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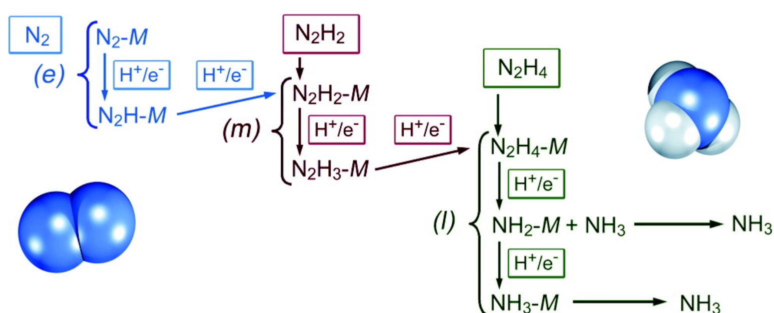

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Intermediates Trapped during Nitrogenase Reduction of N≡N, CH_3−N=NH, and H_2N-NH_2

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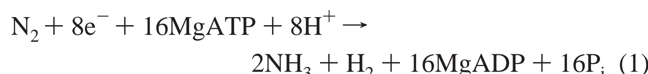
Biological dinitrogen fixation, the reduction of N_2 to $2NH_3$, represents the single largest input of fixed nitrogen into the global biogeochemical nitrogen cycle. This process, which is exclusive to microbes, is catalyzed by the metalloenzyme nitrogenase[1−3] and has an optimal stoichiometry given by eq 1.



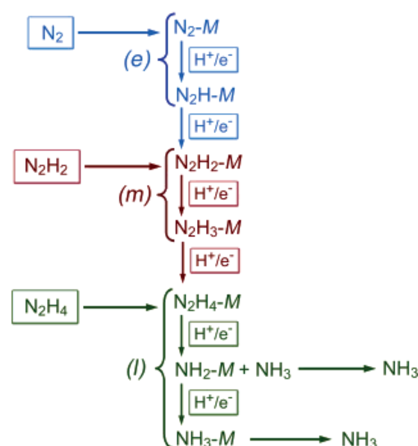
$$N_2 + 8e^- + 16MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

The Mo-dependent nitrogenases have two component proteins. The Fe protein acts as a specific reductant of the MoFe protein in a reaction that requires the hydrolysis of 2 equiv of MgATP per electron transferred. The MoFe protein contains two types of metal clusters: the P cluster ([8Fe−7S]), which mediates electron transfer between the Fe protein, and the FeMo cofactor (M cluster; [7Fe−9S−Mo−X−homocitrate]), which binds and reduces N_2. Until recently,[4] not even the site of N_2 binding on the FeMo cofactor was known, much less the nature of intermediates involved. A combined biochemical−genetic strategy now has indicated that nitrogenous (e.g., N_2 and hydrazine) as well as alkyne (e.g., propargyl alcohol and acetylene) substrates interact at a common FeS face of the FeMo cofactor composed of Fe atoms 2, 3, 6, and 7.[4−8] Progress toward understanding how substrates interact with the FeMo cofactor has been made by freeze-quench trapping substrates[9−11] and inhibitors[12] during turnover of MoFe proteins with specific amino acid substitutions above this face, but only one of these has involved a nitrogenous substrate.[10]

It is proposed that N_2 reduction by nitrogenase involves a series of FeMo cofactor-bound intermediates,[13,14] beginning with bound N_2 and proceeding through the 2-electron/2-proton, semi-reduced intermediates of Scheme 1, but such intermediates have long eluded capture. Here, we present the first report of a high-population intermediate trapped during an early (*e*) stage of N_2 reduction by nitrogenase. In addition, intermediates have been trapped during the reduction of a diazene and of hydrazine (H_2N-NH_2) in an attempt to visualize intermediates corresponding to middle (*m*) and late (*l*) stages of N_2 reduction (Scheme 1). A preliminary characterization by EPR and ENDOR of these intermediates is presented.

Three nitrogenase turnover systems were freeze-quenched during steady-state enzymatic turnover:[4,10] (*e*) wild-type MoFe protein with N_2 as substrate; (*m*) α-195^{Gln} MoFe protein with CH_3−N=NH as substrate;[15−17] and (*l*) α−70^{Ala}/α-195^{Gln} MoFe protein with H_2N-NH_2 [10] as substrate.[18] The substitution of α-195^{His} by Gln has been suggested to disrupt the delivery of protons for reduction of

Scheme 1



nitrogenous substrates,[19] thus allowing an intermediate to be trapped.[10] The substitution of α-70^{Val} by Ala was earlier shown to accommodate the binding of the larger substrate, hydrazine.

Each of these samples shows an EPR signal[10] arising from an $S = 1/2$ state of the FeMo cofactor. The **g** tensors are unique to each intermediate, **g**(*e*) = [2.084, 1.993, 1.969], **g**(*m*) = [2.083, 2.021, 1.993], **g**(*l*) = [2.082, 2.015, 1.987], which suggests that three distinct intermediates may have been trapped. That (*e*) and [(*m*), (*l*)] represent at least two distinct N_2 reduction stages is established by ^{15}N ENDOR measurements on samples prepared with ^{15}N-labeled substrates and ^1H measurements on samples in H_2O and D_2O buffers. The $^{15}N_2$ and $^{15}N_2H_4$ were obtained from Cambridge Isotope Laboratories; $CH_3-N=^{15}NH$ was prepared from ^{15}N-hydroxylamine as had been described.[20, 21]

Figure 1A shows ^{15}N Mims pulsed ENDOR[22] spectra collected at g_1 for $e(^{15}N_2)$, $m(^{15}NH=N-CH_3)$, and $l(^{15}N_2H_4)$. Each spectrum contains a single ^{15}N doublet that is centered at the ^{15}N Larmor frequency and is absent in spectra of samples prepared with ^{14}N-labeled substrates. This demonstrates that each has a substrate-derived species bound to the FeMo cofactor. Each signal has a different hyperfine splitting of its ^{15}N doublet, $A(g_1)$: 0.9 MHz, $e(^{15}N_2)$; 1.5 MHz, $m(^{15}NH=N-CH_3)$; 1.9 MHz, $l(^{15}N_2H_4)$.

The observation of distinct splittings is suggestive that the three states represent distinct stages of N_2 reduction. However, as each intermediate is formed in a different MoFe protein variant, one must consider whether environmental differences have induced differences in an otherwise equivalent common state that accumulates during turnover of all three substrates. The presence of at least two distinct reduction states is established by ^1H ENDOR measurements (Figure 1B).

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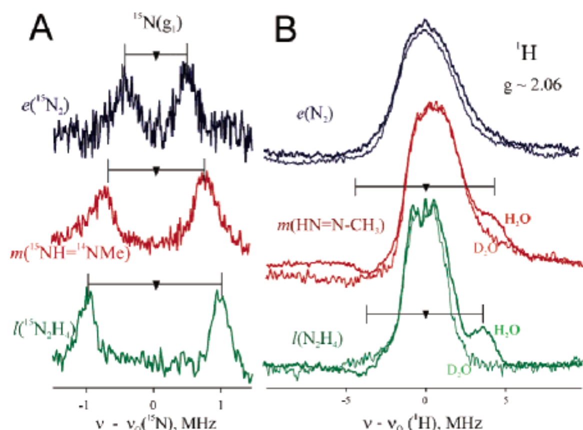


Figure 1. (A) Q-band Mims (e and m) and Re-Mims (l) ^{15}N ENDOR spectra collected at g_1. Conditions: microwave frequency = 34.808−34.819 GHz; B/2 = 52 ns (e, m) and 32 ns (l); RF = 20−30 μs; ϑ = 500 ns (e), 300 ns (m), and 200 ns (l); sampling = ~1000 transients/point; repetition rate = 100 Hz (e and m), 50 Hz (l); 2 K. (B) CW ^{1}H ENDOR. Conditions: microwave frequency = 35.057−35.171 GHz; modulation amplitude = 4 G; RF sweep speed = 1 MHz/s; bandwidth of RF broadened to 100 kHz; 2K.

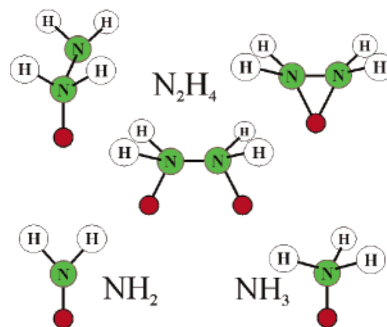
All three intermediates show an unresolved peak at the proton Larmor frequency from the nonexchangeable (unchanged in D_2O buffer) "matrix" protons of nearby residues. However, $l(N_2H_4)$ shows a signal from an exchangeable proton(s), $A(g_1)$ = 8 MHz, and $m(NH{=}N{-}CH_3)$ shows an analogous signal with a slightly larger coupling, $A(g_1) \sim 9$ MHz. These signals are similar to those shown by an alkene bound to FeMo cofactor during alkyne reduction,[11] and presumably arise from an $-NH_x$ moiety bound to the cofactor. In contrast, $e(N_2)$ shows no such exchangeable proton-(s). This absence clearly establishes that $e(N_2)$ is at a distinct and earlier stage of reduction (Scheme 1) than the $m(NH{=}N{-}CH_3)$ and $l(N_2H_4)$ intermediates. Whether or not the modest differences in ^{15}N and ^{1}H ENDOR responses by intermediates m and l arise because they too are at different stages of N_2 reduction must be determined by more detailed studies.

The g_1 spectra of Figure 1 represent a single orientation of the paramagnetic center relative to the magnetic field. As such, the number of ^{15}N doublets in a spectrum is the minimum number of *types* of ^{15}N in the nitrogenous moiety bound to the FeMo cofactor. Hence, the single ^{15}N doublet in each spectrum of Figure 1 is consistent with each substrate-derived species bound to the FeMo cofactor having a single *type* of ^{15}N. This is obvious for the singly labeled $^{15}NH{=}N{-}CH_3$; for the doubly labeled $e(^{15}N_2)$ and $l(^{15}N_2H_4)$, this possibility will be tested by ^{15,14}N and ^{1,2}H ENDOR/ESEEM studies analogous to those we have done with the alkyne reduction intermediates.[5,11]

To illustrate the utility of a limited number of ENDOR-derived constraints in establishing the structure of these metal-bound species, if $l(^{15}N_2H_4)$ is a complex of hydrazine or its reduction product, there are only a few candidates for the structure of the bound species (Chart 1), and if it in fact contains a single *type* of ^{15}N, several of these are eliminated.

We thus have trapped, for the first time, an intermediate formed during reduction of N_2 by nitrogenase, $e(N_2)$, and have trapped two additional intermediates, $m(NH{=}N{-}CH_3)$ and $l(N_2H_4)$, representing later stage(s) of N_2 reduction through the use of partially reduced states of N_2 as substrates. Determination of the structures of the nitrogenous species bound to the FeMo cofactor of the three turnover intermediates reported here, as we have done for other non-nitrogenous substrate intermediates,[5,11,23] coupled with an analysis of their respective "electron inventories", namely, the total number of electrons accumulated in the FeMo cofactor and on the substrate,[24] would represent a major step toward revealing the mysteries of N_2 fixation by nitrogenase.

Chart 1



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