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Insights into substrate binding at FeMo-cofactor in nitrogenase from the structure of an α–70^{Ile} MoFe protein variant

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Abstract

The x-ray crystal structure is presented for a nitrogenase MoFe protein where the alpha subunit residue at position 70 (α-70^{Val}) has been substituted by the amino acid isoleucine (α-70^{Ile}). Substitution of ?-70^{Val} by ?-70^{Ile} results in a MoFe protein that is hampered in its ability to reduce a range of substrates including acetylene and N_2, yet retains normal proton reduction activity. The 2.3 Å structure of the α-70^{Ile} MoFe protein is compared to the α-70^{Val} wild type MoFe protein, revealing that the δ methyl group of ?-70^{Val} is positioned over Fe 6 within the active site FeMo-cofactor. This work provides strong crystallographic support for the previously proposed model that substrates bind and are reduced at a single 4Fe-4S face of the FeMo-cofactor and that when ?-70^{Val} is substituted by ?-70^{Ile} access of substrates to Fe6 of this face is effectively blocked. Furthermore the detailed examination of the structure provides the basis for understanding the ability to trap and characterize hydrides in the variant, contributing significantly to our understanding of substrate access and substrate reduction at the FeMo-cofactor active site of nitrogenase.

Keywords

Nitrogenase; MoFe protein; FeMo-cofactor; Proton reduction; Hydride intermediate

Introduction

The enzyme nitrogenase catalyzes the reduction of N_2 to two ammonia molecules in a reaction having an ideal stoichiometry shown in equation 1.

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§The coordinates for this structure have been submitted to the RCSB Protein Data Bank for release upon submission (PDB code 3K1A).

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$$N_2 + 8e^- + 8\,H^+ + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,P_i \quad \text{Eq 1}$$

In the Mo-dependent nitrogenase, this reaction is catalyzed by two component proteins called the Fe protein and the MoFe protein [1]. The Fe protein delivers electrons to the MoFe protein in a reaction dependent on the hydrolysis of MgATP. The MoFe protein contains the site of substrate binding, which occurs at a heterometallic cofactor called FeMo-cofactor [7Fe-9S-Mo-X-homocitrate] [2,3]. FeMo-cofactor contains many possible sites of N_2 binding including three identical 4Fe-4S faces and Mo [4,5]

The x-ray structure of MoFe proteins shows FeMo-cofactor is covalently bound to the MoFe protein through Cys ligation to an Fe at one end and through His ligation to the Mo on the other end [6,7]. The MoFe protein environment surrounding FeMo-cofactor is asymmetric, with a wide range of different amino acids defining the first shell of non-covalent interactions. In an effort to define where and how substrates interact with FeMo-cofactor, amino acids within this region have been substituted with different amino acids [8–13]. This approach was used to identify ?-70^{Val} as a residue that controls substrate access to FeMo-cofactor [14,15]. The side chain of ?-70^{Val} is in close contact to one Fe-S face of FeMo-cofactor (Fe atoms 2, 3, 6, and 7 in the numbering system established in 1M1N.PDB) [7], indicating that it could control access of small molecules to the site of substrate interaction on FeMo-cofactor. To test this model, α-70^{Val} was substituted by a variety of different amino acids [16]. Substitution of ?-70^{Val} by residues with smaller side chains resulted in a capacity to reduce compounds that are normally very poor substrates for nitrogenase, such as hydrazine ($H_2N\text{-}NH_2$), propyne ($HC{\equiv}C\text{-}CH_3$), propargyl alcohol ($HC{\equiv}C\text{-}CH_2OH$), and 1-butyne ($HC{\equiv}C\text{-}CH_2CH_3$) [17]. In contrast, when the α-70^{Val} is substituted by α-70^{Ile}, with a larger side chain, the resulting MoFe protein showed a significantly lowered activity for reduction of a number of substrates except protons [16]. In the absence of other substrates, nitrogenase reduces protons to make H_2 (eq 1), and thus this reaction is an important control that indicates the overall catalytic function of nitrogenase. In the case of the α-70^{Ile} substituted MoFe protein normal proton reduction activity was observed, confirming that the underlying catalytic functions of the enzyme are undisturbed. The findings with the α-70^{Ala}, α-70^{Gly}, and α-70^{Ile} substituted MoFe proteins were interpreted as evidence that the residue at position α-70 controls access to FeMo-cofactor for binding of both alkyne and nitrogenous substrates, suggesting that substrate binding occurs at a specific FeS cluster face of FeMo-cofactor (Fe atoms 2, 3, 6, 7) [18]. Subsequent studies on a state of the α-70^{Ala} MoFe protein with the substrate propargyl alcohol and propargyl amine trapped suggested that a specific Fe atom (number 6) might be the site of propargyl alcohol binding [19]. The α-70^{Ile} MoFe protein could be freeze trapped during turnover under argon (reducing protons to make H_2) in an EPR active state that was assigned to FeMo-cofactor with one or two hydrides bound [20]. It was not clear why the α-70^{Ile} MoFe protein trapped these likely intermediates during the H_2 evolution reaction.

Here, we report the x-ray crystal structure of the α-70^{Ile} variant MoFe protein at 2.3 Å resolution. A comparison of the structure of the α-70^{Ile} MoFe protein with the wild type MoFe protein reveals a likely explanation for the earlier observations regarding substrate interactions in the α-70^{Ile} MoFe protein, in turn providing new insights into a specific site

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on FeMo-cofactor that could constitute the site of substrate interaction and suggests a role for the α-70^{Val} as a gate keeper. Further, the α-70^{Ile} structure suggests an explanation for why the hydride intermediate is trapped in this variant MoFe protein.

Materials and Methods

The α-70^{Ile} MoFe protein with a polyhistidine tag near the carboxyl terminus of the *nif*D gene was purified from *Azotobacter vinalandii* strain DJ1373 using a metal affinity chromatography method as described earlier [21]. All manipulations were done in the absence of O_2 under an argon atmosphere using gas tight syringes to transfer solutions and gases. The α-70^{Ile} MoFe protein was judged to be > 95% homogeneous from Coomassie blue staining of SDS-gels. The final protein was in Tris-HCl buffer, pH 8.0, with 250 mM NaCl, 2 mM dithionite. The protein had a specific activity for H_2 evolution of greater than 2200 nmol/min/mg MoFe protein (95 % of the wild type specific activity).

For crystallization, the protein was diluted to 38 mg/mL in 50 mM Tris buffer, pH 8.0, and 250 mM NaCl before setting up trials under anaerobic conditions in a nitrogen atmosphere glove box (UniLAB, MBRAUN, NH) using a micro-capillary batch diffusion method [22]. The α-70^{Ile} variant of the MoFe protein crystallized in 30% polyethylene glycol (PEG) 4000, 100 mM Tris (pH 8.0), 170–190 mM sodium molybdate and 1mM dithionite over a period of 3–4 weeks to give dark brown crystals of ~100 × 200 × 200 μm. The crystals were flash frozen in liquid nitrogen on rayon loops before data collection on beam line 11–1 at the Stanford Synchrotron Radiation Laboratory under a continuous flow of liquid nitrogen at 100 K. A single wavelength data set was collected λ = 0.89 up to a resolution 2.3 Å and the data was integrated and scaled using the HKL2000 software package [23]. The data statistics are summarized in Table 1. Calculation of the Matthews coefficient [24,25] and consideration of reasonable solvent content of the crystals suggested that each asymmetric unit of the crystal contained one $\alpha_2\beta_2$ heterotetramer.

AutoMR of CCP4 suite of programs [26] was used to accomplish molecular replacement using the $\alpha_2\beta_2$ MoFe protein heterotetramer (PDB ID: 2MIN) [27] as the starting model. The search resulted in a solution with R_{cryst} of 30.0% and correlation coefficient of 70%, and the model was further refined in REFMAC5 [28] to improve the quality of the model. The refinement runs made use of medium noncrystallographic symmetry (NCS) restraints (medium for main chains and loose for side chains), *B*-factor restraints and 2 screw (Translation/Libration/ Screw) tensors (per polypeptide chain). The final model was refined to R_{cryst} of 22.7% and obeys good stereochemistry with up to 97.3% of all residues in the Ramchandran [29] allowed regions (Table 1). All figures in the manuscript were generated using PyMOL [30].

Results and Discussion

Structure of the α-70^{Ile} MoFe protein

The x-ray structure of α-70^{Ile} MoFe protein from *Azotobacter vinelandii* refined to 2.3 Å shows a very similar overall structure when compared to the wild-type MoFe protein [7]. The protein was crystallized under strict anaerobic conditions in the presence of 2

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mM dithionite to capture the reduced form and has been refined to an R_{cryst} of 22.7%. The structure exhibits good geometry with 97.3% of all residues in the Ramchandran [29] allowed regions (Table 1). Serine 255 in the β-subunit lies in the Ramchandran disallowed region in the structure reported here and this distortion in the protein stereo chemistry has been previously observed in the wild-type structure as well [7]. The distortion in the protein back bone in this region probably arises from steric hindrance from the surrounding residues in chain B. The overall structure of the α-70^{Ile} MoFe protein is quite similar to the wild type protein with the secondary structure elements overlaying with significant similarities. Most of the differences are seen in the FeMo-cofactor binding region and details of the differences are highlighted in the following sections.

Insights into the location of substrate binding on FeMo-cofactor

The secondary structure of the α-70^{Ile} MoFe protein was compared to the structure of the wild-type MoFe protein (1MIN.PDB) using the protein structure comparison service SSM at European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/ssm) [31]. An examination of the root mean square standard deviation (RMSD) among all residues reveals the near identity of the structures, with an average RMSD of 0.2 Å. The close structural agreement between the α-70^{Ile} and wild-type MoFe proteins is evident from the small RMSD values. A short stretch of amino acids in the α-subunit between residues 105–120 shows slightly higher deviations compared to all other residues. These residues are located on the surface of the MoFe protein, and thus some variation in the position of the residues in this region could be expected.

Residues that define the FeMo-cofactor binding pocket between the wild-type and α-70^{Ile} MoFe proteins are shown in Fig. 1. Near identity in the positions of most amino acids in this region is observed, with a few exceptions. The side chains of α-96^{Arg}, α-359^{Arg}, α-195^{His} refine to slightly different positions in the α-70^{Ile} MoFe protein structure relative to the wild-type MoFe protein structure and these differences are supported by the electron density maps in this region. The position of the α-70^{Val} and α-70^{Ile} side chains is seen to be nearly identical, with the methyl group ($C_{\delta 1}$) on the isoleucine approaching Fe 6 of FeMo-cofactor (Fig. 2). The distance between the $C_{\delta 1}$ of α-70^{Ile} and the Fe 6 is found to be 3.9 Å, confirming the close contact between the isoleucine side chain and this Fe atom (Table 2).

The position of $C_{\delta 1}$ in α-70^{Ile} can be considered in light of studies indicating that the same Fe atom is a site of substrate interactions. When α-70^{Val} was substituted by α-70^{Ile}, the MoFe protein showed much lower activity for reduction of most compounds that are effective substrates for the wild-type MoFe protein. Proton reduction rates are unaffected by the α-70^{Ile} substitution. A simple explanation for this observation is that the $C_{\delta 1}$ on α-70^{Ile} has blocked substrates (except protons) from accessing the binding site on FeMo-cofactor. This model was further supported by the observation that when α-70^{Val} is substituted by amino acids having smaller side chains (e.g., alanine or glycine), then larger compounds that are normally poor substrates for nitrogenase can be actively reduced. For example, the α-70^{Ala} MoFe protein is found to reduce larger alkynes such as propyne ($HC{\equiv}C\text{-}CH_3$) and propargyl alcohol ($HC{\equiv}C\text{-}CH_2\text{-}OH$), whereas these compounds are only poor substrates in

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the wild-type MoFe protein [17]. Likewise, the nitrogenous substrate hydrazine (H_2N-NH_2) can be reduced at much higher rates by the α-70^{Ala} MoFe protein when compared to the wild-type MoFe protein [18]. In the α-70^{Gly} MoFe protein, 1-butyne (HC≡C-CH_2-CH_3) is found to be a substrate, whereas this compound is only reduced at very low rates by the wild-type MoFe protein. Taken together, these results can be explained if the side chain at α-70 acts as a gate-keeper, controlling the size of substrates that can gain access to a substrate binding site on FeMo-cofactor. Given the location of the side chain of α-70 over one Fe-S face of FeMo-cofactor, the simplest interpretation of the results is that this FeS face constitutes a substrate binding location.

Other studies also pointed to Fe atom 6 of FeMo-cofactor as a possible substrate binding location. For example, it was found that propargyl alcohol under turnover conditions can be freeze trapped on FeMo-cofactor in the α-70^{Ala} MoFe protein [4]. Using isotopically labeled substrate (^{13}C and $^{1/2}$H) and electron-nuclear double resonance (ENDOR) spectroscopy, it was concluded that the species bound to FeMo-cofactor was the partially reduced allyl alcohol (H_2C=CH-CH_2OH) [4]. It was further concluded that allyl alcohol was bound side-on between the first and second carbons to one or two Fe atoms. Considering the wild-type structure of the MoFe protein, molecular mechanics calculations were done to try to further refine where on the FeS face of FeMo-cofactor the allyl alcohol was likely to be bound [19]. A central observation to these calculations was the proposed role of the side chain of α-195^{His} in H bonding to the OH of allyl alcohol in stabilizing the bound state, favoring binding of substrates to Fe 6 compared to the other Fe atoms on this FeS face.

In a separate study, it was found that the α-70^{Gly} substituted MoFe protein can reduce 1-butyne (HC≡C-CH_2CH_3) but not 2-butyne (H_3C-C≡C-CH_3). To explain these results, both 1-butyne and 2-butyne were modeled into the wild type structure bound side-on to Fe6 of FeMo-cofactor [18], which indicated 2-butyne cannot bind because of steric overlap with the side chain of α-191^{Gln}. This model predicted that substitution of α-191^{Gln} by α-191^{Ala} might allow 2-butyne to fit into a position to bind side-on to Fe6. The α-191^{Ala} substituted MoFe protein was found to reduce 2-butyne at considerable rates, also locating the substrate binding site to Fe 6 [18]. Finally, a density functional theory study favors binding of alkynes such as propargyl alcohol to Fe 6 [19]. The x-ray structure of the α-70^{Ile} MoFe protein presented here, coupled with the earlier studies, provides strong evidence for Fe 6 acting as a specific binding site for substrate interactions on FeMo-cofactor

Insights into a hydride trapped state on the α-70^{Ile} MoFe protein

A spectroscopic investigation of the α-70^{Ile} MoFe protein revealed that when this protein is freeze quenched during turnover conditions, a unique EPR active state is trapped that has been assigned to one or two hydrides bound to the FeMo-cofactor (modeled as bound to one or more Fe atoms). It is not clear why the α-70^{Ile} amino acid substitution should result in the trapping of hydrides to FeMo-cofactor, although there are several possible explanations. One model is that a proton is bound to FeMo-cofactor (formally as a hydride with electrons coming from FeMo-cofactor) and that the addition of the second proton needed to form H_2 is restricted by the additional methyl group of Ile, allowing the hydride bound state to accumulate. The second proton could come either directly from the protein through the

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protein backbone or via a proton relay through amino acid side chains such as the α-195^{His} residue, or from a large pool of waters located around *R*-homocitrate (Fig. 3).

Here we consider the possibility that the side chain of α-70^{Ile} might obstruct the proton flow from the pool of waters near *R*-homocitrate. Other protein components that would be predicted to define the flow of protons from the water pool are the α carbon of α-66^{Gly}, the β carbon of α-191^{Gln}, the ε nitrogen of α-195^{His}, and part of the *R*-homocitrate. Two different models could be considered to explain how the α-70^{Ile} might block the flow of these protons. One model is that the methyl group of isoleucine might block proton transfer from the α-195^{His} residue to a hydride bound to Fe 6. The α-195^{His} residue has been previously implicated as a possible proton donor for the reduction of nitrogenous substrates. Alternatively, if a hydride is bound to Fe2, then the α-70^{Ile} could block the proton flow through waters that reside next to *R*-homocitrate. Both models would explain how a hydride bound state might populate when the α-70^{Ile} MoFe protein is trapped during turnover.

Summary

The 2.3 Å structure of the α-70^{Ile} MoFe protein variant is presented. The localization of the side chain of isoleucine in the structure explains earlier kinetic studies that indicated that the side chain of the amino acid at position α-70 acts as a gate keeper to control access of substrates to a binding location on FeMo-cofactor. Based on the location of the side chain of α-70^{Ile} directly over Fe6 and previous molecular mechanics studies, a model is constructed for substrate interactions at this specific Fe site. Further, the structure offers insights into the observation of trapping of hydrides on the α-70^{Ile} MoFe protein when it is frozen during H_2 evolution.

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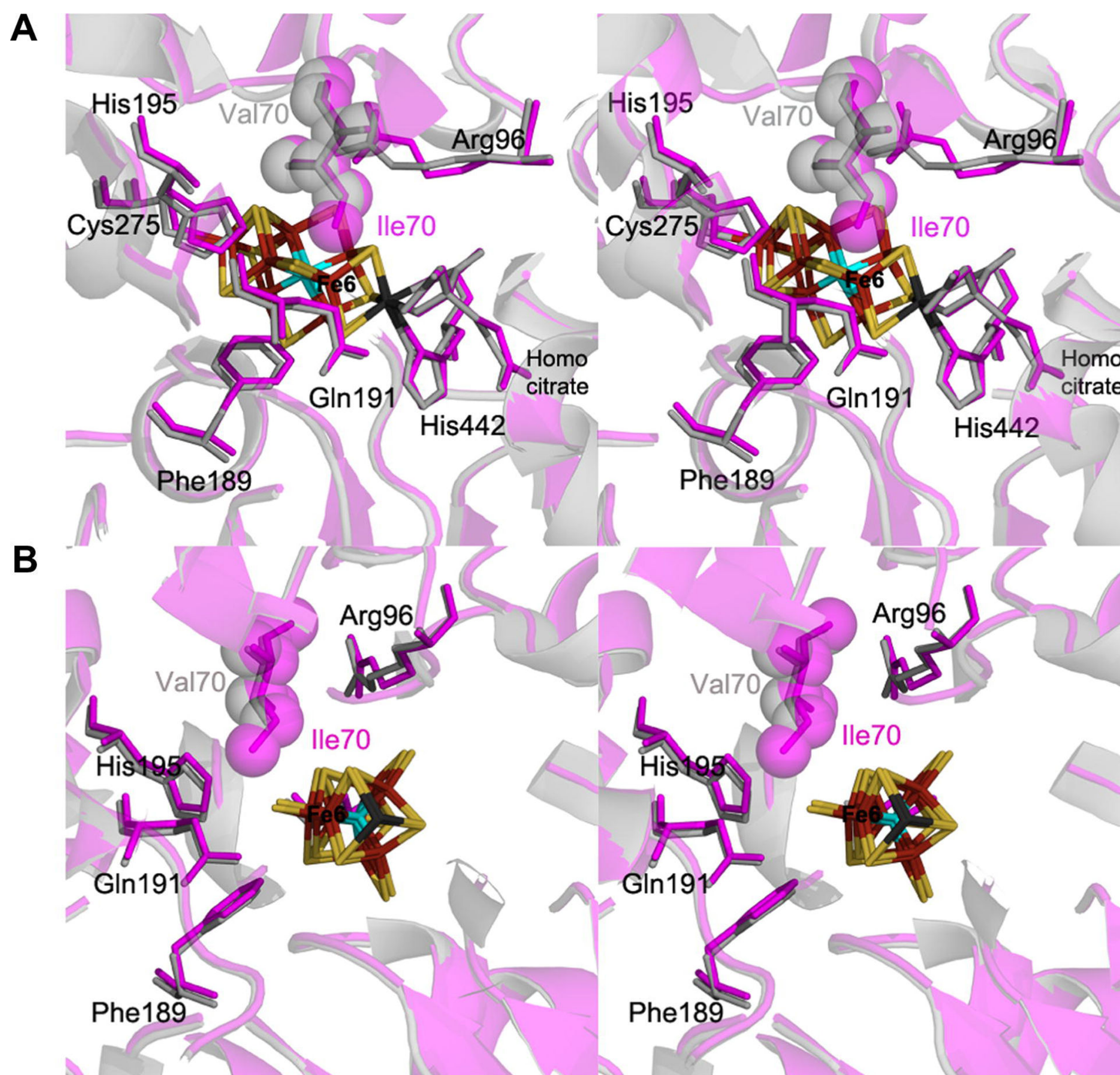

Figure 1:

Stereoviews of the protein environment surrounding the FeMo-cofactor. Shown is a portion of α-carbon trace and select amino acid side chains for both the α-70^{Ile} (magenta) and wild type (cyan) MoFe proteins in two orientations (A and B) separated by an ~90? rotation. The protein backbone is shown as ribbons, with the residues immediately surrounding FeMo-cofactor from the α-subunit and the FeMo-cofactor shown in line angle representations with the exception of either the α-70^{Val} (wild-type) side chain and the α-70^{Ile} (variant) side chain shown in space filling / van der Waals representations. Fe atoms are colored in rust, S in yellow, Mo in black,and X in cyan.

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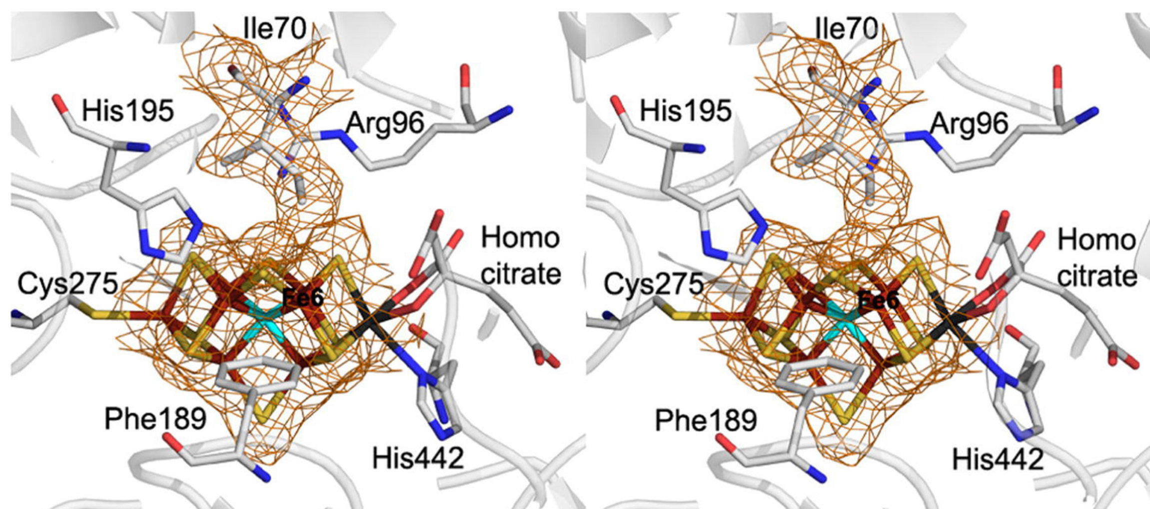

Figure 2:

Stereoview of the 2*F*o-*F*c electron density maps (1.0σ) of the region surrounding the FeMo-cofactor and the adjacent α-70^{Ile} residue. The protein backbone is shown in ribbons with residues in the FeMo-cofactor environment from the α-subunit are shown in line angle representations color coded with Fe in rust, S in yellow, C in grey, N in blue, O in red, and Mo in black.

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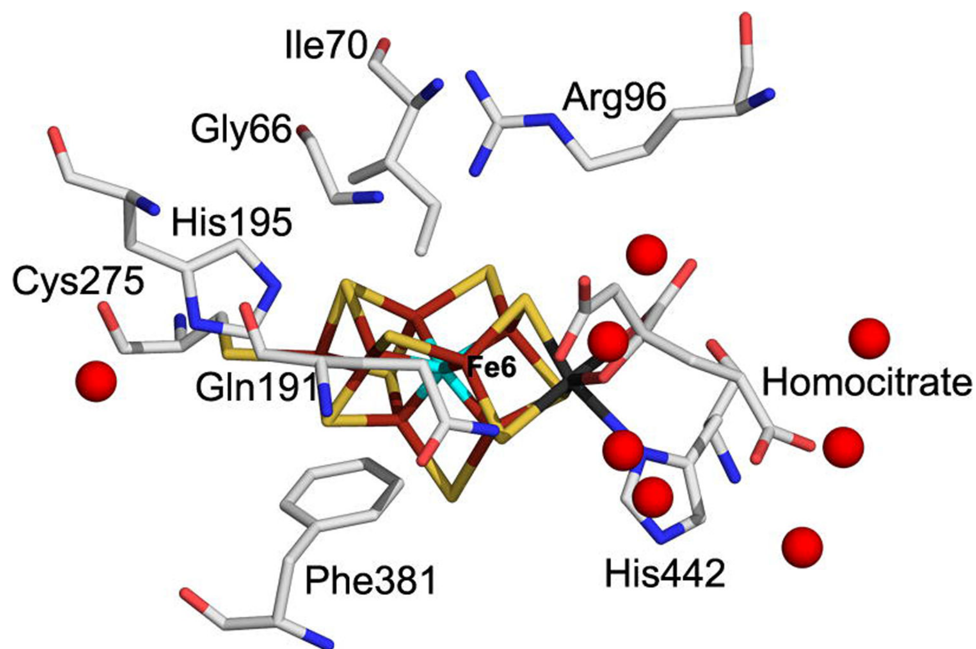

Figure 3:
FeMo-cofactor binding site in the the α-70^{Ile} MoFe protein structure showing the location of the surrounding water molecules represented as red spheres. The residues in the FeMo-cofactor and surrounding protein environment shown with line angle representations color coded as in Figure 2..

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Table 1:

Data Statistics

Cell dimensions	$a = 77.02$ Å
	$b = 129.36$ Å
	$c = 107.09$ Å
	$\alpha = \gamma = 90.00°$
	$\beta = 109.01$
space group	$P2_1$
Wavelength (Å)	$\lambda_1 = 0.88557$
Resolution (Å)	50 – 2.3
Completeness (%)	99.9 (99.9)[a]
Observed reflections	361917
Unique reflections	187881 (18797)
Average redundancy	3.8 (3.5)
I/σ	11.8 (2.7)
R_{sym}[b] (%)	11.5 (23.3)
Refinement Statistics	
Resolution (Å)	26.6 – 2.3
R_{cyrst}[c] (%)	22.7
R_{free}[c] (%)	27.8
Real Space CC[d] (%)	91.1
Mean B Value (Å^2)	26.6
Coordinate Error (based on maximum likelihood, Å)	0.22
RMSD from ideality:	
Bonds (Å)	0.007
Angles (°)	2.812
Ramchandran statistics[e]:	
Most favored (%)	97.3
Additional allowed (%)	2.65
Outliers (%)	0.05

[a]Numbers in parenthesis refer to the highest resolution shell.

[b]$R_{sym} = 100 * \sum_h \sum_i |I_i(h) - < I(h) > | / \sum_h I(h)$ where I_i(h) is the ith measurement of reflection h and <I(h)> is the average value of the reflection intensity.

[c]$R_{cryst} = \sum \| F_o | - | F_c \| / \sum | F_o |$ where F_O and F_C are the observed and calculated structure factor amplitudes used in refinement. R_{free} is calculated as R_{cryst}, but using the "test" set of structure factor amplitudes that were withheld from refinement.

[d]Correlation coefficient (CC) is agreement between the model and 2mF_O-DF_C electron density map.

[e]Calculated using Molprobity [32]

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Table 2 :

Distances between α-70^{Val} and α-70^{Ile} side chain groups and the FeMo-cofactor

Native MoFe protein interactions		α-70^{Ile} MoFe protein interactions	
Interaction	**Distance (Å)**	**Interaction**	**Distance (Å)**
α-70^{Val} ($C_{\gamma1}$) – Fe2	4.4	α-70^{Ile} ($C_{\gamma2}$) – Fe2	4.6
α-70^{Val} ($C_{\gamma1}$) – Fe3	5.2	α-70^{Ile} ($C_{\gamma2}$) – Fe3	5.1
α-70^{Val} ($C_{\gamma2}$) – Fe6	4.4	α-70^{Ile} ($C_{\gamma1}$) – Fe6	4.0
α-70^{Val} ($C_{\gamma2}$) – Fe7	5.5	α-70^{Ile} ($C_{\delta1}$) – Fe6	3.9
		α-70^{Ile} ($C_{\gamma1}$) – Fe7	5.1
		α-70^{Ile} ($C_{\delta1}$) – Fe7	5.5