Structural characterization of the P^{1+} intermediate state of the P-cluster of nitrogenase

Received for publication, February 14, 2018, and in revised form, April 26, 2018. Published, Papers in Press, May 2, 2018. DOI 10.1074/jbc.RA118.002435

Stephen M. Keable, Oleg A. Zadvornyy, Lewis E. Johnson, Bojana Ginovska, Andrew J. Rasmussen, Karamatullah Danyal, Brian J. Eilers, Gregory A. Prussia, Axl X. LeVan, Simone Raugei, Lance C. Seefeldt, and John W. Peters

From the Department of Chemistry and Biochemistry, Montana State University, Bozeman, Montana 59717, Institute of Biological Chemistry, Washington State University, Pullman, Washington 99163, Pacific Northwest National Laboratory, Richland, Washington 99352, and Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322

Edited by Ruma Banerjee

Nitrogenase is the enzyme that reduces atmospheric dinitrogen (N₂) to ammonia (NH₃) in biological systems. It catalyzes a series of single-electron transfers from the donor iron protein (Fe protein) to the molybdenum–iron protein (MoFe protein) that contains the iron–molybdenum cofactor (FeMo-co) sites where N₂ is reduced to NH₃. The P-cluster in the MoFe protein functions in nitrogenase catalysis as an intermediate electron carrier between the external electron donor, the Fe protein, and the FeMo-co sites of the MoFe protein. Previous work has revealed that the P-cluster undergoes redox-dependent structural changes and that the transition from the all-ferrous resting (PN) state to the two-electron oxidized P^{2+} state is accompanied by protein serine hydroxyl and backbone amide ligation to iron. In this work, the MoFe protein was poised at defined potentials with redox mediators in an electrochemical cell, and the three distinct structural states of the P-cluster (P^{2+}, P^{1+}, and PN) were characterized by X-ray crystallography and confirmed by computational analysis. These analyses revealed that the three oxidation states differ in coordination, implicating that the P^{1+} state retains the serine hydroxyl coordination but lacks the backbone amide coordination observed in the P^{2+} states. These results provide a complete picture of the redox-dependent ligand rearrangements of the three P-cluster redox states.

Nitrogenase is the enzyme responsible for the multiple-electron reduction of atmospheric dinitrogen (N₂) to ammonia (NH₃) in biological systems (1). This complex oxygen-sensitive metalloprotein orchestrates a series of ATP-dependent single-electron transfers from the donor iron protein (Fe protein) to the catalytically active molybdenum–iron protein (MoFe protein) (2). During catalysis, the process of electron delivery to the active site involves two types of electron transfer events: one event is the intermolecular electron transfer between the [4Fe-4S] cluster of the Fe protein and the P-clusters of the MoFe protein, and the other is the intramolecular electron transfer between the two P-clusters and two [7Fe-9S-C-Mo-homocitrate] iron–molybdenum cofactor (FeMo-co) active sites within the MoFe protein (3, 4). Recent work has helped delineate the order of these electron transfer events with a proposed “deficit-spending” model (5). This model postulates that the interaction of the Fe protein and the MoFe protein elicits conformational changes that facilitate an initial “slow” and conformationally gated electron transfer step event between the P-cluster and the FeMo-co (5, 6). This event leaves the P-cluster with a “deficit” of one electron (P^{1+}) relative to the all-ferrous resting (PN) state. This deficit is then repaid by a second, faster intermolecular electron transfer event from the reduced [4Fe-4S]^{3+} cluster in the Fe protein to the P^{1+}-cluster, restoring the PN state (5). This fast step is a direct electron transfer step and takes place at rates greater than 1700 s⁻¹, which could explain why the P^{1+} state is difficult to observe during turnover (7). Thus, the deficit-spending model postulates that major conformational changes occur during catalysis, but the conformational changes that regulate the gated unidirectional electron flow are likely short-lived. The mechanistically relevant P^{1+} state has been observed spectroscopically in native and variant MoFe proteins (8–10). However, this state was only achievable using electrochemical mediators. Previous structural work has revealed two distinct conformations of the P-cluster that have been assigned to the PN resting state and the P^{2+} oxidized state.
Structure of nitrogenase P-cluster intermediate

![Diagram of P-cluster structures](image)

Figure 1. Presumed structural representations of the P2⁺ (A) and PN (B) redox states of the P-cluster, highlighting differences in ligation.

The role of alternative electron transfer mechanisms involving direct oscillations between the PN and P2⁺ states has been investigated previously (14); however, various lines of evidence indicate that only single-electron transfer events occur between the reduced PN state of the P-cluster and the active site, suggesting that the P1⁺/PN couple is the predominant oscillation of the P-cluster under turnover conditions (15). The structures of the PN and P2⁺ states have been determined previously, but the structure of the intermediate P1⁺ state is needed for defining the cycle of redox-dependent structural changes that occur during catalysis. To elucidate the structure of the P1⁺ state, structures of the nitrogenase MoFe protein were solved for crystals poised at defined oxidation-reduction potentials with redox mediator solutions in an electrochemical cell. In addition, computational analysis was performed to evaluate the ligation of P-cluster by αCys^{88} and βSer^{188} side chains corresponding to three (P2⁺/P1⁺/PN) oxidation states of P-cluster observed for MoFe protein. Together the results reveal a new state of the P-cluster with coordination distinct from the previously characterized P2⁺ and PN oxidation states.

Results

Crystals of MoFe protein were poised at different potentials in an electrochemical cell containing mother liquor supplemented with redox-active dyes (Fig. 2). Constant stirring maintained a homogenous charge throughout the solution, and a “sandwich-loop” crystal mounting technique was developed to prevent the crystals from washing off the loop during electrochemical poising. After 1 h of incubation in the electrochemical cell at known potential, the nitrogenase crystals were flash frozen in liquid nitrogen. The poised crystals in the presence of redox mediators were highly sensitive to X-rays regardless of potential through the range of poised samples. The plate morphology of the crystals prevented collecting complete data sets in all cases due to the extremely rapid decay observed when data collection was attempted across the long angle of the crystals. Despite being only able to obtain partial data sets (~60%), it was possible to reproduce our previously published results and confirm structural differences between PN and P2⁺ states (Tables S1 and S2).

The role of alternative electron transfer mechanisms involving direct oscillations between the PN and P2⁺ states has been investigated previously (14); however, various lines of evidence indicate that only single-electron transfer events occur between the reduced PN state of the P-cluster and the active site, suggesting that the P1⁺/PN couple is the predominant oscillation of the P-cluster under turnover conditions (15). The structures of the PN and P2⁺ states have been determined previously, but the structure of the intermediate P1⁺ state is needed for defining the cycle of redox-dependent structural changes that occur during catalysis. To elucidate the structure of the P1⁺ state, structures of the nitrogenase MoFe protein were solved for crystals poised at defined oxidation-reduction potentials with redox mediator solutions in an electrochemical cell. In addition, computational analysis was performed to evaluate the ligation of P-cluster by αCys^{88} and βSer^{188} side chains corresponding to three (P2⁺/P1⁺/PN) oxidation states of P-cluster observed for MoFe protein. Together the results reveal a new state of the P-cluster with coordination distinct from the previously characterized P2⁺ and PN oxidation states.

Results

Crystals of MoFe protein were poised at different potentials in an electrochemical cell containing mother liquor supplemented with redox-active dyes (Fig. 2). Constant stirring maintained a homogenous charge throughout the solution, and a “sandwich-loop” crystal mounting technique was developed to prevent the crystals from washing off the loop during electrochemical poising. After 1 h of incubation in the electrochemical cell at known potential, the nitrogenase crystals were flash frozen in liquid nitrogen. The poised crystals in the presence of redox mediators were highly sensitive to X-rays regardless of potential through the range of poised samples. The plate morphology of the crystals prevented collecting complete data sets in all cases due to the extremely rapid decay observed when data collection was attempted across the long angle of the crystals. Despite being only able to obtain partial data sets (~60%), it was possible to reproduce our previously published results and confirm structural differences between PN and P2⁺ states (Tables S1 and S2).
Density functional theory (DFT) calculations were performed for the three oxidation states of the P-cluster to evaluate the relative sequence of binding of the serine and amide ligands. The energy ordering of the possible ligation forms for every given oxidation state is reported in Fig. 5. Consistent with the experimental evidence, in the lowest-energy P$^N$ state structure, both $\alpha$Cys$^{68}$ and $\beta$Ser$^{188}$ are not bound. The P$^N_\text{S}$ is 9 kJ/mol higher than P$^N_\text{C}$, and P$^{1+}_\text{C}$ is 11 kJ/mol higher than P$^{2+}_\text{S}$ (it is 20 kJ/mol higher then P$^N_\text{S}$). Only P$^N_\text{S}$ is serine-ligated. In stark contrast, the doubly ligated P$^{2+}_\text{SC}$ isomer is 174 kJ/mol above the nonligated P$^N$ form. Oxidation of P$^N$ by one or two electrons dramatically alters the ligation preference of the cluster. The $\beta$Ser$^{188}$-ligated state, P$^{1+}_\text{SC}$, is calculated to be the most stable one-electron oxidized form of the P-cluster, −4 kJ/mol lower in energy than the nonligated form, whereas the $\alpha$Cys$^{68}$-ligated form, P$^{1+}_\text{C}$, and doubly ligated P$^{2+}_\text{SC}$ form are far higher in energy. Double oxidation of the P-cluster (P$^{2+}$) favors binding of both $\alpha$Cys$^{68}$ and $\beta$Ser$^{188}$ with the doubly ligated P$^{2+}_\text{SC}$ being significantly more stable than the P$^{2+}_\text{C}$ state, by 55 kJ/mol, and only slightly more stable, by 2 kJ/mol, than P$^{2+}_\text{S}$. When more accurate treatment of the zero-point correction is utilized, the order of the P$^{2+}_\text{S}$ and P$^{2+}_\text{SC}$ inverts with P$^{2+}_\text{SC}$ being more stable by 37 kJ/mol (Fig. S2). Unfortunately, due to numerical issues, this approach was not available for all states.

Taken as a whole, these calculations confirm that the serine side chain has a higher binding affinity than the Cys backbone amide nitrogen and that the serine side chain preferentially binds to the P-cluster after the first oxidation event (P$^{1+}$), whereas double oxidation (P$^{2+}$) greatly increases the binding affinities, resulting in the preferential formation of the doubly ligated form of the P-cluster.

**Discussion**

This newly visualized P$^{1+}$ state is compatible with the proposed deficit-spending mechanism and demonstrates a redox-mediated ligand-exchange mechanism for possibly regulating electron flow for the P$^{1+}$/P$^{N}$ redox couple. Previous mutagenesis studies targeting the $\beta$Ser$^{188}$ P-cluster ligand have utilized the introduction of a stronger ligand, such as cysteine, or removal of coordination by glycine substitution to stabilize the P$^N$ state. This newly visualized P$^{1+}$ state is compatible with the proposed deficit-spending mechanism and demonstrates a redox-mediated ligand-exchange mechanism for possibly regulating electron flow for the P$^{1+}$/P$^{N}$ redox couple. Previous mutagenesis studies targeting the $\beta$Ser$^{188}$ P-cluster ligand have utilized the introduction of a stronger ligand, such as cysteine, or removal of coordination by glycine substitution to stabilize the P$^N$ state.
mediators, which have Eu(II) ligated to polyaniminocarboxylate ligands, whereas substitutions near the Fe5–8 cube (distal side to FeMo-co) do not show this behavior (18, 19). Analyzing this information in light of our current finding, we can assign directional character to the P-cluster. Here, based on the substitution studies, the proximal side of the cubane relative to FeMo-co would be involved in the electron transfer between the P-cluster and FeMo-co during the slow step, whereas the distal cubane undergoes structural conformational changes to accommodate the loss of an electron from the P-cluster (Fig. 6).

We have also recently shown that electron transfer between the Fe protein and the MoFe protein precedes ATP hydrolysis (20). Conformational changes within the P-cluster of the MoFe protein during the deficit-spending events could potentially serve as triggers for the initiation of the ATP-hydrolysis step within the Fe protein. This would result in the Fe protein cycle of electron transfer being a conformationally controlled series of events. Furthermore, it has been previously suggested that the Fe protein–MoFe protein interaction could cause the βSer188 to transiently coordinate to the P-cluster, creating an activated P-cluster state (designated P5N) with a lowering of the potential to facilitate electron transfer between the P-cluster and FeMo cofactor (5). Consistent with this hypothesis, the present DFT calculations predict the ligated P5N state to be only 9 kJ/mol higher in energy than the P5N state and therefore potentially accessible upon slight distortion of the P-cluster environment.

The one-electron ligand exchange also raises the possibility of the involvement of the P-cluster in a proposed proton-coupled electron transfer (PCET) mechanism in nitrogenase. The βSer188 hydroxyl and αCys88 amide would presumably be protonated when not coordinated to the P-cluster. Potential proton donor/acceptors are within hydrogen-bonding distance of the βSer188 hydroxyl group (an ordered water molecule) and the αCys88 backbone amide (αGlu153 side chain). Sequential hydride formation has been postulated as part of the catalytic cycle (21); therefore, the single redox P1+/P0 couple fits well into the most recent proposed deficit-spending mechanism. Additionally, previous spectroscopic studies have also shown residues around the distal side of the P-cluster cubane (relative to FeMo-co) to be involved in PCET (17). Although this unique iron–sulfur cluster has been shown to undergo unprecedented redox-mediated structural changes, a complete understanding of the unidirectional PCET is difficult without more information regarding the complex and dynamic global conformational changes that occur when the Fe protein interacts with the MoFe protein. Current structural techniques have failed to provide details of these transient global conformational changes. By utilizing a new redox crystallography approach, complemented by DFT calculations, we are now able to populate some of these difficult to obtain transient states and piece together the details of one of nature’s most enigmatic catalytic cycles. The work presented here also has relevance to probing electron flow and redox-mediated structural changes in numerous metalloprotein systems, providing possible insights into biological electron transfer.

**Experimental procedures**

**Purification and crystallization of MoFe protein**

All chemicals were purchased from Sigma-Aldrich or Fischer Scientific and were used without further purification. WT MoFe protein from *A. vinelandii* was purified under strict anaerobic conditions according to protocols described previously (22). All proteins were obtained at greater than 95% purity, confirmed by SDS-PAGE analysis using Coomassie Blue staining, and demonstrated maximal specific activity (greater than 2,000 nmol of H2/min/mg of MoFe protein). Handling of proteins was done in septum-sealed serum vials under an argon atmosphere. All transfer of gases and liquids was done using gastight syringes. Protein crystals were grown by capillary batch diffusion in a 100% N2 atmosphere MBRAUN glove box with precipitant solutions reported previously (23).

**Poising crystals of FeMo protein with redox mediators**

Crystals were harvested anaerobically under an argon stream and immobilized on pins using a novel “sandwich” loop designed to prevent the crystals from washing away during electrochemical studies. Two micromesh loops (MiTeGen LLC, Ithaca, NY) were affixed on top of each other to the same pin, and a small piece of monofilament was placed in between to separate them. Crystals were positioned between the loops, and the monofilament was then removed to apply tension from the loops to secure the crystals. The immobilized crystals were immediately submerged in an electrode solution mimicking the precipitant solution composed of 18% PEG 4000, 50 mM Tris-HCl buffer, pH 8.0, 15% glycerol, 100 mM sodium chloride, and 1 mM redox mediator. The mediators used were methylene blue, flavin mononucleotide, and methyl viologen with midpoint potentials (E1/2) of +11, −238, and −488 mV, respectively, versus standard hydrogen electrode (24). The mediator solutions were degassed and placed in an electrochemical cell with a built-in graphite working electrode (2.8-cm2 surface area), a mesh platinum counter electrode separated by a Vycor conductive glass plug, and a saturated calomel electrode reference electrode (Fig. 2). The solutions were kept under a constant stream of humidified argon to maintain anaerobicity without modifying solution volume. The electrode solutions were poised at +11, −238, and −488 mV versus normal hydrogen electrode for methylene blue, flavin mononucleotide, and methyl viologen, respectively. An OMNI-101 microprocessor-controlled potentiostat (Cypress Systems, Lawrence, KS) was used to control potential, and constant stirring was used to achieve a uniform solution. The submerged sandwich looped crystals were
allowed to soak for 1 h and immediately flash cooled in liquid nitrogen to preserve the poised states.

**Data collection and refinement**

Data were collected at Stanford Synchrotron Radiation Lightsource BL12-2 for native *A. vinelandii* MoFe protein poised at three defined potentials. Due to the increased susceptibility to radiation damage caused by the mediator solution treatment, only partial data sets could be collected. Processed data completeness (~60%) and resolution (~2.2 Å) were similar for the poised data sets (Table 1 and Tables S1 and S2) (25). The previously determined MoFe protein structure P1 (Protein Data Bank code 2MIN) was used as an initial model for refinement (26–29). The quantum mechanics backbones were retained, the backbones were methyl-terminated unless the backbone participated in any included hydrogen-bonding interactions. When backbones were retained, the backbones were methyl-terminated beyond the included residues. The quantum mechanics structure was taken from a representative configuration of the enzyme generated as obtained from molecular dynamics simulations of the nitrogenase complex. Details about the molecular dynamics simulation and electronic structure methods are included as supporting information. The structures were optimized in the gas phase, keeping the position of the carbon atoms of the residues that were truncated frozen (Fig. S1). Calculations were performed with the NWChem (30) 6.6 quantum chemistry package using both the BP86 exchange and correlation functional (31, 32) and, for select structures, the B3LYP hybrid functional (33). The calculations adopted the following basis sets: Ahlrichs and co-workers (34) VTZ (valence triple zeta) for iron, 6–311+ + G** (35, 36) for the atoms coordinated to the iron atoms or belonging to moieties engaging direct hydrogen bonds with the P cluster, and 6–31G* (37) for all other atoms (Fig. S1). The effect of the protein environment beyond the outer coordination sphere of the P-cluster explicitly included in the calculation was modeled as a dielectric continuum using the conductor-like screening model (COSMO) framework using a dielectric constant of 10 (38, 39). Due to numerical instabilities in the vibrational analysis for some states, we were only able to obtain reliable zero-point correction for eight of the considered 12 states using normal mode analysis performed within the rigid rotor-harmonic approximation after projecting out spurious imaginary frequencies arising from the positional constraints in the geometries. For this reason, we used an approximation where we correct for the energy of the vibrational mode associated with the lost –OH (41.5 kJ/mol) or –NH bond (40.5 kJ/mol). We benchmarked this approximation against the calculated full zero-point correction energy for the states for which reliable frequencies were available and found it to provide excellent results (Table S4 and Fig. S2). Absolute values for the gas-phase and solvent-phase energies as well as zero-point corrections are provided in Table S4. The relative ordering of states was also conserved if geometries were optimized using the B3LYP hybrid functional (Table S5). In the following, we discuss the BP86 energies only because this functional yields geometries for P\(^{N}\) and P\(^{2+}_{\text{SC}}\) states that are in far better agreement with the crystallographic data than those produced using the B3LYP hybrid functional as reported in Table S5.

Calculations were performed on all three oxidation states of the P-cluster (P\(^{N}\), P\(^{2+}\), and P\(^{1+}\)) with and without the Ser –OH and Cys backbone bond. For the reduced state, P\(^{N}\), Mössbauer studies (9) have shown that all iron atoms are in the +2 state, antiferromagnetically coupled for an overall S = 0 spin. The present calculations reproduce this experimental observation, predicting the S = 0 as the most stable (Table S3). As for the P\(^{1+}\) state, our calculation clearly indicates that it is a doublet (S = 1/2), whereas the P\(^{2+}\) state is a singlet. More details about the stability of the different oxidation states are provided in Table S7.

Ligation of Ser and Cys backbone requires their deprotonation, which implies the knowledge of the pK\(_{a}\) values of these residues and in turn the free energy of the solvated proton in water. High-accuracy extrapolations of the latter are available (40), but their use is problematic because of inconsistencies between the level of theory adopted in the present calculations and those adopted for the extrapolation. Therefore, we avoided

### Table 1

**Data collection and refinement statistics for P\(^{1+}\) data set**

<table>
<thead>
<tr>
<th>Data statistics</th>
<th>P(^{1+}) data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell dimensions</td>
<td>a = 80.79 Å, b = 130.78 Å, c = 107.88 Å, α = γ = 90.00°, β = 110.85°</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
</tr>
<tr>
<td>Wavelength</td>
<td>A₁ = 0.97947</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50–2.10</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>57.06 (60.8)</td>
</tr>
<tr>
<td>Observed reflections</td>
<td>150,869</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>70,560</td>
</tr>
<tr>
<td>Average redundancy</td>
<td>2.1</td>
</tr>
<tr>
<td>I/σ</td>
<td>4.0 (1.3)</td>
</tr>
<tr>
<td>R&lt;sub&gt;SYM&lt;/sub&gt; (%)</td>
<td>14.2 (54.4)</td>
</tr>
<tr>
<td>CC (1/2)</td>
<td>0.992 (0.814)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement statistics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution (Å)</td>
<td>50–2.1</td>
</tr>
<tr>
<td>R&lt;sub&gt;SYM&lt;/sub&gt; (%)</td>
<td>23.2</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt; (%)</td>
<td>26.3</td>
</tr>
<tr>
<td>Real-space CC (%)</td>
<td>24.5</td>
</tr>
<tr>
<td>Mean B value (overall; Å)</td>
<td>0.22</td>
</tr>
<tr>
<td>Coordinate error (based on maximum likelihood; Å)</td>
<td>0.018</td>
</tr>
<tr>
<td>Bonds (Å)</td>
<td>2.291</td>
</tr>
<tr>
<td>Angles (°)</td>
<td>95.08</td>
</tr>
<tr>
<td>Ramachandran plot</td>
<td>4.67</td>
</tr>
<tr>
<td>Most favored (%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Additional allowed (%)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Numbers in parentheses refer to the highest-resolution shell.*
Structure of nitrogenase P-cluster intermediate

the direct use of the free energy of the solvated proton by estimating the overall energetics of ligation for each oxidation state of the P-cluster with respect to methanol (Ser ligation) and methylacetamide (Cys backbone amide) in aqueous solution of which accurate $p_K$ values are available. Details of the calculations are provided in the supporting information.


**Acknowledgments**—Use of the Stanford Synchrotron Radiation Light-source (SSRL), SLAC National Accelerator Laboratory, is supported by the United States Department of Energy (DOE). Office of Science, Basic Energy Sciences under Contract DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research and by the National Institutes of Health, National Institute of General Medical Sciences (including Grant P41GM103393). Computer resources were provided by the W. R. Wiley Environmental Molecular Sciences Laboratory, a DOE Office of Science User Facility, located at Pacific Northwest National Laboratory and sponsored by DOE’s Office of Biological and Environmental Research.

**References**


Structure of nitrogenase P-cluster intermediate