Nitrogenase Structure: Where to Now?

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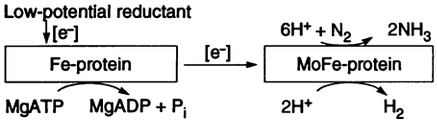
The dramatic contrast between the Haber-Bosch process for ammonia (NH_3) production with its elevated temperature, pressure, and capital costs, and the little soybean nodule or the free-living nitrogen (N_2)–fixing bacterium, plugging away at ambient temperatures and 1 atm, is an irresistible one. Each route accounts for about 10^8 tons per year of N_2 converted to NH_3 (*1*). Nitrogenase uses approximately 34 iron (Fe) atoms and 2 molybdenum (Mo) atoms to catalyze the reaction:

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

where MgATP is magnesium adenosine 5′-triphosphate and P_i is inorganic phosphate. For the quarter century since reproducible cell-free preparations of the enzyme have been available for producing NH_3 from N_2, the problem of determining the mechanism of this catalytic system has been in principle soluble. Two proteins are responsible for the reaction, the Fe-protein and the MoFe-protein (*2*):



The Fe-protein has two subunits and a single Fe_4S_4 unit with the atoms at the corners of a cube (a cubane structure) and can be understood on the basis of the structures of ferredoxins, a well-described class of low-potential Fe-S electron-transfer proteins. The Fe-protein will bind two MgATP units per molecule that are then hydrolyzed when an electron is transferred to the MoFe-protein. The Fe-protein bears at least part of the apparatus needed for reductive dephosphorylation or transduction of ATP hydrolysis energy into very negative electron potentials that initiate the saturation of the triple bond in N_2. The initial 2 e^- reduction is thought to be endothermic by ~49 kcal/mol; the overall reaction is exothermic by ~63 kcal/mol.

The MoFe-protein has been understood to be made of four subunits in an $\alpha_2\beta_2$ arrangement. Shah and Brill (*3*) established that each molecule yields two equivalents of Mo as the MoFe-cofactor, a complex of approximate composition $MoFe_7S_8$·(homocitrate) (*4*–*6*).

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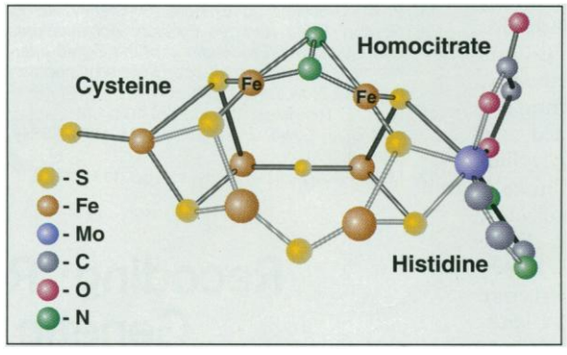
The cofactor is detected by its ability to activate a Mo-deficient protein, which is found in extracts of mutant strains of diazotrophic bacteria. X-ray absorption fine structure (EXAFS), Mössbauer, and electron paramagnetic resonance (EPR) studies have established that the cofactor in solution is essentially identical to the Mo-containing center in the protein, both being sulfur-bridged polynuclear clusters (*7*, *8*). The Mo environment in the protein included coordination to O- or N-containing ligands as well as S bridged to Fe. Studies of a *nif* V^- mutant strain show that the MoFe-cofactor participates directly in the substrate-reduction site of the MoFe-protein (*9*). Attempts to isolate, characterize, and synthesize this cofactor are being actively pursued in several laboratories.

The cofactor, present as two "M centers," accounts for 14 of the 30 Fe atoms in a molecule of MoFe protein. The remaining 16 Fe atoms are present in structures that yield four Fe_4S_4 cores upon displacement with organic thiols and are thought to be the four Fe_4S_4 clusters called P-clusters, which transfer 1 e^- per cluster (*10*).

Thorneley and Lowe have carried out and analyzed a comprehensive pre–steady-state and steady-state kinetics project and derived a kinetic mechanism (*2*, *11*, *12*) in which the Fe-protein pumps one e^- per two ATP molecules hydrolyzed into the MoFe-protein. Each stroke of this pump advances the redox status of the MoFe-protein one step around an eight-step (8 e^-) circular pathway. Hydrazine (N_2H_4) could be detected upon denaturation of MoFe-protein during reduction of N_2. The MoFe-protein acts like an electron buffer, in that the reduction products, H_2 and $2NH_3$, are released before all 8 e^- are aboard. Because a catalytically competent half-molecule does not have to collect all 8 e^- before N_2 is reduced and H_2 and $2NH_3$ are released, forms of the enzyme either further reduced or further oxidized than the form in which the enzyme is isolated may be catalytically important. This concept goes well beyond the notion that 8 e^- do not have to be stuffed into the Mo-Fe-S (M) cofactor center and P-cluster pair all at once; the mechanism is a subtle one.

Two summers ago, Rees and co-workers announced the solution of the atomic structure of the Fe-protein of *Azotobacter vinelandii*, and Bolin showed a low-resolution structure of the structure of the MoFe protein of *Clostridium pasteurianum* (*13*). From the latter, one knew that the M centers were widely separated in the protein. This structure posed two important problems for the field. The electron density ascribed to the P-cluster paired cubanes was seen to be not only adjacent, but most probably merged. Recent Mössbauer and EPR experiments showed that the subcomponent cubanes are tightly coupled pairs that act as 2 e^-/8 Fe redox units (*14*). The nature of the coupling could not be deduced unequivocally from the spectroscopic measurements. Also, the P-cluster and M-center in each half-molecule were 19 Å apart, a distance at which electron transfer is not an easy process to explain. Rees' structure of the Fe-protein depicted a pair of subunits joined by bonds from two pairs of cysteine S atoms to the Fe atoms of a Fe_4S_4 cube.

This summer, Rees' lab proposed an atomic-resolution structure of a MoFe-protein, as well as a further refined structure of the Fe-protein in which a bound nucleotide, suspected from analytical data (*15*), has been located. Bolin's general arrangement of metal clusters in the MoFe-protein was confirmed, and clear atomic models were proposed for both the P-cluster pairs (two Fe_4S_4 cubes bridged by cysteine thiolate S atoms) and the cofactor centers. These proposals were broached at the Enzymes Gordon Conference in early July, at EUROBIC in Newcastle at the end of July, and are described in detail elsewhere in this issue (*16*). Along with a description of further refinements of the MoFe-protein data of



The factory inside the protein. A working model is that N_2 binds to two "privileged" Fe atoms that have open coordination sites. This speculative structure of a side-on N_2 complex with the cofactor is based on the model of Kim and Rees. [Figure by W. H. Orme-Johnson and J. Selengut]

Bolin, these ideas were the centerpiece of a symposium on Mo enzymes at the American Chemical Society (ACS) meeting in Washington, D.C. (23 to 25 August 1992).

The MoFe-cluster structures of Kim and Rees incorporated published chemical composition, EXAFS, and magnetic resonance information as well as a great deal of chemical intuition derived from extensive model Mo-Fe-S and Fe-S chemistry (*17*). However, in the M-center structure, six of the seven Fe atoms are in an unusual three-coordinate state, which invites skepticism and speculation. On the other hand, Bolin's very recent further analysis of his data tended to ignore all but the composition data. Encouragingly, the basic concepts arrived at (conjoined cubes for the P-cluster and elongated Mo-Fe-S structures with Mo at one end for the cofactor) are identical, but the structural details are curiously disparate. In both of Bolin's structures, the metal coordination numbers are orthodox, but he has two P-cluster cubes sharing a S corner atom, which is thus depicted as a six-coordinate S atom (consider SF_6). Similarly, he inserts another six-coordinate S atom inside the Fe_6S_6 cage of the cofactor, to render the coordination of all six Fe atoms as four rather than the three of Rees' and Kim's formulation. In neither structure is there any obvious way for Mo to coordinate N_2 without losing a ligand or two. However, in the Rees and Kim structure two of the Fe atoms are privileged in that they could accommodate a side-on bound N_2 molecule. The kinetic mechanism suggests that the enzyme must be reduced in order to bind N_2. This form of the enzyme becomes important once the structural work on the resting state of enzyme has reached consensus. The irony that the Mo atom may not directly interact with N_2 is not lost on participants in this search.

The field of biological nitrogen fixation and the related areas of synthetic and mechanistic chemistry have undergone a major paradigm shift. The structural characterization of isolated cofactor (*18*), as well as the functions of all 20 *nif* genes (*2*), now take on a new life, as do studies with high-resolution probes like Mössbauer and electron double nuclear resonance (ENDOR) spectroscopies (*10, 19*), as well as gene-centered and protein modification probes (*20, 21*) of every aspect of the action of nitrogenase and its support system. One is keenly appreciative of the work of synthetic chemists (*17, 22*) who in trying to guess the answer to the structures of the nitrogenase clusters have laid down rich veins of metal-dinitrogen and metal-calcogenide cluster chemistry. Nitrogenase is still the only natural substance known to react with the major "inert" constituent of the atmosphere at atmospheric pressure and room temperature.

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Recoding: Reprogrammed Genetic Decoding

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The genetic code dictates how nucleic acid sequence is translated into amino acid sequence. The coded information is the sequential triplets of messenger RNA, each specifying a particular amino acid. In a minority of mRNAs there is another set of instructions contained in the mRNA sequence that specifies an alteration in how the genetic code is to be applied. In some cases these instructions alter the linear mechanism of readout; in other cases the meaning of code words is altered. We suggest that this phenomenon be called "recoding" and that the instructions in the mRNA be called "recoding signals."

There are many examples of redirection of the linear readout mechanism for individual mRNAs. The classical example is that of the *Escherichia coli* gene for release factor 2 (RF2), that encodes a protein needed for termination of translation. The RF2 mRNA programs some 30 percent of the ribosomes to change to the +1 reading frame after codon number 25 in order to complete synthesis of the active protein (*1*). (This is an autoregulatory process: codon 26 is a UGA terminator codon at which there is competition between termination involving RF2 protein and frameshifting.) The signal in the mRNA that causes this recoding has two components, the frameshift site (codons 25 and 26) (*2*) and an upstream sequence, termed a stimulator (*3*), that pairs with 16S RNA in the ribosome to encourage the frameshift event (*4*). This first example establishes the general principle of two crucial components to recoding signals: a site of action and a stimulatory signal.

One class of retroviruses and retroviral-like elements constitutes a large group of mRNA sequences that rely on programmed ribosomal frameshifts to make fusion proteins (*5*). Here, typically, the site of action is a heptanucleotide sequence in the mRNA at which ribosomes can shift to the −1 frame by the tandem slippage of transfer RNAs in the adjacent P and A sites into the new reading frame. The stimulatory sequence is downstream in the form of a stem-loop or pseudoknot structure (*6*) in the mRNA. A similar heptanucleotide mo-

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