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Structural Evidence for Asymmetrical Nucleotide Interactions in Nitrogenase

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Supporting Information

ABSTRACT: The roles of ATP hydrolysis in electron-transfer (ET) reactions of the nitrogenase catalytic cycle remain obscure. Here, we present a new structure of a nitrogenase complex crystallized with MgADP and MgAMPPCP, an ATP analogue. In this structure the two nucleotides are bound asymmetrically by the Fe-protein subunits connected to the two different MoFe-protein subunits. This binding mode suggests that ATP hydrolysis and phosphate release may proceed by a stepwise mechanism. Through the associated Fe-protein conformational changes, a stepwise mechanism is anticipated to prolong the lifetime of the Fe-protein-MoFe-protein complex and, in turn, could orchestrate the sequence of intracomplex ET required for substrate reduction.

Many biological redox reactions involve transient interactions between a catalytic protein and a shuttle protein that donates/accepts the reducing equivalents.[1] The dynamics of these bimolecular electron-transfer (ET) reactions are often governed entirely by structural and physical determinants that are built into the redox partners, such as surface interactions, intervening protein medium between cofactors, and reduction potentials of redox cofactors.[2−4] Consequently, most inter-protein ET reactions occur in short-lived protein-protein encounter complexes. In contrast are a handful of enzyme complexes that utilize nucleotide hydrolysis to regulate the association/dissociation dynamics of redox partners.[5−7] Prominent among them is nitrogenase, which couples ATP hydrolysis to the catalytic reduction of N_2 into NH_3:[8−10]

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

In nitrogenase, intermolecular ET occurs between the iron-protein (FeP) and the catalytic molybdenum-iron protein (MoFeP) and is coupled to hydrolysis of two ATP by FeP. Because only one or two electrons are transferred during each two-protein encounter, multiple cycles of complex formation and ATP hydrolysis are required for N_2 reduction. The mechanistic imperative of ATP-coupled ET remains a central question in biological nitrogen fixation.[11] Namely, why does nitrogenase utilize ATP hydrolysis to enable the thermodynamically favorable N_2 fixation reaction when many other complex, energetically favorable multielectron/multiproton redox reactions, including O_2, H^+, CO_2, SO_3^{2-}, and NO_2^- reduction, do not? Alternatively, why is not an electron donor like a ferredoxin or flavodoxin, some of which have lower potentials than FeP, sufficient to drive nitrogenase catalysis? These questions may be germane to understanding the design principles for other nucleotide-mediated processes as well as for investigating conditions for N_2 reduction without ATP hydrolysis.[12,13]

The MoFeP-FeP complex plays a central role in the nitrogenase mechanism as this is the species in which ATP hydrolysis is coupled to ET. To address the coupling mechanism, we previously reported[14,15] the structure of an ADP-AlF_4^- stabilized FeP-MoFeP complex (*alf*-complex) that documented an intermolecular ET pathway from the [4Fe:4S]-cluster in the FeP homodimer (γ_2) to the [8Fe:7S] P-cluster located between each α/β pair in the MoFeP tetramer. An additional intramolecular ET path within MoFeP was implied between the P-cluster and the active site [7Fe:Mo:9S:1C:R-homocitrate] FeMo-cofactor. We subsequently developed cocrystallization conditions using near-physiological MoFeP and FeP concentrations and ionic strength[16] to capture complexes more reflective of transient interactions under turnover conditions. With this strategy, we obtained crystal structures of three *in situ* formed nitrogenase complexes in different nucleotide states: the nucleotide-free form (*nf*-complex), a form with the nonhydrolyzable ATP analog MgAMPPCP (*pcp*-complex) bound, and a form with MgADP (*adp*-complex) bound.[16] These structures showed that FeP, depending on the nucleotide bound, occupied distinct but overlapping and mutually exclusive docking modes; in each mode, the distance between the [4Fe:4S] cluster and the P-cluster was substantially different. In all of these structures, the two FeP nucleotide binding sites were symmetrically occupied, with MgADP-AlF_4^-, MgAMPPCP, or MgADP, or with both nucleotide-free. However, asymmetrically occupied nucleotide sites are plausible as intermediates should the MgATP be hydrolyzed in a sequential fashion, which is obligatory for the

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functioning of some systems such as the F1-ATPase[17,18] and AAA+ ATPases.[19] Although MgADP is a potent inhibitor of nitrogenase,[20] Mortenson and Upchurch[21] reported the intriguing observation that the ATP/e^- ratio decreases from the canonical value of 2 (eq 1) to ca. 1 at higher MgADP/ATP ratios. While this may be a manifestation of the ability of FeP to transfer two electrons per hydrolysis of two ATPs,[22] it also raises the possibility that FeP bound with one ADP and one ATP might be more energy efficient for ET under some metabolic states of the cell.

To better understand the role of ATP hydrolysis, we now report the structure of a nitrogenase complex with asymmetrical nucleotide binding of MgAMPPCP and MgADP. Together with the previously determined structures, we have an unusually extensive picture of a dynamic protein-protein interaction. Our results highlight the possibility that ATP hydrolysis and phosphate release might proceed in a stepwise fashion as guided by the asymmetry of interactions in the MoFeP-FeP complex, thus yielding long-lived species with discrete intermediates.

Suitable conditions were established that yielded well-diffracting crystals of a MoFeP-FeP complex in the presence of equimolar MgADP and MgAMPPCP, a nonhydrolyzable ATP analog, allowing the structure determination at 1.9 Å resolution (Table S1, PDB accession code 4WZA). These crystals are isomorphous with the previously determined *pcp*-complex,[16] where the asymmetric unit contains a single 2:1 FeP-MoFeP complex of subunit stoichiometry $\alpha_2\beta_2\gamma_4$ (Figure 1A). Remarkably, each FeP dimer is asymmetric with respect to

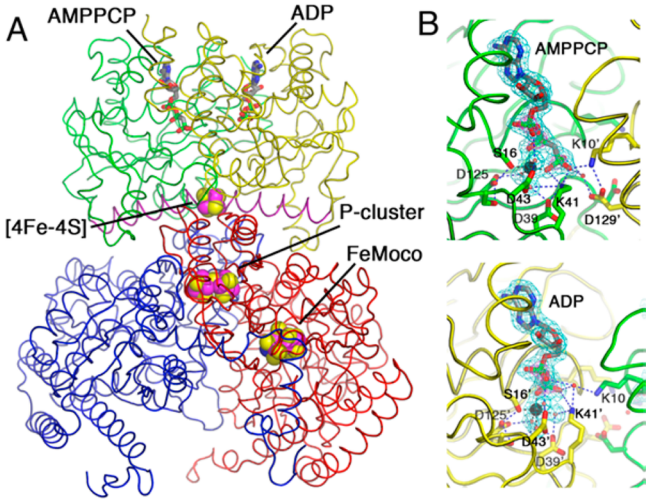

Figure 1. (A) FeP-MoFeP docking geometry in the *pcp*/*adp*-complex (green and yellow, FeP γ1 and γ2 subunits; red and blue, MoFeP α and β subunits). (B) $2F_0-F_c$ electron density maps of the AMPPCP and ADP nucleotides (cyan, 1σ; magenta, 4.5σ) and their interactions with the P-loop and Switch II regions. See Figure S1 for omit electron density maps.

nucleotide binding and constitutes a *pcp*/*adp*-complex: the γ-subunit positioned adjacent to the α-subunit of MoFeP contains well-defined density for MgADP, whereas the γ-subunit interacting most closely with the β-subunit contains a well-defined MgAMPPCP (Figure 1B). The nucleotide asymmetry is striking for two reasons: (1) the affinity of FeP for MgADP is estimated to be at least 2 orders of magnitude tighter than that for the ATP analogues MgAMPPCP[23] and MgAMPPNP,[24] suggesting FeP should predominantly be in the MgADP-bound state in solution; (2) the MgADP and MgAMPPCP-bound γ-subunits are uniquely associated with a specific MoFeP subunit. Because we only observe a mixed nucleotide structure, we can conclude the MoFeP template has selected an otherwise minor species, suggesting that the formation of the *pcp*/*adp* complex is favored under these crystallization conditions.

The overall conformation of the FeP in the *pcp*/*adp*-complex, including the docking geometry with the MoFeP, resembles that observed previously in the *pcp*-complex. Nevertheless, there are tantalizing local structural differences between the two FeP subunits that reflect the asymmetric nucleotide occupancy. Most noticeable is a flip in orientation of the peptide bond between residues Asp 129 and Val 130 in the crucial Switch II region coupling the nucleotide binding segment to the [4Fe:4S] cluster (Figure 2, left panel). The conformation of

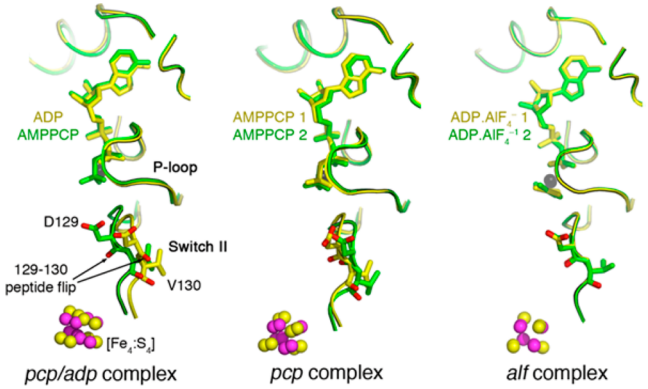

Figure 2. Superpositions of the two FeP subunits in the vicinity of the Switch II region including the [4Fe:4S] cluster and nucleotides binding sites for the *pcp*/*adp*-, *pcp*-, and *alf*-complexes. The flip in the 129−130 peptide bond orientation between the two FeP subunits is highlighted. The yellow and green chains denote the FeP protein predominantly interacting with the MoFeP α-and β- subunits, respectively.

this region in the FeP subunit containing the MgAMPPCP (adjacent to the β-subunit of MoFeP) is similar in the *alf*-, *pcp*-, and *pcp*/*adp*-complexes, while in the subunit binding MgADP in the *pcp*/*adp*-complex, the peptide flip uniquely occurs. Although the consequence of these structural perturbations for nucleotide hydrolysis and ET cannot be assessed here, we note that the reduction potential of iron-sulfur clusters can be sensitive to the orientation of peptide bonds and protein dipoles;[25] hence, the asymmetrical changes observed in the *pcp*/*adp*-complex may be relevant to connecting the nucleotide state to cluster oxidation states.

The FeP conformation in the *pcp*/*adp*-complex was evaluated in the context of the larger ensemble of complexes by principal components analysis (PCA; Figure 3) using methods developed by Berendsen et al.[26] The dominant component (accounting for 88% of the structural variation) closely corresponds to a hinge axis along the FeP subunit-subunit interface, while the second component (accounting for ~half of the remaining structural variation (7% of the total)) more closely represents a twisting motion across the interface. Four distinct conformational categories may be identified, corresponding to "ADP-AlF", "ATP", "ADP", and "nucleotide-free" (NF) states. The distribution of 21 FeP structures into

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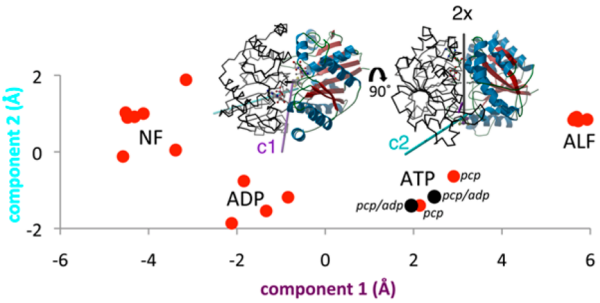

Figure 3. PCA of the crystallographically characterized FeP conformations (complexed or uncomplexed with MoFe-protein). Conformational space associated with the ADP-AlF_4^-, ATP, ADP, and NF conformations are highlighted. (Inset) Two perpendicular views illustrating the orientation of the rotation axes for components 1 (purple) and 2 (cyan), which correspond to hinge bending and twisting along the FeP dimer subunit interface. The conversion factor between distances and rotation angles about an axis is ~3° per Å.

these four groups suggests that they represent well-defined local energy minima, with the FeP conformations dependent upon the form of bound nucleotide, type of complex formation with the MoFeP, and potentially the FeP oxidation state. These conformational states are associated with alternative sets of inter- and intrasubunit salt bridges[14,27] involving Glu154, Arg187 and Arg213 that could serve to differentially stabilize the FeP subunits in defined conformations with distinct subunit-subunit orientations (Figure S2).

While the full course of the ATP hydrolysis reaction cannot be determined solely based on crystal structures, by combining the new *pcp*/*adp*-complex structure with previously determined ones, an outline can be inferred for the evolution of FeP-MoFeP conformational states that are populated during ATP hydrolysis (Figure 4). (1) MgATP binding commits FeP to form an activated, multistate complex with MoFeP that is characterized by extensive and complementary protein-protein interactions (>3500 $Å^2$) that are more typical of high affinity protein complexes[28] than transient protein complexes with smaller, poorly packed interfaces involved in ET.[29] (2) Alternative and mutually exclusive conformations of FeP are populated, differentially stabilized by nucleotide and MoFeP interactions. Differential sets of interfacial salt bridges are generated that may stabilize these distinct species. (3) The selective occupancy of MgADP and MgAMPPCP molecules in the *pcp*/*adp*-complex suggests that ATP hydrolysis and/or phosphate release proceeds through a stepwise sequence of conformational changes[16] reminiscent of the F1-ATPase[17,18] or AAA+ ATPases.[19] (4) A direct implication of multiple discrete conformations is a long-lived encounter complex. From the turnover rate per electron under saturating conditions (~10 s^{-1}),[30,31] the half-life for the FeP-MoFeP complex can be estimated as ~0.1 s. This is considerably longer than the lifetimes of typical encounter complexes formed between simpler ET proteins involved in one-electron exchange ($\tau \sim 10^{-3}$ s).[32−35]

Multiple discrete FeP-MoFeP states populated during ATP hydrolysis provide a mechanism for regulation of ET by controlling the separation between constituent metal centers. Equally important, the lifetimes of these intermediates serve as a timing mechanism for orchestrating underlying reactions, e.g., the rearrangement and reactions at the FeMo-cofactor[36] and ET between P-cluster and FeMo-cofactor. As emphasized by Hopfield[37] for protein biosynthesis, the presence of quasi-irreversible steps (such as ATP hydrolysis) in simple Michaelis−Menten kinetic schemes effectively alters the lifetime of intermediates which allows for proof-reading of multiple potential reaction paths. Similar arguments may be germane to the nitrogenase reaction where ATP hydrolysis would regulate the timing between complex formation and ET processes; a mechanistic feature not available to simpler electron donors such ferredoxins or small molecules. Although FeP reduced by dithionite is a single electron donor for MoFeP, FeP also can be reduced with low potential donors such as flavodoxin to an all-ferrous state which can donate two electrons to MoFeP.[22] The stepwise ATP hydrolysis and phosphate release, in this case, can provide two potential committed steps per cycle of MoFeP-FeP interaction and may very well be an evolutionary requirement for the transfer of two electrons in each interaction cycle. More generally, the ensemble of conformational states observed with the nitrogenase complex is representative of the much broader category of nucleotide-dependent transduction systems, where ATP or GTP hydrolysis is used to enhance the fidelity of a process in the presence of competing outcomes, e.g., protein targeting to the appropriate cellular compartment.[38]

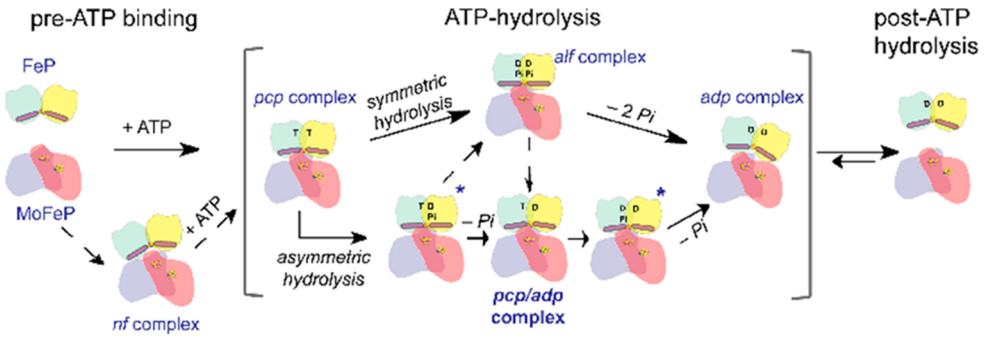

Figure 4. Schematic proposal positioning crystallographically characterized Fe- and MoFe-protein structures along a reaction coordinate for ATP hydrolysis by nitrogenase. The observation of the *pcp*/*adp* complex implicates a pathway for asymmetric ATP hydrolysis and phosphate release and, thereby, the intermediacy of additional structural states compared to a symmetrical pathway. "T" denotes ATP (or AMPPCP). "D" denotes ADP and "D·Pi" denotes the transition state for ATP hydrolysis. The complexes marked with asterisks indicate structurally unobserved but necessary ATP hydrolysis intermediates that would be populated in the asymmetric pathway.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental details and crystallographic data collection/refinement and PCA, and figures showing omit electron density maps and interfacial salt-bridging interactions in FeP. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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