Chapter V

Thiocyanate and Methylamine Binding Sites in the MoFe Protein

V.A. Abstract

One of the most intriguing mysteries of nitrogenase is the mechanism by which it functions. Pieces of this puzzle may be revealed by determining the binding sites of small molecules that mimic substrates and products of nitrogenase. These binding sites yield information on how substrates and products may access and interact with the protein and its metal clusters. The binding sites of two small molecules, thiocyanate (SCN) and methylamine, are reported in this work. Understanding the sulfur binding sites is motivated by the observation that a sulfur atom is displaced from the FeMo-cofactor to accommodate CO-binding during inhibition; a sulfur returns to the belt position upon release of CO from the FeMo-cofactor.[37] Structural data from the MoFe protein of *Azotobacter vinelandii* (Av1) obtained at the resting, CO-inhibited, and re-activated states as well as the Av1-SCN co-crystal structure suggest that the displaced sulfur atom may reversibly bind in a positively charged pocket about 22 Å away from the FeMo-cofactor.[37] The work presented here aims to identify sulfur binding sites that may be present in the MoFe protein from *Clostridium pasteurianum* (Cp1). No sulfur binding sites in Cp1-SCN co-crystals were observed, suggesting that the displaced sulfur may roam about the protein or the water pocket at the active site during inhibition and turnover. Also presented in this work is a methylamine binding site. Methylamine is an analogue of hydrazine, a likely intermediate in the nitrogen fixation mechanism.[8] One methylamine binding site was observed

in Cp1, residing in the interstitial water channel. This provides further support for use of the interstitial water channel as a pathway for substrates and products.[78]

V.B. Introduction

The mechanism of biological nitrogen fixation remains inconclusive despite decades of work. A thorough understanding of the mechanism is highly sought for its potential impact on creating a sustainable alternative to the Haber-Bosch process of industrial ammonia production for fertilizers.[115] A recent breakthrough in the elucidation of the nitrogenase mechanism is the X-ray structure of CO bound to the Av1FeMo-cofactor – the first structure of a non-resting state MoFe protein.[37] CO is usually a non-competitive, reversible inhibitor in nitrogenase for all substrates except for protons.[116–118] In the CO-bound structure, one of the belt sulfur atoms of the Av1 FeMo-cofactor, S2B, is displaced by a CO molecule such that the carbon atom bridges Fe2 and Fe6. Inhibition of nitrogenase activity of this state was confirmed by enzyme activity assays. Activity was regained when the CO atmosphere was removed. The crystal structure of the resulting reactivated nitrogenase shows re-occupation of S2B in the FeMo-cofactor; thus, the displacement of S2B is reversible during inhibition. This study, along with a study on selenium substitution of belt sulfurs in the presence of CO,[38] suggests that substrate reduction may occur by substrate substitution of one or more belt sulfurs. The question remains: to where do the sulfur atoms relocate upon their displacement from the FeMo-cofactor?

By inspection of the anomalous difference Fourier map calculated from X-ray data of the CO-inhibited structure of the MoFe protein obtained at 7100 eV, a sulfur binding site was observed that is usually occupied by a water molecule in the Av1 resting state. This binding site is separated from the FeMo-cofactor by about 22 Å and resides in a small binding pocket between the α- and β-subunits and in a non-continuous water channel connected to the

interstitial water channel. The positive surface charge of this cavity suggests that the sulfur species may be HS^- or S^{2-}. The sulfur binding site is far away from the FeMo-cofactor, and the surrounding residues are poorly conserved among other nitrogenase species, which could indicate that the sulfur binding site is a general anion binding site. However, the density of the sulfur in this site decreased in the reactivated Av1 structure, suggesting that the sulfur binding site could be a sulfur reservoir. The location of the Av1 sulfur binding site was reproduced in co-crystallizations with SCN. To establish if Cp1 exhibits the same or similar sulfur binding sites, Cp1 was co-crystallized with SCN. The results are reported here. Also reported herein is the co-crystallization of Cp1 with methylamine, $H_3C\text{-}NH_2$. Methylamine is an analogue for hydrazine, $H_2N\text{-}NH_2$, which is an intermediate of the nitrogen fixation mechanism.[8] Methylamine binding sites provide clues on how polar substrates and products, such as NH_4^+, move between the protein surface and the active site.

V.C. Results and discussion

V.C.i. Cp1-SCN co-crystallization

X-ray crystallographic data for two Cp1 crystals made in the presence with 10 mM KSCN (called Cp1-SCN1 and Cp1-SCN2) were collected; structures were built by molecular replacement and refined (Table V-1). Inspection of the $2F_o\text{-}F_c$ and anomalous Fourier maps showed no blobs that could be modeled by a sulfur species anywhere within the protein other than in the cofactors or in methionine or cysteine residues. The structural overlay of Av1 and Cp1 (Figure V-1) shows that the Av1 sulfur binding site is blocked in Cp1 by residue sidechains. Cp1 was also crystallized in the presence of KSeCN and Na_2Se. No Se binding sites were observed at physiological pH, which confirms the lack of sulfur binding sites observed in the Cp1-SCN co-crystals.

Table V-1. X-ray crystallographic data collection and refinement statistics for Cp1-SCN1 and Cp1-SCN2

	Cp1-SCN1	Cp1-SCN2
Data Collection Statistics		
Resolution range (Å)	37.87-1.38 (1.38-1.40)*	39.63-2.05 (2.05-2.09)*
Wavelength (Å)	0.9537	0.9537
Space group	P1	P1
Unit cell constants	a = 67.01 Å α = 73.58° b = 72.78 Å β = 87.40° c = 108.33 Å γ = 84.23°	a = 66.99 Å α = 73.49° b = 73.21 Å β = 87.31° c = 108.48 Å γ = 83.92°
Unique reflections	384,397 (18,531)*	121,025 (6,016)*
Completeness (%)	98.9 (92.9)*	98.2 (97.0)*
Redundancy	7.0 (7.6)*	6.8 (6.9)*
$I/\sigma(I)$	15.2 (2.1)*	6.6 (2.0)
R_{merge}	0.076 (0.929)*	6.03 (70.349)*
Refinement Statistics		
Protein residues	1950/1984	1950/1984
Mean *B* value (Å^2)	11.5	14.3
R_{work}	0.146	0.232
R_{free}	0.164	0.274
Ramachandran outliers	9 (0.47%)	8 (0.41%)
RMSD bond lengths (Å)	0.012	0.009
RMSD bond angles (°)	1.54	1.18

*Numbers in parentheses represent data in the highest resolution shell.

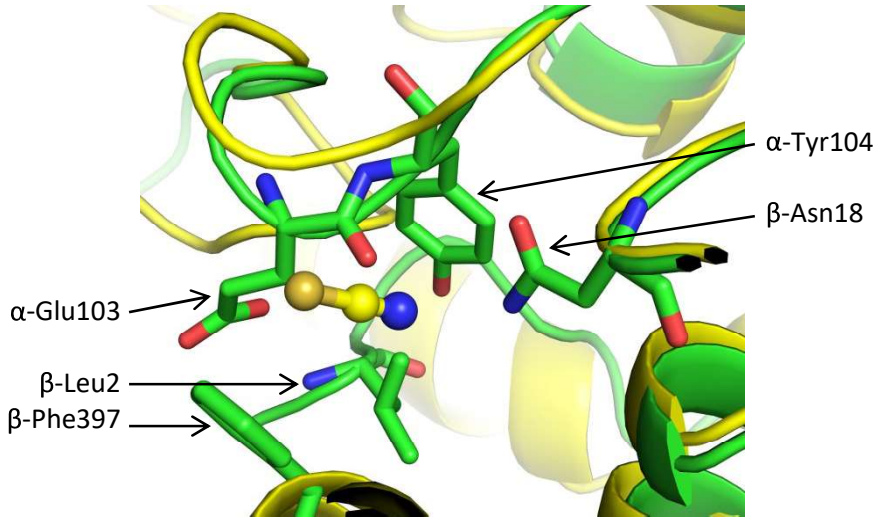

Figure V-1. Overlay of Av1-SCN and Cp1-SCN1. The yellow and green cartoons are that of Av1-SCN and Cp1-SCN1, respectively. The SCN molecule is displayed in ball and stick representation. Cp1-SCN residues blocking the Av1 SCN binding site are shown with sticks and labeled.

V.C.ii. Cp1-methylamine co-crystallization

Cp1-CH_3NH_2 co-crystals were grown in 5 mM methylamine. X-ray crystallographic data was collected, and the structure was built by molecular replacement and refined (Table V-2). Inspection of the $2F_o$-F_c map showed an elongated electron density blob within the α-subunit. A methylamine molecule modeled at this site has *B*-factor of 20 Å^2 – a little larger than the average *B*-factor of all protein molecules, 16.7 Å^2. This is reasonable considering that the methylamine exists in a channel. In this water channel, the methylamine nitrogen atom is 2.9 Å and 3.3 Å from the backbone carbonyl oxygen of α-Arg87 and α-Thr83, respectively (Figure V-2). The methylamine is 7 Å from the homocitrate, 8.5 Å from the FeMo-cofactor, and 11.5 Å from the P-cluster. All measurements were made using the two closest atoms of each species.

Table V-2. X-ray crystallographic data collection and refinement statistics for Cp1-CH_3NH_2

	Cp1-CH_3NH_2	
Data Collection Statistics		
Resolution range (Å)	39.10-1.73 (1.76-1.73)*	
Wavelength (Å)	0.9537	
Space group	P2	
Unit cell constants	a = 69.39 Å	α = 90°
	b = 149.7 Å	β = 103.6°
	c = 116.6 Å	γ = 90°
Unique reflections	235,226 (11,568)*	
Completeness (%)	98.0 (97.9)*	
Redundancy	6.9 (6.9)*	
$I/\sigma(I)$	10.8 (2.0)*	
R_{merge}	0.122 (1.000)*	
Refinement Statistics		
Protein residues	1950/1984	
Mean *B* value (Å^2)	16.7	
R_{work}	0.159	
R_{free}	0.184	
Ramachandran outliers	4 (0.21%)	
RMSD bond lengths (Å)	0.013	
RMSD bond angles (°)	1.46	

*Numbers in parentheses represent data in the highest resolution shell.

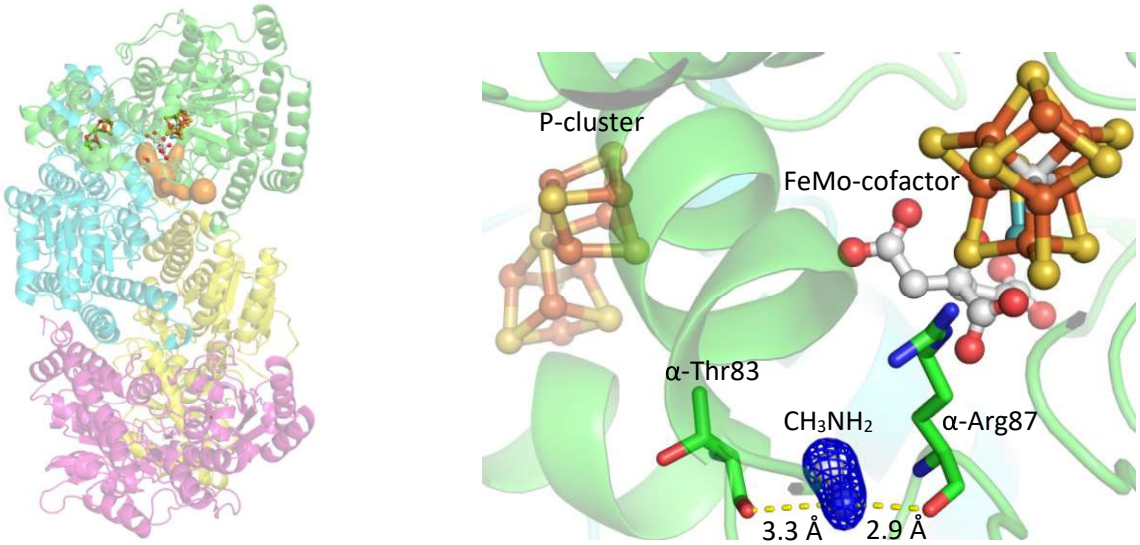

Figure V-2. Ribbon structure of Cp1-methylamine. The α-subunits are colored green and magenta, and the β-subunits are colored cyan and yellow. The cofactors and methylamine are shown with ball and stick and colored by element. The electron density of the methylamine is shown in blue mesh contoured to 1.5 σ. Close contacts to the methylamine are shown in sticks in the right image.

Cp1-CH_3NH_2 was superposed on structures of Av1-SCN, Av1-CO (PDB ID 4KTV), Cp1-Xe (PDB ID 4WN9), and Av1-Xe (PDB ID 4WNA).[37,78] No overlap was observed between the SCN, CO, or Xe atoms with methylamine. Additionally, no overlap was observed with the binding sites of other small molecules, such as sulfur, imidazole, ethane-di-ol, and the Cp1-proline ligand.[78]

V.D. Conclusions

The lack of S and Se binding sites in Cp1 co-crystals indicates that there may not be a defined sulfur binding site in Cp1. Perhaps a sulfur atom displaced from the FeMo-cofactor during turnover in Cp1 roams the water bath surrounding the FeMo-cofactor or elsewhere in the protein. When the MoFe protein returns to the resting state, the S2B binding site may be filled by any nearby sulfur atom, not necessarily by the same sulfur atom that was originally displaced from the FeMo-cofactor. The Cp1-methylamine structure shows a single methylamine

binding site in the interstitial water channel. This provides further support for use of this channel by polar substrates and products traveling between the protein surface and the active site.

V.E. Experimental

V.E.i. Protein purification and crystallization

Purification of Cp1 was performed as previously described.[21] Cp1 was crystallized using sitting-drop vapor diffusion in a Coy anaerobic chamber at room temperature (~295 K). The Cp1-SCN crystals were grown in a solution containing 0.1 M magnesium chloride, 24% (w/v) polyethylene glycol (PEG) with a molecular weight of 3350 g/mol, 10 mM thiocyanate (SCN), 80 mM Tris at pH 8.0, and 5 mM sodium dithionite. The Cp1-methylamine crystals were grown in a solution containing 0.3-0.5 M magnesium chloride, 14-18% (w/v) PEG 3350 g/mol, 5 mM methylamine, 80 mM Tris at pH 8.0, and 5 mM sodium dithionite. For both co-crystallizations, single crystals with block morphology appeared after one day. During crystal harvesting, crystals were first soaked in reservoir solution containing 10% 2-methyl-2,4-pentanediol (MPD) for 15 minutes, after which they were looped and flash-frozen in liquid nitrogen.

V.E.ii. Data collection and processing

Crystallographic diffraction data were collected on beam line 12-2 at the Stanford Synchrotron Radiation Lightsource (SSRL), with a Pilatus 6 M pixel array detector. The data were collected at 12,658 eV using an oscillation angle of 0.15° and 0.25° for Cp1-SCN and Cp1-methylamine, respectively. Because the Cp1-SCN crystals were in the P1 space group, two full data sets (360°) were collected, the second of which was horizontally and vertically offset from the first by 5 mm. In addition, two full sets of anomalous diffraction data (360°) were also

collected at 6.699 eV (the lowest possible energy of beamline 12-2) using an oscillation angle of 0.15° to identify the sulfur atoms. Like with the high energy data sets, the two data sets were offset by 5 mm horizontally and vertically. Although the X-ray wavelength of the low energy data sets is well above the K-edge of sulfur (2.4720 keV), the signal is still strong enough to observe anomalous scattering of the sulfur atoms, using the sulfur of known protein and inorganic atoms as a control. The crystallographic data were integrated with the XDS program package.[57] Scaling and integration were performed with AIMLESS from the CCP4 suite.[58] The model was built using PHASER, a molecular replacement program in CCP4, against the 1.08-Å Cp1 structure, 4WES.[58,64] The model was refined in COOT and with REFMAC5.[60,61,76] PyMOL was used to prepare the figures.[63]