Review

Israel Journal of Chemistry

DOI: 10.1002/ijch.201600020

Electron Transfer Reactions in Biological Nitrogen Fixation

Faith E. H. Katz, Cedric P. Owens, and F. A. Tezcan*[a]

Abstract: In this review, we summarize our recent efforts toward understanding electron transfer (ET) processes in nitrogenase, the only enzyme capable of reducing dinitrogen to ammonia. We discuss new structural and biochemical perspectives on the role of ATP-dependent interactions between the two components of nitrogenase, Fe-protein (FeP) and MoFe-protein (MoFeP), and how these interactions may regulate interprotein ET and catalysis. We also discuss the implications of our work on FeP- and ATP-independent, photoredox-activated substrate reduction by MoFeP. Elucidating why and how ATP-hydrolysis is needed to control electron and proton flow in nitrogenase is not only a fundamentally important question in biological redox chemistry and energy transduction, but it also holds the key to understanding the intimate mechanism of dinitrogen reduction.

Keywords: ATP hydrolysis · electron transfer · encounter complex · metalloproteins · nitrogenases

1. Introduction

Nitrogenase is an extraordinary biological machine. It is the only known enzyme to catalyze the reduction of dinitrogen (N_2) to ammonia (NH_3) (Eq. (1)),[1,2] a reaction that rivals the light-driven water oxidation reaction by Photosystem II (PSII), both in biological importance and mechanistic complexity.[3]

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

There are three known nitrogenases, Mo-, V-, and Fe-only nitrogenase, each named after the contents of their catalytic cofactors. The catalytic component of the most studied nitrogenase, Mo-nitrogenase, is the molybdenum-iron protein (MoFeP). It houses two unique metal clusters, the P-cluster [8Fe:7S] and the FeMoco [1Mo:7Fe:9S:1C], that enable N_2 reduction (Figure 1a).[4,5] Its reductase component, the iron protein (FeP), possesses a canonical [4Fe:4S] cluster for electron (e^-) delivery. FeP is the exclusive e^- donor to the catalytic MoFeP and receives its e^- in *Azotobacter vinelandii* (*Av*) from a flavodoxin, most likely Fld II.[6] With a mass of 60 kDa, FeP is a behemoth, compared with typical electron transfer (ET) proteins like cytochromes, ferredoxins, and flavodoxins (Figure 1b). And very distinctly from these e^- shuttles, FeP couples its reduction of MoFeP to ATP binding and hydrolysis.

The multidimensional complexity and the distinctive features of nitrogenase have engendered many mechanistic questions and inspired and challenged generations of researchers. In an opinion article from 2000, titled *Nitrogenase: standing at the crossroads*, Rees and Howard[7] jokingly asked whether a Faustian bargain might be required to establish the mechanism of this enzyme. Fortunately, the last fifteen years have brought astonishing advances in nitrogenase research without resorting to such a bargain. Through advanced X-ray diffraction and ab-

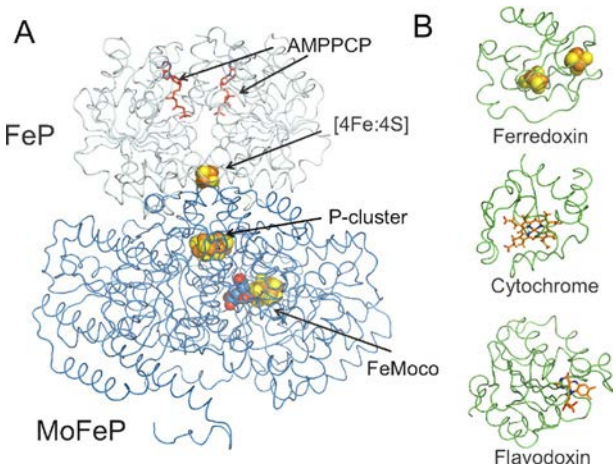

Figure 1. A) Crystal structure of the FeP-MoFeP complex obtained in the presence of AMPPCP (PDB: 4WZB). Only one half of the $(\gamma_2)_2:(\alpha_2\beta_2)$ complex is shown for clarity. B) Structures of e^- shuttle proteins *Av*-ferredoxin (PDB: 1FDA), *Ateles sp.* cytochrome *c* (PDB: 5DFS), and *Av*-flavodoxin II (PDB: 1YOB), which are shown at the same scale as the nitrogenase complex to illustrate the difference in size between them and FeP.

[a] F. E. H. Katz, C. P. Owens, F. A. Tezcan
Department of Chemistry and Biochemistry
University of California, San Diego
9500 Gilman Drive
San Diego CA 92093 (USA)
Tel.: (+1) 858-534-9605
e-mail: tezcan@ucsd.edu

sorption experiments, we now know FeMoco is actually "more unique" than we thought. It contains a carbon atom in its center that stabilizes its core structure,[8–11] while three of its bridging sulfides (the so-called belt sulfides) appear to be labile during enzymatic turnover, likely a critical feature for substrate binding and activation.[12] Through extensive biochemical and spectroscopic studies, it has been possible to characterize the interactions of substrates and intermediates with FeMoco,[13–20] to identify the particular face of FeMoco involved in catalysis,[21] and to put forth informed hypotheses about the mechanism of N_2 reduction.[22–25] Furthermore, we have learned about the remarkable biosynthesis of FeMoco in great detail: how FeMoco is assembled on different scaffolding proteins with the intermediacy of radical SAM chemistry, culminating in the insertion of the mature cluster into apo-MoFeP, aided by none other than FeP.[26–30] Finally, in the same 15-year span, synthetic chemists have created the first examples of both Fe-[31] and Mo-based[32,33] molecular catalysts that can reduce N_2 to NH_3. While the catalytic output of these catalysts is modest, compared with nitrogenase, and they require potent e^- and proton (H^+) donors, they have at last provided model platforms with which to test mechanistic hypotheses about nitrogen fixation by homogeneous, small molecule systems.

It is clear that the nitrogenase field has progressed immensely on all fronts since 2000. Yet, it still faces some of the same old mechanistic challenges:

1) Is it possible to step through the catalytic cycle of nitrogenase one e^-/H^+ at a time and capture reaction intermediates in full structural detail?

2) Why and how is ATP binding and hydrolysis in FeP coupled to substrate reduction at FeMoco? From a fundamental standpoint, the ATP-dependence is a key aspect of biological nitrogen fixation which distinguishes it from essentially all other redox catalytic reactions in nature, including O_2, H^+, CO_2, SO_3^{2-}, and NO_2^- reduction. Why does N_2 reduction require external assistance when these multi-electron/multi-proton redox reactions do not? From a practical standpoint, it is highly challenging to accumulate and capture reaction intermediates of nitrogenase because of the requirement for continuous ATP hydrolysis to maintain e^- flow into FeMoco. This stands in contrast to, for example, the oxygen-evolving complex (OEC) of PSII, which can be conveniently flashed through its catalytic cycle with light pulses.[3,34]

Clearly, to make further headway in elucidating the mechanism of biological nitrogen fixation, we need to understand precisely how ATP binding and hydrolysis regulate e^-/H^+ transfer into FeMoco to enable substrate reduction, and to devise experimental methods that allow controlled e^-/H^+ flow into FeMoco in an ATP-independent fashion. In this review, we summarize our previous efforts toward addressing these goals and describe current and future challenges.

2. Structural Investigations of ATP-dependent FeP-MoFeP Interactions

There are two outstanding features of the FeP-MoFeP partnership. First, ATP-bound FeP is the only known e^- donor to MoFeP that can promote N_2 reduction, and second, ATP hydrolysis by FeP only occurs in the presence of MoFeP (i.e., under enzymatic turnover conditions). Combined, these observations suggest that coupling between ATP hydrolysis and ET/redox catalysis is accomplished through specific FeP-MoFeP interactions. The first picture of this coupling came from the crystal structure of the isolable FeP-MoFeP complex from *A. vinelandii*, stabilized by ADP.AlF_4^-, a transition-state analog for ATP hydrolysis.[35] In this complex, FeP is positioned in a central location on the MoFeP surface with re-

Faith Katz graduated from Scripps College in Claremont, CA in 2013 with her B.A. in chemistry. She then moved to UCSD, where she began her Ph.D. work under Akif Tezcan in bioinorganic chemistry. Currently, she is a third-year graduate student studying the role of protein-protein interactions in biological nitrogen fixation.

Cedric Owens graduated in 2007 from Colby College in Waterville, ME with majors in chemistry/biochemistry and mathematical sciences. After working in the lab of Celia Goulding on bacterial heme uptake, he received his Ph.D. in 2012 from UCI. He currently is a postdoctoral fellow at UCSD in the lab of Akif Tezcan.

Akif Tezcan received his B.A. in chemistry at Macalester College in St. Paul in 1995. He obtained his Ph.D. in 2001 under the direction of Harry Gray at Caltech, where he subsequently was a postdoctoral fellow with Douglas Rees. He has been at UCSD since 2005, where he currently is a professor in the Department of Chemistry and Biochemistry.

spect to the pseudo-two-fold symmetry of MoFeP (that bisects the α and β subunits), placing the [4Fe:4S] cluster of FeP as close to the MoFeP surface and the P-cluster as possible. At the same time, the γ_2-dimeric FeP assumes a much more compact structure relative to uncomplexed FeP,[36] whereby the γ-γ subunit interface tightens and brings necessary residues γLys10 and γAsp129 into contact with the nucleotides bound in the opposing subunit. These findings have established the docking geometry observed in the ADP.AlF_4^--bound nitrogenase complex as the "on" state, competent for both interprotein ET and ATP hydrolysis.

A notable feature of the ADP.AlF_4^- complex is the large size (>3500 Å^2) and the remarkable bonding complementarity of the FeP-MoFeP interface, which is more typical of permanent protein oligomers rather than transient protein complexes. In fact, typical protein complexes involved in biological ET reactions (where one partner is one of the aforementioned e^- shuttles) feature interfaces that cover <1000 Å^2 and are predominantly mediated by polar interactions, enabling fast turnover and recycling. Since each nitrogenase catalytic cycle is an $8e^-/8H^+$-process (Eq. (1)) and FeP and MoFeP associate and dissociate with each ET event,[37] these extensive interfacial contacts lead to a very slow turnover rate of ~6–10 s^{-1}.[38,39] Considering the conservation of N_2 fixation machinery across diazotrophic organisms, there must be one (or more) aspect(s) of N_2 fixation that necessitates such a large e^- shuttle as FeP that forms extensive contacts with its redox partner.

Yet, one practical advantage of the sluggish interactions between FeP and MoFeP is that they afford the opportunity to take crystallographic snapshots of the complexes they form in solution. To this end, we developed cocrystallization protocols using near-physiological MoFeP and FeP concentrations and ionic strength (150–200 mM)[40] to capture complexes that may represent those populated during turnover. Such FeP-MoFeP complexes were obtained and structurally characterized in three different states: in the absence of nucleotides, in the presence of AMPPCP (a non-hydrolyzable ATP analog), and in the presence of ADP (Figure 2a). These structures show that FeP can occupy three distinct docking geometries (DG) on an MoFeP surface in a nucleotide dependent manner, which we refer to as DG1, DG2, and DG3. In the nucleotide-free DG1 state, FeP is positioned largely atop the β-subunit of MoFeP and has an open γ_2 arrangement similar to uncomplexed FeP.[36] In the AMPPCP-bound DG2 state, FeP occupies the same central location as that observed in the ADP.AlF_4^--bound complex and has an γ_2 arrangement that has considerably tightened relative to free FeP, but not to the same extent as the ADP.AlF_4^--bound form.[41] Finally, in the ADP-bound DG3 state, FeP is found in four different conformational states, all occupying the α-subunit side of the MoFeP interaction surface. In all three DGs, the FeP-MoFeP interactions bury extensive surfaces (1600–3700 Å^2), indicating that they likely represent solution conformations and not crystal packing artifacts.

An important structural consequence of multiple DGs is modulation of the ET distance between the FeP [4Fe:4S] cluster and the MoFeP P-cluster: ~23 Å (center-to-center) for DG1 and DG3 and ~18 Å for DG2 (Figure 2b). Based on these distances and the exponential decay of ET rates with a constant of β~1.1 Å^{-1},[42] interprotein ET in DG2 can be estimated to be at least 3 orders of magnitude faster than those in DG1 and DG3, rendering the former to be "on" and the latter to be "off" states. Further, if we posit that these three states are populated in a temporal sequence during turnover, which is implied by the ATP-hydrolysis reaction coordinate (DG1→DG2→DG3), there arises the possibility of a unidirectional motion of FeP on the MoFeP surface, in close analogy to the nucleotide-fueled movement of motor proteins on their tracks.[43–45] Such a unidirectional motion, which would be unusual for protein-protein inter-

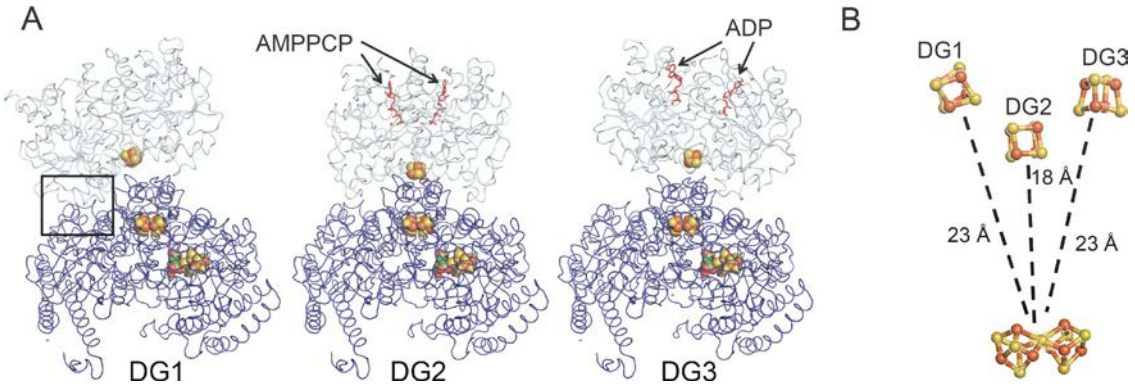

Figure 2. A) Structure of the FeP-MoFeP complex in three docking geometries DG1, DG2, and DG3 (PDBs: 2AFI, 4WZB, 2AFK). The regions on FeP and MoFeP that mediate electrostatic interactions important for encounter complex formation are boxed in the DG1 complex. B) Comparison of the [4Fe:4S] cluster to P-cluster distances in the three docking geometries.

actions involved in biological ET, could be guided by the asymmetric (or pseudosymmetric) nature of the MoFeP surface.

Because of the intimate coupling between ATP hydrolysis and FeP-MoFeP docking, we investigated whether the directionality or asymmetry of FeP-MoFeP interactions may also be manifested in the hydrolysis of ATP by FeP. Using the same cocrystallization conditions as above, but in the presence of equimolar AMPPCP and ADP, we obtained an FeP-MoFeP complex in the DG2 conformation, in which the two nucleotide binding sites of FeP were asymmetrically occupied by AMPPCP and ADP (Figure 3a).[46] We observed that the γ-subunit positioned on top of the α-subunit of MoFeP contained a full-occupancy ADP molecule, while the γ-subunit in contact with the β-subunit contained a full-occupancy AMPPCP molecule. This di- vs. tri-nucleotide discrimination within a symmetric, homodimeric protein scaffold is noteworthy, because in its free (MoFeP-uncomplexed) state, FeP is estimated to have a considerably higher affinity (>100-fold) for ADP than AMPPCP,[47] suggesting that FeP should predominantly bind two ADP molecules in solution. Thus, there appears to be a thermodynamic preference for the asymmetric, site-selective binding of AMPPCP and ADP to FeP, likely induced by the asymmetry of the MoFeP surface acting as a template.

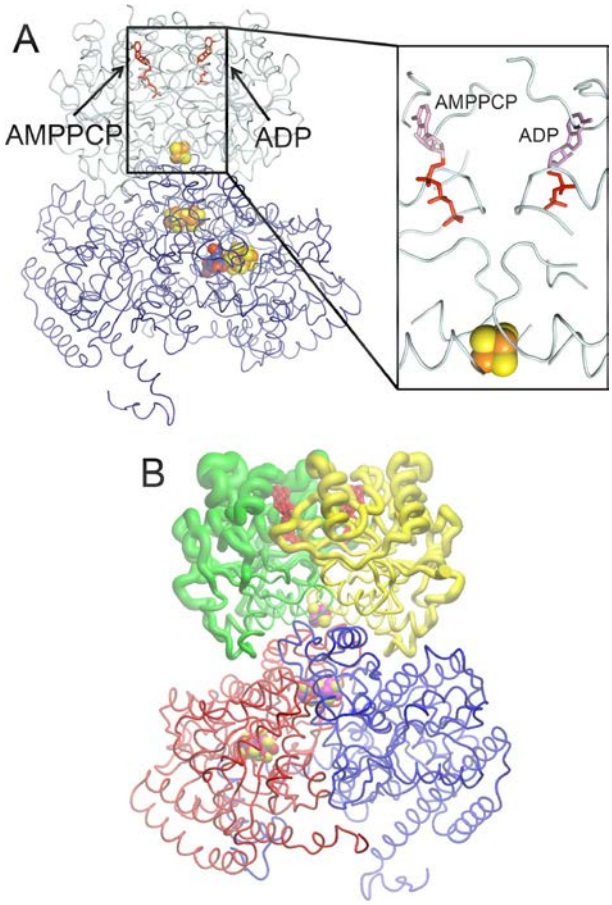

Figure 3. A) DG2 structure of nitrogenase highlighting the asymmetric nucleotide occupancy of the FeP by AMPPCP and ADP. B) Structural superposition of different FeP conformers characterized in the DG2 conformation. The width of the ribbons representing the FeP structure is proportional to the structural flexibility of different regions of FeP.

The selective interaction of AMPPCP and ADP with FeP suggests that ATP hydrolysis and phosphate release by FeP may proceed in a stepwise rather than a concerted fashion. This is reminiscent of other ATP-driven motors, such as the F1-ATPase[48,49] or AAA+ ATPases.[50] The asymmetry further leads to the provocative hypothesis that only one of the two FeP-bound ATP molecules may be hydrolyzed per MoFeP-interaction/ET cycle, though, to date, there is no experimental evidence for such a scenario. A perhaps safer suggestion is that a stepwise mechanism would give rise to a prolonged lifetime of the FeP-MoFeP complex. A superposition of the three complexes obtained in the DG2 state (ADP.AlF_4^-,[41] AMPPCP,[5] and AMPPCP/ADP-bound[46]) indicates that FeP undergoes considerable structural changes in regions away from the MoFeP docking surface, particularly around the nucleotide binding sites, while the position of the [4Fe:4S]-cluster is maintained in the "on" state during the ATP hydrolysis/phosphate release process (Figure 3b). The function of such a long-lived ET-active conformation could provide a timing mechanism (gating) for orchestrating underlying nitrogenase reactions, such as the rearrangement of FeMoco[51] and ET between P-cluster and FeMoco.

3. Functional Relevance of Multiple FeP-MoFeP Docking Modes

These mechanistic suggestions lead to additional questions: if all the action, i.e., ET and ATP hydrolysis, is happening within the DG2 conformation, what is the role of DG1 (or DG3)? Is the DG1 complex, obtained in the absence of any nucleotides, mechanistically relevant or even populated under turnover conditions?

As explained before, the DG1 complex between FeP and MoFeP was obtained in the absence of any nucleotides; this is a physiologically unlikely condition in light of the nucleotide binding affinities of FeP[52] and cellular ATP/ADP concentrations.[53] Yet, there is evidence to suggest that the DG1 complex may not be an artifact. The buried surface between FeP and MoFeP in DG1 is extensive (>2800 Å^2) and contains many H-bonding interactions typical of specific protein-protein complexes. Particular among these interactions are those formed between a highly conserved, negatively charged patch on the FeP surface (γGlu68, γAsp69, γGlu111, γGlu112) and a positively charged patch on the MoFeP β-subunit surface (βAsn399, βLys400, βArg401) (Figure 4a). In fact, Howard and colleagues established that treatment of

Israel Journal of Chemistry

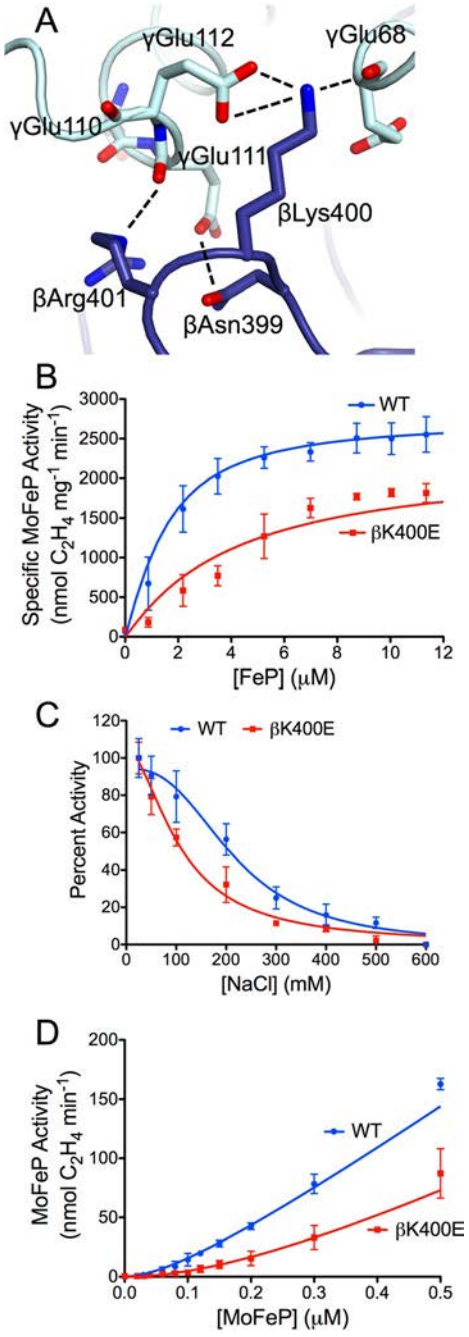

Figure 4. A) Close-up of FeP and MoFeP interactions in DG1 showing key H-bonding interactions between the oppositely charged patches on FeP and MoFeP surfaces (boxed in Figure 2A). B) C_2H_2 reduction assays for βLys400-MoFeP and wt-MoFeP. C) NaCl inhibition of C_2H_2 reduction activity of βLys400-MoFeP and wt-MoFeP. D) Dilution experiments for the C_2H_2 reduction activity of βLys400-MoFeP and wt-MoFeP.

a mixture of FeP and MoFeP with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) led to the formation of a specific isopeptide crosslink between FeP-γGlu112 and MoFeP-βLys400, both in the absence and presence of nucleotides.[54–56] Based on these observations, it was suggested that the crosslinked FeP-MoFeP species could be an encounter complex formed during catalytic turnover.

To investigate the functional relevance of DG1, we prepared three MoFeP mutants (βAsn399Glu, βLys400Glu, and βArg401Glu) aimed at destabilizing the interactions between the oppositely charged patches on FeP and MoFeP surfaces.[57] First, it was established that none of the three variants could form an EDC-mediated linkage to FeP in the presence or absence of ADP and ATP. This is not surprising in the case of the βLys400Glu variant, since this mutation eliminates the amine functionality required for the specific βLys400-γGlu112 linkage. Yet, the lack of crosslinking for βAsn399Glu and βArg401Glu variants suggests the charge-reversal mutations of the β399–401 patch may have significant effects on FeP interactions in solution.

These perturbations are also manifested in the reduced catalytic performance of the variants: the maximal specific activities of the three mutants for C_2H_2 and H^+ reduction are 70–80% of wild-type (wt) MoFeP, with the βLys400Glu variant displaying the most substantial decrease in activity (Figure 4b). In addition, the catalytic activity of βLys400Glu-MoFeP is significantly more sensitive to inhibition by increased ionic strength than wt-MoFeP, with a $IC_{50,NaCl} = 130 \pm 30$ mM as compared with the wt-value of 250 ± 15 mM, further highlighting the role of the electrostatic interactions in enzymatic turnover (Figure 4c). At the same time, the βLys400Glu mutation has no effect on the coupling between ATP hydrolysis and ET, displaying a ratio of ATP/$e^- = 2.1 \pm 0.4$, which is experimentally indistinguishable from that of wt-MoFeP. Thus, the FeP-MoFeP interactions involving the β399–401 patch must be involved in a step that precedes or follows ATP/e^- coupling, such as the formation of an FeP-MoFeP encounter complex.[57]

The determination of the association kinetics between FeP and MoFeP is hampered by the lack of a spectroscopic handle to directly monitor the interactions between the two proteins in solution. While most protein systems are readily amenable to chemical functionalization with molecular tags for fluorescence quenching or energy transfer experiments, FeP and MoFeP are challenging targets for site-selective labeling, the former because of the sensitivity of its [4Fe:4S] cluster to Cys-specific tags, the latter because of its large size, and both proteins because of the difficulty of preparing their site-directed mutants. We therefore carried out "dilution experiments", originally reported by Thorneley and coworkers,[58,59] in which nitrogenase catalytic activity is measured at progressively lower FeP and MoFeP concentrations with constant FeP:MoFeP ratios. At very low protein concentrations, the association between FeP and MoFeP becomes the rate-limiting step of catalysis, which is manifested in sigmoidal concentration-dependent activity profiles at concentrations below the effective dissociation constant of the complex. These activity profiles can be numerically simulated

Israel Journal of Chemistry

by the Thorneley-Lowe model for C_2H_2 (or H^+) reduction to estimate the rate constant for the formation of FeP-MoFeP complex (k_1). Dilution experiments with βLys400Glu- and wt-MoFeP show that the βLys400Glu mutation has a particularly pronounced, negative effect on enzyme activity at low protein concentrations, with a corresponding k_1 of $0.5\times10^7\,M^{-1}s^{-1}$, five-fold lower than that for wt-MoFeP ($2.5\times10^7\,M^{-1}s^{-1}$) (Figure 4d). Since the βLys400Glu mutation lowers the rate of protein association but does not affect the coupling of ATP hydrolysis to ET, this charge-reversal mutation must disrupt encounter complex formation.

Taken together, these findings indicate that interactions involving the β399–401 patch and FeP (and thus the crystallographically observed DG1 complex) are functionally important for nitrogenase catalysis and are populated along a productive reaction pathway toward the formation of the DG2 state. Combining these biochemical data with the previously described crystallographic insights, we propose a detailed picture of nucleotide-dependent FeP-MoFeP interactions (Figure 5). In this updated model, FeP and MoFeP initially form a fluxional ensemble of electrostatically driven encounter complexes centered around the β399–401 patch, which are then steered onto the metastable DG1 complex.[60–62] Formation of DG1 is followed by transition to the "activated" DG2 conformation via a 2D conformational sampling of the MoFeP surface. The complex is thus committed to ET and ATP hydrolysis, which proceed in a stepwise fashion involving different conformations of FeP.[5,35,46] Following ET and phosphate release, FeP is disengaged from the DG2 conformation to start the next ATP hydrolysis/ET cycle,

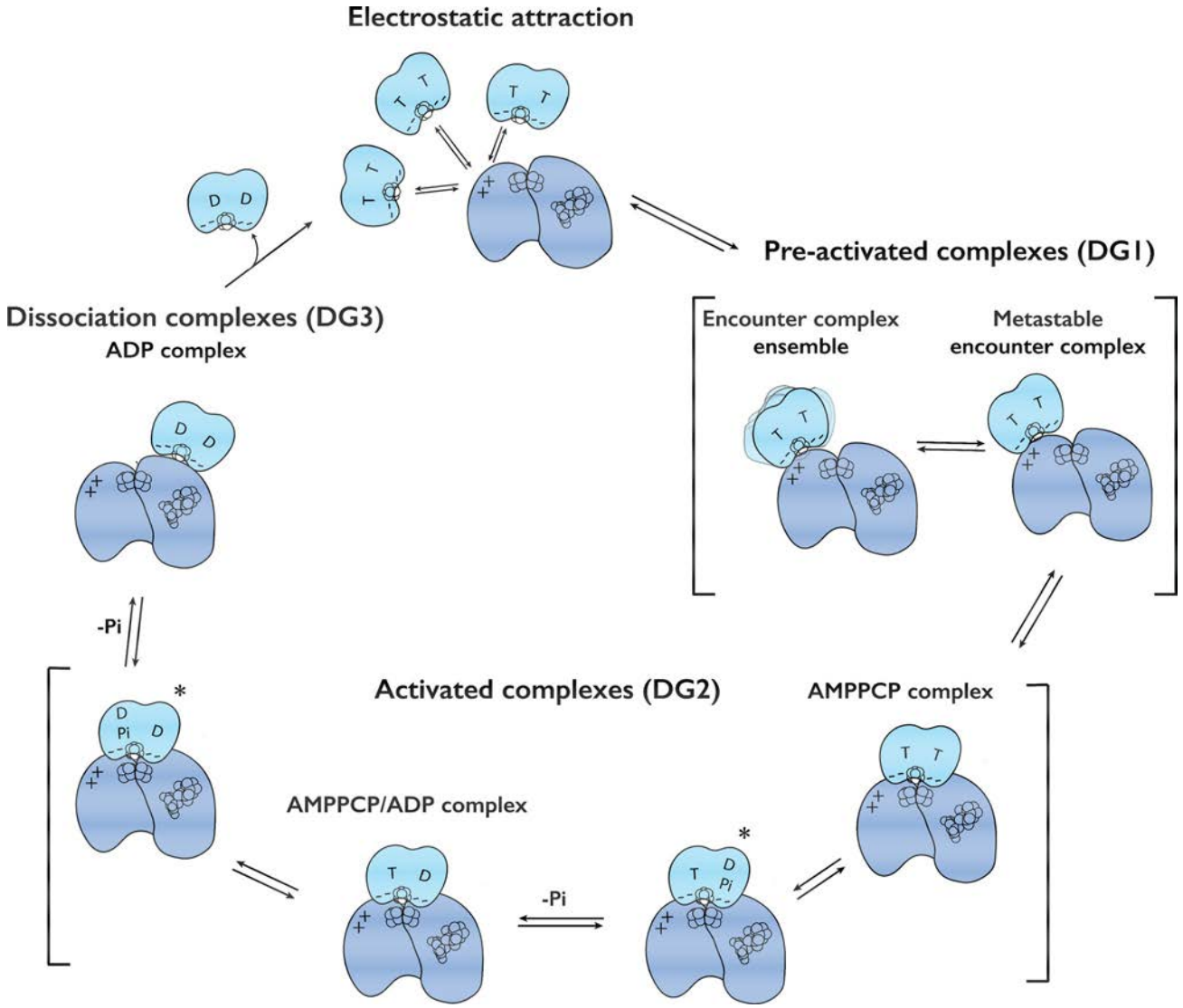

Figure 5. Proposed scheme for ATP-dependent FeP-MoFeP interactions populated during nitrogenase turnover, where T signifies ATP and D signifies ADP. * denotes likely, but experimentally unconfirmed, sub-states.

Israel Journal of Chemistry

likely with an intermediate stop at the DG3 conformation.

While encounter complex formation clearly plays a role in nitrogenase turnover, it is not obvious why this step is necessary. Encounter complex formation may increase e^- flux into FeMoco, which is critical for optimizing the yield of NH_3 production over H_2.[63,64] A dedicated docking site such as β399–401 could minimize the surface area of MoFeP that FeP explores before transitioning into the active DG2 conformation. This effect could be particularly crucial in a cellular environment where the FeP to MoFeP ratios are rather low (1–2)[65,66] and the estimated ionic strength (~150 mM) is considerably higher than that in typical *in vitro* turnover experiments (~50 mM).[67] What is more, the movement of FeP from DG1 to DG2 could activate a conformational change within MoFeP that could prime FeMoco for accepting e^- (i.e., gating) or could provide a mechanism for actively displacing ADP-bound, oxidized FeP from the MoFeP surface for rapid redox recycling. Further work will be needed to test each of these hypotheses.

4. ATP-independent Activation of MoFeP Catalysis by Light

In some of the discussions above, we mentioned that FeP may activate a conformational gate within MoFeP that controls the flow of e^- into FeMoco. The possibility of such a conformational gate has been invoked in part because ATP-bound FeP is the only known e^- donor that enables MoFeP to reduce N_2, whereas ferredoxins, flavodoxins, or potent small-molecule reductants do not. Yet, in all of the diverse FeP-MoFeP complexes characterized thus far (Figure 2), the structure of MoFeP is essentially identical in all mechanistically important regions.[5,35,46,56,68] Since there is no obvious structural indication of an FeP-induced conformational gate, we hypothesized that it might be possible to activate MoFeP catalysis in an FeP- and ATP-independent fashion. To this end, we created an artificial mimic of the DG2 complex, in which MoFeP was functionalized with a redox cofactor positioned where the [4Fe:4S]-cluster of FeP would be located.

To achieve ATP-independent catalysis, we employed an MoFeP variant (αLeu158Cys, originally generated by the Dean Lab at Virginia Tech), in which the αCys158 side chain is located in a surface cleft between the α and β subunits, 14 Å away from the P-cluster (Figure 6a). αLeu158-Cys-MoFeP was quantitatively functionalized with the iodoacetamide derivative of $[Ru(bpy)_2(phenA)]^{2+}$ (IA-RuBP),[69] whose long-lived photoexcited state ($^*Ru^{II}BP$) is readily quenched by sacrificial donors to generate the potent reductant $Ru^{I}BP$ ($E^0 \approx -1.28$ V)[70] species in high yield (Figure 6b). To test the light-driven activity of Ru-labeled αLeu158Cys-MoFeP, we first examined the 2-e^- substrates of nitrogenase, H^+ and C_2H_2, whose reduction

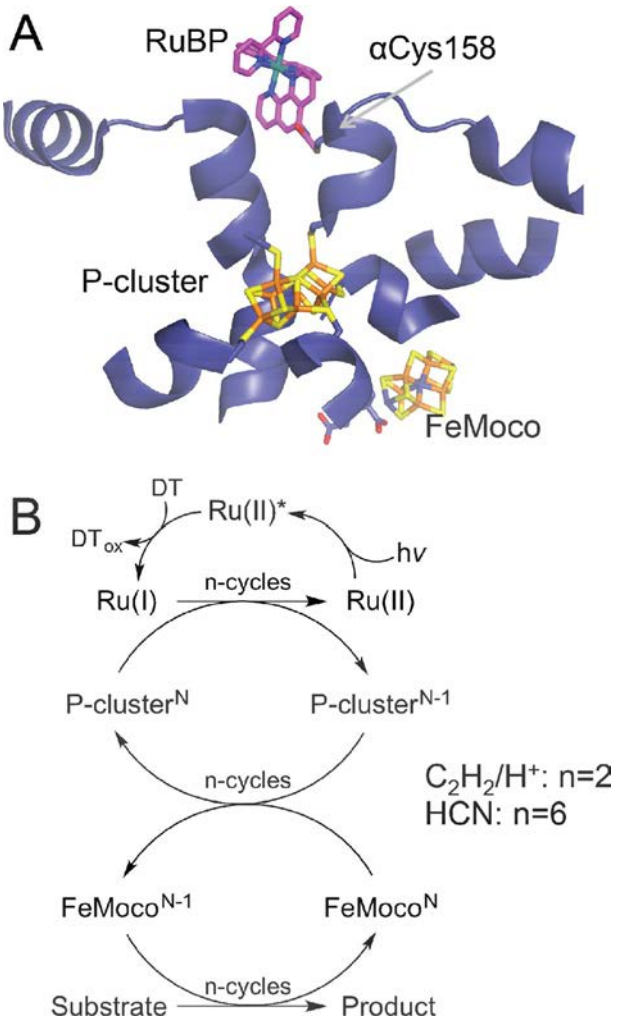

Figure 6. A) Structural model of $Ru(bpy)_2(phenA)$ bound to αLeu158Cys-MoFeP. B) Scheme for the 2-e^- or 6-e^- photoreduction by $Ru(bpy)_2(phenA)$-labeled MoFeP variants.

products (H_2 and C_2H_4) are readily detected by gas chromatography. When large amounts of the sacrificial donor were present (≥200 mM dithionite), substantial quantities of H_2 and C_2H_4 could be generated under white light irradiation, with velocities of 16 nmol C_2H_4 min^{-1} and 14 nmol H_2 min^{-1} per mg MoFeP and a turnover number of ~110 per active site (Figure 7a).[69] Several control studies showed that the H_2 and C_2H_4 production stemmed from the delivery of photo-excited e^- from RuBP to FeMoco (Figure 7a). The key experiment was to measure the activity of Ru-MoFeP under a CO atmosphere, since CO inhibits reduction of every nitrogenase substrate except H^+.[71–73] Indeed, CO fully inhibited the reduction of C_2H_2 but not H^+, confirming FeMoco as the destination of Ru-based e^- and the site of catalysis (Figure 7b).

On one hand, the Ru-MoFeP system challenges the long-standing dogma that ATP-bound FeP is the only reductant that can drive nitrogenase catalysis. On the other hand, Ru-MoFeP only attains about 1 % of the maximum

Israel Journal of Chemistry

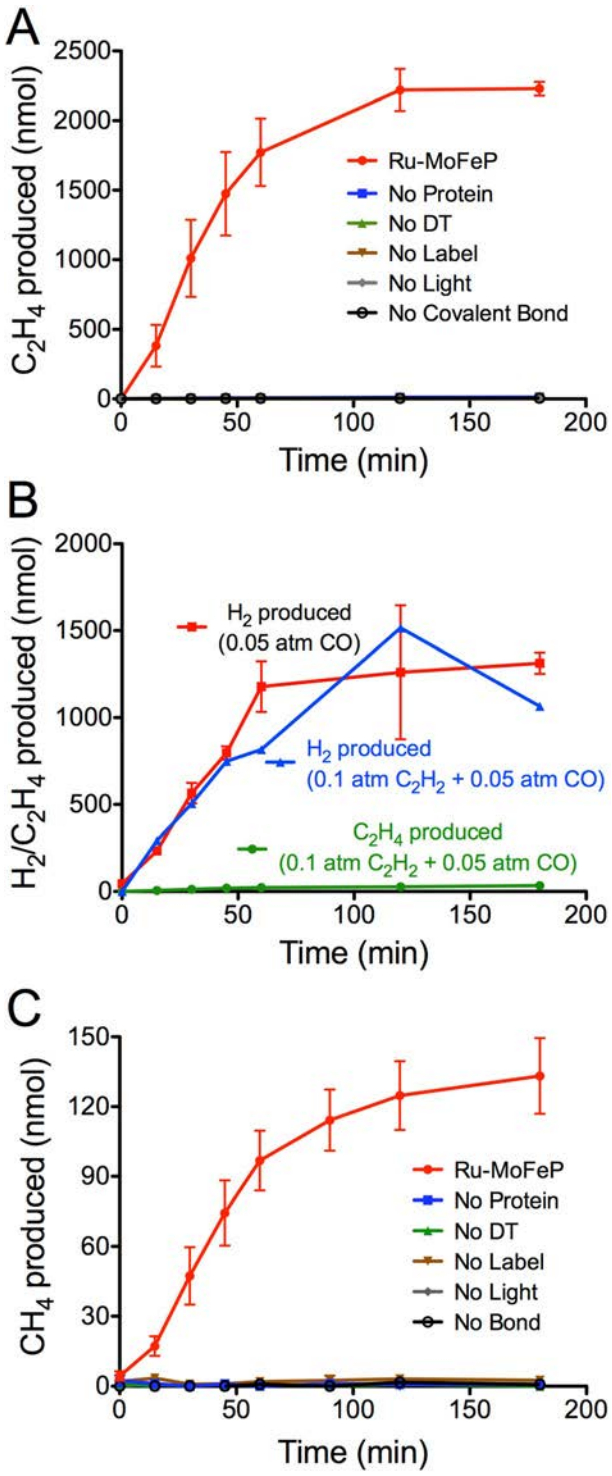

Figure 7. A) C_2H_2 production. B) H_2 or C_2H_2 production under a CO atmosphere. C) CH_4 production from HCN by Ru-MoFeP.

specific activity of the ATP-driven native system for H_2 and C_2H_4 formation. While Ru-MoFeP can also catalyze the $6e^-/6H^+$ reduction of HCN into CH_4,[74] we were unable to observe the similar $6e^-/6H^+$ reduction of the isoelectronic N_2 to NH_3. Seefeldt, Hoffman, Dean, and coworkers have also demonstrated FeP and ATP-independent substrate reduction by MoFeP variants αTyr64-His, βTyr98His, and βPhe99His,[75,76] but have similarly been unable to observe the reduction of N_2 to NH_3. One possible explanation is that N_2 can only bind 3- or 4-electron reduced states of FeMoco,[77] which may be inaccessible in our photosensitized systems or the aforementioned MoFeP variants, whereas HCN can bind the 1- or 2-electron reduced FeMoco.[78,79] Nevertheless, we cannot exclude the possibility that small amounts of NH_3 are produced, but they are below the detection limits of available assays.

As is the norm for nitrogenase, any new observation begets another question: Why is product formation by Ru-MoFeP so low, despite the fact $Ru^{I}BP$ is such a potent reductant and positioned properly for efficient ET to the P-cluster? This question brings us back to the possibility of FeP-induced, conformationally gated ET within MoFeP. The elucidation of such conformational gating would be particularly important for engineering MoFeP variants with increased photocatalytic activities, ultimately leading to PSII-like systems in which catalytically relevant redox states of FeMoco could be efficiently generated *in crystallo* for structural interrogation.

A particular ET scenario that invokes conformational gating in nitrogenase catalysis is the "deficit spending" model,[77,80–82] which has been invoked because the dithionite-reduced resting P-cluster is in an all-ferrous state[7] and presumably cannot be reduced any further by FeP. In this model, complexation of the ATP-bound FeP with MoFeP first induces ET from the P-cluster to FeMoco through a necessarily long-distance structural change, and the now-oxidized P-cluster is then reduced by the docked FeP to restart the next ET cycle. Based on their work with an MoFeP variant with an altered P-cluster (βSer188Cys-MoFeP), Seefeldt, Hoffman, Dean, and colleagues proposed that the intraprotein ET from the P-cluster to FeMoco is a gated,[83,84] slow ET step, which is followed by quick ($>1700\ s^{-1}$) "backfill" of the P-cluster by FeP.[80] The "deficit-spending" hypothesis is compatible with the proposal that stepwise ATP-hydrolysis can prolong the residence time of the ET-activated DG2 state and enable the orchestration of structural rearrangements at or near FeMoco or the P-cluster,[46,85] the latter of which is known to undergo redox-dependent structural changes.[86] How such structural rearrangements would switch ET from the P-cluster to FeMoco on and off is not clear. In our opinion, such a switch – if it exists – must be thermodynamic in origin, that is, it increases the reduction potential of FeMoco or decreases that of the P-cluster (or simultaneously does both), rather than increasing electronic coupling between the two. One possibility we favor is the conformationally induced protonation of FeMoco or deprotonation of the P-cluster, which would make the former a better e^- acceptor and the latter a better e^- donor. Moreover, cluster protonation/deproto-

nation through the movement of side chains or internal water molecules would not necessitate large-scale conformational changes, consistent with structural constancy of MoFeP in all known crystal structures of nitrogenase.

What is not entirely consistent with the deficit spending model and the necessity of FeP-induced structural gating processes are our results with the light-driven Ru-MoFeP system: they show that it is possible to inject catalytically useful e^- into FeMoco via the P-cluster in the absence of FeP, even if with low yields. The possibility of the P-cluster to be super-reduced and accommodate more e^- beyond the all-ferrous state should not be excluded based on lack of precedence (particularly given the many extraordinary aspects of nitrogenase) and represents an exciting avenue to investigate.

5. Outlook

In this review, we emphasized the unique complexity of the ATP-driven FeP-MoFeP redox system. By all accounts, FeP is not a simple e^- shuttle like ferredoxins, flavodoxins, or cytochromes; owing to its ATPase function, it follows a deliberate choreography on the surface of MoFeP and may even orchestrate catalytic events within MoFeP through long-distance effects. In return, MoFeP certainly is a not a passive recipient of e^- like most redox catalytic enzymes; by virtue of its surface architecture, it acts as an effector that dictates the movement and ATPase activity of its e^- donor, FeP. Since nature has evolved no other mechanism for N_2 activation, it is likely that all of the unique features of nitrogenase must be present to meet the distinct challenges of N_2 activation chemistry in a biological system. In this regard, it is useful to note the ability of nitrogenase to carry out the $2H^+ + 2\ e^- \rightarrow H_2$ reaction in the absence of N_2 and compare it with hydrogenases, which specifically execute this reaction. Hydrogenases, like the MoFeP component of nitrogenase, are multidomain enzymes with unusual catalytic metalloclusters buried within the protein scaffold.[87–90] In contrast to nitrogenase, the hydrogenase catalytic clusters are electronically connected all the way to the protein surface via a chain of Fe-S clusters, and they can be readily activated for catalysis by ferredoxins, flavodoxins, or even small molecule reductants. They are structurally optimized to funnel high-energy H^+/e^- to the active site metallocluster as efficiently as possible, without the worry of competing reactions and byproducts. Nitrogenase, on the other hand, must be highly selective regarding when and how e^- and H^+ are delivered to FeMoco in order to maximize N_2 reduction, and minimize the competing, kinetically/thermodynamically more favorable H_2 production. Such selectivity would be hard to accomplish in a system where the catalytic cluster is constitutively hardwired to the protein surface via direct H^+/e^- transfer pathways. In such an architecture, the thermodynamics of H^+/e^- flow would be determined largely by the solution redox potential and pH. We therefore propose that NH_3 vs. H_2 selectivity is the chemical basis for why ATP-hydrolysis and a specialized donor like FeP are required for biological N_2 fixation. Through extensive biochemical and structural studies in the last couple of decades, the coupling between ET and ATP hydrolysis in nitrogenase is now fairly well established. A key challenge now is to determine how ATP hydrolysis also regulates H^+ transfer into FeMoco.

Acknowledgments

The portion of our work conducted at UCSD was funded by NIH grant GM099813 and NSF grant MCB-0643777 to F.A.T. F.A.T. also acknowledges the Frasch Foundation for additional support. C. P. O. is the recipient of a postdoctoral fellowship from the USDA (2015-67012-22895). Along with Harry Gray, to whom this review is dedicated, we thank Doug Rees for many enlightening discussions.

References

[1] B. K. Burgess, D. J. Lowe, *Chem. Rev.* **1996**, *96*, 2983–3012.
[2] J. B. Howard, D. C. Rees, *Chem. Rev.* **1996**, *96*, 2965–2982.
[3] J. P. McEvoy, G. W. Brudvig, *Chem. Rev.* **2006**, *106*, 4455–4483.
[4] R. Y. Igarashi, L. C. Seefeldt, *Crit. Rev. Biochem. Mol. Biol.* **2003**, *38*, 351–384.
[5] F. A. Tezcan, J. T. Kaiser, D. Mustafi, M. Y. Walton, J. B. Howard, D. C. Rees, *Science* **2005**, *309*, 1377–1380.
[6] J. Klugkist, J. Voorberg, H. Haaker, C. Veeger, *Eur. J. Biochem.* **1986**, *155*, 33–40.
[7] D. C. Rees, J. B. Howard, *Curr. Opin. Chem. Biol.* **2000**, *4*, 559–566.
[8] H.-I. Lee, P. M. Benton, M. Laryukhin, R. Y. Igarashi, D. R. Dean, L. C. Seefeldt, B. M. Hoffman, *J. Am. Chem. Soc.* **2003**, *125*, 5604–5605.
[9] J. A. Wiig, C. C. Lee, Y. Hu, M. W. Ribbe, *J. Am. Chem. Soc.* **2013**, *135*, 4982–4983.
[10] K. M. Lancaster, M. Roemelt, P. Ettenhuber, Y. Hu, M. W. Ribbe, F. Neese, U. Bergmann, S. DeBeer, *Science* **2011**, *334*, 974–977.
[11] O. Einsle, F. A. Tezcan, S. L. Andrade, B. Schmid, M. Yoshida, J. B. Howard, D. C. Rees, *Science* **2002**, *297*, 1696–1700.
[12] T. Spatzal, K. A. Perez, O. Einsle, J. B. Howard, D. C. Rees, *Science* **2014**, *345*, 1620–1623.
[13] H.-I. Lee, R. Y. Igarashi, M. Laryukhin, P. E. Doan, P. C. Dos Santos, D. R. Dean, L. C. Seefeldt, B. M. Hoffman, *J. Am. Chem. Soc.* **2004**, *126*, 9563–9569.
[14] B. M. Barney, M. Laryukhin, R. Y. Igarashi, H.-I. Lee, P. C. Dos Santos, T.-C. Yang, B. M. Hoffman, D. R. Dean, L. C. Seefeldt, *Biochemistry* **2005**, *44*, 8030–8037.
[15] B. M. Barney, T.-C. Yang, R. Y. Igarashi, P. C. Dos Santos, M. Laryukhin, H.-I. Lee, B. M. Hoffman, D. R. Dean, L. C. Seefeldt, *J. Am. Chem. Soc.* **2005**, *127*, 14960–14961.
[16] D. Lukoyanov, S. A. Dikanov, Z.-Y. Yang, B. M. Barney, R. I. Samoilova, K. V. Narasimhulu, D. R. Dean, L. C. See-

feldt, B. M. Hoffman, *J. Am. Chem. Soc.* **2011**, *133*, 11655–11664.
[17] R. Y. Igarashi, M. Laryukhin, P. C. Dos Santos, H.-I. Lee, D. R. Dean, L. C. Seefeldt, B. M. Hoffman, *J. Am. Chem. Soc.* **2005**, *127*, 6231–6241.
[18] B. M. Barney, D. Lukoyanov, R. Y. Igarashi, M. Laryukhin, T.-C. Yang, D. R. Dean, B. M. Hoffman, L. C. Seefeldt, *Biochemistry* **2009**, *48*, 9094–9102.
[19] P. M. Benton, M. Laryukhin, S. M. Mayer, B. M. Hoffman, D. R. Dean, L. C. Seefeldt, *Biochemistry* **2003**, *42*, 9102–9109.
[20] B. M. Barney, D. Lukoyanov, T.-C. Yang, D. R. Dean, B. M. Hoffman, L. C. Seefeldt, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 17113–17118.
[21] R. Sarma, B. M. Barney, S. Keable, D. R. Dean, L. C. Seefeldt, J. W. Peters, *J. Inorg. Biochem.* **2010**, *104*, 385–389.
[22] D. Lukoyanov, B. M. Barney, D. R. Dean, L. C. Seefeldt, B. M. Hoffman, *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1451–1455.
[23] Z.-Y. Yang, N. Khadka, D. Lukoyanov, B. M. Hoffman, D. R. Dean, L. C. Seefeldt, *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 16327–16332.
[24] B. M. Hoffman, D. Lukoyanov, Z.-Y. Yang, D. R. Dean, L. C. Seefeldt, *Chem. Rev.* **2014**, *114*, 4041–4062.
[25] R. Bjornsson, F. Neese, R. R. Schrock, O. Einsle, S. DeBeer, *J. Biol. Inorg. Chem.* **2015**, *20*, 447–460.
[26] J. T. Kaiser, Y. Hu, J. A. Wiig, D. C. Rees, M. W. Ribbe, *Science* **2011**, *331*, 91–94.
[27] J. A. Wiig, Y. Hu, C. C. Lee, M. W. Ribbe, *Science* **2012**, *337*, 1672–1675.
[28] J. A. Wiig, Y. Hu, M. W. Ribbe, *Nat. Commun.* **2015**, *6*, 8034.
[29] Y. Hu, M. W. Ribbe, *Annu. Rev. Biochem.* **2016**, *85*, 455–483.
[30] L. M. Rubio, P. W. Ludden, *Annu. Rev. Microbiol.* **2008**, *62*, 93–111.
[31] J. S. Anderson, J. Rittle, J. C. Peters, *Nature* **2013**, *501*, 84–87.
[32] D. V. Yandulov, R. R. Schrock, *Science* **2003**, *301*, 76–78.
[33] K. Arashiba, Y. Miyake, Y. Nishibayashi, *Nat. Chem.* **2011**, *3*, 120–125.
[34] M. Haumann, C. Müller, P. Liebisch, L. Iuzzolino, J. Dittmer, M. Grabolle, T. Neisius, W. Meyer-Klaucke, H. Dau, *Biochemistry* **2005**, *44*, 1894–1908.
[35] H. Schindelin, C. Kisker, J. L. Schlessman, J. B. Howard, D. C. Rees, *Nature* **1997**, *387*, 370–376.
[36] M. M. Georgiadis, H. Komiya, P. Chakrabarti, D. Woo, J. J. Kornuc, D. C. Rees, *Science* **1992**, *257*, 1653–1659.
[37] R. V. Hageman, R. Burris, *Proc. Natl. Acad. Sci. U. S. A.* **1978**, *75*, 2699–2702.
[38] R. Hageman, R. Burris, *Biochemistry* **1978**, *17*, 4117–4124.
[39] R. N. Thorneley, D. Lowe, *Biochem. J.* **1983**, *215*, 393–403.
[40] See Ref. [5].
[41] See Ref. [35].
[42] H. B. Gray, J. R. Winkler, *Annu. Rev. Biochem.* **1996**, *65*, 537–561.
[43] S. Rice, A. W. Lin, D. Safer, C. L. Hart, N. Naber, B. O. Carragher, S. M. Cain, E. Pechatnikova, E. M. Wilson-Kubalek, M. Whittaker, *Nature* **1999**, *402*, 778–784.
[44] R. D. Vale, R. A. Milligan, *Science* **2000**, *288*, 88–95.
[45] K. C. Holmes, I. Angert, F. J. Kull, W. Jahn, R. R. Schröder, *Nature* **2003**, *425*, 423–427.
[46] F. A. Tezcan, J. T. Kaiser, J. B. Howard, D. C. Rees, *J. Am. Chem. Soc.* **2015**, *137*, 146–149.
[47] M. F. Weston, S. Kotake, L. C. Davis, *Arch. Biochem. Biophys.* **1983**, *225*, 809–817.
[48] J. P. Abrahams, A. G. W. Leslie, R. Lutter, J. E. Walker, *Nature* **1994**, *370*, 621–628.
[49] P. D. Boyer, *Annu. Rev. Biochem.* **1997**, *66*, 717–749.
[50] R. T. Sauer, T. A. Baker, *Annu. Rev. Biochem.* **2011**, *80*, 587–612.
[51] See Ref. [12].
[52] J. Cordewener, M. Krüse-Wolters, H. Wassink, H. Haaker, C. Veeger, *Eur. J. Biochem.* **1988**, *172*, 739–745.
[53] C. J. Knowles, L. Smith, *Biochim. Biophys. Acta* **1970**, *197*, 152–160.
[54] A. H. Willing, M. M. Georgiadis, D. C. Rees, J. B. Howard, *J. Biol. Chem.* **1989**, *264*, 8499–8503.
[55] A. Willing, J. B. Howard, *J. Biol. Chem.* **1990**, *265*, 6596–6599.
[56] B. Schmid, O. Einsle, H.-J. Chiu, A. Willing, M. Yoshida, J. B. Howard, D. C. Rees, *Biochemistry* **2002**, *41*, 15557–15565.
[57] C. P. Owens, F. E. H. Katz, C. H. Carter, M. A. Luca, F. A. Tezcan, *J. Am. Chem. Soc.* **2015**, *137*, 12704–12712.
[58] R. N. Thorneley, R. R. Eady, M. G. Yates, *Biochim. Biophys. Acta* **1975**, *403*, 269–284.
[59] D. Lowe, R. Thorneley, *Biochem. J.* **1984**, *224*, 877–886.
[60] G. Schreiber, A. R. Fersht, *Biochemistry* **1993**, *32*, 5145–5150.
[61] G. Schreiber, *Curr. Opin. Struct. Biol.* **2002**, *12*, 41–47.
[62] M. Ubbink, *FEBS Lett.* **2009**, *583*, 1060–1066.
[63] K. R. Schubert, H. J. Evans, *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1207–1211.
[64] J. H. Guth, R. H. Burris, *Biochemistry* **1983**, *22*, 5111–5122.
[65] J. Klugkist, H. Haaker, H. Wassink, C. Veeger, *Eur. J. Biochem.* **1985**, *146*, 509–515.
[66] D. Jacobs, D. Mitchell, G. Watt, *Arch. Biochem. Biophys.* **1995**, *324*, 317–324.
[67] G. Schreiber, G. Haran, H.-X. Zhou, *Chem. Rev.* **2009**, *109*, 839–860.
[68] H.-J. Chiu, J. W. Peters, W. N. Lanzilotta, M. J. Ryle, L. C. Seefeldt, J. B. Howard, D. C. Rees, *Biochemistry* **2001**, *40*, 641–650.
[69] L. E. Roth, J. C. Nguyen, F. A. Tezcan, *J. Am. Chem. Soc.* **2010**, *132*, 13672–13674.
[70] H. B. Gray, A. W. Maverick, *Science* **1981**, *214*, 1201–1205.
[71] L. C. Davis, M. T. Henzl, R. Burris, W. Orme-Johnson, *Biochemistry* **1979**, *18*, 4860–4869.
[72] J. Meyer, *Arch. Biochem. Biophys.* **1981**, *210*, 246–256.
[73] D. J. Lowe, K. Fisher, R. N. Thorneley, *Biochem. J.* **1990**, *272*, 621–625.
[74] L. E. Roth, F. A. Tezcan, *J. Am. Chem. Soc.* **2012**, *134*, 8416–8419.
[75] K. Danyal, B. S. Inglet, K. A. Vincent, B. M. Barney, B. M. Hoffman, F. A. Armstrong, D. R. Dean, L. C. Seefeldt, *J. Am. Chem. Soc.* **2010**, *132*, 13197–13199.
[76] K. Danyal, A. J. Rasmussen, S. M. Keable, B. S. Inglet, S. Shaw, O. A. Zadvornyy, S. Duval, D. R. Dean, S. Raugei, J. W. Peters, *Biochemistry* **2015**, *54*, 2456–2462.
[77] B. M. Hoffman, D. Lukoyanov, D. R. Dean, L. C. Seefeldt, *Acc. Chem. Res.* **2013**, *46*, 587–595.
[78] J. Li, B. K. Burgess, J. L. Corbin, *Biochemistry* **1982**, *21*, 4393–4402.
[79] P. M. Benton, S. M. Mayer, J. Shao, B. M. Hoffman, D. R. Dean, L. C. Seefeldt, *Biochemistry* **2001**, *40*, 13816–13825.
[80] K. Danyal, D. R. Dean, B. M. Hoffman, L. C. Seefeldt, *Biochemistry* **2011**, *50*, 9255–9263.

[81] D. Mayweather, K. Danyal, D. R. Dean, L. C. Seefeldt, B. M. Hoffman, *Biochemistry* **2012**, *51*, 8391–8398.
[82] S. Duval, K. Danyal, S. Shaw, A. K. Lytle, D. R. Dean, B. M. Hoffman, E. Antony, L. C. Seefeldt, *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 16414–16419.
[83] M. G. Duyvis, H. Wassink, H. Haaker, *J. Biol. Chem.* **1996**, *271*, 29632–29636.
[84] K. Danyal, D. Mayweather, D. R. Dean, L. C. Seefeldt, B. M. Hoffman, *J. Am. Chem. Soc.* **2010**, *132*, 6894–6895.
[85] S. M. Mayer, D. M. Lawson, C. A. Gormal, S. M. Roe, B. E. Smith, *J. Mol. Biol.* **1999**, *292*, 871–891.
[86] J. W. Peters, M. H. Stowell, S. M. Soltis, M. G. Finnegan, M. K. Johnson, D. C. Rees, *Biochemistry* **1997**, *36*, 1181–1187.
[87] A. L. De Lacey, V. M. Fernandez, M. Rousset, R. Cammack, *Chem. Rev.* **2007**, *107*, 4304–4330.
[88] D. M. Heinekey, *J. Organomet. Chem.* **2009**, *694*, 2671–2680.
[89] D. W. Mulder, E. M. Shepard, J. E. Meuser, N. Joshi, P. W. King, M. C. Posewitz, J. B. Broderick, J. W. Peters, *Structure* **2011**, *19*, 1038–1052.
[90] M. Winkler, J. Esselborn, T. Happe, *Biochim. Biophys. Acta Bioenerg.* **2013**, *1827*, 974–985.

Received: March 31, 2016
Published online: July 18, 2016