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ATP- and Iron−Protein-Independent Activation of Nitrogenase Catalysis by Light

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Abstract: We report here the light-driven activation of the molybdenum−iron−protein (MoFeP) of nitrogenase for substrate reduction independent of ATP hydrolysis and the iron−protein (FeP), which have been believed to be essential for catalytic turnover. A MoFeP variant labeled on its surface with a Ru-photosensitizer is shown to photocatalytically reduce protons and acetylene, most likely at its active site, FeMoco. The uncoupling of nitrogenase catalysis from ATP hydrolysis should enable the study of redox dynamics within MoFeP and the population of discrete reaction intermediates for structural investigations.

The enzyme nitrogenase performs nitrogen fixation under ambient conditions, in stark contrast to the Haber−Bosch process that requires extreme temperatures and pressures to the same end.[1,2] Biological nitrogen fixation comes at a high energetic cost, however, in that it requires 16 ATP molecules per turnover reaction (2 ATP molecules per electron):

$$N_2 + 8\,e^- + 8\,H^+ + 16\,ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Despite decades of investigations, it is still not understood in detail how ATP hydrolysis is coupled to redox catalysis by nitrogenase and what the mechanistic details of substrate activation by the catalytic core of nitrogenase (the iron−molybdenum cofactor, FeMoco) are.[3,4] The second question is intimately linked to the first: because of the requirement of continuous ATP hydrolysis to maintain the electron flow for the catalytic activation of FeMoco, it has been challenging to populate discrete reaction intermediates bound to the cofactor in sufficient quantities for structural and spectroscopic interrogation. Here we report for the first time the light-driven, ATP-independent activation of nitrogenase toward the catalytic two-electron reduction of alternative nitrogenase substrates, protons (H^+) and acetylene (C_2H_2).[5] Our findings challenge the long-standing assumption that ATP hydrolysis is obligatory for nitrogenase catalysis and open up new avenues for studying the mechanistic intricacies of biological nitrogen fixation.

Mo-nitrogenase comprises the Fe−protein (FeP) and the Mo−Fe−protein (MoFeP) (Figure 1a). FeP is an ATPase that functions as an electron shuttle to MoFeP, where substrate binding and activation take place at FeMoco (a [7Fe:1Mo:9S:1X] cluster). FeP can provide only one or two electrons at a time; hence it has to undergo multiple ATP-dependent association−dissociation cycles with MoFeP before enough electrons accumulate on FeMoco for catalysis.[3,4] Electron transfer (ET) during catalysis is believed to proceed from a [4Fe:4S] cluster located in FeP to a buried [8Fe:7S] cluster (P-cluster) in MoFeP and finally to FeMoco. Association of the two proteins, the ET step, and the dissociation reaction are strictly controlled through the ATPase activity of FeP.

MgATP-bound FeP is the only known electron donor that has been shown to effect the reduction of any substrate by MoFeP.

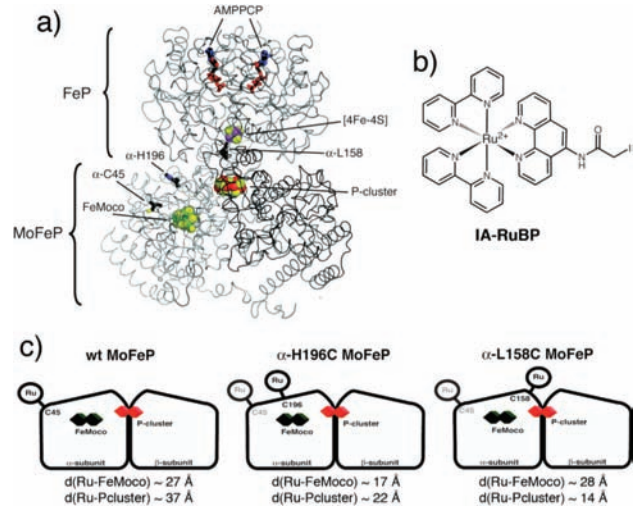

Figure 1. (a) FeP−MoFeP complex structure obtained in the presence of AMPPCP (an ATP analog) highlighting key components. (b) IA-RuBP. (c) Cartoon representations of labeling sites on wt, α-L158C, and α-H196C MoFeP and corresponding edge-to-edge distances to MoFeP clusters.

While many electron donors (e.g., dithionite; $E° = -400$ to -650 mV;[6] $Ti^{IV/III}$[citrate], $E° \leq -800$ mV[7]) with comparable or lower reduction potentials than FeP (-280 mV as isolated, -620 mV as complexed to MoFeP)[8] can reduce oxidized states of the P-cluster (P^{2+} and P^{1+}), they cannot support catalysis alone. This has led to the proposal that MgATP binding to FeP and its hydrolysis within the FeP−MoFeP complex must mechanically gate ET among the three redox centers,[9] explaining the exclusivity of FeP as a catalytic electron donor. Indeed, structures of FeP−MoFeP complexes obtained at various stages of MgATP hydrolysis revealed large conformational changes in FeP that enable it to assume different docking geometries on MoFeP and modulate the distance (thus, electronic coupling) between the [4Fe:4S] cluster and the P-cluster.[10] Nevertheless, these structures also showed that there are no discernible conformational changes within MoFeP that would readily indicate an ET gate.

We set out to probe whether it would be possible to mimic the low-potential, highly coupled state of the FeP [4Fe:4S] cluster formed within the MgATP-activated FeP−MoFeP complex and bypass the need for ATP hydrolysis and FeP to initiate nitrogenase reactivity. Toward this end, we covalently labeled three different *A. vinelandii* MoFeP constructs with a Cys-specific iodoacetamido derivative of $[Ru(bpy)_2(phen)]^{2+}$ (IA-RuBP). These Ru-labeled MoFeP variants (wild type, α-L158C, and α-H196C) are illustrated in Figure 1c. Wild-type (wt) MoFeP contains several Cys residues. However, only one (α-Cys45) is appreciably solvent exposed to be a candidate for functionalization with IA-RuBP. The additional Cys residues on each of the other two mutants, which also include α-C45, are in relatively solvent exposed positions, with α-C196 in

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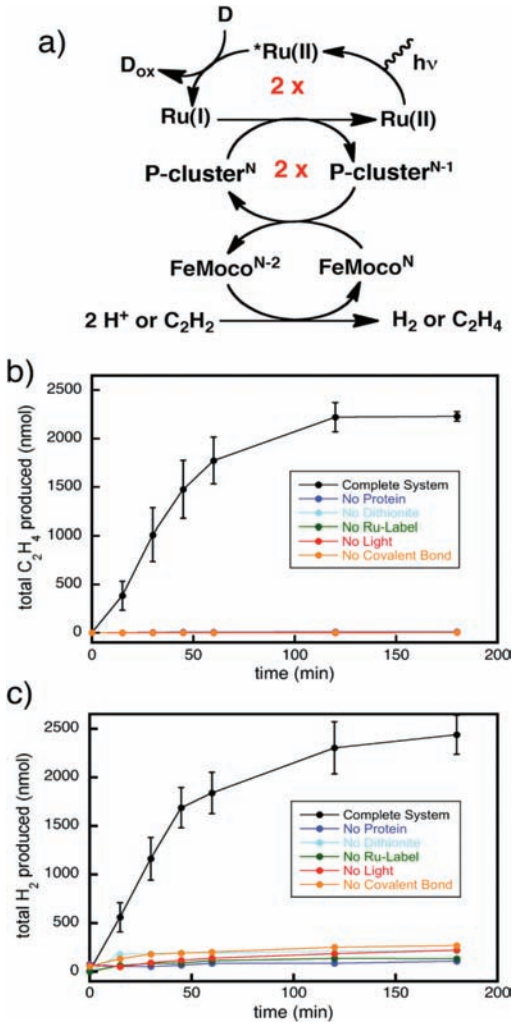

Figure 2. (a) A possible photoreduction scheme for Ru−C158 MoFeP.[11] (b) Acetylene and (c) proton reduction assays and corresponding controls, each of which has one indicated component of the photocatalytic system missing.

the immediate vicinity of FeMoco and α-C158 in a cleft right above the P-cluster. Incubation of the variants (2 h) with a 10-fold molar excess of IA-RuBP leads to quantitative labeling of MoFeP as determined by inductively coupled optical emission spectroscopy, with one label per $\alpha\beta$-dimer on wt-MoFeP and two labels per $\alpha\beta$-dimer on α-C158 and α-C196 variants as planned (Table S1 and Figure S1).

The distances between the Ru-labels on each variant and the MoFeP clusters are listed in Figure 1c. α-C45 is too distant from either cluster to factor in the redox activation of nitrogenase, thus serving as a control. Ru-labels on α-C158 and α-C196, on the other hand, should be coupled to either the P-cluster or FeMoco, respectively, allowing the examination of alternative electron transfer routes to FeMoco. In order to investigate light-driven activation of MoFeP (Figure 2a), we pursued the reduction of the alternative substrates, H^+ and C_2H_2, which require only two electrons and whose products (H_2 and C_2H_4) are readily detected by gas chromatography. A typical reaction included 2.7 mg of RuBP-labeled protein (∼20 nmol of active sites) in a solution of 200 mM dithionite and 200 mM NaCl at pH 7.75. In deoxygenated vials, the reaction solutions were irradiated in a 20 °C water bath with a Xe/Hg lamp using UV- (<300 nm) and IR-cutoff filters under constant stirring, and the headgas was analyzed for products. As a sacrificial electron donor (**D**) in a flash−quench scheme,[12,13] we utilized dithionite, which should allow for diffusion-limited quenching of the $Ru^{II}BP$ excited state ($*Ru^{II}BP$) to generate the reducing $Ru^{I}BP$ ($E° \approx -1.28$ V)[14] species in high yield. Fulfilling a second role, dithionite is a dioxygen scavenger universally employed to protect MoFeP clusters from oxidative damage and to maintain the P-cluster in an all-ferrous state (P^N).

Ru−wt and Ru−C196 MoFeP show little to no production of H_2 or C_2H_4 in the presence of 0.1 atm of C_2H_2 even after 200 min of irradiation (Figure S2). In contrast, irradiation of Ru−C158 leads to the evolution of both products in equal quantities, with average velocities of 16 nmol C_2H_4/min and 14 nmol H_2/min per mg MoFeP over 50 min (Figure 2b,c). Photodriven C_2H_4 and H_2 production reaches a plateau after 50 min despite the presence of excess dithionite, yielding a turnover number of ∼110 per active site for both products. The EPR spectrum of Ru−C158 MoFeP shows no changes in the characteristic $S = 3/2$ feature of FeMoco after turnover, indicating that the cofactor stays intact (Figure S3). On the other hand, the absorption features of Ru−BP steadily disappear during turnover (Figure S4), which may be attributable to Ru−ligand dissocation and explain the loss of activity.

We next investigated whether C_2H_4 and H_2 production indeed stems from our photoreduction scheme and not from an unforeseen reactive site or species. The elimination of any of the reaction components (light, Ru-photosensitizer, MoFeP, or dithionite) leads to a complete abolishment of C_2H_4 and H_2 evolution (Figure 2b,c). When free RuBP is included in solution instead of being covalently linked to α-C158 MoFeP, no activity is observed, indicating that a surface immobilized Ru-photosensitizer is necessary to ensure efficient electronic coupling to the MoFeP clusters and electron injection.

Interestingly, when dithionite is replaced with other typical sacrificial donors like triethanolamine (TEOA, 200 mM), 2-(*N*-morpholino)ethanesulfonic acid (MES, 200 mM), or NADH (40 mM), little photocatalytic activity is observed (Figure S7). In order to test whether this unique ability of dithionite ($\lambda_{max} = 314$ nm) for supporting catalysis is due to its photochemistry, we carried out activity assays and control experiments using monochromatic 455-nm radiation from an LED source that should only excite the Ru-photosensitizer and not dithionite (Figure S8). These experiments show that (a) photocatalytic activity is maintained at this wavelength, albeit with slower kinetics, and (b) Ru−C158 MoFeP is considerably more stable under these conditions with steady activity for at least 300 min of irradiation, likely due to the elimination of UV-based damage. While these findings confirm again that the light-driven reactivity stems from the surface-immobilized Ru-photosensitizer, they also suggest a possible role of dithionite beyond acting as a sacrificial donor. Current studies are underway to probe the potential ability of dithionite to directly reduce a P-cluster intermediate during photocatalysis along with RuBP.

Having established the catalytic competence of the photosensitized Ru−C158 MoFeP system, we sought to determine whether FeMoco is the site of substrate activation. Carbon monoxide (CO) has been shown to interact with FeMoco under turnover conditions and to be a strong inhibitor of all nitrogenase substrates except H^+.[15−17] The inclusion of 0.05 atm of CO alongside 0.1 atm of the substrate C_2H_2 abolishes light-driven C_2H_4 production (Figure 3a). On the other hand, H_2 is produced at high levels in the presence of the same amount of CO or CO/C_2H_2, matching the inhibition profile for ATP-driven nitrogenase catalysis. Similarly, cyanide (CN^-) stalls the photolytic C_2H_2 reduction activity of Ru−C158 MoFeP (Figure 3b), with an apparent inhibition midpoint concentration of ∼0.25 mM CN^- (at 0.05 atm C_2H_2) that agrees well with

a previously reported value ($K_i = 0.5$ mM) obtained for wt MoFeP under ATP-driven turnover conditions.[18] These findings strongly imply that FeMoco is the ultimate destination for the photogenerated electrons and the site of catalysis.[11]

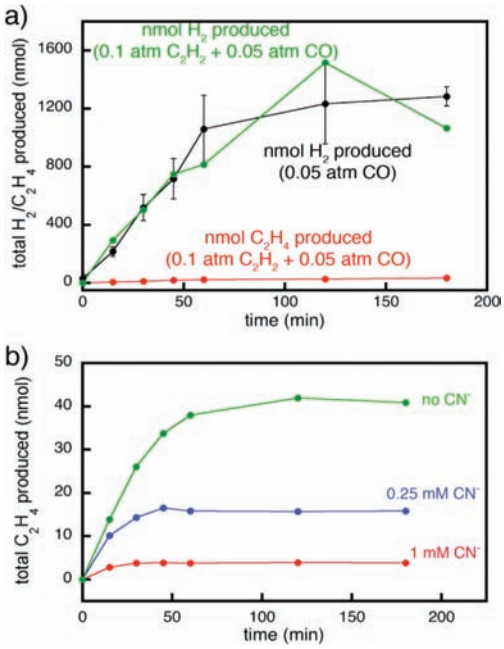

Figure 3. (a) Carbon monoxide and (b) cyanide inhibition of light-driven substrate reduction by Ru−C158 MoFeP. Experiments were conducted under the same conditions as those shown in Figure 1 and described in the text, except that CN^- inhibition experiments were performed with ~0.1 mg Ru−C158 MoFeP.

Presently, the quantum yield (ϕ = catalytically useful electrons/photons absorbed by $Ru^{II}BP$) of our light-driven system is <1%, which likely is the reason why Ru−C158 fails to produce significant levels of ammonia from N_2. We are currently pursuing the optimization of our system for increased yields of electron injection to potentially enable this 6-e^- process. Ultimately, however, our experiments show that ATP hydrolysis and FeP are not absolutely essential for substrate reduction by nitrogenase as commonly assumed.

The study of any major aspect of nitrogenase catalysis has been hampered due to its dependence on continuous ATP hydrolysis and transient FeP−MoFeP interactions, which lead to a heterogeneous mixture of redox and nucleotide-bound states of nitrogenase in solution. While recent studies have provided glimpses into substrate interactions on FeMoco,[4,19] a detailed picture of nitrogenase catalysis is still not available. We have now uncoupled nitrogenase catalysis from ATP hydrolysis and protein−protein interactions by introducing a light-triggered electron delivery system, which should enable the study of ET dynamics within MoFeP and the population of discrete redox intermediates for structural investigations.

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Supporting Information Available: Protein preparation, characterization, additional assays, and materials/methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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