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Electron Transfer in Nitrogenase Catalysis

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Abstract

Nitrogenase is a two-component enzyme that catalyzes the nucleotide-dependent reduction of N_2 to 2 NH_3. This process involves three redox-active metal-containing cofactors including a [4Fe-4S] cluster, an eight-iron P cluster and a seven-iron plus molybdenum FeMo-cofactor, the site of substrate reduction. A deficit-spending model for electron transfer has recently been proposed that incorporates protein conformational gating that favors uni-directional electron transfer among the metalloclusters for activation of the substrate binding site. Also reviewed is a proposal that each of the metal clusters cycles through only two redox states of the metal-sulfur core as the system accumulates the multiple electrons required for substrate binding and reduction. In particular, it was suggested that as FeMo-cofactor acquires the four electrons necessary for optimal binding of N_2, each successive pair of electrons is stored as an Fe-H^--Fe bridging hydride, with the FeMo-cofactor metal-ion core retaining its resting redox state. We here broaden the discussion of stable intermediates that might form when FeMo-cofactor receives an odd number of electrons.

Introduction

Nitrogenase is the complex, two-component metalloenzyme that catalyzes the nucleotide-dependent reduction of N_2 and protons [1–3]. The physiologically relevant reaction ($8H^+ + 8e^- + N_2 \rightarrow H_2 + 2NH_3$), as well as the multi-electron reduction of other artificial substrates, such as acetylene, requires both catalytic partner proteins and the hydrolysis of two MgATP for each electron delivered to the substrate. In the absence of N_2 or artificial substrates, the enzyme continues to catalyze ATP hydrolysis and proton reduction yielding H_2. Key questions related to nitrogenase catalysis are framed in Figures 1 and 2 and include: What is the nature of inter- and intra-molecular electron transfer events associated with the process?; How is nucleotide binding and hydrolysis linked to electron transfer?; Where and how are the multiple electrons necessary for substrate activation and reduction accumulated

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within the system?; and Where do substrates bind? In this brief review, we summarize recent advances in understanding some of the features of the nitrogenase catalytic mechanism.

Nitrogenase electron transfer reactions

During catalysis, the Fe protein [4Fe-4S] cluster cycles between 1+ and 2+ redox states in a dynamic process that involves the association and dissociation of the Fe protein and the MoFe protein and the obligate hydrolysis of two MgATP for each net electron transfer [3,4]. As will become relevant later in our discussion, at least three, and probably four, electrons must be accumulated within the MoFe protein before N_2 can bind to the active site FeMo-cofactor [5].

Previous studies established that delivery of an electron to nitrogenase during catalysis involves two types of electron transfer events (Figure 1): one such event is intermolecular electron transfer between the $[4Fe\text{-}4S]^{2+/1+}$ cluster of the Fe protein and the [8Fe-7S] "P-cluster" located within the MoFe protein; the other is intramolecular electron transfer between the P cluster and FeMo-cofactor that provides the substrate reduction site [5,6]. These studies did not, however, establish the order of events. Two plausible models, designated here as "sequential" and "deficit-spending", can be used to describe the net electron delivery process that occurs during nitrogenase catalysis [7]. A sequential model invokes initial electron transfer from the Fe protein's [4Fe-4S] cluster in the 1+ oxidation state to the P cluster, followed by an electron transfer from the reduced P cluster to the FeMo-cofactor. Although intuitively satisfying, the sequential model is undermined by the finding that all of the Fe atoms in the resting state of the P cluster, designated P^N, are in the ferrous oxidation state [8,9]. Thus, operation of the sequential model would demand that the P cluster have the capacity to transiently exist in a super-reduced oxidation state that appears chemically untenable and for which there is no precedent in known biological FeS clusters [10–13]. In a deficit-spending model, interaction of the Fe protein and the MoFe protein elicits an initial electron transfer event involving intramolecular delivery of an electron from the P cluster to the FeMo-cofactor [7]. This situation would result in the P cluster having a "deficit" of one electron, designated P^{1+}, relative to the all-ferrous P^N resting state. The deficit could then be erased in a second step involving reduction of P^{1+} to yield P^N through intermolecular delivery of an electron from the Fe protein's $[4Fe\text{-}4S]^{1+}$ cluster.

There is evidence that the P^{1+} state is chemically accessible and could be mechanistically relevant because it can be readily generated by treating the P^N form of the resting state MoFe protein with oxidizing reagents [8]. The oxidation of the P^N cluster to the P^{1+} state has been observed to be accompanied by a ligation change of the cluster, with the P^{1+} state having a Ser-O^- group ligated to a Fe atom [14].

The overall rate of electron delivery within the normal nitrogenase system (rate constant k ~ 100 s^{-1}) can be measured with stopped-flow spectrometry by monitoring the change in optical absorption associated with oxidation of the Fe protein's [4Fe-4S] cluster [15–17]. Until recently, however, differentiation between the sequential and deficit-spending models could not be achieved because it has not been possible to resolve the inter- and intra-molecular electron transfer events. This technical challenge was overcome when it was fortuitously discovered that a MoFe protein having an amino acid substitution for a residue that provides a reversible P cluster ligand (α-$188^{Ser\rightarrow Cys}$) is catalytically competent and is significantly populated in the P^{1+} state [18]. Stopped-flow analysis using this substituted MoFe protein revealed that electron transfer is resolved into two phases [7]. These phases include a fast step (k > 1700 s^{-1}), which can be directly correlated with reduction of P^{1+} to yield P^N (the second electron transfer step shown in Figure 1), and a slow step (the first electron transfer step shown in Figure 1) whose rate constant (k = 168 s^{-1}) is the same as the overall rate constant observed in the normal system. The slow electron transfer phase is

reasonably assigned to electron transfer between the P^N cluster and the FeMo-cofactor because this step is eliminated, without affecting the capacity for P^{1+} reduction when a form of the substituted MoFe protein that does not contain the FeMo-cofactor is used in stopped-flow experiments. These observations are consistent with the deficit-spending model in which the P cluster cycles only through two redox states, P^N and P^{1+}, during catalysis.

Other stopped-flow experiments have revealed that the fast electron transfer event associated with the reduction of P^{1+} is unaffected by osmotic pressure [19]. In contrast, the overall electron transfer process in wild-type MoFe protein and the slow electron transfer step in the variant, now suggested to be associated with intramolecular electron transfer between the P cluster and the FeMo-cofactor, are responsive to changes in the solution osmotic pressure [7,19]. These data indicate that large protein conformational changes (> 800 $Å^2$) precede and gate this rate-limiting electron transfer reaction. To ensure unidirectional electron flow, such conformational changes are likely to be ephemeral, which could explain why no insight along these lines has yet been gained by examination of the crystal structures of Fe protein-MoFe protein complexes [20]. Nonetheless, recent studies do hint at a possible role for amino acids within the MoFe protein as participants in gating conformational changes associated with electron transfer events. For example, substitution of histidine for the tyrosine residue located between the P cluster and FeMo-cofactor at the α-98 position within the MoFe protein results in a MoFe protein that can reduce hydrazine, a nitrogenase substrate and possible mimic of an N_2 reduction intermediate, in the absence of the Fe protein [21]. Such reduction only occurs if a powerful small molecule electron donor, such as ligated Eu(II), is used as a substitute for the Fe protein. In this case, it could be that the single amino acid change induces a conformation of the MoFe protein, usually achieved only transiently during docking with the Fe protein that permits substrate reduction. The relative roles of ATP binding and hydrolysis in these processes remains unclear. The recent work is consistent with the need for ATP binding to allow the electron transfer events, but it appears that ATP hydrolysis is not necessary for electron transfer, but rather may be involved in the steps that lead to the dissociation of the Fe protein from the MoFe protein [7]. Clearly, more work is needed to define the roles of ATP and how the energy of hydrolysis is captured in the mechanism. The features of the α-98 substituted MoFe protein provide a starting point for future studies that will be aimed at better defining how the Fe protein, ATP binding, and hydrolysis specifically work to control nitrogenase catalysis.

FeMo-cofactor and substrate reduction

The resting state of the FeMo-cofactor is paramagnetic and exhibits a characteristic EPR signature making it amenable to characterization by a host of advanced spectroscopic techniques [2,22]. In principle, it should also be possible to characterize paramagnetic intermediates formed as the system advances through the catalytic cycle. However, such analyses have historically been frustrated by three technical problems. First, no substrates are known to bind to the resting state of the MoFe protein [3]. Instead, the active site must be activated for substrate reduction by the accumulation of multiple electrons [5]. Second, because nitrogenase has the capacity to reduce protons, the availability of which cannot be controlled, the system is continuously returned to the resting state. Third, because multiple electrons are required for the reduction of any substrate, and electron delivery cannot be synchronized, there are multiple redox states that exist under turnover conditions. These problems have been overcome, at least in part, by using a combination of approaches that involve freeze-quenching the system under turnover conditions [23], the discovery of conditions, or altered forms of the MoFe protein having amino acid substitutions that favor accumulation of intermediate states, and the development of a step-annealing procedure for determining the number of electrons accumulated by a trapped intermediate [24]. The application of these approaches has resulted in the identification of intermediate states

involved in the reduction of protons [23], alkynes [25], hydrazine and diazene [26], and N_2 [27], as well as interaction of the inhibitor, CO [28], with the FeMo-cofactor under turnover conditions. These studies have recently been reviewed [2].

As already mentioned, multiple electrons must accumulate within the MoFe protein before it has the capacity to bind any substrate. For example, careful kinetic studies have revealed that no fewer than three and probably four electrons must be accumulated within the system before N_2 is able to bind and become activated for reduction, whereas only two electrons are required for the activation of alkyne reduction [5]. The ability of different substrates to bind the active site at different redox states explains the unusual non-reciprocity in the mutual inhibition patterns for the reduction of different substrates by nitrogenase [29]. Because electrons are delivered one at a time from the Fe protein to the MoFe protein, Thorneley and Lowe developed a convenient shorthand for describing the successive states formed under turnover conditions [5]. In the Thorneley-Lowe scheme, the resting state of the system is designated as E_0, and the most stable intermediate states resulting from the sequential entry of electrons via nucleotide-dependent Fe protein delivery are denoted E_1, E_2, E_3, and so forth.

In the first section of this review, we described evidence for a deficit-spending model where each single electron transfer results in the net delivery of an electron to the FeMo-cofactor, while leaving the P cluster in the all-ferrous P^N state. Such a transient, gated process provides an elegant way to ensure unidirectional flow of electrons for substrate binding and reduction. However, given that the system must achieve the E_3 or E_4 state before N_2 binding can occur, important questions about the nitrogenase mechanism include where and how those electrons accumulate. Figure 2 presents alternative states of the FeMo-cofactor, represented in the figure as “M”, that might exist as the system accumulates the protons and electrons necessary to achieve the E_4 state. As can be seen, the metal core of the FeMo-cofactor in the E_n states formed during activation to E_4 might be reduced or oxidized by up to four electron equivalents, depending on whether the accumulated electrons reside on the metal ions, on bound H_2, or on bound hydrides. Like the E_0 resting state, the E_2 and E_4 states are also paramagnetic and, therefore, tractable to analysis by advanced electron spin techniques such as ^{1,2}H and metal-ion (^{57}Fe, ^{95}Mo) ENDOR spectroscopy. This feature turned out to be of paramount significance in the analysis of the system.

During the course of the analysis of MoFe proteins having amino acid substitutions that make the active site more accessible [30,31], such that larger alkyne substrates can be reduced, a substituted form of the MoFe protein (α-$70^{Val\rightarrow Ile}$) was isolated that denies access of all substrates, except protons, to the active site [23]. When samples of this substituted MoFe protein were freeze-quenched under turnover conditions, they were found to exist in a paramagnetic state indicating that they had accumulated an even number of electrons relative to the E_0 state and, thus, most likely represented the E_2 or E_4 state. Subsequent temperature annealing experiments established that two H_2 atoms could be successively released from the trapped state, clearly indicating that the trapped form represents the E_4 state having accumulated 4 electrons and 4 protons [24]. Proton [23] and ^{95}Mo [32] ENDOR spectroscopic analysis of this trapped state revealed the presence of two metal-bound hydrides, most likely Fe-H-Fe fragments [32]. In aggregate, these findings permitted an electron inventory analysis [33] that surprisingly revealed the formal redox level of the metal-sulfur core of the FeMo-cofactor in the E_4 state to be the same as that of the resting E_0 state [34]. Namely, the four accumulated electrons reside not on the metal ions but, instead, must be located on the two Fe-bound hydrides. These studies, together with similar analyses of the system having a trapped alkyne reduction intermediate [33] or having the CO inhibitor bound to the metal-sulfur core [33] lead to the remarkable conclusion that throughout the nitrogenase catalytic cycle the FeMo-cofactor might cycle through only a

single redox couple connecting two formal redox levels of the metal-ion core [34]: one corresponding to the resting state, designated M^0 in Figure 2, and the other to the one-electron reduced state of the metal-sulfur core, designated M^-.

The addition of one electron/proton to the MoFe protein results in the E_1 state experimentally observed in Mossbauer experiments [9]. It was presumed that this state contains the reduced metal-ion core of FeMo-co, denoted M^- in Fig 2, with the proton bound to sulfur. Upon delivery of the second electron/proton to form E_2, we proposed that the metal-sulfur core of the FeMo-cofactor shuttles both electrons to one proton to form an Fe-H-Fe hydride, leaving the second proton bound to sulfur and the core at the resting-state, M^0, redox level. A subsequent, analogous two-stage process would then yield the E_4 state, which has two Fe-H-Fe hydrides, two sulfur-bound protons, and the core at the resting-state, M^0, redox level. The E_4 state is optimized for N_2 binding, during which one of the Fe-H-Fe fragments plus a bound proton combine for the obligate evolution of one molecule of H_2 as required by the Thorneley-Lowe kinetic model. Activation thus occurs without formation of a highly reduced metal-ion core.

An alternative description of the E_1 and E_3 states can also be considered. If hydride formation is energetically dominant, then upon addition of an electron/proton to the metal-ion core of the resting state (redox level, E_0), the resulting reduced core M^- might occur only as a transient that converts to a stable E_1 intermediate that contains a bridging hydride bound to an *oxidized* metal-ion core, M^+. Addition of the second electron/proton would then yield E_2 with a hydride bound to the M^0 core plus a sulfur-bound proton, as described above. Analogously, two subsequent stages of electron/proton addition would generate E_4 with its two bridging hydrides, two protons bound to sulfur, and M^0 core, poised for N_2 binding.

Regardless of which redox couple, M^0/M^+ or M^0/M^-, is operative in the stable intermediates throughout the catalytic cycle, or whether FeMo-co utilizes a different couple at different stages (e.g., activation to form E_4 vs hydrogenation of N_2), the proposal that the eight metal ions of the FeMo-cofactor core access no more than two, and likely just one, redox couple, when forming stable intermediates during the eight-electron catalytic cycle might seem counterintuitive. However, other proteins routinely utilize only a single redox couple of those available to their [Fe-S] clusters, including the Fe protein [4Fe-4S] cluster and the eight-iron P cluster [35]. If the FeMo-cofactor does not utilize multiple redox couples during catalysis, then why is it constructed from so many metal ions? Our results suggest that hydride binding and substrate reduction require at least two adjacent Fe ions, and it is further likely that catalysis is modulated by the linkage of these Fe ions to a hemilabile anionic atom C that is centrally located within the metal-sulfur core of the FeMo-cofactor [36,37]. Formation of such a face and the incorporation of C could not occur with a smaller cluster, as no less than a trigonal prism of six Fe ions is needed to generate these structural features. In the FeMo-cofactor, the trigonal prismatic core of six Fe ions plus C is capped, and likely ‘tuned’, by two “anchor” ions--one Fe plus a Mo, or a V or Fe in the alternative nitrogenases.

Another noteworthy advance in the field is the discovery that both the Mo-dependent and V-dependent nitrogenases have the ability to reduce CO, albeit at extremely low levels, to yield CH_3 and short-chain alkenes [38,39]. While an extremely poor substrate, CO is a very powerful inhibitor of the reduction of all nitrogenase substrates, except for proton reduction. Spectroscopic analyses have revealed there are two distinct CO binding sites within nitrogenase with each having a characteristic EPR signature [40]. It seems reasonable to expect that further analysis of the binding of CO by using the advanced spectroscopic approaches already described, together with the manipulation of the enzyme’s capacity to

catalyze short-chain alkene formation, could provide considerable insight concerning exactly where and how substrates and inhibitors interact with the nitrogenase active site. Also of relevance is the intriguing possibility for using the nitrogenase catalytic mechanism to guide the design of inorganic catalysts for hydrocarbon formation using CO feedstocks.

Summary

This commentary summarizes recent studies and proposals that explain how the nitrogenase active site becomes primed for substrate binding. These advances, together with an emergent model for the reaction pathway that describes the reduction intermediates bound to the FeMo-cofactor during the catalytic process [26], now provide the basis for fresh experimental approaches aimed at fully describing the nitrogenase catalytic mechanism. A noteworthy aspect of the recent work summarized here is that the proposed models are consistent with, and grounded by, decades of kinetic, structural, and spectroscopic studies by many investigators.

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Highlights

- The order of electron transfers between nitrogenase metal clusters is described.
- The core of each nitrogenase metal cluster cycles through a single redox couple.
- Hydrides bridging Fe ions of FeMo-cofactor ‘store’ reducing equivalents.

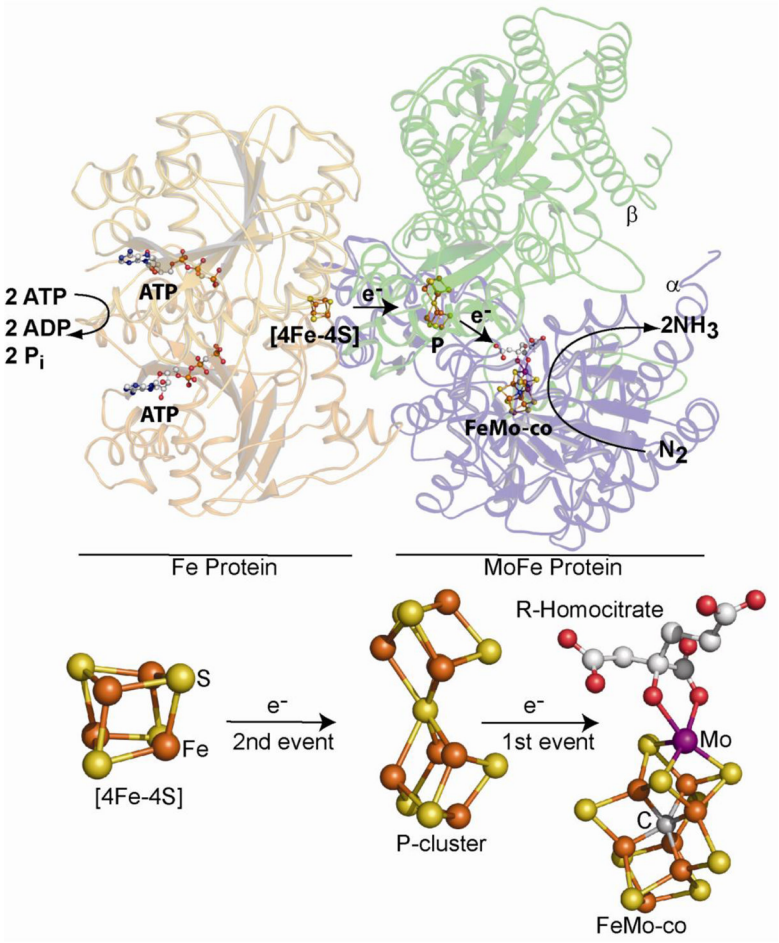

Figure 1.

Nitrogenase proteins and cofactors. Shown are the Fe protein (left) and an αß-unit of the MoFe protein (right) along with two ATP molecules, the [4Fe-4S] cluster of the Fe protein, and the P cluster and the FeMo-cofactor of the MoFe protein. Atom colors are Fe in rust, S in yellow, C in gray, O in red, and Mo in purple. This figure was made using the computer program PyMol from the PDB file 2AFK.

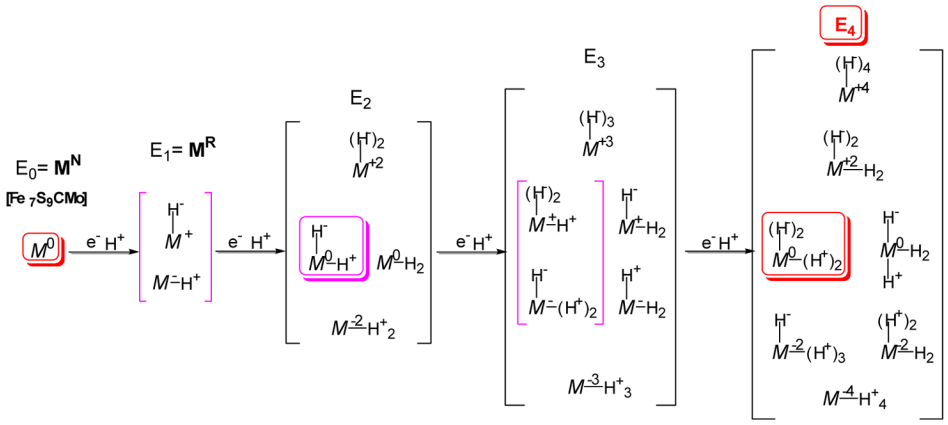

Figure 2.

The possible redox states available to the metal-ion core of the FeMo-cofactor at each stable intermediate of the Lowe-Thorneley scheme during activation by sequential addition to the MoFe protein of up to four [e^-/H^+]. The symbol M^k represents the FeMo-cofactor metal core with the superscript indicating the oxidation state of the metal core relative to that of resting-state FeMo-cofactor. Red symbols represent states that have been characterized by ENDOR spectroscopy; magenta boxes represent the proposed core alternatives in stable Lowe-Thorneley intermediates.