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Spectroscopic Studies of Biological and Synthetic Tetranuclear Iron-Sulfur Clusters.

Yvonne Amalia Onate
Louisiana State University and Agricultural & Mechanical College

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Spectroscopic studies of biological and synthetic tetranuclear iron-sulfur clusters

Oñate, Yvonne Amalia, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1989
SPECTROSCOPIC STUDIES
OF
BIOLOGICAL AND SYNTHETIC
TETRANUCLEAR IRON-SULFUR CLUSTERS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in
The Department of Chemistry

by
Yvonne Amalia Oñate
B.S., Louisiana State University, 1983
December 1989
To my parents

for their constant love, support and encouragement
ACKNOWLEDGEMENTS

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<th>Full Form</th>
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<tr>
<td>(al)₂cat</td>
<td>3,6-Diallyl catecholate</td>
</tr>
<tr>
<td>Ac₁</td>
<td>Conventional MoFe protein from <em>Azotobacter chroococcum</em></td>
</tr>
<tr>
<td>Ac₁⁺</td>
<td>Alternative VFe protein from <em>A. chroococcum</em></td>
</tr>
<tr>
<td>Ac₂</td>
<td>Conventional Fe protein from <em>A. chroococcum</em></td>
</tr>
<tr>
<td>Ac₂⁺</td>
<td>Alternative Fe protein from <em>A. chroococcum</em></td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>Av₁</td>
<td>Conventional MoFe protein from <em>Azotobacter vinelandii</em></td>
</tr>
<tr>
<td>Av₁⁺</td>
<td>Alternative VFe protein from <em>A. vinelandii</em></td>
</tr>
<tr>
<td>Av₂</td>
<td>Conventional Fe protein from <em>A. vinelandii</em></td>
</tr>
<tr>
<td>Av₂⁺</td>
<td>Alternative Fe protein from <em>A. vinelandii</em></td>
</tr>
<tr>
<td>BPS</td>
<td>Bathophenanthroline disulfonic acid</td>
</tr>
<tr>
<td>cat</td>
<td>Catecholate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
</tr>
<tr>
<td>Cp₂</td>
<td>Fe protein from <em>Clostridium pasteurianum</em></td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N Dimethylformamide</td>
</tr>
<tr>
<td>dmpe</td>
<td>1,2-Bis(dimethylphosphino)ethane</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-Diphenyl-1-picylhydrazine</td>
</tr>
<tr>
<td>ENDOR</td>
<td>Electron nuclear double resonance</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Extended X-ray absorption fine structure</td>
</tr>
<tr>
<td>Fd</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>HiPIP</td>
<td>High potential iron protein</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Kp₂</td>
<td>Fe protein from <em>Klebsiella pneumoniae</em></td>
</tr>
<tr>
<td>LP</td>
<td>Linear polarizer</td>
</tr>
<tr>
<td>MCD</td>
<td>Magnetic circular dichroism</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MetMbCN</td>
<td>Metmyoglobin cyanide</td>
</tr>
<tr>
<td>Mg·ADP</td>
<td>Magnesium salt of adenosine diphosphate</td>
</tr>
<tr>
<td>Mg·ATP</td>
<td>Magnesium salt of adenosine triphosphate</td>
</tr>
<tr>
<td>MOPS</td>
<td>(3-[N-Morpholino]propanesulfonic acid)</td>
</tr>
<tr>
<td>mW</td>
<td>milliWatt</td>
</tr>
<tr>
<td>NMF</td>
<td>N-Methylformamide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PEM</td>
<td>Photoelastic modulator</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
</tr>
<tr>
<td>RR</td>
<td>Resonance Raman</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
</tr>
<tr>
<td>Tris/HCl</td>
<td>Tris(hydroxymethyl)aminomethane HCl</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray absorption near-edge structure</td>
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<td>ZFS</td>
<td>Zero-field splitting</td>
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ABSTRACT

Room-temperature UV-visible absorption, low-temperature magnetic circular dichroism (MCD), electron paramagnetic resonance (EPR), and resonance Raman (RR) spectroscopies have been used to characterize the electronic, magnetic and structural properties of the [4Fe-4S] centers in Azotobacter vinelandii Fe proteins from Mo- and V-nitrogenases (Av2 and Av2', respectively), and Bacillus subtilis glutamine phosphoribosylpyrophosphate amidotransferase.

These studies have characterized the S = 1/2 and S = 3/2 forms of the [4Fe-4S]$^{1+}$ cluster in native Av2 and Av2' in the presence and absence of the protein unfold agent urea, glassing solvents, such as ethylene glycol and glycerol, and nucleotides, such as ATP. The results show that in the presence of glassing solvents, the [4Fe-4S]$^{1+}$ cluster in native Av2 is mainly in the S = 1/2 form while in the presence of urea, the cluster is mainly in the S = 3/2 form. The presence of Mg-ATP does not effect this medium-dependent cluster spin state behavior. Av2' has a similar behavior, except that the S = 3/2 form of the cluster is more dominant in all cases. The results afford the first characterization of the MCD properties of an S = 3/2 [4Fe-4S]$^{1+}$ cluster and establish the changes in the electronic structure that accompany S = 3/2 ↔ S = 1/2 spin state conversion. RR studies of oxidized Av2 show that the [4Fe-4S]$^{2+}$ is structurally analogous to similar centers in simple ferredoxins, and show that it can be partially degraded into [2Fe-2S]$^{2+}$ clusters, in the presence of excess thionine.

Spectroscopic studies of photochemically-reduced B. subtilis amidotransferase reveal the presence of S = 3/2 [4Fe-4S]$^{1+}$ and S = 5/2 [4Fe-4S]$^{1+}$ clusters as the major and minor species, respectively, with the latter becoming more pronounced at protein high concentrations. RR studies of the native enzyme show that the structure of the [4Fe-4S]$^{2+}$ clusters is similar to that in simple bacterial
In addition, the properties of cubane-type [MoFe$_3$S$_4$] and [VFe$_3$S$_4$] synthetic clusters, which serve as possible models for the FeMo- and FeV-cofactors in Mo- and V-nitrogenase have been investigated using room-temperature UV-visible absorption, and low-temperature MCD and EPR spectroscopies. The results reveal S = 3/2 ground states for all five clusters investigated. Comparison of spectra from these synthetic clusters with the MoFe and VFe clusters at the active sites of Mo- and V-nitrogenases suggests that the differences in the spectroscopic properties of the protein bound clusters arise from differences in protein ligation.
INTRODUCTION

Fixed dinitrogen is essential for all living things since it is a component in all protein and nucleic acids. The nitrogen consumed by humans and animals is obtained indirectly from the ammonium salts applied to crops as fertilizer. The ammonia used to make these salts is produced industrially via an iron oxide catalyst used in the Haber-Bosch process. Such process requires extreme reaction conditions (100-1000 atm pressure and 400-550 °C) and consumes a considerable amount of energy. This energy required adds significantly to the cost of fertilizer and food. This fact has prompted scientists to look for an alternative, low-energy-consuming method for the synthesis of ammonia for agricultural fertilizer and other industries. A possible solution to this problem has been sought in the enzyme nitrogenase, which catalyzes the conversion of N₂ from the air into ammonia. The mechanism of this enzyme may provide a basis for the design of more efficient industrial catalysts.

Since it was first isolated more than 30 years ago, extensive studies have been conducted on the purification and characterization of nitrogenase. Nitrogenase has been purified from a wide variety of N₂ fixing organisms. In all cases it consists of two proteins, the Fe protein (component 2) and the cofactor-containing protein (component 1). In the conventional enzyme, the component 1 is a molybdenum-iron protein (MoFe) and in the alternative enzyme, it is a vanadium-iron (VFe) protein. The alternative enzyme is produced by mutants in which the structural genes for component 1 and component 2 of conventional nitrogenase have been deleted.

The Fe protein is a dimer of identical subunits and it contains one [4Fe-4S] cluster which may be bridging the two subunits (Mr ≈ 63 kDa). The function of this protein is to transfer electrons to the active site containing MoFe (or VFe)
protein. This electron transfer between the two proteins requires the hydrolysis of Mg-ATP molecules, two of which bind to the Fe protein. In the native state (dithionite reduced), Av2 and Av2' are paramagnetic exhibiting the EPR spectrum and g-values of a [4Fe-4S]^{1+} cluster. However, the electron paramagnetic resonance (EPR) properties of the native Av2 protein show some puzzling aspects. In particular, the signal in the S = 1/2, g = 2 region, was found to yield low spin quantitations and to be dependent on the nature of the medium. The first of these questions was answered by recent investigations which showed that the [4Fe-4S]^{1+} cluster of this protein contains a mixture of two spin states, S = 1/2 and S = 3/2. However, the spectroscopic information available for Av2' was limited to one EPR study. Thus, the fact that these clusters were poorly characterized at the initiation of this work, prompted us to investigate their electronic, magnetic, and structural characteristics in the reduced and oxidized state, using a novel combination of room-temperature UV-visible absorption spectroscopy, and low-temperature EPR, magnetic circular dichroism (MCD), and resonance Raman (RR) spectroscopies. These results facilitated the investigation and characterization of the photochemically-reduced and oxidized [4Fe-4S]^{n+} (n = 1, 2, respectively) cluster in Bacillus subtilis glutamine phosphoribosylpyrophosphate amidotransferase, which was found to have a mixture of high multiplicity ground states in the reduced form.

The nitrogenase MoFe protein contains up to 2 Mo and about 30-32 Fe and acid-labile sulfur atoms which are grouped into two FeMo cofactor centers, of stoichiometry MoFe_{6-8}S_{4-10}, and about four [4Fe-4S] P-clusters of unknown structure. The two VFe proteins that have been isolated thus far are reported to have quite different Fe and V contents. However, they both have a V:Fe ratio of approximately 1:12 and spectroscopic evidence suggests that each has a FeV cluster and P-clusters. Since there is evidence that the nitrogenase cofactor is
the active site for binding and reduction of $\text{N}_2$, considerable efforts have been made to characterize the structural, electronic, and magnetic properties of the cluster in this cofactor, however, the structure of these cofactors remains one of the major unresolved problems in contemporary bioinorganic chemistry since crystals suitable for X-ray crystallographic studies have yet to be obtained. An alternative approach involves the study of synthetic model clusters with similar properties. The present work has involved the spectroscopic investigation of some cubane-type $[\text{MoFe}_2\text{S}_4]$ and $[\text{VFe}_2\text{S}_4]$ synthetic complexes using room-temperature UV-visible absorption spectroscopy, and low-temperature MCD and EPR. It was hoped that these studies would facilitate interpretation of differences in the EPR and MCD properties of the FeMo and FeV clusters in Mo- and V-nitrogenases.

**Low-temperature MCD spectroscopy** provides an optical probe for chromophoric paramagnetic centers. This technique is particularly useful for investigating the electronic and magnetic properties of metal centers in biological systems, and has been applied to the study of Fe-S clusters in several different proteins. The main advantages of low-temperature MCD over other more conventional spectroscopic techniques are that it is not restricted to Fe, and that it can selectively monitor the optical transitions from a particular paramagnetic metal center in a multicomponent system. In addition to the form of the MCD spectrum, *i.e.*, the sign, frequency, and intensity of the bands, information about the ground state can be obtained by detailed investigation of the magnetic field and temperature dependence of different MCD transitions, called magnetization curves. Electronic paramagnetic resonance spectroscopy has been used extensively in the study of metalloproteins. Moreover, most of the current knowledge about the Fe-S centers in nitrogenase has been obtained from this technique. However, this technique can be limited in the case of $S \geq 3/2$ $[\text{4Fe-4S}]^{1+}$ clusters since the
resonances can be weak and difficult to detect, particularly in cases where there is cluster heterogeneities.

Prior to this work, low-temperature MCD spectroscopy had not been applied to the study of nitrogenase Fe proteins or any [4Fe-4S] clusters with high spin-multiplicity. Also, this is the first time that this technique is used in the investigation of synthetic Fe-S clusters. Therefore, this research has been important in the development of low-temperature MCD as a technique for identifying and monitoring the properties of Fe-S clusters in metalloproteins.
1. BACKGROUND

1.1 SPECTROSCOPIC TECHNIQUES

1.1.1 UV-VISIBLE ABSORPTION SPECTROSCOPY

Electronic absorption spectra originate from the promotion of electrons from the ground state to an electronic excited state. Since the theory of electronic absorption spectroscopy is well developed and several good reviews are available, it will not be discussed here. Instead, some aspects of relevance concerning transition metal coordination compounds in general and Fe-S clusters in particular will be covered.

UV, visible, and near IR are the regions of the spectrum were electronic absorption bands for transition metal elements are observed. The transitions observed for transition metal complexes can be explained by crystal field theory and ligand field theory (1-5). The transitions arising from transition metal complexes are usually of two types: 1) ligand field or d-d transitions, which are mainly localized in the transition metal ion and are usually broad and weak, and 2) charge transfer transitions, which involve displacement of charge between the metal ions and the ligands, and are usually stronger and located at higher energy than d-d transitions.

The selection rules that control electronic transitions can be summarized as follows (2):

1. The transition dipole moment integral must be nonzero in order to observe the transition.

2. According to Laporte’s rule, electronic transitions must involve a change in orbital angular momentum.

3. For molecules with a center of symmetry, only g → u or u → g transitions
are allowed.

4. Only transitions between states of the same spin multiplicity are allowed.

5. Only one-electron transitions are allowed.

In accord with the above selection rules, d-d transitions are formally forbidden. However, in transition metal complexes the metal orbitals are not pure d-orbitals and there are factors such as the Jahn–Teller effect, vibronic coupling, and spin-orbit coupling that remove orbital degeneracy and/or cause some of these formally forbidden transitions to become partially allowed.

In general, electronic absorption spectra provide substantial information pertaining to the structure of transition metal compounds. In metalloproteins, in particular, they can provide a description of the electronic and geometrical structure of the metal ion site. Sometimes, they can even contribute to the identification of the ligands at the metal binding site, by comparison with model complexes (6). This work will mainly deal with Fe-S chromophores, which are a very important prosthetic group in numerous metalloenzymes. Their electronic absorption spectra exhibit d-d transitions usually in the near IR region. However, the dominant bands in the electronic spectra, and the reason for their brown coloration, arise from S → Fe charge transfer bands which are generally observed below 600 nm (7). Nevertheless, the UV-visible spectra of metalloenzymes with Fe-S centers are usually broad and featureless and, therefore, are of little diagnostic use in determining the nature and properties of these centers.

1.1.2 RESONANCE RAMAN

In resonance Raman (RR) spectroscopy, laser excitation within an electronic absorption band produces selective enhancement of Raman lines arising from vibrations of the chromophore. The enhancement can be very large, up to $10^2$-$10^6$ fold relative to normal Raman scattering (8). Therefore, RR spectroscopy has
proved to be a very selective and sensitive probe for investigating the structure of chromophoric transition metal centers in biological systems, whatever their physical state (9). In particular, this technique is very useful for investigating the cluster-type and structural variations of the Fe-S centers in metalloproteins in solution (for recent review, see ref. 10). Since these centers contain sulfide bridges between Fe atoms and terminal thiolate Fe coordination, the RR spectra are dominated by Fe-S stretching modes that are enhanced via $S \rightarrow Fe$ charge-transfer transitions in the visible region. These totally symmetric Fe-S bridging modes are observed at frequencies characteristic of the cluster type, e.g., $\sim 335$ cm$^{-1}$ for [4Fe-4S], $\sim 345$ cm$^{-1}$ for [3Fe-4S], and $395$ cm$^{-1}$ for [2Fe-2S] clusters (10). Terminal Fe-S(cys) stretching frequencies are located near 360 cm$^{-1}$ for [4Fe-4S], and a pair of bands, with variable intensities, is found at $\sim 325$ and $\sim 340$ cm$^{-1}$ for [2Fe-2S] clusters(10). The theory of RR spectroscopy will not be discussed here, since it is well developed and several good reviews are available.

1.1.3 MAGNETIC CIRCULAR DICHROISM (MCD)

Plane polarized light can be pictured as the resultant of left and right circularly polarized light propagating in phase with equal frequency and intensity (Fig. 1-la). These components interact equally with matter as long as the medium through which they pass consists of symmetrical molecules. A rotation in the plane of polarization occurs as a result of a difference in the refractive indices of left and right circularly polarized light, when plane polarized light passes through an optically active substance. The measurement of the angle of rotation is called optical rotatory dispersion (ORD) (Fig. 1-1b). Alternatively, optical activity can arise from a difference in the absorption coefficients for left and right circularly polarized light. These results in elliptically polarized light and is termed circular dichroism (CD) (Fig. 1-1c). However, in the presence of a
Figure 1-1. (a) Plane polarized light resulting from two circularly polarized beams. (b) Optical activity resulting from a difference in refractive indices for left and right circularly polarized light. (c) Optical activity resulting from a difference in absorption of left and right circularly polarized light.
longitudinal magnetic field all substances become optically active. The induction of optical activity by a magnetic field in all matter is known as Faraday effect, after its discoverer. In regions of absorption, there is a concomitant differential absorption of left and right circularly polarized light that introduces a wavelength- and field-dependent ellipticity of the resultant beam, and gives rise to the magnetic circular dichroism (MCD) spectrum.

1.1.3.1 BASIC THEORY

The Faraday effect is closely related to the Zeeman effect which also arises from the interaction of magnetic field with matter. A state specified by an angular momentum \( J \), is \((2J+1)\)-fold degenerate in the absence of a magnetic field. A magnetic field causes a removal of the degeneracy of the angular momentum into its \( z \)-components, \( M_J = J, J-1, \ldots, -J \). The relative energies of the states are determined by the Landé \( g \)-factor with the energy of each Zeeman component equal to \( g\beta BM_J \), where \( \beta \) is the Bohr magneton, and \( B \) is the magnetic field strength.

A circularly polarized photon has well defined \( z \)-components of angular momentum, of +1 and -1, that correspond to left and right circularly polarized light, respectively. Thus the selection rules for absorption of left and right circularly polarized light are \( \Delta M_J = +1 \), and \( \Delta M_J = -1 \), respectively. Simply stated, the absorption of circularly polarized light must be accompanied by a change in the quantized component of the angular momentum of the chromophore.

Since there are several reviews of the theory of MCD available (11-19), only a brief summary of the results, with emphasis on the theory of low-temperature spectroscopy concerning the study of metal centers in metalloproteins, is presented here. The formalism of Stephens (see ref. 16) is followed throughout, unless otherwise specified.
In general, there are three stages in the calculation of the optical activity of a substance (18): 1) The observable is related to the difference in refractive indices or absorptions of left and right circularly polarized light; 2) this difference is expressed through Maxwell's equations in terms of the moments induced in individual molecules by the electromagnetic wave; 3) the induced moments are calculated quantum mechanically. Since MCD eliminates problems due to background rotation from other electronic transitions of the system and from other sources such as windows or solvent, it has replaced almost completely the measurement of magnetic optical rotatory dispersion (MORD). Theoretical calculations are also easier for MCD.

Electronic transitions between Zeeman components of the electronic ground and excited states are circularly polarized. MCD employs this phenomena, measuring the differential absorption of left and right circularly polarized light, $\Delta A$, as function of wavelength in the presence of a magnetic field applied parallel to the direction of light propagation. Theoretical treatments (e.g. see ref. 16) lead to a general expression for MCD intensity, $\Delta A = A_L - A_R$, for a transition $A \rightarrow J$ from a ground state $A$ to an excited state $J$, which is the sum of three terms:

$$\Delta A_{IA \rightarrow J} = \gamma \left[ A_1 \left( \frac{\delta f}{\delta E} \right) + \left( B_0 + \frac{C_0}{kT} \right) f \right] \beta B b I \tag{1}$$

with

$$\frac{\int f(E_{IA,E})}{E} dE = 1$$

$$\gamma = \frac{N \pi^2 a^2 \log e}{250 hc n^2}$$

where $\beta$ is the Bohr magneton, $B$ is the magnetic flux, $k$ is the Boltzmann's constant, $b$ is the molar concentration, $l$ is the pathlength, $N$ is Avogadro's
number, c is the speed of light, n is the refractive index, $E_{JA}$ is the energy difference between states A and J, $\alpha$ is the absorption coefficient, $f(E_{JA},E)$ is the line shape function which is a function of both the transition energy $E_{JA} = E_J - E_A$, and the incident photon energy, $E_i$; $\Delta A$ is related to the ellipticity and can be expressed in terms of molar ellipticity $[\theta]_m$ by the equation

$$[\theta]_m = \frac{4500}{\pi \log e} \frac{\Delta \epsilon}{\log e (\Delta A)}$$

The terms $A_1$, $B_0$, and $C_0$ are parameters that depend on the electric dipole selection rules for the absorption of circularly polarized light in a longitudinal magnetic field. The terms $A_1(-\delta f/dE)$, $B_0 f$, and $C_0 f/kT$ are commonly called A, B, and C-terms, respectively. The object of studying MCD through absorption bands is to obtain these terms, which can then be used to provide information about the nature of the states A and J.

In most cases, A, B, and C-terms may be distinguished by their dispersion and temperature dependence. A-terms arise when there is degeneracy in either the excited state only, or both the ground and excited states. For simplicity, an atomic transition in which only the excited state is degenerate will be considered, e.g., $^1S \rightarrow ^1P$ (Fig. 1-2a). In the presence of a magnetic field, Zeeman splitting of the excited state results in three equally spaced sublevels with $M_J = +1, 0, -1$ separated by $g\beta B$ (Fig. 1-2a), where g is the gyromagnetic ratio. Absorption of left and right circularly polarized light occurs, giving rise to two transitions according to the selection rules (Fig. 1-2b). These transitions will be of equal intensity, but will differ in frequency by $2g\beta B$. The differential absorption corresponding to the MCD (Fig. 1-2c) has the form of a derivative-shaped dispersion, with a cross-over point at the energy of the zero-field absorption maximum ($\nu_0$), and is independent of temperature. For light propagating parallel
Figure 1-2. MCD A-term diagram for an atomic $^1S \rightarrow ^1P$ transition in the absence and presence of a magnetic field. (a) energy level diagram; (b) absorption spectrum; (c) absorption spectrum corresponding to absorption of left and right circularly polarized light; (d) MCD spectrum.
to the z-direction, and A-term is mathematically defined as

\[
A_i = \frac{1}{d_A} \sum_{\alpha, \lambda} \left[ \langle \text{A}_\alpha \text{Im.} | \text{J}_\lambda \rangle^* \langle \text{A}_\alpha \text{Im.} | \text{J}_\lambda \rangle - k \langle \text{A}_\alpha \text{Im.} | \text{J}_\lambda \rangle^* \langle \text{A}_\alpha \text{Im.} | \text{J}_\lambda \rangle \right]
\]

\[
x \left[ \langle \text{J}_\lambda | \text{L}_z + 2S_z \rangle^* \langle \text{J}_\lambda \rangle - \langle \text{A}_\alpha \text{Im.} | \text{L}_z + 2S_z \rangle^* \langle \text{A}_\alpha \text{Im.} \rangle \right]
\]  

(3)

where \(d_A\) is the degeneracy of the state \(\text{A}_\alpha\); \(\alpha\) and \(\lambda\) are the vibronic components of states \(\text{A}\) and \(\text{J}\); \(m_+\) and \(m_-\) are the electric dipole moment operators for right and left circularly polarized light, respectively, such that \(m_\pm = (1/\sqrt{2})(m_x \pm im_y)\), and \(L_z\) and \(S_z\) are the z-components of the orbital and spin angular momentum operators, respectively. The superscript * indicates an unperturbed state.

Unlike A-terms, there is no simple depiction of B-terms. They arise from magnetically induced mixing of states which are not necessarily degenerate. The amount of mixing is inversely proportional to the energy separation of the states. Like A-terms, their intensity is generally independent of temperature, except when the mixing state itself becomes populated over the temperature range of the experiment. In such cases temperature-dependent B-terms are observed. However, they exhibit absorption-shaped dispersion. Mathematically, B-terms can be defined as:

\[
B_0 = -\frac{2}{d_A} \sum_{\alpha, \lambda} \text{Re} \left\{ \sum_{\text{K} \neq \text{J}} \left[ \langle \text{A}_\alpha \text{Im.} \text{J}_\alpha \rangle^* \langle \text{K}_\text{K} \text{Im.} \text{A}_\alpha \rangle - \langle \text{A}_\alpha \text{Im.} \text{J}_\lambda \rangle^* \langle \text{K}_\text{K} \text{Im.} \text{A}_\alpha \rangle \right] \right\}
\]

\[
x \left[ \frac{\langle \text{J}_\alpha | \text{L}_z + 2S_z \rangle^* \langle \text{K}_\text{K} \rangle + \sum_{\text{K} \neq \text{A}} \left[ \langle \text{A}_\alpha \text{Im.} \text{J}_\alpha \rangle^* \langle \text{K}_\text{K} \text{Im.} \text{A}_\alpha \rangle - \langle \text{A}_\alpha \text{Im.} \text{J}_\lambda \rangle^* \langle \text{K}_\text{K} \text{Im.} \text{A}_\alpha \rangle \right]}{W_{\text{K}} - W_{\text{A}}^*} \right]
\]

\[
x \frac{\langle \text{K}_\text{K} | \text{L}_z + 2S_z \rangle^* \langle \text{A}_\alpha \rangle}{W_{\text{K}} - W_{\text{A}}^*}
\]  

(4)

where \(K_k\) is any other state which can be mixed with either \(\text{A}_\alpha\) or \(\text{J}_\lambda\) when \(H = 0\). \(W^*\) represents the energy of the states \(K\) and \(A\), and \(\text{Re}\) is the operator that takes the real part of everything to its right.
C-terms require a degenerate or near-degenerate ground state. For simplicity, an atomic transition, \(^1P \rightarrow ^1S\), will be considered (Fig. 1-3a). In the presence of a magnetic field, the ground state is split into three Zeeman components, with \(M_J = +1, 0, -1\) (Fig. 1-3a). Since there is a Boltzmann population distribution over the Zeeman sublevels of the ground state, left and right circularly polarized light are not absorbed equally (Fig. 1-3c). Transitions from the lowest Zeeman component will have a higher intensity. Therefore, the MCD differential absorption exhibits an absorption-shaped dispersion (Fig. 1-3d). Moreover, if the applied magnetic field increases or the temperature decreases, the population of the lowest Zeeman component increases with respect to that of highest Zeeman component, resulting in an increase of differential absorption of circularly polarized light. The net effect is an increase in signal intensity at lower temperatures or higher magnetic field. The mathematical expression that defines C-terms is:

\[
C_0 = -\frac{1}{d_A} \sum_{a,A} \left[ \langle A_a | m - |J_A \rangle^2 \langle A_a | L_z + 2S_zI_{A_a} \rangle \langle A_a | L_z + 2S_zI_{A_a} \rangle \right]
\]

The degeneracy necessary for C-terms can arise either from orbital angular momentum degeneracy, or from spin angular momentum degeneracy. C-terms are very sensitive to interactions which quench the ground state angular momentum, such as Jahn-Teller distortions, low-symmetry crystal field perturbations, and exchange interaction effects that are usually present in transition metal centers. Therefore, C-terms constitute a powerful tool for the study of these systems.

For a given transition, the ratio of the magnitude of the \(A-, B-, \) and \(C-\) terms are inversely related to the ratio of the absorption linewidth at half height (\(\Gamma\)), the energy separation between the mixing state and the ground or excited
Figure 1-3. MCD C-term diagram for an atomic $^1P \rightarrow ^1S$ transition in the absence and presence of a magnetic field. (a) energy level diagram; (b) absorption spectrum; (c) absorption spectrum corresponding to absorption of left and right circularly polarized light; (d) MCD spectrum.
state ($\Delta W$), and $kT$, $A:B:C = \Gamma^{-1} : \Delta W^{-1} : (kT)^{-1}$. For example, at room temperature, when $\Gamma \sim 10^3$ cm$^{-1}$, $kT \sim 200$ cm$^{-1}$, and the energy separation between the state is $\sim 10^4$ cm$^{-1}$, the $A$-, $B$-, and $C$-terms are approximately in the ratio 10:1:50, respectively. As the linewidth and the temperature decrease, the $A$- and $C$-terms become more important, and at liquid He temperatures the ratio becomes 10:1:3400. Therefore, $C$-terms, if present, can be enhanced up to 70-fold on going from room temperature to liquid helium temperatures, and they usually dominate the MCD spectrum at low temperature (20).

For biological chromophores, which generally possess only low symmetry, ground state degeneracy can usually be equated with spin degeneracy, since orbital angular momentum is effectively quenched. Consequently low-temperature MCD provides a selective optical probe for paramagnetic chromophores such as transition metal centers.

1.1.3.2 SATURATION AND MAGNETIZATION CURVES

The general expressions for $\Delta \varepsilon = \varepsilon_L - \varepsilon_R$ given above apply only to paramagnetic chromophores in the Curie law-limit, i.e., $kT$ is much greater than the ground state Zeeman splitting, $g\beta B$. At these temperatures, the population change between the Zeeman components is linear in $B/T$, according to Curie's law. As the Zeeman energy becomes comparable to or greater than $kT$, the population change becomes non-linear as a function of $B/T$. Eventually, only the lowest level is populated and a further increase in $B/T$ does not cause any population change. The system is then said to be magnetically saturated, and the paramagnetic center is fully magnetized. At this point, $A$- and $B$-terms need not be considered since they are temperature independent and can therefore be subtracted out by extrapolation to infinite temperature.

Plots of the MCD intensity, $\Delta A$, as a function of the magnetic field and
reciprocal temperature, B/T, in both the Curie law region \( i.e. \) \( g\beta B \ll kT \) and at saturation \( i.e. \) \( g\beta B \gg kT \) are known as magnetization or saturation curves (Fig. 1-4). To a first approximation, magnetization curves are only dependent on the ground state. All transitions arising from the same ground state should exhibit similar magnetization behavior, provided that the ground state is not highly anisotropic, in which case the magnetization behavior is largely dependent on the polarization of the transition.

Schatz, Mowery, and Krausz (21) developed saturation theory for a Kramers' doublet \( S = 1/2 \) ground states, and the results have been applied to a variety of simple and well characterized systems (22-25). A brief summary of the most important equations is given here.

For a transition between an \( S = 1/2 \) ground state and an \( S = 1/2 \) excited states, the form of the magnetization curve depends on both the effective ground state \( g \)-values and the polarization of the electronic transition. For a randomly oriented paramagnetic chromophore exhibiting axial symmetry, the mathematical expression for saturation is:

\[
\frac{\Delta \varepsilon}{K} = m_+ \left( \int_0^{\pi/2} \frac{\cos^2 \theta \sin \theta}{\Gamma} g_{\parallel} \frac{\tanh \left( \frac{\Gamma \beta B}{2kT} \right)}{2kT} \cos \theta \sin \theta \right) \left( \sqrt{2} \frac{m_z}{m_+} \int_0^{\pi/2} \sin^3 \theta \frac{g_{\perp} \tanh \left( \frac{\Gamma \beta B}{2kT} \right)}{2kT} \sin \theta \right) \tag{6}
\]

where \( \Delta \varepsilon \) is the temperature dependent MCD intensity after correcting for any temperature independent contributions, \( K \) is a constant \( (8\pi^3N\times10^{-3} / h\ln 10) \), \( \theta \) is the angle between the molecular \( z \)-axis and the applied magnetic field, \( m_z \) and \( m_+ \) are the transition dipole moment operators for the molecular \( z \)- and \( xy \)-polarized transitions, respectively, and \( \Gamma = (g_{\parallel}^2 \cos^2 \theta + g_{\perp}^2 \sin^2 \theta)^{1/2} \). Since the sample
Figure 1-4. A typical MCD magnetization plot. The solid and dashed lines represent experimental curves for two systems differing in ground state $g$-values ($g_{av}$ greater for the solid line).
consists of an assembly of molecules in a frozen glass, it is necessary to average over all angles by integration. This integration cannot be performed analytically but may be readily accomplished numerically using a microcomputer.

Clearly equation 6 is the sum of two terms, the first of which is the contribution to the MCD of the xy-polarized component. From eq. 6, it is apparent that magnetization data for an axial chromophore can vary as a function of wavelength of the measurement as a result of varying amounts of z- and xy-polarized transitions. This indicates that the magnetization can vary as a function of the wavelength of measurement. However, there are three special cases in which the form of the magnetization curves is independent of the wavelength of measurement. The first case is where the electronic spectrum is dominated by transitions of a particular polarization, such as xy-polarized transitions in hemes. In this case, the second term is eq. 6 can be neglected. The second case is when the symmetry of the ground state is completely isotropic, i.e. \( g_\parallel = g_\perp = g \). In this case eq. 6 simplifies to a simple tanh function:

\[
\frac{\Delta \varepsilon}{K} = \frac{1}{3} m_\perp^2 \left(1 - 2 \sqrt{2} \frac{m_\perp}{m_\parallel}\right) \tanh \frac{gB B}{2kT}
\]

The polarization ratio, \( m_\perp/m_\parallel \), attenuates the MCD intensity but leaves the form of the magnetization curve unchanged. Equation 7 is also applicable for analyzing magnetization data from \( S = 1/2 \) ground states with small g-value anisotropy. The third case is a ground state doublet with \( g_\perp = 0 \) and \( g_\parallel \neq 0 \), which can arise as a result of axial zero field splitting of non-Kramers' ground states with \( S > 0 \). In this case, eq. 6 reduces to
Therefore, when $g_{\perp} = 0$ all temperature dependent MCD transitions from the ground state become $xy$-polarized and magnetization data are independent of the wavelength of measurement.

As mentioned before, eqs. 6, 7, and 8 are only applicable to paramagnetic chromophores with isolated $S = 1/2$ ground states. However, when a paramagnet contains more than one unpaired electron, the interaction between the unpaired electrons and the ligand field results in the removal of the spin degeneracy even in the absence of a magnetic field. This splitting is termed zero field splitting (1,26). Paramagnetic transition metal centers with $S > 1/2$ ground states are readily recognizable by their MCD magnetization characteristics. First, the curves deviate substantially from theoretical data for $g_{av} = 2$. Second, data points measured at different temperatures do not necessarily lie on a smooth curve, due to the thermal population and/or field-induced mixing of low-lying components, giving rise to "nested" magnetization curves. Third, plots of MCD intensity as a function of $1/T$ only become linear at high temperatures when the spread of zero-field components is very much less than $kT$. Complete analysis of magnetization data from $S > 1/2$ chromophores presents a complex theoretical problem, requiring the inclusion of field-induced mixing of zero-field components, as well as rhombic zero-field splitting parameters, effective principal g-values and polarization of the transitions for each doublet. The problem is usually simplified by fitting the data at a temperature such that only the lowest doublet is significantly populated, using expressions derived for an isolated doublet ground state. Eq. 6 can then be used to estimate effective g-values and the polarization of the transitions from the lowest doublet. Certain assumptions are implicit. First, the ground state is approximated to axial symmetry with axial polarization of the electronic

$$\frac{\Delta \varepsilon}{K} = m_{+}^{2} \int_{0}^{\pi/2} \cos \theta \sin \theta \tanh \frac{g_{\beta} B \cos \theta}{2kT} d\theta$$  

Therefore, when $g_{\perp} = 0$ all temperature dependent MCD transitions from the ground state become $xy$-polarized and magnetization data are independent of the wavelength of measurement.
transitions. Secondly, the field-induced mixing of zero field components is neglected. This assumption is only valid if the Zeeman splitting, $g_0 \beta B$, where $g_0$ is the $g$-value in the Zeeman term of the spin Hamiltonian, is much less than the zero-field splitting. Thirdly, only the lowest doublet is assumed to be populated at the lowest temperature. The validity of the latter two approximations can be experimentally tested by evaluating the magnitude of the zero field splitting parameters from analysis of plots of MCD intensity versus $1/T$, considering that each zero-field component contributes to the MCD C-terms proportionally to the fractional population of each component. The fractional population, in turn, depends on the absolute temperature and the energy separation between each doublet, according to the Boltzmann distribution (23,27).

The estimation of the spin state of the complexes and clusters studied in this work was made by the simulation of the experimental curves at the lowest temperatures available ($\approx 1.5$ K) by means of eq. 6-8, using $g$-values corresponding to those of the lowest zero-field doublet of the ground state manifold.

1.1.3.3 APPLICATIONS OF MAGNETIC CIRCULAR DICHROISM TO BIOLOGICAL SYSTEMS

MCD constitutes an alternative optical technique to absorption spectroscopy for detecting electronic excited states, since transitions that are weak or obscured in the absorption spectrum are often readily observed in the MCD spectrum. A transition buried under a stronger transition in the absorption spectrum can be observed in the MCD spectrum if $\Delta A/A$ is larger for the weaker transition and/or of opposite sign. In the near-IR region, MCD can be useful in distinguishing electronic transitions from vibrational transitions since the latter exhibit weaker MCD intensity. Therefore, MCD facilitates the resolution and assignment of electronic transitions and is applicable to any chromophoric metal center.
MCD has proven very effective in determining the coordination geometry of metal centers in metalloproteins (17,19,20,28) and gives information about the symmetries, angular momentum, electronic splittings, and vibrational-electronic interactions of excited electronic states. When paramagnetic metal centers are present, ground state properties can also be investigated by MCD magnetization curves along with MCD temperature-dependence studies, which yield information about the spin state, effective g-values, zero-field splitting parameters, and magnetic coupling constants. While similar information is often attainable from EPR, Mössbauer, or magnetic susceptibility studies, MCD offers certain advantages over each of these techniques in the study of metalloproteins. For example, unlike Mössbauer, it is not limited to Fe and does not require isotopic enrichment. Weak magnetic interactions or zero-field splittings that can prevent the observation of EPR resonances, do not prevent characterization of paramagnets by MCD spectroscopy. Moreover, magnetic susceptibility studies can be difficult to interpret for multicentered metalloproteins particularly when paramagnetic impurities are present, whereas with MCD spectroscopy the magnetic properties of individual centers can be investigated independently.

Since biological systems usually exhibit low symmetry, assignment of the transitions revealed in the MCD spectrum is often difficult. However, the characterization of metal centers in metalloproteins is made easier by comparison with model compounds. Heme proteins constitute the group most extensively studied by this technique, since MCD is sensitive to the redox and spin state of the Fe, as well as to the nature of the axial ligands (19,22). Recently, MCD has found applications in the study of non-heme iron proteins, especially Fe-S clusters (25). In particular, MCD has proven to be valuable for determining Fe-S cluster types in multicomponent enzymes (for recent reviews, see refs. 28 and 29). Copper-containing proteins such as superoxide dismutase and hemocyanin have also
been extensively studied (30,31). A complete review of the applications of MCD to the study of biological transition metal centers (except hemes) has been published by Dooley and Dawson (20).

1.1.4 ELECTRON PARAMAGNETIC RESONANCE (EPR)

Electron paramagnetic resonance (EPR) is a spectroscopic technique that probes the environment of a paramagnetic center by defining the size and shape of the magnetic moment produced by the unpaired electron, and by characterizing any magnetic fields which might be produced by the parent molecule in the vicinity of the paramagnetic center (17). The technique involves the reorientation of the magnetic moment of an electron in a strong magnetic field. According to the Pauli exclusion principle, it is only possible to reorientate the spin of an electron if it is unpaired, therefore EPR is restricted to ions and molecules with unpaired electrons, i.e., free radicals and transition metal compounds.

The EPR spectra of transition metal compounds provide information about the electronic structure and local environment of the paramagnets, hence EPR spectroscopy constitutes a very powerful tool for the study of metal centers in biological system (26,32,33). The technique can give information about ground state g-values, spin state, and zero-field splitting and thereby afford insight into the geometry and redox state of the metal. In addition, EPR spectra provide quantitative information on the concentration of the paramagnetic center.

Several books and reviews of the theory of EPR are available (1,7,26,32,34-37). Some of the most important aspects concerning the theory of EPR in transition metal compounds, with emphasis on those which are relevant to metalloproteins are discussed below.

1.1.4.1 BASIC THEORY
When a substance with unpaired electrons is placed in a magnetic field $B$, the Zeeman interaction causes a splitting of the energy levels corresponding to the spins, which align parallel or antiparallel to the applied field (Fig. 1-5). The Hamiltonian that describes the system is given by

$$\hat{H} = g \beta B \hat{S}$$

where $\hat{S}$ is the spin operator, $g$ is the gyromagnetic ratio or g-factor, $\beta$ is the Bohr magneton, and $B$ is the magnetic field strength.

Transitions between the energy levels characterized by the quantum number $M_\sigma$, arising from the Zeeman interaction, can be induced by a microwave frequency applied perpendicular to the magnetic field. The energies of the two states are given by

$$E = g \beta B M_\sigma$$

where $E$ is the energy, and $M_\sigma$ is the quantum number for a spin quantized about the magnetic field axis. In order for a resonance to occur, the energy difference $\Delta E$ must be equal to the applied energy, which for an electron is generally in the microwave region. Therefore, the energy necessary for the transition is given by

$$h\nu = \Delta E = g \beta B \Delta M_\sigma$$

where $h$ is Planck's constant and $\nu$ is the frequency of the microwave radiation. The selection rule for absorption of microwave radiation in EPR is $\Delta M_\sigma = \pm 1$.

The position of the resonance can be calculated from eq. 11, in terms of $g$, which measures the ratio of the resonance frequency to the applied field. If $\nu$ is expressed in GHz and $B$ is in gauss, the numerical expression for the observed $g$-value is given by

$$g = \frac{714.45 \nu}{B}$$

The net absorption depends on the difference in population between the upper and lower states, which in turn is dependent on temperature, according to the Boltzmann distribution.
Figure 1-5. Illustration of the EPR transition for an $S = 1/2$ ground state. $\Delta E$ corresponds to the energy required for the EPR transition.
**g-Factor Anisotropy for S = 1/2 Systems**

For systems with negligible effective orbital angular momentum, the g-factor is equal to the free electron value of 2.00232 (34). In transition metal systems, the unpaired electron is relatively well localized. The nucleus has a large atomic number and electronic excited states are relatively accessible. As a consequence, the magnetic moment contains an additional contribution from the orbital angular momentum. The orbital paramagnetism reflects the ability of the unpaired electron to migrate from its initial orbital into other orbitals related to the original by rotations about a coordinate axis (35). The total magnetic moment will then have the combined contribution from both spin and orbital angular momenta and thus the observed g-value deviates from the free electron value and is often strongly anisotropic. The interaction between the spin and orbital angular momenta is called spin-orbit coupling. The magnitude of the spin-orbit coupling is determined by the symmetry of the paramagnet and the energy separation between the ground and excited states. The spin-orbit coupling has spatial dependence, and thus the value of g depends on the molecular orientation relative to the applied field and is described by a tensor. Although the ground state orbital angular momentum of most first row transition metals is quenched, as a result of low-symmetry ligand fields, significant deviations from the free electron value are still commonly observed. This is due to the field induced mixing with low lying excited states that have orbital angular momentum leading to anisotropy in g. The spin-orbital Hamiltonian is given by

$$\hat{H}_{s.o.} = \lambda (\hat{S} \cdot \hat{L})$$  \hspace{1cm} (13)

where $\hat{S}$ and $\hat{L}$ are the spin and orbital angular momentum operators, respectively and $\lambda$ is the spin-orbit coupling constant. The magnitude of the spin-orbit coupling will be determined by the symmetry and the energy separation of the
ground and excited states. This coupling often has a spatial dependence in the value of $g$ in a given direction which depends on the orientation relative to the applied magnetic field. Consequently, the $g$-value is a third-rank tensor. By prudent selection of an orthogonal coordinate system, so that the crystal axes coincide with the molecular coordinate system, the off-diagonal contributions of the matrix that describes $g$ can be eliminated, and the system can be defined by the three diagonal, or principal, $g$-values $g_{xx}$, $g_{yy}$, and $g_{zz}$ (often abbreviated as $g_x$, $g_y$, and $g_z$).

In an anisotropic system, the resonant field is related to the orientation of the molecular axes with respect to the applied magnetic field and this orientation dependence of the $g$-factor has a significant effect on the line shape of the EPR spectrum. Three basic types of symmetry exist— isotropic, axial, and rhombic (Fig. 1-6a). In isotropic systems, $g_x = g_y = g_z$ and a single resonant frequency is observed regardless of sample orientation. For axial systems, $g_x = g_y \neq g_z$ and the absorption envelop permits determination of the two principal $g$-values. These are designated as $g_\parallel = g_z$ and $g_\perp = g_x$ and $g_y$. Rhombic symmetry ($g_x \neq g_y \neq g_z$) gives rise to a more complex absorption envelop which facilitates evaluation of all three principal $g$-values, $g_x$, $g_y$, and $g_z$. The EPR absorption spectra of these limiting cases are shown in Fig. 1-6b. Due to the broadness of the EPR absorption spectra, better resolution is obtained when the spectra are plotted as the first derivative of the absorption spectra, as shown in Fig. 1-6c; the principal $g$-values refer then to the positions of the maxima, cross-over, and minima of the spectra.

Usually for metalloproteins, EPR spectra are taken in frozen solutions, in which the protein molecules adopt unique orientations so that all possible orientations of the protein molecules are present, and then the effective $g$-value is given by
Figure 1-6. Schematic representation of the idealized powder EPR spectra for an isotropic, axial, and rhombic, $S = 1/2$ system. (a) Geometric shapes associated with isotropic, axial, and rhombic magnetic moments; (b) absorption curves with a finite linewidth; (c) EPR first derivative curves. Figure taken from ref. (35).
\[ g_{\text{eff}}^2 = g_x^2 l_x^2 + g_y^2 l_y^2 + g_z^2 l_z^2 \]  

(14)

where \( l_x^2, l_y^2, l_z^2 \) are the normalized direction cosines between the principal axis of the g-tensor and the applied field. The extreme g-values are exhibited when the applied field lies parallel to any one of the coordinate axis, in which case \( l_x^2 = 1 \) (or \( l_y^2 \) or \( l_z^2 \)) and the other two will be zero. The g-values for all other molecules will be intermediate between \( g_x \) and \( g_z \), assuming \( g_x < g_y < g_z \). From eq. 14 it can be seen that \( g_y \) is adopted not only when the applied field is parallel to the y-axis, but also in some other orientations. As a consequence, the absorption in the y-direction is more intense than in the other directions in the rhombic spectra.

1.1.4.2 S > 1/2 SYSTEMS

In systems with two or more unpaired electrons, the degeneracy of the spin states may be removed by low symmetry ligand fields, even in the absence of a magnetic field, as a result of mixing of excited states with orbital angular momentum with the orbitally nondegenerate ground state via spin-orbit coupling. This phenomenon is called zero-field splitting. In systems with an odd number of electrons, \( e.g., S = 3/2 \), Kramers' rule states that in the absence of a magnetic field, there exists at least a two-fold degeneracy that can only be removed by an applied magnetic field. Therefore, in principle, regardless of symmetry an EPR spectrum should always be observable. However, for systems with even number of electrons (known as non-Kramers' systems), \( e.g., S = 1 \), the degeneracy may be completely removed by a low-symmetry crystal field, so that only singlet levels remain. The presence of zero-field splitting will frequently lead to a separation between the energy states which is larger than the energy of the incident microwave radiation. Thus, EPR spectra are frequently not observed for these systems.
Systems with $S > 1/2$ are best treated by considering each doublet as an effective $S = 1/2$ system and determining effective $g$-values using the spin Hamiltonian. The spin Hamiltonian that describes the zero field splitting for systems with $S > 1/2$ is given by

$$
\hat{H} = D\hat{S}_z^2 - S(S + 1)/3 + E\hat{S}_x^2 - \hat{S}_y^2 + g_0 B \hat{S}
$$

(15)

where $D$ and $E$ are the axial and rhombic zero field splitting parameters, respectively, and $g_0$ is the $g$-value assuming an isotropic Zeeman interaction. It is important to note that the values of $D$ and $E$ are not unique but rather depend on which axis is chosen as the $z$ axis. For completely rhombic symmetry, $E/D = 1/3$. $D$ and $E$ may be positive or negative and may have opposite signs. Their absolute signs are not relevant, since line positions depend only on their relative signs. The effect of the ZFS is illustrated in Fig. 1-7, where the energy level diagrams and the EPR allowed transitions are shown for an $S = 5/2$ ion in a completely isotropic symmetry (octahedral ligand field) i.e., $E = D = 0$ (Fig. 1-7a), in axial symmetry with small ZFS i.e., $D << h\nu$, $E = 0$ (Fig. 1-7b), and in an axial symmetry with large ZFS i.e., $D >> h\nu$ (Fig. 1-7c). If $D >> h\nu$, only EPR transitions between the $\pm 1/2$ states will be observed, and the system can be treated in terms of a fictitious spin $S' = 1/2$. Large ZFS is usually present for $S > 1/2$ Fe-S clusters, causing the transitions to occur usually within each doublet and not between them.

When rhombic distortions are applied to the system, the zero field splitting parameters $D$ and $E$ are both non zero. Again, three Kramers' doublets are produced for an $S = 5/2$ system as a result of linear mixing of the energy levels. Thus, transitions within each Kramers' doublet become allowed. In this case, a field and frequency dependence in the effective $g$-value is induced, and different
Figure 1-7. Illustration of the energy level diagram and EPR allowed transitions for an $S = 5/2$ system with magnetic field parallel to the principal axis. (a) Isotropic symmetry; (b) axial symmetry in a weak magnetic field (small zero-field splitting); (c) axial symmetry in a strong magnetic field (large zero-field splitting). Figure taken from reference (34).
resonances can be observed, as illustrated in Fig. 1-8. The intensity of the signal arising from each doublet is temperature dependent, according to the Boltzmann population distribution, and this effect can be used to determine the zero-field splitting in \( S > 1/2 \) systems. These effects are treated in detail for the \( S = 5/2 \) and \( S = 3/2 \) systems discussed in chapters 3-5.

1.1.4.3 NUCLEAR HYPERFINE INTERACTIONS

The presence of nearby nuclei which possess an intrinsic spin angular momentum (represented by the spin quantum number \( I \)) modifies the electron energy states in a magnetic field. The magnetic moment associated with the nuclear spin angular momentum causes a local magnetic field that will either oppose or augment the magnetic field experienced by the unpaired electron. The interaction of an unpaired electron and a magnetic nucleus is called nuclear hyperfine interaction, and can cause splitting of certain lines of the EPR spectrum by a factor of \( 2I + 1 \). The hyperfine interaction may be either isotropic or anisotropic leading to very complex spectra. EPR transitions will be observed according to the selection rule \( \Delta M_I = 0 \). The nuclear hyperfine interactions can be caused either by the nucleus of the atom that contains the unpaired electron (hyperfine interaction), or by a nearby nucleus from the ligand atoms that surround the metal center (superhyperfine interaction).

Nuclear hyperfine interactions are usually not observed in Fe-S proteins, unless isotopic enrichment with \(^{57}\text{Fe} \) (\( I = 1/2 \)) is used, since the naturally abundant isotopes of Fe and S, \(^{56}\text{Fe} \) and \(^{32}\text{S} \), have \( I = 0 \). However, hyperfine interactions might be expected for biological or synthetic clusters containing Mo (\(^{95}\text{Mo}, I = 5/2 \) 15.8% n. a., and \(^{97}\text{Mo}, I = 5/2 \) 9.6% n. a.) or V (\(^{51}\text{V}, I = 7/2 \) 99.8% n. a.).
Figure 1-8. Energy level diagram for an \( S = 5/2 \) ground state in rhombic symmetry, for three different orientations of the magnetic field. The effective \( g \)-values for each doublet are given in the figure. Figure taken from reference (35).
1.1.4.4 SPIN RELAXATION

As mentioned previously, the fractional population of the levels within Kramers' doublets is determined by the Boltzmann distribution. Thus, for an $S = 1/2$ system,

$$\frac{N_a}{N_b} = \exp\left(-\frac{g\mu B}{kT}\right)$$  \hspace{1cm} (16)

where $N_a$ and $N_b$ are the population of the upper and lower states, respectively. The net absorption depends on the population difference between both states, which is maintained by relaxation processes (1,36,39-41). For transition metal ions, spin-lattice relaxation ($T_1$) is the dominant relaxation process.

Spin-lattice relaxation results from interaction of the paramagnet with the thermal vibrations of the lattice. In general, transition metal complexes exhibit a very fast spin-lattice relaxation, due to the presence of low-lying excited states. As a consequence of the Heisenberg Uncertainty Principle,

$$\Delta E \Delta r \geq \frac{\hbar}{2\pi}$$  \hspace{1cm} (17)

which requires the linewidth to be inversely proportional to the relaxation time, transition metal complexes generally exhibit very broad EPR spectra at room temperature. Spin-lattice relaxation can be slowed by lowering the temperature, thereby reducing the thermal motion. Hence, almost all paramagnetic transition metal centers, including Fe-S, Fe-Mo-S, and Fe-V-S clusters, are usually studied at temperatures below 70 K. Lowering the temperature has the additional benefit of increasing the differential population of the Zeeman levels, resulting in an increase in signal intensity.

The decrease in spin-lattice relaxation time can lead to microwave power saturation, which occurs at high microwave powers when to the rate of
promotion to the upper state exceeds the rate of relaxation to the lower state. This will result in nearly equal populations of spins in ground and excited states, precluding net absorption. Thus, the intensity of the microwave power that can be used decreases at lower temperatures. In order to obtain quantitative results from an EPR spectrum, it must be recorded under non-saturating conditions. The saturation behavior can be assessed by a plot of the signal intensity as a function of the square root of the microwave power \((I \text{ vs. } P^{1/2})\) which should give a straight line in the region where there is no microwave power saturation. More often, a plot of \(\log (I/P^{1/2}) \text{ vs log } P\) is used to obtain the same information. Such a plot gives a line parallel to the abscissa as long as the signal is not saturated, and slopes downwards with increasing power when power saturation is reached. The half-saturation power, \(P^{1/2}\), which provides an estimate of the relaxation rate \((39,42)\), can be determined by extrapolation of the two straight-line segments until the intersection. This experimental parameter indicates the incident power at which the signal is half as intense as it would be in the absence of microwave power saturation.

1.1.4.5 SPIN-SPIN INTERACTION

Spin-spin interactions are only pertinent to those metalloproteins which contain more than one paramagnet. The local magnetic field at a paramagnetic center is affected by bringing it in contact with a second paramagnetic species. Due to the large matrix, metalloproteins are necessarily dilute, therefore, interactions generally occur only between centers of the same protein molecule. Spin-spin interactions can have two effects on a EPR spectrum. First, spin-spin interaction sometimes results in enhancement of the spin-relaxation time for an individual paramagnetic center, and hence different power saturation characteristics. Secondly, the dipolar interactions between both paramagnetic
species affect both the line width and the spin-lattice relaxation time. The extent of interaction depends on the distance between the paramagnets. Dipolar coupling between the paramagnets results in broadening of the EPR spectrum. In some cases, splitting of the spectrum is observed, which may become very complex depending on the anisotropy of the g-tensors.

1.1.4.6 QUANTITATION OF EPR SPECTRA

One of the most useful features of EPR spectroscopy is that the signals can be quantified in terms of the concentration of the paramagnetic species giving rise to the signal. The intensity of the EPR absorption signal is proportional to the number of paramagnetic centers in the sample, provided the sample is not power saturated. While it is not possible to correlate the absolute intensity to the spin concentration, very accurate quantitations can be obtained by comparison to a standard, run under identical conditions of temperature, modulation amplitude, and non-saturating microwave power. EPR spectra, which are obtained as the first derivative of the absorption, must be doubly integrated. This can be accomplished manually or using computer software supplied with the EPR instrument using the method derived by Wyard (38). The area is corrected for g-value dependence according to the methods described by Aasa and Vanngard (43). The spin concentration in the sample is then related to that in the reference by the equation

$$ C_s = \frac{A_s}{A_r} \cdot \frac{G_r}{G_s} \cdot \frac{g_r}{g_s} \cdot C_r $$

(18)

where the subscripts r and s refer to the reference and sample, respectively, A represents the area under the absorption curve calculated by double integration, G is the spectrometer gain, C is the concentration, and g corresponds to the
average g-factor. The area under the absorption curve is manually calculated according to

\[ A = \frac{1}{2} \hbar^2 \sum_{i=1}^{n} (n-2i+1)Y_i \]  \hspace{1cm} (19)

where \( \hbar \) is the magnetic field interval, \( n \) is the number of intervals, and \( Y_i \) is the intensity of the EPR signal for the \( i^{\text{th}} \) interval. The average g-value is given by

\[ g = \frac{2}{3} \left[ \frac{1}{3} \left( g_x^2 + g_y^2 + g_z^2 \right) \right]^{1/2} + \frac{1}{3} \left[ \frac{1}{3} \left( g_x + g_y + g_z \right) \right] \]  \hspace{1cm} (20)

Aasa and Vanngard (43) also devised a useful method for obtaining the total intensity of a EPR spectrum from a single, isolated absorption-shaped component of an anisotropic signal. The area, \( A \), of the isolated band is evaluated as

\[ A = \hbar \sum_{i=1}^{n} Y_i \]  \hspace{1cm} (21)

and \( g \) is given by

\[ g = \frac{g_x^2 + g_y^2}{2g_z B_{\text{max}} \left[ (1-\rho_x)(1-\rho_y) \right]^{1/2}} \]  \hspace{1cm} (22)

where

\[ \rho_{x,y} = \frac{g_{x,y}^2}{g_z} \]

and \( B_{\text{max}} \) corresponds to the magnetic field at which the maximum of the absorption band occurs. Then the concentration of the sample is calculated from eq. 18. This method can be applied provided that the linewidth is smaller than the g-value anisotropy, and is useful for integrations of very broad spectra and of spectra arising from different species whose signals overlap.
1.2 IRON-SULFUR PROTEINS

The importance of iron for living organisms is emphasized by its role in a large number of proteins that require its presence for their activity (44). Based on the type of ligands surrounding this metal center, the International Union of Biochemistry has divided Fe-containing proteins into three major categories: heme proteins (in which the iron has any derivative of the porphyrin ring as ligand), iron-sulfur proteins, and other Fe-containing proteins (such as oxygenases and hydroxylases) (45). The term iron-sulfur proteins (Fe-S proteins) refers only to those proteins in which a non-heme iron is ligated with inorganic (acid labile) sulfur or cysteine sulfur. There are two major categories of these proteins: simple Fe-S proteins, which contain only one or more Fe-S clusters, and complex Fe-S proteins, which in addition, contain other active groups such as other metal centers, hemes, flavins, etc. Fig. 1-9 illustrates the classification of Fe-S proteins.

Fe-S proteins are found in a wide variety of organisms, such as, anaerobic, aerobic and photosynthetic bacteria, fungi, algae, higher plants, and mammals. Primarily, they function as electron transfer agents, and as such they are found in both the respiratory and photosynthetic electron transport chain. They are also involved in numerous redox enzymes catalyzing the oxidation or reduction of a wide range of substrates. However, there are other Fe-S proteins, such as aconitase and glutamine phosphoribosylpyrophosphate amidotransferase, that catalyze reactions with no apparent redox involvement. Other roles proposed for Fe-S proteins include structural, regulatory, and storage. Over the years, these proteins have been the subject of several reviews (46-49). Table 1-1 contains a partial list of some of the most important Fe-S proteins and the reaction they catalyze. The names of these proteins are derived from their functions, such as
Figure 1-9. Classification of Iron-containing proteins. According to the nomenclature recommended by the International Union of Biochemistry.
TABLE 1-1

EXAMPLES OF IRON-SULFUR PROTEINS

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Process Catalyzed</th>
<th>Centers Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogenase</td>
<td>$N_2 + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2$</td>
<td>Fe-S\textsuperscript{a}, Fe-Mo-S\textsuperscript{b}, Fe-V-S\textsuperscript{c}</td>
</tr>
<tr>
<td>Hydrogenase</td>
<td>$2H^+ + 2e^- \rightarrow H_2$</td>
<td>Fe-S, Ni</td>
</tr>
<tr>
<td>Sulfite Reductase</td>
<td>$SO_3^{2-} + 6H^+ + 6e^- \rightarrow S^{2-} + 3H_2O$</td>
<td>Fe-S, siroheme, flavin</td>
</tr>
<tr>
<td>Nitrate Reductase</td>
<td>$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$</td>
<td>Fe-S, Mo, heme</td>
</tr>
<tr>
<td>Nitrite Reductase</td>
<td>$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$</td>
<td>Fe-S, siroheme, flavin</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>xanthine + $H_2O \rightarrow$ uric acid + $2H^+ + 2e^-$</td>
<td>Fe-S, Mo, flavin</td>
</tr>
<tr>
<td>Aconitase</td>
<td>citrate $\rightarrow$ isocitrate</td>
<td>Fe-S</td>
</tr>
<tr>
<td>NADH Dehydrogenase</td>
<td>$NADH + H^+ \rightarrow NAD^+ + 2H^+ + 2e^-$</td>
<td>Fe-S, flavin</td>
</tr>
<tr>
<td>Succinate Dehydrogenase</td>
<td>succinate $\rightarrow$ fumarate + $2H^+ + 2e^-$</td>
<td>Fe-S, flavin</td>
</tr>
<tr>
<td>Rubredoxin</td>
<td>electron transfer</td>
<td>Fe-S</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>electron transfer</td>
<td>Fe-S</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Fe-S = Iron-Sulfur cluster
\textsuperscript{b}Fe-Mo-S = Iron-Molybdenum-Sulfur cluster
\textsuperscript{c}Fe-V-S = Iron-Vanadium-Sulfur cluster
nitrogenase and hydrogenase, whereas those without classical enzymatic functions are generally termed rubredoxins (Rd) or ferredoxins (Fd) (Fig. 1-9). The term rubredoxin is used for those proteins with one iron atom, while those containing clusters of Fe and $S^{2-}$ are generally called ferredoxins.

1.2.1 IRON-SULFUR CLUSTERS

The structural characterization of Fe-S proteins has established five basic types of biological Fe-S centers (Fig. 1-10), all of which involve approximately tetrahedral coordination of Fe by S. These centers include: [Fe-Scys$_4$] or 1Fe center, [2Fe-2S] or 2Fe cluster, [3Fe-4S] or 3Fe cluster, and [4Fe-4S] or 4Fe clusters. The recommended IUB-IUPAC nomenclature for these centers indicates the cluster type in square brackets with the net charge of the centers as superscript. Oxidation states are calculated assuming that the cysteine residues are formally present as mercaptide, without contributing to the net charge, and the labile sulfur as sulfide. The number of clusters present per protein molecule is indicated by an integer prefix. There are some other Fe-S clusters of unknown structure, that have different properties to those of the structurally characterized centers (e.g. novel Fe-Mo-S and Fe-V-S and P-clusters in the MoFe and VFe proteins of nitrogenase and the cluster at the active site of Fe-only hydrogenases).

The 1Fe centers include such proteins as rubredoxins, which are the simplest type of Fe-S protein having molecular weights around 6000. Their physiological role is still unknown. X-ray crystallographic studies of Rds from *Clostridium pasteurianum* and *Desulfovibrio vulgaris* indicated that the iron atom is in a D$_{2d}$-distorted tetrahedral arrangement of sulfur atoms from four cysteinyl residues (Fig. 1-10A) (50,51). The metal center cycles between the $+3$ ($S = 5/2$), which is EPR active, and $+2$ ($S = 2$) oxidation states (52). The protein
Figure 1-10. Schematic representation of the structures of Fe-S proteins. (A) [Fe-Scys₄] center; (B) [2Fe-2S] cluster; (C) [3Fe-4S] cluster; (D) linear [3Fe-4S] cluster; (E) [4Fe-4S] cluster.
desulforedoxin is rubredoxin-like but with slightly different spectral properties (53), having a more axial EPR spectrum than rubredoxin in the oxidized state.

The 2Fe centers are frequently present in proteins isolated from plant sources. They are found in chloroplasts and they are involved in photosynthesis. X-ray diffraction of the [2Fe-2S] cluster of *Spirulina platensis* (54) confirmed that the center has a pair of bridging sulfide ions and two terminal cysteine ligands on each Fe (Fig. 1-10B). The cluster has two oxidation states, +2 (S = 0, diamagnetic) and +1 (S = 1/2, paramagnetic). Based on low-temperature MCD (55) and EPR (56-58) spectroscopic studies, there are at least three types of 2Fe clusters that have been identified: the plant-type ferredoxin, such as spinach Fd (rhombic EPR spectrum with $g_{av} = 1.96$); the hydroxylase-type cluster, such as adrenodoxin (axial EPR spectrum with $g_{av} = 1.96$); and the Rieske center (rhombic EPR spectrum with $g_{av} = 1.91$).

The existence of [3Fe-4S] clusters was first suggested by Mössbauer studies of *Azotobacter vinelandii* Fdl (59), and later by MCD (24) and EXAFS (60). However, it was not until recently, that the structure of *A. vinelandii* Fdl was redetermined and the structure depicted in Fig. 1-10C was confirmed (61-62). This structure consists of a cubane-like cluster with one corner missing. In the oxidized state, [3Fe-4S] clusters are paramagnetic (S = 1/2 ground state) and in the oxidized state, MCD spectroscopy determined an S = 2 ground state. This structure is also present in *Thermus thermophilus* Fd, and *D. gigas* FdlII, and aerobically isolated aconitase. The [3Fe-4S] cluster in aconitase has been shown to convert to a linear (Fig. 1-10D) form at high pH (63).

The 4Fe centers appear to be more common than the 2Fe centers and have more complex behavior. The structure of [4Fe-4S] centers can be visualized as a distorted cube with four tetrahedral Fe atoms bridged by acid labile sulfides (Fig. 1-10E). This structure is supported by X-ray diffraction studies on the so called
high potential iron protein (HiPIP) from *Chromatium vinosum* (64) and *Peptococcus aerogenes* Fd (66).

These centers are known to occur in three different redox states: +3, +2, and +1, but in any given system, [4Fe-4S] clusters cycle between either +3 and +2 or +2 and +1 states. The clusters that cycle between the +3/+2 oxidation state (+350 mV) are found in high potential iron proteins (HiPIPs), whereas those that cycle between +2/+1 oxidation states (between -350 and -425 mV) are most common and are found in ferredoxins.

Oxidized HiPIP [4Fe-4S] centers have $S = 1/2$ ground state and formally consists of three Fe(III) and one Fe(II) atoms antiferromagnetically coupled (66,67). EPR studies show the present of an axial signal with $g_{av} > 2$. Upon one-electron reduction, HiPIP 4Fe centers become diamagnetic, and formally consist of two Fe(III) and two Fe(II) ions (57,67,68).

In the oxidized state, the ferredoxin-type clusters are identical to the HiPIP-type in the reduced state. In the reduced state, the clusters formally consist of one Fe(III) and three Fe(II) ions, coupled to produce an $S = 1/2$ ground state (68). The species give rise to a rhombic EPR signal with $g_{av} = 1.94$ (69). More complex EPR signals, arising from weak intercluster spin coupling, are observed for proteins that contain more than one [4Fe-4S] cluster, such as the 8-Fe Fd from *C. pasteurianum* (70). In addition to the $S = 1/2$ spin states, some [4Fe-4S]$^{1+}$ clusters have an additional $S = 3/2$ species. This the case of the nitrogenase Fe proteins, in which the $S = 3/2$ and $S = 1/2$ forms of the cluster interconvert depending on the media (71-74, and chapter 3 of this thesis). Moreover, $S = 5/2$ species have been reported as minor components in native nitrogenase Fe proteins in the presence of ATP (75), and in photochemically-reduced *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase (see chapter 4 of this thesis).
Table 1-2 gives a summary of the electronic and magnetic properties of simple Fe-S centers.

1.3 NITROGEN FIXATION

An area in which Fe-S proteins play a vital role is N₂ fixation. Fig. 1-11 shows a schematic representation of the N₂ cycle. The processes involved in this cycle are described below. Assimilation is the process by which plants and microbes make their component proteins from nitrates via nitrites and ammonia, then animals make protein directly or indirectly from plants or microbes, but this organic nitrogen is inaccessible to plants. Therefore, other processes are necessary to make this nitrogen available. By a process called ammonification or mineralization, death, decomposition, and putrefaction lead to release of the protein nitrogen as NH₃. Most of this NH₃ is oxidized back to NO₃⁻ by nitrifying bacteria. Then, denitrifying bacteria may reduce the nitrate to N₂, causing a net loss of N₂ to the atmosphere. This net loss is reversed by activities of nitrogen-fixing bacteria (76). In addition to biological dinitrogen fixation (commonly called nitrogen fixation), fixation is also accomplished by natural processes in the atmosphere, and industrially. These processes are vital to the persistence of life on this planet, and they are very important in world agriculture.

Industrially, ammonia is produced by the Haber-Bosch process. This process involves the reaction of N₂ with H₂ at elevated temperatures (450° to 500° C) and pressures (100 to 1000 atm) in the presence of an iron catalyst (77). The energy required to achieve these high temperatures and pressures elevates significantly the cost of ammonia, and therefore, the cost of fertilizers and food. Biologically, nitrogen fixation is accomplished by the enzyme nitrogenase, which catalyzes the reduction of N₂ to ammonia, at ambient temperature and pressure.
**TABLE 1-2**

**ELECTRONIC AND MAGNETIC PROPERTIES OF IRON-SULFUR CLUSTERS**

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Redox State</th>
<th>Ground State</th>
<th>EPR</th>
<th>Temp. Dep. MCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>[FeScys₄]</td>
<td>+3</td>
<td>5/2</td>
<td>g~4.3</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>+2</td>
<td>2</td>
<td>silent</td>
<td>yes</td>
</tr>
<tr>
<td>[2Fe-2S]</td>
<td>+2</td>
<td>0</td>
<td>silent</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>+1</td>
<td>1/2</td>
<td>g~1.94</td>
<td>yes</td>
</tr>
<tr>
<td>[3Fe-4S]</td>
<td>+1</td>
<td>1/2</td>
<td>g~2.01</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>silent</td>
<td>yes</td>
</tr>
<tr>
<td>[4Fe-4S]</td>
<td>+3</td>
<td>1/2</td>
<td>g~2.06</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>+2</td>
<td>0</td>
<td>silent</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>+1</td>
<td>1/2</td>
<td>g~1.94</td>
<td>yes</td>
</tr>
</tbody>
</table>
Figure 1-11. Illustration of the \( \text{N}_2 \) cycle. The numbers beneath each step are orders of magnitude of turnover in tonnes of \( \text{N}_2 \) per year. Figure taken from reference (76).
Just as the industrial process requires energy in the form of high pressures and temperatures, the enzymatic reaction requires energy in the form of ATP. Therefore, understanding the mechanism of nitrogenase may provide a basis for the design of more efficient industrial catalysts.

1.4 NITROGENASE

Several reviews on the properties and function of nitrogenase have been published (76–80 and refs. in chapters 3 and 5 of this thesis). As discussed above, the enzyme nitrogenase catalyzes the reduction of atmospheric dinitrogen to ammonia. The reaction requires energy obtained through the hydrolysis of Mg·ATP to Mg·ADP, a source of electrons, and protons from water. In vivo the source of electrons is either a reduced ferredoxin or a reduced flavodoxin, and in vitro, the electrons are provided by sodium dithionite. This discussion will focus only on the general properties of the enzyme nitrogenase.

Nitrogenase is found in blue-green algae, cyanobacteria, in free-living bacteria, including the strains of Rhizobia, Azotobacter, and Klebsiella, and photosynthetic bacteria. Nitrogenases from a number of diverse biological sources have now been isolated and characterized in some detail (78–80). All are comprised of two oxygen-sensitive proteins, the Fe protein (component 2) (see chapter 3 for detailed discussion) and the cofactor-containing protein (component 1) (76–80). In the conventional enzyme, component 1 is a molybdenum-iron protein (MoFe) and in the alternative enzyme, it is a vanadium-iron (VFe) protein (81,82). In this text, the nomenclature of Eady et. al. (83) will be used for the two component proteins as isolated from individual diazotrophs. This nomenclature uses a capital letter for the genus and a lower case letter for the species, followed by the number 1 for the MoFe protein, and 2 for the Fe protein. Thus, for example, MoFe protein isolated from C. pasteurianum is Cp1,
and the Fe protein isolated from *A. vinelandii* is Av2. The function of the component 2 is to transfer electrons to the component 1, with concomitant hydrolysis of ATP. N\textsubscript{2} (as well as other substrates, such as, acetylene, cyanide, azide, protons, cyclopropene, nitrous oxide, etc.) binding and reduction take place on component 1. Since the characteristics of the alternative nitrogenase will be discussed in chapter 3, only the characteristics of the MoFe protein will be covered here. Component 1 is an $\alpha_2\beta_2$ tetramer of $M_r \sim 240,000$. It contains 2 atoms of Mo, approximately 30 Fe atoms, and $\sim$ 30 acid-labile sulfides (76-80) which are arranged in two different clusters, the P-clusters and FeMo or cofactor cluster. The P-clusters are probably [4Fe-4S] clusters and their function and structure are not known. The cofactor or FeMo cluster is believed to be the site of N\textsubscript{2} binding and reduction. The properties of the latter clusters will be discussed in detail in chapter 5.

In its native (as isolated) form, the MoFe protein has temperature-dependence MCD (84) and EPR (78) spectra which arises from the FeMo cluster, which is paramagnetic with an $S = 3/2$ ground state under these conditions. MCD studies on thionine-oxidized MoFe protein (84) gave insight into the electronic and magnetic properties of the oxidized P-clusters, suggesting they are a novel class of [4Fe-4S] clusters with an $S = 5/2$ ground state. More recently, EPR studies have indicated an $S = 7/2$ ground state in MoFe for oxidized P-clusters in samples of MoFe protein treated with excess solid thionine (85).
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2. MATERIALS AND METHODS

2.1 UV-VISIBLE ABSORPTION SPECTROSCOPY

UV-visible absorption spectra were recorded at room temperature using either a Hewlett Packard 8452A diode array spectrophotometer interfaced to an IBM PC-XT computer using Hewlett Packard 89530A MSDOS-UV-VIS operating software or a Cary 219 UV-visible absorption spectrophotometer. The spectra were recorded under anaerobic conditions in serum-capped 0.1 cm or 1 cm quartz cuvettes.

2.2 MAGNETIC CIRCULAR DICHROISM SPECTROSCOPY

2.2.1 INSTRUMENTATION

Magnetic circular dichroism spectra were obtained using an Oxford Instruments SM3, split coil, superconducting magnet mated to a Jasco J500C spectropolarimeter (Fig. 2-1). The system allows measurements of MCD spectra in the wavelength range of 200 to 1000 nm, at temperature ranges between 1.5 and 300 K, and at magnetic fields between 0 and 5 Tesla.

The Jasco J500C employs a xenon arc lamp as the radiation source. The radiation is passed through a conventional double monochromator and a linear polarizer. The linear polarizer converts light into polarized light. A photoelastic modulator (PEM) alternately converts linearly polarized light into left and right circularly polarized light at a frequency of 50 KHz. The circularly polarized light is transmitted directly through the magnet and the sample, and finally to the photomultiplier tube (PMT), which is phase locked to the PEM frequency for measuring the differential absorption of right and left circularly polarized light. Phase sensitive detection greatly increases the signal-to-noise ratio.

Since a strong magnetic field can affect the operation of the PMT and the
Figure 2-1. Schematic representation of the Jasco J500 spectropolarimeter mated to an Oxford Instruments SM3 superconducting magnet.
PEM, the magnet is spatially separated from both the optical system and the PMT by at least one meter. The dichrograph exit slit, the optical compartment of the magnet and the PMT grid were aligned using an optical rail, with the direction of light propagation being parallel to the magnetic field. The windows of the optical chamber of the magnet are connected to the dichrograph by means of an optical tube to eliminate stray light. The optical tube is rigidly fixed in position to ensure maximum light intensity impinging on the PMT. The divergence of the light beam upon passage through the tube was negligible, therefore a refocusing lens was not required.

2.2.2 MAGNET AND TEMPERATURE CONTROL

A variable, longitudinal magnetic field is applied to the sample mounted in the optical compartment of the split coil superconducting solenoid, immersed in a liquid He bath (Fig. 2-2). The liquid He is insulated by a liquid nitrogen jacket and a vacuum compartment. The sample compartment of the magnet is centered between the split coils and can be filled with liquid He from the main helium reservoir by opening a needle valve. The magnet is equipped with an Oxford Instruments MK3 power supply, and magnetic fields are ramped by using an Oxford Instruments SG3 sweep generator. The magnetic fields at the sample are calibrated using a transverse Hall probe (Lake Shore Cryogenics) mounted in place of the sample on the sample probe. The magnet current is digitally measured as a voltage across a two milliohm resistor, using a Dynasan Corporation 2830 digital multimeter connected directly to the magnet power supply. The magnetic field as a function of magnet current is linear over the range 0-5 T. The accuracy of the magnetic field measurements is ± 0.03 T.

The sample probe (Fig. 2-3), with the sample positioned at the lower end, is placed directly into the sample compartment filled with liquid He. This probe
Figure 2-2. Schematic representation of an Oxford Instruments SM3 superconducting magnet. (S) Sample holder; (W) optical windows; (T) needle valve; (M) coils of superconducting magnet.
Figure 2-3. Schematic representation of the MCD sample probe.
contains two carbon glass resistors (Lake Shore Cryogenics) as temperature sensors, and a heater. The two carbon glass resistors are placed directly above and below the sample and were calibrated by Lake Shore Cryogenics over a temperature range of 1.5-300 K to give accurate temperature readings within 0.5%. A Rh/Fe resistor, used to control the temperature above 4.2 K, is also located directly above the sample.

Temperatures between 1.5 and 2.2 K are obtained by reducing the pressure with a two-stage rotary pump. Temperatures above the boiling point of liquid He (4.218 K) are obtained by passing cold He gas through the sample compartment, and are controlled by the Rh/Fe resistor and a heater connected to an Oxford Instruments DTC2 temperature controller. MCD spectra are generally not collected between the lambda point of He (2.2 K) and 4.218 K due to light scattering, caused by bubbling of He, which results in a decrease of the signal-to-noise ratio. Below 2.2 K, liquid He becomes optically clear and also exhibits anomalously high thermal conductivity, eliminating problems with thermal gradients.

2.2.3 SAMPLE CELLS

Two types of MCD cells were used during the course of this work. The first type of cell is constructed from two square polished quartz plates (1.5 x 1.5 x 0.1 cm) separated by a rubber spacer. Aerobic cells are sealed on three sides by epoxy resin. Anaerobic cells are sealed on all four sides, leaving two small gaps for injection of the sample into the cell through the rubber spacer. The pathlength of each cell is determined by subtracting the thickness of both quartz plates from the total cell thickness. The second type of MCD cells used, are regular UV-visible absorption cuvettes (0.1-0.2 cm pathlength) cut to fit the sample holder (2.0 cm in height), and polished. Anaerobic cells are sealed with a
rubber gasket and epoxy resin.

The anaerobic MCD cells, mounted on the probe, are repeatedly pumped and flushed with oxygen-scrubbed Ar prior to the introduction of the sample. Whenever aerobic cells were used for anaerobic samples, they were also flushed on top of the magnet and filled under a He gas stream. The cells are filled with the sample using Hamilton gas-tight syringes and are rapidly frozen by immersing the probe in the liquid He bath.

2.2.4 MCD SAMPLES

Frozen MCD samples must be in the form of optical quality glass to allow transmittance of light. For protein samples, the addition of glassing agents, such as ethylene glycol, glycerol or sucrose, is required. Throughout this work, either 50% (v/v) ethylene glycol or 40% (v/v) glycerol, as indicated in figure legends, was added to the protein solutions. Although the addition of glassing agent did not show major effects on the activity or UV-visible absorption characteristics of the proteins studied in this work, it affected the EPR characteristics of the nitrogenase Fe-proteins.

For the inorganic complexes, the solvents or solvent mixtures must meet three requirements i.e., the complexes must be soluble, the solvent must not alter the complex, and it must form an optical quality glass upon freezing at liquid He temperatures. A mixture of 50:50 (v/v) DMF:toluene was satisfactory for all the VFe and MoFe complexes.

Strain in the frozen glass or magnet windows may cause some depolarization of the light beam, inducing a decrease in the signal intensity. Depolarization was assessed and strain birefringence corrected for by measuring the decrease in the intensity of the natural CD of a standard sample of D-tris(ethylenediamine)cobalt(III) chloride. The natural CD of the standard was
measured in the absence of the sample, and with the standard placed after the sample, in the absence of a magnetic field. The depolarization in the samples studied was generally less than 5% and never more than 10%. No residual strain birefringence was found in the magnet windows.

2.2.5 DATA COLLECTION AND MANIPULATION

MCD spectra were recorded digitally either using an OKI IF 800 model 30 microcomputer, interfaced via a Jasco IF500 interface, or using a Jasco DP-501N data processor interfaced to an IBM PC-XT computer. Data collection and manipulation were performed using the software provide by Jasco Corp. and modified by Drs. M. K. Johnson, D. E. Bennett, and A. T. Kowal.

In a typical MCD spectrum, the data were sampled every 0.4 nm at a scan rate of 50 nm/min and a time constant of 2 s. Unless otherwise stated, each spectrum was scanned once. In the range of 800-512 nm, the slit openings were fixed at 100 μm and at higher energies were programmed for a constant resolution of 2 nm.

MCD spectra are measured with the field aligned both parallel (positive field) and antiparallel (negative field) to the direction of light propagation, as well as in the absence of an applied magnetic field (zero field). The data were considered of good quality when the positive and negative field traces formed mirror images through the zero field trace, which corresponds to the natural CD spectrum. MCD spectra corrected for contributions from natural CD and for any magnetic effects are obtained as one-half of the difference between the positive and the negative field traces. The recording of the signal in this manner increases the signal-to-noise ratio by a factor of the square root of 2. The MCD intensity is expressed as the difference in the molar extinction coefficients for left and right circularly polarized light \( \Delta \varepsilon = \varepsilon_{\text{LCP}} - \varepsilon_{\text{RCP}} \), in units of \( \text{M}^{-1}\text{cm}^{-1} \).
after applying a correction for depolarization of the light beam.

MCD magnetization plots are generated by measuring the MCD intensity at a given wavelength, at several fixed temperatures, as a function of the magnetic field strength. Magnetization data are presented as plots of the percentage of magnetization vs $\beta B/2kT$, where $\beta$ is the Bohr magneton, $B$ is the magnetic flux, $k$ is the Boltzmann constant and $T$ is the absolute temperature. The percentage magnetization refers to the MCD intensity as a percentage of the intensity at magnetic saturation. The experimental data were corrected for any temperature-independent diamagnetic contributions by extrapolating the plots of MCD intensity vs $1/T$ to infinite temperature and then subtracting the proportionate diamagnetic contribution from the data at each individual magnetic field. The theoretical plots used to simulate the experimental data are generated from expressions given in section 1.1.3.2. The software for calculating and plotting magnetization curves was developed by Drs. M. K. Johnson and D. E. Bennett (1) and modified by Dr. A. T. Kowal.

2.3 ELECTRON PARAMAGNETIC RESONANCE

EPR spectra were recorded on an Bruker ER200D spectrometer interfaced to either an ASPECT 2000, an IBM 9000, or a Bruker 1600 computer for data handling and manipulation. Low temperatures were achieved using either an Air Products LTR-3-110E flow cryostat (2 K to 300 K) or an Oxford Instruments ESR-9 flow cryostat (4.2 K to 300 K) positioned in a TE$_{102}$ cavity, resonating at X-band frequencies. A calibrated silicon diode resistance thermometer (Lake Shore Cryogenics), immersed in buffer solution inside an EPR tube and positioned in place of the sample in the cryostat, was used to assess the temperature within 2% accuracy.

EPR spectra are recorded as the first derivative of the absorption as a
function of the magnetic field at a given microwave frequency. Quantitation of EPR signals was carried out by double integration, using the equations given in section 1.1.4.6, with software written by Drs. M. K. Johnson and A. T. Kowal. A solution of either 1 mM CuEDTA or 1 mM metmyoglobin cyanide (MetMbCN), prepared as described in reference (1), was used as standard for quantitations. The spin quantitation values are precise within ± 10%.

The EPR samples were prepared simultaneously with MCD samples, to facilitate comparison of the results from both spectroscopic techniques. Since, as mentioned earlier, the presence of glassing agents affects the EPR characteristics of the nitrogenase Fe-protein, samples with just buffer were also prepared at the same concentration of the MCD samples. The EPR samples of the VFe and MoFe complexes were at least 3-fold more concentrated than the MCD samples. The protein and synthetic inorganic complex solutions were injected into Ar-flushed EPR tubes inside a Vacuum Atmospheres glove box and frozen by rapid immersion in liquid nitrogen.

2.4 RESONANCE RAMAN

Raman spectra were recorded using an Instruments SA Ramanor U1000 spectrometer fitted with a cooled RCA 31034 photomultiplier tube with 90° scattering geometry. Spectra were recorded digitally using photon counting electronics interfaced to an IBM PC-XT microcomputer and HP 7470A plotter. Improvements in signal to noise were achieved by multiple scanning; no smoothing function was applied to the data. Typically, each spectrum is the sum of 4 to 20 scans, each involving photon counting for 1 s every 0.2 cm⁻¹, with 5 cm⁻¹ resolution. Band positions were calibrated using the excitation frequency and are accurate to ±0.1 cm⁻¹.

Lines from a Coherent Innova 100 10W Argon Ion Laser were used for
excitation. Plasma lines were removed using a Pellin Broca Prism premonochromator. Laser powers at the sample ranged from 20 to 80 mW. To avoid damage due to heating, samples were placed on the end of a cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator. This enabled the sample to be cooled down to 16 K, which facilitated improved spectral resolution and prevented laser-induced sample degradation. A sample holder suitable for handling air-sensitive compounds was designed for use with the closed cycle refrigerator (2). For proteins and solutions of inorganic compounds about 10 μL of a concentrated solution are placed on the gold plated sample holder.

2.5 SAMPLE PREPARATION AND HANDLING

Since all the samples used in this work were very oxygen sensitive, they were handled either on a gas/vacuum line, in which argon is passed over a BASF copper catalyst at 120°C to remove oxygen, or in a Vacuum Atmospheres inert atmosphere glove box equipped with a model AO-316-C oxygen analyzer, a model AM-2 moisture analyzer, a model HE-493 dri-train, and a pedatrol pressure control. Oxygen levels in the inert atmosphere box and in the gas/vacuum line were below 1 ppm. All solvents and solutions employed were degassed by three freeze-pump-thaw cycles. All solids were degassed by multiple pump-flush cycles. All solvents and solutions were contained in Schlenk flasks or in serum-capped vials, and transferred using Hamilton gas-tight syringes.

Proteins were dissolved in degassed buffer solutions of tris(hydroxymethyl) aminomethane hydrochloride (Aldrich), hereafter referred as Tris/HCl, adjusted to the appropriate pH with a concentrated solution of hydrochloric acid. All solutions were prepared using water which was purified by glass-distillation and then passed through an ion exchange column.

The reduction of samples with dithionite was carried using a saturated
solution of sodium dithionite and was added anaerobically to the proteins up to a concentration of 2 mM in dithionite. The dithionite solution was prepared in degassed Tris/HCl buffer immediately prior to its use. The reagent employed had a purity greater than 90% and was purchased from Kodak Chemicals.

For the iron analyses, extreme care was taken to eliminate Fe contamination and all the glassware was cleaned by soaking in 30% HNO₃, followed by rinsing with deionized water. The use of syringe needles was avoided when possible and instead, plastic pipettes were used, to eliminate contamination.

The synthetic inorganic complexes were dissolved in 50:50 (v/v) DMF:toluene mixture. HPLC grade, 99.9% pure, N, N-dimethylformamide (DMF) was purchased from Alfa Products, and Baker Analyzed toluene was purchased from J. T. Baker Chemical Co. These solvents were purified by distillation followed by drying with 5A molecular sieves, and then degassed and stored inside the glove box with Na-Hg amalgam. All synthetic complexes were stored in an inert atmosphere box, under Ar, until used.

2.5.1 **AZOTOBACTER VINELANDII NITROGENASE IRON-PROTEINS**

*A. vinelandii* Fe-proteins from wild type (strain UW) and alternative (strain LS15) nitrogenase were generously provided by Dr. Brian J. Hales from Louisiana State University. Detail of the growth of the bacteria, isolation, purification of the enzymes, and activity assays are given in references (3-5).

The specific activity from the Av2 samples used was 1500 nmol C₂H₂ reduced min⁻¹ mg⁻¹ protein. All the protein samples used during this work were concentrated to at least one-half of their original concentration before adding either glassing agent or buffer. The concentration was performed under Ar using either a 3 mL or 10 mL Amicon ultrafiltration stirred cell, fitted with a YM30 membrane, inside a Vacuum Atmospheres glove box, using Ar. An oxysorb LP
cartridge (MG Scientific Gases) was attached to the outlet side of the pressure
gauge of the Ar to remove residual traces of O₂. Sample concentrations are
based on the Fe analysis described herein.

As isolated:

Since the proteins are isolated and purified in the presence of dithionite,
which acts as O₂ scavenger, the "as isolated" proteins are in the reduced state.
After concentration, each batch of samples was divided in two portions, one of
the portions was diluted with 50% (v/v) ethylene glycol, degassed and saturated
with solid dithionite immediately before adding it to the protein. The second
portion was diluted with 50mM Tris/HCl buffer, pH 7.5, up to the same
concentration as the ethylene glycol sample. Dithionite solution was added to the
samples to keep the dithionite concentration up to 2 mM.

Treatment with Urea:

A 9.5 M urea (purchased from Sigma) and 2mM dithionite solution in 50 mM
Tris/HCl, pH 7.5, was added to the protein to a final urea concentration of 0.8 M,
and then either glassing agent or buffer was added to give a final urea
concentration of 0.4 M. The urea solution was prepared immediately before
addition to the sample.

Treatment with MgATP:

A 0.1 M MgATP solution containing 0.1 M ATP (Sigma) and 0.15 M
MgCl₂.6H₂O (Fisher) was made up and the pH adjusted to 7.7 using a
concentrated solution of KOH. The solution also contained 2 mM dithionite, and
was added to the protein, before dilution with glassing solvent or buffer, to give
a final concentration of 10-fold excess MgATP.
Treatment with MgATP and urea:

A 9.5 M urea solution was added to a sample containing 10-fold excess MgATP and 50 mM Tris/HCl, to a final urea concentration of 0.4 M. Only an EPR sample was prepared to check spin quantitation.

Oxidation with thionine:

The samples were concentrated by blowing Ar over the sample inside a Vacuum Atmosphere glove box to a final concentration of ~ 2 mM. A saturated thionine solution was added to the sample using a microliter gas-tight syringe until a weak but stable blue color was observed. A droplet of the sample was placed on the anaerobic resonance Raman cell and frozen immediately on the cold finger of the closed cycle refrigerator.

Fe analyses:

Fe analyses of all Av2 and Av2' samples were performed following published procedures by Ljones et al (6) and modifications by Howard et al (7). The samples were thawed anaerobically inside an inert atmosphere glove box, and all the solutions were degassed previously and added using precision microliter pipettes (Pipetman, Rainin Instrument Co.) with disposable plastic tips.

2.5.2 BACILLUS SUBTILIS GLUTAMINE PHOSPHORIBOSYLPYRROPHOSPHATE AMIDOTRANSFERASE

Purified B. subtilis glutamine PRPP amidotransferase samples, hereafter called amidotransferase, were generously supplied by Dr. Robert L. Switzer from University of Illinois, Urbana-Champaign, Illinois, and shipped to Baton Rouge in a liquid nitrogen dewar. The enzyme was isolated and purified as described in
references 8 and 9. Enzyme concentrations were based on absorbance at 278 nm using $E_{1\%}^{1\text{cm}} = 9.6$, which corresponds to $e_{410} = 13,000 \text{ M}^{-1}\text{cm}^{-1}$ (10). The samples used for spectroscopic investigations contained $3.5 \pm 0.1$ atoms of Fe/subunit and specific activities of 47 IU/mg using the assay previously described (7).

Oxidized enzyme:

Since the Fe-S cluster is known to be degraded in the presence of oxygen, the enzyme was isolated and handled under anaerobic conditions. The enzyme was thawed anaerobically and diluted with 50% (v/v) ethylene glycol under an argon atmosphere. The presence of ethylene glycol had no effect on the room-temperature absorption of CD spectra. Moreover, exposure to 50% (v/v) ethylene glycol for up to one hour at 0°C did not affect the activity. An approximate 50% reduction in activity was observed for assays conducted in the presence or 50% (v/v) ethylene glycol. However, in light of the spectroscopic results, this effect is attributed to changes in solvent polarity and viscosity, rather than changes in the environment of the Fe-S cluster. The samples for resonance Raman studies were concentrated using YM30 centricon units or by blowing Ar over the sample.

Photochemically reduced enzyme:

Since the reduced cluster is extremely oxygen-sensitive, residual traces of oxygen were removed from samples used for reduction by dialysis with at least 3 volumes of degassed 50 mM Tris/HCl buffer, pH 7.5, using an Amicon ultrafiltration cell fitted with a YM30 membrane, inside a Vacuum Atmosphere glove box under Ar (< 1 ppm O$_2$). Ethylene glycol was added dropwise at 0°C with continuous agitation to 50% (v/v), followed by 10mM sodium oxalate and 10μm deazaflavin, and the sample was placed in an anaerobic MCD cell or serum-capped EPR tube prior to illumination. The photoreduction was carried with the
sample cooled by either cold N\textsubscript{2} or inside an ice/water bath to avoid damage by the light beam. The apparatus for illumination consisted of an Oriel Corporation Xe arc lamp connected to an arc lamp supply, and a focusing lens. The illumination time is indicated on figure legends.

2.5.3 SYNTHETIC INORGANIC COMPLEXES

All the synthetic inorganic complexes used in this work were generously supplied by Dr. Richard H. Holm from Harvard University. The samples arrived at the University of Georgia in sealed ampules under Ar and they were stored inside a Vacuum Atmosphere glove box.

The following cubane clusters, with their preparation references following, were studied: (Me\textsubscript{4}N)[VFe\textsubscript{3}S\textsubscript{4}Cl\textsubscript{3}(DMF)\textsubscript{3}].2DMF (11), (Ph\textsubscript{4}P)[VFe\textsubscript{3}S\textsubscript{4}(S-p-C\textsubscript{6}H\textsubscript{4}CH\textsubscript{3})\textsubscript{3}(DMF)\textsubscript{3}] (11), (n-Pr\textsubscript{4}N)[VFe\textsubscript{3}S\textsubscript{4}Cl\textsubscript{5}(dmpe)(CH\textsubscript{3}CN)].3MeCN (11), (Et\textsubscript{4}N)[MoFe\textsubscript{3}S\textsubscript{4}Cl\textsubscript{4}(dmpe)] (12), and (Et\textsubscript{4}N)[MoFe\textsubscript{3}S\textsubscript{4}(SEt)\textsubscript{4}(dmpe)] (12); (dmpe=(CH\textsubscript{3})\textsubscript{2}P(CH\textsubscript{2})\textsubscript{2}P(CH\textsubscript{3})\textsubscript{2}).
2.6 REFERENCES

3. NITROGENASE Fe PROTEINS

As mentioned in chapter 1, the process of nitrogen fixation is an essential part of the nitrogen cycle on our planet (1). The enzyme that catalyzes this process is called nitrogenase. This enzyme is comprised of two components, the Fe protein and the MoFe protein.

The fact that the Fe protein is an essential component of nitrogenase and that the number and properties of the constituent Fe-S cluster were poorly characterized at the initiation of this work, prompted us to investigate the electronic, magnetic, and structural characteristics of this protein in the conventional (Mo) and alternative (V) enzymes using room-temperature UV-Visible absorption spectroscopy, and low-temperature MCD, EPR, and RR spectroscopies.

3.1 INTRODUCTION

3.1.1 THE Fe PROTEIN IN Mo NITROGENASES

Protein Structure:

The Fe protein (60 - 65 kDa) is a γ2 dimer protein, possessing two identical subunits (2) each having Mr ≈ 30,000 (3,4). The dimeric nature of the protein was determined by gel filtration chromatography and ultracentrifugation measurements (5-8). The Fe protein is generally accepted as the specific electron donor for the MoFe protein (9-13). In addition to the MoFe and Fe proteins, a source of reducing equivalents, Mg\text{ATP}, protons, and an anaerobic environment are required for all substrate reductions catalyzed by nitrogenase (14).

It is generally accepted that the Fe protein of any nitrogenase enzyme complex has one [4Fe-4S] cluster (13,15,16). This is based upon iron and sulfide determinations of various Fe proteins isolated from different bacteria, such as Clostridium pasteurianum (5,8,17,18), Azotobacter chroococcum (19), Klebsiella
pneumoniae (6), and Azotobacter vinelandii (20), yielding an average of 3.7 iron atoms per protein molecule, and 3.3 sulfides per protein molecule. Further evidence for the presence of one [4Fe-4S] center comes from cluster extrusion experiments which indicate that the iron in Cp2 preparations can be quantitatively extruded as one [4Fe-4S] cluster (21-24). In contrast, Braaksma et al. (25) reported that Av2 samples with high specific activity contained at least 8 Fe and 8 S per molecule. However, it is difficult to reconcile their values with the activity, iron quantitation, and proposed ligands reported by others.

Howard et al. (26) reported that the active Fe protein is encoded by the nifH and nifM genes. While the former codes for the Fe protein subunits, the latter product acts on the subunits to form active Fe protein (26). The protein homology in the Fe protein sequences is unusually high, Av2 and Kp2 are 90% homologous, while other species show about 60% homology (27-30).

There is no sequence homology between the nitrogenase Fe proteins and other iron-sulfur proteins whose sequence has been published (30). However, there are five completely conserved cysteines in all known Fe protein sequences (31). They are located at residues 38, 85, 97, 132, and 184 (numbering according to Av2), and are in highly homologous regions (31). On the basis of a labeling study (31), it was proposed that cysteines 97 and 132 are the probable ligands for the [4Fe-4S] cluster, and that the cluster is bound symmetrically between the subunits. By comparison with other [4Fe-4S]-containing proteins, the five cysteines are not organized in closely spaced groups (28,30). The labeling study (31) also showed that Av2 contains no disulfides, hyperactive thiols, or surface thiols.

The Fe protein binds two Mg·ATP (and Mg·ADP) molecules (32,33). Hausinger and Howard (31), and Robson (34) have shown evidence that at least two regions, constructed from residues 1-30 and 85-110, may be involved in the
Figure 3-1 Comparison of the Fe protein sequence from *Clostridium pasteurianum*, *Anabaena*, *Klebsiella pneumonia*, and *Azotobacter vinelandii*. The sequences are aligned for maximum identity. The cysteine residues are circled, differences are boxed, and the numbering is according to Av2. (Taken from Ref. 29)
binding of the adenylate portion of ATP and/or ADP, since these residues are somewhat homologous to the nucleotide binding residues of adenylate kinase. A sulfur from a cysteine may serve as the nucleophile for ATP hydrolysis (31), and therefore, it could be a part of the nucleotide binding site. It has been proposed that cysteiny1 residue 85 is associated with the ATP/ADP binding site (31).

The most highly conserved region of the amino acid sequence among Fe proteins of *Clostridium pasteurianum*, *Klebsiella pneumoniae*, *Anabaena*, and *Azotobacter vinelandii* occurs as a mainly hydrophobic region (residues 79-187 in the Av2 sequence) that contains 4 of the 5 conserved cysteiny1 residues. Out of the 10 completely conserved cysteine residues per dimer, only 6 can be assigned functional roles, two for Mg-ATP binding (cysteines 85) and four for cluster binding (cysteines 97 and 132) (31) (see Fig. 3-2 for a depiction of the [4Fe-4S] cluster coordination in Av2, as proposed by Hausinger and Howard (31)). Of the remaining four residues, two are thought to be near the protein surface (cysteines 184) and two are thought to be close to the cluster (cysteines 38) (31).

*Spectroscopic Studies of the Fe-S Center:*

EPR studies of reduced Fe protein show the presence of a signal with resonances at \( g_x = 2.06 \), \( g_y = 1.94 \), and \( g_z = 1.87 \) (35,36), hereafter called the "g = 1.94" signal, similar to the g = 1.94 spectra of reduced [2Fe-2S]\(^{1+}\) and [4Fe-4S]\(^{1+}\) clusters in simple Fe-S proteins. This signal cannot be observed at temperatures higher than about 35 K, due to relaxation broadening, which is a characteristic of [4Fe-4S]\(^{1+}\) signals (6,37) of the ferredoxin type. However, there are two features of this EPR signal that are unusual (23,38), first, the g-value anisotropy is somewhat atypical for ferredoxin-type [4Fe-4S]\(^{1+}\) centers, and second, the double integrations of the g = 1.94 signal give low values for the spin quantitation. These spin quantitations consistently yield about 0.3-0.4
Figure 3-2 Proposed [4Fe-4S] cluster coordination in Av2 from thiol reactivity studies. Taken from Ref. 31.
spins/molecule (23,35,36,39-43), rather than the expected 1 spin/molecule (44) suggested by reports of potentiometric studies of Cp2 (45) and Av2 (4), that show a transfer of one electron per molecule.

Before 1985, there were two explanations offered for the unusual EPR properties of the Fe-S cluster in nitrogenase Fe proteins. The first explanation, proposed by Lowe (46), suggested that the low EPR spin quantitations and the rather distinctive line shape were due to interactions between two paramagnetic centers in the protein, one of them being EPR-silent. The proposed simulations were phenomenologically reasonable. However, no other direct evidence of the second paramagnet has been found and analyses by Eady et al. (6) and Haaker et al. (43) for Mo, Cu, Mg, Mn, Zn, Cd, Co, and Ca could not detect any significant amounts of these elements; Fe was the only metal found in the protein (6,43). The room temperature absorption, CD, and MCD studies by Stephens et al. (47) also failed to show any contributions from chromophores other than the [4Fe-4S] cluster, and the spectra were typical of those from [4Fe-4S]1+ clusters (47). Similarly, the spectra of the oxidized protein were typical of a [4Fe-4S]2+ center. The Mössbauer spectrum of reduced Kp2, reported by Smith and Lang (48), is consistent with the presence of reduced [4Fe-4S] clusters, however, these results did not address the number of these clusters (48). The second explanation was advanced by Braaksma et al. (12). They proposed that the spin quantitation correlated with the activity and that samples with high specific activity afford quantitations of at least 1 spin/molecule. However, in a subsequent paper from the same group (43), spin quantitations of 0.51 spins/molecule, that increased to 1 spin/molecule in the presence of 50% ethylene glycol, were reported. Zumft et al. (41) had reported earlier that in the presence of 0.5 M urea, the EPR signal almost disappears. However, no explanation for this phenomenon was given. Hagen et al. (49) showed that the intensity of the EPR signal at g = 1.94 follows
Curie's Law, its power saturation characteristics are standard for \( S = \frac{1}{2} \) \([4\text{Fe}-4\text{S}]^{1+}\) clusters, and its dependence on frequency is characteristic for an isolated \( S = \frac{1}{2} \) ground state. Therefore, the low spin quantitation was attributed to either multiple spin systems in some of the protein molecules or a fraction of the clusters having an \( S > \frac{1}{2} \) ground state.

The effect of urea and ethylene glycol were more fully characterized by Lindahl \textit{et al.} (50). They confirmed the EPR results described above and, in addition, they reported Mössbauer and magnetic susceptibility data which facilitated more definitive analysis. They found that the "EPR-silent" urea state described by Zumft \textit{et al.} (41) shows low-field EPR resonances with \( g \)-values of 5.8 and 5.15, attributable to a cluster with a \( S = 3/2 \) ground state (50). The ethylene glycol state, described by Haaker \textit{et al.} (43), yields Mössbauer spectra like those from standard \( S = \frac{1}{2} \) \([4\text{Fe}-4\text{S}]^{1+}\) clusters and the EPR quantitates to 1 spin/molecule. They also found that the native Av2 Mössbauer spectrum can be simulated by summing a 40\% contribution from the ethylene glycol spectrum and a 60\% contribution from the urea spectrum. Therefore, the low spin integrations observed before in the Fe protein were attributed to a mixture of spin-states for the \([4\text{Fe}-4\text{S}]^{1+}\) cluster, with 40\% in the \( S = 1/2 \) form that exhibits the \( g = 1.94 \) signal and 60\% in the \( S = 3/2 \) form that exhibits the \( g = 5 \) signal (50). Their EPR and Mössbauer studies indicated a zero-field splitting of \( 2D \approx -5 \text{ cm}^{-1} \) for this \( S = 3/2 \) state (50).

Watt and McDonald (51) also reported the presence of the \( g = 5 \) signal. Furthermore, they noted an unusual line shape for the \( g = 1.94 \) signal at low temperatures which they attributed to dispersion. By comparing the mixture of spin states in Av2, with some synthetic \([4\text{Fe}-4\text{S}]^{1+}\) clusters that exhibit similar behavior, they confirmed that the \( g = 5 \) resonances come from an \( S = 3/2 \) ground state. The same conclusions concerning the presence of the \( S = 3/2 \) spin state
were made, independently, by Hagen et al. (52) in EPR studies of reduced Av2, Kp2, and Ac2.

Most recently, Lindahl et al. (53) published EXAFS studies of the cluster in Av2, in the reduced and oxidized state. They found that the average Fe-S bond length increases by 0.02 to 0.03 Å on going from the oxidized to the reduced form (53). This change is close to that observed by x-ray crystallographic studies for the oxidized (n = 2) (54) and reduced (n = 3) (55) synthetic Fe₄S₄(SR)₄ⁿ⁺ clusters (0.025 Å). They also found no observable structural differences in the S = 1/2 and S = 3/2 forms of reduced Av2 (53). Therefore, the clusters in both spin states are structurally very similar, with no major changes in the average Fe-S bond length.

The Mössbauer, magnetic susceptibility, EPR, and X-ray crystallographic studies reported by Carney et al. (56-58) demonstrate that synthetic model clusters with the cubane [4Fe-4S]¹⁺ core can also have different spin ground states, S = 1/2 and S = 3/2, depending on the nature of the thiolate group. According to their ground spin state behavior, these clusters can be classified in three different categories: pure spin S = 1/2 or 3/2 clusters; physical mixtures of S = 1/2 and 3/2 clusters; and spin admixed (S = 1/2 + S = 3/2 states) ground states. The first category is recognized by Curie behavior and saturation magnetization at low temperatures and characteristic magnetic EPR and Mössbauer spectra. The second category exhibits intermediate Curie constants and saturation magnetization values and composite EPR and Mössbauer spectra. The third category is distinguished by the absence of Mössbauer and EPR spectra from S = 1/2 and S = 3/2. Their results also show that the ground spin state of [4Fe-4S]¹⁺ synthetic clusters can be changed going from the solid state to a solution and all polycrystalline cluster compounds in solution exist as physical mixtures of S = 1/2 and S = 3/2 spin states. Thus, these results clearly indicate that [4Fe-
4S\(^{1+}\) cores of reduced synthetic analogues and proteins, like in Av2, are subject to variations in their structural and magnetic properties due to factors external to the clusters, such as crystalline packing forces acting on thiolate substituents and protein conformation (56). In addition, they concluded that there is no strong relationship between core structure or terminal ligand conformation and spin state.

Finally, the origin of spin state heterogeneity of the reduced [4Fe-4S] center in the Fe protein has been addressed by room-temperature proton NMR studies (59). The results suggest that the paramagnetically shifted proton resonances of Cp2 were independent of solvent composition and, in particular, of conditions that changed the relative concentrations of the S = 1/2 and S = 3/2 spin states displayed by the Fe-S cluster at temperatures below 30 K. In addition, the bulk room-temperature magnetic susceptibility of Cp2 solutions was found to remain largely constant over a wide range of solvent compositions. From these observations it was concluded that the [4Fe-4S]\(^{1+}\) cluster in nitrogenase Fe proteins is exclusively S = 1/2 at room temperature. Thus, it is suggested that the spin-state mixture in nitrogenase Fe proteins at low temperature is an artifact of the freezing process (59).

**Nucleotide-binding:**

As mentioned before, Mg-ATP is involved in the process of nitrogen fixation. Tso and Burris (60) reported binding studies by gel equilibration, indicating that there are two binding sites for Mg-ATP in the Fe protein. They also concluded that Mg-ADP binds more tightly than Mg-ATP and that it competes for at least one of the two binding sites (60). There are several studies involving the effects of nucleotide-binding on the properties of the cluster. The changes in properties have been used to measure the end point in studies where Mg-ATP is titrated.
against the Fe protein. Some of these changes are discussed below.

Mg·ATP lowers the redox potential of the Fe-S cluster from -290 to -400 mV (61). The midpoint potential shifts to approximately -380 mV when Mg·ADP is bound (61). The presence of the nucleotide appears to affect the association of the two components (components 1 and 2), because the Mg·ATP molecules are bound tighter in the presence of the MoFe protein, and it has been suggested that the nucleotide may bridge the Fe protein and the MoFe protein during catalysis (62,63).

Another change that Mg·ATP or Mg·ADP binding induces is a change in the EPR spectrum. It converts the g = 1.94 signal from rhombic to more axial symmetry (35,40,41), presumably as a result of a conformational change in the vicinity of the [4Fe-4S]^{1+} cluster (61,63). Mg·ADP produces the same change in the EPR spectrum in Cp2 and it has therefore been concluded that the terminal phosphate group is not required for the conversion of the signal (41). Lindahl et al. (64) and Hagen et al. (52) reported that the [4Fe-4S]^{1+} cluster of the nucleotide-bound (Mg·ATP or Mg·ADP) Fe protein also exists in an S =1/2, S = 3/2 mixture, with the latter exhibiting a broad EPR signal at g = 5. The former group of workers also claim the presence of a S = 5/2 state in the Mg·ATP-bound protein, exhibiting a resonance around g = 4.3, with zero-field splitting parameters of D = 1 - 3 cm^{-1} and E/D ~ 0.32 (64). The same group in another paper (53) reported that there were no noticeable changes in the EXAFS data of the Mg·ATP-bound protein, therefore, the conformational change probably causes very little structural changes in the cluster. Morgan et al. (65) characterized the redox properties of both states in the presence and absence of ATP and ADP, and found that the cluster in the S = 3/2 form has identical redox properties to those previously determined for the S = 1/2 form. In addition, they reported that the conversion to axial line shape in the presence of both Mg·ATP and ethylene glycol
is incomplete, suggesting that ethylene glycol may interfere with the Mg·ATP binding or that conformational changes of the protein in the glassing solvent are not the same as in buffer.

When Mg·ATP is bound to the Fe protein, it increases the oxygen sensitivity (66,67) and increases the reactivity of the cluster to iron chelators such as bathophenanthroline sulfonate or α,α'-dipyridyl (10,39). These effects appear to be unique to Mg·ATP, since in the presence of Mg·ADP and chelators, there is no significant cluster degradation (11,17,30). Anderson and Howard (39) reported that the Fe-S center of oxidized Av2 is decomposed by α,α'-dipyridyl in a biphasic process, in the presence of Mg·ATP. In the first phase, two Fe atoms are immediately removed by chelation while the additional Fe atoms are lost only after several hours. Mg·ADP prevented the Fe release by chelator. The UV-visible absorption spectrum of the intermediate was similar to that of oxidized [2Fe-2S] ferredoxins. On reduction, EPR studies showed a rhombic resonance, g = 2.00, 1.94, and 1.92, which was detectable up to 70 K, and is indicative of [2Fe-2S]¹⁺ centers (39). The oxidized [4Fe-4S]²⁺ cluster in the Fe protein, therefore, appears to be unique among biological [4Fe-4S] clusters in its ability to undergo oxidative conversion to a [2Fe-2S] center. This may well be a consequence of the cluster bridging between the 2 subunits.

Upon Mg·ATP binding, there are significant changes in the protein structure. X-ray crystallographic studies of reduced Av2 show that the protein has the orthorhombic space group P2₁2₁2, with five or six Fe protein monomers present in the asymmetric unit (68). When the crystals were added to a solution of Mg·ATP, the crystals were disrupted and dissolved (68). This behavior is consistent with previous observations suggesting that ATP binding to Fe protein is accompanied by significant conformational changes. In contrast, the crystals were stable in a solution of Mg·ADP (68). Binding of Mg·ATP (or Mg·ADP) to the Fe protein
alters the room temperature CD spectrum when the protein is in the oxidized state (47). The effect of Mg\textcdot ATP or Mg\textcdot ADP on the CD of reduced Av2 is negligible (47). Room-temperature $^1$H NMR studies (59) showed that Mg\textcdot ATP and Mg\textcdot ADP induce different modifications of the paramagnetically shifted proton resonances and that some resonances remained largely unmodified in the presence of either nucleotide. All these results suggest that although nucleotide-binding affects the cluster, there are no drastic structural changes. The above discussion also indicates that there are differences in the way ATP and ADP bind to the protein, since the changes produced by ADP-binding are not as pronounced as the ones caused by ATP-binding.

3.1.2 THE Fe PROTEIN IN V NITROGENASES

More than half a century ago, Bortels (69) gave evidence for the existence of a vanadium-containing nitrogenase, however no other efforts were made to investigate this system, until 1980 when Bishop and co-workers proposed that an alternative nitrogen-fixing enzyme exists in *Azobacter vinelandii* (70) and then proposed that it contained vanadium (71). It was not until 1986 that Robson and co-workers reported purification of the alternative component 1 of nitrogenase from *Azotobacter chroococcum*, Ac1*, and showed that it contained vanadium (72) instead of molybdenum. Independently, Hales *et al.* (73) have shown that vanadium is also found in the alternative component 1 of nitrogenase from *Azotobacter vinelandii*, Av1'. These V-containing nitrogenases are encoded by different genes. Most recently, a second alternative enzyme in *Azotobacter vinelandii* has been reported by Chisnells *et al.* (74). While the nature of this enzyme is still under investigation, initial results indicate that it does not contain V or Mo, and therefore, appears to be an Fe-only system.
In common with the Mo-nitrogenase, the V-nitrogenase contains two components (75,76). Component 1 has a molecular weight of 210 kDa in Ac1* (76) and a molecular weight of 200 kDa in Av1' (75). Like the Mo-nitrogenase, V-nitrogenase requires Mg·ATP and a low potential electron donor to reduce N₂ or C₂H₂ (75,76). In the absence of an added reducible substrate (e.g. under Ar) protons are reduced to H₂, a reaction which also occurs to a lesser extent in the presence of N₂ or C₂H₂ (75,76). A general feature of V-nitrogenase is the decreased effectiveness of N₂ or C₂H₂ to compete with H⁺ as a reducible substrate when compared with Mo-nitrogenase (75,76). In addition to C₂H₄, the V-nitrogenase, in contrast with the Mo-nitrogenase, produces C₂H₆ as a minor product of C₂H₂ reduction (77). Only the characteristics of component 2, the Fe protein of V-nitrogenase will be discussed in detail here.

While the Fe proteins associated with V-nitrogenases have not been extensively investigated thus far, the overall biochemical properties of Ac2* and Av2' are similar to those of Ac2 and Av2, the Fe proteins from the Mo-nitrogenases. They are both oxygen sensitive (75,76), γ₂ dimers with Mr \( \approx 63 \) kDa, and contain approximately 4 Fe atoms and 4 acid-labile sulfides per mole (75,76).

The dithionite-reduced Fe protein from V-nitrogenase exhibits the characteristic g = 1.94 EPR signal arising from a spin S = 1/2 system typical of proteins containing a [4Fe-4S] center, with spin quantitations of 0.2 for Av2' (75) and 0.5 for Ac2* (78). In addition, they also show resonances in the g = 5 region arising from a S = 3/2 spin system (75,78). Upon addition of Mg·ATP or Mg·ADP, the g = 1.94 signal changes from rhombic to axial, consistent with a change in protein conformation around the [4Fe-4S] cluster, similar to the one observed in the Mo-nitrogenase Fe proteins (75,78). The redox potential of the Mg·ADP-bound form of Ac2* is -463 mV (79), similar to that of Ac2 (80). The binding of
Mg-ATP also results in a conformational change which increases the rate of chelation of Fe of the Fe-S center with bathophenanthroline sulfonate (75). Therefore, while more extensive spectroscopic studies are required, the EPR characteristics of these Fe proteins reported thus far appear to be similar to those of the Mo-nitrogenase Fe proteins.

Eady et al. (78) determined the amino acid composition of Ac2* and compared it with that of Ac2 (refer to Table 3-1). Acidic residues are approximately twice as abundant as basic residues, and tryptophan is absent, both features that are characteristic of the Fe proteins associated with Mo-nitrogenases (78).

The Fe proteins in the Mo-nitrogenase, in the presence of Mg-ATP, act as specific electron donors to the MoFe protein. Since the Fe proteins associated with V-nitrogenase are functional in heterologous activity cross-reactions with MoFe proteins (75,76) the topology of the contact surface between the VFe proteins or the MoFe proteins with the Fe protein must be sufficiently similar to allow complex formation and subsequent electron transfer coupled to the hydrolysis of Mg-ATP to occur (79).

3.2 OBJECTIVES:

The objective of this research was to gain further insight to the electronic, magnetic, and structural properties of the [4Fe-4S]1+,2+ clusters in Fe proteins from V- and Mo-containing nitrogenases (Av2 and Av2') in order to characterize the function of the cluster in the electron-transfer to the VFe- and MoFe-proteins. To achieve this goal, room-temperature UV-visible absorption in addition to low-temperature MCD, EPR, and resonance Raman (RR) studies have been carried out.

At the outset of this project, the major obstacle to a better understanding
# TABLE 3-1

AMINO ACID COMPOSITION OF Ac2* AND Ac2 (78)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>From nifH* sequence</th>
<th>Ac2*</th>
<th>Ac2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>26</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>Threonine</td>
<td>9</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Serine</td>
<td>13</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>35</td>
<td>37</td>
<td>41</td>
</tr>
<tr>
<td>Proline</td>
<td>9</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Glycine</td>
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<td>31</td>
<td>29</td>
</tr>
<tr>
<td>Alanine</td>
<td>32</td>
<td>39</td>
<td>32</td>
</tr>
<tr>
<td>Valine</td>
<td>22</td>
<td>24</td>
<td>28</td>
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<tr>
<td>Methionine</td>
<td>13</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
<td>22</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Histidine</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>17</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Arginine</td>
<td>11</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>7</td>
<td>8</td>
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</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proportion of Gly + Asx (%)</td>
<td>21.1</td>
<td>21.81</td>
<td>22.54</td>
</tr>
<tr>
<td>Proportion of Lys + Arg (%)</td>
<td>9.6</td>
<td>9.4</td>
<td>10.9</td>
</tr>
<tr>
<td>Total residues</td>
<td>289</td>
<td>306</td>
<td>304</td>
</tr>
</tbody>
</table>
of the cluster in the Fe protein was the low $S = 1/2$ EPR spin quantitations, but reports published in 1985 suggest this to arise from $S = 3/2 / S = 1/2$ spin state heterogeneity for the $[\text{4Fe-4S}]^{1+}$ center. The spin interconversion between these two forms is dependent upon the nature of the medium. The fact that there were no low-temperature MCD studies published describing the electronic and magnetic properties of $S = 3/2 [\text{4Fe-4S}]^{1+}$ clusters, prompted us to study the medium effects described in the literature to alter the spin state of the cluster in native Av2. It was hoped that this would establish MCD as a technique for identifying and characterizing this type of center in other Fe-S metalloproteins, including the alternative Fe protein, Av2'.

3.3 RESULTS:

3.3.1 Av2

3.3.1.1 Native Av2

As isolated, native Av2 is in the reduced state, since it is purified in the presence of dithionite to ensure completely anaerobic conditions.

EPR Studies: Native Av2 in 50 mM Tris/HCl buffer, pH 7.5, is paramagnetic exhibiting a rhombic EPR signal with $g$-values of 2.05, 1.94, and 1.86 ($g = 1.94$ resonance) (spectrum A, Fig. 3-3), with shape and intensity characteristic of nitrogenase Fe proteins. The $g$-values and relaxation characteristics are typical of $S = 1/2$, reduced $[\text{4Fe-4S}]^{1+}$ centers of the ferredoxin type. Also, there is an additional low-field EPR signal in the $g = 5$ region (spectrum A, Fig. 3-4), similar to that reported by Lindahl et al. (50), Watt and McDonald (51), Hagen et al. (52), and Morgan et al. (65) with $g$-values of 5.85, and 4.64. The weak derivative signal centered at $g = 4.30$ most probably originates from adventitiously bound Fe.

EPR spin quantitations of the $S = 1/2$ resonance at 15 K and 0.20 mW indicate that the $g = 1.94$ signal corresponds to about 0.35 ±0.05 spins/4Fe (Table
Figure 3-3  $S = 1/2$ EPR spectra of Native Av2. (A) Protein (0.39 mM) in 50 mM Tris/HCl buffer, pH 7.5, and 2 mM dithionite. (B) Protein (0.39 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 1 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 10 K. Both spectra were recorded at the same spectrometer gain.
Figure 3-4  $S = 3/2$ EPR spectra of Native Av2. (A) Protein (0.39 mM) in 50 mM Tris/HCl buffer, pH 7.5, and 2 mM dithionite. (B) Protein (0.39 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 4.6 K. Both spectra were recorded at the same spectrometer gain.
TABLE 3-2

*g = 1.94 EPR SPIN QUANTITATIONS FOR NITROGENASE Fe PROTEINS
(UNDER NON-SATURATING CONDITIONS)

<table>
<thead>
<tr>
<th>SAMPLE CONDITIONS</th>
<th>SPIN CONCENTRATION/4Fe&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Av&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Native</td>
<td>0.35 ±0.05</td>
</tr>
<tr>
<td>+ ethylene glycol</td>
<td>0.85 ±0.05</td>
</tr>
<tr>
<td>+ glycerol</td>
<td>N.D.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ urea</td>
<td>0.12 ±0.05</td>
</tr>
<tr>
<td>+ urea + ethylene glycol</td>
<td>0.45 ±0.05</td>
</tr>
<tr>
<td>+ urea + glycerol</td>
<td>N.D.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Mg·ATP</td>
<td>0.30 ±0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Mg·ATP + ethylene glycol</td>
<td>0.80 ±0.05&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Mg·ATP + urea</td>
<td>0.20 ±0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fe concentrations determined by BPS colorimetric method developed by Ljones and Burris (11).

<sup>b</sup>Average of at least 4 measurements at 12 K and 0.2 mW, and 15 K and 0.2 mW.

<sup>c</sup>Average of at least 3 measurements at 12 K and 0.2 mW, and 19 K and 1 mW.

<sup>d</sup>Not determined.

<sup>e</sup>Spin concentration/molecule
suggesting that the form of the protein with a $S = 3/2$ [4Fe-4S]$^{1+}$ cluster dominates in the native protein at low temperature.

We have studied the temperature dependence of the $g = 5.85$ and 4.64 resonances over the range 4.2 K to 20 K, as indicated in Fig. 3-5. The relative intensity of the resonances at $g = 5.85$ and 4.64 is strongly temperature dependent, with the low-field signal dominating at lower temperatures. These characteristics are indicative of an $S = 3/2$ spin system that is split into two Kramers doublets as a result of a zero-field splitting that is large compared to the Zeeman interaction. The magnetic properties of the $S = 3/2$ ground state can be described in terms of the spin Hamiltonian:

$$
\hat{H} = D[S_x^2 - S(S + 1)/3] + E[S_x^2 - S_y^2] + g_0 B \cdot \hat{S} \quad (1)
$$

where $D$ and $E$ are the axial and rhombic zero-field splitting parameters and $g_0$ is the real $g$-value for an assumed isotropic Zeeman interaction. In zero magnetic field the spin quartet is split into two doublets, separated in energy by $\Delta = 2D(1 + 3(E/D)^2)^{1/2}$. Provided that the zero-field splitting is much greater than the Zeeman interaction, each doublet can be treated as an effective $S = 1/2$ spin system and diagonalization of the energy matrix for external fields along each of the three principal axes enables computation of effective $g$-values. The observed temperature dependence and "absorption-shape" indicate that the $g = 5.85$ and 4.64 signals are the low-field components of resonances from the lower and upper doublets, respectively. The $g$-values and temperature dependence are consistent with intermediate rhombicity and a negative value for $D$. For example, the best fit to the observed $g$-values occurs for $E/D = 0.13$ and $g_0 = 1.98$, when the theory predicts $g_x = 3.19$, $g_y = 4.64$, and $g_z = 1.89$ for one doublet and $g_x = 0.77$, $g_y = 0.68$, and $g_z = 5.85$ for the other (see Fig. 3-6). As is the case for many other
Figure 3-5  Temperature-dependence of the $S = 3/2$ EPR signal of Native Av2. Protein (0.39 mM) in 50 mM Tris/HCl buffer, pH 7.5, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperatures as indicated. Spectra were recorded at the same spectrometer gain. Insert: plot of the natural logarithm of the ratio of the intensities of the $g = 5.85$ and 4.64 inflections versus the reciprocal of the absolute temperature.
Figure 3-6  Plot of g-values for an $S = 3/2$ system as a function of $E/D$ according to equation 1. (a) $M_s = \pm 3/2$; (b) $M_s = \pm 1/2$. Figure taken from ref. 50.
rhombic high-spin Fe centers, we have not observed the predicted high field resonances. This is probably a result of substantial broadening as a result of small heterogeneity in the Fe-S cluster in individual protein molecules. The energy separation between these two zero-field doublets, $\Delta$, can be estimated from the slope of a plot of the natural logarithm of the ratio of the intensity of the two field resonances versus the reciprocal of the absolute temperature, which is a straight line within experimental error of the measurement of the absolute temperature (see insert Fig. 3-5). From this plot we estimate $\Delta = 4.2 \pm 0.6$ cm$^{-1}$, which corresponds to $D = -[2.1 \pm 0.3]$ cm$^{-1}$.

Assuming that the $[4\text{Fe}-4\text{S}]^{1+}$ cluster exists only with either a $S = 1/2$ or $S = 3/2$ ground state, the EPR data for native Av2 clearly indicate that approximately 35% of the clusters have $S = 1/2$ ground states and 65% have $S = 3/2$ ground states.

3.3.1.2 Native Av2 + Ethylene Glycol

EPR Studies: As shown in spectrum B, Fig. 3-3, the rhombic $g = 1.94$ signal for Av2 in 50 mM Tris/HCl buffer, pH 7.5, with 50% (v/v) ethylene glycol, has $g$-values of 2.05, 1.94, and 1.87 and its intensity is significantly greater than the one observed in the absence of ethylene glycol. This $g = 1.94$ signal integrates to $0.85 \pm 0.05$ spins/4Fe (see Table 3-2). The low-field signals observed in native Av2 are much less intense in the sample with ethylene glycol (see spectrum B, Fig. 3-3).

The shape of the $g = 1.94$ signal in Av2 in presence of ethylene glycol ($g = 2.05, 1.94, \text{ and } 1.87$) is similar to the $g = 1.94$ signal in the native Av2 ($g = 2.05, 1.94, \text{ and } 1.86$), but there are some notable differences. Besides small position changes, there is less resolution between the $g = 2.05$ and $g = 1.94$ features and the $g = 1.94$ resonance is narrower, in the sample with ethylene glycol (Fig. 3-3).
Figure 3-7 shows the temperature dependence of the EPR spectra of Av2 in ethylene glycol at 1 mW, at temperatures between 4.2 K and 30 K. In contrast to native Av2 in the absence of ethylene glycol, the form of the spectrum displays marked temperature dependence. At temperatures below 12 K the $g = 2.05$ and 1.87 signals start to broaden, and are no longer clearly discernible at 4.2 K and high powers. New features, i.e. broad positive component at $g = 1.97$ and negative absorption shaped component at $g = 1.82$, are apparent at the lower temperatures. The spectrum broadens and disappears at temperatures higher than 40 K as a result of relaxation broadening.

Figure 3-8 shows the EPR spectra of native Av2 in the presence of ethylene glycol at 4.6 K with very high (100 mW) and very low (0.02 mW) microwave powers. On taking the difference between the two spectra, the resultant spectrum resembles the normal $g = 1.94$ signal obtained under non-saturating conditions at 15 K. This indicates that an additional fast-relaxing $S = 1/2$ species contributes the EPR spectrum. This feature was not apparent in temperature dependence studies of samples in the absence of ethylene glycol. Consequently, in addition to heterogeneity of the $[4\text{Fe}-4\text{S}]^{1+}$ cluster in terms of ground state spin, $S = 1/2$ or $S = 3/2$, there appears to be heterogeneity in the $S = 1/2$ form, with a more axial species with $g_\perp \approx 1.94$ and $g_\parallel \approx 1.82$ being induced by ethylene glycol.

**UV-Visible Absorption and MCD studies:** Low-temperature MCD spectra at 1.61, 4.22, 10.0, and 92.0 K and 4.5 T, for reduced Av2 with 50% (v/v) ethylene glycol are shown in Fig. 3-9. The MCD spectra are rich in detail compared to the featureless room-temperature absorption spectrum, consisting of positive bands at 720, 534, 440, and 354 nm, and negative bands at 580 and 660 nm. The positive band at 410 nm corresponds to a small (< 1%) heme impurity in the sample and varied in intensity from sample to sample. The overall form and intensity of the
Figure 3-7  Temperature-dependence of $S = 1/2$ EPR signal of native Av2 in the presence of ethylene glycol. Protein (0.39 mM) in 50 mM Tris/HCl, pH 7.5, with 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 1 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperatures and multiplication factors as indicated.
Figure 3-8  Microwave power-dependence of the $S = 1/2$ EPR spectra of native Av2 in the presence of ethylene glycol. Protein sample as in Fig. 3-6. (A) 0.02 mW; (B) 100 mW; (C) Difference (A) - (B). Conditions: modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 4.5 K. All spectra at same relative gain.
Figure 3-9  Room-temperature UV-visible absorption and low-temperature MCD spectra of native Av2. Protein sample as in Fig. 3-6. Conditions for UV-visible spectra: pathlength, 0.10 cm. Conditions for MCD spectra: pathlength, 0.17 cm; magnetic field and temperatures as indicated (intensity of transitions increasing with decreasing temperature).
spectra resemble those of a single [4Fe-4S]$^{1+}$ center (81), confirming the presence of one [4Fe-4S]$^{1+}$ cluster.

MCD magnetization data were collected at the prominent peaks, i.e. 720, 534, and 354 nm (Fig. 3-10), at temperatures between 1.62 and 10.1 K and magnetic fields between 0 and 4.5 T. These magnetization data, after correction for contributions from temperature-independent MCD bands, show some wavelength dependence. At 534 and 354 nm data collected at different temperatures lie on the same curve within experimental error, indicating that a transition with a $S = 1/2$ ground state is the dominant contributor. However, at 720 nm the data collected at 1.62 K, 4.22 K, and 10.1 K lie on separate curves. Such nesting is consistent with significant contribution at this wavelength from a transition originating from a chromophore with a $S > 1/2$ ground state that is subject to zero-field splitting.

More detailed analysis involving simulating magnetization curves based on EPR-determined g-values suggests that at all three wavelengths, a contribution from a $S = 3/2$ paramagnet in addition to that of the $S = 1/2$ Fe-S center is apparent. In Fig. 3-10, the solid line is the theoretical curve for a system with 90% of the MCD intensity originating from an axial $S = 1/2$ and 10% from an axial $S = 3/2$ ground states, with $g\parallel = 2.04$ and $g\perp = 1.90$ and $g\parallel = 5.75$ and $g\perp = 1.2$ respectively, and $m_\|/m_\perp = 0$. These results concur with the EPR quantitations of 0.85 ±0.05 spins/4Fe for the $S = 1/2$ signal, indicated in Table 3-2, and confirm that in the presence of ethylene glycol, the [4Fe-4S]$^{1+}$ cluster is mainly in the form with a $S = 1/2$ ground state. Analogous results were found in five different preparations of Av2 with 50% (v/v) ethylene glycol, and are consistent with the EPR and Mössbauer data reported by Lindahl et al. (50). The intensities of the MCD spectra under identical conditions were in good agreement in all five preparations with the exception of the 354 nm band which varied by ±25%
Figure 3-10 MCD magnetization plots for native Av2. Protein sample as in Fig. 3-8. Conditions: temperatures and wavelengths as indicated; magnetic fields between 0 and 4.5 T.
MCD Magnetization Plots (534 nm)
Dithionite Reduced Av2 (50% (v/v) Ethylene Glycol)

Circle — 1.62 K
Square — 4.22 K
Triangle — 10.1 K

Solid line: theoretical curve for $S=1/2$ (90%) and $S=3/2$ (10%)
Dashed lines: theoretical curve for $S=1/2$ (lower)

Fig. 3-10 (Continued)
MCD Magnetization Plots (354 nm)
Dithionite Reduced Av2 (50% (v/v) Ethylene Glycol)

Circle - 1.62 K
Square - 4.22 K
Triangle - 10.1 K

Solid line: theoretical curve for $S = \frac{1}{2}$ (90%) and $S = \frac{3}{2}$ (10%)
Dashed lines: theoretical curve for $S = \frac{1}{2}$ (lower)

Fig. 3-10 (Continued)
compared with that in Fig. 3-10. We attribute this inconsistency to low transmission of light by the sample as a result of absorption by dithionite and the polypeptide backbone. We have observed that MCD intensities for samples with OD > 1 in a 1 mm pathlength cell vary considerably with the quality and depolarization characteristics of the glass.

3.3.1.3 Native Av2 + Urea

*EPR Studies:* Addition of urea to native Av2 to a final urea concentration of 0.4 M, causes a marked decrease of the intensity of the $g = 1.94$ signal (see spectrum A, Fig. 3-11) and an increase in the $g = 5$ signal (see spectrum A, Fig. 3-12). The residual $g = 1.94$ signal has essentially the same shape as that of native Av2, with slightly different $g$-values (2.04, 1.94, and 1.87). Quantitation of the $g = 1.94$ signal yielded a spin concentration of $0.12 \pm 0.05$ spins/4Fe compared to $0.35 \pm 0.05$ spins/4Fe for native Av2 (see Table 3-2).

The form of the $S = 3/2$ EPR signal is somewhat perturbed in the presence of urea, with positive features centered at $g = 5.80$ and 5.15, as opposed to $g = 5.85$ and 4.64 in the absence of urea. Based on the $S = 1/2$ spin quantitation data, approximately 88% of the $[4Fe-4S]^{1+}$ clusters have $S = 3/2$ ground states and 12% have $S = 1/2$ ground states in the presence of 0.4 M urea. The weak signal at $g = 4.3$ is most likely due to adventitiously bound Fe.

We describe the magnetic properties of the system with the $S = 3/2$ spin Hamiltonian given in Eq. 1. Following the same analysis made for native Av2, the best fit to the observed $g$-values occurs when $E/D = 0.22$ and $g_0 = 2.02$, when theory predicts $g_x = 2.67$, $g_y = 5.15$, and $g_z = 1.76$ for one doublet and $g_x = 1.37$, $g_y = 1.11$, and $g_z = 5.80$ for the other. By determining which resonance results from the ground doublet, the sign of $D$ can be determined.

We have studied the temperature dependence of the $g = 5.8$ and 5.15
Figure 3-11  S = 1/2 EPR spectra of native Av2 in the presence of 0.4 M urea.
(A) Protein (0.30 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 0.4 M urea, and 2 mM dithionite. (B) Protein (0.30 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 0.4 M urea, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 1 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 10 K. The spectrometer gains are the same for both spectra.
Figure 3-12  S = 3/2 EPR spectra of native Av2 with 0.4 M urea. (A) Protein (0.30 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 0.4 M urea, and 2 mM dithionite. (B) Protein (0.30 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 0.4 M urea, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 4.6 K. Both spectra were recorded at the same spectrometer gain.
resonances between 4.7 and 20.2 K (see Fig. 3-13). The intensity of the $g = 5.80$ resonance increases relative to that of the $g = 5.15$ peak as the temperature is lowered, showing that $D$ is negative and indicating that its magnitude is such that noticeable repopulation of the states occurs between 4.7 and 10.2 K, similarly to native Av2. From our EPR studies, the estimated energy separation, $\Delta$, has a value of $3.7 \pm 0.6 \text{ cm}^{-1}$, which corresponds to $D = -|1.8 \pm 0.3| \text{ cm}^{-1}$ (see insert on Fig. 3-13), in good agreement with the value of $D = -1.5$ to $-3 \text{ cm}^{-1}$ reported by Lindahl et al. (50) for native Av2 plus urea.

While the presence of urea increases the $S = 3/2$ to $S = 1/2$ ground state ratio in native Av2, the subsequent addition of 50% (v/v) ethylene glycol results in a shift in favor of the $S = 1/2$ species. The $g = 1.94$ signal increases in intensity (see Fig. 3-13) and the spin concentration increases to $0.45 \pm 0.05$ spins/4Fe (see Table 3-2). The $g$-values for this signal are 2.04, 1.94, and 1.87, with a shape that is similar to native Av2 with ethylene glycol. The $g = 5$ region shows a $S = 3/2$ resonance very similar to that of native Av2 with or without ethylene glycol (compare spectra B, Fig. 3-12 and Fig. 3-4). The effective $g$-values for the $S = 3/2$ species have reverted back to those of Av2' in the presence of urea and glassing solvent, and close to those of native Av2, i.e. $g = 5.90$ and 4.74.

**UV-Visible Absorption and MCD Studies:** The upper panel in Fig. 3-13 shows the room-temperature UV-visible spectrum for native Av2 with 0.4 M urea and 50% (v/v) ethylene glycol, over the wavelength region of 800-300 nm. The low-temperature MCD spectra at 4.5 T and temperatures between 1.61 and 91.8 K, are shown in the lower panel of Fig. 3-14. Analogous data were obtained for 3 distinct samples. The MCD spectra is temperature dependent with pronounced positive bands at 736, 524, and 358 nm. While the spectra clearly resemble those
Figure 3-13  Temperature-dependence of $S = 3/2$ EPR signal of native Av2 with 0.4 M urea. Protein (0.30 mM) in 50 mM Tris/HCl, pH 7.5, with 0.4 M urea, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperatures as indicated; all spectra were recorded using the same spectrometer gain. Insert: plot of the natural logarithm of the ratio of the intensities of the $g = 5.80$ and 5.15 inflections versus the reciprocal of the absolute temperature.
Figure 3-14  Room-temperature UV-visible absorption and low-temperature MCD spectra of native Av2 with 0.4 M urea. Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 0.4 M urea, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions for UV-visible absorption spectrum: pathlength, 0.10 cm. Conditions for MCD spectra: pathlength, 0.20 cm; magnetic field and temperatures as indicated (intensity of transitions increasing with decreasing temperature).
obtained in the absence of urea (Fig. 3-10), the bands are significantly broader and the region between 570 nm and 690 nm no longer exhibits negative features.

In an attempt to ascertain the nature of the electronic ground state of the Fe-S center, MCD magnetization data were collected at 358 nm, 524 nm, and 736 nm at temperatures between 1.62 and 10.1 K. The data at 524 nm are shown in Fig. 3-15 and very similar plots were obtained at the two other wavelengths investigated (data not shown). The magnetization data clearly show the presence of significant contribution from a chromophore with an $S = 3/2$ ground state. This is apparent from the pronounced nesting and the observation that the data obtained at the lowest temperature magnetizes with a much steeper initial slope than that expected for a chromophore with an $S = 1/2$ ground state. The lowest temperature plot at 524 nm can be adequately simulated with theoretical data for a mixture of 65% $S = 1/2$ and 35% $S = 3/2$ ground states, using the EPR-determined g-values. Since the absolute intensity of the contribution from the $S = 3/2$ system to the MCD spectrum is unknown, it is not possible to use these fits to assess the $S = 1/2$ to $S = 3/2$ ground states ratio. However, the result is qualitatively in agreement with the EPR data on the same sample which showed $S = 1/2$ and $S = 3/2$ [4Fe-4S]$^{1+}$ clusters in an approximately 1:1 ratio.

3.3.1.4 Native Av2 + Mg:ATP

*EPR Studies:* Upon addition of 10-fold excess Mg:ATP to native Av2 in 50 mM Tris/HCl, pH 7.6, the rhombic $g = 1.94$ signal in native Av2 becomes more axial, with $g_{\parallel} = 2.05$ and $g_{\perp} = 1.94$ (see spectrum A, Fig. 3-16). The intensity of the $g = 1.94$ signal showed no significant change, compared to native Av2, integrating to 0.30 ±0.03 spins/molecule (see Table 3-2).

EPR signals indicative of $S = 3/2$ [4Fe-4S]$^{1+}$ centers are observed in the $g = 5$ region (spectrum A, Fig. 3-17). Compared to native Av2 the low field
MCD Magnetization Plots (524 nm)
Dithionite Reduced Av2 in 0.4 M Urea
(50% (v/v) Ethylene Glycol)

Circle — 1.62 K
Square — 4.22 K
Triangle — 10.1 K
Solid line: theoretical curve for S=1/2
and S=3/2
Dashed lines: theoretical curve for S=1/2 (lower)

Figure 3-15 MCD magnetization plots for native Av2 with 0.4 M urea. Protein sample as in Fig. 3-13. Conditions: temperatures and wavelength as indicated; magnetic fields between 0 and 4.5 T.
Figure 3-16  $S = 1/2$ EPR spectra of native Av2 with 10-fold excess of Mg·ATP. (A) Protein (0.33 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 10-fold excess of Mg·ATP, and 2 mM dithionite. (B) Protein (0.33 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 10-fold excess of Mg·ATP, 2 mM dithionite, and 0.4 M urea. (C) Protein (0.33 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 10-fold excess of Mg·ATP, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 1 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 16 K. Multiplication factors indicate relative gains.
Figure 3-17  S = 3/2 EPR spectra of native Av2 in the presence of 10-fold excess of Mg\textsuperscript{2+}ATP. (A) Protein (0.33 mM) in 50 mM Tris/HCl buffer, pH 7.5, 10-fold excess of Mg\textsuperscript{2+}ATP, and 2 mM dithionite. (B) Protein (0.33 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 10-fold excess of Mg\textsuperscript{2+}ATP, 2 mM dithionite, and 0.4 M urea. (C) Protein (0.33 mM) in 50 mM Tris/HCl buffer, pH 7.5, with 10-fold excess of Mg\textsuperscript{2+}ATP, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 4.5 K. Multiplication factors indicate relative gains.
resonance from the upper doublet is shifted to $g = 5.15$ and is very similar to that observed for native Av2 plus urea. The $S = 1/2$ EPR spin quantitations, therefore, suggest a similar mixture of spin states to that observed in native Av2, i.e. 30% of the clusters with $S = 1/2$ ground states and 70% with $S = 3/2$ ground states.

The presence of 10-fold excess of Mg:ATP and 0.4 M urea produces a similar $g = 1.94$ axial signal (see spectrum B, Fig. 3-16) to that observed in the absence of urea. This signal quantifies to $0.20 \pm 0.03$ spins/molecule (see Table 3-2) which is similar to the value observed for native Av2 plus urea ($0.12 \pm 0.05$ spins/4Fe). Likewise, the $g = 5$ resonance is very similar to that of native Av2 plus urea (compare spectrum A, Fig. 3-13 and spectrum B, Fig. 3-17), with the same $g$-values of 5.80 and 5.15.

Upon addition of 50% (v/v) ethylene glycol to native Av2 with 10-fold Mg:ATP, the $g = 1.94$ signal reverts to the rhombic line-shape observed in the absence of Mg:ATP, with $g$-values of 2.05, 1.94, and 1.88 (see spectrum C, Fig. 3-16). These results suggest that ethylene glycol either interferes with Mg:ATP binding or that the conformational changes of the protein in the presence of glassing solvent are not the same as in its absence. The EPR spin quantitation for this signal is $0.80 \pm 0.05$ spins/molecule (see Table 3-2), comparable to the quantitation obtained for the sample in the absence of Mg:ATP. The low field resonances are similar to the ones observed in the sample in the absence of Mg:ATP (compare spectrum C, Fig. 3-17 and spectrum B, Fig. 3-4).

**UV-Visible Absorption and MCD Studies:** The room-temperature UV-visible absorption and variable-temperature MCD spectra (not shown) of native Av2 with 10-fold excess Mg:ATP and 50% (v/v) ethylene glycol are identical to the spectra obtained for the sample in the absence of Mg:ATP. Magnetization plots at three
different wavelengths (not shown), 715, 528, and 352 nm, also show similar behavior when compared to the sample in the absence of Mg-ATP. In summary, the MCD spectra and magnetization data of native Av2 in the presence of ethylene glycol are not significantly perturbed by the addition of Mg-ATP, which is consistent with the EPR results reported above.

3.3.1.5 Thionine-Oxidized Av2

On oxidation with excess thionine, the Fe-S cluster in native Av2 exhibited no EPR signals and no temperature-dependent MCD bands (data not shown). This is consistent with oxidation to a diamagnetic, \( S = 0 \) \([4\text{Fe-4S}]^{2+}\) cluster. Since this diamagnetic chromophore is not amenable to investigation by these techniques, RR spectroscopy was used to investigate the structural properties of the oxidized cluster.

**RR Studies:** Fig. 3-18 compares the RR spectrum of thionine-oxidized Av2, and the oxidized *Clostridium pasteurianum* ferredoxin which is composed of two identical \([4\text{Fe-4S}]^{2+}\) clusters. Although the spectra are not identical, they show several similarities in the position of the major bands, which are a fingerprint for \([4\text{Fe-4S}]^{2+}\) clusters. Based on the band assignments reported by Czernuszewicz et al. (82) for the \([4\text{Fe-4S}]^{2+}\) clusters in *C. pasteurianum* ferredoxin, *Chromatium vinosum* "high potential" iron protein (HiPIP), and the synthetic cluster \((\text{Et}_4\text{N})_2\text{Fe}_4\text{S}_4(\text{SCH}_2\text{Ph})_4\) it can be concluded that in oxidized Av2 the 249, 335, and 390 cm\(^{-1}\) bands can be assigned predominantly to bridging Fe-S vibrations, and the 353 cm\(^{-1}\) band to terminal (cysteinyl) Fe-S vibrations.

In an attempt to get better quality spectrum than the one showed in Fig. 3-18, the Av2 sample with thionine was concentrated further by blowing Ar over the sample inside an inert-atmosphere glove box. The resulting sample exhibited additional bands in the RR spectrum, see Fig. 3-19. In addition to well-resolved
Figure 3-18 Low-temperature resonance Raman spectra of the [4Fe-4S]^{2+} clusters in thionine-oxidized Av2 and oxidized *Clostridium pasteurianum* ferredoxin. *Upper spectrum:* thionine-oxidized Av2 (~ 2 mM) in 50 mM Tris/HCl buffer, pH 7.5. *Lower spectrum:* oxidized *Cp* ferredoxin in 100 mM Tris/HCl buffer, pH 7.9. Conditions of measurement: excitation wavelength, 457.9 nm; laser power at sample, ~ 50 mW for Av2 and ~ 100 mW for *Cp* ferredoxin; spectral resolution, 5 cm\(^{-1}\); both spectra are the sum of 10 scans, after 25-points smoothing; sample temperature, 17 K. Starred bands indicate spectral contribution of lattice modes of ice.
bands at 249, 333, 352, 368, and 393 cm\(^{-1}\) that are assigned to the \([4\text{Fe-4S}]^{2+}\) cluster by comparison with Fig. 3-18, additional bands appeared at 287 and 425 cm\(^{-1}\). Bands with similar frequencies have been observed for all biological \([2\text{Fe-2S}]^{2+}\) clusters examined thus far (see ref. 83 for the most recent data and assignments). To facilitate comparison, the spectrum of spinach ferredoxin under analogous conditions is shown in Fig. 3-19. Therefore, it is concluded that partial breakdown of the \([4\text{Fe-4S}]^{2+}\) clusters has occurred to yield \([2\text{Fe-2S}]^{2+}\) centers. Whether this occurs as a result of the freezing and thawing procedure, or oxidative degradation due to traces of oxygen and/or prolonged exposure to thionine is unclear at present. However, it is clear that RR spectroscopy offers an excellent method for monitoring this novel cluster degradation and, contrary to previous investigations (39), the presence of Mg-ATP and a chelating agent are not required to effect the conversion.

In summary, RR spectroscopy confirms the presence of a \([4\text{Fe-4S}]^{2+}\) cluster in thionine-oxidized Av2, with a structure similar to the clusters present in Cp ferredoxin and HiPIP. Also, it shows that prolonged exposure to oxidants and/or freezing and thawing results in partial \([4\text{Fe-4S}]\) to \([2\text{Fe-2S}]\) cluster degradation. Unfortunately no Raman bands were discernible for native Av2. This is due in part to strong background fluorescence and the inherently weak Raman scattering of \([4\text{Fe-4S}]^{1+}\) centers (unpublished observations).

### 3.3.2 Av2’

#### 3.3.2.1 Native Av2’

As for Av2, the isolation of Av2’ is performed in the presence of dithionite to ensure anaerobicity, therefore the Fe-S center in native Av2’ is in the reduced state.

_EPR Studies_: Native Av2’, in 50 mM Tris/HCl, pH 7.6, contains a paramagnetic
Figure 3-19 Low-temperature resonance Raman spectra of the [2Fe-2S]^{2+} clusters in oxygen-damaged thionine-oxidized Av2 and oxidized spinach ferredoxin. Upper spectrum: oxygen-damaged thionine-oxidized Av2 (~ 3 mM) in 50 mM Tris/HCl buffer, pH 7.5. Lower spectrum: oxidized spinach ferredoxin. Conditions of measurement: excitation wavelength, 457.9 nm; laser power at sample, ~ 50 mW for Av2 and ~ 100 mW for ferredoxin; spectral resolution, 5 cm^{-1}; upper spectrum is the sum of 20 scans, and lower spectrum is the sum of 5 scans, after 25-points smoothing; sample temperature, 17 K. Starred bands indicate spectral contribution of lattice modes of ice.
Fe-S cluster exhibiting EPR characteristics and g-values similar to Mo-nitrogenase Fe proteins, indicating the presence of a [4Fe-4S]^{1+} cluster. The rhombic g = 1.94 signal has a similar shape to that of native Av2, albeit with slightly different g-values (2.06, 1.94, and 1.87) (spectra A, Figs. 3-20 and 3-21). This S = 1/2 resonance integrates to 0.15 ±0.05 spins/4Fe, as compared to 0.35 ±0.05 spins/4Fe in native Av2 (see Table 3-2). In addition, resonances at g = 5.90 and 5.15 (spectra A, Figs. 3-22 and 3-23) are observed, which are indicative of [4Fe-4S]^{1+} centers with S = 3/2 ground states. These resonances are similar to the ones observed in native Av2 plus 0.4 M urea. The g = 5 region exhibits a temperature dependence very similar to that of native Av2, suggesting a value of 2D close to -3.7 cm^{-1}. Clearly, Av2' also exhibits heterogeneity in terms of the ground state spin of the reduced [4Fe-4S]^{1+} center. However, the results indicate that native Av2' contains a greater proportion of clusters with S = 3/2 ground states than native Av2.

3.3.2.2 Native Av2' + Glassing Solvents

EPR Studies: The presence of glassing solvents, such as 50% (v/v) ethylene glycol or 40% (v/v) glycerol, in native Av2' in 50 mM Tris/HCl, pH 7.6, increases the intensity of the rhombic g = 1.94 signal (see spectra B in Figs. 3-20 and 3-21) g-values of 2.06, 1.94, and 1.87, slightly different to the ones observed for native Av2 in the presence of ethylene glycol. The intensity of this S = 1/2 signal is significantly greater than the one observed in the absence of glassing solvents, and it integrates to 0.40 ±0.05 spins/4Fe for the sample with ethylene glycol, and 0.30 ±0.05 spins/4Fe for the sample with glycerol (see Table 3-2). These two values are comparable within experimental error, indicating a shift in the ground state of the cluster to the S = 1/2 form, although not to the same extent as in native Av2 in the presence of ethylene glycol. Also, there is no indication in the
Figure 3-20  S = 1/2 EPR spectra of native Av2' in the presence and absence of urea and glycerol. (A) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, and 2 mM dithionite. (B) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 40% (v/v) glycerol, and 2 mM dithionite. (C) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, and 2 mM dithionite. (D) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, 40% (v/v) glycerol, and 2 mM dithionite. Conditions: microwave power, 5 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 19 K. Multiplication factors indicate relative gains.
Figure 3-21 S = 1/2 EPR spectra of native Av2' in the presence and absence of urea and ethylene glycol. (A) Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, and 2 mM dithionite. (B) Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 50% (v/v) ethylene glycol. (C) Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, and 2 mM dithionite. (D) Protein (0.35 mM) in 50 mM Tris/HCl, pH 7.6, with 0.4 M urea, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 5 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 19 K. Multiplication factors indicate relative gains.
EPR spectra of native Av2' with glassing solvents of a fast-relaxing $S = 1/2$ species such as that observed at low temperatures and high powers for native Av2 in the presence of ethylene glycol. Consequently, there is no observable heterogeneity in the $S = 1/2$ form of the cluster in native Av2' in the presence of glassing solvents.

The resonances observed in the $g = 5$ region (spectra B, Figs. 3-22 and 3-23) are more intense than those observed in native Av2 with ethylene glycol which is in accord with quantitations of the $g = 1.94$ signal. The observed $g$-values for this region are $g = 5.84$ and 5.15 for the sample with glycerol, and $g = 5.87$ and 5.10 for the sample with ethylene glycol, which are similar to the values observed for native Av2' in the absence of glassing solvents (see spectra A on Figs. 3-22 and 3-23), suggesting similar ground state properties of the $S = 3/2$ species.

In light of the $S = 1/2$ spin quantitations, we can conclude that the $[4\text{Fe}-4\text{S}]^{1+}$ centers in native Av2' in the presence of glassing solvents have approximately 35% $S = 1/2$ and 65% $S = 3/2$ ground states, suggesting that the $S = 3/2$ form is the major species in the presence of glycerol and ethylene glycol, and differing from native Av2 whose dominant species is the $S = 1/2$ form, under analogous conditions.

**UV-Visible Absorption and MCD Studies:** The room-temperature UV-visible absorption spectrum (upper panel, Fig. 3-24 and 3-25) for native Av2' with either 50% (v/v) ethylene glycol or 40% (v/v) glycerol is broad and featureless, and indistinguishable to that observed for native Av2 with ethylene glycol. MCD spectra (lower panel, Figs. 3-24 and 3-25) at 4.5 T and at temperatures in the range 1.6 to 100 K, comprise temperature-dependent bands throughout the visible region and are indicative of a paramagnetic Fe-S center. The MCD spectra
Figure 3-22  $S = 3/2$ EPR spectra of native Av2' in the presence and absence of urea and glycerol. (A) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, and 2 mM dithionite. (B) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 40% (v/v) glycerol, and 2 mM dithionite. (C) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, and 2 mM dithionite. (D) Protein (0.25 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, 40% (v/v) glycerol, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 4.5 K. All spectra were recorded at the same spectrometer gains.
Figure 3-23 $S = \frac{3}{2}$ EPR spectra of native Av2' in the presence and absence of urea and ethylene glycol. (A) Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, and 2 mM dithionite. (B) Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 50% (v/v) ethylene glycol. (C) Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, and 2 mM dithionite. (D) Protein (0.35 mM) in 50 mM Tris/HCl, pH 7.6, with 0.4 M urea, 50% (v/v) ethylene glycol, and 2 mM dithionite. Conditions: microwave power, 20 mW; modulation amplitude, 0.63 mT; microwave frequency, 9.42 GHz; temperature, 4.5 K. All spectra were recorded at the same spectrometer gains.
Figure 3-24 Room-temperature UV-visible absorption and low-temperature MCD spectra of native Av2' in the presence of ethylene glycol. Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 50% (v/v) ethylene glycol, and 2 mM dithionite. Condition for UV-visible absorption spectrum: pathlength, 0.10 cm. Conditions for MCD spectra: pathlength, 0.17 cm; magnetic field and temperatures as indicated (intensity of transitions increasing with decreasing temperatures).
Figure 3-25 Room-temperature UV-visible absorption and low-temperature MCD spectra of native Av2' in the presence of glycerol. Protein (0.35 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 40% (v/v) glycerol, and 2 mM dithionite. Condition for UV-visible absorption spectrum: pathlength 0.10 cm. Conditions for MCD spectra: pathlength, 0.10 cm; magnetic field and temperatures as indicated (intensity of transitions increasing with decreasing temperature).
consist of broad, positively signed bands throughout the wavelength range 350-800 nm, with dominant bands centered at 730, 534, 440, and 360 nm (the 410 nm band in Fig. 3-24 is due to a trace of heme impurity in the sample). The form and intensity of these spectra resemble those of native Av2 in the presence of urea, which is in accord with the presence of [4Fe-4S]$^{1+}$ clusters with predominantly S = 3/2 ground states.

To study further the ground-state properties of the [4Fe-4S]$^{1+}$ in native Av2', magnetization data were collected at 730, 534, and 360 nm, at temperatures between 1.65 and 10.0 K for the sample with ethylene glycol, and at 730, 524, and 364 nm in the temperature-range of 1.65 to 10.5 K for the sample with glycerol, and magnetic fields between 0 and 4.5 T. The data at these wavelengths behave similarly and only the plots at 534 nm (Fig. 3-26) and 524 nm (Fig. 3-27) are presented. After correction for diamagnetic contributions, the 534 nm and 524 nm magnetization data collected at different temperatures all lie on separate curves. Moreover, the lowest temperature data magnetizes much more rapidly than would be expected for an isolated S = 1/2 ground state. Such behavior is only interpretable in terms of the presence of an Fe-S center with an S > 1/2 ground state that is subject to zero-field splitting.

The simulation of magnetization curves based on EPR-determined g-values suggests that at all wavelengths there is a contribution from an S = 3/2 paramagnet in addition to that of the S = 1/2 Fe-S center. The lowest temperature plots at 534 nm and 524 nm are well fit by simulations using a mixture of 55% S = 1/2 and 45% S = 3/2 ground states, and 60% S = 3/2 and 40% S = 1/2 ground states, respectively. Thus, the MCD magnetization and EPR data concur in finding a much greater S = 3/2 to S = 1/2 ratio of [4Fe-4S]$^{1+}$ ground states for Av2' as compared to Av2 in the presence of glassing solvents.
Figure 3-26  MCD magnetization plots for native Av2' in the presence of ethylene glycol. Protein sample as in Fig. 3-21. Conditions: temperatures and wavelength as indicated; magnetic fields between 0 and 4.5 T.
**Figure 3-27** MCD magnetization plots for native Av2' in the presence of glycerol. Protein sample as in Fig. 3-25. Conditions: temperatures and wavelength as indicated; magnetic fields between 0 and 4.5 T.
3.3.2.3  Native Av2' + Urea

**EPR Studies:** Upon addition of 0.4 M urea to native Av2' in 50 mM Tris/HCl buffer, pH 7.6, there is an increase of the intensity of the \( g = 5 \) signal (see spectra C, Figs. 3-22 and 3-23) and a decrease in the \( g = 1.94 \) signal (see spectra C, Figs. 3-20 and 3-21). The weak \( g = 1.94 \) signal has essentially the same shape and \( g \)-values as native Av2'. EPR quantitations of this signal rendered a spin concentration of 0.06 ±0.02 spins/4Fe (see Table 3-2), compared to 0.15 ±0.05 spins/4Fe for native Av2' and 0.12 ±0.05 spins/4Fe for native Av2 in the presence of 0.4 M urea.

In the presence of urea, the form of the \( g = 5 \) signal is slightly perturbed, with positively signed features centered at \( g = 5.85 \) and 5.18 (spectra C, Figs. 3-22 and 3-23). The temperature dependence of this signal indicates that these \( g \)-values correspond to the low field features of the lower and upper doublets, respectively, and the magnitude of D is close to the value of -1.8 cm\(^{-1}\) obtained for native Av2 plus urea.

As shown in spectra D, Figs. 3-20 and 3-21, the presence of either 40\% (v/v) glycerol or 50\% (v/v) ethylene glycol in Av2' plus urea slightly increases the intensity of the \( S = 1/2 \) signal, giving spin quantitation of 0.10 ±0.02 spins/4Fe (see Table 3-2), but without affecting the \( g \)-values. Therefore, as for native Av2 plus urea, the presence of glassing solvents in native Av2' with urea slightly perturbs the mixture of spin states in favor of the \( S = 1/2 \) form. However, the resulting samples still contain \( \approx 90 \% \) of the clusters in the \( S = 3/2 \) form and hence present the best opportunity for obtaining the MCD characteristics of this type of center without significant contributions from the \( S = 1/2 \) form. As for Av2, the form of the \( g = 5 \) signal is somewhat changed in the presence of urea and glassing solvents (spectra D, Figs. 3-22 and 3-23), giving low field resonances at \( g = 5.92 \) and 4.90, similar to those observed in native Av2 in the presence of...
urea and ethylene glycol. Following the same analysis made for Av2, the best fit to the observed g-values occurs with \( E/D = 0.16 \) and \( g_0 = 2.02 \), when theory predicts \( g_x = 3.04, g_y = 4.90, \) and \( g_z = 1.88 \) for one doublet and \( g_x = 1.00, g_y = 0.86, \) and \( g_z = 5.92 \) for the other. From the temperature dependence (not shown) of the low-field signal, it can be concluded that the resonances at \( g = 5.92 \) and 4.90 arise from the lower and upper doublets, respectively, and they are consistent with a negative D.

**UV-Visible Absorption and MCD Studies:** The room-temperature UV-visible absorption spectra for native Av2' in the present of urea and glassing solvents are shown on the upper panel of Figs. 3-28 and 3-30. Low-temperature MCD spectra for these samples at 4.5 T and temperatures in the range 1.6 to 100 K are shown in the lower panels of Figs. 3-28 and 3-30. The spectra exhibit broader bands than the samples in the absence of urea, with positive bands centered at 730, 528, and 360 nm. These features are now seem to be the characteristics of a [4Fe-4S]\(^{1+}\) cluster with an \( S = 3/2 \) ground state. The spectra in the presence of ethylene glycol and glycerol show some minor differences in the 600-800 nm region but are generally in excellent agreement.

MCD magnetization plots recorded at 730, 528, and 360 nm for the sample with ethylene glycol, and 725, 520, and 364 nm for the sample with glycerol, show similar behavior, and only the plots at 528 and 520 nm are shown in Figs. 3-29 and 3-31. In light of the EPR data, the magnetization characteristics shown in Figs. 3-29 and 3-31 can be attributed, almost exclusively, to \( S = 3/2 \) [4Fe-4S]\(^{1+}\) centers. The lowest temperature data can be fit using the approximate effective g-values for the lowest doublet \( g_{\parallel} = 5.90 \) and \( g_{\perp} = 0.93 \) with the polarization ratio \( m_x/m_{xy} = -0.8 \). This theoretical magnetization data has been used above in assessing the relative contributions from \( S = 3/2 \) and \( S = 1/2 \) species contributing
Figure 3-28  Room-temperature UV-visible absorption and low-temperature MCD spectra of native Av2' in the presence of urea and ethylene glycol. Protein (0.24 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, 50% (v/v) ethylene glycol, and 2 mM dithionite. Condition for UV-visible absorption spectrum: pathlength 0.10 cm. Conditions for MCD spectra: pathlength, 0.17 cm; magnetic field and temperatures as indicated (intensity of transitions increasing with decreasing temperature).
Figure 3-29  MCD magnetization plots for native Av2' in the presence of urea and ethylene glycol. Protein sample as in Fig. 3-23. Conditions: temperatures and wavelength as indicated; magnetic fields between 0 and 4.5 T.
Figure 3-30  Room-temperature UV-visible absorption and low-temperature MCD spectra of native Av2' in the presence of urea and glycerol. Protein (0.20 mM) in 50 mM Tris/HCl buffer, pH 7.6, with 0.4 M urea, 40% (v/v) glycerol, and 2 mM dithionite. Condition for UV-visible absorption spectrum: pathlength 0.10 cm. Conditions for MCD spectra: pathlength, 0.17 cm; magnetic field and temperatures as indicated (intensity of transitions increasing with decreasing temperature).
Figure 3-31  MCD magnetization plots for native Av2' in the presence of urea and glycerol. Protein sample as in Fig. 3-27. Conditions: temperatures and wavelength as indicated; magnetic fields between 0 and 4.5 T.
to the MCD intensity at this wavelength.

3.4 DISCUSSION:

Our investigations are in good agreement with reported results by Lindahl et al. (50), Watt and McDonald (51), Hagen et al. (52), and Morgan et al. (65), in finding that the reduced [4Fe-4S] cluster of Av2 can exist in two forms: one with $S = 1/2$ ground state that gives rise to the rhombic $g = 1.94$ signal, and the other with an $S = 3/2$ ground state that yields low-field EPR resonances at around $g = 5$. Our studies also confirm that these two cluster forms interconvert when certain agents are added to the native protein. For instance, glassing solvents, such as ethylene glycol, shift the equilibrium to the $S = 1/2$ form (~ 90%) whereas denaturing agents, such as urea, shift the equilibrium to the $S = 3/2$ form (> 80%).

While the studies that appear in the literature during the course of this work have focused on EPR, Mössbauer, and magnetic susceptibility measurements, the data presented above constitutes the first time that low-temperature MCD has been used to study the properties of the Fe-S cluster in Av2. The MCD results confirm the assignment of cluster-type and provide the first insight into the differences in the electronic properties of $S = 1/2$ and $S = 3/2$ [4Fe-4S]$^{1+}$ clusters. The MCD and EPR results presented herein concur within experimental uncertainties. Native Av2 in the presence of ethylene glycol shows EPR characteristics consistent with 85% of molecules having an $S = 1/2$ [4Fe-4S]$^{1+}$ cluster. The MCD spectra are characterized by temperature-dependent positive bands centered at 720, 534, 440, and 354 nm, and negative bands at 580 and 660 nm. The magnetization data shows that the cluster magnetizes as a system with mainly $S = 1/2$ ground state. On the other hand, the presence of urea and ethylene glycol gives rise to a weaker $g = 1.94$ signal, which quantitates to only
45% of the [4Fe-4S]^{1+} clusters having an S = 1/2 ground state. The MCD spectra show changes that are indicative of a larger S = 3/2 to S = 1/2 ratio and the magnetization curves are nested, indicating substantial contribution from the S = 3/2 form.

The observed S = 3/2 EPR resonances in native Av2 (D ~ -2.1 cm^{-1} and E/D = 0.13) are similar to those observed for native Av2 in the presence of urea, except for slight differences in their g-values. The values of the zero-field splitting parameters for these S = 3/2 species agree within experimental error (D ~ -2.1 cm^{-1} and E/D = 0.13 and D ~ -1.8 cm^{-1} and E/D = 0.22 for native and native plus urea, respectively). This indicates significant population of the upper doublet at temperatures > 4.2 K. Lindahl et al. (50) reported an estimate of D for the S = 3/2 for Av2 plus urea (-1.5 to -3 cm^{-1}) which is in excellent agreement with the values observed in this work.

A new observation that has arisen from this work is that the g = 1.94 signal of Av2 is composed of two different species in the presence of ethylene glycol. These two species have different g-value anisotropy and can readily be distinguished by their different relaxation properties. Consequently, in addition to the heterogeneity of the [4Fe-4S]^{1+} cluster in terms of ground state spin, S = 1/2 or S = 3/2, there appears to be heterogeneity in the S = 1/2 cluster in the presence of ethylene glycol. Watt and McDonald (51) have reported distortion in the g = 1.94 signal of native Av2 at temperatures < 12 K and low powers, which they attributed to dispersion. We were unable to confirm this observation. Moreover, it should be noted that the effects they reported are completely different to those reported herein for Av2 in the presence of ethylene glycol. Our results for this sample cannot be attributed to dispersion effects.

One of the objectives of the MCD studies was to investigate changes in the electronic properties of the cluster on binding Mg-ATP. Unfortunately, this has
not proven possible. While EPR studies do show that Mg\textsuperscript{2+} binding perturbs the reduced [4Fe-4S]\textsuperscript{1+} cluster, they also show that these changes are reversed on addition of glassing agents such as ethylene glycol. Consequently, it is not surprising that MCD studies, which by necessity are performed in the presence of the glassing agent, appear to be unaffected by the presence of Mg\textsuperscript{2+}ATP. This result suggests that the presence of ethylene glycol interferes with Mg\textsuperscript{2+}ATP binding, which may explain the reversible loss of activity of nitrogenase in an assay mixture containing 50% (v/v) ethylene glycol.

The spectroscopic data obtained for the Fe protein from the conventional nitrogenase, Av2, during the course of this work, have been fundamental to enhancing our understanding of the electronic and magnetic properties of the Fe protein from the alternative nitrogenase, Av2'. The EPR results reported herein agree closely with preliminary data reported by Hales et al. (75) for native Av2'. Av2' is clearly very similar to Av2 in terms of the Fe-S cluster. The S = 1/2 / S = 3/2 spin state heterogeneity is apparent for both Fe proteins. However the proportions of the S = 3/2 form are invariably greater for Av2' than Av2 under comparable conditions. For instance, Av2' in the presence of glycerol or ethylene glycol has 40% of the clusters in the S = 1/2 form, compared to 85% for Av2; Av2' in the presence of urea and glassing solvent has only 10% of the clusters in the S = 1/2 form, compared to 45% for Av2.

These results indicate that the low-temperature MCD spectra for native Av2' with urea are dominated by transitions from [4Fe-4S]\textsuperscript{1+} clusters having a S = 3/2 ground state. These spectra are the first to be reported for a S = 3/2 [4Fe-4S]\textsuperscript{1+} cluster. They are quite distinct from those exhibited by S = 1/2 [4Fe-4S]\textsuperscript{1+} clusters and are characterized by broad-positive bands centered at 730, 528, and 360 nm. The magnetization data are characteristic of an S = 3/2 ground state. These results establish low-temperature MCD as an extremely useful method for
detecting or distinguishing $S = 1/2$ and $S = 3/2$ [4Fe-4S]$^{1+}$ centers in metalloproteins. This will be particularly useful for $S = 3/2$ centers which can exhibit EPR signals that are very difficult to detect as a result of broad resonances due to minor heterogeneities (e.g., see results for *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase, chapter 4).

Several examples of biological [4Fe-4S]$^{1+}$ centers with $S > 1/2$ ground states have recently emerged: the [4Fe-4Se]$^{1+}$ of Se-reconstituted *Clostridium pasteurianum* ferredoxin can have ground states with $S \geq 3/2$ (84,85); the P-clusters in the MoFe- and VFe-protein of nitrogenase which are most likely centers with $S = 5/2$ or $7/2$ (86-90); the [4Fe-4S]$^{1+}$ clusters in reduced Cp hydrogenase I that have a mixture of $S = 1/2$ and $S = 3/2$ ground states (93); the [4Fe-4S]$^{1+}$ centers in dithionite-reduced *Pyrococcus furiosus* ferredoxin (92); and the $S = 3/2$ [4Fe-4S]$^{1+}$ centers in *Bacillus subtilis* glutamine phosphoribosylpyrophosphate amidotransferase (see chapter 4 of this thesis). In addition, the presence of $S = 3/2$ [4Fe-4S]$^{1+}$ has also been observed in synthetic analog complexes containing a [Fe$_4$S$_4$(SR)$_4$]$^{3-}$ core, and a very detailed analysis of the characteristics of these synthetic clusters was recently published by Carney et al. (56). They examined the electronic properties of these clusters by Mössbauer, EPR, magnetic susceptibility, and X-ray crystallography, and they found that there are three categories of ground spin state behavior: pure spin $S = 1/2$ or $3/2$ clusters; physical mixtures of $S = 1/2$ and $3/2$ clusters; and spin admixed ($S = 1/2 + S = 3/2$ states) ground states (86). From this classification, we can conclude that the clusters in native Av2 and Av2' correspond to the second category. From this discussion one can conclude that the presence of $S > 1/2$ [4Fe-4S]$^{1+}$ centers, particularly $S = 3/2$, in biological systems is no longer a rare occurrence. Rather, it has been overlooked in the past due to lack of sensitive spectroscopic probes.
The origin of the $S = 1/2$ and $S = 3/2$ spin mixture in Av2 and Av2' is still uncertain. Lindahl et al. (30) showed that there was no obvious correlation between cluster spin state and activity, but this conclusion may be invalid if the $S = 3/2$ form is not present at room temperature. In this regard, it is noteworthy that the room-temperature magnetic susceptibility and NMR studies conducted recently by Meyer et al. (59) suggest it may be a freezing artifact. However, we do not regard this as definitive evidence in the absence of assignment and quantitation of the contact shifted proton resonances. These authors also report that the spin state of Cp2 in the presence of urea shifts to the $S = 1/2$ form upon warming the frozen sample to 230 K without thawing and then bringing it back to liquid $N_2$ temperatures, which if verified is convincing evidence that the spin mixture is a freezing artifact. We performed similar experiments for both Av2 and Av2' samples, and found no significant differences in the $S = 1/2$ EPR quantitation (data not shown). Therefore, we conclude that the question of whether or not the heterogeneity of spin states exists under physiological conditions is still unresolved.

The structural basis for the spin state change is also still unknown. The fact that the Fe protein is thus far unique among Fe-S proteins in having $[4\text{Fe}-4\text{S}]^{1+}$ centers with easily interconvertible spin states may originate from the cluster linking the two protein subunits. However, X-ray crystallographic studies of $[\text{Fe}_4\text{S}_4(\text{SR})_4]^{3-}$ clusters with different ground state spin do not indicate a unique structural perturbation that can be associated with the spin state change. Furthermore, EXAFS studies could not detect significant differences between the $S = 3/2$ and $S = 1/2$ forms of the cluster in Av2 (53).

The vibrational spectrum should be most sensitive to small structural changes and this was the driving force for the RR studies reported herein. Unfortunately, we could not observe any Fe-S RR bands in the spectrum of native
Av2. This is due in part to the inherent weakness of the resonance enhancement of Fe-S stretching modes of [4Fe-4S]$^{1+}$ clusters using excitation in the visible region (83) and a large background fluorescence. We have been able to observe and resolve for the first time weak Raman bands from oxidized Av2, albeit superimposed on large sloping, fluorescence background. The RR spectrum is very similar to those of [4Fe-4S]$^{2+}$ clusters in simple ferredoxins and does not suggest any major difference in structure for this center in the Fe protein. The RR results also indicate that the oxidized [4Fe-4S]$^{1+}$ cluster is susceptible to degradation under oxidizing conditions to yield a [2Fe-2S]$^{2+}$ center. This novel cluster conversion has been reported before for the nitrogenase Fe protein but only in the presence of Mg-ATP and an Fe-chelator (39). This type of oxidative cluster conversion, as opposed to the now well-established [4Fe-4S]$^{2+}$ to [3Fe-4S]$^{1+}$ conversion (93), may well be a consequence of a [4Fe-4S]$^{2+}$ cluster that bridges two subunits. Clearly, RR experiments offer an excellent method of monitoring this type of conversion between diamagnetic clusters and more experiments are planned on this aspect. This type of cluster conversion may not be unique to the nitrogenase Fe protein. Recent RR studies of Clostridium pasteurianum bidirectional hydrogenase found evidence for [2Fe-2S] centers that do not appear to be present in the reduced, as isolated on thionine-oxidation (94). While these authors attributed this cluster to the novel hydrogen-activating center, the work presented herein suggests it could arise from thionine-induced degradation of one of the constituent ferredoxin-type [4Fe-4S]$^{1+}$ clusters.

In summary, parallel MCD, EPR, UV-visible absorption, and RR studies have proven to be fundamental in the investigation of the structural, electronic, and magnetic properties of Av2 and Av2'. The results are of particular importance to the development of low-temperature MCD as a useful probe for the electronic ground state and excited state properties of [4Fe-4S]$^{1+}$ centers in biology.
3.5 REFERENCES


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4.  

**BACILLUS SUBTILIS GLUTAMINE PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE**

4.1 INTRODUCTION

Glutamine amidotransferases are a family of enzymes that utilize the amide NH$_2$ of glutamine as the immediate source of nitrogen atoms in the biosynthesis of amino acids (e.g., tryptophan, histidine, asparagine, glutamic acid, and arginine), purine and pyrimidine nucleotides, coenzymes, and amino sugars (1,2). It is highly likely that these amination reactions initially occurred in evolution with ammonia rather than glutamine as the direct reactant. Indeed, these enzymes all have dual substrate specificity and NH$_3$ can replace glutamine when assayed *in vitro* (3,4). There are now 13 enzymes known that catalyze the utilization of the amide nitrogen atom of glutamine (5). Each of the purified and characterized glutamine amidotransferases, has an active site cysteine specifically required for glutamine-dependent activity (3,4). A mechanism employing covalent glutamyl and glutaminyl intermediates has been proposed to explain the role of the active-site cysteine (3,4).

Glutamine phosphoribosylpyrophosphate amidotransferase (also known as amidophosphoribosyltransferase, EC 2.4.2.14), hereafter called amidotransferase, catalyzes the reaction:

\[
\text{glutamine} + 5\text{-phosphoribosyl-1-pyrophosphate} \rightarrow 5\text{-phosphoribosyl-1-amine } + \text{glutamate} + \text{PP}_i
\]

which is the first reaction of purine nucleotide biosynthesis *de novo*. Similar to other glutamine amidotransferases, NH$_3$ can substitute for glutamine *in vitro* and *in vivo* (1,2,6), in which case the products are phosphoribosylamine and PP$_i$. Interest in this enzyme has focused on the mechanism for glutamine utilization (7,8) and on the role of the non-heme iron center in avian (9,10), human (11,12),
and *Bacillus subtilis* (13-15) amidotransferase. The function of non-heme iron in these enzymes is not understood but a direct role in catalysis or feedback inhibition is unlikely, since comparable amidotransferases from *Escherichia coli* and *Saccharomyces cerevisiae* do not contain iron or other metals (7,16,17).

In common with other glutamine amidotransferases (1), glutamine phosphoribosylpyrophosphate amidotransferase uses an active site cysteine in the mechanism of glutamine amide transfer (7). This active site cysteine has been identified as the NH$_2$-terminal residue in the mature amidotransferase isolated from *E. coli* (16), *B. subtilis* (18), and *S. cerevisiae* (6). While the *B. subtilis* enzyme is synthesized with an NH$_2$-terminal undecapeptide leader sequence which is processed to expose the active site cysteine residue at the NH$_2$-terminus (19,20), in the *E. coli* (19,20) (see Fig. 4-1) and *S. cerevisiae* (17) only the initiator methionine is removed to yield a mature enzyme having an NH$_2$-terminal active site cysteine. This active site cysteine is thought to function in catalysis by formation of a covalent glutaminyl intermediate (6,7,16). Therefore, the integrity of the cysteine residue is required for the glutamine-dependent activity. The NH$_3$-dependent activity is retained when the active site cysteine is affinity-labeled (7,18) or replaced (6). These results document the role of the active site cysteine in activation of glutamine by amidotransferase in various organisms.

Gene *purF* encoding amidotransferase has been cloned and sequenced from *E. coli* (21) and *B. subtilis* (20). An alignment of the derived amino acid sequences (Fig. 4-1) from these two bacteria indicates approximately 39% identity, demonstrating that these two sequences are homologous (20).

The present work will only be concerned with the amidotransferase from *B. subtilis*. Interest in this enzyme has focused on the role of the Fe-S cluster in a metalloenzyme that does not catalyze a redox process. Native *B. subtilis* amidotransferase, as isolated in aqueous buffer solution, exists in equilibrium
Figure 4-1: Alignment of the amino acid sequences of *E. coli* and *B. subtilis* amidotransferase. Identical amino acids are overlined or underlined. Cysteine residues that are involved in binding the [4Fe-4S] center are marked with dots. Taken from Ref. 20.
among monomeric, dimeric, and tetrameric forms of identical subunits with a $M_r \sim 50,000$ each (13). The distribution among these forms at low concentrations of the enzyme is markedly affected by allosteric inhibitors, e.g. AMP and GMP stabilize the dimeric form, and GDP stabilizes the tetrameric form (13). The enzyme contains equimolar non-heme iron and acid-labile sulfur in the range 3-4 atoms per subunit, depending on preparation (15). Fe-S core extrusion (15), and Mössbauer (15,22) studies, as well as the arrangement of cysteine residues in the amino acid sequence (20) indicate the presence of one diamagnetic $[4\text{Fe}-4\text{S}]^{2+}$ cluster per subunit, suggesting partial degradation of the cluster during enzyme isolation. Furthermore, site-directed mutagenesis experiments (19) have identified Cys-445, Cys-448, Cys-451, and Cys-393 as the likely ligands to the $[4\text{Fe}-4\text{S}]$ center. This binding site is identified in Fig. 4-1.

Although the role for the $[4\text{Fe}-4\text{S}]$ cluster in B. subtilis amidotransferase is not understood, it is known to be essential for catalytic activity without being directly involved with glutamine utilization (10,13,18,23), and recent evidence points to a regulatory role in which the cluster determines the stability of the enzyme to degradation in vivo, via oxygen-dependent inactivation (23,24).

Due to its low midpoint potential ($< -600$ mV), the native cluster is poorly reduced by dithionite (except at extremely alkaline pH values or under denaturing conditions), however, it can be reduced to the +1 state using photochemical reduction mediated by 5-deazaflavin with oxalate as the electron donor (22). Mössbauer and magnetic susceptibility studies (22) suggested that the reduced cluster is in an $S \geq 3/2$ spin state, but this assignment was uncertain because the reduction of samples was less than 100% and was accompanied by partial cluster degradation. Consequently, while the available evidence points to a novel $S > 1/2$ $[4\text{Fe}-4\text{S}]^{1+}$ cluster in B. subtilis amidotransferase, a detailed characterization of the magnetic and electronic properties of this cluster was not available at the
onset of these investigations.

4.2 OBJECTIVES

In this work, structural, electronic and magnetic properties of the oxidized \( n = 2 \) and reduced \( n = 1 \) forms of the \([4\text{Fe}-4\text{S}]^{n+}\) cluster in \( B. \ subtilis \) amidotransferase were investigated by means of low-temperature EPR, MCD, and resonance Raman (RR) spectroscopies. Used together, these techniques constitute sensitive and discriminating probes for the properties Fe-S centers in biological systems. The results facilitate comparison of this \([4\text{Fe}-4\text{S}]\) center with those involved in oxidation-reduction reactions in other enzymes and proteins. Of particular interest is the characterization of \( S \geq 3/2 \) spin states for the \([4\text{Fe}-4\text{S}]^{1+}\) cluster in the reduced enzyme using EPR and MCD spectroscopies.

4.3 RESULTS

4.3.1 Native Amidotransferase

In agreement with previous studies (22), the four samples of amidotransferase used in this work exhibited a weak EPR signal at \( g = 2.01 \) (data not shown) indicative of the presence of a \([3\text{Fe}-4\text{S}]^{1+}\) center with an \( S = 1/2 \) ground state. This EPR signal corresponded to < 0.02 spins/subunit for all samples, both in the presence and absence of 50% (v/v) ethylene glycol and hence represents only a minor species.

Low-temperature MCD studies show no temperature-dependent MCD bands in the region between 300 nm and 800 nm. These results are consistent with the presence of mainly diamagnetic Fe-S clusters with \( S = 0 \) ground state, as reported from Mössbauer studies (15). Since resonance Raman (RR) spectroscopy has proven to be a very effective and sensitive structural probe for this type of centers (25), RR spectra were recorded for native amidotransferase to facilitate
structural comparison with similar clusters in simple redox proteins. Fig. 4-2 compares the RR spectra of native amidotransferase from \textit{B. subtilis} and reduced HiPIP from \textit{Chromatium vinosum}, obtained using the 457.9 nm line of an Ar ion laser. Although the native amidotransferase spectrum is more noisy, due to high background fluorescence, the relative intensities and frequencies of the bands are very similar to those observed for \([4\text{Fe}-4\text{S}]^{2+}\) clusters in general, and reduced HiPIP in particular (25). The observed stretching vibrations, for amidotransferase, at 250, 281, 333, 357, 385, and 397 cm\(^{-1}\) correlate very well with the assignments reported by Czernuszewicz \textit{et al.} (25) for the \([4\text{Fe}-4\text{S}]^{2+}\) clusters in \textit{Clostridium pasteurianum} ferredoxin, \textit{C. vinosum} HiPIP, and the cluster \((\text{Et}_4\text{N})_2\text{Fe}_4\text{S}_4(\text{SCH}_2\text{Ph})_4\). As for thionine-oxidized Av2 (cf. Fig. 3-17), it can be concluded that the bands at 250, 333, and 397 cm\(^{-1}\) can be assigned predominantly to bridging Fe-S vibrations, and the 357 cm\(^{-1}\) band to terminal (cysteinyl) Fe-S vibrations. In summary, the RR studies show that the structure of the Fe-S cluster in native amidotransferase is very similar to that of biological \([4\text{Fe}-4\text{S}]^{2+}\) clusters with conventional redox roles.

4.2.2 Photochemically Reduced Amidotransferase

The magnetic and electronic properties of the \([4\text{Fe}-4\text{S}]^{1+}\) in photochemically reduced amidotransferase have been investigated by parallel UV-visible absorption, EPR and MCD studies. Fig. 4-3 shows the changes in the room-temperature absorption spectrum that occur upon 5-deazaflavin-mediated photoreduction under rigorously anaerobic conditions. The absorption in the visible region progressively decreased with illumination time. Four samples were investigated and in each case the absorption at 410 nm decreased by 25\% to 30\% after 11 mins, and no change in the spectrum was observed on further illumination or incubation at room temperature for an additional 23 mins. Under these conditions, we estimate
Figure 4-2. Low-temperature resonance Raman spectrum of native *B. subtilis* amidotransferase and reduced *C. vinosum* HiPIP. Upper panel: reduced *C. vinosum* HiPIP. The protein concentration was ~ 6 mM, in 50 mM Tris/HCl buffer, pH 7.8. The spectrum is the sum of 6 scans and bands arising from the frozen buffer solution have been subtracted. Lower panel: native *B. subtilis* amidotransferase. The sample, ~ 2 mM in the 50 kDa subunit, was in 50 mM Tris/HCl buffer, pH 7.8. The spectrum is the sum of 43 scans and is corrected for a linear sloping baseline after subtraction of bands arising from the frozen buffer solution. Conditions of measurement: excitation wavelength, 457.9 nm; laser power at sample ~ 30 mW; spectral bandwidth, 6 cm⁻¹; photon counting for 1 sec every 0.2 cm⁻¹ for each scan; sample temperature, 16 K.
Figure 4-3  Photochemical reduction of *B. subtilis* amidotransferase monitored by room-temperature UV-visible absorption spectrometry. Enzyme (0.17 mM in the 50 kDa subunit) in 50 mM Tris/HCl, pH 7.8, with 50% (v/v) ethylene glycol, 10 μM deazaflavin, and 10 mM sodium oxalate. The spectra were recorded after photoreduction for 0, 3, 11, and 34 mins. The extinction coefficient at all wavelengths is decreasing with increasing time, except for the spectra after 11 and 34 mins which are superimposable. Condition: pathlength, 0.10 cm.
that approximately 50% to 75% of the clusters are reduced; this is based on the magnitude of analogous absorption changes that are observed upon one-electron reduction of [4Fe-4S]$^{2+}$ clusters in bacterial ferredoxins.

The low-temperature MCD spectra for photochemically reduced amidotransferase recorded at temperatures between 1.58 K and 75 K with a magnetic field of 4.5 T are shown in Fig. 4-4. Almost identical spectra were obtained for all four samples investigated. The MCD spectra show temperature-dependent bands throughout the visible region that are indicative of a paramagnetic Fe-S center. These spectra consist of positive bands with maxima at 740, 520, 430, and 360 nm. The overall form of the spectrum is characteristic of a [4Fe-4S]$^{1+}$ cluster. Moreover, with the exception of the band at 430 nm which is more pronounced in this case, the wavelength, sign, and relative intensity of the major MCD bands are very similar to those observed for the $S = 3/2$ [4Fe-4S]$^{1+}$ centers in Av2 and Av2' (cf. Figs. 3-13, 3-23, and 3-24).

Further support for the suggestion that $S = 3/2$ [4Fe-4S]$^{1+}$ clusters are the dominant paramagnetic chromophore in photochemically reduced amidotransferase comes from MCD magnetization studies conducted at 520 nm, at temperatures between 1.68 K and 15.0 K, and magnetic fields between 0 and 4.5 T (see Fig. 4-5). Previous studies have shown that MCD magnetization data for [4Fe-4S]$^{1+}$ clusters with $S = 1/2$ ground states are well fit at all temperatures by theoretical curves constructed for an isolated doublet ground state with g-values close to 2 (26,27). Such behavior is shown by the dashed line in Fig. 4-5. Clearly the experimental data for photochemically reduced amidotransferase has a much steeper initial slope and this observation can only be rationalized in terms of a ground state with $S \geq 3/2$. Moreover, the experimental data at the lowest temperatures investigated (1.68 K and 4.22 K) are well fit by theoretical data constructed for an xy-polarized transition from a doublet with effective g-values,
Reduced B. s. Glutamine PRPP Amidotransferase

Figure 4-4 Low-temperature MCD spectra of reduced B. subtilis amidotransferase. The enzyme corresponds to that described in Fig. 4-3, after 34 mins of photolysis. Conditions of measurement: pathlength, 0.32 cm; magnetic field, 4.5 T; temperatures as indicated (intensity of transitions increasing with decreasing temperature).
MCD Magnetization Plot (520 nm)
Reduced B. s. Amidotransferase

Circle - 1.68 K
Square - 4.22 K
Triangle - 15.0 K

Solid line: theoretical curve for $S = 3/2$
Dashed line: theoretical curve for $S = 1/2$

Beta B/2kT

Figure 4-5 MCD magnetization plot for photochemically reduced B. subtilis amidotransferase. The enzyme sample is the same as that used for Fig. 4-4. Conditions of measurement: temperatures and wavelengths as indicated; magnetic fields between 0 and 4.5 T. The solid line is theoretical magnetization data for an xy-polarized transition from a ground state doublet with $g_\perp = 4$ and $g_\parallel = 2$, and the dashed line is theoretical magnetization for an isotropic doublet ground state with $g = 2$. 
$g_\perp = 4$ and $g_\parallel = 2$. These are the g-values expected for the $M_s = \pm 1/2$ doublet of an $S = 3/2$ ground state with purely axial zero-field splitting. The MCD magnetization data is, therefore, consistent with an $S = 3/2$ ground state with predominantly axial and positive zero-field splitting ($D > 0$, $E \sim 0$, where $D$ and $E$ are the axial and rhombic zero-field splitting parameters, respectively), leaving the $M_s = \pm 1/2$ doublet lying lowest in energy. The close coincidence of the data collected at 1.68 K and 4.22 K suggests little or no population of the upper $M_s = \pm 1/2$ doublet at 4.22 K, which translates to $D > 3 \text{ cm}^{-1}$.

Variable-temperature EPR studies have confirmed the results from MCD investigations. Spectra were recorded for identical samples and samples of comparable concentration without ethylene glycol that were photochemically reduced in serum-capped EPR tubes (see Fig. 4-6). EPR spectra were recorded at temperatures between 4 K and 70 K and they consist of a number of overlapping resonances with different relaxation properties. There are weak signals in the $g = 2$ region that can be attributed to two overlapping $S = 1/2$ species. One of them is a slower relaxing species with an axial resonance at $g_\perp = 1.95$ and $g_\parallel = 2.02$. This signal is still observed at 70 K without substantial broadening and, hence, has the properties usually associated with [2Fe-2S]$^{1+}$ clusters. The other feature is a more rapidly relaxing species with a rhombic resonance at $g = 2.11, 1.95$ and 1.80, that is only observed below 30 K and, therefore, is characteristic of a [4Fe-4S]$^{1+}$ cluster. At low temperatures ($< 10$ K) and high microwave powers ($> 20$ mW), this resonance becomes broader and less well resolved as a result of power saturation. The intensities of these $S = 1/2$ resonances varied considerably from sample to sample and, in some instances, they were not observed. Unfortunately, we have been unable to correlate their intensity with the presence of ethylene glycol or the sample concentration. However, in all the samples they appear to be minor components, since their spin quantitations under non-
Figure 4-6  Temperature-dependence EPR spectra of photochemically reduced *B. subtilis* amidotransferase at low concentration. The sample, 0.39 mM in the 50 kDa subunit, was in 50 mM Tris/HCl buffer, pH 7.8, with 10 μM deazaflavin, and 10 mM sodium oxalate. The sample was photoreduced for 45 mins prior to freezing in liquid N₂. Conditions of measurement: microwave power, 100 mW; microwave frequency, 9.42 GHz; modulation amplitude, 1 mT; modulation frequency, 100 kHz. All spectra were recorded using the same relative gain. Temperatures and g-values as indicated.
saturating conditions maximally accounted for 0.01 and 0.05 spins/subunit for the [2Fe-2S]$^{1+}$ and [4Fe-4S]$^{1+}$ signals, respectively.

In addition to the $S = 1/2$ resonances in the $g = 2$ region (Fig. 4-6), there is a very broad derivative-shaped feature centered at $g = 4$. While the width of the resonance suggests considerable heterogeneity in ground state properties, the $g$-value and temperature-dependence, under non-saturating conditions, indicate that it originates from the lower ($M_s = \pm 1/2$) doublet of an axial $S = 3/2$ ground state with $D > 0$ and $g_\perp = 4$ and $g_\parallel = 2$. Observation of the negative, absorption-shaped component at $g = 2$ is precluded by overlap with resonances in the $S = 1/2$ region. Thus, it is clear that this resonance arises from the $S = 3/2$ [4Fe-4S]$^{1+}$ cluster that is responsible for the temperature-dependent MCD intensity. Accurate spin quantitation of this resonance is complicated due to overlapping signals and the absence of a precise value of $D$. However, preliminary attempts for samples exhibiting very weak $S = 1/2$ signals indicate quantitations of approximately 0.4 spins/subunit for the $S = 3/2$ species. Since the UV-visible absorption studies indicate incomplete cluster reduction, the EPR and MCD studies, therefore, agree in finding [4Fe-4S]$^{1+}$ clusters with $S = 3/2$ ground states as the dominant paramagnetic species in photoreduced amidotransferase. In addition, these results indicate that the ground state properties of the $S = 3/2$ [4Fe-4S]$^{1+}$ clusters in reduced amidotransferase are somewhat different from those found in native Av2 and Av2' (see section 3.3 of this thesis). The $S = 3/2$ forms of the [4Fe-4S]$^{1+}$ clusters in the latter proteins have $D < 0$ and a substantial rhombic component to the zero-field splitting.

The EPR spectrum of photochemically reduced amidotransferase also shows a derivative-shaped resonance centered at $g = 4.3$ and a sharp absorption-shaped band at $g = 9.8$ that is only apparent at very low temperature and high microwave powers, see Fig. 4-6. These resonances were more apparent in highly
concentrated samples (2 mM in the 50 kDa subunit) where they are the dominant features in the low-field EPR spectra, see Figs. 4-7 and 4-8. Such signals are characteristic of a rhombic $S = 5/2$ species and are frequently observed for Fe-containing metalloproteins where they are usually attributable to adventitiously bound high spin Fe(III) ion. In light of the ease of oxidative degradation of the reduced cluster in amidotransferase (22), such an assignment is possible in this case. However, two pieces of evidence argue against this line of reasoning. First, the $g = 4.3$ feature is not isotropic as is the case for the weak adventitious Fe(III) signal in the native enzyme or the air-reoxidized reduced enzyme. Rather it has well-resolved features to high and low field and has $g = 4.42, 4.27,$ and $4.18$ at the maximum, cross-over, and minimum, see Figs. 4-6, 4-7, and 4-8. The observed $g$-values are readily interpreted in terms of the conventional $S = 5/2$ spin Hamiltonian, assuming an isotropic Zeeman interaction. For example, for $D < 0$ and $E/D = 0.31$ the lowest doublet would exhibit a low field resonance at $g = 9.73,$ and the middle doublet would have $g = 4.15, 4.27,$ and $4.42.$ These resonances are clearly seen in Fig. 4-7, which shows the temperature dependence of the $S = 5/2$ signal for the 2 mM sample at 20 mW. Second, the resonance centered around $g = 4.3$ in the photochemically reduced enzyme was invariably more intense than the isotropic $g = 4.3$ resonance that is attributed to adventitious Fe(III) in the native enzyme prior to reduction. This is illustrated in Fig. 4-8, which shows low-field EPR spectra for the 2 mM sample before and after photochemical reduction under identical conditions. Therefore, it seems likely that the $S = 5/2$ species corresponds to another high-multiplicity spin state of the $[4\text{Fe-4S}]^{1+}$ cluster, which becomes the dominant species at high concentrations. Changes in the mixture of spin states may well reflect changes in the aggregation state of the enzyme, since it is known to exist in equilibrium among monomeric, dimeric, and tetrameric forms (13). Unfortunately, it was not
Figure 4-7 Temperature-dependence EPR spectra of the low-field region of photochemically reduced *B. subtilis* amidotransferase at high concentration. The sample, ~ 2 mM in the 50 kDa subunit, was in 50 mM Tris/HCl buffer, pH 7.8, with 20 μM deazaflavin, and 20 mM sodium oxalate. The sample was photoreduced for 1 hour and 55 mins prior to freezing in liquid N₂. Conditions of measurement: microwave power, 20 mW; microwave frequency, 9.42 GHz; modulation amplitude, 0.63 mT; modulation frequency, 100 kHz. All spectra were recorded using the same relative gain, except when otherwise indicated. Temperatures and g-values as indicated.
Figure 4-8 Low-field EPR spectra of concentrated sample of *B. subtilis* amidotransferase before and after photochemical reduction. The enzyme sample is the same as that used for Fig. 4-7. Spectrum A is without photoreduction, and spectra B and C after photoreduction for 1 hour and 55 mins at 0°C. Conditions of measurement are as given in Fig. 4-7, except: microwave power, 1 mW (A and B) and 20 mW (C); temperature, 10 K (A and B) and 4 K (C). The spectrometer gain for spectra A and B was 4x that of spectrum C.
possible to carry out MCD experiments on such highly concentrated samples, since addition of glycerol or ethylene glycol resulted in enzyme precipitation.

4.4 DISCUSSION

In agreement with previous studies (22), the low-temperature EPR, MCD and resonance Raman studies presented above show that native \textit{B. subtilis} amidotransferase contains a diamagnetic ($S = 0$) $[4\text{Fe}-4\text{S}]^{2+}$ cluster. Since this cluster is diamagnetic, resonance Raman proved to be the most effective in investigating the structural properties of this center. In common with other biological $[4\text{Fe}-4\text{S}]^{2+}$ clusters, optimal resonant enhancement was obtained using the 457.9 nm line of an Ar ion laser, and the spectrum closely resembles that of the $[4\text{Fe}-4\text{S}]^{2+}$ cluster in reduced HiPIP from \textit{C. vinosum}. Although the spectrum for native amidotransferase can be assigned under idealized $T_d$ symmetry for the $[4\text{Fe}-4\text{S}]^{2+}$ cluster, the quality of the data is not sufficient to rule out a $D_{2d}$-distorted cube. X-ray crystallographic studies for \textit{Peptococcus aerogenes} ferredoxin (28) and \textit{C. vinosum} HiPIP (29) indicate $D_{2d}$-distortions for the $[4\text{Fe}-4\text{S}]^{2+}$ clusters, although this distortion is not clearly apparent in the frozen solution resonance Raman spectra of the latter protein (25). In summary, our RR studies show that the structure of the Fe-S center in native amidotransferase is very similar to those present in proteins with conventional redox roles.

From previous spectroscopic studies it could only be established that the clusters in reduced amidotransferase were $S \geq 3/2$ $[4\text{Fe}-4\text{S}]^{1+}$ clusters (22). Our low-temperature EPR and MCD studies show that the Fe-S centers in reduced amidotransferase are $[4\text{Fe}-4\text{S}]^{1+}$ clusters having a mixture of $S = 1/2$, $3/2$, and $S = 5/2$ ground states, with the $S = 3/2$ form dominating at low concentrations, and the $S = 5/2$ form dominating at very high concentrations. While further studies are required, it seems probable that these concentration-dependent spin state
changes may be due to different aggregation states of the enzyme. Amidotransferase is not unique in its ability to stabilize three different spin states of a $[4\text{Fe}-4\text{S}]^{1+}$ cluster. The same mixture of spin states has been proposed for nucleotide-bound Av2 (30) and a mixture of $S = 1/2$, $3/2$, and $7/2$ states has been observed for the two $[4\text{Fe}-4\text{Se}]^{1+}$ clusters in selenium-substituted clostridial ferredoxins (31). In contrast with other proteins with mixtures of $S = 3/2$ and $S = 1/2$ $[4\text{Fe}-4\text{S}]^{1+}$ centers, such as native Av2 and Av2' (see chapter 3 of this thesis), the ferredoxin from the extreme thermophile Pyrococcus furiosus (32), C. pasteurianum hydrogenase (33), and synthetic clusters of the type $[\text{Fe}_2\text{S}_4\text{(SR)}_4]^{3-}$ (34), the $S = 3/2$ $[4\text{Fe}-4\text{S}]^{1+}$ in reduced amidotransferase have $D > 0$ with purely axial symmetry. This fact makes the $S = 3/2$ $[4\text{Fe}-4\text{S}]^{1+}$ clusters in reduced amidotransferase unique among biological $[4\text{Fe}-4\text{S}]^{1+}$ clusters.

In addition, the finding of high-multiplicity spin states for the $[4\text{Fe}-4\text{S}]^{1+}$ cluster in photochemically reduced amidotransferase which, in the native form has the structurally unexceptional $[4\text{Fe}-4\text{S}]^{2+}$ cluster coordinated by a ferredoxin-type arrangement of cysteinyl residues, supports the conclusion that very subtle structural changes are likely to be associated with a change in ground state. This is in accord with EXAFS studies of reduced Av2 (35) and X-ray crystallographic studies of a wide range of synthetic clusters (34) that failed to find a distinctive structural feature of the Fe-S center that could be related with a change in ground spin state.

In summary, the combination of RR, MCD, EPR and UV-visible absorption spectroscopies have been very useful in the physiochemical characterization of the oxidized ($n = 2$) and reduced ($n = 1$) forms of the $[4\text{Fe}-4\text{S}]^{n+}$ cluster in B. subtilis amidotransferase. The results presented here make it clear that the possibility of $[4\text{Fe}-4\text{S}]^{1+}$ clusters with high-multiplicity spin states must always be explicitly considered in studies aimed at identifying the number and type of Fe-S
centers in reduced metalloproteins. Moreover, EPR signals from these $S \geq 3/2$
spin systems can be difficult to detect, particularly in the event of cluster
heterogeneity, making parallel low-temperature MCD and/or Mössbauer studies
essential for complete characterization.
4.5 REFERENCES


5. SYNTHETIC MODEL CLUSTERS FOR THE ACTIVE SITE OF NITROGENASE

For the scientists, biological nitrogen fixation is usually viewed in two ways. The first is described as a functional approach in which the concern is how the chemistry of the \( \text{N}_2 \) molecule (and the other alternative substrates) and that of any possible binding site on the enzyme nitrogenase might meld (1). The second approach is the structural alternative which involves the synthesis of Mo-Fe-S and V-Fe-S clusters in attempts to simulate the atomic composition, arrangement, reactivity, and spectroscopic properties of the FeMo- and FeV-cofactor (1).

Using the structural approach, the present work involves the spectroscopic investigation of some cubane-type \([\text{MoFe}_3\text{-S}_4]\) and \([\text{VFe}_3\text{-S}_4]\) synthetic complexes as possible models for the cofactor clusters in Mo- and V-nitrogenases using room-temperature UV-visible, and low-temperature EPR and MCD.

5.1 INTRODUCTION

This discussion will focus on the studies of the composition of FeMo- and FeV-cofactor (FeMo-co and FeV-co, respectively) of nitrogenase, a brief review on the development of synthetic model complexes for these two cofactors, and the properties of the synthetic complexes studied during the course of this work.

5.1.1 FeMo-cofactor

The molybdenum-iron (MoFe) protein of nitrogenase contains up to two Mo and about 30-32 Fe and acid-labile sulfur atoms which are grouped into two FeMo-co centers (also called M clusters), of stoichiometry \( \text{MoFe}_{6-8}\text{S}_{4-10} \), and about four \([4\text{Fe-4S}] \) P-clusters of unknown structure (2,3). FeMo-co was first obtained by Shah and Brill (4) in the form of an NMF extract of acid-denatured FeMo protein. The composition and properties of FeMo-co are very similar in the
extracted cofactor and in the native MoFe protein of the enzyme. The extracted FeMo-co samples from all species seem to be identical and all can activate the MoFe protein polypeptides in mutants of *Klebsiella pneumoniae* or *Azotobacter vinelandii* that are unable to synthesize FeMo-co (5-7).

FeMo-co is believed to be the site of N₂-binding in Mo-containing nitrogenase (8). Consequently, it has been thoroughly studied by a variety of biochemical and spectroscopic techniques (for a recent review, see ref. 3). These results, together with analytical data, indicate the extracted FeMo-co is anionic with the atomic composition discussed above. The means by which FeMo-co is coordinated to the MoFe protein is unknown. EPR (9-11) (which shows resonances with g-values of 4.3, 3.7, and 2.01 for the dithionite-reduced M center, and 4.8, 3.3, and 2.0 for the NMF-extracted and dithionite-reduced FeMo-co), ⁵⁷Fe Mössbauer (10-13), and ⁶⁷Fe (14,15) and ⁶⁵Mo (16) ENDOR spectroscopic studies have demonstrated that FeMo-co has an S = 3/2 ground state and that three unpaired electrons are shared by the Mo and at least 5 (probably 6) Fe atoms. Recent electrochemical studies (17) have indicated that isolated FeMo-co, as does its protein-bound form, undergoes two redox processes and, as a result, there are three forms of the center, the semireduced (s-r) redox state produced in the presence of excess dithionite, the more reduced form (red) and the oxidized form (ox). The oxidized to semireduced change in isolated FeMo-co is a reversible, one-electron transfer, which correlates with the appearance and loss of the biologically unique S = 3/2 EPR signal (17).

Most of the knowledge of the structure of FeMo-co has been deduced from X-ray absorption spectroscopic (XAS) studies, using X-ray absorption near edge structure (XANES), which includes the X-ray absorption edge and near-edge regions, and the extended X-ray absorption fine structure (EXAFS) region. Analysis of improved Mo K-edge EXAFS data on the MoFe protein (18,19), and on
the extracted FeMo-co (19), and of Mo K-edge XANES data (20) give a fairly consistent view of the immediate environment of the Mo atoms in the FeMo-co centers. These studies have shown the Mo in FeMo-co in the presence of excess dithionite to be coordinated by three O (N or C) atoms and three S (or Cl) atoms at about 2.12 and 2.37 Å, respectively, with about two to four Fe atoms neighbors at an average distance of about 2.7 Å. These data suggest a roughly octahedral ligand arrangement around the Mo atoms. The ligand environment of the Mo atom changes when FeMo-co is extracted into NMF with an apparent exchange of one S for and additional O ligand (19). In addition, the number of Fe-Mo interactions apparently decreases. However, thiophenol or selenophenol bind to FeMo-co, although not directly to the Mo, and restore the S and Fe interactions without removing the additional O ligand (19). Extracted FeMo-co seems to have a relatively mobile structure, perhaps existing in more than one conformation, but becomes more constrained when it is bound by ligands like thiophenol or to the MoFe protein polypeptides (19). In contrast to previous Fe K-edge EXAFS data (21), recent studies (22) have shown that most of the iron centers in FeMo-co are not coordinated to light (O or N) atoms, however, these two studies agree that FeMo-co contains rhombs of metal-sulphur atoms, as found in a wide variety of Fe-S and Fe-Mo-S clusters (23). Sulfur K- and Mo L-XANES studies (24) have recently indicated that three types of sulfur are associated with FeMo-co(ox), one is present as sulfide, and the other two represent thiolate-like and an oxidized sulfur species. In addition, the results show that Fe and Mo have no Cl ligands in FeMo-co(ox) and suggest an oxidation state for Mo of 4+. However, the Mo L-edges of both the oxidized and semireduced states of FeMo-co show that the oxidation state of molybdenenum is unchanged in this redox reaction (24). This last observation suggests that, if Mo has a role in N₂ binding and reduction, the production of the reducing (red) state probably involves electron
transfer to Mo aided by an energy-dependent conformational changes within the MoFe protein (24).

In summary, the FeMo-co cluster has some general characteristics: sulfur-rich Fe and Mo coordination sites, $S = 3/2$ ground state resulting from antiferromagnetic interactions of the Fe atoms, delocalized electronic structure of the Fe-S portion, three oxidation levels, and capable of binding one thiolate ligand in its isolated form. However, despite its importance and the application of a wide variety of spectroscopic techniques, the structure of FeMo-co remains one of the major unresolved problems in modern bioinorganic chemistry. Fig. 5-1 shows the recent proposals for the structure of FeMo-co (25). Two distinct types of models, the cubane (I) and the linear (II), were originally proposed to account for the EXAFS data (26,27), and subsequently other structures (III-VI) for the Mo environment have been proposed (28-31). Since crystals of FeMo-co suitable for X-ray crystallographic studies have yet to be obtained, the synthesis of model clusters for this center plays a very important role in its structure elucidation.

5.1.2 FeV-cofactor

Thus far VFe proteins have only been isolated to homogeneity from Azotobacter vinelandii (Avl') (32) and Azotobacter chroococcum (Acl') (33). While these preparations are in good agreement in terms of the V:Fe ratio, approximately 1:12, they differ in terms of the absolute metal contents. Avl' is reported to contain 0.7 V and 9.3 Fe atoms/molecule (32), whereas Acl' is reported to contain 2 V and 23 Fe atoms/molecule (33). However, the clusters seem to be arranged in a similar manner as in the MoFe protein, with P-like clusters (as suggested by MCD of thionine-oxidized Avl' (34)) and a FeV-cofactor (FeV-co) cluster.

EPR studies of dithionite-reduced Acl' (33) and Avl' (35), as well as MCD
Figure 5-1  Recent proposals for the structure of FeMo-co. Figure taken from ref. 25.
(34), indicate the presence of an $S = 3/2$ spin system in the FeV protein. This $S = 3/2$ spin system is the dominant species, and it has different zero-field splitting parameters than the one observed in both dithionite-reduced forms of Avl and Acl. In addition, the EPR spectra of Acl* and Avl* have a minor component due to an $S = 1/2$ spin system such as that found in reduced Fe-S clusters (33,35).

FeV-co has been extracted by NMF from acid-treated precipitated Acl* (36) and it contains V, Fe, and acid-labile S atoms in the approximate proportions 1:6:5. The EPR of the extracted FeV-co exhibits a weak signal with g-values of 4.5, 3.6, and 2.0, which are characteristic of an $S = 3/2$ FeMo-co center, and it is able to activate the MoFe proteins from mutants deficient in FeMo-co (36).

The only direct information on the environment of V in the VFe protein has come from X-ray spectroscopic studies, which indicate a similar coordination geometry to that of Mo in the MoFe protein. The V K-edge EXAFS studies of dithionite-reduced (37,38) and thionine-oxidized Acl* (38) indicated that V is surrounded by 3 O (or N), 3 S, and 3 Fe atoms at average distances of 2.15 Å, 2.31 Å, and 2.75 Å, respectively. These data are consistent with V being octahedrally coordinated in a similar environment as in the synthetic compound $(\text{Me}_4\text{N})[\text{VFe}_2\text{S}_4\text{Cl}_9(\text{DMF})_3].2\text{DMF}$. As for Mo in MoFe protein (24), the V K-edge studies show no appreciable differences in V oxidation state upon oxidation of the VFe protein (38). EXAFS studies of dithionite-reduced Avl* (39) show similar results on the V coordination as Acl*. They also found that the Avl* absorption edge and EXAFS are similar to those of $(\text{Me}_4\text{N})[\text{VFe}_2\text{S}_4\text{Cl}_9(\text{DMF})_3].2\text{DMF}$, and that V is surrounded by 3 Fe atoms at 2.76 Å, 2-3 O (or N) atoms at 2.33 Å, and 3-4 S (or Cl) at 2.15 Å. These distances and type of neighbors of V in VFe protein from Avl* and Acl* are similar to those obtained for Mo in MoFe proteins, suggesting that a FeV-cofactor, analogous to FeMo-co is present in V-nitrogenase.
5.1.3 Synthetic models for FeMo-co and FeV-co

Since the initial EXAFS reports on the MoFe protein (26,27), a wide variety of inorganic MoFeS clusters have been synthesized (23,40,41). These include the one with a cubane framework (MoFe$_5$S$_4$) and linear arrangement (MoS$_2$Fe$_2$) (23,40,42), the more recently reported triply-bridged MoS$_2$Fe$_2$ unit (43), the S$_2$Fe$_3$S$_2$Mo unit containing a discrete 3Fe core (44), and the doubly-capped hexamers with Mo$_2$Fe$_6$S$_6$ cores (45-48) that closely approach the core stoichiometry of FeMo-co. These synthetic clusters have been obtained mainly by three approaches: 1) spontaneous self-assembly of appropriate reagents (23), in which the inherent kinetic lability of the FeMoS system allows for formation of various thermodynamically stable, heteronuclear products (e.g., the double and single cubanes), and 2) the coordination of the MoS$_4^{2-}$ ligand to certain Fe(L)$_2$ units (49), used in the synthesis of simple MoFeS complexes containing the MoS$_2$ ligand, and 3) the use of preformed Fe-S clusters (e.g. [Fe$_6$S$_6$(L)$_6$]$^{3-}$ (45-48) and [Fe$_2$S$_2$(CO)$_6$]$^{2-}$ (44)) and Mo reagents other than MoS$_4^{2-}$. As yet, none of the many attempts to produce synthetic analogues of FeMo-co has resulted in the preparation of clusters possessing the correct stoichiometry, redox and spectroscopic properties, and none has yet duplicated its ability to reconstitute activity in mutants deficient in the FeMo-co center (50). However, some of them have magnetic and/or structural features that resemble those of FeMo-co, and based on these properties, the closest models yet are the single cubane clusters with the [MoFe$_5$S$_4$]$^{8+}$ core (51-53). These complexes have the following characteristics: a) they react with nitrogenase substrates and inhibitors, such as CO and CN$^-$, b) they exhibit a $S = 3/2$ signal, and c) they have Mo coordination spheres containing Fe, S, and O donors, similar to that of FeMo-co as determined by XAS (19,20). With the isolation of the V-nitrogenase, a new family of cubane clusters (54-56) has been synthesized, with a [VFe$_5$S$_4$]$^{2+}$ core that is isoelectronic
and nearly isostructural with the [MoFe₅S₄]³⁺ clusters, as possible analogs for FeV-co, based on the characteristics of the latter. Obviously, these clusters do not match the metal and sulfur content of FeMo-co and FeV-co, therefore, a new class of clusters with potentially closer stoichiometry is arising from the third synthetic approach, mentioned before, which consists of binding Mo(CO)₅ moieties to preformed Fe₆S₆ prismane clusters (57).

Structurally, there are six types of MFe₅S₄ (M = Mo, W, or V) cubane-like clusters (23): a) the heterogroup, where the two M atoms are bridged by one (µ₂-S) and two (µ₂-SR), e.g. [Mo₂Fe₆S₉(SC₂H₅)₈]²⁺; b) the homogroup, where the two M atoms are bridged by three (µ₂-SR), e.g. [Mo₂Fe₆S₈(SR)₉]²⁺; c) Fe (II,III) octahedrally bridged double cubanes containing trigonal subclusters (e.g., [Mo₂Fe₇S₄(SR)₂]²⁻), d) the doubly bridged double cubanes with subclusters of mirror symmetry (e.g., [Mo₂Fe₆S₈(SR)₉(3,6-R₂)₂]⁴⁺), e) the single cubanes with trigonal (e.g., [MoFe₅S₄(SR)₂(cat)₃]⁴⁺) (cat = catecholate dianion) or f) mirror symmetry (e.g., [MoFe₅S₄Cl₃(al₂cat)(THF)]²⁻) (al₂cat = 3,6-diallylcatecholate dianion) (23). Of these, the former have the most diversified reaction chemistry, including the ability to bind certain substrates of nitrogenase, such as azide and cyanide.

In the following discussion, a review of all model clusters for FeMo-co and FeV-co will not be attempted (for recent reviews, see refs. 23,58,59), instead only the properties of the single cubane clusters studied during the course of this work will be included. These clusters have the same spin state, and coordination sites resembling those in the MoFe proteins and FeMo-co, and in FeV-co and VFe proteins of nitrogenase.

[MoFe₅S₄]³⁺ Single-Cubane Clusters (60,61)

The single-cubane clusters having the core [MoFe₅S₄] can be only
synthesized by cleavage reactions of preassembled doubled-cubane clusters (60). 
$\text{(Et}_4\text{N)}[\text{MoFe}_3\text{S}_4(\text{SEt})_4(\text{dmpe})]$ is derived from the reaction of the Fe(III)-bridged double-cubane cluster $[\text{Mo}_3\text{Fe}_7\text{S}_8(\text{SEt})_{12}]^{3+}$ with ~6 equivalents of 1,2-bis(dimethylphosphino)ethane ($\text{dmpe} = (\text{CH}_3)_2\text{P(CH}_2)_2\text{P(CH}_3)_2$) in benzene at 40 °C. The reaction of $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4(\text{SEt})_4(\text{dmpe})]$ with 5.6 equiv of PhCOCl causes thiolate substitution at all metal sites, giving as a product, $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4\text{Cl}_4(\text{dmpe})]$ (60). These two clusters are black microcrystalline solids and are easily oxidized in air either in solution or in the solid state. The electronic absorption spectrum (60) for $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4(\text{SEt})_4(\text{dmpe})]$ (see Fig. 5-2), in acetonitrile, exhibits bands with maxima at 402 and 283 nm, with extinction coefficients of 26300 and 15200 M$^{-1}$ cm$^{-1}$, respectively. The absorption maxima (60) of $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4\text{Cl}_4(\text{dmpe})]$ (see Fig. 5-3) dissolved in acetonitrile occur at 560, 334 (shoulder), and 258 nm with extinction coefficients of 1550, 6700, and 22300 M$^{-1}$ cm$^{-1}$, respectively.

X-ray diffraction studies (60) indicate that both of these clusters contain cubane-type $[\text{MoFe}_3(\mu_3-\text{S})_4]^{3+}$ cores (see Fig. 5-2 for schematic representation of structures), their core dimensions vary irregularly, and they do not conform well to idealized $C_S$ symmetry. Generally, the terminal Fe-SR/Cl distances in cubane-type clusters are indicative of oxidation states intermediate between +2 and +3 (23) and increased Fe$^{3+}$ character results in relatively shorter bond distances (55, 56, 61). Since these distances for $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4(\text{SEt})_4(\text{dmpe})]$ (2.250 Å) and $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4\text{Cl}_4(\text{dmpe})]$ (2.205 Å) are slightly less than those in Fe-S clusters (63) containing Fe$^{2.5+}$ with thiolate and chloride ligands (~2.26 and 2.21 Å, respectively), the mean oxidation state is > +2.5. The Mo-SR (2.486 and 2.566 Å) and Mo-Cl (2.490 Å) distances in $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4\text{Cl}_4(\text{dmpe})]$ are consistent with an oxidation state of +3 for Mo. In summary, these two clusters contain trigonally distorted Fe sites, and the Mo($\mu_3$-S)$_3$P$_2$L coordination units (where L = RS$^-$, Cl$^-$)
Figure 5-2  Schematic representation of molybdenum single-cubane clusters.
Figure 5-3  Electronic absorption spectra of (Et₄N)[MoFe₃S₄(SEt)₄(dmpe)] and (Et₄N)[MoFe₃S₄Cl₄(dmpe)] in acetonitrile solutions. Figure taken from ref. 60
are severely distorted octahedra (60). Also, the two clusters showed asymmetric skew conformations for the Mo-dmpe chelate rings.

Mössbauer studies (61) of these two clusters indicate that there are two Fe subsites in the ratio 2:1. Furthermore, the solution magnetic moments (60) at room temperature (obtained from NMR measurements) of (Et4N)[MoFe5S4(SEt)4(dmpe)] (3.97μB in DMSO) and (Et4N)[MoFe5S4Cl4(dmpe)] (4.10μB in acetonitrile), and low-temperature magnetization and magnetic susceptibility measurements (61), in the crystalline phase, are consistent with an S = 3/2 ground state, as do all known clusters with [MoFe5S4]3+ core. This property and the fact that these clusters undergo chemically reversible one-electron reductions (60) at -1.48 ((Et4N)[MoFe5S4(SEt)4(dmpe)]) and -0.71 V ((Et4N)[MoFe5S4Cl4(dmpe)]), vs. SCE in acetonitrile solution, make these clusters possible analogs for FeMo-co.

/[VFe5S4]2+ Single-cubane Clusters

Single-cubane clusters with the [VFe5S4] core are synthesized by spontaneous assembly reactions. The first step in the synthesis of (Me4N)[VFe5S4Cl3(DMF)3].2DMF is the formation of the linear-cluster [VFe2S4Cl4]3- by reacting (NH4)3[VS4] with 2 equiv of FeCl2. Then, the cubane cluster is formed from the linear cubane core by Fe(II) insertion, rearrangement and reduction using an additional 2 equivalents of FeCl2 in DMF (54,55).

A unique feature of (Me4N)[VFe5S4Cl6(DMF)3].2DMF (54,55) is the presence of labile ligands at all terminal binding sites, this property makes it a very reactive cluster that undergoes substitution at all metal sites, and solvolysis at the V site (55). The order of solvent binding affinity at the V site is MeCN < DMF ≤ DMSO. The black crystalline solid dissolved in DMF has an electronic absorption spectrum essentially featureless, with a gradual rise from 800 to 250
Figure 5-4 Schematic representation of vanadium single-cubane clusters.
When this cluster is reacted with dmpe in acetonitrile, it gives, as a product, \((n-Pr_4N)[VFe_3S_4Cl_3(dmpe)(CH_3CN)].3MeCN\). In acetonitrile this cluster has an absorption spectrum with shoulders at 530, 450, and 370 nm, with extinction coefficients of 1400, 2300, and 4500 M\(^{-1}\) cm\(^{-1}\), respectively (55). When \((Me_4N)[VFe_3S_4Cl_3(DMF)]_2\).2DMF is treated with p-CH\(_3\)C\(_6\)H\(_4\)S\(^-\) in DMF, it results in substitution at the Fe sites and the formation of \((Ph_4P)[VFe_3S_4(S-p-C_6H_4CH_3)_3(DMF)]_3\) (55). The electronic absorption spectrum of this cluster, in DMF, has features at 433, 360 (shoulder), and 263 (shoulder) nm with extinction coefficients of 15600, 17000, and 51300 M\(^{-1}\) cm\(^{-1}\), respectively (55) (see Fig. 5-5).

Crystallographic data (56) (refer to Fig. 5-4 for schematic representation of structures) indicates that the cores of \((Me_4N)[VFe_3S_4Cl_3(DMF)]_2\).2DMF and \((n-Pr_4N)[VFe_3S_4Cl_3(dmpe)(CH_3CN)].3MeCN\) have idealized C\(_{3v}\) and C\(_{5v}\) symmetry, respectively. In each cluster, trigonally distorted tetrahedral coordination at the Fe sites is completed by terminal binding of Cl\(^-\). Both clusters have terminal Fe-Cl bond lengths of 2.266 Å, in contrast to 2.204 Å for \((Et_4N)[MoFe_3S_4Cl_4(dmpe)]\). This suggests that the mean oxidation state of Fe in these V-clusters \(<\) 2.5+. The V atoms exhibit distorted octahedral coordination involving one or three solvent molecules. The mean V-O bond length in \((Me_4N)[VFe_3S_4Cl_3(DMF)]_2\).2DMF is 2.310 Å, and based on a similar analysis as for the Mo-clusters, this information suggests that the oxidation state of V does not exceed +3. Since \((n-Pr_4N)[VFe_3S_4Cl_3(dmpe)(CH_3CN)].3MeCN\) contains a chelating dmpe ligand, it has a VS\(_3\)P\(_2\)N coordination unit that is distorted from octahedral geometry. The dmpe bite distance of 3.148 Å is close to that in \((Et_4N)[MoFe_3S_4Cl_4(dmpe)]\), and in both clusters the V-dmpe ring adopts an asymmetric skew (near-half-chair) conformation. Unfortunately, no crystallographic data has been reported in the literature for \((Ph_4P)[VFe_3S_4(S-p-C_6H_4CH_3)_3(DMF)]_3\), however NMR data in solution indicate that its core structure is similar to the one in
Figure 5-5 Electronic absorption spectra of \((\text{Ph}_4\text{P})[\text{VF}_{8}\text{S}_4(\text{S-}p\text{-C}_6\text{H}_4\text{CH}_3)\text{(DMF)}_3]\) in DMF solution. Taken from ref. 55.
Mössbauer, magnetization and magnetic susceptibility measurements of crystalline samples of \((\text{Me}_4\text{N})[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{DMF})_3].2\text{DMF}\) (63), \((n-\text{Pr}_4\text{N})[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{dmpe})(\text{CH}_3\text{CN})].3\text{MeCN}\) (61), and \((\text{Ph}_4\text{P})[\text{VFe}_3\text{S}_4(\text{S}-p-\text{C}_6\text{H}_4\text{CH}_3)_3(\text{DMF})_3]\) (61) indicate an \(S = 3/2\) ground state. This spin state was also confirmed by EPR (61) studies of \((\text{Ph}_4\text{P})[\text{VFe}_3\text{S}_4(\text{S}-p-\text{C}_6\text{H}_4\text{CH}_3)_3(\text{DMF})_3]\) that show a signal with \(g\)-values of 5.50, 3.27, and 2.04 in DMF solution, and the broader signal with \(g = 4.77, 3.03,\) and 1.95 in the solid state (refer to Fig. 5-6). In addition, the hyperfine structure, centered at \(g = 2.04\) in the solution spectrum, was attributed to \(^{51}\text{V} \ (I = 7/2)\) (61).

In summary, the structural differences between the \([\text{VFe}_3\text{S}_4]^{2+}\) and \([\text{MoFe}_3\text{S}_4]^{3+}\) core units in the various clusters are small and unsystematic. The cores are nearly isostructural and isoelectronic. The effect of thiolate vs. chloride coordination in the V clusters can be assessed by comparing \((\text{Ph}_4\text{P})[\text{VFe}_3\text{S}_4(\text{S}-p-\text{C}_6\text{H}_4\text{CH}_3)_3(\text{DMF})_3]\) and \((\text{Me}_4\text{N})[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{DMF})_3].2\text{DMF},\) and by comparing the two Mo clusters discussed here. The correlation between isoelectronic V and Mo clusters can be best investigated by comparing \((n-\text{Pr}_4\text{N})[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{dmpe})(\text{CH}_3\text{CN})].3\text{MeCN}\) and \((\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4\text{Cl}_4(\text{dmpe})]\) whose clusters differ only in one terminal ligand at the V and Mo site. From the Mössbauer and X-ray diffraction data, it can be concluded that the Fe atoms in the isoelectronic cores \([\text{VFe}_3\text{S}_4]^{2+}\) and \([\text{MoFe}_3\text{S}_4]^{3+}\) are in very nearly the same oxidation state, with the Fe atoms in the V clusters slightly more reduced. However, these clusters are similar in their spin state, existence and extent of electron delocalization, parallel vs. antiparallel spin coupling, as well as geometric structures.

5.2 OBJECTIVES
Figure 5-6 EPR spectra of \((\text{Ph}_4\text{P})\text{[VF}_8\text{S}_4\text{(S-p-C}_8\text{H}_4\text{CH}_2)_3\text{(DMF)}_3]\). \textit{Upper:} in DMF solution (~ 30 mM), \textit{lower:} in the solid state. Conditions: temperature, 10 K, microwave power, 1 mW, modulation amplitude, 32 G. The inset is an expansion of the \(g = 2\) region. Figure taken from ref. 61.
The objectives of this work were to characterize the changes in the electronic and magnetic properties associated with a) the replacement of Mo by V, and b) the replacement of thiolate terminal Fe ligation with chloride in mixed metal single-cubane clusters. The combination of room-temperature UV-visible absorption spectroscopy, low-temperature EPR and MCD spectroscopies were used to achieve these objectives. It was hoped that such investigations would facilitate the interpretation of differences in the EPR and MCD properties of the FeMo and FeV clusters in Mo- and V-nitrogenases (32-36). The following complexes were investigated: \((\text{Et}_4\text{N})\)[MoFe\(_3\)S\(_4\)(SEt)\(_4\)(dmpe)\], (\(\text{Et}_4\text{N}\))[MoFe\(_3\)S\(_4\)Cl\(_4\)(dmpe)], (\(\text{n-Pr}_4\text{N}\))[VFe\(_2\)S\(_4\)Cl\(_3\)(dmpe)(CH\(_3\)CN)]·3MeCN, (\(\text{Me}_4\text{N}\))[VFe\(_3\)S\(_4\)Cl\(_3\)(DMF)\(_3\)]·2DMF, and (\(\text{Ph}_4\text{P}\))[VFe\(_2\)S\(_4\)(S-p-C\(_6\)H\(_4\)CH\(_3\))\(_3\)(DMF)\(_3\)].

5.3 RESULTS

The EPR spectra presented herein were obtained on samples that were ~ 3-fold more concentrated than those used for UV-visible absorption and MCD studies to obtain better quality data. However, in all cases, analogous EPR spectra, albeit of poorer quality, were obtained for the samples used in optical investigations.

5.3.1 \((\text{Et}_4\text{N})\)[MoFe\(_3\)S\(_4\)(SEt)\(_4\)(dmpe)\]

**EPR Studies:** The EPR spectra of \([\text{MoFe}_3\text{S}_4(\text{SEt})_4(\text{dmpe})]^{1-}\) in 50:50 (v/v) DMF/toluene consist of resonances with \(g = 5.97, 4.88, 3.85,\) and 2.00 (see Fig. 5-7). The temperature dependence (4.4 K to 50 K) of these inflections (refer to Fig. 5-7) show that their relative intensities slightly change, with the low-field signal dominating at lower temperatures. These \(g\)-values and relaxation properties are indicative that the resonances at \(g = 5.85\) and 4.88 originate from the lower and upper zero-field doublet of an \(S = 3/2\) ground state, respectively, with
Figure 5-7  EPR temperature-dependedence spectra of (Et₄N)[MoFe₂S₄(SEt)₄(dmpe)]. Cluster (~ 2 mM) in 50:50 (v/v) DMF/toluene. Conditions: microwave power, 5.0 mW; microwave frequency, 9.41 GHz; modulation amplitude, 1.0 mT; temperatures as indicated; relative gains, x2, except for 4.4 K spectrum (x1). Inset: plot of the natural logarithm of the ratio of the intensities of the g = 5.97 and 4.88 inflections versus the reciprocal of the absolute temperature.
intermediate rhombicity and $D < 0$. The observed $g$-values can be interpreted in terms of the $S = 3/2$ spin Hamiltonian (see Eq. 1, chapter 3), assuming an isotropic Zeeman interaction. For example, the best fit to the observed $g$-values occurs for $E/D = 0.15$ and $g_0 = 2.03$, when the theory predicts $g_x = 3.12$, $g_y = 4.88$, and $g_z = 1.90$ for one doublet and $g_x = 0.94$, $g_y = 0.82$, and $g_z = 5.97$ for the other. The energy separation between these two zero-field doublets, $\Delta = 2D(1 + 3(E/D)^2)^{1/2}$, can be approximated from the slope of a plot of the natural logarithm of the ratio of the intensity of the two field resonances vs. the reciprocal of the absolute temperature, which is a straight line within experimental error of the measurement of the absolute temperature (see inset Fig. 5-7). From this plot we estimate $\Delta = 1.3 \pm 0.3 \text{ cm}^{-1}$, which corresponds to $D = -0.7 \pm 0.2 \text{ cm}^{-1}$. These spin Hamiltonian parameters are significantly different from those deduced from magnetic susceptibility studies solid samples (i.e. $|D| = 1.8 \text{ cm}^{-1}$ and $E/D = 0.1$) (61). The differences presumably relate to perturbations of the cluster in the solid state as a result of crystal packing effects or the relative insensitivity of the magnetic susceptibility data to the $E/D$ parameter (61). In this connection, it is noteworthy that the rhombicity ($E/D$) parameters determined from solution EPR measurements for all the single-cubane clusters investigated in this work (see below) are all very different from those deduced from magnetic susceptibility studies for the identical samples in the solid states (61).

**UV-Visible Absorption and MCD Studies:** The room-temperature UV-visible spectrum in a 50:50 (v/v) mixture of DMF/toluene (Fig. 5-8) is very similar to the published spectra in acetonitrile (Fig. 5-3) (60), indicating that the sample is intact in this solvent mixture. The spectrum, as described in the section 5.1.2, shows the typical $S \rightarrow Fe$ charge transfer band at 402 nm.

The low-temperature MCD spectra of the corresponding sample at 4.5 T and
temperatures between 1.62 and 87.8 K (Fig. 5-8) show multiple temperature dependent transitions throughout the visible region. The spectra are clearly indicative of the presence of a paramagnetic center with positive bands centered at 750, 624, 515, 474, and 376 nm, and a negative band at 510 nm.

MCD magnetization data, collected at 515 nm, are shown in Fig. 5-9. These data were recorded at temperatures between 1.61 and 10.1 K and magnetic fields between 0 and 4.5 T, and have been corrected for contributions from temperature-independent MCD bands. The experimental data magnetize much steeper than that for an isolated $S = 1/2$ ground state and are generally in accord with the $S = 3/2$ ground state deduced from the parallel EPR studies. However, since the EPR analysis indicates that the two zero-field split doublets are very close in energy ($\Delta = 1.3 \text{ cm}^{-1}$), we have not attempted fitting the data at 1.61 K to the $g$-values for the lowest doublet. Both doublets will be significantly populated even at 1.6 K and fitting of the data as a sum of $C$-terms from both doublets would not be a meaningful exercise, because of the large number of parameters involved (i.e. $C$-terms and polarization ratios for each doublet).

5.3.2 (Et$_4$N)[MoFe$_3$S$_4$Cl$_4$(dmpe)]

EPR studies: Replacement of the RS$^-$ ligands by Cl$^-$ causes a substantial change in the EPR spectra. The temperature-dependence of this signal at temperatures between 4.4 and 50 K, and 5 mW is shown in Fig. 5-10. These spectra show a very fast-relaxing, approximately axial signal with $g_{||} = 5.57$ and $g_{\perp} \approx 2.00$ as the dominant species. Such $g$-values are indicative of a rhombic $S = 3/2$ ground state which would have effective $g$-values of 5.5, 2.0 and 1.4 for both of the zero-field doublets. Since both doublets exhibit identical $g$-values in the rhombic limit, it is not possible to obtain an estimate of the zero-field splitting based on the EPR spectrum. In addition to the above signal, there is a weak feature centered at $g$.
Figure 5-8  Room-temperature UV-visible absorption and low-temperature MCD spectra of \((\text{Et}_4\text{N})\text{MoFe}_5\text{S}_4(\text{SEt})_2(\text{dmpe})\)_2. Cluster (0.39 mM) in 50:50 (v/v) DMF/toluene. Conditions: pathlength, 0.10 cm (for both absorption and MCD spectra); magnetic field and temperatures as indicated. The intensity of the transitions increases with decreasing temperature.
Figure 5-9 Magnetization Plot for (Et₄N)[MoFe₃S₄(SEt)₄(dmpe)]. Sample as in Fig. 5-8. Conditions: temperatures as indicated; wavelength, 515 nm.
Figure 5-10  Temperature-dependence of EPR signal of \((\text{Et}_4\text{N})\text{MoFe}_2\text{S}_4\text{Cl}_4(\text{dmpe})\). Cluster (~ 4 mM) in 50:50 (v/v) DMF/toluene. Conditions of measurement: microwave power, 5 mW; microwave frequency, 9.42 GHz; modulation amplitude, 1.0 mT; temperatures as indicated; relative gains, x1 for 4.4 and 7.0 K spectra, and x2 for 13-50 K spectra.
that is most apparent at high temperatures. We tentatively attribute this feature to a minor contribution from an axial $S = 3/2$ component ($g_\perp = 4$, $g \parallel = 2$). In addition there is a very weak $S = 1/2$ signal ($g \parallel = 2.04$ and $g_\perp = 1.91$) indicative of an $[4\text{Fe}-4\text{S}]^{1+}$ cluster. These minor species suggest some cluster heterogeneity.

**UV-visible and MCD studies:** The room-temperature UV-visible absorption spectrum for this cluster in a mixture of 50:50 (v/v) DMF/toluene (Fig. 5-11) is identical to that in acetonitrile reported previously (see Fig. 5-3). The spectrum has a weak band at 560 nm and a shoulder at 334 nm, and lacks the 400 nm band indicating complete Cl$^-$ coordination at the Fe sites.

The change in ligation at the metal sites also produces marked changes in the low-temperature MCD spectra. The spectra (Fig. 5-11) at 4.5 T and temperatures in the range 1.62 to 92.5 K show temperature-dependent bands, indicative of a paramagnetic chromophore, with maxima at 800, 680, 594, 520, 486, and 360 nm. There are also two negative bands at 570 and 396 nm. The only similarities observed between these MCD spectra and those of $(\text{Et}_4\text{N})[\text{MoFe}_3\text{S}_4(\text{SEt})_4(\text{dmpe})]$ are the negative band at 396 and the positive band at 360 nm, suggesting that those features are indigenous to the $[\text{MoFe}_3\text{S}_4]$ core.

MCD magnetization data were recorded at 520 nm (Fig. 5-12). The presence of low lying zero-field splitting components that become thermally populated with increasing temperature is indicated by the "nesting" of the magnetization data. This MCD behavior is consistent with the presence of a zero-field split $S = 3/2$ ground state. In the absence of a good estimate for the magnitude of the zero-field splitting, analysis of this data has been confined to fitting the lowest temperature data (1.60 K) to theoretical curves using the EPR $g$-values of $g \parallel = 5.57$ and $g_\perp = 2.00$, and $m_x/m_{xy} = -5$ suggesting a significant $z$-polarized
Figure 5-11  Room-temperature UV-visible absorption and low-temperature MCD spectra of (Et₄N)[MoFe₅S₄Cl₄(dmpe)]. Cluster (1.0 mM) in 50:50 (v/v) DMF/toluene. Conditions: 0.10 cm (for both absorption and MCD spectra); magnetic field and temperatures as indicated (intensity of the transitions increasing with decreasing temperature).
Figure 5-12 MCD Magnetization plot for (Et₄N)[MoFe₃S₄Cl₄(dmpe)]. Sample as in Fig. 5-11. Conditions: temperatures as indicated; wavelength, 520 nm. Solid line is the theoretical magnetization data for an $S = \frac{3}{2}$ with effective $g$-values of $g_\parallel = 5.60$, and $g_\perp = 2.0$, and polarization ratio of $m_-/m_+ = -5$. 
component at this wavelength.

5.3.3 \((n-Pr_4N)[VF_{3}S_4Cl_3(dmpe)(CH_3CN)]\cdot 3MeCN\)

**EPR studies:** Fig. 5-13 shows the low-temperature EPR spectrum for \((n-Pr_4N)[VF_{3}S_4Cl_3(dmpe)(CH_3CN)]\cdot 3MeCN\) in a mixture of 50:50 \((v/v)\) DMF/toluene. The spectrum consists of a fast relaxing, rhombic signal with \(g = 5.66, 2.26,\) and \(1.60\) at the maximum, crossover, and minimum of the spectrum. The signal corresponds to a very fast-relaxing species, and it exhibits significant broadening at temperatures higher than 13 K. These data closely resemble the EPR signal observed for \((Et_4N)[MoFe_3S_4Cl_4(dmpe)]\) and are similarly analyzed in terms of a rhombic \(S = 3/2\) ground state. The similarity in these EPR spectra suggests that a conservative replacement of V for Mo does not significantly perturbs the ground state properties as revealed by EPR spectroscopy.

**UV-visible absorption and MCD studies:** The upper panel in Fig. 5-14 shows the room-temperature UV-visible spectrum in a mixture of 50:50 DMF/toluene. The spectrum has shoulders at 530, 450 and 370 nm, and is similar to the data reported in the literature with acetonitrile as the solvent (55).

The lower panel of Fig. 5-14 shows the low-temperature MCD spectra for the same sample, at temperatures between 1.63 and 93.5 K. The intensity of the spectra increases with decreasing temperature, indicating the presence of a paramagnetic species, as indicated by EPR. Except for the absence of pronounced negative band at \(\approx 390\) nm, the major features clearly resemble those of \([MoFe_3S_4Cl_4(dmpe)]^{1-}\) showing that the electronic structure as revealed by low-temperature MCD is not markedly perturbed by the substitution of Mo by V. The MCD magnetization data at 520 nm (Fig. 5-15) are very nested and the best fit to the lowest temperature data was obtained for parameters very similar to those
Figure 5-13  EPR spectrum of (n-Pr₄N)[VF₆S₄Cl₄(dmpe)(CH₂CN)].3MeCN. Cluster (~ 4 mM) in 50:50 (v/v) DMF/toluene. Conditions of measurement: microwave power, 5 mW; microwave frequency, 9.42 GHz; modulation amplitude, 1.0 mT; temperature, 13.0 K.
Figure 5-14 Room-temperature UV-visible absorption and low-temperature MCD spectra of \((\text{n-Pr}_4\text{N})\text{[VFe}_5\text{S}_4\text{Cl}_2\text{(dmpe)}\text{(CH}_3\text{CN)}]\text{Cl}_3\text{MeCN} \). Cluster (1.3 mM) in 50:50 (v/v) DMF/toluene. Conditions: 0.10 cm (for both absorption and MCD spectra); magnetic field and temperatures as indicated (intensity of the transitions increasing with decreasing temperature).
Figure 5-15 MCD magnetization plot for (n-Pr₄N)[(VF₃S₄Cl₃(dmpe)(CH₃CN)].3MeCN. Sample as in Fig. 5-14. Conditions: temperatures as indicated; wavelength, 520 nm. Solid line is the theoretical magnetization data for an S = 3/2 with effective g-values of g∥ = 5.70, and g⊥ = 2.0, and polarization ratio of m⁺/m⁻ = -6.
used in simulating equivalent data for [MoFe₅S₄Cl₄(dmpe)]⁺.

5.3.4 (Me₄NXVFe₃S₄Cl₃(DMF)₃]·2DMF

**EPR studies:** Low-temperature EPR spectrum of (Me₄N)[VFe₃S₄Cl₃(DMF)₃]·2DMF (Fig. 5-16) shows low-field resonances with maximum, crossover and minimum g-values of 5.70, 2.00, and 1.40. This resonance can be attributed to a rhombic (E/D ~ 0.33) S = 3/2 ground state. The hyperfine pattern observed at g = 2 is assigned to a VO²⁺ impurity, as opposed to V hyperfine on the g = 2 component of the cluster S = 3/2 signal. This impurity varied in intensity from sample to sample and depended on the sample history. There appear to be an additional component with a low-field g-value of 5.4. The ratio of the g = 5.4 and 5.7 low-field features was not found to be dependent on temperature and hence, they appear to correspond to distinct species arising from cluster heterogeneity, as opposed to originating from different doublets of the same S = 3/2 ground state.

**UV-visible Absorption and MCD studies:** The room-temperature absorption spectrum (shown in Fig. 5-17) is essentially featureless with a gradual rise from 800 to 300 nm. Since no extinction coefficient was reported in the literature, one was experimentally measured at 450 nm (ε = 1800 M⁻¹ cm⁻¹) and was used to calculate the concentrations given in the figure legends.

Variable temperature MCD spectra at 4.5 T and temperatures between 1.58 and 84 K are shown in Fig. 5-17. The spectra consist of several bands with maxima at 800, 712, 606, 520, 354, 330 and 304 nm. The general features of the spectrum (particularly above 400 nm) are very similar to those of [VFe₃S₄Cl₃(dmpe)(CH₃CN)]⁺ indicating that the nature of the terminal ligands on V is not an important factor in determining the electronic structure of the cluster. MCD magnetization data collected at 712 nm (Fig. 5-18) are similar to
Figure 5-16 Low-temperature EPR spectrum of (Me₄N)[VF₆S₄Cl₆(DMF)₆]2DMF. Cluster (~ 4 mM) in 50:50 (v/v) DMF/toluene. Conditions of measurement: microwave power, 5 mW; microwave frequency, 9.42 GHz; modulation amplitude, 1.0 mT; temperature, 12.0 K.
Figure 5-17 Room-temperature UV-visible absorption and low-temperature MCD spectra of (Me₄N)₂[VFe₂S₄Cl₆(DMF)₆]·2DMF. Cluster (1.3 mM) in 50:50 (v/v) DMF/toluene. Conditions: 0.10 cm (for both absorption and MCD spectra); magnetic field and temperatures as indicated (intensity of the transitions increasing with decreasing temperature).
MCD magnetization plot for (VFe₃S₄Cl₃(DMF)₃)¹⁻ in 50% DMF + Toluene

Circle — 1.58 K
Square — 4.22 K
Triangle — 10.3 K

Solid line: theoretical curve for g=5.7,1.7 and (Mz/M+)=−1

Figure 5-18 MCD magnetization plot for (Me₄NXVFe₃S₄Cl₃(DMF)₃)₂DMF. Sample as in Fig. 5-17. Conditions: temperatures as indicated; wavelength, 712 nm. Solid line is the theoretical magnetization data for an S = 3/2 with effective g-values of g∥ = 5.70, and g⊥ = 1.70, and polarization ratio of m₀/m⁺ = −1
those observed for [VFe₃S₄Clₙ(dmpe)(CH₂CN)]⁺ and are analyzed in a similar manner. The lowest temperature data can be simulated with theoretical data using the EPR-values (g∥ = 5.70 and g⊥ = 1.70). Therefore, this cluster shows a similar rhombic S = 3/2 ground state as the other clusters studied herein.

5.3.5 (Ph₄P)[VFe₃S₄(S-p-C₆H₄CH₂)₉(DMF)₃]

**EPR studies:** The first-derivative EPR spectrum shown in Fig. 5-19 is very similar to that previously published (Fig. 5-6) except in the g = 2 region. In this spectrum (Fig. 5-19), the hyperfine structure in the g = 2 region is not due to V, instead, this is indicative of Mn(II). This impurity was not apparent in the EPR spectrum of the less concentrated sample used for MCD experiments (data not shown). Moreover, neither sample was observed to have any significant evidence of V hyperfine structure. In fact, a close inspection of the data in Fig. 5-6 indicates that the V-hyperfine reported by Carney et. al. (61) is not indigenous to the cluster but rather is a trace VO²⁺ impurity (cf. Fig. 5-16). The EPR spectrum, g = 5.5, 3.2, and 2.0, originates from the lower doublet (D > 0) of an S = 3/2 system with intermediate rhombicity (with g₀ > 2, as is the case of Co²⁺ complexes (65)). It is noteworthy that these EPR characteristics are quite distinct from the two other [VFe₃S₄] clusters investigated, both of which had chloride as the terminal Fe ligand.

**UV-visible absorption and MCD studies:** The electronic absorption spectrum in DMF/toluene (see Fig. 5-20) is identical to that previously reported in DMF solution (Fig. 5-5). MCD studies (Fig. 5-20) show temperature dependent transitions, confirming the presence of a paramagnetic chromophore, with all bands positive in sign, resembling the spectra of (Et₄N)[MoFe₃S₄(SEt)₄(dmpe)] in the 500-800 nm region. The MCD magnetization data at 466 nm are shown in Fig.
Figure 5-19 Low-temperature EPR spectrum of (Ph4P)2[VFe3S4(S-p-C6H4CH3)2(DMF)3]. Cluster (~ 1 mM) in 50:50 (v/v) DMF/toluene. Conditions of measurement: microwave power, 5 mW; microwave frequency, 9.42 GHz; modulation amplitude, 1.0 mT; temperature, 12.0 K.
Figure 5-20 Room-temperature UV-visible absorption and low-temperature MCD spectra of \((\text{Ph}_4\text{P})\text{[VFe}_2\text{S}_4\text{(S-p-C}_6\text{H}_4\text{CH}_3)_2\text{(DMF)}_2]\). Cluster (0.31 mM) in 50:50 (v/v) DMF/toluene. Conditions: 0.10 cm (for both absorption and MCD spectra); magnetic field and temperatures as indicated (intensity of the transitions increasing with decreasing temperature).
5-21 and are broadly consistent with the $S = 3/2$ ground state parameters obtained from EPR spectroscopy.

Figs. 5-22 and 5-23 provide a summary of the MCD data presented above in that they compare the lowest temperature MCD spectra of all the models studied herein, and compare them with MCD spectra for $S = 3/2$ MoFe and VFe clusters in *A. vinelandii* nitrogenases. Unfortunately, no samples of the isolated cofactors from these proteins were available for direct comparison.

5.4 DISCUSSION

The results discussed herein are the first study of the electronic and magnetic properties of the isoelectronic and isostructural $[\text{MoFe}_3\text{S}_4]^{3+}$ and $[\text{VFe}_3\text{S}_4]^{2+}$ single-cubane clusters using low-temperature MCD spectroscopy. In fact, they represent the first report of low-temperature MCD spectra for any synthetic Fe-S cluster. Such measurements are only now becoming possible as a result of improvements in the anaerobicity of sample handling techniques. As mentioned before, these mixed-metal single-cubane clusters are possible analogs for cofactor clusters in nitrogenase VFe and MoFe proteins. These synthetic clusters have some common characteristics with the mixed-metal clusters at the active site of nitrogenases, *e.g.* an $S = 3/2$ spin state resulting from antiferromagnetic interactions of the Fe atoms, delocalized electronic structure of the Fe-S portion, octahedral coordination at the Mo or V, and three oxidation levels. However, these clusters do not have the stoichiometry reported for the nitrogenase clusters.

Based on the synthetic complexes investigated herein, the following generalities can be made. First, only minor changes in the electronic structure and ground state magnetic properties are associated with a conservative change of Mo for V, as long as the nature of the terminal ligands (thiolate or chloride) on
Figure 5-21 MCD magnetization plot for (Ph4P)[VFe3S4(S-p-C6H4CH3)3(DMF)3]. Sample as in Fig. 5-20. Conditions: temperatures as indicated; wavelength, 466 nm. Solid line is the theoretical magnetization data for an S = 3/2 with effective g-values of \(g_\perp = 4.50\), and \(g_\parallel = 2.0\), and polarization ratio of \(m_\perp/m_+ = -1\).
Figure 5-22 Comparison of low-temperature MCD spectra of Avl with Mo single-cubane clusters. Temperatures and samples as indicated.
Figure 5.23 Comparison of low-temperature MCD spectra of Av1' with V single-cubane clusters. Temperatures and samples as indicated.
the Fe atoms remain constant. In other words, similar EPR and MCD spectra were observed for all [MFe$_3$S$_4$] clusters (M = Mo or V) with chloride completing tetrahedral coordination about Fe and these spectra were quite distinct from those obtained for analogous clusters with thiolate Fe coordination. Unfortunately it has not yet proven possible to synthesize synthetic mixed-metal single-cubane clusters involving Mo and V with identical thiolate ligands on the Fe. Therefore, the differences that are apparent in the MCD and EPR properties of the Mo and V clusters with thiolate coordination must originate in part from the aliphatic and aromatic thiolate ligands (SEt$^-$ and S-p-tol$^-$). Second, it is clear that changing the terminal Fe ligands from chloride to thiolate has a dramatic effect on both the EPR and MCD properties. For example, all three clusters investigated with chloride terminal coordination for Fe exhibited completely rhombic S = 3/2 ground states and hence, analogous EPR signals; whereas both clusters with thiolate ligation exhibited EPR spectra indicative of intermediate rhombicity. Third, there is no evidence of $^{51}$V (I = 7/2) hyperfine structure on any of the EPR spectra of the [VFe$_3$S$_4$] clusters investigated thus far. This suggests that little of the cluster 3d electron density is localized on V. Such a conclusion is consistent with the apparent insensitivity of the EPR and MCD spectra to the conservative substitution of Mo with V. Further studies on a wider range of similar synthetic clusters will clearly be necessary to substantiate these generalizations.

Do the observations discussed above permit any conclusions concerning the nature of the MoFe and VFe clusters in nitrogenases? Unfortunately MCD spectra of both of the isolated cofactors in aprotic solvents are not available for comparison. Thus the discussion must be confined to the clusters in their biological environments. As seen in Figs. 5-22 and 5-23 there is not close correspondence between the MCD spectra of the biological and synthetic clusters. Moreover, both the MCD and EPR characteristics of the S = 3/2 MoFe and VFe
clusters in Avl and Avl', respectively, are quite different (34). For example, the biological VFe clusters exhibit rhombic $S = 3/2$ EPR resonances resembling those of $[\text{MFe}_3\text{S}_4]$ ($\text{M} = \text{Mo}$ or $\text{V}$) clusters with chloride ligation for Fe, whereas the MoFe clusters exhibit almost axial $S = 3/2$ EPR resonances (32,33,35). Clearly the above results for the synthetic clusters make it very unlikely that the markedly different MCD spectra and EPR properties of the $S = 3/2$ VFe and MoFe clusters in nitrogenase can originate solely from a conservative replacement of Mo by V. However, in light of the available XAS data which suggests analogous environments for both V (37–39) and Mo (18–22, 24–28), the similar Fe–Mo/V–S stoichiometries of the isolated FeMo-co (2,3) and FeV-co (36) clusters, and the ability of FeV-co to activate Mo nitrogenase mutants deficient in the FeMo cluster (36), it seems unlikely that these spectroscopic differences reside in gross structural differences in the FeMo and FeV clusters. Therefore, in view of the sensitivity of the EPR and MCD spectra to the nature of terminal Fe ligands, it is tentatively concluded that the spectroscopic differences are a result of differences in the protein ligation of the FeMo and FeV clusters. Spectroscopic studies on the isolated FeMo-co and FeV-co clusters in identical aprotic solvents would enable a direct test of this conclusion and such studies are planned in future work.
5.5 REFERENCES


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