Published in final edited form as:

J Am Chem Soc. 2016 February 3; 138(4): 1320–1327. doi:10.1021/jacs.5b11650.

Reversible Photoinduced Reductive Elimination of H₂ from the Nitrogenase Dihydride State, the E₄(4H) Janus Intermediate

Dmitriy Lukoyanov[†], Nimesh Khadka[§], Zhi-Yong Yang[§], Dennis R. Dean[#], Lance C. Seefeldt^{§,*}, and Brian M. Hoffman^{†,*}

[†]Department of Chemistry, Northwestern University, Evanston, IL 60208

[§]Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322

[#]Department of Biochemistry, Virginia Tech, 110 Fralin Hall, Blacksburg, VA 24061

Abstract

We recently demonstrated that N₂ reduction by nitrogenase involves the obligatory release of one H₂ per N₂ reduced. These studies focused on the E₄(4H) ‘Janus intermediate’, which has accumulated four reducing equivalents as two [Fe-H-Fe] bridging hydrides. E₄(4H) is poised to bind and reduce N₂ through reductive elimination (*re*) of the two hydrides as H₂, coupled to the binding/reduction of N₂. To obtain atomic-level details of the *re* activation process, we carried out *in situ* 450 nm photolysis of E₄(4H) in an EPR cavity at temperatures below 20 K. ENDOR and EPR measurements show that photolysis generates a new FeMo-co state, denoted E₄(2H)*, through the photoinduced *re* of the two bridging hydrides of E₄(4H) as H₂. During cryoannealing at temperatures above 175 K, E₄(2H)* reverts to E₄(4H) through the oxidative addition (*oa*) of the H₂. The photolysis quantum yield is temperature invariant at liquid helium temperatures and shows a rather large kinetic isotope effect, KIE = 10. These observations imply that photoinduced release of H₂ involves a barrier to the combination of the two nascent H atoms, in contrast to a barrierless process for mono-metallic inorganic complexes, and further suggest that H₂ formation involves nuclear tunneling through that barrier. The *oa* recombination of E₄(2H)* with the liberated H₂ offers compelling evidence for the Janus intermediate as the point at which H₂ is necessarily lost during N₂ reduction; this mechanistically coupled loss must be gated by N₂ addition that drives the *re/oa* equilibrium toward reductive elimination of H₂ with N₂ binding/reduction.

Graphical Abstract

Corresponding author: bmh@northwestern.edu; lance.seefeldt@usu.edu.

Supporting Information: Two EPR and ENDOR figures; analysis of the rate constant for photolysis in a scattering medium; Table of specific activities of MoFe protein variants; Table of rate parameters for photoinduced *re* of E₄(4H).

Introduction

Biological nitrogen fixation — the reduction of N_2 to two NH_3 molecules — is primarily catalyzed by the Mo-dependent nitrogenase. This enzyme comprises two component proteins, denoted the Fe protein and the MoFe protein. The former delivers electrons one-at-a-time to the MoFe protein, where they are utilized at the active site iron-molybdenum cofactor ([7Fe-9S-Mo-C-R-homocitrate]; FeMo-co), Fig 1, to reduce substrate.^{1,2} Kinetic studies of N_2 reduction by nitrogenase, carried out in the 1970's and 1980's by many groups, especially by Lowe and Thorneley and their coworkers, culminated in the Lowe-Thorneley (LT) kinetic model for nitrogenase function.^{1,3,4} It describes the kinetics of transformations among catalytic intermediates, denoted E_n where n is the number of electron/proton deliveries to the catalytic FeMo-co, with electron transfer from the partner Fe protein in each of these steps being driven by the binding and hydrolysis of two MgATP within the Fe protein.⁵ A central defining feature of this scheme is a mysterious and puzzling, obligatory (mechanistic) requirement for the formation of one H_2 for each N_2 reduced. This, in turn, leads to a limiting enzymatic stoichiometry for enzyme-catalyzed nitrogen fixation, given by eq 1,

a conclusion in agreement with stoichiometric experiments by Simpson and Burris.⁶ However, obligatory requirement for H_2 formation has not been universally accepted.⁷ Most tellingly, in their magisterial review, Burgess and Lowe themselves questioned this requirement: “Thus the data that support the obligatory evolution of one H_2 for every N_2 reduced are much less compelling than the data that require us to believe that some H_2 will always be evolved during N_2 reduction.”¹

We recently proposed^{8,9} that obligatory H_2 formation was required to explain the multitude of mechanistic observations by numerous investigators that had accumulated over decades.¹ This proposal focuses on the $E_4(4H)$ ‘Janus intermediate’ (See Fig 2 for notation), which has accumulated four of the eight required reducing equivalents, storing them as two [Fe-H-Fe] bridging hydrides.^{10–12} $E_4(4H)$ sits at a transition in the N_2 reduction pathway, poised to ‘fall back’ to E_0 by release of two H_2 , but equally poised to bind and reduce N_2 through the accumulation of four more equivalents, hence the appellation.⁹ The bridging mode of hydride binding plays a key mechanistic role. Bridging hydrides are less susceptible to protonation than terminal hydrides, and so they diminish the tendency of FeMo-co to ‘fall back’ by losing reducing equivalents through the formation of H_2 . However, the bridging mode also lowers hydride reactivity, relative to that of terminal hydrides.^{13,14} How this

‘deactivated’ intermediate becomes activated through the release of H_2 coupled to N_2 binding forms part of the ‘mystery’ of dinitrogen fixation by nitrogenase.

We proposed that the $E_4(4H)$ state becomes activated for the binding of N_2 and its hydrogenation to a N_2H_2 -level moiety through the reductive elimination (*re*) of the two hydrides as H_2 , the forward direction of the equilibrium in Fig 2.^{8,9} This proposal was initially supported^{8,9} by showing that the behavior of nitrogenase during the reverse of this equilibrium, the oxidative addition (*oa*) of H_2 with loss of N_2 , explains the key constraints on nitrogenase mechanism that had been revealed over the years.¹ In particular it explains the previously baffling observation that D_2 can only react with nitrogenase during turnover with N_2 present, and then is stoichiometrically reduced to two HD.¹ Promptly thereafter we confirmed the mechanistic prediction that during turnover under N_2/D_2 , the reverse of the equilibrium of Fig 2, the *oa* of D_2 by the $E_4(2N_2H)$ intermediate with the loss of N_2 , must generate the $E_4(2D_2H)$ isotopologue with D_2 having been converted selectively into two bridging deuterides, a state which could form in no other way.¹⁵ This observation established the *re/oa* equilibrium is thermodynamically reversible. More recently, we demonstrated that the (*re/oa*) activation equilibrium of Fig 2 is not only thermodynamically, but also kinetically reversible. The overall result of these several findings is to establish the mechanistic requirement for the formation of one H_2 per N_2 . This in turn implies the limiting stoichiometry of eight electrons/protons for the reduction of N_2 to two NH_3 (eq 1).¹⁶

But these efforts, while establishing the *re/oa* mechanism for nitrogenase activation, do not provide atomic-level details of the *re* activation process. For example, in rough analogy to nucleophilic substitution in organic chemistry, we can imagine a spectrum of reaction pathways for *re/oa*, as illustrated in Fig 3: Is the conversion concerted? associative? dissociative? If a discrete intermediate (*e.g.* **Y**, or **Z**) exists, what are its properties? To obtain atomic-level details of the *re* activation process requires deeper understanding of the inorganic chemistry of the bridging hydrides of $E_4(4H)$: what are their properties and what properties do they confer on this enzyme state? We here present the initial results of a photochemical approach inspired by the properties of inorganic dihydride complexes.

The photolysis of transition metal dihydride complexes with mutually *cis* hydride ligands commonly results in the release of H_2 .^{17–2526} As noted by Perutz,¹⁷ “The photochemical reaction causes a reduction in oxidation state of two and is a typical example of reductive elimination. The reverse reaction will usually proceed thermally and is the prototype example of an oxidative addition reaction.” Regardless of the precise nature of the thermal *re/oa* equilibrium process in nitrogenase, Fig 2, photo-induced *re* would cleanly give an activated version of the doubly reduced $E_2(2H)$ intermediate, which we denote $E_4(2H)^*$, that would be analogous to the intermediate that would form upon thermal dissociative *re* loss of H_2 prior to N_2 binding (**Z**; Fig 3). Our cartoon depictions of $E_4(4H)$ frequently have shown the two [Fe-H-Fe] hydrides with a common vertex, Chart 1, in order to emphasize the analogy between *re* of H_2 by mononuclear metal di-hydrides, and the activation of FeMo-co through *re* of H_2 . However, we do not yet know their exact disposition, and know of no precedent for *re*, either thermal or photochemical, for the ‘parallel’ hydrides drawn in the cartoon, Fig 2, a geometry that is suggested by preliminary DFT computations.²⁷ On the

other hand, no inorganic multimetallic dihydride of which we are aware exhibits a 4Fe 'face', as does FeMo-co, and therefore none could have the adjacent, parallel hydrides drawn in the cartoon. Thus, photolysis of the Janus $E_4(4H)$ intermediate embeds FeMo-co even more deeply within the body of organometallic chemistry, yet breaks new ground.

Materials and Methods

Materials and Protein purifications

All the reagents were obtained from SigmaAldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ) and were used without further purification. Argon, N_2 , and acetylene gases were purchased from Air Liquide America Specialty Gases LLC (Plumsteadville, PA).

Remodeling the active site of MoFe protein by the α -70^{Val→Ile} mutation permits the freeze trapping of MoFe with high populations of $E_4(4H)$.¹⁰ Experiments were carried out both with the singly substituted, α -70^{Ile} MoFe protein and with the doubly-substituted α -70^{Val→Ile}/ α -195^{His→Gln} MoFe proteins. As shown in Table S1, this protein functions similarly to the single mutant. The two proteins were obtained from the corresponding *Azotobacter vinelandii* strains. They were grown, and the corresponding nitrogenase MoFe proteins were expressed and purified as described elsewhere.²⁸ The handling of all buffers and proteins were done anaerobically under Ar atmosphere or under Schlenk vacuum line unless stated otherwise.

EPR and ENDOR samples

The $E_4(4H)$ intermediate and its deuterated analogue, $E_4(4D)$, are prepared by turnover of the MoFe protein in H_2O and D_2O buffers, respectively. Depending on the buffer used, all exchangeable sites are thereby populated with H or D, not only the hydride bridges. Thus, measured kinetic isotope effects associated with *re* and *oa* steps of the equilibrium of Fig 2^{29,30} are a composite of primary isotope effects associated with hydride *re* or H_2 *oa* plus any, smaller, solvent isotope effects.

EPR samples were prepared in a dioxygen free buffer containing a MgATP regeneration system with final concentrations of 13 mM ATP, 15 mM $MgCl_2$, 20 mM phosphocreatine, 2.0 mg/mL bovine serum albumin, and 0.3 mg/mL phosphocreatine kinase in a 200 mM MOPS buffer at pH 7.3 (H_2O buffer) or pD=7.3 (D_2O buffer; pH meter reading of 6.9)³¹ with 50 mM dithionite. MoFe protein was added at ~50 μ M final concentration and the reaction was initiated by addition of Fe protein at ~36 μ M concentration. The reaction was allowed to run at room temperature under Ar atmosphere for 20–25 s before freeze-quenching the samples. The samples for ENDOR experiments were prepared similarly, but typically with three-fold higher concentrations.

EPR and ENDOR Measurements

X-band EPR spectra were recorded on a Bruker ESP 300 spectrometer equipped with an Oxford Instruments ESR 900 continuous He flow cryostat. To allow illumination, a Bruker ER4104R cavity was employed. This cavity allows front-face optical access through a waveguide beyond cutoff (microwave non-transmitting) on the cavity front face, with a

4×10 mm optical transmission path. *In situ* photolysis of a sample held within the cryostat at the chosen temperature initially employed a 15 mW blue LED inserted into this illumination port. Subsequently it employed a Thorlabs Inc (Newton, New Jersey) PL450B, 450 nm, 80 mW Osram Laser Diode mounted on the port through use of the corresponding diode mount with focusing lenses. Thermal relaxation was monitored by the step-annealing procedure in which the sample was quickly warmed to a desired temperature, held there for a fixed time, then promptly returned to 77K, and then examined by EPR at a still lower temperature.

Q-band CW EPR and ^1H ENDOR spectra were collected on a spectrometer with a helium immersion Dewar as previously reported.³² The stochastic field-modulation detected ENDOR technique, first reported by Brueggeman and Niklas,³³ was also utilized. In the stochastic ENDOR sequence, RF is randomly hopped over the frequency range of the spectrum, with subtraction of a background signal (RF off) at each frequency. All measurements were done at 2 K. As desired, Q-band samples were photolyzed by placing them in liquid nitrogen in an X-band finger Dewar and illuminating them with a 1000 mW blue LED. The samples were then transferred to a liquid helium cryostat for EPR/ENDOR study.

Results and Discussion

Figures 4 and S1 present X-band and Fig S2 Q-band EPR spectra of the $S = \frac{1}{2} E_4(4H)$ intermediate freeze-trapped during turnover of MoFe protein that was remodeled with the $\alpha\text{-70}^{\text{Val}\rightarrow\text{Ile}}$ substitution; similar results are seen for MoFe with the double substitution, $\alpha\text{-70}^{\text{Val}\rightarrow\text{Ile}}, \alpha\text{-194}^{\text{His}\rightarrow\text{Gln}}$ (Fig 1). Both single and double substitutions prevent access of substrates other than protons without perturbing FeMo-co function (Table S1), and enhance the accumulation of $E_4(4H)$.^{10–12}

Figure 4 includes spectra collected during *in situ* photolysis with 450 nm light with the sample held at 12 K; equivalent spectra are obtained by photolysis at 77 K (e.g., Fig S2). Although it has not yet proven possible to create optically transparent freeze-quenched samples of this intermediate, the figures show that irradiation nonetheless causes the progressive loss of the $E_4(4H)$ signal, $g = [2.15, 2.007, 1.965]$, and accompanying appearance of a new signal, S , with $g = [2.098, 2.0, 1.956]$. EPR spectra collected over temperatures between 12 K and 50 K (Fig S1) show the $E_4(4H)$ signal disappears by ~40–50 K because of rapid spin relaxation, whereas for S , the EPR signal is clearly visible at 50 K, demonstrating differences between excited spin-state manifolds of S and $E_4(4H)$.

Careful examination of the timecourse of photolysis shows no new signals that are generated, other than S . In particular, Figures 4, S1, S2 show *no* other photo-induced change in the spectrum in the vicinity of $g \sim 2$; likewise, there is *no* change at lower fields (not shown), where the signal from residual resting state (E_0) appears and where signals would appear from the state which has accumulated two $[\text{e}^-/\text{H}^+]$ ($E_2(2H)$).³⁴ The photolysis product, S , completely relaxes back to $E_4(4H)$ when annealed for 2 minutes at 217 K, with *no* other change in the EPR spectrum, either in the $g \sim 2$ region, Fig 4, or the high- g (low-field) region (not shown). *Thus, photolysis/annealing cause the reversible, and kinetically direct, conversion, $E_4(4H) \rightleftharpoons S$.*

^1H ENDOR spectroscopy was used to determine the fate of the [Fe-H-Fe] bridging hydrides during the photolytic $\text{E}_4(4\text{H}) \Rightarrow \text{S}$ conversion. Fig 5 shows 2 K Q-band stochastic CW ^1H ENDOR spectra collected at two different g -values from $\text{E}_4(4\text{H})$ before and after photolysis; Fig S2 presents corresponding ‘conventional’ CW spectra. In both figures, the spectra represent components of the 2D field-frequency pattern of spectra collected across the EPR envelope, which has been thoroughly analyzed in terms of two hydrides with anisotropic hyperfine tensor components that are virtually identical, but with tensors that are differently oriented with respect to $\mathbf{g}$.¹⁰ In the $g = 2.01$ spectrum of $\text{E}_4(4\text{H})$ in Fig 5, the strongly-coupled ^1H signals from the two hydrides ($A \lesssim 40$ MHz) are completely overlapped; at $g = 2.00$, the signals again overlap, but distinct peaks also are seen from the individual hydrides, most noticeably the two doublets with couplings of $A \sim 22$ and 28 MHz.^{10,27}

For the sample that gave the ENDOR spectra in Fig 5, the photolysis had reduced the intensity of the $\text{E}_4(4\text{H})$ EPR signal by $\sim$ threefold, with the corresponding appearance of EPR intensity from S . Fig 5 shows that the photolysis decreases the intensity of the ^1H ENDOR signals from the strongly-coupled hydrides by a comparable amount, *without* the appearance of new strongly-coupled signals that can be associated with a metal-hydride ‘isomer’. At $g = 2.01$ *no* new signal appears; at $g = 2.0$ a signal appears with the relatively small coupling, $A \sim 10$ MHz, which likely is associated with one of the protonated sulfides that must be present in S . CW ENDOR spectra taken on a sample with an even greater extent of photolysis show essentially complete loss of the ENDOR signals at a field where the two hydrides show distinct features (Fig S2). Correspondingly, ^2H ENDOR measurements of S created by photolysis of $\text{E}_4(4\text{D})$ prepared in D_2O buffer show the loss of the [Fe-D-Fe] signals associated with $\text{E}_4(4\text{D})$, again with no new strongly-coupled signal appearing (not shown).

The absence of new strongly-coupled signals in the $^1,^2\text{H}$ ENDOR responses further rules out the possibility that S is an H_2 complex of FeMo-co, either with strongly-hindered or free rotation of H_2 , rather than having released H_2 . A bound H_2 complex with strongly hindered rotation would show new strongly-coupled ^1H ENDOR signals, contrary to observation.³⁵ Quantum statistical arguments show that freely rotating H_2 , which can occur even at 2 K, would not show ^1H ENDOR signals, but would show new strongly-coupled ^2H ENDOR signals for the corresponding D_2O sample.³⁶ The absence of such new signals for S means that it does not contain a freely-rotating bound D_2 .

We thus infer that the state S generated by photolysis of $\text{E}_4(4\text{H})$ has indeed lost the metal-bound hydrides through the release of one or two H_2 .

Mechanism of Photoconversion

Three mechanisms must be considered for the $\text{E}_4(4\text{H}) \Rightarrow \text{S}$ photo-conversion through loss of both hydrides and release of H_2 , Fig 6, which we denote, *re*, *hp*, and *L*. The first would be precisely the photo-induced reductive-elimination of one H_2 , as discussed above, yielding a non-thermal form of the doubly-reduced state of the cluster, which we denote $\text{E}_4(2\text{H})^*$. The second postulates two steps of photo-induced hydride protonolysis (*hp*), each with loss of H_2 , in which case S must be a low-spin ($S = 1/2$) ‘spin-isomer’ of the E_0 , $S = 3/2$ resting state, which we can denote $\text{E}_0^\dagger$. Thermal *hp* processes are responsible for H_2 formation by

nitrogenase,^{1,8,9} as well as by hydrogenases.^{37,38} The third mechanism differs from the other two in that it does not involve generation of H_2 . Instead it would involve two steps of photo-induced *re* of a hydride, with transfer of the proton 'released' to a bridging sulfide. We denote this the '*L* mechanism' because it is inspired by the photoinduced conversion of the Ni-C state of the Ni-Fe hydrogenases.^{37,38} Ni-C exhibits a [Ni(III)-H-Fe(II)] bridging hydride and a cysteinyl thiolate bound to Ni. Photolysis generates a state, denoted Ni-L, which contains [Ni(I), Fe(II)] metal ions, and with the proton formed by photoinduced reductive elimination of the hydride having been transferred to the bound sulfur. Any one of these three imagined processes would yield a final photoproduct without metal hydrides.

The observation that *S* thermally relaxes to $E_4(4H)$ immediately rules out the *hp* mechanism for photo-induced $E_4(4H) \Rightarrow S$ conversion. This conversion would involve the loss of four reducing equivalents as two H_2 molecules, and its reverse must involve two steps of reduction of FeMo-co by H_2 : $E_0^+ + H_2 \Rightarrow E_2(2H)$; $E_2(2H) + H_2 \Rightarrow E_4(4H)$. However, it is one of the foundational facts about nitrogenase mechanism that H_2 cannot react with any thermally generated state of FeMo-co except the N_2 -bound state produced by *re* of H_2 ; as we have explained, the reactions are uphill by ~ 30 kcal/mol.⁸ Even if the E_0^+ photo-generated spin isomer were sufficiently activated as to react with H_2 , the first step of reduction would necessarily produce the thermally equilibrated $E_2(2H)$, which could not react with H_2 : hence $E_4(4H)$ could not be regenerated from *S* during cryoannealing.

The complete absence during photolysis of a second new EPR signal in addition to *S* (see above) in fact not only independently rules out *hp*, but also rules out *L*. Both mechanisms involve sequential steps of photon absorption. Under the constant low-level illumination of this experiment this necessarily implies the buildup then loss of the EPR signal from the intermediate stage that has absorbed one photon. *Thus we conclude that photolysis indeed generates a FeMo-co state, $S = E_4(2H)^*$, through the photoinduced reductive elimination of the two bridging hydrides of $E_4(4H)$ with accompanying production of one H_2 , and that the relaxation of $E_4(2H)^*$ to $E_4(4H)$ during cryoannealing corresponds to the oxidative addition of H_2 to the photo-generated state, $E_4(2H)^*$.*

Kinetics of H_2/D_2 *oa*

To characterize the isotope dependence of the thermal *oa* process, $E_4(2H)^* + H_2 \Rightarrow E_4(4H)$ versus $E_4(2D)^* + D_2 \Rightarrow E_4(4D)$,^{29,30,39} we measured the kinetics of the 193 K relaxation of $E_4(2H)^*$ and $E_4(2D)^*$ in samples prepared, respectively, in H_2O and D_2O buffers. In these experiments the sample was annealed at 193 K for multiple time intervals, with cooling to 12 K for collection of EPR spectra between intervals. For both H_2O and D_2O buffers the relaxation is well-modeled as a one-step process, Fig 7. This is consistent with, but not proof of, the absence of an intermediate state(s), for example an H_2 complex.⁴⁰

With *S* in H_2O buffer, the exponential decay time-constant is, $\tau = 10$ min; decay is slowed in D_2O buffer, to the decay time, $\tau = 55$ min. The $E_4(4H)$ state recovers in synchrony with the loss of *S*, as the recovery also is exponential, and exhibits the same time-constants, $\tau = 9$ min for H_2O sample and $\tau = 47$ min for D_2O . This kinetic isotope effect during *oa*, KIE ~ 5.4 , is larger than typical for closed-shell monometallic complexes.^{41,42} Combined with a strong temperature dependence in the time-constant (not shown), this KIE implies that *oa* of

H₂ involves traversal of an energy barrier associated with H₂ binding and/or bond cleavage. The exponential decay of *S* and appearance of E₄(4H) suggest that in the frozen solution the H₂ formed by photoinduced *re* is trapped adjacent to FeMo-co and undergoes *oa* ‘intramolecularly’, presumably in some part because the incorporation of isoleucine over the active face of FeMo-co prevents H₂ diffusion away. Indeed, although we presume that the relatively weakly coupled, but clearly resolved ¹H ENDOR signal seen for *S* is associated with a sulfur-bound proton, we cannot rule out the possibility that it comes from the H₂ trapped nearby.

KIE of photoinduced *re*

We know of only two studies of the KIE for photoinduced *re* of H₂/D₂, and these found small⁴³ or negligible isotope effects.²¹ For completeness, we nonetheless used *in situ* photolysis to compare the time-dependent loss of the E₄(4H) and corresponding E₄(4D) signals as a function of temperature, Fig 8. In a clear solution of low optical density, photolysis under constant illumination would cause an exponential loss of the signal with rate constant (*k*) proportional to the light intensity (I₀) and quantum yield (φ), for *re*, $k(\phi) = \tau^{-1}(\phi) \propto I_0\phi$, where $\tau(\phi)$ is the corresponding time-constant for decay. However, it proved impossible to prepare clear frozen samples with high population of E₄(4H), only frozen ‘snows’. The decay of the signal during photolysis of a ‘snow’ is *necessarily* non-exponential because light scattering diminishes the photon flux across the sample (See **SI**); instead, as shown in Fig 8, it can be described with a stretched exponential, $\exp(-[t/\tau]^n)$,⁴⁴ with φ-dependent ‘1/e’ decay time, $\tau(\phi)$, whose inverse is proportional to the average decay rate constant (*K*)⁴⁴ and is thus proportional to the quantum yield for *re*: $\tau^{-1}(\phi) \propto K(\phi) \propto \phi$ (See **SI**).

The decay time for photoinduced *re* of dihydrogen from E₄(4H) measured in *both* H₂O and D₂O buffers is temperature invariant, within error, from 4–12 K, Fig 8, Table S2, a variation in the thermal energy (k_BT) by a factor of ~ 3. Contrary to expectation, the decay slows markedly in D₂O buffer; the KIE for *re* over this range, defined as the ratio of the ‘1/e’ decay times for D₂O and H₂O buffers is large, KIE ~ 10. *These observations together imply that photoinduced re involves a barrier to the combination of the two nascent H atoms, in contrast to the barrierless process inferred for mono-metallic metal complexes,^{17,21} and suggest that the photoinduced formation of H₂ involves nuclear tunneling through that barrier.* Whether the process involves an actual intermediate H₂ complex remains to be determined. In combination with the evidence for a barrier crossing in the *oa* of H₂ to E₄(2H)* to regenerate E₄(4H), this leads us to the picture of the energy surfaces for photoinduced *re/oa* for the Janus intermediate presented in Fig 9.

A Second Channel for Photoinduced Reaction

Intriguingly, the slowed conversion of E₄(4D) to E₄(2D)* in D₂O buffer uncovered a second channel for the photoinduced loss of E₄(4D), one which could not compete with the more rapid conversion to E₄(2H)* in H₂O. During photolysis of E₄(4D) in D₂O buffer at ~ 10 K we observed a very weak photoinduced spin – ½ EPR signal in addition to that of E₄(2D)* (not shown). This state is much less stable than E₄(2D)*, and decays completely within two minutes upon raising the temperature to 77 K. Its low population and instability have

precluded detailed analysis, and it has proven impossible to determine its mechanism of formation (for example, whether it is generated by the *L* mechanism).

Excited-State Molecular Orbitals

The photo-induced *re* of mutually *cis* hydrides on a single metal ion (M) has been studied computationally,^{45,46} and is thought to occur from a ligand-field excited state that is H-H bonding and that weakens M-(H-H) bonding and/or is actually M-(H-H) (σ^*) antibonding, as shown in Fig 10. The process is thought to be concerted, and to occur on a barrierless repulsive energy surface, with the H's freely coming together for release as H₂.^{17,21} This view is compatible with measurements showing prompt release of H₂ after photolysis (< 6 ps),¹⁸ and with the absence of a KIE for H₂/D₂ photorelease.²¹ The large KIE and tunneling-like behavior for the photo-*re* of E₄(4H) in contrast imply that its excited energy surface must instead have a barrier to H₂ formation, and suggest that at the low temperature of our experiments H₂ is formed by H-atom tunneling through the barrier. This behavior in turn suggests to us that the hydrides of E₄(4H) do not have a common vertex.

What type of excited state can generate the release of bridging hydrides? Regardless of whether the two bridging hydrides share a vertex (Chart 1) or are otherwise displayed, as in the 'parallel' arrangement in Figs 2, 3, 6, by analogy to the mononuclear case we would suggest that the optical excitation generates a M₂-(H-H) (σ^*) antibonding ligand-field excited state of one of the two bridging hydrides, as exemplified in Fig 11. This could couple to the equivalent state of the other bridging hydride, delocalizing the excitation and generating a *cluster* excited state from which H₂ is liberated, or alternatively, the excited hydride state might undergo reductive elimination through an *L*-like mechanism, with the 'liberated' proton attacking its neighbor hydride, liberating H₂. In any case, the formation of H₂ appears to involve nuclear tunneling through a barrier, as discussed above.

Summary and Prospects

These measurements have shown that *in situ* 450 nm photolysis of the Janus Intermediate, E₄(4H), in an EPR cavity at temperatures below 20 K generates a new FeMo-co state, denoted E₄(2H)*, that has lost the hyperfine couplings characteristic of the two bridging hydrides. We have concluded that this state is neither a 'hydride isomer' nor an H₂ complex, but instead E₄(4H) has undergone photoinduced *re* of the two bridging hydrides of E₄(4H) to generate E₄(2H)* with the release of H₂. During cryoannealing at temperatures above 175 K, E₄(2H)* reverts to E₄(4H) through the oxidative addition (*oa*) of H₂. Our observations further imply that photoinduced release of H₂ involves a barrier to the combination of the two nascent H atoms, in contrast to a barrierless process for mono-metallic inorganic complexes, and further suggest that H₂ formation involves nuclear tunneling through that barrier. The *oa* recombination of E₄(2H)* with the liberated H₂ offers compelling evidence for the Janus intermediate as the point at which H₂ is necessarily lost during N₂ reduction; this mechanistically coupled loss must be gated by N₂ addition that drives the *re/oa* equilibrium toward reductive elimination of H₂ with N₂ binding/reduction.⁴⁷

There are many issues to be addressed concerning this remarkable observation of photo-induced *re* of the bridging hydrides of E₄(4H) and its thermal reverse, *oa* of H₂ by the

photo-generated $S = E_4(2H)^*$ intermediate. Most obvious, is further characterization of the energy surfaces for photoinduced *re/oa* of the Janus intermediate, $E_4(4H)$, through experiments that address the indications of tunneling during photoinduced *re* and the possibility of stable intermediates during either or both, *re* and *oa*, as noted in the legend to Fig 9. In addition, we anticipate characterizing $E_4(2H)^*$ by ^{57}Fe and ^{95}Mo ENDOR spectroscopy.

Expanding the perspective, it is of particular interest to examine the reactivity of the photogenerated state $E_4(2H)^*$. We know that $E_4(2H)^*$ is in some way more reactive than all thermally generated states of FeMo-co *except* the $E_4(2N_2H)$ state produced by *re* of H_2 (Fig 2), as this latter is the only turnover state that can react with H_2 , through the *oa* reverse of the *re/oa* equilibrium with loss of N_2 . It seems unlikely that this equilibrium in fact involves the Dissociative pathway of Fig 3, as $E_4(4H)$ *re* does not occur unless N_2 is present. Nevertheless, it is important to test whether $E_4(2H)^*$ binds and reacts with N_2 , as would be the case if *re* did occur in this fashion and $E_4(2H)^*$ were equivalent to **Z**, Fig 3. Indeed, as there is keen interest in using nitrogenase to reduce substrates other than N_2 ,^{48–50} it is of fundamental importance to test if $E_4(2H)^*$ can react with other possible substrates (CO_2 ; C_2H_2 , C_2H_4 , etc), as occurs with the coordinatively unsaturated states produced by photo-*re* of single-metal complexes.^{17,20,51}

Of course, the use of $\alpha\text{-}^{70}\text{Ile}$ MoFe protein precludes such experiments. Although this active-site change is central to the present report in allowing the accumulation of high populations of $E_4(4H)$, it does so at the cost of blocking access to the FeMo-co active site to all substrates *but* protons.¹⁰ There is, however, an excellent prospect of overcoming this limitation. We find that under certain conditions of turnover with the wild-type enzyme, the $E_4(4H)$ EPR signal can be observed. Efforts are under way to maximize its population; photochemical studies of this state will be undertaken subsequently. These may help us to distinguish between the alternative Concerted and Associative mechanisms for *re/oa*, Fig 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the NIH (GM 111097;BMH), NSF (MCB 1118613 to BMH) and the Department of Energy, Office of Basic Energy Sciences (LCS and DRD). DL dedicates this work to his teacher, Dr. Igor N. Kurkin, who has been conducting EPR research at Kazan University since the beginning of the 1960s and is celebrating his 75th birthday in 2016.

References

1. Burgess BK, Lowe DJ. Chem Rev. 1996; 96:2983. [PubMed: 11848849]
2. Seefeldt LC, Hoffman BM, Dean DR. Annu Rev Biochem. 2009; 78:701. [PubMed: 19489731]
3. Thorneley RNF, Lowe DJ. Met Ions Biol. 1985; 7:221.
4. Wilson PE, Nyborg AC, Watt GD. Biophys Chem. 2001; 91:281. [PubMed: 11551440]
5. Duval S, Danyal K, Shaw S, Lytle AK, Dean DR, Hoffman BM, Antony E, Seefeldt LC. PNAS. 2013; 110:16414. [PubMed: 24062462]
6. Simpson FB, Burris RH. Science. 1984; 224:1095. [PubMed: 6585956]

7.
For example, during the decades after the definitive formulation of the LT scheme (ref 3), with its obligatory loss of H₂ and resulting eight-electron/proton stoichiometry (eq 1), numerous computational studies described ‘mechanisms’ with only six electrons/protons. Indeed, one investigator published multiple six-electron/proton mechanisms without even acknowledging or addressing the contradiction to LT: eg., Dance I. *Chem Asian J.* 2007; 2:936. [PubMed: 17614310]
8. Hoffman BM, Lukoyanov D, Yang ZY, Dean DR, Seefeldt LC. *Chem Rev.* 2014; 114:4041. [PubMed: 24467365]
9. Hoffman BM, Lukoyanov D, Dean DR, Seefeldt LC. *Acc Chem Res.* 2013; 46:587. [PubMed: 23289741]
10. Igarashi RY, Laryukhin M, Dos Santos PC, Lee HI, Dean DR, Seefeldt LC, Hoffman BM. *J Am Chem Soc.* 2005; 127:6231. [PubMed: 15853328]
11. Lukoyanov D, Yang ZY, Dean DR, Seefeldt LC, Hoffman BM. *J Am Chem Soc.* 2010; 132:2526. [PubMed: 20121157]
12. Lukoyanov D, Barney BM, Dean DR, Seefeldt LC, Hoffman BM. *PNAS.* 2007; 104:1451. [PubMed: 17251348]
13. Crabtree, RH. *The organometallic chemistry of the transition metals.* 5. Wiley; Hoboken, N.J: 2009.
14. Peruzzini, M.; Poli, R., editors. *Recent Advances in Hydride Chemistry.* Elsevier Science B.V; Amsterdam, Netherlands: 2001.
15. Yang Z-Y, Khadka N, Lukoyanov D, Hoffman Brian M, Dean Dennis R, Seefeldt Lance C. *PNAS.* 2013; 110:16327. [PubMed: 24062454]
16. Lukoyanov D, Yang ZY, Khadka N, Dean DR, Seefeldt LC, Hoffman BM. *J Am Chem Soc.* 2015; 137:3610. [PubMed: 25741750]
17. Perutz RN. *Pure Appl Chem.* 1998; 70:2211.
18. Colombo M, George MW, Moore JN, Pattison DI, Perutz RN, Virrels IG, Ye TQ. *J Chem Soc, Dalton Trans.* 1997:2857.
19. Whittlesey MK, Mawby RJ, Osman R, Perutz RN, Field LD, Wilkinson MP, George MW. *J Am Chem Soc.* 1993; 115:8627.
20. Ballmann J, Munha RF, Fryzuk MD. *Chem Commun.* 2010; 46:1013.
21. Ozin GA, Mccaffrey JG. *J Phys Chem.* 1984; 88:645.
22. Dugan TR, Holland PL. *J Organomet Chem.* 2009; 694:2825.
23. Yu Y, Smith JM, Flaschenriem CJ, Holland PL. *Inorg Chem.* 2006; 45:5742. [PubMed: 16841977]
24. Smith JM, Sadique AR, Cundari TR, Rodgers KR, Lukat-Rodgers G, Lachicotte RJ, Flaschenriem CJ, Vela J, Holland PL. *J Am Chem Soc.* 2006; 128:756. [PubMed: 16417365]
25. Yu Y, Sadique AR, Smith JM, Dugan TR, Cowley RE, Brennessel WW, Flaschenriem CJ, Bill E, Cundari TR, Holland PL. *J Am Chem Soc.* 2008; 130:6624. [PubMed: 18444648]
26.
We particularly note the extensive reference collection in ref 22
27.
Manuscript in preparation.
28. Christiansen J, Goodwin PJ, Lanzilotta WN, Seefeldt LC, Dean DR. *Biochemistry.* 1998; 37:12611. [PubMed: 9730834]
29. Parkin G. *J Labelled Compd Radiopharm.* 2007; 50:1088.
30. Parkin G. *Acc Chem Res.* 2009; 42:315. [PubMed: 19133745]
31. Glasoe PK, Long FA. *J Phys Chem.* 1960; 64:188.
32. Davoust CE, Doan PE, Hoffman BM. *J Magn Reson.* 1996; 119:38.
33. Brueggemann W, Niklas JR. *J Magn Reson, Ser A.* 1994; 108:25.
34. Lukoyanov D, Yang ZY, Duval S, Danyal K, Dean DR, Seefeldt LC, Hoffman BM. *Inorg Chem.* 2014; 53:3688. [PubMed: 24635454]

35. Lee Y, Kinney RA, Hoffman BM, Peters JC. *J Am Chem Soc.* 2011; 133:16366. [PubMed: 21954981]
36. Gunderson WA, Suess DL, Fong H, Wang X, Hoffmann CM, Cutsail GE III, Peters JC, Hoffman BM. *J Am Chem Soc.* 2014; 136:14998. [PubMed: 25244422]
37. Lubitz W, Ogata H, Rudiger O, Reijerse E. *Chem Rev.* 2014; 114:4081. [PubMed: 24655035]
38. Tai HL, Nishikawa K, Suzuki M, Higuchi Y, Hirota S. *Angew Chem Int Ed.* 2014; 53:13817.
39. Bullock, RM.; Bender, BR. *Encyclopedia of Catalysis.* Horváth, IT., editor. Vol. 4. Wiley; Hoboken, New Jersey: 2003. p. 281
40. Mas-Ballesté, R.; Lledos, A. *Comprehensive Inorganic Chemistry II.* Alvarez, S., editor. Vol. 9. Elsevier Ltd; 2013. p. 736
41. Abu-Hasanayn F, Goldman AS, Krogh-Jespersen K. *J Phys Chem.* 1993; 97:5890.
42. Campian MV, Perutz RN, Procacci B, Thatcher RJ, Torres O, Whitwood AC. *J Am Chem Soc.* 2012; 134:3480. [PubMed: 22276646]
43. Wang WH, Narducci AA, House PG, Weitz E. *J Am Chem Soc.* 1996; 118:8654.
44. Berberan-Santos MN, Bodunov EN, Valeur B. *Chem Phys.* 2005; 315:171.
45. Daniel C. *J Phys Chem.* 1991; 95:2394.
46. Wang WH, Weitz E. *J Phys Chem A.* 1997; 101:2358.
- 47.
- We thank an anonymous reviewer for helping us to articulate this point.
48. Hu Y, Lee CC, Ribbe MW. *Science.* 2011; 333:753. [PubMed: 21817053]
49. Yang ZY, Moure VR, Dean DR, Seefeldt LC. *PNAS.* 2012; 109:19644. [PubMed: 23150564]
50. Seefeldt LC, Yang ZY, Duval S, Dean DR. *Biochim Biophys Acta.* 2013; 1827:1102. [PubMed: 23597875]
51. Calladine JA, Torres O, Anstey M, Ball GE, Bergman RG, Curley J, Duckett SB, George MW, Gilson AI, Lawes DJ, Perutz RN, Sun XZ, Vollhardt KPC. *Chem Sci.* 2010; 1:622.

Figure 1.

Crystal structure of FeMo-co. Fe is shown in rust, Mo in magenta, S in yellow, carbide in dark-grey, carbon in gray, N in blue and O in red. The Fe atoms of catalytic 4Fe-4S face are labelled as 2, 3, 6, and 7. Two amino acids, α -70^{Val} and α -195^{His}, around the FeMo-co are also shown; the former or both are modified in enzyme used in this study (see text). The image was created using PDB coordinate 2AFI.

Figure 2. Schematic of *re/oa* Equilibrium

The cartoon represents the Fe 2,3,6,7 face of FeMo-co, and the ‘2N2H’ implies a species at the diazene reduction level of unknown structure and coordination geometry. In the indicated equilibrium the binding and activation of N_2 is mechanistically coupled to the *re* of H_2 , as described in the text. In the E_n notation, n = number of $[e^-/H^+]$ added to FeMo-co; parenthesis denotes the stoichiometry of H/N bound to FeMo-co.

Figure 3.
Schematic of alternative limiting mechanisms for *re/oa* equilibrium.

Figure 4.

X-band EPR spectra of MoFe protein (α -70^{Ile}) freeze-trapped during Ar turnover in H₂O before (*black*) and during irradiation with 450 nm diode laser at 12 K (*blue* (2.5 min) and *red* (20 min) traces); *Red* arrows highlight the conversion of E₄(4H) to the photoinduced *S* state. *Dashed Green* spectrum shows that annealing (Ann) the illuminated sample at 217 K for 2 minutes causes complete reversion of *S* to E₄(4H). *EPR conditions*: T = 12 K; microwave frequency, 9.36 GHz; microwave power, 10 mW; modulation amplitude, 13 G; time constant, 160 ms; field sweep speed, 20 G/s.

Figure 5.

Q-band stochastic ^1H CW ENDOR spectra showing loss of signals from hydrides, H1 and H2, through photolysis. (*Black*) Before and (*red*) after 450 nm photolysis of MoFe protein (α -70 $^{\text{Ile}}$ / α -195 $^{\text{Gln}}$) trapped during Ar turnover in H_2O buffer. ENDOR conditions: microwave frequency, ~ 34.99 GHz; modulation amplitude, 6.3 G; RF duration 3 ms; RF cycle, 200 Hz; bandwidth of RF broadened to 100 kHz; 2000 scans; temperature, 2 K.

Figure 6.

Alternative mechanisms for the $E_4(4H) \Rightarrow S$ photo-conversion through loss of both hydrides and release of H_2 and thermal reverse.

Figure 7.

Decay during 193 K annealing of $E_4(2H)^*$ photoinduced in MoFe (α -70^{IIe}) freeze-trapped during turnover in H_2O (red) and D_2O (blue), along with the parallel recoveries of $E_4(4H)$. Data points obtained as intensities of g_1 feature of the corresponding $S=1/2$ EPR signals, normalized to the maximum signal; they were fit with an exponential function, with time constants shown in the figure. *EPR conditions:* as in Figure 4.

Figure 8.

Timecourse of *in situ* 450 nm photoinduced conversion of $E_4(4H)$ intermediate trapped during MoFe protein (α -70^{IIe}) turnover in H_2O (lower) and D_2O (upper). Photolysis at 3.8 K (green), 8 K (red) and 12 K (blue). Signal measured directly as intensity of the g_1 feature of the $E_4(4H)$ $S=1/2$ EPR signal, normalized to the maximum signal and fit with a stretched exponential decay function, $I = \exp(-[t/\tau]^n)$, with '1/e' time constant, τ ; $0 < n \leq 1$ equals unity for exponential decay and decreases with the spread of the distribution. Time constants for fits (white dashed lines) are given in figure; in all cases $n \sim 0.4$ (see SI for details). *EPR conditions*: microwave frequency, 9.36 GHz; microwave power, 10 mW (1 mW for measurements at 3.8 K); modulation amplitude, 13 G; time constant, 160 ms.

Figure 9.

Idealized energy surfaces for photoinduced *re/oa* of the Janus intermediate, $E_4(4H)$. Among issues to be resolved are the possibility of tunneling on the excited-state surface, '(?)', and whether there are stable intermediates along *re* or *oa* paths.

Figure 10.

Cartoon showing nodal properties of an excited MO for an $M(H)_2$ complex that is bonding between the two hydrides and antibonding between each one and the metal dz^2 orbital.

Figure 11. Cartoon showing nodal properties of the excited MO's for bridging hydrides (parallel arrangement), each antibonding between the hydride and the two d_{z^2} orbitals on the Fe ions it bridges.

Chart 1.