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A Journey into the Active Center of Nitrogenase

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Abstract

Nitrogenase catalyzes the reduction of N_2 to NH_3, a key step in the global nitrogen cycle. This article describes our journey toward the definition of a complete molecular structure of the active site of nitrogenase, with an emphasis on the discovery of the interstitial carbide and the radical SAM-dependent insertion of this atom into the active FeMo cofactor site of nitrogenase.

Keywords

Nitrogenase; FeMo cofactor; interstitial carbide; NifB; radical SAM enzyme

Introduction

It took the Greek hero Odysseus a ten-year journey to reach his home Ithaca after the fall of Troy. Who would have thought that our journey from the discovery of the nitrogenase to the unveiling of structure of this enzyme would take more than a century, exceeding the Odyssey by a whole magnitude? Not Hellriegel and Wilfarth, who reported in 1888 that legumes could utilize atmospheric nitrogen (N_2) for growth and that the “fixation” of nitrogen by plants depended on bacteria that colonize their rood nodules (1); not Beijerinck and Winogradsky, who isolated nitrogen-fixing bacteria from root nodules and soil between 1888 and 1901 (2-4); and not even Carnahan and his colleagues, who made the first cell-free extracts that consistently fixed N_2 in 1960 (5, 6). The enzyme responsible for N_2 fixation is nitrogenase, which breaks the strong triple bond in N_2 to form ammonia (NH_3), thereby turning nitrogen into a bioavailable form which is then accessible to all life forms on Earth. Amazingly, it took nearly 80 years from the time nitrogen fixation was discovered till Bulen and LeComte successfully purified the first nitrogenase enzyme in 1966 (7) and another 30 years till Rees and coworkers reported the first xray crystallographic structures of the component proteins and the active site of nitrogenase in 1992 and 1993—a remarkable milestone in the area of nitrogenase research (8-10). But, just like our Greek hero Odysseus, whose journey was far from over after he overcame one of his early obstacles, Polyphemus,

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on the island of the Cyclops, the initial crystallographic analysis only marked the beginning of our journey into the active center of nitrogenase. What's more, Odysseus would eventually arrive home and take his place as king after his journey, but our journey into the center of nitrogenase is still ongoing and additional hurdles must be surmounted on the way till the reaction mechanism of this enzyme can be fully deciphered.

Piecing together the structure of the FeMo cofactor

The successful purification of nitrogenase from *Azotobacter vinelandii* and *Clostridium pasteurianum* (11, 12) led to the realization that this enzyme consisted of two component proteins, namely, the iron (Fe) protein (the reductase component) and the molybdenum-iron (MoFe) protein (the catalytic component). Subsequently, it was demonstrated that a metal cluster, designated the iron-molybdenum (FeMo) cofactor, could be extracted from the MoFe protein into an organic solvent, *N*-methylformamide (NMF) (13). Moreover, the extracted FeMo cofactor could then be inserted into the apo MoFe protein, leading to the activation of this protein upon reconstitution. This discovery not only established the FeMo cofactor as the active site of nitrogenase, but also enabled early structural analyses of this cofactor both in the protein-bound and in the NMF-extracted states. In 1978, Hodgson and coworkers presented the very first Mo K-edge x-ray absorption spectroscopy (XAS)/ extended x-ray absorption fine structure (EXAFS) analysis of the FeMo cofactor (14, 15), which permitted the modeling of partial structures of this unique metal cluster (Figure 1). Remarkably, their favored model 1 consisted of a $MoFe_3S_3$ cuboidal fragment (Figure 1), which was later confirmed by the x-ray crystallographic analysis of the MoFe protein (10). Following this pioneering work, Mo and Fe K-edge XAS/EXAFS analyses further demonstrated the presence of a sulfur-rich coordination environment for both Mo and Fe atoms, as well as a possible long Fe-Fe scattering path at >3.5 Å, in the structure of the FeMo cofactor, which would be consistent with an extended structure of this metal cluster beyond the Fe-Fe interaction at 2.7 Å (16-20). A rough "sketch" of the FeMo cofactor has come to light through these studies; however, it would still take another few years till the unique structure of this cofactor was finally unveiled.

Discovering a "void" in the center of the FeMo cofactor

The first complete structure of the FeMo cofactor was obtained through the crystallographic studies of the nitrogenase from *A. vinelandii*. These studies defined the Fe protein as a γ_2-dimer containing a subunit-bridging $[Fe_4S_4]$ cluster and an ATP-binding site within each subunit (8) and the MoFe protein as an $\alpha_2\beta_2$-tetramer containing a P-cluster ($[Fe_8S_7]$) at each α/β-subunit interface and a FeMo cofactor ($[MoFe_7S_9$-homocitrate]) within each α-subunit (9, 10). The subsequent structural analysis of an $ADP{\bullet}AlF_4^-$-stabilized complex between the Fe protein and the MoFe protein (21) revealed an electron transfer pathway that extended from the $[Fe_4S_4]$ cluster of the Fe protein, through the P-cluster, to the FeMo cofactor of the MoFe protein (Figure 2). This observation, in combination with the outcome of biochemical studies during this time period (22), suggested a possible mechanism of nitrogenase involving the complex formation between the Fe protein and the MoFe protein, the ATP-dependent, inter-protein transfer of electrons from the former to the latter, and the eventual reduction of N_2 to NH_3 at the active FeMo cofactor site of the MoFe protein. With

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these major structural findings in hand, the enthusiasm of the nitrogenase community was enormous, as many assumed that a complete understanding of nitrogenase catalysis was within close reach. However, this optimism was soon replaced by the realization of the complexity of this binary enzyme system and, in particular, the complexity of the structure of the active FeMo cofactor site, which precluded a straightforward assignment of the site for substrate binding and reduction. Indeed, this [$MoFe_7S_9$-homocitrate] cluster—the core structure of which comprises [$MoFe_3S_3$] and [Fe_4S_3] subcubanes bridged by three μ_2-sulfides (Figure 3A)—offers a number of possible sites as the reaction center. Most strikingly, there is an empty space in the center of the initial structure of the FeMo cofactor, which is enclosed by six μ_3-coordinated Fe atoms (Figure 3A). The observation of a "void" in the center of the FeMo cofactor led to the attractive hypothesis that it could serve as a "reaction cavity" of nitrogenase; however, the anomalous Fe geometry of such a structure (23) would pose a major challenge to the overall stability and reactivity of the FeMo cofactor, prompting a continuation of efforts to explore the center of this cofactor.

Unveiling the presence of carbide in the center of the FeMo cofactor

The presence of a μ_6-coordinated monoatomic ligand in the center of the FeMo cofactor (Figure 3B) was first revealed through the high-resolution crystallographic analysis of MoFe protein at 1.16 Å (24). The discovery of this core ligand, designated "X" then, generated great excitement in the nitrogenase field because not only did this ligand "fill" the void and normalize the previously observed anomalous iron geometry of the FeMo cofactor, it also could serve as an "anchor" for substrate binding and processing during turnover. Unfortunately, the level of refinement at this time did not allow the conclusive identification of the central ligand "X", although the electron density and bond distances of "X" narrowed it down to a light (2*p*) element, such as nitrogen, oxygen or carbon (24). These structural data, along with density function theory (DFT) calculations (25, 26), suggested that "X" could be best described as a nitrogen—an appealing proposal given the nitrogenous nature of the substrate. However, there were other opinions with regard to the identity of "X", with some disfavoring nitrogen (27), some not excluding oxygen (28), and some favoring carbon (29) as the central ligand of the FeMoco factor. The uncertainty of the identity of "X" had remained a major stumbling block toward deciphering the molecular structure of this cofactor till 2011, when two independent studies (30, 31) identified "X" as a carbide ion at the "heart" of the cofactor (Figure 3C). One study used x-ray emission spectroscopy (XES) to pinpoint the carbide-specific features of the FeMo cofactor (30), while the other employed a combination of crystallographic analysis of MoFe protein at a further improved resolution (1.0 Å) and electron spin echo envelope modulation (ESEEM) analysis of ^{13}C-labeled MoFe protein (31) to reach the same conclusion. The success in defining the full atomic-resolution structure of the FeMo cofactor provided an excellent platform to address two key questions regarding the active site of this enzyme: how is carbon inserted into the cofactor and can this atom undergo exchange with substrates during turnover?

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Discovering a “radical” pathway for carbide insertion into the FeMo cofactor

The discovery of a unique carbide insertion pathway was facilitated by knowledge accumulated on the biosynthesis of the FeMo cofactor in the recent years (32-35). This biosynthetic event involves the sequential actions of NifB, NifEN and the Fe protein in a highly coordinated manner (Figure 4): first, two $[Fe_4S_4]$ clusters are coupled on NifB into a $[Fe_8S_9]$ precursor, which closely resembles the core structure of the FeMo cofactor (36-39); then, the $[Fe_8S_9]$ precursor is processed on NifEN into a fully matured cofactor upon Fe protein-mediated insertion of Mo and homocitrate (40); finally, the matured FeMo cofactor is transferred to MoFe protein and inserted into its final binding site in this protein (41, 42). The observation of a SAM-binding domain in the primary sequence of NifB led to the hypothesis that that NifB might use SAM as a carbon donor and insert a carbon atom via radical chemistry concomitant with the fusion/rearrangement of two $[Fe_4S_4]$ clusters into a $[Fe_8S_9]$ precursor (43). This hypothesis was subsequently proven by biochemical experiments, which demonstrated the incorporation of ^{14}C labels into the cofactor when it was matured in the presence of [methyl-^{14}C] *S*-adenosylmethionine (SAM) (44). Furthermore, as observed in the cases of radical SAM methyltransferases RlmN and Cfr (45, 46), a deuterated form of 5′-deoxyadenosine (5′-dAD) could be detected in the presence of [methyl-d_3] SAM, suggesting the involvement of hydrogen atom abstraction by 5′-deoxyadenosyl radical (5′-dA·) in the process of carbide insertion (44). Combined outcome of these analyses not only identified the methyl group of SAM as the origin of the interstitial carbide, but also led to the proposal of two plausible pathways of carbide insertion (Figure 5). While they differ in the mechanism of carbon radical intermediate formation, both pathways suggest a pivotal role played by the carbon radical intermediate, which initiates radical chemistry for the bond rearrangement that is required for the coupling/restructuring of two $[Fe_4S_4]$ clusters into a $[Fe_8S_9]$ precursor. The discovery of such a “radical” pathway for carbide insertion defined a novel synthetic route to bridged metalloclusters; perhaps more importantly, it provided an effectively means to label the interstitial carbide and trace the fate of this atom during substrate turnover.

Exploring the function of the interstitial carbide of the FeMo cofactor

The question of whether the interstitial atom could be exchanged upon catalysis was tackled even before the identity of this atom was unveiled, with one electron-nuclear double resonance (ENDOR)/ESEEM study suggesting that if X was a nitrogen, it could not be exchanged during catalysis (27). A recent study of ^{14}C- and ^{13}C-labeled FeMo cofactor demonstrated that the interstitial carbide could not be exchanged upon turnover, nor could it be used as a substrate and incorporated into the products (47). This observation seemed to support a role of the interstitial carbide in stabilizing the structure of the FeMo cofactor, but, does this cofactor really have a “heart of steel”? Maybe not. Recently, an interesting study of iron model complexes suggested that the modulation of the belt Fe-C interaction in the FeMo cofactor could serve as a “molecular spring” and facilitate the binding and reduction of substrates (48). As such, a function of the interstitial carbide in indirectly modulating the reactivity of the cofactor or directly interacting with the substrate cannot be ruled out.

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Clearly, we are not at our "destination Ithaca" of nitrogenase research yet, but we may be slowly approaching the shores of the island with the knowledge of the complete molecular structure of the active site of this fascinating enzyme.

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Abbreviations

FeMo cofactor	iron-molybdenum cofactor
Fe protein	iron protein
MoFe protein	molybdenum-iron protein
NMF	*N*-methylformamide
XAS	x-ray absorption spectroscopy
EXAFS	extended x-ray absorption fine structure
XES	x-ray emission spectroscopy
ENDOR	electron-nuclear double resonance
ESEEM	electron spin echo envelope modulation
DFT	density function theory

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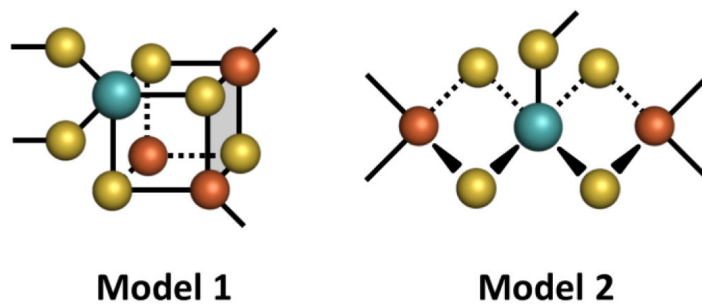

Fig. 1.
Structural models of the molybdenum coordination environment in the FeMo cofactor based on Mo K-edge XAS/EXAFS analysis in 1978.

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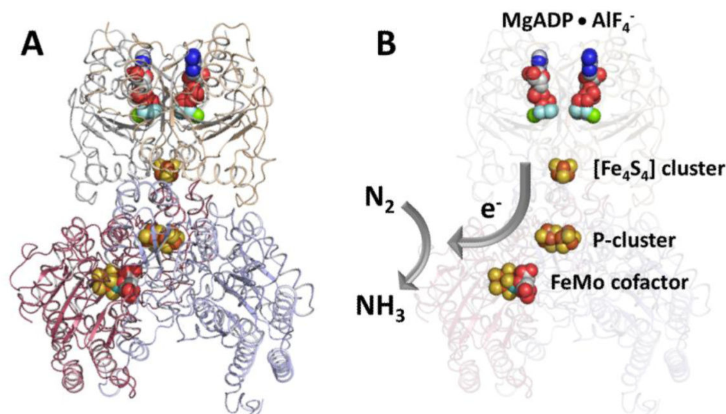

Fig. 2.

Crystal structure of the ADP•AlF_4^--stabilized Fe protein/MoFe protein complex (**A**) and the relative positions of components involved in electron transfer during catalysis (**B**). One "catalytic unit", which consists of one αβ-half of the complex, is shown. The two subunits of the Fe protein are colored gray and light brown, respectively, and the α- and β-subunits of the MoFe protein are colored red and light blue, respectively, in **A**. The protein peptides of one αβ-dimer are rendered transparent in the background in **B**. All clusters and ADP•AlF_4^- are shown as space-filling models. Atoms are colored as follows: Fe, orange; S, yellow; Mo, cyan; O, red; C, gray; N, blue; Mg, dark green; Al, light green; F, light blue. PYMOL was used to create this figure (PDB ID: 1N2C).

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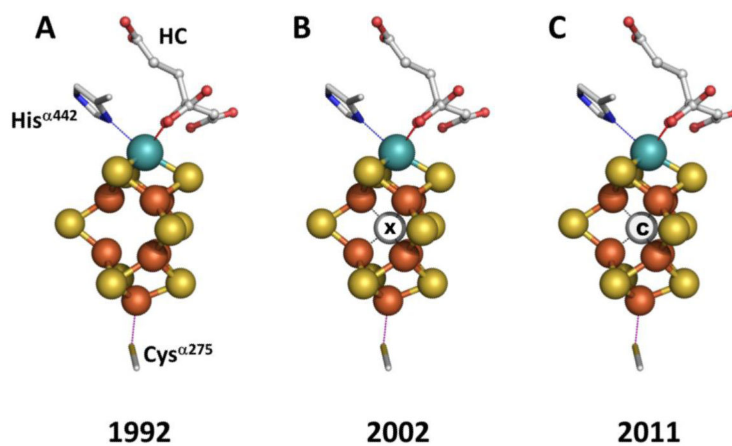

Fig. 3.

Structure of the FeMo cofactor based on data from 1992 (**A**), 2002 (**B**), and 2011 (**C**). The atoms are colored as those in Figure 2. His$^{\alpha442}$ and Cys$^{\alpha275}$, the two protein ligands of the FeMo cofactor, are indicated. HC, homocitrate. PYMOL was used to create this figure (PDB IDs: 1N2C, 1M1N, 3U7Q).

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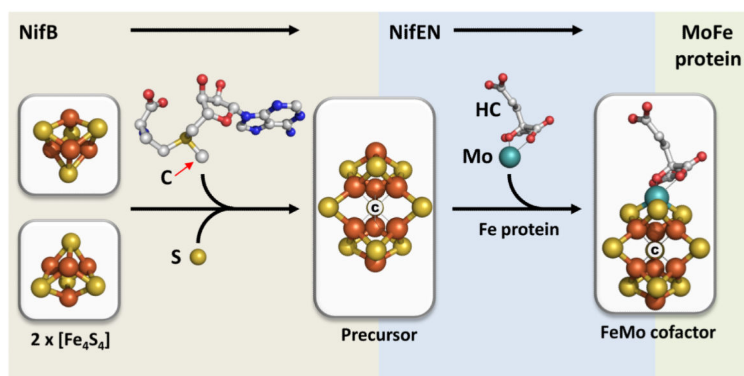

Fig. 4.
Biosynthesis of the FeMo cofactor. Shown are the coupling of two $[Fe_4S_4]$ clusters into a $[Fe_8S_9]$ precursor concomitant with carbide insertion (on NifB) and the subsequent conversion of the precursor to a fully matured FeMo cofactor upon Fe protein-mediated insertion of Mo and homocitrate (on NifEN).

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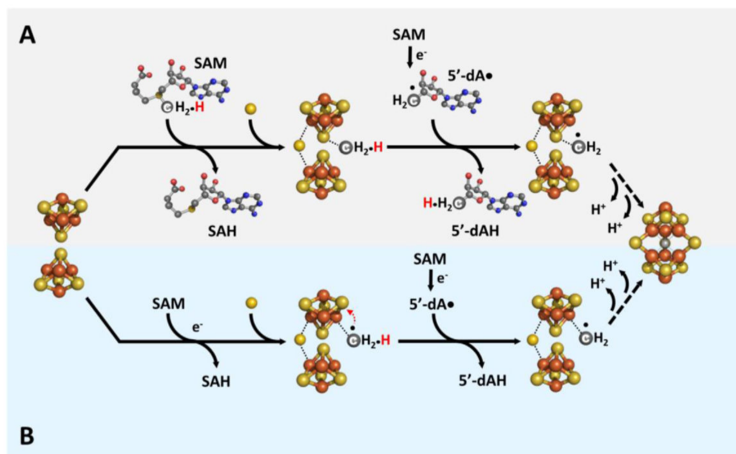

Fig. 5.

Proposed pathways of carbon insertion by NifB into the precursor of the FeMo cofactor. Both pathways involve hydrogen atom abstraction from a SAM-derived methyl group, radical-mediated methyl transfer to a $[Fe_4S_4]$ cluster, and continued deprotonation till an interstitial carbide ion is formed in the center of the precursor. However, the pathway in **A** begins with the transfer of methyl group via an SN2 mechanism, and it is followed by the formation of a methylene radical upon hydrogen atom abstraction by 5′-dA• and the subsequent transfer of this radical intermediate to a sulfur atom of the $[Fe_4S_4]$ cluster; whereas the pathway in **B** begins the formation of a methyl radical via reductive cleavage of SAM, and it is followed by the transfer of this transient intermediate to an iron atom of the $[Fe_4S_4]$ cluster and the subsequent processing of this intermediate into a methylene radical. The clusters are shown as ball-and-stick models, with the atoms colored as those in Figure 2. PYMOL was used to create this figure (PDB ID: 3PDI).