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## Control of electron transfer in nitrogenase

Lance C Seefeldt<sup>1</sup>, John W Peters<sup>2</sup>, David N Beratan<sup>3</sup>,  
Brian Bothner<sup>4</sup>, Shelley D Minter<sup>5</sup>, Simone Raugel<sup>6</sup> and  
Brian M Hoffman<sup>7</sup>

The bacterial enzyme nitrogenase achieves the reduction of dinitrogen ( $N_2$ ) to ammonia ( $NH_3$ ) utilizing electrons, protons, and energy from the hydrolysis of ATP. Building on earlier foundational knowledge, recent studies provide molecular-level details on how the energy of ATP hydrolysis is utilized, the sequencing of multiple electron transfer events, and the nature of energy transduction across this large protein complex. Here, we review the state of knowledge about energy transduction in nitrogenase.

### Addresses

<sup>1</sup> Department of Chemistry and Biochemistry, Utah State University, 0300 Old Main Hill, Logan, UT 84322, USA

<sup>2</sup> Institute of Biological Chemistry, Washington State University, 287 Clark Hall, Pullman, WA 99164, USA

<sup>3</sup> Departments of Chemistry, Physics, and Biochemistry, Duke University, Durham, NC 27708 and 27710, USA

<sup>4</sup> Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, USA

<sup>5</sup> Departments of Chemistry and Materials Science & Engineering, University of Utah, 315 S 1400 E, Salt Lake City, UT 84112, USA

<sup>6</sup> Physical and Computational Sciences, Pacific Northwestern National Laboratory, 602 Battelle Blvd, Richland, WA 99352, USA

<sup>7</sup> Department of Chemistry, Northwestern University, Evanston, IL 60208, USA

Corresponding author: Seefeldt, Lance C ([lance.seefeldt@usu.edu](mailto:lance.seefeldt@usu.edu))

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### Introduction

The bacterial enzyme nitrogenase, which is known to occur in Mo, V, and Fe forms [1–5], reduces dinitrogen ( $N_2$ ) to ammonia ( $NH_3$ ) utilizing protons, electrons, and the hydrolysis of ATP. Since the discovery of these enzymes, there has been great interest in understanding their mechanism in order to gain insights into how nature solved the problem of reducing one of the most stable molecules,  $N_2$ , at ambient temperatures and pressure. Recent progress provides significant new insights into the

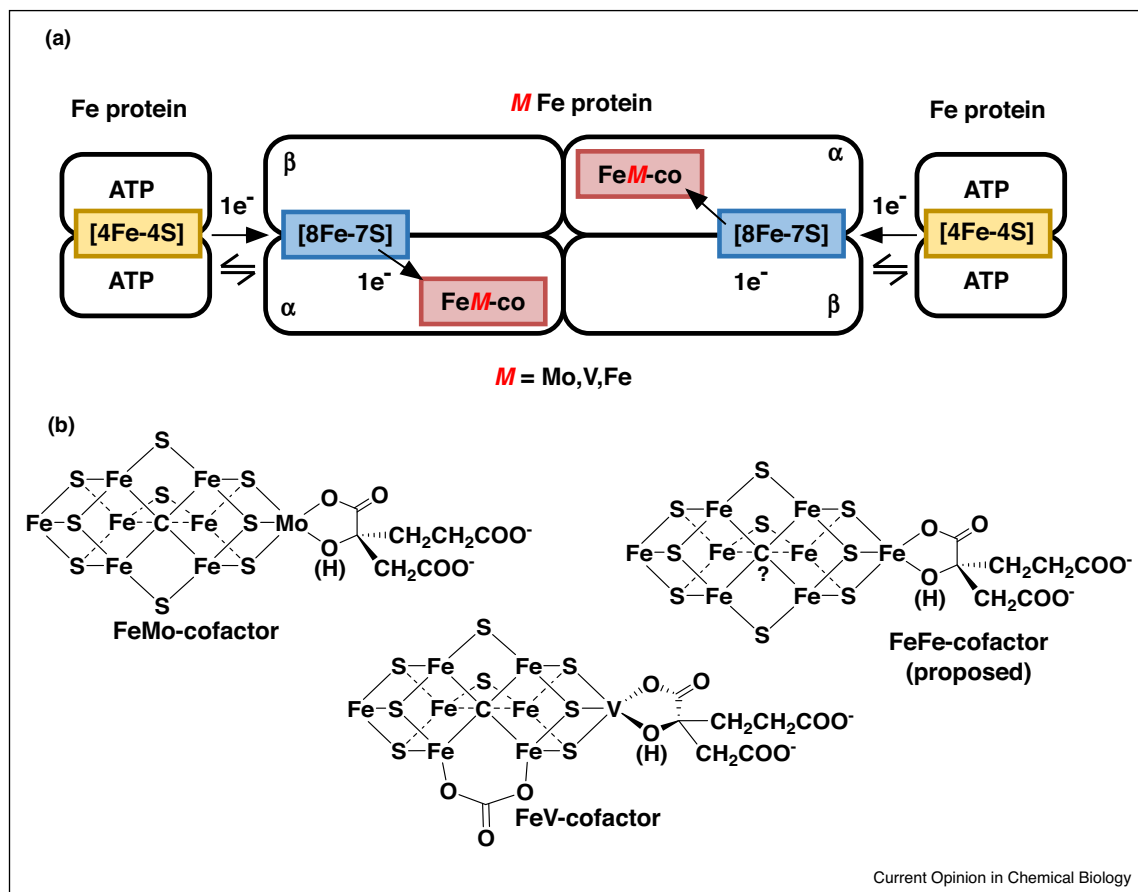
$N_2$  binding event and how the reduction reaction is achieved [6,7–10]. Here, we review new findings that are unravelling the complexities of how electrons traverse the nitrogenase two-component system to achieve the reduction of bound  $N_2$  and how energy is transduced from the hydrolysis of nucleotides in the process.

Nitrogenases comprised two proteins, one being a reductase component known as the Fe protein, that contains a single 4Fe-4S cluster and two ATP binding sites. A second catalytic protein, known as the MoFe-protein, VFe-protein or FeFe-protein, houses an electron transfer P cluster as well as the active-site metal cofactor (FeMo-co, FeV-co, and FeFe-co) (Figure 1). The Mo-nitrogenase system is the focus of this review, and insights into this system are likely to be applicable to the other systems. We know that an Fe protein, with two bound ATP molecules, associates with one-half of the MoFe protein, and that this association initiates a series of events that result in the transfer of an electron from the Fe protein to FeMo-co, the hydrolysis of two ATP molecules to two ADP and two  $P_i$ , release of phosphate, and finally the dissociation of the Fe protein (oxidized and with two bound ADP) from the MoFe protein (reduced by one electron) [1,11]. This cycle of events is repeated in order to accumulate electrons on the active site, with four cycles needed to achieve the state that binds  $N_2$  and eight cycles to achieve the complete reduction of  $N_2$  and release of one  $H_2$ . The order and nature of the events that occur during this transient association of the two component proteins, often referred to as the Fe protein cycle, is one of the great mysteries of nitrogenase function. Studies during the last several years have added to the considerable body of knowledge about the Fe protein cycle [1], filling in and refining our understanding of nitrogenase function.

### ATP hydrolysis and electron transfer

Recent studies have contributed four significant insights into the key events that occur in the Fe protein cycle, including that: firstly electron transfer occurs ahead of ATP hydrolysis [12<sup>\*\*</sup>]; secondly the overall rate limiting step is not component protein dissociation, but rather  $P_i$  release [13<sup>\*\*</sup>]; thirdly the electron transfer events are staged to occur in the order, conformationally gated P cluster  $\rightarrow$  FeMo-co first, followed by rapid Fe protein  $\rightarrow$   $P^{ox}$  (together referred to as ‘deficit-spending’ electron transfer) [14]; and finally the two halves of nitrogenase

Figure 1



Nitrogenases. Schematic diagram for the three forms of nitrogenase, where *M* is Mo, V, or Fe (a). Shown are the structures for the active site FeMo-cofactor [33] and FeV-cofactor [34] determined by X-ray crystallography and a proposed structure for the FeFe-cofactor (b). It is noteworthy that some recent work is indicating that changes in the structure of FeMo-co and FeV-co can occur upon addition of electrons and/or upon binding of substrates and inhibitors, suggesting structural dynamics of the cofactor is part of the mechanism.

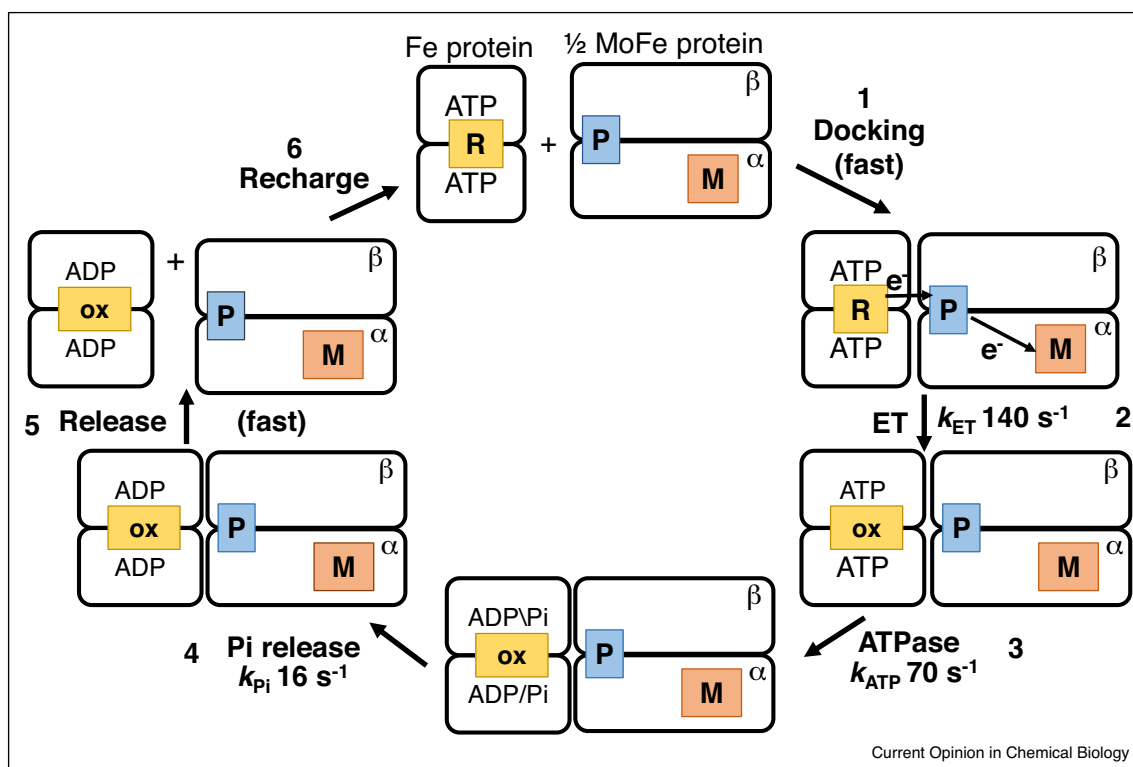
MoFe protein function through negative cooperativity, with each side influencing activity on the opposite side [15<sup>••</sup>].

The earliest studies of nitrogenase revealed that the hydrolysis of 2 ATP to 2 ADP + 2 Pi was coupled to the transfer of one electron from the Fe protein into the MoFe protein [16–18]. This prompted the widely accepted model wherein the energy from hydrolysis of ATP was being used to drive electron transfer in nitrogenase [1]. Studies over the last few years have upended this model by showing that ATP hydrolysis occurs after the electron transfer events [12<sup>••</sup>]. Indeed, an early theoretical study by Beratan *et al.* [19] concluded that ATP hydrolysis energy should come at the end of the cycle, to drive the proteins apart. That model is consistent with the new experimental studies placing electron transfer ahead of ATP hydrolysis. The key steps of the new model are summarized in Figure 2. It shows that ATP binding to the Fe protein is essential to activating Fe protein for

binding to the MoFe protein, while the energy of hydrolysis of ATP is used to weaken the interaction between the Fe protein and the MoFe protein for dissociation at the end of the cycle, with Pi release being the rate limiting step [13<sup>••</sup>].

On the basis of continuum electrostatics analysis, it was earlier proposed that ATP binding to the Fe protein energized the 4Fe-4S cluster for electron transfer by desolvation of the 4Fe-4S cluster caused by docking of the Fe protein with the MoFe protein, and that the protein-protein complex is made more stable by about the same amount [19]. ATP binding to the Fe protein also drives the 4Fe-4S cluster several Angstroms closer to the protein surface [20<sup>•</sup>, 21–23] and strengthens its electronic coupling to the P cluster of the MoFe protein. It is possible that the docking of the Fe protein to the MoFe protein induces a conformational change within the MoFe protein that opens the gate for electron transfer from the P cluster to FeMo-co. The docking may further

Figure 2



Scheme for the key events during electron transfer between the nitrogenase Fe protein and the MoFe protein. The MoFe protein is shown as one  $\alpha\beta$  pair (right side) containing a P cluster (P) and FeMo-co (M). The Fe protein dimer (left side), with one [4Fe-4S] cluster (R or ox) and two bound ATP, associates with the MoFe protein (top). This association initiates a gated electron transfer event from the P cluster to FeMo-co with a rate constant ( $k_{ET}$ ) of  $140\text{ s}^{-1}$  at  $25^\circ\text{C}$ , with rapid follow up electron transfer from the Fe protein to the oxidized P cluster. The next event is ATP hydrolysis with a rate constant  $k_{ATP}$  of  $70\text{ s}^{-1}$ , followed by Pi release with a rate constant  $k_{Pi}$  of  $16\text{ s}^{-1}$ , and then the fast dissociation ( $k_{dis}$ ) of the two proteins.

accelerate electron tunneling from the Fe protein to the MoFe protein beyond simple distance shortening. Alternatively, the gating may also be accomplished by a Marcus free energy effect created by changing solvent exposure or cofactor structure (or both effects may be at play). Given the osmotic pressure dependence of the gating process, it is likely that water molecules at this dynamical interface play a role in electron flow between the cofactors (as well as through their influence on coupling or redox potentials).

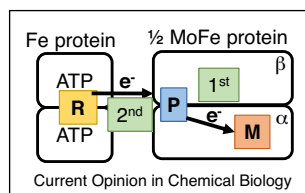
### Deficit-spending ET

One of the most intriguing aspects of electron transfer within nitrogenase is the order of the two electron transfer events involving the FeS cluster of the Fe protein, the P cluster, and FeMo-co. Recent work showed that following the association of the Fe protein with the MoFe protein, one electron is transferred from the Fe protein to the MoFe protein in a process that is conformationally gated [24]. From structures of nitrogenase complexes of the two component proteins [25,26], it is clear that the P cluster in the MoFe protein is involved as an intermediate in the

transfer of the electron to the FeMo-cofactor [27,28]. Early models assumed that the Fe protein would first transfer an electron to the P cluster [1]. A problem with this model is that the resting P cluster (designated as  $P^N$ ) has all Fe atoms in the ferrous oxidation state [29]. Since there are no known examples of reduction of enzyme Fe-S clusters below the ferrous oxidation state, instead, gated P cluster to FeMo-co electron transfer occurs first, followed by rapid reduction of the oxidized P cluster by the Fe protein. The ‘deficit-spending’ electron transfer model was given support by studies on MoFe proteins using amino acid substitutions that were consistent with such a model (Figure 3) [14].

While a reasonable model, the details of how this ‘deficit-spending’ process would be achieved remain unknown. To a first approximation, the ‘deficit-spending’ electron transfer model would require that Fe protein docking to the MoFe protein activates electron transfer from the P cluster to the FeMo-cofactor. Given that the Fe protein docking interface is far from the P cluster [25,26], the activation of electron transfer from the P cluster initiated

Figure 3



Deficit spending ET in nitrogenase. Shown is the Fe protein (left) and  $\frac{1}{2}$  of the MoFe protein (right) with the metal clusters R for the 4Fe-4S cluster, P for the 8Fe-7S cluster, and M for FeMo-cofactor. The first ET event is proposed to be from the P cluster to the M cluster, while the second electron transfer event is from the Fe protein to the oxidized P cluster.

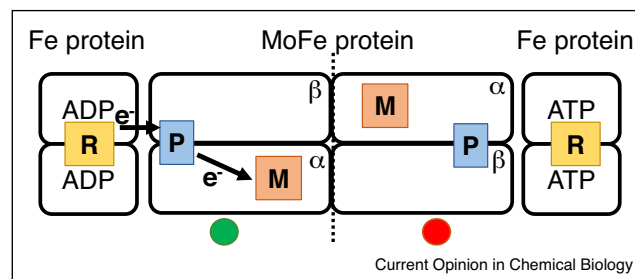
at the protein-protein interface, must be achieved through protein conformational changes that are propagated deep in the MoFe protein. Such changes could activate electron transfer by changing parameters to favor the electron transfer event. Parameters that seem most likely to be changed would be the driving force for the electron transfer (the difference in the midpoint reduction potential  $E^{\circ}$  for the donor and acceptor) or the coupling between the donor and acceptor [30,31]. A model of this kind can be refined by computation to examine possible conformational changes that could be used as 'levers' to communicate from the Fe protein-MoFe protein docking interface to control the electron transfer events at the P cluster.

Examination of the environment of the P cluster suggests models to explain the transduction of mechanical energy from protein-protein docking to electrochemical energy to drive electron transfer, through changes in the ligation or environments of the P cluster [32] or FeMo-co.

### Half-sites reactivity in nitrogenase

The complexity of the overall energy transduction landscape in nitrogenase is enhanced by a recent unexpected finding showing negative cooperativity between the two symmetric halves of nitrogenase [15<sup>••</sup>]. Quench-flow studies find during pre-steady state, that the entire nitrogenase complex only shows the hydrolysis of 2 ATP (not 4) and the transfer of one electron (not two) in the initial time after mixing (Figure 4), contrary to the expectation if the two halves of the complex functioned independently. Even though a fully charged Fe protein is already bound on the opposite end of the MoFe protein, only the first side proceeds through the ET and ATP hydrolysis cycle [15<sup>••</sup>]. The second side is partially 'held up' by 'negative cooperative' control across the entire complex. The effective outcome of this control is that only after the first side has completed the Fe protein cycle is the second side partially released to proceed through a full Fe protein cycle. It is not clear if this back and forth mechanism

Figure 4



Negative cooperativity in nitrogenase. The left half of the complex is shown after ET, ATP hydrolysis, and Pi release. The right side is held up with the Fe protein bound, but with ET and ATP hydrolysis suppressed through negative cooperativity.

continues for many cycles or if the system proceeds stochastically at longer times. Coarse grained modeling, based on a Gaussian network, reveal connection between the Fe protein on one end, through the MoFe protein, to the Fe protein on the other end [15<sup>••</sup>]. Thus, the beginnings of a mechanism for coupling of events on the two opposite ends of the nitrogenase complex can be envisioned, although why such communication occurs remains unknown. It seems reasonable that the communication across the complex is part of the energy transduction in the system. Resolving the nature and potential advantages of this cross-protein communication will be an important topic for future research, as it may reveal how nitrogenase manipulates free-energy to control electron transfer and to accomplish a challenging multi-electron substrate reduction.

### Conclusions and prospects

Understanding how nitrogenase couples the energy from ATP binding and hydrolysis to catalyze electron transfer and substrate binding/reduction continues to be a significant challenge. Building on decades of foundational work [1,11], recent studies have created the framework for an understanding of the Fe protein cycle. It is now clear that the energy from ATP hydrolysis is used at the end of the cycle [12<sup>••</sup>], likely to drive the dissociation of the Fe protein from the MoFe protein. Further, it is clear that the events associated with Pi release after ATP hydrolysis are rate limiting for the entire cycle [13<sup>••</sup>]. Recent studies also support a deficit-spending electron transfer model, wherein the binding of the Fe protein to the MoFe protein stimulates gated electron transfer from the P cluster to FeMo-co, with follow-up electron transfer from the Fe protein to the oxidized P cluster [14]. Significant work remains to develop a molecular-level understanding of how Fe protein binding activates the electron transfer reactions and how ATP hydrolysis and Pi release destabilize the complex allowing spent Fe protein to dissociate. Finally, the observation of negative cooperativity associated with conformational coupling across the

nitrogenase complex [15\*\*] opens an important new frontier for furthering our understanding of energy transduction in nitrogenase.

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