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Fresh insight to functioning of selected enzymes of the nitrogen cycle

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The global nitrogen cycle is the process in which different forms of environmental N are interconverted by microorganisms either for assimilation into biomass or in respiratory energy-generating pathways. This short review highlights developments over the last 5 years in our understanding of functionality of nitrogenase, Cu-nitrite reductase, NO reductase and N_2O reductase, complex metalloenzymes that catalyze electron/proton-coupled substrate reduction reactions.

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Introduction

Despite the different chemistries occurring at the active site the efficient utilisation of protons (H^+) and electrons (e^-) is a common feature of the enzymes considered in this review. Electron input to each of these enzymes is to an electron acceptor site of varying complexity that is distinct from the catalytic active site. Here we endeavour to correlate enzyme structures with what is known about these processes and their involvement in catalysis occurring at the active sites.

Mo nitrogenase

The reduction of N_2 to NH_3 by Mo-nitrogenase does not involve free intermediates and is accompanied by the reduction of H^+ to H_2, with the limiting stoichiometry:

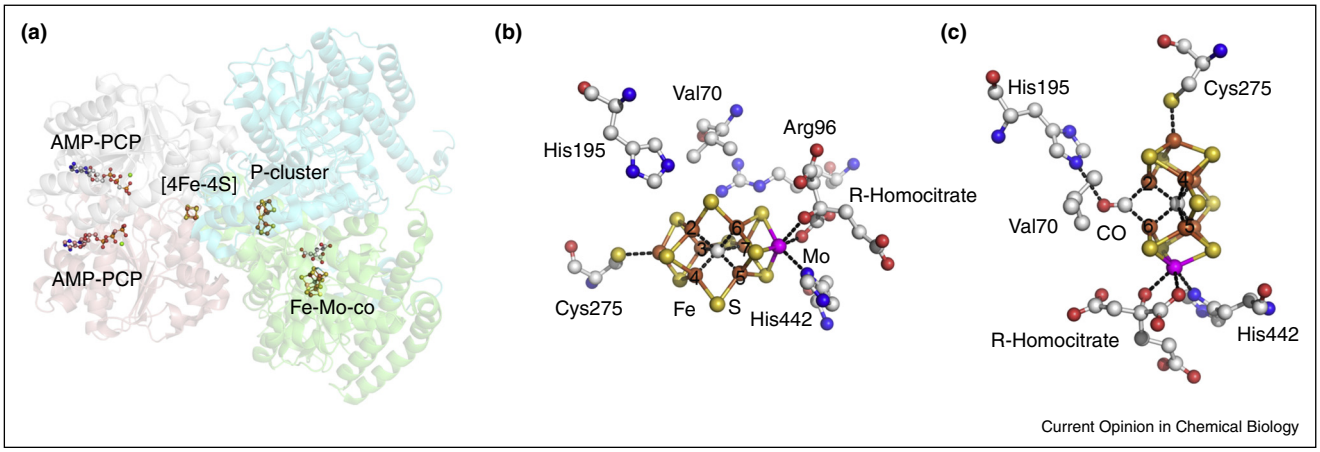
$$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

Nitrogenase is a two-protein component enzyme system comprised of an Fe protein that acts as a specific ATP-dependent electron donor to the MoFe protein (Figure 1a). The latter is an $\alpha_2\beta_2$ heterotetramer containing two [Fe_8-S_7] P clusters that transfer an electron to the catalytic FeMo-cofactor [Mo-C-Fe_7-S_9-homocitrate] sites. These unique clusters have complex biosynthetic pathways with P cluster assemblies *in situ*, and FeMoco on scaffold proteins before transfer to apo-MoFe protein [1]. One of the steps in FeMoco synthesis involves the radical *S*-adenosyl-L-methionine in the insertion of a carbide atom into the central core of the [Fe_8S_9] cluster. The unequivocal identification of the central atom is an important advance in consolidating spectroscopic and structural data [2,3].

Electron transfer events: With dithionite as reductant, turnover involves transfer of a single electron in a Fe protein–MoFe protein complex that subsequently dissociates. Counter-intuitively, the initial ET reaction is internal. The all-ferrous P cluster reduces FeMo-cofactor, followed by re-reduction via transfer of an electron from the ATP-bound Fe protein. This 'deficit spending' model was recently reviewed in detail here [4]. Subsequently, inter-protein ET was shown to occur before ATP hydrolysis and that P_i release is followed by dissociation of the two proteins in the rate-limiting step of turnover. These data demonstrate that ET is driven by the free energy of protein–protein association and suggest that ATP hydrolysis and Pi release promote dissociation of the Fe_{ox}(-ADP)$_2$ protein from reduced MoFe protein [5]. Structural studies suggest release of ADP/P_i and associated conformational changes account for the relatively long half-life of ~0.1 s of the ET complex (compared with typical values of ~10^{-3} s) required to allow control of the rate and timing of ET [6]. The initial encounter complexes involve electrostatic interactions with the β subunit that increase the association rate [7] before attaining the ATP-activated species in which the initial ET event is conformationally gated [8•].

Substrate binding to FeMo-cofactor: CO has long been known to be a potent non-competitive inhibitor of N_2 but not H^+ reduction. It has recently been shown to be a substrate being reduced to CH_4 and a variety of C2 and C3 products, suggestive of the activation of two CO molecules at adjacent sites [9]. Spectroscopic and computational studies assigned to Fe–CO and a partially reduced –CHO ligand suggests a flexible iron–carbon interaction may be important for FeMo-cofactor reactivity [10]. In a recent landmark study Rees and colleagues overcame the technical hurdle of crystallizing a transient two-component enzyme during turnover to determine the

Figure 1



Overall view of Mo nitrogenase complex and details of the FeMo-cofactor. **(a)** Structure of the MoFe and Fe protein complex stabilized by the ATP analogue AMPPCP. The structure clearly shows the route for transfer of electrons from the [4Fe-4S] cluster located in the Fe protein to the [8Fe-7S] P cluster located at the αβ subunit interface to the [Mo-C-Fe_7-S_9-homocitrate] catalytic FeMo-cofactor centre within the α subunit of the MoFe protein. Formation of the complex causes a conformational change in the Fe protein bringing the [Fe-4S] cluster 4 Å closer to the P cluster compared with a rigid-body docking model. **(b)** FeMo-cofactor showing the proximity of residues $\alpha70^{Val}$ and $\alpha195^{His}$ to the reactive Fe2-S2B-Fe6 face where substrate/inhibitors bind. **(c)** This structure of FeMo-cofactor during turnover under CO shows a single CO molecule bridging the Fe2 and Fe6 atoms of the central prismatic cage of FeMo-cofactor, in a μ_2-binding mode. This is enabled by the reversible displacement of the bridging sulfur atom S2B. CO is close to residue $\alpha70^{Val}$ and is H-bonded to residue $\alpha195^{His}$. Atoms in (b) and (c) are shown as spheres and sticks and coloured as: C – light grey, N – blue, O – red, S – yellow, Fe – orange and Mo–magenta. Pictures were generated with Pymol using coordinates from PDB 4wzb (for a and b) and PDB 4tkv (for c).

1.50 Å resolution structure of MoFe protein with the inhibitor CO bound [11••]. A single CO molecule bridged the Fe2 and Fe6 atoms of the central prismatic cage of FeMo-cofactor, in a μ_2-binding mode, enabled by the reversible displacement of a bridging sulfur atom. The CO is close to the catalytically important residues $\alpha70^{Val}$ and $\alpha195^{His}$ such that the oxygen atom of CO is in H-bonding distance of $\alpha195^{His}$ (Figure 1c). This study revealed flexibility of FeMo-cofactor on ligand binding and provides structural evidence supporting Fe as the binding site.

Reduction of N_2: Substrates require FeMo-cofactor to be more reduced than the 'as isolated' state in order to bind. The long-standing Lowe-Thorneley kinetic scheme for nitrogenase turnover [12] defined the rate constants for the transformation of intermediates during the reduction of N_2 as eight [e^-/H^+] are accumulated during successive reduction cycles by the Fe protein. The characterization of such reduced intermediates is hindered by H_2 evolution resulting in a relaxation of the reduced states of FeMo-cofactor, and it is only in the last 10 years that methods have been developed to allow accumulation and characterization of these species. The recognition of the roles of residues $\alpha70^{Val}$ and $\alpha195^{His}$ in control of substrate access and proton delivery to FeMo-cofactor respectively, enabled Dean, Seefeldt, Hoffman and coworkers to construct mutants and use freeze-quench and annealing techniques to trap intermediates. Together with isotopic labeling of ^{57}Fe and ^{95}Mo this allowed advanced spectroscopic techniques to be used to assign identities to them, and redefine the mechanism for N_2 reduction [13••,14•].

The revised mechanism allows the E_4, E_7 and E_8 states of the kinetic scheme of Lowe and Thorneley to be correlated with characterized intermediates, and favours Fe rather than Mo as the binding and reactive site of FeMo-cofactor, a notion for which early evidence emerged from XAFS data some 20 years ago. Substrate binding and catalysis primarily involve the Fe2-S2B-Fe6 region beneath the side chain of the $\alpha70^{Val}$ residue (Figure 1b). To bind N_2, FeMo-cofactor has to accumulate four electrons and protons (the pivotal 'Janus' E_4 state), beyond which the enzyme is committed to N_2 reduction. The E_4 state was assigned as having two hydrides bridging Fe atoms [Fe2-H-Fe6] with two protons presumed to bind to the sufides of FeMo-cofactor. Central to this mechanism is the hydride chemistry of the 4Fe face of FeMo-cofactor, which is activated for N_2 hydrogenation by the reversible reductive elimination of two hydrides to form H_2 on binding of N_2. The bound N_2 rapidly reduced to yield the characterized diazene (NH=NH) species. Subsequently reductive cycles lead to E_6 (N_2H_4-bound), and on delivery of the seventh [e^-/H^+], N–N bond cleavage occurs. The release of NH_3 forms the characterized $[NH_2]^-$-bound intermediate, and following the final [e^-/H^+], addition, E_8 with the product NH_3 bound and FeMo-cofactor restored to its resting redox state. This