Title:
*A study of the electronic structure and co-ordination chemistry of Iron-Sulfur clusters: Nitrogenase as a case study.*

INTRODUCTION:

WHAT ARE IRON-SULFUR CLUSTERS?
Biological iron-sulfur clusters are very important chemically versatile inorganic structures attached to several proteins. They came into focus around 1960 when studies of photosynthetic organisms, nitrogen fixing bacteria and sub mitochondrial fractions of mammalians cells showed the presence of proteins involved in oxidoreductive functions containing complexes of iron and cysteinate sulphur. The clusters commonly found in proteins are: Fe$_2$S$_2$, Fe$_3$S$_4$, Fe$_4$S$_4$ with ligands completing the Fe co-ordination$^1$(fig 1).

![Iron-Sulfur Clusters](image)

Iron-sulfur clusters were originally encountered as electron transfer agents in photosynthetic bacteria, in nitrogen fixation and mitochondrial electron-transport chain. They undergo core conversions, like from 2(2Fe-2S) to (4Fe-4S) in nitrogenase iron-protein$^1$. Such spontaneous cluster conversions are important in self activation of enzymes and restoration of their catalytic sites. The facts that Fe-S clusters are sensitive to dioxygen and can assume different oxidation states are exploited by nature in regulation and sensory functions. The oxidation state of Fe-S cluster of SoxR, a transcriptional regulator is used to sense the physiological superoxide level$^{1,3}$. In case of Iron-regulatory protein, IRP, the cluster assembly and disassembly signals levels of iron in the cell. These clusters may also serve as storage devices for iron and possibly for sulphide$^{1,3}$. 
Several metalloproteins like hydrogenase and nitrogenase have complex clusters which in addition to iron co-ordinated to sulfur has another metal, like molybdenum or nickel. Given the fact that these clusters are so important to the function of such complex proteins, it is of utmost importance to understand what governs their function and reactivity. The spin state of the cluster, charge delocalization over the central metal and the ligands, the co-coordinating ligands completing the co-ordination of Fe play a central role in determining the events involved in functioning of the Fe-S cluster. The most obvious function of these clusters is electron transfer in oxidoreductive reactions and a classic case to demonstrate this function is the nitrogenase enzyme.

NITROGENASE: CASE STUDY.
The enzyme nitrogenase catalyses the very last step in biological nitrogen fixation, namely the conversion of dinitrogen to ammonia. The enzyme consists of two separate proteins; the iron-protein (Fe-P) which acts as the electron transfer component to the substrate reduction protein called the molybdenum-iron protein (MoFe-P)\(^2\). The MoFe-P is a heterotetramer that has two distinct types of metal clusters, a (8Fe-7S) cluster called the P-cluster and a (7Fe-9S-Mo) cluster co-ordinated by homocitrate (Fe-Mo-cofactor)\(^2\). The Fe-P is a homodimer that has two chemically identical subunits bridged by a (4Fe-4S) cluster\(^2\). For catalysis to take place, the two components of the enzyme have to associate which in turn allows electron transfer from Fe-P to Mo-Fe-P\(^2\). During catalytic cycle, the two components assemble to form the nitrogenase complex to allow electron transfer from the Fe-P to the Mo-Fe-P.
The general mechanism of nitrogenase enzyme can be represented by the following sequence of reactions.

\[
\begin{align*}
N_2 + 8H^+ + 8e^- + 16MgATP & \rightleftharpoons 2NH_3 + H_2 + 16MgADP + 16Pi
\end{align*}
\]

The above equation represents the overall stoichiometry of the nitrogenase-catalyzed reduction of dinitrogen to ammonia.
Each component of the nitrogenase complex has its own unique structure which gives each of the proteins, its signature function.

The Mo-Fe-protein.
This protein has a $\alpha_2\beta_2$- subunit structure with a $M_r \sim 230kD$. It is comprised of two molybdenum atoms, 30-34 iron atoms and an equivalent number of sulfide atoms per molecule, making up the two metal clusters: P-Cluster and M-cluster. The P-cluster is located and the interface of $\alpha$ and $\beta$ subunit; it acts as a channel for electron transfer from the Fe-P to the M-cluster and also provides docking for the Fe-P. The M-cluster is the substrate reduction site for enzyme complex, and is located in the $\alpha$ subunit.

The P-cluster exists as [8Fe-7S]$^2$ cluster which is diamagnetic in the dithionite reduced form of the protein. It undergoes large scale conformational change going from the native to the oxidized form, exchanging the ligands that complete the co-ordination of the iron atoms. In the oxidized form, the cluster is comprised of a [4Fe-4S] cluster bound to the $\alpha$ subunit and a [4Fe-3S] cluster bound to the $\beta$ subunit. In this form, the central sulfur is bound to four iron atoms, three from the [4Fe-4S] cluster and one from the [4Fe-3S] cluster (fig3a). When the protein is reduced (fig3b), conformational changes result in the central sulfur to be surrounded by a distorted octahedral arrangement of the six Fe atoms (fig3).

The M-cluster is called the Mo-Fe cluster because of the presence of the Mo atom in one of the metal clusters making up this co-factor cluster. It is comprised of two partial
cubanes; [4Fe-3S] and [Mo-3Fe-3S], bridged by three non-protein sulfurs\(^2\). The co-factor cluster in *Aztobacter vinelandii* is co-ordinated by only two residues; \(\alpha\)-Cys257 (Fe1) and \(\alpha\)-His442 (to Mo)\(^2\). The octahedral co-ordination of Mo is completed by binding to hydroxyl and carboxyl oxygen of a homocitrate molecule\(^2\). The six iron atoms in the Mo-Fe cluster are attached to only the bridging non-protein sulfur atoms and remain in approximate triogonal geometry\(^2\). The complete cluster (MoFe\(_7S_9\)) has an idealized \(C_{3v}\) symmetry, with a Fe\(_6\) trigonal prism capped by an iron and a molybdenum atom on its \(C_3\) axis\(^2\) (fig4).

![Fig.4 Mo-Fe-co-Factor(1)](image)

The Fe-Mo and the Fe-Fe distances get slightly shortened when the protein goes from reduced to oxidized state\(^2\).

**The Fe-Protein:**
This protein is a homodimer covalently bridged by a single [4Fe-4S] cluster. It is important for electron transfer to the Mo-Fe-cofactor\(^2\). This cluster exists as a regular cubane coordinated to each dimer by two cysteine residues; Cys97 and Cys132\(^2\) (fig5). The cluster is positioned between the two subunits and is exposed to the solvent, making it sensitive to dioxygen.
For the electron transfer to take place, one MgATP is hydrolyzed by each of the subunits of the Fe-P. The binding of the MgATP to the Fe-P induces a conformational change in the proteins that alters the properties of the Fe-S cluster, notably a 100-mV decrease in the midpoint potential, a change in the anisotropy of the $S = \frac{1}{2}$ EPR signal of the [4Fe-4S]$^{2+}$ cluster and a dramatic change in the CD spectrum of the oxidized protein. This nucleotide dependent conformational change of the Fe-P also increases the reactivity of the cluster to chelators, so that the [4Fe-4S]$^{2+}$ cluster undergoes degradation via a discrete [2Fe-2S]$^{2+}$ intermediate.

Because of such large scale changes in properties, the [4Fe-4S] cluster of the iron protein serves a good example to probe the different oxidation states and charge localization in such clusters under the influence of protein environment or otherwise. The different oxidation states of the cluster can be used to probe the electronic structure and the ligand environment of the iron using various spectroscopic methods. Each of the methods, probes one different aspect of the electronic structure; for example EPR probes the spin state of the cluster. Other methods commonly used to study clusters are CD, Mossbauer and Resonance Raman. This review will discuss few of the spectroscopic studies done on the Fe-S cluster using the above mentioned spectroscopic methods.

**METHODS:**

**PROTEIN:**
Most of the spectroscopic studies covered in the review carried out on the iron protein (Fe-P) of nitrogenase was to probe the spin states of the cluster in different oxidation
states. The protein is studied both in its MgATP bound state in which case the Fe-S cluster is reduced; and MgADP bound state where the cluster is oxidized. This ensures that signature signals of the Fe-S cluster in all its oxidized forms are obtained. These signals can then be used to quantify the spin state of similar cluster structure and also to deduce functional characteristics of proteins contains the same.

SPECTROSCOPIC TECHNIQUES:
The most commonly used spectroscopic methods are:

- **EPR or ESR (Electron Paramagnetic Resonance):** This method probes the ground state of the cluster. For getting an EPR signal from the cluster, it is mandatory that the irons in the cluster have net spin. In their oxidized state, the [2Fe-2S] and the [4Fe-4S] clusters have Fe$^{3+}$ pairs, antiferromagnetically coupled. Only when the cluster gets reduced, does it show up in EPR. This method looks at only odd electron metals and can probe the orbital that has the unpaired electron.

- **Mossbauer:** This method is used to measure the s-electron density at the nucleus of a Fe atom using the isomer shift from normal Fe to $^{57}$Fe. It examines the oxidation and spin states of Fe; and also gives a picture of the electron configuration. Because the $d$ and $s$ electron cloud have a very good overlap, this method is most useful to probe the $d$ electron population and is a good indicator of the oxidation state of iron.

- **Resonance Raman:** this method probes the valence-excited state of the metal in the cluster by combining Electron absorption and Raman spectroscopic techniques. It is useful to probe the transient geometrical changes brought about in the electronic orbitals of the metal in the excited state. It is also used to assign CT bands for charge transfer between ligand and metal, which in turn would allow interpreting the strength of the metal-ligand interaction.
ANALYSIS:

Extensive EPR studies on the Fe-P from *Aztobactoer vinelandii* have shown that the $[4\text{Fe}-4\text{S}]^+$ in the native protein exists in two separate forms characterized by the different spin states of the cluster; one has $S=1/2$ state and exhibits the typical $g = 1.94$ type EPR signal and the other has $S = 3/2$ and exhibits $g \sim 5.8$ and $5.1^5$. Some exceptional properties noted form such studies are that $S =3/2$ cluster has a broad range of EPR signal at $g \sim 4.8$ and also, in the MgATP-bound protein spectra at $g \sim 4.8$ an $S =5/2$ spin state is observed (following representative fig from 5). The MgADP bound Fe-P has a rhombic EPR signal, intermediate between native Fe-P and the axial signal from MgATP bound Fe-P$^6$. Quantitation of the signals shows that there is $\sim 0.4$ spins/4Fe$^6$.

Representative figure from (5) showing EPR signals obtained form different oxidised states of Fe-P.
In the above figure, a and b are signals from native Fe-P, showing characteristic signal at \( g \approx 1.94 \). c and d are from ATP bound state of Fe-P which has been exposed to creatine phosphate (which hydrolyses ATP). e and f are from ATP bound state which has not been exposed to creatine phosphate; this is the reduced form of Fe-P. g and h are from ADP bound state of the Fe-P; i and j are unresolved native Fe-P and k and l are resolved ATP samples. The results from the above figure were summarized by Orme-Johnson et al.\(^5\), in the following table:

<table>
<thead>
<tr>
<th>Protein form</th>
<th>( S = 1/2 )</th>
<th>( S = 3/2 ) (urea)</th>
<th>( S = 3/2 ) (ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Av2/native</td>
<td>45</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>Av2/urea</td>
<td>15</td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td>Av2/ethylene glycol</td>
<td>90</td>
<td>0</td>
<td>10(^*)</td>
</tr>
<tr>
<td>Av2/ADP</td>
<td>45</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>Av2/ATP</td>
<td>60</td>
<td>0</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^*\) Based on the shape and intensity of the Av2/ethylene glycol low field EPR signals.

The studies form Mossbauer spectra and circular dichroism indicates that the charge localization in a cluster can be explained for different oxidation states. In the 2+ state of the 2Fe cluster, there are two Fe\(^{3+}\) atoms. These spins are 5/2 for each iron, and are antiferromagnetically coupled, resulting in a net spin of zero. As an additional electron is added to the cluster, it settles on one of the irons, making it ferrous and giving the cluster a typical EPR signal of \( S = 1/2 \) or \( g = 1.94 \). However, this electron tends to hop from one iron to another, making both the irons 2.5+ resulting in a mixed valence state with parallel spins. This kind of spin-coupled high-spin pair is always observed in a 4Fe cluster, in which case there is a pair of Fe\(^{3+}\) and Fe\(^{2+}\). When an extra electron is added to this system, the most stable state arises from a mixed valence pair of irons and a ferrous pair which are each antiferromagnetically coupled to spins 9/2 and 8/2. Following is a representative figure from (4) showing the localization and delocalization of charges in a [4Fe-3S] cluster.
Such charge delocalization and mixed spin states is seen in [2Fe-2S] and [3Fe-4S] clusters (fig.6)³

NMR studies on the oxidized high-potential iron protein (HiPP), the 3+ state with one mixed valence pair, give information how the valence pair shifts from one pair to another. It is seen that in the following figure³ (fig.7) it can be seen that the mixed valence pair shifts position from one face of the cluster to another.
Spectroscopic studies of the Fe-S cluster of nitrogenase is also used to typify variants of the protein to the different oxidised state. Study of the MgATP bound state of the Fe-P has been very difficult because the ATP in the complex tends to get hydrolysed. One of the available variants, the deletion mutant L127, has proved to be very useful for the study of the “on state” of the protein which mimics the MgATP bound form. However, the characterization of this variant to the MgATP bound state was made by crystallographic and spectroscopic studies. It was the characterization of the Fe-S cluster of this variant using EPR and resonance Raman spectroscopy which in turn facilitated the mutant protein’s assignment to the ATP bound state.

EPR studies of the ditionite-reduced samples of L127 show that its Fe-S cluster has a near axial $g = 2.03, 1.92, 1.87$ and a weak feature at $g \sim 5^2$. The first three features of the EPR signal account for 0.6 spin/dimeric Fe protein and the weak signal at $g \sim 5$ is indicative of a minor spin, $S = 3/2^2$. These EPR properties are in agreement with previously characterized mixed spin state of $[4\text{Fe-4S}]^{2+}$ cluster in the ATP bound state. Resonance Raman shows the presence of the both $[4\text{Fe-4S}]^+$ and $[2\text{Fe-2S}]^+$ clusters in the thionine oxidised samples. Following is the representative Resonance Raman spectra of thionine oxidised L127 samples (Fig.8 (a), from 8) compared to wild type (Fig.8 (ba), from 8) under the same conditions. The spectrum of $[2\text{Fe-2S}]^-$ in isolation is obtained after treating the oxidised sample of MgATP-bound-L127 with EDTA$^2$. One of the conclusions drawn from the analysis for the Resonance Raman spectra for L127 and wild type was that ATP binding to the Fe-P results in the degradation of the $[4\text{Fe-4S}]^{2+}$ to
the semistable $[2\text{Fe}-2\text{S}]^{2+}$ clusters. For the L127 mutant, the cleavage of the regular cubane $[4\text{Fe}-4\text{S}]^{2+}$ cluster into two $[2\text{Fe}-2\text{S}]^{2+}$ rhombs is induced by glycerol (Fig. 9, from 8).

**Fig. 8** (a) Resonance Raman spectra of thionin-oxidized samples of the L127 deletion variant of *A. vinelandii* Fe protein: (a) oxidized after removal of glycerol; (b) sample in (a) treated with a 5-fold stoichiometric excess of EDTA; (c) difference spectrum, (a) minus (b); (d) oxidized in the presence of 30% (v/v) glycerol. Samples were all ~3 mM in Fe protein and were oxidized anaerobically after removal of thionin using a 2-fold stoichiometric excess of thionin. Spectra were recorded at 17 K, using 457.9 nm excitation, by photon counting for 1 s every 1 cm$^{-1}$. Each spectrum is the sum of 90–100 scans. Bands originating from lattice modes of ice, glycerol, and oxidized thionin have been subtracted.

**Fig. 9(b)** Resonance Raman spectra of thionin-oxidized samples of wild-type *A. vinelandii* Fe protein: (a) oxidized after removal of glycerol; (b) sample in (a) treated with a 4-fold stoichiometric excess of MgATP; (c) difference spectrum, (b) minus contribution from $[2\text{Fe}-2\text{S}]^{2+}$-containing wild-type Fe protein (analogous to the spectrum shown in Figure 4b); (d) sample in (a) treated with 30% (v/v) glycerol and a 4-fold stoichiometric excess of MgATP; (e) difference spectrum, (d) minus (c). Samples were all ~3 mM in Fe protein and were oxidized anaerobically after removal of thionin using a 2-fold stoichiometric excess of thionin. Spectra were recorded at 17 K, using 457.9 nm excitation, by photon counting for 1 s every 1 cm$^{-1}$. Each spectrum is the sum of 90–100 scans. Bands originating from lattice modes of ice, glycerol, and oxidized thionin have been subtracted.

*(The conditions of the experiments for the resonance raman spectra are mentioned in the foot note of each figure, taken from 8.)*
DISCUSSION:
Spectroscopic studies show that for metal sites containing high spin transition metals like iron, there exists a major spin state and a minor spin state, having different spin polarization\(^7\). At any time, there exists a greater number of spin-up (\(\alpha\)) electrons than spin-down (\(\beta\)) electrons, which results in a difference in electron densities and exchange-correlation potential\(^7\), resulting in an inverted energy level (fig.10)\(^7\).
For any mononuclear or polynuclear system, the ligand will split the above energy level into different patterns (fig.11) depending upon the co-ordination geometry. Such spin splitting is important for Fe-S clusters.

![Energy level scheme (fig.10)](image)

The spins on adjacent metal centers are spin coupled, usually antiferromagnetically. This is favored by the Heisenberg exchange coupling and represents weak metal-metal bonding (fig.12).

**Fig.11** (a) tetrahedral or distorted tetrahedral iron co-ordination. (b) triogonal or distorted triogonal iron co-ordination.

A single minority spin electron is placed in the lowest ligand field orbital in each case.

![Spin coupling diagram (fig.12)](image)
These spectroscopic studies, along with theoretical calculations using density functional theory on various simple and complex clusters; it is seen that one of the contributing factors to the chemistry of the cluster is the metal-ligand covalency. This covalency is a function of the ligand environment and changes when the protein goes from oxidized to reduced state; as seen in the P-cluster of nitrogenase. It is also notable that the metal-ligand covalency, especially for sulfur, is higher for Fe$^{3+}$ sites than Fe$^{2+}$ sites. It has also been seen that the bridging sulfur are better donors in a Fe-S bond than terminal thiolates are, and more importantly, the bridging sulfurs are better donors in a [2Fe-2S] system than in a [4Fe-4S] system. When the protein goes from oxidized to the reduced state, sulfur charges become more negative because of the increased hydrogen bonding with the peptide backbone and/or the side chains, which in turn results in higher redox potential observed for the protein. The hydrogen bond competes with the bridging sulfurs for charge transfer to the metal. The higher redox potentials result from two contributing factors, namely, the hydrogen bond potential and the charge polarization over sulfur resulting due charge transfer into the NH-S bond.

Such studies on nitrogenase system shows that the magnetochemistry of the Fe-S clusters can be extensively used to understand the very functioning of these amazing inorganic
catalysts. The different co-ordination properties of iron are useful not only to make several proteins functional but also to probe the spin states of the clusters. This makes it easy to study and characterize the Fe-S clusters. It also makes it possible for us probe the core transition of cluster having one geometry to one with another geometry, e.g from cuboidal [4Fe-3S] to cubane [4Fe-4S]. We can also study the ligand swapping which changes the spin state of the metal and determine what ligand is co-coordinating to the central metal. The study of the [Fe-S] clusters holds promise not only for basic research but also for industrial application of their synthetic analogs.
References:


2. Sen.,S., Nucleotide dependent Conformational Changes in the Nitrogenase Fe Protein PhD dissertation submitted to Montana State University in the Department of Chemistry and Biochemistry (2005) 1-54


