 6 before rapid freezing in a pentane slurry and stored in liquid nitrogen before measurement.

Evidence for the presence of $E_4(2H)^*$ During Ar Turnover of α-V70I MoFe Protein

To support the identification of the g_1 = 2.11 S = 1/2 EPR signal observed for α-V70I under Ar turnover (**Fig 10**) with the $E_4(2H)^*$ state we used as a probe its oxidative-addition reaction with D_2, to form the $E_4(2H2D)$ intermediate with two bridging deuterides. We had shown that the activated $E_4(2H)^*$ state generated photochemically oxidatively adds H_2/D_2 to form $E_4(4H)/E_4(2H2D)$, **Scheme S1**.[3] In contrast, it has long been established that the only state that participates in the LT N_2-reduction catalytic cycle for WT enzyme of **Fig 1**, and that is capable of reacting with H_2/D_2 is the $E_4(2N2H)$ state, which only forms during turnover under N_2, and which can undergo oxidative addition of H_2/D_2 coupled with loss of N_2 to generate $E_4(4H)/E_4(2H2D)$. Thus, if the S = ½ EPR signal observed in low levels during Ar turnover of α-V70I is indeed $E_4(2H)^*$, then when α-V70I is turned over under D_2 a small steady-state population of the dideuteride $E_4(2H^+,2D^-)$ intermediate should develop along with the majority of $E_4(4H)$, **Scheme S1**.



We previously showed that the apparent EPR linewidth of the $E_4(4H)$ isotopologue is actually generated by hyperfine coupling to the bridging hydrides, and the apparent linewidth of $E_4(4D)$ dideuteride state is correspondingly decreased.[4] The hyperfine-broadening effect for $E_4(4H)$ EPR is particularly well exhibited in the shape of the g_3 feature, which reveals resolved splitting arising from hyperfine couplings to two hydrides. Thus, if the observed g_1 = 2.11 S = ½ signal indeed is associated with $E_4(2H)^*$, the E_4 state accumulated during turnover in H_2O under D_2 should exhibit a decrease in linewidth caused by the presence of the dideuteride isotopologue $E_4(2H^+,2D^-)$ and in the opposite case of turnover in D_2O under H_2 the observed EPR linewidth of E_4 state should slightly increase due to formation of the dihydride state $E_4(2D^+,2H^-)$. As shown in **Figure S1**, the EPR signal of E_4 trapped during

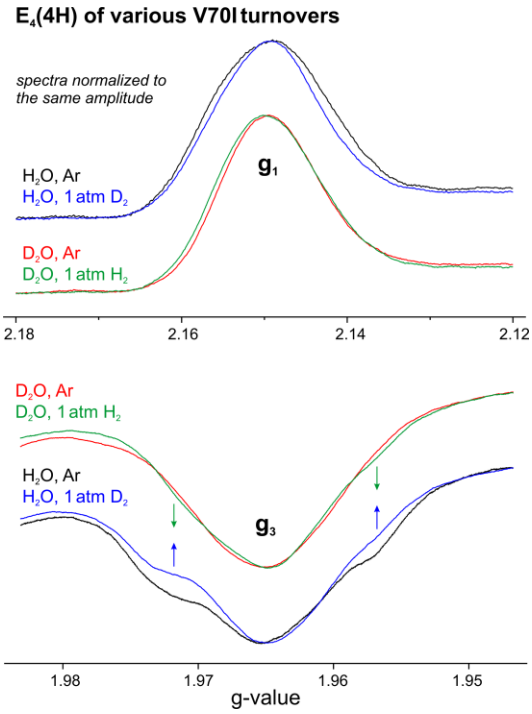


Figure S1. Expanded g_1 and g_3 portions of X-band EPR spectra of the E4(4H) level intermediates showing effect of α-V70I turnover conditions (H_2O vs D_2O; Ar vs D_2 and H_2 atmosphere) on signal linewidth of trapped E_4 state. ***EPR conditions***: temperature, 12 K; microwave frequency, ~9.36 GHz; microwave power, 10 mW; modulation amplitude, 3 G; time constant, 160 ms; field sweep speed, 5 G/s.

α-V70I turnover in H_2O under D_2 indeed is narrower than that trapped under Ar, while E_4 trapped in D_2O under H_2 exhibits hyperfine broadening, in particular a splitting of g_3 feature caused by hydrides of the $E_4(2D^+,2H^-)$ isotopologue formed during turnover. Thus it can be concluded that the newly observed g_1 = 2.11 S = ½ signal is truly associated with $E_4(2H)$*.

With this identification, we confirm that the $E_4(2H)$* intermediate accumulates in low occupancy during turnover of α-V70I under Ar. Indeed, this has led us to recognize that the signal was present in earlier α-V70I turnover spectra.[5,6] As noted above, WT nitrogenase does not react with H_2/D_2 unless N_2 is present, establishing that the $E_4(2H)$* state, which has that ability, does not occur during turnover of the WT enzyme: N_2 binding/reduction and hydride *re* in WT enzyme are fully concerted, **Fig 1**.

References

(1) Kim, S. H.; Perera, R.; Hager, L. P.; Dawson, J. H.; Hoffman, B. M. Rapid Freeze-Quench ENDOR Study of Chloroperoxidase Compound I: The Site of the Radical. *J. Am. Chem. Soc.* **2006**, *128*, 5598-5599.

(2) Danyal, K.; Shaw, S.; Page, T. R.; Duval, S.; Horitani, M.; Marts, A. R.; Lukoyanov, D.; Dean, D. R.; Raugei, S.; Hoffman, B. M.; Seefeldt, L. C.; Antony, E. Negative Cooperativity in the Nitrogenase Fe Protein Electron Delivery Cycle. *PNAS* **2016**, *113*, E5783-E5791.

(3) Lukoyanov, D.; Khadka, N.; Yang, Z. Y.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. Reversible Photoinduced Reductive Elimination of H_2 from the Nitrogenase Dihydride State, the $E_4(4H)$ Janus Intermediate. *J. Am. Chem. Soc.* **2016**, *138*, 1320-1327.

(4) Lukoyanov, D.; Khadka, N.; Yang, Z. Y.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. Reductive Elimination of H_2 Activates Nitrogenase to Reduce the N≡N Triple Bond: Characterization of the $E_4(4H)$ Janus Intermediate in Wild-Type Enzyme. *J. Am. Chem. Soc.* **2016**, *138*, 10674-10683.

(5) Igarashi, R. Y.; Laryukhin, M.; Dos Santos, P. C.; Lee, H. I.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. Trapping H^- Bound to the Nitrogenase FeMo-Cofactor Active Site During H_2 Evolution: Characterization by ENDOR Spectroscopy. *J. Am. Chem. Soc.* **2005**, *127*, 6231-6241.

(6) Lukoyanov, D.; Barney, B. M.; Dean, D. R.; Seefeldt, L. C.; Hoffman, B. M. Connecting Nitrogenase Intermediates with the Kinetic Scheme for N_2 Reduction by a Relaxation Protocol and Identification of the N_2 Binding State. *PNAS* **2007**, *104*, 1451-1455.