Localization of a Catalytic Intermediate Bound to the FeMo-cofactor of Nitrogenase*§

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Nitrogenase catalyzes the biological reduction of N₂ to ammonia (nitrogen fixation) as well as the reduction of a number of alternative substrates, including acetylene (HC≡CH) to ethylene (H₂C≡CH₂). It is known that the metallocluster FeMo-cofactor located within the nitrogenase MoFe protein component provides the site of substrate reduction, but the exact site where substrates bind and are reduced on the FeMo-cofactor remains unknown. We have recently shown that the α-70 residue of the MoFe protein plays a significant role in defining substrate access to the active site; α-70 approaches one face of the FeMo-cofactor, and when valine is substituted by alanine at this position, the substituted nitro-arginine-directed binding of nitrogenase to include larger alkynes (12–15). For example, substitution of the α-70Val residue by alanine has been shown to expand the substrate range of nitrogenase to include propyne (HC≡CCH₃) or propargyl alcohol (HC≡CH₂OH, propargyl-OH). During this reduction, a substrate-derived intermediate can be trapped on the FeMo-cofactor resulting in an S = 1/2 spin system with a novel electron paramagnetic resonance spectrum. In the present work, trapping of the propargyl-OH-derived or propargyl amine (HC≡CCH₂NH₂, propargyl-NH₂)-derived intermediates is shown to be dependent on pH and the presence of histidine at position α-195. It is concluded that these catalytic intermediates are stabilized and thereby trapped by H-bonding interactions between either the –OH group or the –NH₂ group and the imidazole ε-NH of α-195NH₂. Thus, for the first time it is possible to establish the location of a bound substrate-derived intermediate on the FeMo-cofactor. Refinement of the binding mode and site was accomplished by the use of density functional and force field calculations pointing to an S² coordination at Fe-6 of the FeMo-cofactor.

Nitrogenase is comprised of two component proteins, called the iron protein and the MoFe protein, which together catalyze the nucleotide-dependent reduction of N₂ to ammonia (Equation 1).

\[
\text{N}_2 + 8e^- + 16\text{MgATP} + 8\text{H}^+ \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{MgADP} + 16\text{P}_i
\]

(Eq. 1)

During catalysis, electrons are delivered one at a time from the iron protein to the MoFe protein in a reaction coupled to the hydrolysis of 2 eq of MgATP for each equivalent of electrons transferred (1, 2). The MoFe protein contains two metalloclusters called the P-cluster [8Fe–7S] and the FeMo-cofactor [7Fe–9S–Mo–X–homocitrate], where X is proposed to be nitrogen, carbon, or oxygen (3). The P-clusters are thought to mediate electron transfer from the iron protein to the FeMo-cofactor, which in turn provides the site for substrate binding and reduction. The structure of the FeMo-cofactor has been elucidated from the solution of x-ray structures of MoFe proteins (3–7), yet where and how substrates interact with the FeMo-cofactor is still unknown. Different models for where substrates bind to the FeMo-cofactor have been developed; they were built on evidence from model compounds, theoretical calculations, and kinetic and biophysical studies on the wild-type (WT)³ and genetically altered MoFe proteins (8). Some models propose binding and reduction of substrates at the molybdenum atom, whereas others suggest binding and reduction of substrates at one or more of the six iron atoms that constitute the central portion of the FeMo-cofactor. Models have also been proposed that involve substrate binding of both molybdenum and iron at different steps during the reduction reaction (9–11).

Recently, we have pursued genetic and biophysical approaches on nitrogenase to localize the substrate binding site on the FeMo-cofactor (12–16). It has been demonstrated that substitution of α-70Val by alanine has been shown to expand the substrate range of nitrogenase to include propyne (HC≡CCH₂) or propargyl alcohol (HC≡CCH₂OH, propargyl-OH) (14). When the α-70Val MoFe protein is freeze-trapped during the reduction of propargyl-OH, a reduction intermediate bound to the FeMo-cofactor is captured (15). Using ¹³C- and ¹⁵N-labeled propargyl-OH and electron nuclear double resonance spectroscopic methods, we have recently deduced that the trapped intermediate has two hydrogen atoms added (i.e. allyl alcohol, H₂C≡CH₂OH) and is bound to iron such that the two terminal hydrogen atoms are spectroscopically indis-

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§ The on-line version of this article (available at http://www.jbc.org) contains a supplemental QuickTime movie.

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³ The abbreviations used are: WT, wild-type; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; TAPS, 3-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]-1-propanesulfonic acid.
Substrate Localization on the FeMo-cofactor

Protein Purification and Activity Assays—Azotobacter vinelandii strains DM1510 and DM1518 expressing the α-70\textsuperscript{His} and α-70\textsuperscript{His}-195\textsuperscript{Val} variant MoFe proteins, respectively, were constructed using site-directed mutagenesis and gene replacement techniques as described previously (12, 13). The α-70\textsuperscript{His} and α-70\textsuperscript{His}-195\textsuperscript{Val} variant MoFe proteins were purified using a poly(His)-metal affinity chromatography system described earlier (15). The wild-type iron protein component of nitrogenase was purified essentially as described previously (19). All manipulations of proteins were conducted in septum-sealed glass vials under an argon atmosphere, and all anaerobic liquid and gas transfers were performed using gas-tight syringes. Acetylene reduction, H\textsubscript{2} evolution, and \textsubscript{N} \textsubscript{2} reduction activities were determined as described earlier (20, 21). \textsubscript{N} \textsubscript{2} was quantified using a liquid chromatographic fluorescence method with α-phthalaldehyde mercaptobenzothiazole as described previously (22). Thirty \textsubscript{μ}l of an assay reaction producing \textsubscript{N} \textsubscript{2} was added to 1 ml of a solution containing 19 mM phthalic dicarboxylic acid, 3.4 mM 1,2-mercaptobenzothiazole, 5% (v/v) ethanol, and 190 mM potassium phosphate, pH 7.3, and allowed to react in the dark for 30 min. The mixture was injected and separated on a C-18 guard column and detected by fluorescence (\textlambda\textsubscript{excitation}=370 nm, \textlambda\textsubscript{emission}=440/472 nm). The \textsubscript{N} \textsubscript{2} quantification was standardized with NH\textsubscript{4}Cl. The K\textsubscript{m} for propargyl-OH was estimated from the apparent affinity for acetylene (K\textsubscript{m(app)} in the presence of varying propargyl-OH concentrations and then plotting K\textsubscript{m(app)} versus propargyl-OH concentration (23).

Preparation of Non-turnover and Turnover MoFe Protein Electron Paramagnetic Resonance Samples—Non-turnover (resting state) MoFe protein (100 \textsubscript{μ}M) samples were made in 100 mM MOPS buffer, pH 7.0, with 30 mM sodium dithionite (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}) under 1 atm of argon. Nitrogenase turnover samples were prepared by the addition of the MoFe protein (100 \textsubscript{μ}M) to a buffer mixture (100 mM MES, 100 mM MOPS, 100 mM Tris, and 100 mM TAPS) at defined pH values and containing 30 mM sodium dithionite, 10 mM ATP, 15 mM MgCl\textsubscript{2}, 20 mM phosphocreatine, 2 mg/ml bovine serum albumin, and 0.3 mg/ml creatine phosphokinase. The reaction was initiated by the addition of iron protein (50 \textsubscript{μ}M) and was allowed to react for ~20 s at room temperature before being frozen in EPR tubes in liquid nitrogen. Proteins were added from a concentrated stock solution and did not significantly alter the reaction pH. Where appropriate, propargyl-OH (3 mM) or propargyl-NH\textsubscript{2} (15 mM) was included in the initial reaction mixture. Standard 4-mm quartz EPR tubes were used for all samples.

EPR Spectroscopy—X-band EPR spectra were recorded on a Bruker ESP-300 E spectrometer equipped with an ER 4116 dual-mode X-band cavity including an Oxford Instruments ESR-900 helium flow cryostat. EPR spectra were recorded at a modulation frequency of 100 kHz, a modulation amplitude of 1.58 milliteslas (12.8 gauss), a sweep rate of 10 milliteslas/s, and a microwave frequency of ~9.65 GHz (with the precise value recorded for each spectrum to ensure exact g alignment). All spectra were recorded at 8000 and a microwave power of 2.0 mW with each trace being the sum of five scans. The software program IGR Pro (WaveMetrics, Lake Oswego, OR) was used for all subsequent manipulation of spectral data.

Theoretical Calculations—Spin-unrestricted all-electron density functional calculations of the FeMo-cofactor with bound intermediates used the blyp functional with numerical basis sets as implemented in the software program DMol\textsuperscript{3} (24–26). Calculations of the FeMo-cofactor with bound intermediates within the protein involved a large protein component composed of 1692 amino acid residues, 1532 associated water molecules, and the P-cluster together with the FeMo-cofactor. This large protein component was selected as all of chains A and B of α-70\textsuperscript{Val}-70\textsuperscript{His}-195Val MoFe protein (100 \textsubscript{μ}M) and did not significantly alter the reaction pH. This large protein component was selected as all of chains A and B of α-70\textsuperscript{Val}-70\textsuperscript{His}-195Val MoFe protein (100 \textsubscript{μ}M) and did not significantly alter the reaction pH.

RESULTS AND DISCUSSION

Relevant Features of the α-70\textsuperscript{His}-substituted MoFe Protein—Substitution of the MoFe protein α-70\textsuperscript{Val} residue by alanine expands the substrate range for nitrogenase to include the short chain alkynes propyne (HC≡CH\textsubscript{2}) and propargyl alcohol (14). When propargyl-OH is used as a nitrogenase substrate and freeze-quenched under turnover conditions, a paramagnetic intermediate is observed that results from conversion of the resting state S = 3/2 spin system to S = 1/2 spin system having a rhombic EPR signal with g values of 2.123, 1.998, and 1.986 (Fig. 1, trace 2). The lower set of traces (3, 4, and 5) is of the α-70\textsuperscript{His} MoFe protein under turnover conditions with protons as substrate at pH 7.0 (trace 3) or with 3 mM propargyl-OH as substrate at pH 6.7 (trace 4) and pH 8.7 (trace 5). All samples contained 100 \textsubscript{μ}M α-70\textsuperscript{His} MoFe. Other conditions are presented under “Experimental Procedures.”

In contrast to propargyl-OH, when propyne is used as the substrate, freeze-quenching under turnover conditions does not result in the formation of a trapped adduct that can be observed by EPR. An obvious explanation for this difference is that the –OH group of propargyl-OH stabilizes the bound intermediate through hydrogen bonding interactions with a functional group.

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provided by an amino acid located within the vicinity of the substrate binding site. Identification of the proposed functional group thus provided an unprecedented opportunity to identify where and how an alkyne substrate might interact with the nitrogenase active site. Toward this end, the environment around α-70Val and the FeMo-cofactor in the resting state x-ray structure of the MoFe protein (3) was examined and the imidazole of α-195His identified as the most likely candidate to provide the hydrogen bonding interaction with propargyl-OH. This possibility suggested two predictions that could be experimentally tested. First, if a propargyl-OH reduction intermediate is stabilized by a hydrogen bond interaction with the imidazole group of α-195His, such stabilization should be pH-dependent having a pK_a value near that expected for a histidine residue. Second, an ability to trap the EPR-active intermediate should be dependent on having a histidine residue at the α-195 residue position.

Appearance of the Freeze-trapped Propargyl-OH Reduction Intermediate Is pH-dependent—Fig. 1 illustrates the pH dependence of the intensity of the propargyl-OH-derived EPR signal. The maximal EPR signal intensity, which is interpreted to indicate the maximal concentration of the trapped adduct, is observed at a pH ~6.7. As the pH value increases from 6.7 to 8.7, the intensity of the EPR signal steadily decreases (Fig. 1, traces 4 and 5). No significant changes in EPR lineshape are observed over this pH range, indicating a simple depopulation of the intermediate bound state as pH rises. A more complete data set for the intensity of the propargyl-OH-dependent EPR signal versus pH is presented in Fig. 2, also illustrating a depopulation of the trapped adduct as the pH value increases from 6.7 to 9.0. This result is consistent with the deprotonation of a group having a pK_a ~7.5 and the fact that a protonated state is required for formation of a hydrogen bond necessary to elicit the EPR signal associated with the trapped species. In proteins, histidine residues can have pK_a values ranging from 5 to 9, depending on the protein environment (27). The NH underline portion of α-195His is surrounded by the hydrophobic residues α-65Ala, α-66Gly, α-70Ala, α-71Val, and α-381Pro together with the negatively charged sulfur atoms of the FeMo-cofactor, generating a net negative electrostatic potential. This environment would be predicted to increase the pK_a of the imidazole of α-195His (28). Thus, one explanation of the results is that the deprotonated state of the NH underline of α-195His acts as a hydrogen bond donor to a non-bonded electron pair of the oxygen of bound allyl-OH (Fig. 3, panel A). As the NH underline of α-195His becomes deprotonated (with an observed pK_a of 7.5), the hydrogen bond with the oxygen of allyl-OH would greatly diminish.

The pH dependence for the formation of the propargyl-OH trapped intermediate can be contrasted with the previously reported pH dependence for acetylene reduction by nitrogenase. In the latter case, maximal activity is around pH 7.3 with a decline in activity at pH values higher and lower than pH 7.3 (29). The pH optimum value of 6.7 observed here for the formation of the propargyl-OH-trapped species is significantly lower than the pH optimum value of 7.3 for activity, suggesting that a specific deprotonation event is controlling the trapping of the intermediate.

Appearance of a Propargyl-NH underline Reduction Intermediate Is Also pH-dependent—More convincing evidence that the appearance of the trapped EPR-active species is the result of specific hydrogen bonding between a reduction intermediate and an active site residue (rather than a nonspecific effect on enzyme activity) was obtained by using propargyl amine (propargyl-NH underline) as a substrate instead of propargyl-OH. If the appearance of the trapped species derived from propargyl-OH is dependent on hydrogen bonding provided by the protonated imidazole group of α-195His, it is predicted that the appearance of a trapped species when propargyl-NH underline is used as substrate would require a deprotonated imidazole group (Fig. 3B) because the pK_a of propargyl-NH underline is higher than that of imidazole. In control experiments, it was shown that, like propargyl-OH, propargyl-NH underline is an inhibitor of acetylene reduction (50%
inhibition observed by inclusion of 20 mM propargyl-NH₂ in an assay with 0.003 atm of acetylene at pH 8.0) and therefore interacts with the active site. When the α-70⁸⁸⁸² MoFe protein is freeze-quenched during turnover using propargyl-NH₂ as substrate, an EPR-active species is also detected with the same lineshape as the propargyl-OH-derived species with apparent g values of 2.12, 2.00, and 2.00 (Fig. 4). Given the similarity in lineshape of the EPR spectra elicited under freeze-quench conditions when either propargyl-OH or propargyl-NH₂ is used as substrate, it is presumed that an allyl-NH₂ adduct is bound to an iron atom of the FeMo-cofactor in a way similar to that proposed for propargyl-OH (17). One difference in behavior between these two substrates is the lower intensity of the propargyl-NH₂ (15 mM)-elicited EPR signal (approximately one-fourth) when compared with propargyl-OH (3 mM)-dependent EPR signal intensity. Another important difference is the pH profile for the formation of the respective intermediates (Fig. 2). As can be seen, the population of the trapped propargyl-NH₂ reduction intermediate EPR-active species increases with rising pH (maximizing at about pH 8.2) followed by a rapid decline. The important observation is that the pH required for maximizing the propargyl-NH₂-elicited EPR signal is significantly shifted to a higher pH than required for maximizing the propargyl-OH-elicited EPR signal. This feature is in line with a requirement for a protonated imidazole group for hydrogen bonding to propargyl-OH and a deprotonated imidazole group for hydrogen bonding to propargyl-NH₂ (Fig. 3). The rapid decline in population of the trapped species when propargyl-NH₂ is used as substrate can be explained by the deprotonation of both the imidazole group of α-19⁵⁵⁵α and the amino group of propargyl-NH₂ (30). Taken together, the different pH dependences required to populate intermediate states when either propargyl-OH or propargyl-NH₂ is used as substrate provides a compelling case for the protonation or deprotonation of an imidazole group of α-19⁵⁵⁵α as controlling the stabilization of bound intermediates by hydrogen bonding interactions.

The Imidazole Group of α-19⁵⁵⁵α Is Required for Intermediate Stabilization—Previous work has shown that substitution of the MoFe protein α-19⁵⁵⁵α residue by glutamine results in an altered MoFe protein that can bind N₂ as effectively as wild-type MoFe protein but is not able to effectively reduce N₂ (<2% N₂ reduction) (31–33). In contrast, the α-19⁵⁵⁵α-substituted MoFe protein is unaffected in its ability to reduce acetylene or protons (32, 33). The explanation offered for these features is that the imidazole group of α-19⁵⁵⁵α is specifically required to stabilize an N₂ reduction intermediate or is required as a proton donor for N₂ reduction (32, 33). These observations taken together indicate that α-19⁵⁵⁵α is the most likely candidate for providing the ionizable group responsible for stabilizing propargyl-OH or propargyl-NH₂ intermediates. If α-19⁵⁵⁵α is responsible for hydrogen bonding interactions necessary to stabilize a propargyl-OH reduction intermediate, such an interaction should be lost by substitution of glutamine for the MoFe protein α-19⁵⁵⁵α residue. This prediction was tested by the construction and characterization of a doubly substituted MoFe protein where α-19⁵⁵⁵α is substituted by glutamine and α-70⁰⁰⁰α is substituted by alanine. The α-70⁰⁰⁰α/α-19⁵⁵⁵α doubly substituted MoFe protein retains ~65% of the wild-type acetylene and proton reduction activities (Table 1), indicating no major disruption in the binding or reduction of these substrates. Like the α-19⁵⁵⁵α MoFe protein, the doubly substituted MoFe protein shows very low N₂ reduction activity (~1%). Further, propargyl-OH remains an effective inhibitor of acetylene reduction for the doubly substituted MoFe protein with a Kᵢ of 8 mM compared with the published value of 4 mM for the α-70⁰⁰⁰α MoFe protein (14), clearly indicating that propargyl-OH continues to associate with the doubly substituted MoFe protein. However, no EPR-detectable adduct is observed when the α-70⁰⁰⁰α/α-19⁵⁵⁵α MoFe protein is freeze-quenched during turnover when either propargyl-OH or propargyl-NH₂ is used as substrate (data are shown in Fig. 2 for propargyl-OH).

Refined Identification of the Substrate Binding Site by Density Functional and Force Field Calculations—The above results all point to the formation of a hydrogen bond between the –OH or –NH₂ group of the propargyl-OH- or propargyl-NH₂-bound reduction intermediate and the imidazole of α-19⁵⁵⁵α, thereby localizing the position of the –OH or –NH₂ groups to be within ~2 Å of the eN of α-19⁵⁵⁵α. Further, electron nuclear double resonance spectroscopic characterization (17) has indicated that the C=C portion of allyl-OH is bound to a single iron atom in an η⁶ configuration (Scheme 1). With these constraints, the location of the bound intermediate is largely defined. To further define the likely binding site, theoretical calculations were used. The strategy employed density functional methods to elucidate the detailed geometry of bonding of the intermediate to the FeMo-cofactor and then to test the fit into the α-70⁰⁰⁰α protein using force field methods with all hydrogen atoms explicitly included. The density functional calculations were made on a model that includes the essential coordination features of the FeMo-cofactor, namely Fe₃MoS₉N³(SCH₃)₂(OCH₂COO)(C₃H₂N₂H₄), with net charge −3 corresponding to the resting state (34). The resulting structures with bound intermediate were then substituted (in silico) for FeMo-cofactor in the protein with α-70⁰⁰⁰α and relaxed to assess their ability to meet two criteria, which are hydrogen bonding with eN of α-19⁵⁵⁵α and accommodation by the α-70⁰⁰⁰α protein but not the wild-type α-70⁰⁰⁰α protein.

From these calculations, the best binding modes for allyl-OH and allyl-NH₂ were deduced, where the alkene portion is bound η⁶ at Fe-6 in a position that is closer to exo than endo. For both allyl-OH and allyl-NH₂, a good H-bond is formed with the eN of α-19⁵⁵⁵α and with a μ-S (SZB) of FeMo-cofactor (Fig. 5). Normal van der Waals contact occurs between the methyl group of α-70⁰⁰⁰α and the bound intermediates, but as expected there is impossible conflict with the side chain of α-70⁰⁰⁰α in the wild-

![Fig. 4. X-band EPR spectra of α-70⁰⁰⁰α MoFe protein in the resting and turnover states with propargyl-NH₂ at different pH values.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ on July 24, 2018.
Table I

| MoFeP               | C2H2 (0.1 atm) | Argon (1 atm) | N2 (1 atm) |
|---------------------|----------------|---------------|------------|
|                     | Specific activitya, C2H4 | WTb | % | % | % |
| Wild-type           | 1860 ± 40      | 100           | 0.005 ± 0.001 | 2250 ± 70 | 100 | 940 ± 60 | 100 |
| α-70Ala             | 1680 ± 60      | 89            | 0.003 ± 0.001 | 2200 ± 50 | 98  | 790 ± 20  | 54  |
| α-70Ala/α-195Gln    | 1240 ± 60      | 67            | 0.007 ± 0.001 | 1460 ± 60 | 65  | 8 ± 2     | <1  |

* Specific activities are reported in units of nmol of product/min/mg of MoFe protein and were determined with an iron protein:MoFe protein molar ratio of 40:1.

† % WT were calculated by a ratio of the activity of the altered MoFe protein to the activity of wild-type.

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