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Structural characterization of the nitrogenase molybdenum-iron protein with the substrate acetylene trapped near the active site

Stephen M. Keable[a], Jacopo Vertemara[b], Oleg A. Zadvornyy[c], Brian J. Eilers[a], Karamatullah Danyal[d], Andrew J. Rasmussen[d], Luca De Gioia[b], Giuseppe Zampella[b], Lance C. Seefeldt[d,*], John W. Peters[a,c,**]

[a] Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, United States
[b] Department of Biotechnology and Life Sciences, University of Milano-Bicocca, Milan, Italy
[c] Institute of Biological Chemistry, Washington State University, Pullman, WA 99163, United States
[d] Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322, United States

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ABSTRACT

The biological reduction of dinitrogen (N_2) to ammonia is catalyzed by the complex metalloenzyme nitrogenase. Structures of the nitrogenase component proteins, Iron (Fe) protein and Molybdenum-iron (MoFe) protein, and the stabilized complexes these component proteins, have been determined, providing a foundation for a number of fundamental aspects of the complicated catalytic mechanism. The reduction of dinitrogen to ammonia is a complex process that involves the binding of N_2 followed by reduction with multiple electrons and protons. Electron transfer into nitrogenase is typically constrained to the unique electron donor, the Fe protein. These constraints have prevented structural characterization of the active site with bound substrate. Recently it has been realized that selected amino acid substitutions in the environment of the active site metal cluster (Iron-molybdenum cofactor, FeMo-co) allow substrates to persist even in the resting state. Reported here is a 1.70 Å crystal structure of a nitrogenase MoFe protein α-$96^{Arg \rightarrow Gln}$ variant with the alternative substrate acetylene trapped in a channel in close proximity to FeMo-co. Complementary theoretical calculations support the validity of the acetylene interaction at this site and is also consistent with more favorable interactions in the variant MoFe protein compared to the native MoFe protein. This work represents the first structural evidence of a substrate trapped in the nitrogenase MoFe protein and is consistent with earlier assignments of proposed substrate pathways and substrate binding sites deduced from biochemical, spectroscopic, and theoretical studies.

1. Introduction

Nitrogenase, which catalyzes the reduction of atmospheric dinitrogen (N_2) to ammonia (NH_3) in bacteria and archaea [1], is an integral component of the global nitrogen cycle [2]. The most abundant nitrogenase, the molybdenum-dependent enzyme, contains two metalloprotein components, Molybdenum-iron (MoFe) protein and Iron (Fe) protein [2,3]. The MoFe protein is an $\alpha_2\beta_2$ heterotetramer that contains 2 [8Fe-7S] P clusters and 2 active site [7Fe-9S-C-Mo-homocitrate] Iron-molybdenum cofactor (FeMo-co), and must undergo multiple cycles of MgATP-dependent association and dissociation events to deliver electrons from the Fe protein to the substrate binding and catalytic sites in the MoFe protein [4,5]. The Fe protein is a homodimer bridged by a [4Fe-4S] cubane and has two sites for MgATP binding and hydrolysis, one in each subunit [6]. The proposed substrate binding site of nitrogenase is located in the MoFe protein at the FeMo-co [1,7]. Several amino acid sequence comparisons [8] and site-directed mutagenesis studies [9,10] have shown that certain amino acid residues are important for substrate interactions at the FeMo-co including α-70^{Val}, α-195^{His}, and α-96^{Arg} [10–13]. It has been shown that the substitution of α-70^{Val}, by amino acids with small side chain, resulted in the ability of the MoFe protein from *Azotobacter vinelandii* to reduce larger organic substrates such as propyne, or 1-butyne, which are normally poor substrates for nitrogenase. On the other hand, the substitution of the α-70^{Val} by longer side chain, for example isoleucine, decrease the ability of the MoFe protein reduce a number of substrates including dinitrogen and acetylene, while maintaining normal proton reduction activity, indicating an important role of the α-70^{Val} at the substrate binding site at the MoFe protein [11].

The structural characterization of nitrogenase with bound substrate

* Corresponding author.
** Corresponding author at: Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717, United States
E-mail addresses: lance.seefeldt@usu.edu (L.C. Seefeldt), jw.peters@wsu.edu (J.W. Peters).

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has been a challenging problem due to the complex interplay between the component proteins and the corresponding high turnover rate of the substrates. The binding of substrates (e.g., acetylene) to the FeMo-co was detected only under turnover conditions where all components of the nitrogenase complex and a reducing agent are present [14]. Previous biochemical and Electron Paramagnetic Resonance (EPR) spectroscopy studies have demonstrated the ability of several site directed variants to trap acetylene and cyanide substrates at the active site of MoFe protein under turnover conditions [12,13] and in the resting state [10,15]. Acetylene-derived species bound to the FeMo-co in α-195$^{His \rightarrow Gln}$ MoFe protein variant had been characterized under turnover conditions using Q-band EPR and Electron Nuclear Double Resonance (ENDOR) spectroscopy [12,13]. It has been shown that the EPR signals of the dithionite-reduced MoFe protein variants where α-96^{Arg} were substituted by leucine, glutamine, alanine, or histidine are similar to that from native MoFe protein. The spectroscopic characterization of these MoFe protein variants incubated with acetylene or cyanide resulted in changes of the EPR signal, showing interaction of the acetylene and cyanide with FeMo-co in the resting state of the MoFe proteins [10]. To capitalize on this finding, the α-96$^{Arg \rightarrow Gln}$ variant of MoFe protein was crystallized in the presence of acetylene. Reported here are the first crystal structure and computational analysis of the nitrogenase MoFe protein variant α-96$^{Arg \rightarrow Gln}$ with acetylene captured near the FeMo-co active site.

2. Experimental

2.1. Cell growth and protein purification

Nitrogenase MoFe protein was expressed in *Azotobacter vinelandii* strain DJ1264 (α-96$^{Arg \rightarrow Gln}$) and grown as previously described [16]. The variant MoFe protein was purified to > 95% purity, confirmed by SDS gel electrophoresis. All protein manipulations were carried out in sealed serum vials under argon atmosphere or in an anaerobic MBraun glovebox (M.Braun Inc., USA) under 100% of nitrogen.

2.2. Crystallization, data collection and refinement

MoFe protein crystals were grown by the capillary batch diffusion method [17] in a solution containing 30% (*w/v*) polyethylene glycol (PEG) 4000, 0.1 M Tris (pH 8.0), 0.17 M Na_2MoO_4, and 0.001 M sodium dithionite [18]. Prior to crystallization, both the protein solution and the precipitant solution were saturated with acetylene gas by sparging for 5 min. Crystals of dark brown colour with dimensions of ~200 μm × 200 μm × 200 μm were observed within 4–6 weeks. Crystals were treated with acetylene-saturated mother liquor supplemented with 15% (v/v) glycerol prior to flash cooling in liquid nitrogen. Data collection was carried out under a continuous nitrogen stream (~100 K) on Beam Line 12-2 at the Stanford Synchrotron Radiation Laboratory (Table SI 1).

Data collected from nitrogenase variant crystals were processed and scaled by XDS [19] and HKL2000 [20]. The unit cell parameters of the collected data were isomorphous to the published native MoFe protein structure (PDB ID: 3U7Q [21]). Initial rigid body refinement was carried out using REFMAC5 [22,23]. The solutions were refined and improved by phenix.refine [24] with final R_{work}/R_{free} 15.7/19.7 (Table SI 1). Model building was subsequently completed manually using COOT [25]. Figures were prepared using PyMol [26]. The coordinates of the α-96$^{Arg \rightarrow Gln}$ nitrogenase variant structure were deposited in the PBD databank with code 6BBL.

2.3. Theoretical basis and theoretical calculations

Density functional theory (DFT) calculations have been carried out using the TURBOMOLE 7.0.1 suite of programs [27]. The following mixed basis sets has been used: 1) a split valence triple-ζ basis set with polarization (TZVP) [28] for FeMo-co (E0, S = 3/2 state), α-442^{His}, α-275^{Cys}, homocitrate (that are all covalently bound to Fe-S cluster) and acetylene; 2) a triple-ζ basis set with double polarization and diffuse functions (def2-TZVPD) [29] for Mo atom; 3) a single-ζ basis set (SVP) [30] for amino acid side chain atoms. The pure exchange-correlation functional Becke Lee Yang Parr (BLYP) [31] has been adopted in that it was already validated in literature for Quantum mechanics (QM) nitrogenase investigations [32]. Dispersion correction to Self -Consistent Field (SCF) energy has been performed using the approach proposed by Grimme for DFT [33]. Polarization effects of both solvent and active site amino acid side chains have been evaluated according to the conductor-like screening model (COSMO, ε = 40) [34–36]. Spin configurations of iron atoms within FeMo-co (determined by a Broken Symmetry approach) [36] have been verified to correspond to that generally accepted for E0 i.e., all iron ions are reciprocally antiferromagnetically coupled, in line with previous investigations [37].

DFT geometry optimization included the FeMo-co/acetylene complex and relevant amino-acid residues (α-65^{Ala}, α-69^{Gly}, α-70^{Val}, α-96^{Gln}, α-191^{Gln}, α-195^{His}, α-229^{Tyr}, α-231^{Ile}, α-275^{Cys}, α-278^{Ser}, α-359^{Arg}, α-381^{Phe}, α-442^{His}) starting from crystal structure of α-96$^{Arg \rightarrow Gln}$ MoFe variant. Amino acid residues have been truncated at Cα so all the atoms of side chains are present in the model. To our knowledge, this is the largest size setup related to QM-only (i.e., no atom in the model is treated by semi-empiric and/or classic Force Field based methods) investigations on nitrogenase. The original purpose of maintaining zwitterionic forms of amino acids has been subsequently dropped as it yields unrealistic bond dissociations during simulations. Twelve molecules of water identified by crystallization have been included in calculation to simulate explicitly the presence of solvent. Both possible protonation states of homocitrate covalently bound to Fe-S cluster have been considered. This system is studied considering two charge state assignments: the first state has a total charge equal to − 4 according to the FeMo-co and amino acids present in the model; the other one is a neutral charge model. The effect of neutralizing the − 4 charge of the computational model that we have set up in the present study has been investigated by placing 4 K^+ ions at the exact position where it is located the centroids of the ammonium and the three guanidinium groups belonging to α-304^{Lys}, α-28^{Arg}, α-203^{Arg}, and α-439^{Arg} in the X-ray structure.

The computational model of native MoFe protein active site has been obtained starting from X-ray structure 1M1N [38]; the amino acids residues taken into explicit account were exactly the same used for the α-96$^{Arg \rightarrow Gln}$ MoFe variant structure, with the (trivial) sole exception of α-96^{Arg}. Two sets of calculations have been performed for native MoFe protein: one is with total charge equal to − 3 (arising from FeMo-co and amino acids present in the DFT model of native system); the second is with total charge 0 (− 3 charge has been neutralized by three K^+, placed according to criteria previously described). The same corrections to pure DFT energy that were used for the α-96$^{Arg \rightarrow Gln}$ MoFe variant protein have also been applied to the native MoFe protein model (dispersion correction, implicit solvation model and counterpoise correction). Different rotamers of α-96^{Arg} have been energetically sampled [39].

2.4. Refinement levels used in DFT calculation

A first level refinement has been performed including effects of dispersive forces in the model. This was necessary to treat the attractive component of the potential energy curve associated with London-type interactions [33,40], which is normally missing in standard density functionals, due to the fact that the stabilizing effect of dispersion occurs from medium- to long-range distance (where electron densities based on truncated basis sets do not overlap). A second level refinement has required single-point corrections including effect of polarizing environment of the active site pocket (ε = 40, as often used for a large number of protein active sites [34]). A third level refinement has been