Transition-metal/sulfide sites, especially those containing iron, are present in all forms of life and are found at the active centers of a wide variety of redox and catalytic proteins. These proteins include simple soluble electron-transfer agents (the ferredoxins), membrane-bound components of electron-transfer chains, and some of the most complex metalloenzymes, such as nitrogenase, hydrogenase, and xanthine oxidase.

In this chapter we first review the chemistry of the Fe-S sites that occur in relatively simple rubredoxins and ferredoxins, and make note of the ubiquity of these sites in other metalloenzymes. We use these relatively simple systems to show the usefulness of spectroscopy and model-system studies for deducing bioinorganic structure and reactivity. We then direct our attention to the hydrogenase and nitrogenase enzyme systems, both of which use transition-metal-sulfur clusters to activate and evolve molecular hydrogen.

I. IRON-SULFUR PROTEINS AND MODELS

Iron sulfide proteins involved in electron transfer are called ferredoxins and rubredoxins.* The ferredoxins were discovered first, and were originally classified as bacterial (containing Fe₄S₄ clusters) and plant (containing Fe₃S₂ clusters) ferredoxins. This classification is now recognized as being not generally useful, since both Fe₃S₂ and Fe₄S₄ ferredoxins are found in plants,¹⁴,¹⁵ animals,²,⁶,¹⁶ and bacteria.⁴ Ferredoxins are distinguished from rubredoxins by their possession of acid-labile sulfide; i.e., an inorganic S²⁻ ion that forms H₂S gas upon denaturation at low pH. Rubredoxins have no acid-labile sulfide, and generally have a single iron in a more or less isolated site. Despite their lack of acid-labile sulfide, rubredoxins are included in this chapter because they have se-

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* For review articles, see References 1–11. For a discussion of nomenclature, see References 12 and 13.
quences much like those of the ferredoxins, and because their simple mononuclear Fe$^{2+}$ and Fe$^{3+}$ sites provide convenient illustrations of key structural and spectroscopic features.

In most ferredoxins, and in all rubredoxins, the protein ligands are cysteines, which provide four thiolate donors to the 1Fe, 2Fe, or 4Fe center. Additionally, the existence of 3Fe centers and of Fe-S sites that contain a second metal (i.e., heteronuclear clusters) make the Fe-S class a broad and multifunctional one.

Simple cytochromes and simple iron-sulfide proteins are similar, in that both can undergo one-electron transfer processes that are generally uncoupled from proton-, atom-, or group-transfer processes. Some of these proteins, such as cytochrome $c_3$ from Desulfovibrio with four hemes or ferredoxin from Clostridium pasteurianum with two Fe$_3$S$_4$ centers, can transfer more than one electron, because they have multiple copies of a one-electron transfer group. The cytochromes were discovered in 1886 by McMunn, and their role in metabolism was discovered in the 1920s by Keilin (Chapter 6). The intense optical absorbance of these heme-containing proteins contributed singularly to their discovery and biochemical characterization. In contrast, the iron-sulfur proteins, although red to red-brown, absorb far more weakly in the visible region than do the cytochromes. Their presence is sometimes obscured by the cytochromes, and their frequent air instability made their initial recognition and isolation more difficult. It was not until the early 1960s that discoveries by several research groups led to the isolation, recognition, and characterization of the ferredoxins. The use of EPR spectroscopy and its application to biological systems had a profoundly stimulating effect on the field (see below).

Although cytochromes were discovered first, the ferredoxins are likely to be the older proteins from an evolutionary perspective. Ferredoxins have relatively low-molecular-weight polypeptide chains, require no organic prosthetic group, and often lack the more complex amino acids. In fact, the amino-acid composition in clostridial ferredoxin is close to that found in certain meteorites.

The various Fe-S sites found in electron-transfer proteins (ferredoxins) are also found in many enzymes, where these centers are involved in intramolecular electron transfer. For example, sulfite reductase contains a siroheme and an Fe$_4$S$_4$ center, which are strongly coupled and involved in the six-electron reduction of SO$_3^{2-}$ to H$_2$S. Xanthine oxidase (see Figure 7.1) has two identical subunits, each containing two different Fe$_2$S$_2$ sites plus molybdenum and FAD sites. In xanthine oxidase, the Mo(VI) site carries out the two-electron oxidation of xanthine to uric acid, being reduced to Mo(IV) in the process. The Mo(VI) site is regenerated by transferring electrons, one at a time, to the Fe$_2$S$_2$ and flavin sites, thereby readying the Mo site for the next equivalent of xanthine. Although the Fe$_2$S$_2$ sites do not directly participate in substrate reactions, they are essential to the overall functioning of the enzyme system. The Fe$_2$S$_2$ centers in xanthine oxidase play the same simple electron-transfer role as the Fe$_2$S$_2$ ferredoxins play in photosynthesis.
Figure 7.1
A schematic drawing of xanthine oxidase illustrating the Mo, flavin, and Fe$_2$S$_2$ sites and interaction of the enzyme with substrate and oxidant(s).

Structurally, all the iron-sulfur sites characterized to date are built up of (approximately) tetrahedral iron units (see Figure 7.2). In rubredoxins the single iron atom is bound in tetrahedral coordination by four thiolate ligands provided by cysteine side chains. In two-iron ferredoxins the Fe$_2$S$_2$ site consists of two tetrahedra doubly bridged through a pair of sulfide ions, i.e., Fe$_2$(μ$_2$-S)$_2$, with the tetrahedral coordination of each Fe completed by two cysteine thiols. In four-iron or eight-iron ferredoxins, the ‘thiocubane’ Fe$_4$S$_4$ cluster consists of four tetrahedra sharing edges with triply bridging S$^{2-}$ ions, i.e., Fe$_4$(μ$_3$-S)$_4$, with each Fe completing its tetrahedron by binding to a single cysteine thiolate. Finally, for Fe$_3$S$_4$ clusters, which are now being found in more and more proteins, the well-established structure has one triply bridging and three doubly bridging sulfide ions, Fe$_3$(μ$_3$-S)(μ$_2$-S)$_3$. The Fe$_3$S$_4$ unit can be thought of as derived from the ‘thiocubane’ Fe$_4$S$_4$ unit by the removal of a single iron atom.

In what follows we will introduce these structures in the order 1Fe, 2Fe, 4Fe, and 3Fe. For each, we will first discuss the physiological role(s) of the particular proteins, then the structural features, followed by the spectroscopic properties and model systems.

A. Rubredoxin: A Single-Fe Tetrathiolate Protein

The physiological role of rubredoxins (sometimes abbreviated as Rd) is not always known with certainty. In particular, although rubredoxin was first identified in the anaerobe *Clostridium pasteurianum*, its role in anaerobic metabolism re-
Figure 7.2
Structural systematics of Fe-S units found in proteins:
(A) rubredoxin single Fe center; (B) Fe$_2$S$_2$ unit;
(C) Fe$_4$S$_4$ unit; (D) Fe$_3$S$_4$ unit.
Figure 7.3
Diagram illustrating the redox changes that occur in the rubredoxin-dependent ω-hydroxylase of *Pseudomonas oleovorans*.27

mains obscure. Some rubredoxins, such as that from the aerobe *Pseudomonas oleovorans*, participate in fatty acid ω-hydroxylation, i.e., hydroxylation at the end of the hydrocarbon chain farthest from the carboxylic acid.27 Like the Fe2S2 proteins putidaredoxin28 and adrenodoxin,29 the rubredoxin provides electrons to the hydroxylase, which acts as a monooxygenase forming the ω-alcohol product and water (see Figure 7.3). In a reaction catalyzed by rubredoxin reductase, rubredoxin is reduced by NADH to the ferrous state and reoxidized by the ω-hydroxylase to the ferric form during the catalytic cycle.

Most rubredoxins contain a single Fe atom, which can exist in the ferrous or ferric state. For the rubredoxin from *Clostridium pasteurianum*,26 the E0' value is −57 mV, which is much more positive than that of ferredoxins from the same organism (see below). The 6-kDa clostridial protein has only 54 amino acids in its polypeptide chain, and has a very low isoelectric point of 2.93. The rubredoxin from *P. oleovorans*27 has one or two iron atoms in a single polypeptide chain of MW ~ 20 kDa. Its redox potential is −37 mV for the Fe3+/2+ couple. Rubredoxins as a class show considerable sequence identity, and the larger 2Fe members of the class show evidence, involving internal-sequence homology, that they may have evolved through gene duplication.

A protein from *Desulfovibrio gigas*, called desulforedoxin,30,31 appears to resemble rubredoxins in some respects, but the two Fe atoms in the 7.6-kDa protein appear to be spectroscopically and structurally distinct from the Fe atoms in rubredoxins.31 A protein from *Desulfovibrio vulgaris* called ruberythrin has a single rubredoxin site as well as a strongly coupled 2Fe site resembling that of hemerythrin. Its physiological function is unknown. Table 7.1 lists some of the known rubredoxins and their properties.

The x-ray crystal structures of the rubredoxins from *C. pasteurianum*32 and *D. vulgaris*33 have been determined.33a The *C. pasteurianum* protein structure is known to a resolution of 1.2 Å, placing it among the metalloproteins whose structures are known with greatest precision. The individual Fe and S atoms are clearly resolvable. As shown in Figure 7.4, the single iron is coordinated by four S ligands provided by Cys-6, Cys-9, Cys-39, and Cys-42. The sequence Cys-x-y-Cys is a common one in Fe-S proteins, because it allows both cysteine residues to bind to the same metal site or cluster. The Fe-S distances and angles
<table>
<thead>
<tr>
<th>Protein source</th>
<th>Molecular weight (subunits)</th>
<th>Fe-S composition</th>
<th>Redox potential mV (pH)</th>
<th>EPR g values</th>
<th>References</th>
</tr>
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<td><strong>Rubredoxins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>6,000</td>
<td>Fe</td>
<td>-58 (7)</td>
<td>9.3</td>
<td>4.3</td>
</tr>
<tr>
<td><em>Pseudomonas oleovorans</em></td>
<td>6,000</td>
<td>Fe</td>
<td>9.42</td>
<td>0.9</td>
<td>1.25*</td>
</tr>
<tr>
<td><em>Fe₂S₂ proteins</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach ferredoxin</td>
<td>11,000</td>
<td>[2Fe-2S]</td>
<td>-420 (7.0)</td>
<td>2.05</td>
<td>1.96</td>
</tr>
<tr>
<td>Parsley ferredoxin</td>
<td>11,000</td>
<td>[2Fe-2S]</td>
<td>-300 (7.5)</td>
<td>2.00</td>
<td>1.96</td>
</tr>
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<td>Euglena ferredoxin</td>
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<td>[2Fe-2S]</td>
<td>-420 (7.0)</td>
<td>2.02</td>
<td>1.93</td>
</tr>
<tr>
<td>Adrenal cortex ferredoxin (pig)</td>
<td>16,000</td>
<td>[2Fe-2S]</td>
<td>-270 (7.0)</td>
<td>2.02</td>
<td>1.93</td>
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<td><em>Pseudomonas putida</em> ferredoxin [Putidaredoxin]</td>
<td>12,500</td>
<td>[2Fe-2S]</td>
<td>-240 (7.0)</td>
<td>2.02</td>
<td>1.93</td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
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<td>[2Fe-2S]</td>
<td>-300 (7.5)</td>
<td>2.00</td>
<td>1.96</td>
</tr>
<tr>
<td>Xanthine Oxidase</td>
<td>280,000 (2)</td>
<td>2 × [2Fe-2S] I</td>
<td>-343 (8.2)</td>
<td>2.02</td>
<td>1.94</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>2 × [2Fe-2S]</td>
<td>-303 (8.2)</td>
<td>2.12</td>
<td>2.01</td>
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<td><em>Thermus thermophilus</em> Rieske</td>
<td>20,000</td>
<td>2 × [2Fe-2S]</td>
<td>+150 (7.8)</td>
<td>2.02</td>
<td>1.90</td>
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<td><strong>Fe₄S₄ proteins</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>6,000</td>
<td>2 × [4Fe-4S]</td>
<td>-420 (8.2)</td>
<td>2.06</td>
<td>1.92</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em></td>
<td>9,100</td>
<td>[4Fe-4S]</td>
<td>-280 (8.0)</td>
<td>2.06</td>
<td>1.92</td>
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<tr>
<td><em>Desulfovibrio gigas</em> ferredoxin I</td>
<td>18,000 (3)</td>
<td>[4Fe-4S]</td>
<td>-455 (8.0)</td>
<td>2.07</td>
<td>1.94</td>
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<td><em>Aconitase</em> (beef heart) [active]</td>
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<td>[4Fe-4S]</td>
<td>-343 (8.2)</td>
<td>2.02</td>
<td>1.94</td>
</tr>
<tr>
<td><em>Chromatium vinosum</em> HiPIP</td>
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<td>[4Fe-4S]</td>
<td>+356 (7.0)</td>
<td>2.12</td>
<td>2.04</td>
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<td><em>Paracoccus sp.</em></td>
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<td>2.04</td>
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<tr>
<td>Protein</td>
<td>Molecular Mass</td>
<td>[Fe-S] Cluster</td>
<td>G values</td>
<td>Other Notes</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Azotobacter vinlandii</em> Fd I</td>
<td>14,500</td>
<td>[3Fe-4S]</td>
<td>2.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4Fe-4S]</td>
<td>1.93</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>1.89</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>360</td>
<td></td>
</tr>
<tr>
<td><em>Thermus aquaticus</em></td>
<td>10,500</td>
<td>[3Fe-4S]</td>
<td>2.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4Fe-4S]</td>
<td>1.99</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1.92</td>
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<td></td>
<td></td>
<td>353, 361</td>
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<tr>
<td>Fe₃S₄ proteins</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td><em>Desulfovibrio gigas</em> Fd II</td>
<td>6,000 (4)</td>
<td>[3Fe-4S]</td>
<td>-130</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4Fe-4S]</td>
<td>2.02</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>359</td>
<td></td>
</tr>
<tr>
<td><em>Azotobacter vinlandii</em> Fd I</td>
<td>14,500</td>
<td>[3Fe-4S]</td>
<td>-450</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4Fe-4S]</td>
<td>2.01</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>360</td>
<td></td>
</tr>
<tr>
<td><em>Thermus aquaticus</em></td>
<td>10,500</td>
<td>[3Fe-4S]</td>
<td>-260</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>[4Fe-4S]</td>
<td>2.02</td>
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<td>1.99</td>
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<td>353, 361</td>
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<tr>
<td>Aconitase (beef heart) [inactive]</td>
<td>81,000</td>
<td>[3Fe-4S]</td>
<td>2.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a $g'$-tensors for $\pm \frac{1}{2}$ and $\pm \frac{3}{2}$ Kramers doublets, respectively, of the $S = \frac{1}{2}$ system. The values of 0.9 and 1.25 are calculated (not observed). 44
b The fully reduced protein has a complex spectrum due to magnetic coupling between the two identical Fe₃S₄ clusters. The $g$-values are those for partly reduced samples, and represent a magnetically isolated cluster.
c The reported spectrum is complex because of magnetic interaction with the reduced Fe₃S₄ cluster.
d Recent evidence suggests that *Thermus thermophilus* and *Thermus aquaticus* are actually the same species. 362 EPR parameters of the homologous *Thermus thermophilus* ferredoxin estimated from computer simulations. 361 In this protein a signal originating from the Fe₃S₄ cluster at $g' = 12$, attributable to $\Delta M_s = \pm 4$ transitions, is observed for the reduced ($S = 2$) cluster.
in the clostridial rubredoxin are shown in Table 7.2. The range of distances and angles reveals a slightly distorted tetrahedral structure.

The initial structural results on \textit{C. pasteurianum} rubredoxin were reported at a slightly lower resolution than those displayed in Table 7.2. In fact, the early study\textsuperscript{34} reported a range of Fe-S distances from 2.05 to 2.34 Å. Prior to the higher-resolution refinement, a synchrotron-radiation x-ray-absorption spectroscopy study of the iron-absorption edge of rubredoxin was reported.\textsuperscript{35,36} Using the technique of Extended X-ray Absorption Fine Structure,\textsuperscript{*} EXAFS, the average Fe-S distance was found\textsuperscript{35,36} to be 2.26 Å, in agreement with the average distance from the x-ray crystallographic study. However, the EXAFS indicated a much narrower permissible range of Fe-S distances than did the early crystallographic study. The later, more highly refined crystallographic treatment\textsuperscript{32} agreed nicely with the EXAFS result, illustrating the importance of applying more than one technique to the elucidation of key parameters. Here, as with the 3Fe proteins we will discuss later, EXAFS proved a useful complementary technique to x-ray crystallography.

The tetrahedral iron sites in rubredoxins offer an interesting glimpse of ligand-field theory in action, and illustrate the use of various physical methods in

\textsuperscript{*} X-ray absorption spectroscopy is most commonly (and conveniently) used with the K-edges of transition-metal ions, such as Fe or Mo. It can be split up into two distinct types; X-ray Absorption Near-Edge Structure (or XANES), and Extended X-ray Absorption Fine Structure (or EXAFS) spectroscopy. The former consists of features near the absorption edge itself, which are due to transitions of the photoelectron to bound states and also to other, more complex, phenomena (e.g., the so-called shape resonances). Although the spectra are highly dependent on the nature of the site, they are quite difficult to interpret, and most analyses are based upon simple comparisons with spectra from model compounds. The EXAFS are oscillations of the absorption coefficient at rather higher x-ray energies, and arise from scattering of the emitted photoelectron by surrounding atoms. In contrast to the XANES, EXAFS spectra are relatively simple to interpret in a quantitative manner, yielding a local radial structure. With proper interpretation of the spectra, very accurate interatomic distances (e.g., to ± 0.02 Å), plus more approximate ligand coordination numbers and atomic numbers can be obtained.
Table 7.2
Bond distances and bond angles around Fe in rubredoxin from *Clostridium pasteurianum* (W1).32

<table>
<thead>
<tr>
<th>Bond距离 (Å)</th>
<th>Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-S[Cys(6)]</td>
<td>2.333 (11)</td>
</tr>
<tr>
<td>Fe-S[Cys(9)]</td>
<td>2.288 (15)</td>
</tr>
<tr>
<td>Fe-S[Cys(39)]</td>
<td>2.300 (15)</td>
</tr>
<tr>
<td>Fe-S[Cys(42)]</td>
<td>2.235 (12)</td>
</tr>
</tbody>
</table>

In contrast, the 3d⁶ Fe²⁺ state, with one additional electron, has four unpaired electrons, as confirmed by its magnetic moment of 5.05 Bohr magnetons. In exact tetrahedral symmetry, a single, low-energy, low-intensity d-d absorption of designation ⁵E → ⁵T [(e)³(t₂)³ → (e)²(t₂)⁴] is expected for the high-spin ferrous site (Figure 7.5). Indeed, reduced rubredoxin displays a band in the near-infrared region at 6,250 cm⁻¹ that arises as a component of the ⁵E → ⁵T₂ transition.40 This band stands out particularly vividly in the low-energy circular dichroism (CD) spectrum of reduced rubredoxin.41 Moreover, magnetic circular dichroism (MCD) has proven valuable in dissecting electronic transitions in several rubredoxins and metal-sulfide proteins.38,39,42,43

![Figure 7.5](image-url)

**Figure 7.5**
Splitting of the 3d orbitals of Fe by the tetrahedral ligand field of four coordinated cysteine residues: (A) Fe³⁺; (B) Fe²⁺.
The EPR spectrum of oxidized rubredoxin (Figure 7.6) shows characteristic peaks at \( g = 4.31 \) and 9.42 (\( P. \) oleovorans), which have been assigned\(^{44}\) to transitions within excited and ground-state Kramers doublets, respectively, of a nearly completely rhombic \( S = \frac{5}{2} \) site, with \( D = 1.8 \) and \( E = 0.5 \text{ cm}^{-1} \). These values for the mononuclear \( \text{Fe}^{3+} \) ion stand in sharp contrast to those for other iron-sulfur proteins, which are usually \( S = \frac{1}{2} \) (when reduced) and have \( g \) values close to 2. The even-electron \( \text{Fe}^{2+} \) state \((S = 2)\) in reduced rubredoxin has no detectable EPR when conventional instruments are used.*

Mössbauer spectroscopy has proven to be a particularly powerful complementary tool to EPR in probing the iron sites in Fe-S proteins.\(^{3,37,51,52}\) It is a nuclear spectroscopy that can give valuable information not available from other techniques.\(^{\dagger}\) Unlike EPR, where only paramagnetic centers are “seen,” every \( ^{57}\text{Fe} \) atom in the sample will contribute to the Mössbauer spectrum. For rubredoxin, the high-spin nature of the ferric and ferrous sites are clearly seen in the Mössbauer spectra.\(^{53}\) The high-spin \( \text{Fe}^{3+} \) sites show a small quadrupole splitting of roughly 0.7–0.8 mm/s due to the almost spherical distribution of the five \( d \) electrons in the five \( d \) orbitals (Figure 7.7A). In contrast, the high-spin \( \text{Fe}^{2+} \) ion with an additional \( d \) electron has a significant asymmetry, and thus displays

\* But see Reference 45. EPR spectroscopy uses magnetic fields to split the electron spin states into levels that differ by energy in the microwave region of the spectrum. For an \( S = \frac{1}{2} \) system, the \( g \) value (and its anisotropy) and the \( a \) values (hyperfine splitting from various nuclei and their anisotropy) are the major parameters reported. EPR spectroscopy has played a role in the development of Fe-S biochemistry akin to the role played by optical spectroscopy in the development of the biochemistry of the cytochromes.\(^{46-49}\) particularly for mitochondria\(^{57}\) and chloroplasts,\(^{50}\) where the \( g = 1.9 \) EPR signal has facilitated the monitoring of electron flow through these redox systems. Although EPR has been a powerful tool, it does have some important limitations. A necessary but not sufficient condition for EPR is that the center to be observed must be in a paramagnetic state. Fortunately, this condition is met for at least one member of each one-electron redox couple, i.e., the odd-electron species. However, even when the even-electron species is paramagnetic, it is usually not observed in the EPR, because of the presence of large zero-field splittings. Moreover, relaxation effects and/or the population of excited states often cause the EPR of proteins to be unobservable at room temperature, necessitating the use of liquid \( \text{N}_2 \) or liquid He temperatures to observe the signals in the frozen state. The need to freeze samples prior to observation can lead to artifacts involving the observation of nonphysiological states and processes. On the positive side, the low temperature increases the signal intensity by altering the Boltzmann distribution of the spin population, and allows various quenching techniques to be used with EPR to evaluate kinetic and electrochemical parameters. Nevertheless, one cannot usually observe real-time kinetics or be certain that one is observing a physiologically relevant state. Despite these caveats, EPR has proven a valuable and, in some cases, indispensable tool for identification and monitoring of Fe-S sites. Recently, the advanced EPR techniques ENDOR (Electron Nuclear Double Resonance) and ESEEM (Electron Spin Echo Envelope Modulation) have allowed the extraction of additional information from the EPR signal.

\( ^{\dagger} \) Mössbauer spectroscopy measures nuclear absorption of light at \( \gamma \)-ray energies, and can be used to probe nuclear energy levels (usually of \( ^{57}\text{Fe} \)). The splitting of these levels is influenced by the \( (s) \) electron density at the nucleus, and by the electric-field gradient that is set up by nearby atoms. These factors affect the isomer shift and the quadrupole splitting of the Mössbauer spectrum, respectively. Information on nuclear hyperfine couplings is also available when experiments are conducted in the presence of an external (usually applied) magnetic field. Fortunately, the nucleus most commonly (and easily) studied by this technique is present in all the proteins discussed in this chapter, although the level of \( ^{57}\text{Fe} \) (2 percent natural abundance) must be increased by isotopic enrichment to achieve a high-enough signal-to-noise ratio. For spectra containing one type of site, the spectra are relatively straightforward to interpret. For multisite systems deconvolution is required to get data on individual centers. When possible, selective labeling of sites with \( ^{57}\text{Fe} \) is extremely helpful in the deconvolution process.
Figure 7.6
EPR spectra of various Fe-S proteins:
(A) oxidized *Desulfovibrio gigas* rubredoxin; (B) reduced spinach ferredoxin [Fe₃S₄]⁺; (C) reduced *Bacillus stearothermophilus* ferredoxin [Fe₃S₄]⁺; (D) oxidized *Thermus aquaticus* ferredoxin [Fe₃S₄]⁺; (E) oxidized *Chromatium vinosum* HiPIP [Fe₄S₄]⁺. (Spectra courtesy of S. J. George.)
Mössbauer spectra of various Fe-S sites: (A) oxidized and (B) reduced Fe(SR)$_4$ (Fe$^{3+}$ and Fe$^{2+}$, respectively) rubredoxin models (data from Reference 347); (C) oxidized and (D) reduced Scenedesmus Fe$_2$S$_2$ ferredoxin (2Fe$^{3+}$ and [Fe$^{3+}$, Fe$^{2+}$], respectively, data from Reference 348); (E) oxidized and (F) reduced Desulfovibrio gigas Fe$_3$S$_4$ ferredoxin II (3Fe$^{3+}$ and [2Fe$^{3+}$, Fe$^{2+}$], respectively, data from Reference 158); (G) oxidized and (H) reduced Bacillus steroother-mophilus Fe$_4$S$_4$ ferredoxin (data from Reference 349).

Figure 7.7

a large and quite characteristic quadrupole splitting of 3.1–3.4 mm/s (Figure 7.7B). The isotope shift also distinguishes between Fe$^{2+}$ and Fe$^{3+}$, although not as dramatically.$^{37}$ Finally, the observation$^{33}$ of magnetic hyperfine interaction in the Mössbauer spectrum at low temperature in the Fe$^{3+}$ state directly reveals the presence of unpaired electrons, i.e., magnetic coupling with a hyperfine field of 370 ± 3 kG. Although in rubredoxins with a single Fe atom, this observation of magnetic coupling does not reveal any new information, similar magnetic coupling is particularly useful in unraveling the Fe sites in more complex multiiron proteins.
NMR studies on both the oxidized and the reduced states of rubredoxins have been reported. The strongly paramagnetic iron atoms have a profound effect on the NMR spectra of protons in the vicinity of the iron. The iron drastically affects the relaxation behavior of such protons, causing line-broadening, sometimes so much that the protons become nonobservable. If observed, the protons are shifted far from the values found in diamagnetic proteins by combinations of Fermi contact (through overlap/through bond) and pseudo-contact (through space/dipolar) coupling. In the rubredoxins, the reduced state shows resolved spectra, which can be assigned with the help of data from model systems.

Resonance Raman spectroscopy provides information involving molecular vibrations that is not dependent on either nuclear or magnetic properties. Electronic excitation of bands involving $\text{S} \rightarrow \text{Fe}$ charge transfer often leads to resonance enhancement of Fe-S stretching modes. In rubredoxin, the Fe-S stretching vibrations are located between 300–400 cm$^{-1}$. Deviations of the expected two-band tetrahedral pattern ($T_2$ and $A_1$ modes) are attributable to coupling of the Fe-S vibrations with S-C-C bending modes. This coupling makes for greater variability, and the detailed vibrational assignment is thus more difficult for bands involving the cysteiny1 sulfur atoms. In contrast, for sites containing inorganic $S^{2-}$, the Fe-S vibrations involving the inorganic core are less variable and therefore more characteristic of the core type.

In theory, each of the spectroscopic techniques applied to rubredoxins can give useful information about the other iron-sulfur proteins. In practice, some techniques have proven more useful than others in particular situations, and combined use of several techniques is necessary to draw meaningful conclusions.

Chemical studies of rubredoxins have led to the replacement of the Fe$^{2+}$ with an Fe$_4$S$_4$ center, with Co$^{2+}$, and with Ni$^{2+}$. The Co$^{2+}$ replacement of Fe$^{2+}$, in *P. oleovorans* rubredoxin, leads to a stable protein that displays reduced (but not trivial) reactivity in the $\omega$-hydroxylation reaction. The spectral properties of the cobalt(II) site show the expected changes in d-d bands and the expected shifts in charge-transfer transitions. Interestingly, when Ni$^{2+}$ is substituted into rubredoxins from desulfovibrio species, the resultant proteins show hydrogenase activity.

B. Rubredoxin Model Systems

The simple mononuclear tetrahedral site of Rd has been chemically modeled in both its reduced and its oxidized forms. The bidentate o-xylyl-α,α′-dithiolate ligand forms bis complexes of Fe(II) and Fe(III) that have spectroscopic features

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* NMR is a technique whose great utility in the study of low-molecular-weight proteins and model systems has not (yet) carried over to the study of larger proteins. Slower tumbling rates, rapid electronic relaxation, multiple paramagnetic sites, large numbers of protons, and more dilute solutions conspire to make the observation and/or interpretation of NMR spectra a daunting task in multisite redox proteins of >50 kDa.
were quite similar to those of the protein. The preparative procedure is relatively straightforward (Equation 7.1).

\[
\text{FeCl}_{4}^{2-} + \text{HS-CH}_{2}-\text{phen} + \text{NaOEt} \rightarrow \text{Fe}^{2+}\left(\text{S-CH}_{2} \text{-phen}\right)_{2}^{2-} + \text{EtOH}
\]  

(7.1)

The UV-visible-NIR spectra, Mössbauer spectra, and magnetic susceptibility differ only slightly from those of oxidized and reduced rubredoxins.

The monodentate benzenethiolate (thiophenolate) ligand, C\textsubscript{6}H\textsubscript{5}S\textsuperscript{-}, similarly forms the ferrous Fe(SC\textsubscript{6}H\textsubscript{5})\textsubscript{4}\textsuperscript{2--} complex. Although for some time it was felt that the oxidized form, Fe(SC\textsubscript{6}H\textsubscript{5})\textsubscript{4}\textsuperscript{-}, was inherently unstable, the sterically hindered monothiolate ligand 2,3,5,6-tetramethylbenzenethiolate was found to form a stable, quite symmetric Fe(III) tetrathiolate anion. Armed with this information, the preparation of the tetrakis(benzenethiolate) Fe(III) complex was reinvestigated, and the complex successfully synthesized (Equation 7.2).

\[
\text{Fe} \left(\text{S-CH}_{2} \text{-phen}\right)_{4} + 4 \text{HSC}_{6}\text{H}_{5} \overset{0\degree \text{C}}{\longrightarrow} \text{Fe(SC}_{6}\text{H}_{5})_{4}^{2-} + 4 \text{HOC}_{6}\text{H}_{3}(\text{CH}_{3})_{2}
\]  

(7.2)

The Fe(III) and Fe(II) tetrathiolate species now serve as excellent structural models for the Fe sites of both oxidized and reduced Rd.

The structural parameters for the oxidized rubredoxin analogues are very similar to those of the oxidized Rd iron site. The reduced complexes reveal a lengthening of the average Fe-S bond from 2.27 to 2.36 Å, consistent with the change in oxidation state from ferric to ferrous. The addition of an electron has a more profound structural effect in this single-iron center than in some of the multiiron clusters, where electrons are more delocalized.

Clearly, for the single-Fe sites, the dominant structural feature is their near-tetrahedral tetrathiolate coordination. The dominant electronic structural feature is the presence of high-spin Fe\textsuperscript{3+} and Fe\textsuperscript{2+} sites. The important mode of chemical reactivity is a simple one-electron transfer. Each of these features carries over to the 2Fe, 4Fe, and 3Fe sites discussed below.

C. Fe\textsubscript{2}S\textsubscript{2} Ferredoxins

The simple 2Fe-2S proteins are sometimes referred to as "plant" or "plant-type" ferredoxins. The protein from spinach, which serves as an electron accep-
tor in the photosynthetic apparatus, was among the first to be well-characterized and widely studied, and could be considered the prototypical 2Fe-2S ferredoxin. However, 2Fe-2S proteins are also well-known in bacteria. The protein from the cyanobacterium (blue-green alga) *Spirulina platensis* has been structurally elucidated by x-ray crystallography. Putidaredoxin, from *Pseudomonas putida*, which serves as a donor to the P-450 camphor monoxygenase system, has been extensively studied. Fe2S2 centers are also well-established in mammalian proteins. Adrenodoxin serves as the electron donor to the P-450 monoxygenase system that carries out the 11-β-hydroxylation of steroids. The so-called ‘Rieske proteins’ are found in the *bc1* complex of mitochondria as well as in the *bof* complex of the photosynthetic apparatus of plants. In addition, Fe2S2 centers are well-known constituents of such redox proteins as xanthine oxidase, CO oxidase, succinate dehydrogenase, and putidamonooxin. Table 7.1 lists some of the Fe2S2 proteins and their properties.

The x-ray crystal structure of only the single 2Fe-2S protein mentioned above has been determined; the 2Fe-2S ferredoxin from the blue-green alga *Spirulina platensis* shows significant sequence identity with chloroplast ferredoxins typical of higher plants. As Figure 7.8 shows, the Fe2S2 unit in this 11-kDa protein is bound by Cys-41, Cys-46, Cys-49, and Cys-79. The binuclear iron cluster is found in a largely hydrophobic region of the protein, but is within 5 Å of the protein surface. The sulfur atoms of the cluster, both inorganic and cysteinyl, are hydrogen-bonded to six peptide NH groups and one serine OH group, which presumably stabilize the cluster/protein complex. The serine involved in the H-bonding, Ser-40, is conserved in all plant and algal 2Fe-2S ferredoxins sequenced, which implies that it plays a crucial structural or functional role.

The structure of the 2Fe-2S core in Figure 7.2 reveals a tetrahedron of S ligands surrounding each Fe atom. The two tetrahedra share an edge defined by the two bridging sulfide ions, and the core structure is designated Fe2(μ2-S)2. Fe-S distances and angles cannot be measured accurately in the structure at the present 2.5-Å resolution; so we will later discuss these details in terms of model compounds.

The Fe2S2 center shows nicely how spectroscopy can be used to deduce the structure of an active site. Indeed, in this case the now well-established active-site structure was deduced by a combination of chemical, spectroscopic, and magnetic methods, and the site was successfully modeled long before the first protein crystallographic study was reported.

The presence of acid-labile, inorganic sulfide is a key feature of both the Fe2S2 and the Fe4S4 centers. The 1:1 stoichiometry between iron and acid-labile sulfide was eventually established analytically for Fe2S2 centers. Care must be taken to ensure that both the protein and its active-site complement are homogeneous. Although protein homogeneity is usually established by electrophoretic methods, these methods may not distinguish between pure proteins and those with absent or incomplete active centers. Fortunately, absorption at 420 nm
is due solely to the Fe$_2$S$_2$ cluster, whereas the 275-nm absorption is dominated by the protein. Therefore a good criterion for active-site saturation and homogeneity is the ratio of the absorbances at 420 and 275 nm, $A_{420\text{ nm}}/A_{275\text{ nm}}$, which is $\sim 0.48$ for pure spinach ferredoxin.$^8$ Once homogeneous protein is obtained, the Fe$_2$S$_2$ composition of the "plant" ferredoxins can be correctly deduced analytically.

The Fe$_2$S$_2$ center displays two redox states that differ by a single electron. The potential range for the couple is $-250$ to $-420$ mV, revealing the highly reducing nature of the ferredoxin. The correct structure of the Fe$_2$S$_2$ center was first proposed in 1966 based on EPR studies.$^{82}$ The reduced state of the cluster shows a rhombic EPR signal with $g$ values of 1.88, 1.94, and 2.04 (Figure 7.6B) characteristic of an $S = \frac{1}{2}$ center. The oxidized state is EPR-silent. The weakness of the sulfur ligand field causes the iron atoms to be high-spin. But how can two sulfur-ligated iron atoms, each with a tendency to be high-spin, produce a state with a single unpaired electron?

The individual Fe atoms in the Fe$_2$S$_2$ cluster resemble those in rubredoxin quite closely. The two redox states of the Fe$_2$S$_2$ protein correspond to an Fe$^{3+}$-Fe$^{3+}$ and an Fe$^{3+}$-Fe$^{2+}$ pair, respectively, as shown in Figure 7.9. In the all-
ferric oxidized state, the two Fe$^{3+}$ sites are antiferromagnetically coupled; i.e.,
the spins of the five d electrons on the two iron atoms are oppositely aligned,
such that their pairing produces an effective $S = 0$, diamagnetic ground state.
In the reduced form, a single unpaired electron is present, because the $S = \frac{3}{2}$
Fe$^{3+}$ and $S = 2$ Fe$^{2+}$ sites are antiferromagnetically coupled, leaving one net
unpaired spin and an $S = \frac{1}{2}$ ground state. The profound difference between the
electronic properties of rubredoxin and Fe$_2$S$_2$ ferredoxin arises because the latter
has two Fe atoms in close proximity, which allows for their magnetic coupling.

Strong support for the spin-coupling model in Fe$_2$S$_2$ ferredoxins comes from
a detailed analysis of their absorption and circular dichroism spectra. As with
rubredoxin (see Figure 7.5), we expect no low-energy spin-allowed d-d bands
for the ferric site in either the oxidized or the reduced state. Indeed, the oxidized
state containing all Fe$^{3+}$ shows no low-energy bands; the reduced state contain­
ing a single Fe$^{2+}$ displays low-energy, low-intensity bands in the region 4,000–
9,000 cm$^{-1}$, in close analogy to the situation in reduced rubredoxin. The com­
bined EPR and optical spectra leave little doubt about the structural assignment:
two coupled high-spin ferric ions in the oxidized state, and coupled high-spin
ferric and ferrous ions in the reduced state. Moreover, the spectra are consistent
only with a localized model, i.e., one in which the Fe(II) site is associated with
a single iron. The Fe$_2$S$_2$ site is inherently asymmetric, and inequivalence
of the Fe(III) sites is spectroscopically detectable in the all-ferric oxidized form.

In fact, the localized valence trapping is present in reduced model compounds
that contain no ligand asymmetry.

Mössbauer spectra provide additional and striking confirmation of the struc­
tural assignment. The spectrum of the oxidized ferredoxin (Figure 7.7) resem­
bles strongly that of oxidized rubredoxin, indicating the presence of high-spin
Fe$^{3+}$, even though the net spin is zero. In the reduced form, the Mössbauer
spectrum involves the superposition of signals from a high-spin Fe$^{2+}$ and a
high-spin Fe$^{3+}$, i.e., a reduced and an oxidized rubredoxin, respectively. Clearly,
the simplest interpretation of this result consistent with the $S = \frac{1}{2}$ spin state
required by the EPR is the localized Fe$^{2+}$-Fe$^{3+}$ antiferromagnetic coupling model
discussed above.

NMR studies of oxidized Fe$_2$S$_2$ proteins reveal broad isotropically shifted
resonances for the CH$_2$ protons of the cysteine ligands. Despite the coupling
of the irons, the net magnetism at room temperature is sufficient to lead to large
contact shifts (−30 to −40 ppm downfield from TMS). The assignment of the
resonance was confirmed with the synthesis and spectroscopic analysis of model
compounds. Extensive NMR studies of the Fe$_3$S$_2$ proteins have been re­
ported.

Resonance Raman spectra of Fe$_2$S$_2$ sites reveal many bands attributable to Fe-S stretching. Detailed assignments have been presented for the four
bridging and four terminal Fe-S modes. A strong band at $\sim$390 cm$^{-1}$, which
shifts on $^{34}$S sulfide labeling, is assigned to the A$_{1g}$ "breathing" mode; another
band at 275 cm$^{-1}$ is assigned to B$_{3u}$ symmetry in point group D$_{2h}$. Spectro­
scopic differences in the terminal, Fe-S(Cys) stretches between plant ferredoxins
and adrenodoxin (which also differ somewhat in redox potential) seem to reflect
different conformations of the cysteine ligands in the two classes. Evidence for
asymmetry of the iron atoms is found in the intensity of the resonance enhance­
ment of certain modes.

D. Rieske Centers

Within the class of Fe₂S₂ ferredoxins there is a subclass called the Rieske pro­
teins, or the Rieske centers. The Rieske iron-sulfur centers are found in
proteins isolated from mitochondria and related redox chains. In addition,
the phthalate dioxygenase system from Pseudomonas cepacia contains one
Fe₂S₂ Rieske center as well as one additional nonheme Fe atom. Although the
Rieske centers appear to contain an Fe₂S₂ core, there is extensive evidence for
nonsulfur ligands coordinated to at least one of the Fe atoms. The proposed
model in Scheme (7.3) has two imidazole ligands bound to one Fe atom. The
nitrogen atoms are seen in ENDOR (Eelectron Nuclear Double Resonance) ex­
periments, and are manifest in EXAFS spectra, which are consistent with the
presence of a low-Z (atomic number) ligand bound to iron. The potentials for
the Rieske proteins range from +350 to −150 mV, in contrast to the plant­
type Fe₂S₂ centers, which range from −250 to −450 mV. The strong depen­
dence of redox potential on pH suggests a possible role in coupling proton­
and electron-transfer processes.

Although spectroscopic studies led to the correct deduction of the structure of
the Fe₂S₂ core, the synthesis of model compounds containing this core provided
unequivocal confirmation. The model compounds allowed detailed structural
analysis unavailable for the proteins. Moreover, by using a uniform set of pe­
ripheral ligands, properties inherent to the Fe₂S₂ core could be discerned.

The Fe₂S₂ core has been synthesized by several routes (see Figure 7.10). For example, the reaction of Fe(SR)₂²⁻, the ferrous rubredoxin model,
with elemental sulfur produces the complex Fe₂S₂(SR)₄²⁻. In this reaction the
sulfur presumably oxidizes the Fe²⁺ to Fe³⁺, being reduced to sulfide in the
process. The Fe₂S₂ core has been prepared with a variety of peripheral S-donor
ligands. Metrical details for Fe₂S₂(SC₆H₄-p-CH₃)₂²⁻ are given in Table 7.3.
Notable distances are the Fe-S (bridging) distance of 2.20 Å, the Fe-S (terminal)
distance of 2.31 Å, and the Fe-Fe distance of 2.69 Å.
Figure 7.10
Preparative schemes leading to complexes containing the Fe₂S₂ core.¹²⁸

Table 7.3
Structural parameters for Fe₂S₂(SC₆H₄-p-CH₃)₄²⁻.

<table>
<thead>
<tr>
<th>Atoms</th>
<th>Distance Å</th>
<th>Atoms</th>
<th>Angle °</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-Fe</td>
<td>2.691 (1)</td>
<td>Fe-S-Fe</td>
<td>75.3</td>
</tr>
<tr>
<td>Fe-S1 (bridge)</td>
<td>2.200 (1)</td>
<td>S-Fe-S</td>
<td>104.6</td>
</tr>
<tr>
<td>Fe-S2 (bridge)</td>
<td>2.202 (1)</td>
<td>S-Fe-S</td>
<td>115.1</td>
</tr>
<tr>
<td>Fe-S3</td>
<td>2.312 (1)</td>
<td>S-Fe-S</td>
<td>105.4</td>
</tr>
</tbody>
</table>

¹ Data from Reference 211.
To date, all analogue systems structurally characterized contain the Fe$^{3+}$-Fe$^{3+}$ fully oxidized form. Attempts to isolate the Fe$^{3+}$-Fe$^{2+}$ form have so far failed. However, the mixed-valence Fe$_2$S$_2$ form can be generated and trapped by freezing for spectroscopic examination.$^{97,98}$ Mössbauer spectroscopy reveals the presence of distinct Fe$^{2+}$ and Fe$^{3+}$ ions, as found in the proteins, clearly showing that “trapped” valence states are an inherent characteristic of the Fe$_2$S$_2$$^{2+}$ core and are not enforced by the protein.$^{97,98}$

The existence of noncysteine-bound Fe$_2$S$_2$ cores in Rieske-type proteins has led to attempts to synthesize complexes with oxygen and nitrogen ligands.$^{99-101}$ Characterized species include Fe$_2$S$_2$(OC$_6$H$_5$)$_4$$^{2-}$, Fe$_2$S$_2$(OC$_6$H$_4$-p-CH$_3$)$_4$$^{2-}$, Fe$_2$S$_2$(C$_4$H$_4$N)$_4$$^{2-}$, and Fe$_2$S$_2$(L)$_2$$^{2-}$, where L is a bidentate ligand.

The potentially tridentate ligand $^{7.4}$

acts in a bidentate fashion, binding through S and O but not N.

No Fe$_2$S$_2$ complexes containing mixed S,N terminal ligands, such as those suggested for the Rieske site, have been prepared. The Se$^{2-}$ bridged analogue has been prepared for some of the complexes.$^{102,103}$

F. Fe$_4$S$_4$ Ferredoxins (including HiPIPs)

We now turn our attention to proteins containing the Fe$_4$S$_4$ center. Historically, within this class a strong distinction was made between the “ferredoxins,” which are low-potential (as low as $-600$ mV in chloroplasts) iron-sulfur proteins, and the “HiPIPs” = High Potential Iron Proteins, which have positive redox potentials (as high as $+350$ mV in photosynthetic bacteria). Although the HiPIP designation is still useful, proteins of both high and low potential are considered ferredoxins, whose key defining feature is the presence of iron and acid-labile sulfide.$^{13}$

The Fe$_4$S$_4$ proteins participate in numerous electron-transfer functions in bacteria, and in some organisms (such as Clostridium) are the immediate electron donors for the nitrogenase and/or hydrogenase enzymes. The function of the HiPIPs seems obscure at present. In addition, Fe$_4$S$_4$ centers have been shown or postulated to occur in numerous microbial, plant, and mammalian redox en-
zymes, including nitrate reductase, sulfite reductase, trimethylamine dehydrogenase, succinate dehydrogenase, hydrogenase, and, possibly, in altered forms, nitrogenase. Table 7.1 lists some of the Fe₄S₄ ferredoxins and their properties.

In the Fe₂S₂ ferredoxins, combined spectroscopic, analytical, and model-system work led to an unequivocal assignment of the structural nature of the active site long before the crystallography was done. In contrast, for Fe₄S₄ systems and in particular the 8Fe-8S = 2Fe₄S₄ systems from bacteria, the initial chemical suggestions were fallacious, and even the number and stoichiometry of the clusters were in doubt. In these cases, crystallography provided the definitive structural information.

The first indication of the presence of the "thiocubane" structure came in 1968, when a 4-Å resolution study indicated a compact cluster of potentially tetrahedral Fe₄ shape in the HiPIP from *Chromatium vinosum*. This finding did not lead to much excitement, since it was not yet appreciated that HiPIPs and ferredoxins were structurally similar. In 1972, the high-resolution structure solution of both *Chromatium* HiPIP and the 8Fe ferredoxin from *Peptococcus aerogenes* (formerly *Microbacter aerogenes*) confirmed the presence of virtually identical thiocubane clusters in the two proteins. Moreover, the structures for both oxidized and reduced HiPIP were deduced, and these revealed that the Fe₄S₄ cluster remained intact during the redox interconversion. Subsequently, four-iron clusters have been crystallographically confirmed in an Fe₄S₄ ferredoxin from *Bacillus thermoproteolyticus*, in *Azotobacter vinelandii* ferredoxin I (also previously called Shethna Fe-S protein II), which also contains a 3Fe-4S cluster, in the active form ofaconitase, and in sulfite reductase, where the cluster is probably bridged by cysteine sulfur to a siroheme.

In all the proteins characterized to date, the Fe₄S₄ clusters adopt the thiocubane structure, which is discussed at greater length in the section on models. The clusters are usually bound to their proteins by four cysteine residues. As shown in Figure 7.11, in the *P. aerogenes* protein the two Fe₄S₄ clusters are bound by cysteines numbered 8, 11, 14, 18, 35, 38, 41, and 45. However, this sequence seems prominent in all Fe-S proteins, and so is not specific for a particular Fe-S site. At first glance one might expect one cluster to be bound by cysteines 8, 11, 14, and 18, the other by cysteines 35, 38, 41, and 45. Actually, one cluster is bound by cysteines 8, 11, 14, and 45, the other by cysteines 35, 38, 41, and 18. The binding of a given cluster by cysteine residues from different portions of the polypeptide chain apparently helps stabilize the tertiary structure of the protein and brings the two clusters into relatively close proximity, the center-center distance being 12 Å.

The *C. pasteurianum* protein displays weak magnetic coupling, which leads to an unusual EPR spectrum consistent with the 12-Å cluster-cluster separation. However, the redox potentials for the two sites seem virtually identical at −412 mV, thus allowing the 8Fe ferredoxin to deliver two electrons at this low
Figure 7.11
The x-ray crystal structures of (A) Chromatium vinosum HiPIP\textsuperscript{108} and (B) the 8Fe-8S ferredoxin from Peptococcus aerogenes.\textsuperscript{101}

redox potential.\textsuperscript{115} Significant sequence identity indicates the likelihood that other 8Fe ferredoxins, such as the well-studied one from \textit{C. pasteurianum},\textsuperscript{116–118} have quite similar structures.

The thiocubane unit of Fe\textsubscript{4}S\textsubscript{4} proteins can exist in proteins in at least three stable oxidation states. This so-called three-state model\textsuperscript{74,109,119,120} contrasts dramatically with the situation for Rd(1Fe), Fe\textsubscript{2}S\textsubscript{2}, and Fe\textsubscript{3}S\textsubscript{4} systems, in which only two oxidation states are accessible through simple electron transfer for each center. For the thiocubane structure, the three accessible states can be designated Fe\textsubscript{3}S\textsubscript{4}\textsuperscript{3+}, Fe\textsubscript{3}S\textsubscript{4}\textsuperscript{2+}, and Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{+}, corresponding to [Fe(III)\textsubscript{3}Fe(II)], [Fe(III)\textsubscript{2}Fe(II)\textsubscript{2}], and [Fe(III)Fe(II)\textsubscript{3}] valence-state combinations, respectively. It is crucial to note that, in sharp contrast to the Fe\textsubscript{2}S\textsubscript{2} and Fe\textsubscript{3}S\textsubscript{4} sites, the oxidation states are not localized in the Fe\textsubscript{4}S\textsubscript{4} clusters. In most cases, each Fe atom behaves as if it had the same average oxidation level as the other Fe atoms in the cluster. The redox interconversion of the Fe\textsubscript{4}S\textsubscript{4} sites is shown in Figure 7.12. The Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{3+} \rightarrow Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{2+} couple is the high-potential redox couple characteristic of HiPIPs; the Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{2+} \rightarrow Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{+} couple is responsible for the low-potential process characteristic of the classical ferredoxins. In any given protein under physiological conditions, only one of the two redox couples appears to be accessible and functional.

Both the Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{+} and the Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{3+} states of the thiocubane cluster are paramagnetic and display characteristic EPR spectra (Figure 7.6C,D). The Fe\textsubscript{4}S\textsubscript{4}\textsuperscript{3+}
Figure 7.12
The redox interconversions of Fe₄S₄ sites illustrating the three-state model. The states are found in (A) oxidized HiPIP; (B) reduced HiPIP and oxidized ferredoxin; (C) reduced ferredoxin.

The site in reduced ferredoxins displays a rhombic EPR signal (Figure 7.6C) with \( g = 1.88, 1.92, \) and 2.06. The oxidized form of low-potential ferredoxins is EPR-silent, and attempts to “superoxidize” it to achieve the Fe₄S₄³⁺ state invariably lead to irreversible cluster decomposition, probably through a 3Fe-4S structure. The Fe₄S₄³⁺ signal is usually referred to as the HiPIP signal (Figure 7.6D) and shows distinct \( g \) values at 2.04(\( g_\perp \)) and 2.10(\( g_\parallel \)); it is present in oxidized HiPIP but absent in reduced HiPIP. Reduction of HiPIP to a “super-reduced” state apparently occurs under partially denaturing conditions in aqueous DMSO. The observed axial EPR signal with \( g = 1.94 \) and 2.05 is assigned to the Fe₄S₄⁺ state characteristic of reduced ferredoxins. This result is consistent with structural and spectroscopic identity of the HiPIP and Fd sites, as required by the three-state model of the Fe₄S₄ proteins (Figure 7.12).

In Fe₄S₄ centers at each level of oxidation, electronic transitions give rise to characteristic visible and UV spectra, although the delocalized nature of the electronic states makes detailed assignment difficult. MCD spectra of clusters in the three states of oxidation are clearly distinguishable from each other and from MCD of Fe₂S₂ clusters. MCD, magnetic susceptibility, and Mössbauer spectra provide evidence that the \( S = \frac{1}{2} \) state, whose EPR signal is so distinct in reduced ferredoxins, may coexist at higher \( T \) with \( S = \frac{3}{2} \) and perhaps even higher spin states. Indeed, recent studies with model systems and theoretical treatments clearly support the ability of the Fe₄S₄ cluster to display a number of spin states that are in labile equilibria, which are influenced, perhaps quite subtly, by local structural conditions. The iron protein of nitrogenase also displays this behavior.

The Mössbauer spectra of Fe₄S₄ centers of ferredoxins reveal the equivalence of the Fe sites, and quadrupole splittings and isomer shifts at averaged values for the particular combination of oxidation states present. Representative spectra are shown in Figure 7.7. Magnetic coupling is seen for the paramagnetic states.

Resonance Raman spectra (and IR spectra) have been extensively investigated in C. pasteurianum ferredoxin and in model compounds. Selective labeling of either thiolate sulfur or sulfide sulfur with \(^{34}\text{S}\) allows modes associated with the Fe₄S₄ core to be distinguished from modes associated with the Fe-SR ligands. The band at 351 cm⁻¹ is assigned to Fe-SR stretching, and Fe₄S₄ modes occur at 248 and 334 cm⁻¹ in reduced ferredoxin from C. pasteurianum. There is little difference between the oxidized and reduced spectra, although an
extra band at 277 cm\(^{-1}\) seems present in the oxidized protein. The Fe\(_4\)Se\(_4\) substituted protein has also been studied.\(^{125}\)

As in the 1Fe and 2Fe proteins, \(^1\)H NMR spectra reveal resonances from contact-shifted -CH\(_2\)- groups of cysteinyl residues.\(^{125a}\) However, unlike the other proteins, where all states are at least weakly magnetic, only the reduced ferredoxin and the oxidized HiPIP states show contact shifts.\(^{87a,125a,b,c}\)

EXAFS studies on proteins and on model compounds clearly identify the Fe-S distance of \(\sim 2.35\) Å and an Fe-Fe distance of 2.7 Å. These distances, as expected, vary only slightly with state of oxidation.\(^{125d}\)

G. Fe\(_4\)S\(_4\) Models

Judging from the ease with which models of Fe\(_4\)S\(_4\) are prepared under a variety of conditions and their relative stability, the Fe\(_4\)S\(_4\)^{2+} core structure seems to be a relatively stable entity, a local thermodynamic minimum in the multitude of possible iron-sulfide-thiolate complexes. The initial preparation and structural characterization\(^{126,127}\) of the models showed that synthetic chemistry can duplicate the biological centers in far-simpler chemical systems, which can be more easily studied in great detail.

The general synthetic scheme for Fe-S clusters is shown in Figure 7.13. Many different synthetic procedures can be used to obtain complexes with the Fe\(_4\)S\(_4\) core.\(^{126-138,138a,b}\) The multitude of preparative procedures is consistent with the notion that the Fe\(_4\)S\(_4\)^{2+} core is the most stable entity present and "spontaneously self-assembles" when not limited by stoichiometric constraints.

\[\begin{align*}
\text{FeCl}_2 & \xrightarrow{RS^-} \text{Fe(SR)}_2^{2+} \\
\text{FeCl}_2 & \xrightarrow{RS^-} \text{Fe(SR)}_3 \\
\text{Fe}_2\text{S}_4\text{(SR)}_4^{2-} & \xrightarrow{\text{ROCl}} \text{Fe}_2\text{S}_4\text{Cl}_4^{2-} \\
\text{Fe}_4\text{(SR)}_2^{2-} & \xrightarrow{\text{Br}^-} \text{FeS}_2\text{Br}_2^{2-} \\
\text{FeS}_2\text{(SR)}_2^{2-} & \xrightarrow{\text{Br}^-} \text{FeSO}_7\text{H}_5\text{O} \\
\end{align*}\]

\([\text{S}] = \text{S}_8\text{ or RSSSRR}\)

Figure 7.13
Preparative schemes leading to complexes containing the Fe\(_4\)S\(_4\) core.\(^{128}\)
The thiocubane structure can be viewed as two interpenetrating tetrahedra of 4Fe and 4S atoms. The 4S tetrahedra are the larger, since the S-S distance is \( \sim 3.5 \) Å, compared with the Fe-Fe distance of \( \sim 2.7 \) Å. The S4 tetrahedron encloses \( \sim 2.3 \) times as much volume as does the Fe4 tetrahedron.\(^{128}\) Key distances and angles for Fe4S4(SCH2C6H5)4\(^{2-}\) given in Table 7.4 are extremely similar to those found in oxidized ferredoxin and reduced HiPIP centers in proteins.\(^{127}\)

The idealized symmetry of Fe4S4\(^{2+}\) model systems is that of a regular tetrahedron, i.e., \( T_d \). Though the distortion of the cube is quite pronounced, all known examples of the Fe4S4\(^{2+}\) core show distortion, which lowers the symmetry at least to \( D_{2d} \). In most Fe4S4\(^{2+}\) core structures, this distortion involves a tetragonal compression, which leaves four short and eight long Fe-S bonds.

Complexes with non-S-donor peripheral ligands have been prepared and studied. The halide complexes Fe4S4X4\(^{2-}\) (\( X = \text{Cl}^-, \text{Br}^-, \text{I}^- \)) have been prepared, and serve as useful starting points for further syntheses.\(^{129-133}\) The complex Fe4S4(OC6H5)4\(^{2-}\) can be prepared\(^{134}\) from the tetrachloride (or tetrathiolate) thiocubane by reaction with NaOC6H5 (or HOC6H5). There are a few examples of synthetic Fe4S4\(^{2+}\) cores in which the peripheral ligands are not identical. For example, Fe4S4Cl2(OC6H5)2- and Fe4S4Cl2(SC6H5)2- have structures characterized by \( D_{2d} \) symmetry.\(^{135}\) The complexes Fe4S4(SC6H5)2[S2CN(C2H5)2]2\(^{2-}\) and Fe4S4(SC6H4OH)4\(^{2-}\) are similarly asymmetric, containing both four- and five-coordinate iron.\(^{136-138}\) The presence of five-coordinate iron in the Fe4S4 cluster is notable, since it offers a possible mode of reactivity for the cluster wherever it plays a catalytic role (such as in aconitase). Complexes with Fe4Se4\(^{2+}\) and Fe4Te4\(^{2+}\) cores have also been prepared.\(^{138e,d}\)

One structural analysis of Fe4S4(SC6H5)4\(^3-\), which contains the reduced Fe4S4\(^+\) core, revealed a tetragonal elongation\(^{139}\) in the solid state. In contrast,
analysis of Fe₄S₄(SCH₂C₆H₅)₄³⁻ revealed a distorted structure possessing C₂ᵥ symmetry.²⁰² It would appear that the Fe₄S₄⁺ clusters maintain the thiocubane structure, but are nevertheless highly deformable. Interestingly, when the solid-state C₂ᵥ structure, Fe₄S₄(SCH₂C₆H₅)₄³⁻, is investigated in solution, its spectroscopic and magnetic behavior change to resemble closely those of the Fe₄S₄(SC₆H₅)₄³⁻ cluster,³⁹⁰ which does not change on dissolution. The simplest interpretation assigns the elongated tetragonal structure as the preferred form for Fe₄S₄⁺ cores with deformation of sufficiently low energy that crystal packing (or, by inference, protein binding forces) could control the nature of the distortions in specific compounds.¹²⁸ The elongated tetragonal structure has four long and eight short bonds in the core structure. The terminal (thiolate) ligands are 0.03–0.05 Å longer in the reduced structure, consistent with the presence of 3Fe(II) and 1Fe(III) in the reduced form, compared to 2Fe(II) and 2Fe(III) in the oxidized form. There is no evidence for any valence localization.¹²⁸

The oxidized Fe₄S₄⁺ core defied isolation and crystallization in a molecular complex prior to the use of sterically hindered thiolate ligands. With 2,4,6 tris(isopropyl)phenylthiolate, the Fe₄S₄L₄⁻ complex could be isolated and characterized.¹⁴¹ The structure is a tetragonally compressed thiocubane with average Fe-S and Fe-SR distances 0.02 and 0.04 Å shorter than the corresponding distances in the Fe₄S₄L₄²⁻ complex. Again, there is no evidence for Fe inequivalence or more profound structural distortion in this 3Fe(III)-1Fe(II) cluster. Clearly, the Fe₄S₄ clusters have highly delocalized bonding.

Evidence from model systems using sterically hindered thiolate ligands indicates the existence of an Fe₄S₄⁴⁺, i.e., all-ferric fully oxidized cube."¹⁴² The existence of the complete series Fe₄S₄[(Cy)₃C₆H₂S]₄⁺ (Cy = cyclohexyl; n = 0, −1, −2, −3) is implied by reversible electrochemical measurements. Clearly, five different states of the Fe₄S₄ core—including the (at least) transient fully oxidized state and the all-ferrous fully reduced state—may have stable existence. Although only the central three states have been shown to exist in biological contexts, one must not rule out the possible existence of the others under certain circumstances.

Recently, specifically designed tridentate ligands have been synthesized that bind tightly to three of the four Fe atoms in the thiocubane structure.¹⁴³,¹⁴³a,b The remaining Fe atom can then be treated with a range of reagents to produce a series of subsite-differentiated derivatives and variously bridged double-cubane units. These derivatives illustrate the potential to synthesize complexes that mimic the more unusual features of Fe₄S₄ centers that are bound specifically and asymmetrically to protein sites. The recently synthesized complex ion [(Cl₁Fe₄S₄S₂)S]⁴⁻, containing two Fe₄S₄ units bridged by a single S²⁻ ligand, illustrates the potential coupling of known clusters into larger aggregates.¹⁴³c

The model-system work has made an important contribution to our understanding of the Fe₄S₄ centers. The existence of three states, the exchange of ligands, the redox properties, the metrical details of the basic Fe₄S₄ unit, and the subtleties of structural distortion can each be addressed through the study of models in comparison with the native proteins.
H. Core Extrusion/Cluster Displacement Reactions

Synthetic model-system work led to the realization that the cluster cores can exist outside the protein and undergo relatively facile ligand-exchange reactions.\(^{128}\) This behavior of the purely inorganic complexes allowed core extrusion reactions\(^{128,144}\) to be developed. The basic assumption behind these reactions is that the cluster core retains its integrity when it is substituted by low-MW thiolates, especially aryl thiolates, which replace the cysteinyl ligands that bind it to the protein. In order to free the cluster from the protein, one must at least partially denature the protein, usually by using \(\sim 80\) percent aqueous solution of a polar aprotic solvent, such as DMSO or HMPA. The resulting inorganic clusters can be identified and quantified by measurement of their characteristic electronic absorption or NMR spectra. An alternative approach involves transferal of the unknown cluster in question to an apoprotein that binds a cluster of known type.\(^{145}\)

Since the 1Fe, 2Fe, and 4Fe sites are each usually bound to the protein by four cysteine residues, it is perhaps not surprising that there have been reports\(^{58}\) of interconversion of cluster types bound to a given protein. Specifically, in 90 percent aqueous DMSO, the single Fe site in rubredoxin from \textit{C. pasteurianum}\(^{90}\) is converted to an Fe\(_4\)S\(_4\) cluster by the addition of sodium sulfide, ferrous chloride, and ferric chloride in ratio 4:2:1. Presumably the spacing and geometric disposition of the cysteines are suitable to bind a single Fe or the Fe\(_4\)S\(_4\) cluster, which is readily formed under the reaction conditions. Another example of cluster rearrangement involves the three-iron center discussed below that does not extrude as an Fe\(_3\)S\(_4\) center. Rather, at least under certain conditions, the Fe\(_3\)S\(_x\) center rearranges to form Fe\(_2\)S\(_2\) centers.\(^{146}\) The facile interconversion of the Fe clusters demonstrated the lability of Fe-S systems, and indicates that caution must be exercised in interpreting the results of cluster-displacement reactions.

I. Fe\(_3\)S\(_4\) Centers

Three-iron centers are a comparatively recent finding,\(^{119,146}\) and the full scope of their distribution is not yet known. Although they have now been confirmed in dozens of proteins, it often remains uncertain what physiological role these centers play. Indeed, since Fe\(_3\)S\(_4\) centers can be produced as an artifact upon oxidation of Fe\(_4\)S\(_4\) centers, it has been suggested that 3Fe centers may not be truly physiological, and could be side products of aerobic protein isolation. This caveat notwithstanding, the 3Fe sites are being found in more and more proteins and enzymes. Their physiological \textit{raison d'être} may be more subtle than that of their 1, 2, and 4Fe cousins; we should certainly try to find out more about them. Some proteins containing Fe\(_3\)S\(_4\) centers are listed in Table 7.1.

The 3Fe center was first recognized\(^{147}\) in the protein ferredoxin I from the anaerobic nitrogen-fixing bacterium \textit{Azotobacter vinelandii}. The protein is called \textit{Av} Fdl for short. It is instructive to sketch historically the evolution of our understanding of this protein. \textit{Av} ferredoxin I was reported to have 6 to 8 Fe
atoms and was first thought to resemble the clostridial 8Fe ferredoxins. However, unlike the clostridial protein, the Av FdI clusters appeared to have two quite different redox couples at +320 and −420 mV. Although it might have been thought that this protein contained one HiPIP-type and one Fd- or “ferredoxin”-type Fe₄S₄ cluster, the protein as isolated had an EPR signal with g = 2.01, which differed significantly (Figure 7.6) from that of an oxidized HiPIP or a reduced Fd. Cluster extrusion reactions also seemed to indicate the presence of an unusual cluster type.

Fortunately, the protein was crystallized, and could be studied by x-ray diffraction. Unfortunately, the initial conclusions and subsequent revisions of the crystal-structure analysis have proven to be wrong, teaching us in the process that protein x-ray crystallography, taken alone, does not always provide definitive results. Specifically, the first crystallographic report suggested the presence of a conventional Fe₄S₄ cluster and a smaller packet of electron density that was assigned as a 2Fe-2S center. However, upon further refinement, and following the formulation of the 3Fe center by Mössbauer spectroscopy, a 3Fe-3S center was identified and refined. The “refined” Fe₃S₃ center was a six-membered alternating iron-sulfide ring with an open, almost flat, twisted-boat conformation (Figure 7.14). The Fe-Fe separation of 4.1 Å and the struc-

![Figure 7.14](image)

Fe₃S₃ structures: (A) open Fe₃S₃ structure proposed from initial x-ray studies (now shown to be in error); (B) the thiocubane fragment structure believed present in most Fe₃S₄ proteins; (C) open Fe₃S₄ structure (not found to date).
tural type was unprecedented, and did not agree with the results of resonance Raman spectroscopy, with x-ray absorption spectroscopy on the native protein or on samples from which the Fe₄S₄ center was removed, or even with stoichiometry, which eventually led to the reformulation of the cluster as Fe₃S₄. The x-ray absorption studies (EXAFS) clearly led to the assignment of a 2.7 Å Fe-Fe distance for the 3Fe cluster.

In parallel with the studies on Av FdI, two additional proteins played key roles in the resolution of the nature of the Fe₃ cluster. These are FdII from Desulfovibrio gigas and aconitase from beef heart. Each contains (under certain conditions) only Fe₃S₄ sites, thus enabling more definitive structural, stoichiometric, and spectral information to be acquired. Studies on these proteins using EXAFS, Mössbauer, EPR, and resonance Raman (to which we will return briefly) clearly favor the closed structure shown in Figure 7.14C. Indeed, x-ray crystallography on aconitase by the same group that did the initial x-ray work on Av FdII revealed the compact structure in agreement with the spectroscopy.

In 1988 the structural error in the crystallography of Av FdI was found by two groups, and a new refinement in a corrected space group led to a structure in agreement with the spectroscopy. The Fe₃S₄ cluster has the apoFe thiocubane structure, with each iron atom bound to the protein by a single cysteinyl thiolate. Clearly, even x-ray crystallography is potentially fallible, and its findings must be critically integrated with the data from other techniques in arriving at full structural definition of metalloenzyme sites.

The studies on the ferredoxins from D. gigas present an interesting lesson on the lability of the Fe-S cluster systems. Two distinct proteins from D. gigas, FdI and FdII, contain the same polypeptide chain (6 kDa) in different states of aggregation. Whereas FdI is a trimer containing three Fe₄S₄ clusters, FdII is a tetramer that contains four Fe₃S₄ clusters. The ferredoxins differ in their redox potentials and appear to have different metabolic functions in D. gigas. The oxidation of D. gigas FdI with Fe(CN)₆³⁻ leads to FdII, and treatment of FdII with iron salts leads to FdI. The D. gigas system reveals the lability and interconvertibility of Fe-S clusters. The recently reported crystal structure of D. gigas FdII shown in Figure 7.15 confirms the partial (apoFe) thiocubane Fe₃S₄ center. The iron atoms are ligated by three cysteinyl residues from protein side chains. The cube missing an iron is now firmly established as a viable structural type.

The aconitase system presents yet another fascinating story. Aconitase is a key enzyme in the Krebs cycle, catalyzing the conversion of citrate and isocitrate through the intermediacy of cis-aconitate, as shown in Equation (7.6).
This is a hydrolytic nonredox process, and for some time it was thought that aconitase was a simple Fe$^{2+}$ protein wherein the ferrous iron was involved in the Lewis-acid function of facilitating the hydrolytic reaction. Indeed, aconitase is inactive when isolated from mitochondria, and requires the addition of Fe$^{2+}$ to achieve activity.

Surprisingly, the isolated aconitase was found by analysis and Mössbauer spectroscopy to possess an Fe$_3$S$_4$ site in its inactive form. Low-resolution crystallographic study supports the presence of an apo-Fe thiocubane, Fe$_3$S$_4$ structure in aconitase. Resonance Raman and EXAFS studies clearly fingerprint the Fe$_3$S$_4$ cluster. The current hypothesis for aconitase activation involves the Fe$_3$S$_4$ thiocubane fragment reacting with Fe$^{2+}$ to complete the cube, which is the active form of the enzyme. Recent crystallographic studies confirm the presence of a complete cube in the activated aconitase. The dimensions and positioning of the Fe$_3$S$_4$ and Fe$_4$S$_4$ centers in the cube are virtually identical. The added Fe$^{2+}$ iron atom is ligated by a water (or hydroxide) ligand, consistent with the absence of any cysteine residues near the exchangeable iron. Since this water (or a hydroxide) is also present in the Fe$_3$S$_4$ system, one wonders whether a small ion such as Na$^+$ might be present in the Fe$_3$S$_4$ aconitase system.

Questions of detailed mechanism for aconitase remain open. ENDOR spectroscopy shows that both substrate and water (or OH$^-$) can bind at the cluster. Does one of its Fe-atom vertices play the Lewis-acid role necessary for aconitase activity? Is the Fe$_3$S$_4$ ⇌ Fe$_4$S$_4$ conversion a redox- or iron-activated switch, which works as a control system for the activity of aconitase? These and other questions will continue to be asked. If aconitase is indeed an Fe-S enzyme with an iron-triggered control mechanism, it may be representative of a
large class of Fe₃/Fe₄ proteins. Other hydrolytic enzymes containing similar Fe-S centers have recently been reported.¹⁶⁶,¹⁶⁶ᵃ

Spectroscopically, the Fe₄S₄ center is distinct and clearly distinguishable from 1Fe, 2Fe, and 4Fe centers. The center is EPR-active in its oxidized form, displaying a signal (Figure 7.6) with \( g = 1.97, 2.00, \) and 2.06 \((D. \text{gigas FdII})\).¹⁵⁸

The Mössbauer spectrum (Figure 7.7) shows a single quadrupole doublet with \( \Delta Q = 0.53 \text{ nm/s} \) and isomer shift of 0.27 nm, suggesting the now familiar high-spin iron electronic structure.¹⁵⁸,¹⁵⁹ In its reduced form, the center becomes EPR-silent, but the Mössbauer spectrum now reveals two quadrupole doublets of intensity ratio 2:1. The suggestion of the presence of a 3Fe center was first made based on this observation.¹⁴⁷ The picture of the reduced Fe₄S₄ state that has emerged involves a coupled, delocalized Fe²⁺/Fe³⁺ unit responsible for the outer doublet, with a single Fe³⁺ unit responsible for the inner doublet of half the intensity. The oxidized state contains all Fe³⁺ ions, which are coupled in the trinuclear center.

EXAFS studies were consistent and unequivocal in finding an Fe-Fe distance of ~2.7 Å in all putative Fe₄S₄ proteins.¹⁰³,¹⁵⁵,¹⁶⁷ Resonance Raman spectra compared with those of other proteins and of model compounds with known structures¹⁶⁸,¹⁶⁹ for other metals also favored the structure of Figure 7.14B. Clearly, what has been termed the spectroscopic imperative¹⁷⁰ has been crucial in the successful elucidation of the 3Fe structure.

An interesting excursion has led to isolation of what are presumed to be ZnFe₃S₄, CoFe₃S₄, and NiFe₃S₄ thiocubane structures by adding Zn²⁺, Co²⁺, or Ni²⁺, respectively, to proteins containing reduced Fe₄S₄ cores.¹⁷⁰ᵃ,ᵇ,c These modified proteins provide interesting electronic structural insights and, potentially, new catalytic capabilities.

J. Fe₃ Model Systems

To date, the Fe₃S₄ center is the only structurally characterized biological iron-sulfide center that does not have an analogue in synthetic Fe chemistry. In fact, the closest structural analogue²¹,¹⁷⁰ᵈ is found in the Mo₃S₄⁴⁺ or V₃S₄³⁺ core in clusters such as Mo₃S₄(SCH₂CH₂S)₃²⁻, whose structure is shown in Figure 7.16.

Figure 7.16
The x-ray crystal structure of Mo₃S₄(SCH₂CH₂S)₃²⁻. (From Reference 10a.)
The resonance Raman spectrum\textsuperscript{169} of this complex bears a close resemblance to that of the \textit{D. gigas} ferredoxin II. Since the vibrational bands responsible for the resonance Raman spectrum are not strongly dependent on the electronic properties, it is not surprising that an analogue with a different metal can be identified using this technique.

In synthetic Fe chemistry, although there is no precise structural analogue, it is instructive to consider three types of trinuclear and one hexanuclear center in relation to the three-iron biocenter.

The trinuclear cluster Fe$_3$S[(SCH$_2$)$_2$C$_6$H$_4$]$_3^{2-}$ is prepared\textsuperscript{171,172} by the reaction of FeCl$_3$, C$_6$H$_4$(CH$_2$SH)$_2$, Na$^+$OCH$_3^-$, and p-CH$_3$C$_6$H$_4$-SH in CH$_3$OH. As shown in Figure 7.17A, this cluster has, like the bicluster, a single triply bridging sulfide ion but, unlike the bicluster, it uses the sulfur atoms of the ethane 1,2-dithiolate as doubly bridging, as well as terminal, groups. The inorganic ring Fe$_3$(SR)$_3$Se$_3^{3-}$($X = Cl, Br$) has a planar Fe$_3$(SR)$_3$ core, which resembles the now-discredited structure for \textit{Av FdI}\textsuperscript{173} (Figure 7.14A).

The complex Fe$_3$S$_4$(SR)$_4^{3-}$ is prepared\textsuperscript{174,175} by reaction of Fe(SR)$_4^{2-}$ with sulfur. The x-ray-determined structure reveals two tetrahedra sharing a vertex with the linear Fe-Fe-Fe array shown in Figure 7.17B. This complex has distinctive EPR, Mössbauer, and NMR spectra that allow it to be readily identified.\textsuperscript{174,175,175a} Interestingly, after the complex was reported, a study of (denatured) aconitase at high pH ($>$9.5) revealed that the thiocubane fragment Fe$_3$S$_4$ site in that enzyme rearranged to adopt a structure that was spectroscopically almost identical with that of the linear complex.\textsuperscript{176} Although the state may not have any physiological significance, it does show that Fe-S clusters different

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure717.png}
\caption{The x-ray crystal structures of trinuclear Fe complexes: (A) Fe$_3$S$_4$(SR)$_4^{3-}$; (B) Fe$_3$S[(SCH$_2$)$_2$C$_6$H$_4$]$_3^{2-}$. (Data from References 171, 172, 174, 175.)}
\end{figure}
from the common (conventional) ones already discussed could be important in proteins under certain physiological conditions or in certain organisms; i.e., iron centers first identified synthetically may yet prove to be present in biological systems.

A synthetic cluster that displays features related to the biological Fe$_3$S$_4$ cluster is the hexanuclear cluster Fe$_6$S$_9$(SR)$_2^{4-}$ shown in Figure 7.18. This cluster contains two Fe$_3$S$_4$ units bridged through their diiron edges by a unique quadruply bridging S$^{2-}$ ion ($\mu_4$-S$^{2-}$) and by two additional $\mu_2$-S$^{2-}$ bridges. The inability of synthetic chemists to isolate an Fe$_3$S$_4$ analogue may indicate that in proteins this unit requires strong binding. Significant sequestration by the protein may be needed to stabilize the Fe$_3$S$_4$ unit against oligomerization through sulfide bridges or, alternatively, rearrangement to the stable Fe$_4$S$_4$ center.

K. Fe-S Chemistry: Comments and New Structures

The first successful model system for an iron-sulfur protein was an analogue of the Fe$_4$S$_4$ system, i.e., the system with the largest presently established biological Fe cluster. The reactions used to synthesize the cluster shown in Figure 7.13 are said to involve self-assembly, meaning that starting materials are simply mixed together, and thermodynamic control causes the cluster to assemble in its stable form. Interestingly, the Fe$_4$S$_4$-containing proteins, such as those of *C. pasteurianum*, are considered to be among the most ancient of proteins. Perhaps on the anaerobic primordial Earth, Fe-S clusters self-assembled in the presence of protein ligands to form the progenitors of the modern ferredoxins.

Much progress has been made in synthetic chemistry, and it is clear that both understanding and control of Fe-S chemistry are continuing to grow. New
preparations for known clusters continue to be found, and new clusters continue to be synthesized. Although many of the new clusters appear to be abiological, we should not ignore them or their potential. They add to our understanding of Fe-S chemistry in general, and serve as starting points in the study of heteronuclear clusters. There is also the distinct possibility that one or more of these synthetic clusters represent an existing biological site that has not yet been identified in an isolated system.

Among the "nonbiological" structures that have been synthesized are complexes with Fe$_6$S$_6^{3+/2+}$ cores including the thioprismanes, the octahedron/cube Fe$_6$S$_8^{3+}$ cores, the Fe$_6$S$_9^{2-}$ cores discussed above, the adamantane-like Fe$_6$(SR)$_{10}^{4-}$ complexes related to Zn and Cu structures in metallothioneins, basket Fe$_6$S$_6^{2+}$/Fe$_6$S$_6^{+}$ cores, monocapped prismatic Fe$_7$S$_6^{3+}$ cores, the cube/octahedron Fe$_8$S$_6^{5+}$ cores, and the circular Na$^+$.binding Fe$_{18}$S$_{30}^{10-}$ unit. Representative ions are shown in Figure 7.19. Some of these cores are stabilized by distinctly nonbiological phosphine ligands. Nevertheless, one should not a priori eliminate any of these structures from a possible biological presence. Indeed, recently a novel, apparently six-iron protein from Desulfovibio gigas has been suggested to have the thioprismane core structure first found in model compounds.

L. Detection of Fe-S Sites

Several recent reviews have concentrated on the ways in which the various Fe-S centers can be identified in newly isolated proteins. It is instructive to summarize the central techniques used in the identification of active sites. Optical spectra are usually quite distinctive, but they are broad and of relatively low intensity, and can be obscured or uninterpretable in complex systems. MCD spectra can give useful electronic information, especially when the temperature dependence is measured. EPR spectra, when they are observed, are distinctive, and are usually sufficiently sharp to be useful even in complex systems. Mössbauer and resonance Raman spectroscopies have each been applied with good effect when they can be deconvoluted, and NMR and magnetic susceptibility have given important information in some simple, lower-MW protein systems. X-ray absorption spectra, especially EXAFS, give accurate Fe-S and Fe-Fe distances when a single type of Fe atom is present. Analytical and extrusion data complement the spectroscopic and magnetic information. Extrusion data must be viewed with considerable caution, because of possible cluster-rearrangement reactions. Even x-ray crystallography has led to incorrect or poorly refined structures. In general, no one technique can unequivocally identify a site except in the very simplest systems, and there is continued need for synergistic and collaborative application of complementary techniques to a given system.

M. Redox Behavior

Figure 7.20 shows the ranges of redox behavior known for Fe-S centers. Clearly, the Fe-S systems can carry out low-potential processes. The rubredoxins cover the mid-potential range, and the HiPIPs are active in the high-potential region.
Figure 7.19
Structures of "to date nonbiological" Fe-S clusters: (A) the thioprismane structure;\textsuperscript{177} (B) basket-handle structure;\textsuperscript{182a} (C) monocapped prismatic structure;\textsuperscript{183} (D) adamantane structure;\textsuperscript{182} (E) circular Fe\textsubscript{10}S\textsubscript{30}\textsuperscript{10–} core unit.\textsuperscript{183a,b}
II. MULTISITE REDOX ENZYMES

In making the transition from the relatively simple electron-transfer proteins to the far more complex Fe-S-containing enzymes, we must recognize that the difference in our degree of understanding is enormous. Not only are the catalytic proteins ten to twenty times larger than the redox proteins, but they often also have several subunits and multiple copies of prosthetic groups. Moreover, very few crystal structures are known for the redox enzymes, and none is known at high resolution. In the absence of three-dimensional protein structural information, we do not know the arrangement, relative separation, or orientation of the prosthetic groups. Finally, studies on model systems have not yet approached the sophisticated state that they have for the structurally known centers.

In general, multicomponent redox enzyme systems appear to be organized in two distinct ways to effect their substrate reactions. In the first mode, the enzyme is designed to bring the oxidant and reductant together so that they may directly interact. For example, oxygenases bring O₂ and an organic molecule together, and activate one or both of these reactants to cause them to react directly with each other. This mode can be called proximation, as the reactants are brought near each other by the enzyme catalyst.

In contrast to proximation, many redox enzymes keep the oxidant and reductant well-separated, and use rapid (usually long-distance) internal electron transfer to bring electrons from the reductant to the oxidant. We can term their

The lack of extensive Fe-S proteins in the positive potential region may reflect their instability under oxidizing conditions and their preemption by Mn, Cu, or heme-iron sites (such as in cytochrome c), which function in this region.
mode of action *electrochemical*. The oxidant and reductant are separated spatially. The enzyme provides the "anode" site to interact with the reductant, the "cathode" site to interact with the oxidant, and the wire to allow electronic flow between the "anode" and "cathode" sites. Hydrogenases and nitrogenases adopt the electrochemical mode of redox activation. In hydrogenases, the electron acceptor, even if it must formally take up hydrogen (e.g., NAD$^+$ → NADH), does not interact at the same site as the H$_2$. There is no direct transfer of H$^-$ from H$_2$ to NAD$^+$. Rather, H$_2$ reduces the enzyme at one site, and NAD$^+$ or other acceptors, such as methylene blue, retrieve the electrons at other sites following internal electron transfer. For nitrogenase, the redox partners are even more removed, as a separate protein, the Fe protein, delivers electrons to the FeMo protein, that eventually end up at the FeMoco site ready to reduce N$_2$ to NH$_3$. These enzymes work much like electrochemical cells.

A. Hydrogenase and Nitrogenase

Hydrogenase is the enzyme responsible for the uptake or evolution of H$_2$. Nitrogenase is the enzyme that catalyzes the ATP-dependent reduction of N$_2$ to NH$_3$, with concomitant evolution of H$_2$.

The relationship between H$_2$ and N$_2$ in biology is intricate. Metabolically, H$_2$ use and N$_2$ use are tightly coupled in many nitrogen-fixing organisms, with H$_2$ serving indirectly as the reductant for N$_2$. Moreover, H$_2$ and N$_2$ react in related ways with various transition-metal complexes, which are at present the closest (albeit quite imperfect) models of the enzyme active sites. The biological fixation of molecular nitrogen is dependent on iron-sulfide proteins that also contain molybdenum or vanadium. The biological production or uptake of H$_2$ depends on the presence of iron-sulfide proteins, which often also contain nickel and sometimes selenium. Spectroscopic and model-system studies, which have played such a key role in advancing the understanding of simple Fe-S sites, are now helping to foster an understanding of these more complex enzyme sites, although we have much yet to learn about structure and mechanism in these enzymes. The remainder of this chapter seeks to convey the state of our rapidly evolving knowledge.

B. Hydrogenases

1. Physiological significance

Molecular hydrogen, H$_2$, is evolved by certain organisms and taken up by others. For either process, the enzyme responsible is called hydrogenase. The *raison d'être* for hydrogenases in particular organisms depends on the metabolic needs of the organism. Properties of some representative hydrogenases are given in Table 7.5. Hydrogenases are found in a wide variety of anaerobic bacteria, such as the eubacterial *C. pasteurianum* and *Acetobacterium woodii* and the archaeabacterial
### Table 7.5
Properties of some representative hydrogenases.

<table>
<thead>
<tr>
<th>Organism (designation)</th>
<th>MW (subunits)</th>
<th>Approximate composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>60,000 (1)</td>
<td>12Fe</td>
<td>191</td>
</tr>
<tr>
<td><em>(Hydrogenase I)</em></td>
<td></td>
<td>22Fe</td>
<td>194</td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>53,000 (1)</td>
<td>8Fe</td>
<td>191</td>
</tr>
<tr>
<td><em>(Hydrogenase II)</em></td>
<td></td>
<td>17Fe</td>
<td>194</td>
</tr>
<tr>
<td><em>Acetobacterium woodii</em></td>
<td>15,000</td>
<td>Fe</td>
<td>192</td>
</tr>
<tr>
<td><em>Megasphaera elsdenii</em></td>
<td>50,000 (1)</td>
<td>12Fe</td>
<td>188</td>
</tr>
<tr>
<td><em>Desulfovibrio vulgaris</em></td>
<td>56,000 (2)</td>
<td>12Fe</td>
<td>171</td>
</tr>
<tr>
<td><em>(periplasmic)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Desulfovibrio gigas</em></td>
<td>89,000 (2)</td>
<td>11Fe, 1Ni</td>
<td>363</td>
</tr>
<tr>
<td><em>Desulfovibrio africanus</em></td>
<td>92,000</td>
<td>11Fe, 1Ni</td>
<td>364</td>
</tr>
<tr>
<td><em>Methanobacterium thermoautotrophicum</em></td>
<td>200,000</td>
<td>Fe, 1Ni</td>
<td>145</td>
</tr>
<tr>
<td><em>Methanosarcina Barkeri</em></td>
<td>60,000</td>
<td>8–10Fe, 1Ni</td>
<td>77</td>
</tr>
<tr>
<td><em>Methanococcus variabilis</em></td>
<td>340,000</td>
<td>Fe, Ni, 1Se, FAD</td>
<td>365</td>
</tr>
<tr>
<td><em>Desulfovibrio baculatus</em></td>
<td>85,000 (2)</td>
<td>12Fe, 1Ni, 1Se</td>
<td>365</td>
</tr>
</tbody>
</table>

*Methanosarcina barkeri.* Interestingly, *C. pasteurianum* sometimes evolves H₂ during its growth on sugars. This H₂ evolution is required for continued metabolism, since it allows the organism to recycle (reoxidize) cofactors that are reduced in the oxidation of sugars (or their metabolic descendants, lactate or ethanol). In effect, H⁺ is acting as the terminal oxidant in clostridial metabolism, and H₂ is the product of its reduction. In contrast, methanogens such as methanosarcina take up H₂ and in effect use it to reduce CO₂ to CH₄ and other carbon products. Clearly, either H₂ uptake or H₂ evolution may be important in particular anaerobic metabolic contexts. The hydrogenases of the anaerobic sulfate-reducing bacteria of the genus *Desulfovibrio* have been particularly well-studied (see Table 7.5).

In nitrogen-fixing organisms, H₂ is evolved during the nitrogen-fixation process, and hydrogenase is present to recapture the reducing equivalents, which can then be recycled to fix more nitrogen. In N₂-fixing organisms, such “uptake” hydrogenases can make an important contribution to the overall efficiency of the nitrogen-fixation process. In fact, certain species of rhizobia lacking the hydrogen-uptake system (*hup*⁻ strains) can be made more efficient by genetically engineering the *hup* activity into them.

Aerobic bacteria such as *Azotobacter vinelandii*, *Alcaligenes eutrophus*, and *Nocardia opaca*, and facultative anaerobes, such as *Escherichia coli* and various species of *Rhizobium* and *Bradyrhizobium* (the symbionts of leguminous plants), also contain hydrogenase, as do photosynthetic bacteria such as *Chromatium vinosum*, *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulata*), and *Anabaena variabilis* (a filamentous cyanobacterium). The thermophilic hydro-
gen oxidizer *Hydrogenobacter thermophilus*, which grows in alkaline hot springs above 70°C, obviously has a critical requirement for hydrogenase.

In certain aerobic organisms, such as hydrogenomonas, \( \text{H}_2 \) and \( \text{O}_2 \) are caused to react (but not directly) according to the Knallgas reaction:\[185\]

\[
2\text{H}_2 + \text{O}_2 \longrightarrow 2\text{H}_2\text{O} \quad \Delta G^o = -54.6 \text{ kcal/mol}
\] (7.7)

These organisms break up this thermodynamically highly favorable redox process by using intermediate carriers, thereby allowing the large negative free-energy change to be captured in biosynthetic capacity.

Hydrogenases seem to be especially prevalent in anaerobic, nitrogen-fixing, and photosynthetic organisms. However, although hydrogenases are obviously found widely among prokaryotes, unlike nitrogenase, their domain is not restricted to prokaryotes. Eukaryotic green algae such as *Chlorella fusca* and *Chlamydomonas reinhardtii* possess hydrogenase. The anaerobic protozoan *Trichomonas vaginalis*, which lacks typical aerobic organelles, such as mitochondria and peroxisomes, has an organelle called a hydrogenosome, whose function is to oxidize pyruvate to acetate, producing \( \text{H}_2 \), via hydrogenase, in the process.

The various hydrogenase enzymes are all transition-metal sulfide proteins. However, before we discuss these enzymes, we turn briefly to the dihydrogen molecule and its physical and chemical properties.

2. Dihydrogen: the molecule

Diatominc \( \text{H}_2 \) has a single \( \text{H—H} \) bond formed by overlap of the two 1s orbitals of the two hydrogen atoms. In molecular orbital terms, this overlap forms bonding \( \sigma \) and antibonding \( \sigma^* \) orbitals, shown in the energy-level diagram in Figure 7.21A and displayed spatially in Figure 7.21B. The \( \text{H—H} \) distance is 0.74 Å, and the bond dissociation energy is 103.7 kcal/mole. The isotopes of hydrogen \( ^1\text{H} \), \( ^2\text{H} = ^2\text{D} \), \( ^3\text{H} = ^3\text{T} \) are called protium (a designation seldom used), deuterium, and tritium, respectively. Deuterium, at natural abundance of 0.015 percent, is a stable isotope with nuclear spin \( I = 1 \), whereas both \( ^1\text{H} \) and \( ^3\text{T} \) have nuclear spin \( I = \frac{1}{2} \). NMR has been fruitfully applied to all the hydrogen isotopes, including tritium. Tritium is radioactive, decaying to \( ^3\text{He}_2 \) by \( \beta^- \) emission with a half-life of 12 years. The nuclear properties of deuterium and tritium make them useful as labels to probe structure and mechanism in hydrogen-containing compounds. “Exchange” reactions involving the formation of HD or HT have played a significant role in mechanistic studies of both hydrogenase and nitrogenase.

In molecular hydrogen, the existence of nuclear-spin energy levels is responsible for the distinction between *ortho* and *para* hydrogen, which correspond to the triplet and singlet (i.e., parallel and antiparallel) orientations, respectively, of the two nuclei in \( \text{H}_2 \). Because of the coupling of the rotational and spin levels, *ortho* and *para* hydrogen differ in specific heat and certain other properties. The correlated orientation of the nuclear spins in *para* \( \text{H}_2 \) has re-
recently been shown to constitute a powerful mechanistic probe, wherein NMR may be used to trace the relative fate of the two H nuclei in the original molecule.\textsuperscript{186,187} Although this technique has not yet been applied to any enzyme systems, hydrogenase is known to catalyze the interconversion of the \textit{ortho} and \textit{para} forms of H\textsubscript{2} (as does the hydrogenase analogue Pd).

Dihydrogen is a reducing agent. The H\textsubscript{2}/H\textsuperscript{+} couple at [H\textsuperscript{+}] = 1 M defines the zero of the potential scale. At pH = 7 the hydrogen half-reaction

\[
H_2 \longrightarrow 2H^+ + 2e^- \tag{7.8}
\]
II. MULTISITE REDOX ENZYMES

has \( E_0 = -420 \text{ mV} \). Dihydrogen is therefore one of the strongest biological reductants.

Although many hydrogenases are reversible, some "specialize" in the uptake of \( H_2 \). One hydrogenase has been reported\(^{188}\) to specialize in the evolution of \( H_2 \). This "specialization" seems curious, since it appears to contradict the notion of microscopic reversibility, and seems to violate the rule that catalysts increase the speed of both forward and backward reactions without changing the course (direction) of a reaction. In fact, there is no contradiction or violation, since the overall reactions catalyzed by the various types of hydrogenases are fundamentally different. The electron acceptor in uptake hydrogenases differs from the electron donor/acceptor in the reversible hydrogenases. The difference involves structure and, more importantly, redox potential. The reaction catalyzed by the uptake hydrogenase involves an acceptor of such high positive redox potential that its reaction with \( H_2 \) is essentially irreversible. The enzyme appears to be designed so that it can transfer electrons only to the high potential acceptor.

A selection of hydrogenases from various organisms is given in Table 7.5. All hydrogenases contain Fe-S centers. The hydrogenases from more than 20 organisms\(^{189}\) have been found to contain Ni by analysis and/or spectroscopy. Many more Ni hydrogenases are likely to be found, given the nutritional requirements\(^{189}\) for hydrogenase synthesis or growth on \( H_2 \). Hydrogenases may be cytoplasmic (as in \( C. \) pasteurianum), membrane-bound (as in \( E. \) coli), or located in the periplasmic space (as in \( D. \) vulgaris). The isolation of hydrogenases is sometimes complicated by their air sensitivity or membrane-bound nature. Many hydrogenases have now been isolated and studied in detail; they can be divided into two categories, the iron hydrogenases and the nickel-iron hydrogenases.

3. Iron hydrogenases

The iron hydrogenases\(^{189 a}\) generally have higher activities than the NiFe enzymes, with turnover numbers approaching \( 10^6 \text{ min}^{-1} \). Iron hydrogenases from four genera of anaerobic bacteria have been isolated: \( D. \) vulgaris,\(^{190}\) \( M. \) elsdenii,\(^{188}\) \( C. \) pasteurianum, and \( A. \) woodii.\(^{192}\) Of these, the enzymes from \( D. \) vulgaris, \( M. \) elsdenii, \( C. \) pasteurianum (which contains two different hydrogenases), and \( A. \) woodii have been well-characterized (especially the \( D. \) vulgaris and \( C. \) pasteurianum enzymes). Although \( A. \)木 and \( C. \) are closely related, the other organisms are only distant cousins.\(^{193}\) Nevertheless, their hydrogenases display significant similarities; all contain two different types of iron-sulfur cluster, called F and H clusters,\(^{194}\) and carbon monoxide is a potent inhibitor (although this has not been reported for the \( M. \) elsdenii enzyme). The F clusters are thought to be of the \( \text{Fe}_4\text{S}_4^{+}/^{+2} \) thiocubane type, and give \( S = \frac{7}{2} \) EPR signals when the enzyme is in the reduced form. On the other hand, the H cluster, which is thought to be the hydrogen-activating site, gives an EPR signal only when the
enzyme is in the oxidized form. The H-cluster EPR signals of all the enzymes are quite similar \( (g = 2.09, 2.04, 2.00) \), and are quite unlike the signals from other oxidized iron-sulfide clusters (such as Fe\(_3\)S\(_4\) clusters and HiPiPs), in that they are observable at relatively high temperatures \( (>100 \text{ K}) \). Inhibition of the \( D. \text{ vulgaris} \) and both \( C. \text{ pasteurianum} \) enzymes by carbon-monoxide yields a photosensitive species that has a modified H-cluster EPR signal.\(^{195,196}\)

The two different hydrogenases of \( C. \text{ pasteurianum} \), called hydrogenase I and II, have both been quite extensively studied, and can be regarded as prototypical iron-only hydrogenases. Hydrogenase I is active in catalyzing both \( \text{H}_2 \) oxidation and \( \text{H}_2 \) evolution, whereas hydrogenase II preferentially catalyzes \( \text{H}_2 \) oxidation.\(^{188}\) The two enzymes differ in their iron contents: hydrogenase I contains about 20 iron atoms, 16 of which are thought to be involved in four F clusters,\(^{194}\) while the remainder presumably constitute the H cluster, which may contain six Fe atoms.\(^{194}\) Hydrogenase II contains about 14 iron atoms as two F clusters and one H cluster.\(^{194}\) These estimates of iron content result from a recent reappraisal of the metal contents (based on amino-acid analysis) that indicated a rather higher Fe content than previously realized.\(^{188,194}\) It is important to note that much of the spectroscopic work, which will be discussed below, was initially interpreted on the basis of the earlier, erroneous, iron analysis. Of particular interest is the possibility (first suggested\(^{197}\) for the \( D. \text{ vulgaris} \) enzyme) that the H cluster contains six iron atoms.

Carbon-monoxide treatment of the \( D. \text{ vulgaris} \) and both \( C. \text{ pasteurianum} \) enzymes yields a photosensitive species that has a modified H-cluster EPR signal.\(^{195,196}\) Interestingly, the \( C. \text{ pasteurianum} \) enzymes also form complexes with \( \text{O}_2 \), in a process that is distinguishable from the deactivation of hydrogenase by \( \text{O}_2 \), which results from a much more prolonged exposure to \( \text{O}_2 \) than that required to form the \( \text{O}_2 \) complex. The \( \text{O}_2 \) complexes have (photosensitive) EPR signals much like those of the CO complex.\(^{196}\) It is important to note that although CO, when in excess, is a potent inhibitor of the enzymes, the hydrogenase I-CO complex is actually quite active.\(^{196}\) With hydrogenase II, the CO complex is dissociated on exposure to \( \text{H}_2 \), restoring the “active” enzyme.\(^{196}\)

The EPR spectrum of reduced hydrogenase I is typical of (interacting) Fe\(_4\)S\(_4^+\) clusters, and integrates to 3 or 4 spins/protein.\(^{194,198}\) Electrochemical studies\(^{199}\) show that these clusters possess indistinguishable reduction potentials. Recently, MCD and EPR spectroscopies have been used to demonstrate the presence of significant quantities of an \( S = \frac{3}{2} \) species in reduced hydrogenase I. This signal apparently integrates to about one spin per molecule, and probably originates from an \( S = \frac{3}{2} \) state of an Fe\(_4\)S\(_4\) cluster.\(^{198}\) No information is yet available on the reduction potential of the \( S = \frac{3}{2} \) species. However, based on analogy with the nitrogenase iron protein,\(^{200}\) we might expect the \( S = \frac{3}{2} \) form to have electrochemistry indistinguishable from the \( S = \frac{1}{2} \) form. EPR signals with high \( g \) values \( (g = 6.1 \text{ and } 5.0) \) have also been observed in \( C. \text{ pasteurianum} \) hydrogenase I, and in the \( D. \text{ vulgaris} \) enzyme.\(^{198,201}\) Since there is some uncertainty about the nature and origin of these signals,\(^{198}\) we will not discuss them further. The F clusters of hydrogenase II, on the other hand, give two different EPR signals that integrate to one spin each per protein molecule, and that correspond to sites with different redox potentials.\(^{194,198,199}\) This suggests that hydrogenase II con-
tains two different F clusters, called F and F' (note that the presence of F' was in fact first suggested by Mössbauer spectroscopy\textsuperscript{202}). The EPR spectrum from the F' cluster is unusually broad. The H-cluster EPR signals of active hydrogenase I and II are quite similar and have essentially identical redox potentials.\textsuperscript{199}

The redox behavior of the F and H centers in hydrogenases I and II is nicely consistent with their respective modes of function. As shown in Figure 7.22, the F clusters are presumed to transfer electrons intermolecularly with the exter-

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Figure 7.22
Redox schemes illustrating proposed action of Fe hydrogenases: (A) \textit{Clostridium pasteurianum} hydrogenase I; (B) \textit{Clostridium pasteurianum} hydrogenase II.\textsuperscript{189a,199}
nal electron carrier and intramolecularly with the H center. In the reversible hydrogenase I, the F and H centers have the same redox potentials (about \(-400\) mV at pH 8), similar to that of the hydrogen electrode (\(-480\) mV at pH 8). Thus, electrons may flow in either direction when a mediator such as methyl viologen (E^{o'} = \(-440\) mV at pH 8) is used as the external electron acceptor (methyl viologen is the 4,4'-bipyridinium ion). On the other hand, for hydrogenase II, the F clusters have E^{o'} (pH 8) = \(-180\) mV and E^{o'} > \(-300\) mV for F and F', respectively. In hydrogenase II, therefore, electrons can only move favorably from H\(_2\) to the H cluster, through F', F, and then to a lower-potential acceptor, such as methylene blue [for which E^{o'} (pH 8) = 11 mV].

Mössbauer spectroscopic studies of both hydrogenase I and II have been reported.\(^{202,203}\) Our discussion focuses primarily on the H cluster. The results are similar for the two enzymes, but better defined for hydrogenase II because of the smaller number of clusters. The H cluster apparently contains only two types of iron, in the ratio of 2:1, with quadrupole splittings reminiscent of Fe\(_3\)S\(_4\) clusters. The oxidized cluster is confirmed to be an S = \(\frac{1}{2}\) system, also reminiscent of Fe\(_3\)S\(_4\) clusters, and the reduced H cluster is an S = 0 system; this contrasts with reduced Fe\(_3\)S\(_4\) clusters, which have S = 2. In agreement with the Mössbauer studies, ENDOR spectroscopy of \(^{57}\)Fe-enriched protein indicates at least two different types of iron in the H cluster, with metal hyperfine couplings of about 18 and 7 MHz in hydrogenase II. Rather-less-intense ENDOR features were also observed at frequencies corresponding to couplings of about 11 and 15 MHz.\(^{204}\) The H-cluster EPR signals of hydrogenase II and hydrogenase I change on binding carbon monoxide. Although the signals of the uncomplexed enzymes are quite similar, the signals of the CO-bound species are very different (note, however, that \(C.\) \textit{pasteurianum} CO-bound hydrogenase I has EPR similar to that of the CO-bound \(D.\) \textit{vulgaris} enzyme). When produced with \(^{13}\)C-enriched CO, the EPR signal of hydrogenase II shows resolved \(^{13}\)C hyperfine coupling (A_{av} = 33 MHz) to a single \(^{13}\)C nucleus, indicating that only a single CO is bound, presumably as a metal carbonyl. A slightly smaller coupling of 20 MHz was obtained using ENDOR spectroscopy for the corresponding species of hydrogenase I.\(^{205}\)

Recent ESEEM spectroscopy of hydrogenase I indicates the presence of a nearby nitrogen, which may be a nitrogen ligand to the H-cluster. This nitrogen possesses an unusually large nuclear electric quadrupole coupling and a rather novel structure, involving an amide amino-acid side chain connected to an H-cluster sulfide via a bridging proton ligand, has been suggested for it.\(^{206}\) Although the nitrogen in question must come from a chemically novel species, the proposed proton bridge might be expected to be exchangeable with solvent water. The ENDOR-derived result\(^{205}\) that there are no strongly coupled exchangeable protons in the oxidized H cluster may argue against such a structure.

Rather weak MCD\(^{198,207}\) and resonance Raman spectra\(^{208}\) have also been reported for iron hydrogenases. The lack of an intense MCD spectrum\(^{198,207}\) contrasts markedly with results for other biological FeS clusters. The resonance Raman spectra of hydrogenase I resemble, in some respects, spectra from Fe\(_2\)S\(_2\)
sites. These results further emphasize that the hydrogenase H clusters are a unique class of iron-sulfur clusters.

Perhaps most tantalizing of all are the recent EXAFS results on hydrogenase II. It is important to remember that for enzymes with multiple sites, the EXAFS represents the sum of all sites present (i.e., the iron of the F, F', and H clusters). Despite this complication, useful information is often forthcoming from these experiments. The EXAFS of oxidized hydrogenase I showed both iron-sulfur and iron-iron interactions; the latter, at about 2.7 Å, is at a distance typical of Fe₂S₂, Fe₃S₄, and Fe₄S₄ clusters, and thus is not unexpected. The reduced enzyme, however, gave an additional, long Fe-Fe interaction at 3.3 Å. This Fe-Fe separation is not found in any of the FeS model compounds reported to date. The appearance of the 3.3-Å interaction indicates a change in structure on reduction of the H cluster, again revealing a cluster of unique structure and reactivity. The large structural change of this H cluster on H₂ reduction is likely to have significant mechanistic implications.

4. Nickel-iron hydrogenases

The presence of nickel in hydrogenases has only been recognized relatively recently. Purified preparations of the active enzymes were the subject of quite intensive studies for years before the Ni content was discovered by nutritional studies (see Reference 189 for a history). Some workers even tried (in vain) to purify out “impurity” EPR signals that were later found to be from the Ni. In contrast to the Fe hydrogenases discussed in the previous section, the Ni enzymes possess a variety of compositions, molecular weights, activation behavior, and redox potentials. As Table 7.5 shows, some of the Ni hydrogenases contain selenium, likely in the form of selenocysteine, some contain flavin (FMN or FAD), and all contain iron-sulfur centers, but in amounts ranging from 4 to 14 iron atoms per Ni atom.

Among the different Ni hydrogenases there is a common pattern of protein composition, to which many, but not all, seem to conform (especially those enzymes originating from purple eubacteria). There are two protein subunits, of approximate molecular masses 30 and 60 kDa, with the nickel probably residing in the latter subunit. The hydrogenase of the sulfate-reducing bacterium Desulfovibrio gigas is among the best investigated, and we will concentrate primarily on this enzyme. D. gigas hydrogenase contains a single Ni, two Fe₄S₄ clusters, and one Fe₃S₄ cluster. Of primary interest is the Ni site, which is thought to be the site of H₂ activation.

EPR signals attributable to mononuclear Ni [as shown by enrichment with ⁶¹Ni (I = ⁹/₂)] have been used in numerous investigations of the role of Ni in hydrogenases. Three major Ni EPR signals are known, which are called Ni-A, Ni-B, and Ni-C. The principal g values of these signals are: 2.32, 2.24, and 2.01 for Ni-A; 2.35, 2.16, and 2.01 for Ni-B; and 2.19, 2.15, and 2.01 for Ni-C. Of these, Ni-C is thought to be associated with the most active form of the enzyme (called active); the other two are thought to originate from less-active enzyme forms.
In the enzyme as prepared (aerobically) the Ni-A EPR is characteristically observed. On hydrogen reduction the Ni-A EPR signal disappears, and the enzyme is converted into a higher-activity form (Ni-B arises from reoxidation of this form). Further progressive reduction of the enzyme gives rise to the Ni-C EPR signal, which also finally disappears. These redox properties show that Ni-C arises from an intermediate enzyme oxidation state. Although the Ni-A and Ni-B EPR signals almost certainly originate from low-spin Ni(III), the formal oxidation state of Ni-C is rather less certain. Both an Ni(I) site and an Ni(III) hydride have been suggested, with the former alternative currently favored because of the apparent absence of the strong proton hyperfine coupling expected for the latter. In the fully reduced enzyme, Ni-C is converted to an EPR-silent species. This has variously been suggested to be Ni(0), Ni(II), or an Ni(II) hydride. One possible reaction cycle is shown in Figure 7.23.

Information on the coordination environment of the nickel has been obtained from both x-ray absorption spectroscopy and EPR spectroscopy. The Ni K-edge EXAFS of several different hydrogenases, and EPR spectroscopy of 35S enriched Wolinella succinogenes hydrogenase, clearly indicate the presence of sulfur coordination to nickel. A recent x-ray absorption spectroscopic investigation of the selenium-containing D. baculatus hydrogenase, using both Ni and Se EXAFS, suggests selenocysteine coordination to Ni.

ESEEM spectroscopy of the Ni-A and Ni-C EPR signals indicate the presence of 14N coupling, which probably arises from a histidine ligand to Ni.

Figure 7.23
Proposed activation/reactivity scheme for Ni hydrogenases.
Interestingly, Ni-C, but not Ni-A, shows coupling to a proton that is exchangeable with solvent water. Although this coupling is too small to suggest a nickel-hydride (consistent with conclusions drawn from EPR), the proton involved could be close enough to the Ni to play a mechanistic role.

Despite the extensive studies reported to date, there are still many unanswered questions about the mechanism of the NiFe hydrogenases, which remain as exciting topics for future research. Despite our lack of detailed knowledge of enzyme mechanism, it is nevertheless not premature to seek guidance from inorganic chemistry.

5. Insights from inorganic chemistry

Recent years have brought insights into the way dihydrogen can be bound at a transition-metal site. Unexpectedly, it has been shown that molecular H₂ forms simple complexes with many kinds of transition-metal sites. This finding contrasts with the classical situation, in which H₂ interacts with a transition-metal site by oxidative addition to form a dihydride complex. The H—H bond is largely maintained in the new/nonclassical structures. The dihydrogen and dihydride complexes can exist in simple equilibrium, as in Equation (7.9).

\[
\begin{align*}
M - N≡N + H₂ &\rightarrow M \quad \overset{H}{\rightleftarrows} \quad M \quad \overset{H}{\rightleftarrows} \\
\text{(7.9)}
\end{align*}
\]

The bonding of dihydrogen to a metal occurs via the \( \sigma^b \) orbital of the H—H bond acting as a donor, with the \( \sigma^* \) level of H₂ acting as a weak acceptor. If the back donation is too strong, sufficient electron density will build up in the \( \sigma^* \) level to cause cleavage of the H—H bond, leading to the formation of a dihydride. Dihydrogen complexes therefore require a delicate balance, in which the metal coordination sphere facilitates some back-bonding, but not too much.

The proclivity of a metal center to form H₂ complexes can be judged by the stretching frequency of the corresponding N₂ complexes: N₂ can usually displace H₂ from the H₂ complex to form an N₂ complex without changing the remainder of the coordination sphere. If \( \nu(N≡N) \) is between 2060 and 2160 cm\(^{-1}\), H₂ complexes form upon replacement of N₂. If \( \nu(N≡N) \) is less than 2060 cm\(^{-1}\), indicative of electron back-donation from the metal center, a dihydride complex should form. For example, \( \nu(N≡N) = 1950 \text{ cm}^{-1} \) in MoN₂(PC₃)₅ and MoH₂(PC₃)₅ is a dihydride complex, but \( \nu(N≡N) = 2090 \text{ cm}^{-1} \) in Mo(Ph₂PCH₂CH₂PPh₂)₂CO(N₂) and Mo(Ph₂PCH₂CH₂PPh₂)H₂(CO)₂ is a dihydrogen complex. By comparison, Mo(Et₂PCH₂CH₂PPEt₂)₂(CO)(N₂) has \( \nu(N≡N) = 2050 \text{ cm}^{-1} \) and forms a dihydride complex, Mo(Et₂PCH₂CH₂PPEt₂)₂H₂(CO). The correlation between \( \nu(N≡N) \) and the type of hydrogen complex formed seems quite useful.

Since Fe-S and Ni-S sites are implied for hydrogenase, the reactivity of transition-metal/sulfide systems with H₂ may also be relevant. Interestingly, H₂
can react with metal-sulfide systems at S instead of at the metal site. For example, \(^\text{225}\) \((\text{Cp'})_2\text{Mo}_2\text{S}_4\) reacts with \(\text{H}_2\) to form \((\text{Cp'})_2\text{Mo}_2(\text{SH})_4\). Here the dihydrogen is cleaved without any evidence for direct interaction with the metal center, and the resulting complex contains bridging SH groups and no direct metal-H bonding. \(^\text{225}\) In recent work, \(^\text{226}\) the binuclear rhodium-sulfur complex \(\{\text{RhS}[\text{P}(\text{C}_6\text{H}_5)_2\text{CH}_2\text{CH}_2\text{CH}_3]\}_2\) was reported to react with two equivalents of dihydrogen to yield the complex \(\{\text{Rh}(\text{H})(\text{SH})[\text{P}(\text{C}_6\text{H}_5)_2\text{CH}_2\text{CH}_2\text{CH}_3]\}_2\), in which two SH groups bridge the two Rh centers, each of which contains a single hydrido ligand. Figure 7.24 illustrates the possibilities for hydrogen activation. Each of these types of reactivity must be considered as possibilities for the hydrogen activation process of hydrogenase.

\[ \text{H}_2 \rightarrow \text{H}_2 \rightarrow \text{H}_2 \]

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\[ \text{H}_2 \rightarrow \text{H}_2 \rightarrow \text{H}_2 \]

Recently, a great deal of attention has been given to the chemistry of nickel-sulfur systems, inspired in part by the results showing that many hydrogenases are nickel-sulfur proteins. \(^\text{227–230}\) A particularly interesting finding is that Ni thiolates can react with \(\text{O}_2\) to produce sulfinate complexes. \(^\text{231,232}\) The oxygenated thiolate can be regenerated, thus providing a potential model for the \(\text{O}_2\) inactivation of Ni hydrogenases.

C. Nitrogenases

Nitrogen fixation is a key reaction of the biological nitrogen cycle. \(^\text{233}\) Fixed nitrogen, in which N is in molecules other than \(\text{N}_2\), is frequently the limiting factor in plant growth. \(^\text{234}\) Since natural systems often cannot provide enough fixed nitrogen for agriculture or animal husbandry, industrial processes have been developed to "fix nitrogen" chemically. The major process in use, often referred to as ammonia synthesis, is the Haber-Bosch process, in which \(\text{N}_2\) and \(\text{H}_2\) are reacted at temperatures between 300–500°C and pressures of more than 300 atm, using catalysts (usually) based on metallic iron. \(^\text{235}\) Hundreds of massive chemical plants are located throughout the world, some producing more than 1,000 tons of \(\text{NH}_3\)/day. In contrast, in the biological process, \(\text{N}_2\) is reduced...
locally as needed at room temperature and $\sim 0.8$ atm by the enzyme system called nitrogenase (variously pronounced with the accent on its first or second syllable).

1. The scope of biological nitrogen fixation

Biological nitrogen fixation occurs naturally only in certain prokaryotic organisms (sometimes called diazotrophs). Although the majority of bacterial species are not nitrogen fixers, the process of nitrogen fixation has been confirmed in at least some members of many important phylogenetic groups. Nitrogen fixation occurs in strict anaerobes such as *Clostridium pasteurianum*, in strict aerobes such as *Azotobacter vinelandii*, and in facultative aerobes such as *Klebsiella pneumoniae*. Much of the established biochemistry of $N_2$ reduction has been gleaned from studies of these three species. However, nitrogen fixation has a far broader range, occurring in archaebacterial methanogens such as *Methanobacillus omelianskii*, which produce methane, and eubacterial methanotrophs such as *Methyllococcus capsulatus*, which oxidize methane. Photosynthetic organisms ranging from the purple bacterium *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulata*) to the cyanobacterium (blue-green alga) *Anabaena cylindrica* fix nitrogen. Nitrogen fixation occurs mostly in mesophilic bacteria (existing between 15 and 40°C), but has been found in the thermophilic archaebacterial methanogen *Methanococcus thermolithotrophicus* at 64°C.

Many organisms fix $N_2$ in nature only symbiotically. Here the most studied systems are species of *Rhizobium* and *Bradyrhizobium*, which fix nitrogen in the red root nodules of leguminous plants such as soybeans, peas, alfalfa, and peanuts. The red color inside the nodules is due to leghemoglobin, a plant $O_2$-binding protein analogous to animal myoglobins and hemoglobins (Chapter 4). Other symbioses include that of blue-green algae such as *Anabaena azollae* with *Azola* (a water fern); actinomycetes such as *Frankia* with trees such as alder; and *Citrobacter freundii*, living in the anaerobic hind gut of termites. The distribution of nitrogenase clearly points to its adaptability as a metabolic option for species occupying widespread ecological niches.

The absence of nitrogen fixation in eukaryotes therefore seems somewhat puzzling. There would appear to be no fundamental limitation to the existence of nitrogen fixation in higher organisms. Indeed, *nif* genes have been transferred to yeast, where they work effectively under anaerobic conditions. Furthermore, the problem of the simultaneous presence of nitrogen fixation and aerobiosis has been solved effectively by aerobic bacteria such as *Azotobacter*, *Gleocapsa*, and *Anabaena*. Indeed, the lack of a fundamental limitation has encouraged researchers to propose the construction of nonsymbiotic nitrogen-fixing plants (whose niche to date is limited to the grant proposal).

Due to the mild conditions under which it occurs, the biological nitrogen-fixation process may seem inherently simpler than the industrial one. However, it is not; the biological process displays a complexity$^{235,235a}$ that belies the simplicity of the chemical conversion of $N_2 \rightarrow 2NH_3$. Genetic analysis reveals that
at least twenty genes are required for nitrogen fixation in the bacterium *Klebsiella pneumoniae*.236,237 These *nif* genes (illustrated in Figure 7.25) specify proteins that are involved in regulation (*nif* A and L), pyruvate oxidation/flavin reduction (*nif* J), electron transfer (*nif* F for flavodoxin), the subunits of the structural proteins of the nitrogenase (*nif* H, D, K), Fe-S cluster assembly (*nif* M) and biosynthesis of the iron-molybdenum cofactor, FeMoco (*nif* N, B, E, Q, V, H).238 The last two functions specify proteins that are responsible for the incorporation of unusual transition-metal sulfide clusters into the nitrogenase proteins. These clusters have allowed nitrogenase to be studied by biophysical and bioinorganic chemists to establish aspects of its structure and mechanism of action.

We will first discuss the N\(_2\) molecule and focus on its reduction products, which are the presumed intermediates or final product of nitrogen fixation. We then present what has been called239,240 the "Dominant Hypothesis" for the composition, organization, and function of molybdenum-based nitrogenases. Until 1980, it was thought that molybdenum was essential for nitrogen fixation. However, work starting in 1980 led finally in 1986 to the confirmation of vanadium-based nitrogen fixation. The newly discovered vanadium-based nitrogenases dif-
fer in reactivity from the Mo-based enzyme in having "alternative" substrate specificity. The distinct reaction properties of the different nitrogenases point to the importance of the study of alternative substrate reactions in probing the mechanism of nitrogen fixation.

2. Dinitrogen: The molecule and its reduced intermediates

The N\textsubscript{2} molecule has a triple bond with energy 225 kcal/mole, a \(\nu(\text{N==N})\) stretch of 2331 cm\(^{-1}\), and an N==N distance of 1.098 Å. The stable isotopes of nitrogen are \(^{14}\text{N}(I = 1)\) with natural abundance of 99.64 percent and \(^{15}\text{N}(I = \frac{1}{2})\) with an abundance of 0.36 percent.

The challenge to which nitrogenase rises is to break and reduce at a reasonable rate the extremely strong N==N triple bond. The kinetic inertness of N\textsubscript{2} is highlighted by the fact that carrying out reactions "under nitrogen" is considered equivalent to doing the chemistry in an inert atmosphere. Despite this kinetic inertness, thermodynamically the reduction of N\textsubscript{2} by H\textsubscript{2} is a favorable process,

\[\text{N}_2 + 8\text{H}^+ + 6\text{e}^- \rightarrow 2\text{NH}_3\quad \Delta G^o = -3.97 \text{ kcal/mole}\]  

and at pH = 7 the reaction

\[\text{N}_2 + 8\text{H}^+ + 6\text{e}^- \rightarrow 2\text{NH}_4^+\quad E^{o'} = -280 \text{ mV}\]  

has an \(E^{o'}\) value that makes it easily accessible to biological reductants such as the low-potential ferredoxins discussed earlier in this chapter.

What, then, is the cause of the kinetic inertness of the N\textsubscript{2} molecule? The thermodynamically favorable reduction of N\textsubscript{2} to 2NH\textsubscript{3} is a six-electron process. Unless a concerted 6e\(^-\), 6H\(^+\) process can be effected, intermediates between N\textsubscript{2} and NH\textsubscript{3} must be formed. However, all the intermediates on the pathway between N\textsubscript{2} and NH\textsubscript{3} are higher in energy than either the reactants or the products. The \(E^{o'}\) values for the formation of N\textsubscript{2}H\textsubscript{2} (=diimine, diazene, diamide) or N\textsubscript{2}H\textsubscript{4} (hydrazine) are estimated\(^{24}\) as

\[\text{N}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{N}_2\text{H}_2\quad E^{o'} \sim -1000 \text{ to } -1500 \text{ mV}\]  

\[\text{N}_2 + 5\text{H}^+ + 4\text{e}^- \rightarrow \text{N}_2\text{H}_5^+\quad E^{o'} = -695 \text{ mV}\]  

Clearly, these potentials are sufficiently negative that the normal biological reductants cannot effect the reaction. The difficulty of reaching these intermediates is indicated in Figure 7.26.

Several factors may allow this barrier to be overcome. First, the six-electron reduction might be carried out in a concerted or near-concerted manner to avoid the intermediates completely. Alternatively, the intermediates could be complexed at metal centers to stabilize them to a greater extent than either the reactants or products. Finally, the formation reaction for the unfavorable intermedi-
ate could be coupled with ATP hydrolysis or with the evolution of dihydrogen, each a favorable process, so that the overall process is favorable. Which of the above strategies is used by nitrogenase is unknown, but it seems likely that some combination of the last two of these is used to effect the difficult reduction of N₂ to NH₃. To probe the possibilities, a variety of complexes of N₂, diazenes, and hydrazines has been prepared and chemically characterized, and these are discussed toward the end of this section.

3. The Dominant Hypothesis for molybdenum nitrogenase²³⁹,²⁴⁰,²⁴²,²⁴³

The action of the Mo-nitrogenase enzyme involves the functioning of two separately isolatable component proteins, as sketched in Figure 7.27A. The larger of the two proteins, sometimes incorrectly²⁴⁴ designated²⁴⁵ dinitrogenase has, in the past, been called molybdoferredoxin, azofermo, or component I. More often this protein is called the MoFe or FeMo protein ([MoFe] or [FeMo]). The smaller protein, formerly called azoferredoxin or component II, is sometimes incorrectly²⁴⁴ referred to²⁴⁵ as dinitrogenase reductase. * This protein is properly

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* The nomenclature proposal²⁴⁵ that [FeMo] be designated as dinitrogenase and [Fe] as dinitrogenase reductase, although sometimes used in the literature, is incorrect or, at best, premature.²⁴⁴ The suggested nomenclature implies that both [FeMo] and [Fe] are enzymes. However, neither protein can function catalytically in the absence of the other. [FeMo] will not reduce N₂ or C₂H₂ or evolve H₂ in the absence of [Fe]. The iron protein will not hydrolyze MgATP in the absence of [FeMo]. Nitrogen fixation requires the simultaneous presence of both proteins. Although mechanistic considerations²³⁵ point to [FeMo] as the substrate binding and reducing protein, and [Fe] as the ATP binding locus, catalytic reactions characteristic of this enzyme system have never been consumated by one protein in the absence of the other (but see later for the uptake of H₂). In this chapter, we use the [FeMo] and [Fe] designations in accord with most workers in the field.
Table 7.6
Properties of some representative nitrogenases.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Component</th>
<th>MW</th>
<th>Metal content</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>[MoFe]</td>
<td>234,000</td>
<td>2Mo, 34–38Fe, 26–28S</td>
<td>366, 367</td>
</tr>
<tr>
<td></td>
<td>[Fe]</td>
<td>64,000</td>
<td>3.4Fe, 2.8S</td>
<td></td>
</tr>
<tr>
<td><em>Azotobacter chroococcum</em></td>
<td>[MoFe]</td>
<td>227,000</td>
<td>2Mo, 22Fe, 20S</td>
<td>366, 368</td>
</tr>
<tr>
<td></td>
<td>[Fe]</td>
<td>65,400</td>
<td>4Fe, 3.9S</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium pasteurianum</em></td>
<td>[MoFe]</td>
<td>221,800</td>
<td>2Mo, 24Fe, 24S</td>
<td>366, 369</td>
</tr>
<tr>
<td></td>
<td>[Fe]</td>
<td>55,000</td>
<td>4Fe, 4S</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>[MoFe]</td>
<td>229,000</td>
<td>2Mo, 32Fe, 24S</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td>[Fe]</td>
<td>66,800</td>
<td>4Fe, 3.8S</td>
<td></td>
</tr>
<tr>
<td><em>Anabaena cylindrica</em></td>
<td>[MoFe]</td>
<td>223,000</td>
<td>2Mo, 20Fe, 20S</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>[Fe]</td>
<td>60,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>[MoFe]</td>
<td>215,000</td>
<td>2Mo, 25–30Fe, 19–22S</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>[Fe]</td>
<td>60,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

called the Fe protein or [Fe]. A useful nomenclature for discussions of kinetics and comparative biochemistry designates the FeMo protein as Xyl, where X and y are the first letters of the first and second name of the bacterial source, respectively. For example, *Cpl* is the FeMo protein from *Clostridium pasteurianum*. Similarly, for the Fe protein the designation Xy2 is given; for example, the Fe protein of *Azotobacter vinelandii* is called Av2. This system will be used where appropriate to distinguish the protein source. Properties of representative Mo nitrogenases are given in Table 7.6.

The schematic diagram in Figure 7.27 shows some of the compositional and functional relationships of the nitrogenase proteins. The iron protein contains two identical subunits of MW ~ 30 kDa. The subunits are products of the *nif* H gene. A single Fe₄S₄ center is present in the protein and appears to be bound between the subunits. A recent x-ray structure of the iron protein confirms this picture. As shown in Figure 7.27A, the single Fe₄S₄ center is located at one end of the molecule, in the only region of significant contact between the two subunits. In vivo, the Fe protein is reducible by flavodoxin or ferredoxin. In vitro, artificial reductants such as dithionite or viologens are generally used. The single Fe₄S₄ center undergoes a single one-electron redox process, wherein the reduced form is EPR-active and the oxidized form is diamagnetic. As such, this center resembles four-iron-cluster-containing ferredoxins. Its redox potential is dependent on the ATP or ADP level in the solution. For example, *Cp2* (the Fe protein from *Clostridium pasteurianum*) shows E°⁺ = −294 mV in the absence and −400 mV in the presence of MgATP. Two equivalents of MgATP and MgADP each bind to [Fe].

Until recently there was a major mystery over the number of Fe₄S₄ centers in [Fe] as deduced by EPR quantitation of the Fe₄S₄ centers compared to the number derived analytically or by extrusion experiments. However, it has now been clearly established that the single Fe₄S₄ center can exist in
Figure 7.27
(A) Preliminary x-ray crystal structure of the *Azotobacter vinelandii* nitrogenase Fe protein.\(^{246b}\)
(B) Schematic of the nitrogenase proteins illustrating their composition and mode of action.

- *Alternative nitrogenase contains FeVco*
this protein in two spin states, $S = \frac{1}{2}$ and $S = \frac{3}{2}$. Only that part of the EPR signal corresponding to the $S = \frac{1}{2}$ form, with its $g$ values near 2, was considered in earlier spin quantitations. When the $S = \frac{3}{2}$ center, with $g$ values between 4 and 6, is included, the EPR spin integration shows one paramagnetic site per Fe$_4$S$_4$ unit. Model systems$^{121,122}$ and theoretical studies$^{123,124,251}$ strongly support the ability of Fe$_4$S$_4$ to exist in various spin states. During enzyme turnover, the single Fe$_4$S$_4$ of the Fe-protein center transfers electrons to the FeMo protein in one-electron steps. There is no evidence for any difference in redox behavior between the $S = \frac{1}{2}$ and $S = \frac{3}{2}$ states of the protein.$^{200}$

The Fe protein binds two molecules of MgATP.$^{252}$ The recent structure$^{246b,378}$ suggests that a cleft between the two subunits may serve as the ATP binding site. As the enzyme system turns over, a minimum of two molecules of MgATP are hydrolyzed to MgADP and phosphate in conjunction with the transfer of each electron to the FeMo protein.$^{253}$ The ATP/2e$^-$ ratio is generally accepted to have a minimum value of 4. Higher numbers represent decreased efficiency, often attributed to "futile cycling," where back electron transfer from [FeMo] to [Fe] raises the effective ratio.$^{248,253}$ Except for an as-yet-unconfirmed report of reduction by thermalized electrons produced by pulse radiolysis,$^{254}$ there is no evidence that the FeMo protein can be reduced to a catalytically active form without the Fe protein present.

Even though [Fe] must be present for catalysis to take place, the Dominant Hypothesis$^{239}$ designates [FeMo] as the protein immediately responsible for substrate reduction, and genetic/biochemical evidence supports this view. The FeMo protein contains an $\alpha_2\beta_2$ subunit structure, where $\alpha$ and $\beta$ are coded by the nif D and nif K genes,$^{15,229}$ respectively. The overall molecular weight of about 230 kDa reflects the 50- to 60-kDa MW of each of its four subunits. In addition to protein, a total of 30 Fe, 2 Mo, and 30 S$^{2-}$, all presumed to be in the form of transition-metal sulfide clusters, add relatively little to the molecular weight, but are presumed to be major parts of the active centers of the protein. Figure 7.27, which is highly schematic, displays the cluster types in accord with the Dominant Hypothesis.

4. Protein purity and active sites

It has been almost 20 years since the first relatively pure preparations of nitrogenase became available. Indeed, homogeneous preparations are a sine qua non for progress in our understanding of the chemical nature and reactivity of any active site. In metalloproteins, there are two levels of homogeneity. The first involves purity with respect to the protein/subunit composition. This type of purity is achieved by conventional protein-purification techniques, and can be monitored by gel electrophoresis under native and denaturing conditions. In the language of polymer science, the macromolecular portion of the protein can be said to be monodisperse, corresponding to a single molecular weight for the polypeptide chain(s). However, even if the protein is homogeneous by this criterion, it may be inactive or only partially active because it does not have a full
complement of active metal sites. The metal sites may be empty, filled with the wrong metals, or otherwise imperfect. Often the apo or inactive enzyme has chromatographic, electrophoretic, and centrifugal behavior very much like that of the holo protein, and therefore copurifies with it. Therefore, purification to electrophoretic homogeneity is only the first step. It is then necessary to ensure the chemical homogeneity of the active site. Very often activity is the major criterion for the approach toward such purity; i.e., the most homogeneous preparations are usually those in which the activity is highest. Several studies done on preparations that lacked active-site homogeneity were, as a result, not meaningful.

The two types of centers present in the nitrogenase FeMo protein are designated P clusters and FeMoco (or M) centers. Both types of centers display unique spectroscopic properties, but only FeMoco continues to display most of those properties when it is extracted from the protein.

5. FeMoco

The presence of the FeMo cofactor within the FeMo protein of nitrogenase, i.e., the M center, is revealed through spectroscopic and redox studies. In the resting state of [FeMo], as isolated in the presence of dithionite, the M center has a distinct $S = \frac{3}{2}$ EPR signal, which is discussed below (see Figure 7.28).

Figure 7.28
EPR spectra: (A) the $S = \frac{3}{2}$ M center in Clostridium pasteuriunum nitrogenase FeMo protein; and (B) the FeMoco extracted into NMF from the protein. (Spectra courtesy of R. Bare and G. N. George.)
When the enzyme is turning over the EPR signal essentially disappears, leaving an EPR-silent state in which the FeMoco site is super-reduced to what is presumed to be its catalytically active form. In addition, a third state in which the $S = \frac{3}{2}$ EPR signal disappears is produced upon oxidation under non-turnover conditions. Thus the M center within the protein shows three states of oxidation, and these appear to have been reproduced in the FeMoco extracted from the protein: 255a

$$\text{FeMoco (oxidized)} \rightarrow \text{FeMoco (reduced)} \rightarrow \text{FeMoco (super-reduced)} \quad (7.14)$$

The detailed characterization of the FeMoco site has involved parallel studies of the site within the protein and in its extracted form. The authentication of the extracted FeMoco involves the production and use of mutant organisms that make an inactive FeMo protein that contains all subunits and P clusters, but lacks the FeMoco sites. 255,256 A mutant of *Azotobacter vinelandii* called UW-45 (UW = University of Wisconsin) was first used to assay for isolated FeMoco. 257 Since several genes are involved in specifying FeMoco biosynthesis, mutants lacking these genes produce FeMo protein either lacking FeMoco or having a defective version of FeMoco. Mutants such as *Nif B* of *Klebsiella pneumoniae* 172 lack cofactor, and an inactive "apo" protein can be isolated from them.

The breakthrough in this field 257 came in 1976, when FeMoco was extracted from [FeMo] into N-methylformamide 258 after the protein was acidified and then neutralized. The acidification removes most of the acid-labile P clusters, and partially denatures the protein. Renumeralization precipitates the protein (near its isoelectric point) and the precipitated denatured protein can then be extracted.

It has been shown that FeMoco can be extracted into many organic solvents, 10,257,259–259b provided proper combinations of cations and anions 259a are present in the solvent. The role of the cation is to balance the charge of the negatively charged cofactor. The role of the anion is to displace the cofactor from anion-exchange columns, such as DEAE cellulose or TEAE cellulose, to which the cofactor and/or its protein source had been adsorbed. The ability to dissolve cofactor in such solvents as CH$_3$CN, acetone, THF, and even benzene should facilitate attempts at further characterization and crystallization. 259,259a

The biochemical authenticity of FeMoco has been assayed by its ability to activate the FeMo protein from the cofactor-less mutant organism. 258 The stoichiometry of the cofactor is MoFe$_6$–S$_7$–O$_{10}$, with the variability likely due to sample inhomogeneity. The extracted cofactor resembles the M-center unit spectrosopically and structurally as shown in Table 7.7. The differences are presumed to result from differences in the peripheral ligands of the metal-sulfide center between the protein and the organic solvent. 260

Strong evidence to support FeMoco as the site of substrate binding and reduction comes from the study of *nif V* mutants, 261–263 (The V designation is somewhat unfortunate, as *nif V* has nothing to do with vanadium.) The *Nif V* mutants do not fix nitrogen in vivo, and have altered substrate specificity in
Dihydrogen evolution by isolated \textit{nif V} nitrogenase is inhibited by CO, in contrast to the wild type, where H\textsubscript{2} evolution is insensitive to CO. FeMoco can be extracted from the \textit{nif V} protein and used to reactivate the FeMoco-deficient mutants, such as \textit{nif B} or UW-45. Remarkably, the reconstituted FeMo protein has CO-sensitive H\textsubscript{2} evolution, which is characteristic of \textit{nif V}; i.e., the \textit{nif V} phenotype is a property of FeMoco and not of the protein.\textsuperscript{263} This result clearly implicates the FeMoco site as an important part of the substrate reactions of the nitrogenase enzyme complex.

Recently, a heat-stable factor called the V-factor has been discovered that restores the wild-type phenotype when added to \textit{nif V} mutants during \textit{in vitro} FeMoco assembly reactions.\textsuperscript{264} The V-factor has been shown to be homocitrate (see Scheme 7.15) and \textsuperscript{14}C labeling strongly suggests that homocitrate (or a part of it) is a component of the cofactor center. Interestingly, the far more metabolically common citrate appears to be present in the \textit{nif V} mutant.\textsuperscript{265a} Replacement of homocitrate by analogues that differ in structure or stereochemistry yields modified FeMoco sites that have altered substrate specificities.\textsuperscript{265b} Thus, as is true for many cofactors (e.g., heme = porphyrin + iron; B\textsubscript{12} = corrin + cobalt; F430 = corphin + nickel; Moco, the molybdenum cofactor = Mo + molybdopterin), both inorganic and organic components are present in FeMoco.

\begin{equation}
\begin{array}{c}
\text{Homocitrate} \\
\text{CH}_2\text{CH}_2\text{-COOH} \\
\text{HO-C-COOH} \\
\text{CH}_2\text{-COOH} \\
\end{array} \quad \begin{array}{c}
\text{Citrate} \\
\text{CH}_2\text{CH}_2\text{-COOH} \\
\text{HO-C-COOH} \\
\text{CH}_2\text{-COOH} \\
\end{array} \quad (7.15)
\end{equation}

The biosynthesis of the cofactor and its insertion into [FeMo] apparently requires the presence of [Fe] and ATP in \textit{A. vinelandii}.\textsuperscript{266,266a} Whether this involves redox or conformational change in [FeMo] induced by [Fe] is unknown, but the fact that inactive versions of [Fe] are effective would seem to favor the nonredox mechanism. An attractive idea\textsuperscript{266} is that [Fe]·MgATP binds to [FeMo], producing a state that is conformationally accessible for cofactor insertion.

Recently, site-directed mutagenesis studies\textsuperscript{266b,c} have shown that cysteine residues are involved in binding FeMoco to the subunits of [FeMo]. Moreover, these studies again implicate FeMoco in the substrate-reducing site.

6. The P-clusters

Evidence has been presented\textsuperscript{229} for the presence of four Fe\textsubscript{4}S\textsubscript{4}-like clusters (designated as P-clusters) in [FeMo]. The P-clusters are, however, by no means ordinary Fe\textsubscript{4}S\textsubscript{4} clusters, and may not be Fe\textsubscript{4}S\textsubscript{4} clusters at all. P-clusters are manifest\textsuperscript{239,248} in electronic absorption and, especially, MCD and Mössbauer spectra of [FeMo]. These spectra are clearly not conventional; i.e., they are not
like those found in ferredoxins and have not yet been seen in model compounds. In their oxidized forms, the P-clusters are high-spin, probably $S = \frac{5}{2}$, according to EPR studies. Mössbauer spectra reveal decidedly inequivalent Fe populations, indicating that the putative Fe$_4$S$_4$ clusters are highly distorted or asymmetric. The four P-clusters do not appear to behave identically under many circumstances, and it is clear that they form at least two subsets. There is open disagreement over the redox behavior of these sets. Furthermore, an additional Mössbauer signal sometimes designated as $S$ may also be part of the P-cluster signal.

Although spectroscopic studies of the P-clusters do not unequivocally reveal their structural nature, extrusion of these clusters from the protein leads to the clear identification of three or four Fe$_4$S$_4$ clusters. As discussed previously, the extrusion technique has inherent uncertainties, because it may be accompanied by cluster rearrangement. Nevertheless, the experimental result does support the Dominant Hypothesis, which designates the P centers as highly unusual Fe$_4$S$_4$ clusters.

The P-clusters are thought to be involved in electron storage and transfer, and presumably provide a reservoir of low-potential electrons to be used by the M center (FeMoco) in substrate reduction. Attractive as it may seem, there is no direct evidence to support this notion.

7. EPR, ENDOR, and ESEEM studies

The FeMoco or M center has been identified spectroscopically within the FeMo protein; it has a distinctive EPR signal with effective $g$ values of 4.3, 3.7, and 2.01, and originates from an $S = \frac{3}{2}$ state of the M center. The signal arises from transitions within the $\pm \frac{1}{2}$ ground-state Kramers doublet of the $S = \frac{3}{2}$ system ($D = +5.1$ cm$^{-1}$, $E/D = 0.04$). The isolated cofactor (FeMoco) gives a similar EPR signal, but with a rather larger rhombicity ($E/D = 0.12$). Spectra from the C. pasteurianum nitrogenase and cofactor are shown in Figure 7.28, and comparative data are given in Table 7.7. The M-center EPR signal has proved useful in characterizing the nature of the site, especially when more sophisticated magnetic resonance techniques, such as ENDOR or ESEEM, are used.

Extensive ENDOR investigations have been reported using protein samples enriched with the stable magnetic isotopes $^2$H, $^{33}$S, $^{57}$Fe, $^{95}$Mo, and $^{97}$Mo. The $^{57}$Fe couplings have been investigated in the most detail. Individual hyperfine tensors of five coupled $^{57}$Fe nuclei are discernible, and were evaluated by simulation of the polycrystalline ENDOR spectrum. The data from $^{33}$S and $^{95}$Mo were analyzed in less detail; $^{33}$S gave a complex ENDOR spectrum, evidently with quite large hyperfine couplings, although no quantification was attempted because of the complexity of the spectrum. On the other hand, $^{95}$Mo was shown to possess a small hyperfine coupling, indicating that the molybde-

*Recent x-ray crystallographic results show that, if Fe$_3$S$_4$ clusters are present, they are very close together in two pairs, which may account for their unusual properties.
Table 7.7
Comparison of the FeMo protein and isolated FeMoco.

<table>
<thead>
<tr>
<th></th>
<th>FeMo protein (M center)</th>
<th>FeMoco (in NMF)</th>
</tr>
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<tbody>
<tr>
<td>EPR g' values</td>
<td>4.27 4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.79 3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.01 2.0</td>
<td></td>
</tr>
<tr>
<td>EXAFS a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mo-S</td>
<td>2.36 (4)b</td>
<td>2.37 (3.1)c</td>
</tr>
<tr>
<td>Mo-Fe</td>
<td>2.69 (3)b</td>
<td>2.70 (2.6)c</td>
</tr>
<tr>
<td>Mo-O or N</td>
<td>2.18 (1)b</td>
<td>2.10 (3.1)c</td>
</tr>
<tr>
<td>Fe-S</td>
<td>2.25 (3.4)d 2.20 (3.0)e</td>
<td>3.68 (0.8)e</td>
</tr>
<tr>
<td>Fe-Fe</td>
<td>2.66 (2.3)d 2.64 (2.2)c</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-Mo</td>
<td>2.76 (0.4)d 2.70 (0.8)e</td>
<td>2.25 (3.0)d</td>
</tr>
<tr>
<td>Fe-O or N</td>
<td>1.81 (1.2)d</td>
<td>2.64 (2.2)e</td>
</tr>
<tr>
<td>XANES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MoO₅S₃ fits best f</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Distance in Å with number of atoms in parentheses.
b From 287; earlier study reported in 286.
c Data from 373; earlier study reported in 372.
d Data from 290.
e Data from 291.
f Data from 288, 289.

Numerous possess very little spin density (although the quantitative aspects of the conclusions of the ⁵⁷Mo ENDOR study have recently been shown to be in error ²⁷⁶). Although no nitrogen splittings were reported in any of the ENDOR studies, evidence for involvement of nitrogen as a cluster component has been forthcoming from ESEEM spectroscopy.²⁷⁷–²⁷⁹ ¹⁴N modulations are observed in the ESEEM of the M center. The observed ¹⁴N is not from the substrate (N₂), or from an intermediate or product of nitrogen fixation, because enzyme turnover using ¹⁵N as a substrate does not change the ESEEM spectrum. The isolated cofactor (FeMoco) does not show the modulation frequencies observed for the M center in the protein. These experiments suggest that the M-center ¹⁴N ESEEM arises from a nitrogen atom that is associated with the M center, and probably from an amino-acid side chain (most likely a histidine) ligated to the cluster.²⁷⁹ Recent evidence from site-directed mutagenesis of the Azotobacter vinelandii protein²⁸⁰ provides strong support for the presence of histidine ligation, and points specifically to His-195 of the α subunit as the N ligand.

8. Mössbauer studies

Extensive Mössbauer investigations of nitrogenase²⁷¹,²⁸¹–²⁸³ and FeMoco²⁸³ have been reported. Unlike EPR and EPR-based spectroscopies, which can be
used to investigate only the EPR-active $S = \frac{3}{2}$ oxidation state, all three available M-center oxidation states are accessible to Mössbauer spectroscopy. The fully reduced site was found to be diamagnetic with $S = 0$ (but see Reference 284), whereas the oxidized site was found to have $S \geq 1$. The zero-field spectrum of reduced C. pasteurianum nitrogenase is shown in Figure 7.29; the spectrum is comprised of four quadrupole doublets, one of which was concluded to originate from the M site.282 Mössbauer spectra taken in the presence of applied magnetic fields were used to deduce the presence of four types of $^{57}$Fe hyperfine coupling; these were called sites A1, A2, and A3, which have negative hyperfine couplings, and B sites, which have positive hyperfine couplings. The A sites were quantitated as a single Fe each; the B sites were estimated to contain three irons. These conclusions were largely confirmed and extended by later ENDOR investigations,274 although the B sites were resolved as two inequivalent, rather than three equivalent, sites. ENDOR is rather more sensitive to the nature of the hyperfine couplings than Mössbauer, although it cannot usually be used to count numbers of exactly equivalent sites. Thus the number of iron atoms in the M center is minimally five, although larger numbers cannot be excluded. Note also that some of the quantitative aspects of the earlier Mössbauer investigations have been criticized.285

![Figure 7.29](image-url)

Mössbauer spectrum of C. pasteurianum nitrogenase FeMo protein,282 indicating the various components (quadrupole doublets) and their assignments. The doublet labeled M is the cofactor signal; those labeled D, S, and Fe$^{2+}$ are attributed to the P-clusters.
9. X-ray absorption studies

One of the early triumphs of biological x-ray absorption spectroscopy was the deduction that the nitrogenase M center is an Mo-Fe-S cluster.\textsuperscript{286} (It is also worth noting that nitrogenase was the first enzyme to be studied by x-ray absorption spectroscopy.) Early work on lyophilized protein samples indicated the presence of two major contributions to the Mo K-edge EXAFS, which were attributed to Mo-S ligands, plus a more distant Mo-Fe contribution.\textsuperscript{286} Subsequently, these conclusions have been confirmed and extended, using samples in solution and with much more sensitive detection systems.

Most EXAFS studies to date have been on the molybdenum K-edge of the protein or of FeMoco, and indicate a very similar Mo environment in both (Table 7.7, Figure 7.30). A consensus of the best available analyses\textsuperscript{287} indicates that Mo is coordinated by three or four sulfur atoms at 2.4 Å, one to three oxygens or nitrogens at 2.2 Å, with approximately three nearby iron atoms at 2.7 Å. Of these, the EXAFS evidence for the oxygen/nitrogen contribution is weakest. However, comparison of Mo K-edge\textsuperscript{288} and Mo L-edge XANES\textsuperscript{289} spectra with model compounds indicates strong similarities with MoFe\textsubscript{3}S\textsubscript{4} thio­
cubane model compounds possessing MoS\textsubscript{3}O\textsubscript{3} coordination, and provides some support for the presence of O/N ligands.

The iron EXAFS of FeMoco has been independently examined by two groups.\textsuperscript{290,291} Both groups agree that the iron is coordinated largely to sulfur at about 2.2 Å, with more distant Fe-Fe interactions at about 2.6 Å. They differ, however, concerning the presence of short (1.8 Å) Fe-O interactions. Such interactions were apparently observed in the earlier study,\textsuperscript{290} but not in the later study.\textsuperscript{291} One possible explanation for this discrepancy is that the short Fe-O interactions of the earlier study were due to extraneous iron coordinated to solvent, contaminating the FeMoco preparation.\textsuperscript{291} A final resolution of this discord must, however, await the results of further experiments. Interestingly, a long Fe-Fe interaction at 3.7 Å was also observed in the later study.\textsuperscript{291}

Largely on the basis of the Mo K-edge EXAFS results and model studies discussed below, several proposals for the structure of the M center have been put forward. These are illustrated in Figure 7.31.

The MoFe proteins from \textit{Clostridium pasteurianum}\textsuperscript{292} and from \textit{Azotobacter vinelandii}\textsuperscript{293} have been crystallized. For the former protein, crystals of space group P2\textsubscript{1} are obtained, with two molecules per unit cell of dimensions 70 × 151 × 122 Å. There is good evidence for a molecular two-fold axis, which presumably relates equivalent sites in the two αβ dimers that make up the protein molecule.\textsuperscript{294} Preliminary refinement reveals that the two FeMoco units per protein are about 70 Å apart and the four P clusters are grouped in two pairs.

Single crystal EXAFS studies\textsuperscript{295} have provided important structural information on the molybdenum site. For different crystal orientations (relative to the polarized x-ray beam), the amplitude of the Mo-Fe EXAFS changes by a factor of 2.5, but the Mo-S EXAFS changes only slightly. Analysis of the an-
Figure 7.30
Mo K-edge EXAFS spectrum (left panel) and EXAFS Fourier transform (right panel) of Klebsiella pneumoniae nitrogenase MoFe protein. The solid line is the processed experimental spectrum and the dashed line a calculated one.287
isotropy of the Mo-Fe EXAFS using the available crystallographic information is consistent with either a tetrahedral MoFe₃ geometry such as that found in thiocubanes (Figure 7.32) or a square-based pyramidal MoFe₄ arrangement of metals. This interpretation tends to rule out some of the structural proposals shown in Figure 7.33. The observed orientation-dependence of the iron amplitudes is too small for clusters containing a linear or planar arrangement of iron and molybdenum (e.g., Figure 7.33B,C), and too large for arrangements that involve regular disposition of iron about molybdenum. Moreover, the lack of anisotropy of the sulfur EXAFS (which was apparently not considered in the original interpretation) argues against an MoS₃ (O/N)₃ model that has molybdenum coordinated by sulfur atoms that bridge only to Fe atoms disposed to one side of the molybdenum. Significant anisotropy for the Mo-S EXAFS (of opposite polarization, and smaller than that for Mo-Fe) would be expected for
Figure 7.32
Structures of thiocubanes that display Mo-S and Mo-Fe distances similar to FeMoco:
(A) (Fe₃MoS₄)₂(SR)₉⁻; (B) (MoFe₃S₄)₂Fe(SR)₁₂⁻/₁₄⁻; (C) MoFe₃S₄(SEt)₃(cat)CN⁻³⁻.
(Data on A and B from References 328, 330a; data on C from References 331, 332.)
Figure 7.33
FeMoS and FeWS structures of potential interest with respect to nitrogenase. 

\(^{331,332,332a-j}\)
such an arrangement of sulfur atoms. However, the cubane model of Figure 7.33, which provides the best model of both geometric and electronic structure, remains viable if one of the nonbridging ligands to molybdenum is a sulfur atom (rather than oxygen or nitrogen) with a bond length similar to that of the bridging sulfides.

10. Substrate reactions

The two-component Mo-nitrogenase enzyme catalyzes the reduction of $N_2$ to $2NH_4^+$ as its physiological reaction. Concomitant with the reduction of $N_2$, $H_2$ evolution occurs, with electrons supplied by the same reductants that reduce $N_2$. The limiting stoichiometry appears to be

$$N_2 + 10H^+ + 8e^- \rightarrow 2NH_4^+ + H_2$$  \hspace{1cm} (7.16)

If $N_2$ is omitted from the assay, all the electrons go to $H_2$ evolution. Indeed, to a first approximation the rate of electron flow through nitrogenase is independent of whether the enzyme is producing only $H_2$, producing both $NH_4^+$ and $H_2$, or reducing most of the alternative substrates.

As displayed in Table 7.8, many alternative substrates are known for this enzyme.\textsuperscript{240,243,296} The most important of these from a practical perspective is

<table>
<thead>
<tr>
<th>Table 7.8</th>
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<tbody>
<tr>
<td>Nitrogenase substrate reactions.\textsuperscript{296,374–376}</td>
</tr>
</tbody>
</table>

**Two-electron reductions**

- $2e^- + 2H^+ \rightarrow H_2$
- $C_2H_2 + 2e^- + 2H^+ \rightarrow C_2H_4$
- $N_2^- + 2e^- + 3H^+ \rightarrow NH_3 + N_2$
- $N_2O + 2e^- + 2H^+ \rightarrow H_2O + N_2$

\[
\begin{array}{c}
\text{CH}_2 - \text{C-CH} + 2e^- + 2H^+ \rightarrow \text{CH}_2=\text{CH}-\text{CH}_3 + \\
\text{H}_2\text{C-CH}_2
\end{array}
\]

**Four-electron reductions**

- $HCN + 4e^- + 4H^+ \rightarrow CH_3NH_2$
- $RNC + 4e^- + 4H^+ \rightarrow RNHCH_3$

**Six-electron reductions**

- $N_2 + 6e^- + 6H^+ \rightarrow 2NH_3$
- $HCN + 6e^- + 6H^+ \rightarrow CH_4 + NH_3$
- $HN_3 + 6e^- + 6H^+ \rightarrow NH_3 + N_2H_4$
- $RNC + 6e^- + 6H^+ \rightarrow RNH_2 + CH_4$
- $RCN + 6e^- + 6H^+ \rightarrow RCH_3 + NH_3$
- $NCNH_2 + 6e^- + 6H^+ \rightarrow CH_3NH_2 + 2NH_3$
- $NO_2^- + 6e^- + 6H^+ \rightarrow NH_3$

**Multielectron reductions**

- $RNC \rightarrow (C_2H_4, C_3H_6, C_3H_8) + RH_2$
- $NCNH_2 + 8e^- + 8H^+ \rightarrow CH_4 + 2NH_3$
Acetylene, C₂H₂, which is reduced by the Mo nitrogenase exclusively to ethylene, C₂H₄. Acetylene can completely eliminate H₂ evolution by nitrogenase. Many of the substrates in Table 7.8 have a triple bond. Indeed, the only triple-bonded molecule not reduced by nitrogenase is CO, which nevertheless inhibits all substrate reactions, but not H₂ evolution (in the wild type). Triple-bonded molecules such as acetylene (H—C≡C—H) are useful probe molecules for related reactivity as discussed below for simple inorganic systems. All substrate reductions involve the transfer of two electrons or multiples thereof (i.e., 4, 6, 8 . . .). Multielectron substrate reductions may involve the stepwise execution by the enzyme of two-electron processes. Further, about as many protons as electrons are usually transferred to the substrate. One way of viewing the nitrogenase active site is that it can add the elementary particles (H⁺ and e⁻) of H₂ to the substrate. This may have mechanistic implications. 297

It is potentially fruitful to pursue the intimate connection between H₂ and the N₂ binding site in nitrogenase. It has been shown unequivocally²⁹⁸,²⁹⁹ that one H₂ is evolved for each N₂ “fixed” even at 50 atm of N₂, a pressure of N₂ well above full saturation. Moreover, H₂ is a potent inhibitor of N₂ fixation, and under D₂, HD is formed, but only in the presence of N₂. These complex relationships between N₂ and H₂ (D₂) have elicited a variety of interpretations.²⁵⁵,³⁰⁰-³⁰²

Recently, it has been demonstrated that the FeMo protein alone acts as an uptake hydrogenase.³⁰³ Dihydrogen in the presence of [FeMo] causes the reduction of oxidizing dyes such as methylene blue or dichlorophenolindophenol in the absence of Fe protein. This is the only known catalytic reaction displayed by the FeMo protein alone. The hydrogen evolution and uptake by [FeMo] suggest that understanding hydrogen interaction with transition-metal/sulfur centers may be crucial to understanding the mechanism of nitrogenase action.

11. The role of ATP

ATP hydrolysis appears to be mandatory, and occurs during electron transfer from [Fe] to [FeMo]. Dissociation of [Fe] and [FeMo] following electron transfer is probably the rate-limiting step in the overall turnover of the enzyme.²⁵⁵ The fact that reductant and substrate levels do not affect turnover rates is consistent with this finding.

The role of ATP on a molecular level remains one of the great mysteries of the mechanism of nitrogen fixation. As discussed above, the overall thermodynamics of N₂ reduction to NH₃ by H₂ or by its redox surrogate flavodoxin or ferredoxin is favorable. The requirement for ATP hydrolysis must therefore arise from a kinetic necessity. This requirement is fundamentally different from the need for ATP in other biosynthetic or active transport processes, wherein the free energy of hydrolysis of ATP is needed to overcome a thermodynamic limitation.

What is the basis for the kinetic requirement of ATP hydrolysis in nitrogen fixation? To answer this question, we again look at the potential reduction prod-
ucts of the N₂ molecule. Of these, only N₂H₂ (diimide, three potential isomers), N₂H₄ (hydrazine and its mono and dications), and NH₃ (and its protonated form, NH₄⁺) are isolable products. (In the gas phase, other species such as N₂H, N₂H₃, or NH₂ also have a “stable” existence.) In the presence of H₂, only the formation of ammonia is thermodynamically favored (Figure 7.26). Clearly, the formation of the intermediate species in the free state cannot occur to any reasonable extent. However, this does not mean that nitrogenase must form NH₃ directly without the formation of intermediates. It is possible for these reactive intermediates to be significantly stabilized by binding to a metal-sulfur center or centers.

Detailed kinetic studies²⁵⁵,³⁰⁴ have suggested a scheme in which intermediates with bound and probably reduced nitrogen are likely to be present. Rapid quenching experiments in acid solution lead to the detection of hydrazine during nitrogenase turnover.³⁰⁵ Likewise, studies of inhibition of N₂ fixation by H₂ and the formation of HD under D₂ have been interpreted in terms of a bound diimide intermediate.³⁰⁶,³⁰⁷ Although a bound “dinitrogen hydride” is likely to be present, its detailed structure remains unknown.

D. The Alternative Nitrogenases

1. Vanadium nitrogenase

The “essentiality” of molybdenum for nitrogen fixation was first reported by Bortels in 1930.³⁰⁸ This finding led ultimately to the characterization of the molybdenum nitrogenases discussed in the preceding section. Bortels’ work has been cited many times, and is often referred to without citation. Following this seminal work, many other Mo-containing enzymes were subsequently sought and found.²⁵,³⁰⁹ At present more than a dozen distinct Mo enzymes are known, and new ones are continually being discovered.

In addition to the classic 1930 paper, Bortels³¹⁰ reported in 1935 that vanadium stimulated nitrogen fixation. In contrast to the 1930 paper, the 1935 paper languished in obscurity. Then, starting in the 1970s, attempts were made to isolate a vanadium nitrogenase. In 1971, two groups reported isolating a vanadium-containing nitrogenase from A. vinelandii.³¹¹,³¹² The interesting notion at this time was that V might substitute for Mo in nitrogenase, not that there was a separate system. The isolated enzyme was reported to be similar to the Mo enzyme, but had a lower activity and an altered substrate specificity. One of the groups carefully reinvestigated their preparation, and found small amounts of molybdenum, which were presumed to be sufficient to account for the low activity, although the altered selectivity was not addressed.³¹³ The vanadium was suggested to play a stabilizing role for [FeMo], allowing the small amount of active Mo-containing protein to be effectively isolated. Apparently the possibility was not considered that a truly alternative nitrogenase system existed, whose protein and metal centers both differed from that of the Mo nitrogenase.
The unique essentiality of molybdenum for nitrogenase fixation went unchallenged until 1980, when it was demonstrated that an alternative nitrogen-fixation system could be observed in *A. vinelandii* when this organism was starved for molybdenum. Despite skepticism from the nitrogenase research community, it was eventually shown that even in a mutant from which the structural genes for the Mo nitrogenase proteins (*nif* H, D, and K) had been deleted, the alternative system was elicited upon Mo starvation. In 1986, two groups isolated the alternative nitrogenase component proteins from different species of *Azotobacter*, and demonstrated unequivocally that one component contained vanadium and that neither component contained molybdenum.

One of the two components of the V-nitrogenase system is extremely similar to the Fe protein of nitrogenase. This similarity is evident in the isolated proteins from *A. vinelandii* and in the genetic homology between *nif* H (the gene coding for the subunit of the Fe protein in the Mo-nitrogenase system) and *nif* H* (the corresponding gene in the V-based system). Both Fe proteins have an α2 subunit structure, and contain a single Fe₄S₄ cluster that is EPR-active in its reduced state.

The FeV proteins from *Azotobacter vinelandii* and *Azotobacter chroococcum* each have an α₂β₂δ₂ subunit structure. Metal composition and spectroscopic comparisons between the FeMo and FeV proteins are shown in Table 7.9. Although there is the major difference involving the presence of V instead of Mo in the FeV protein and in the probable presence of the small δ subunits (13 kDa), the two nitrogenase systems are otherwise quite similar. In each, a system of two highly oxygen-sensitive proteins carries out an ATP-dependent N₂ reduction with concomitant H₂ evolution. The Fe proteins have the same subunit structure and cluster content, and are spectroscopically very similar. The V versions of the larger protein have somewhat lower molecular weights than their Mo analogues, and by MCD spectroscopy seem to contain P-like clusters. The FeV site still may be an S = 3/2 center (by EPR, although its EPR differs significantly from that of the FeMo center). The V-S and V-Fe distances as measured by EXAFS are similar to those in thiocubane VFe₃S₄ clusters and to Mo-S and Mo-Fe distances like those in [FeMo], which are in turn similar to those in MoFe₃S₄ thiocubanes. Likewise, XANES indicates VS₃O₅ type coordination in [FeV] nitrogenase similar to the MoS₃O₃ coordination suggested by XANES for FeMoco. The “FeV cofactor” is extractable into NMF, and can reconstitute the *nif* B⁻, FeMoco-deficient mutant of the Mo system. Despite the substitution of V for Mo, the proteins and their respective M-Fe-S sites do not differ drastically. However, the compositional changes do correlate with altered substrate reactivity.

A major difference between the V and Mo enzymes lies in substrate specificity and product formation. As is clearly shown in Table 7.9, the FeV nitrogenase has a much lower reactivity toward acetylene than does the Mo system. Furthermore, whereas the FeMo system exclusively produces ethylene from acetylene, the FeV system yields significant amounts of the four-electron reduction product, ethane. The detection of ethane in the acetylene assay may
Table 7.9
Comparison of alternative nitrogenase proteins

<table>
<thead>
<tr>
<th>Property</th>
<th>Avl \textsuperscript{17}</th>
<th>Avl* \textsuperscript{17}</th>
<th>Ac1* \textsuperscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>240,000</td>
<td>200,000</td>
<td>210,000</td>
</tr>
<tr>
<td>Molybdenum\textsuperscript{b}</td>
<td>2</td>
<td>&lt;0.05</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Vanadium\textsuperscript{b}</td>
<td>—</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>Iron\textsuperscript{b}</td>
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<td>9.3</td>
<td>23</td>
</tr>
<tr>
<td>Activity\textsuperscript{c}</td>
<td>H\textsuperscript{+}</td>
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<td>1400</td>
</tr>
<tr>
<td></td>
<td>C\textsubscript{2}H\textsubscript{2}</td>
<td>2000</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>N\textsubscript{2}</td>
<td>520</td>
<td>330</td>
</tr>
<tr>
<td>EPR g values</td>
<td>4.3</td>
<td>5.31</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>4.34</td>
<td>4.35</td>
</tr>
<tr>
<td></td>
<td>2.01</td>
<td>2.04</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>1.93</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Avl is the FeMo protein of Azotobacter vinelandii, Avl* is the FeV protein of A. vinelandii and Ac1* is the FeV protein of A. chroococcum. Data from References 317, 377, and 319, respectively.

\textsuperscript{b} Atoms per molecule.

\textsuperscript{c} nmol product/min/mg of protein.

prove a powerful technique for detecting the presence of the V nitrogenase in natural systems.\textsuperscript{322} Moreover, this reactivity pattern is found in the nif B\textsuperscript{−} mutant reconstituted with FeVco, indicating that the pattern is characteristic of the cofactor and not the protein.\textsuperscript{326} The reactivity change upon going from Mo to V in otherwise similar protein systems clearly adds weight to the implication of the M-Fe-S center (M = V or Mo) in substrate reduction.

2. The all-iron nitrogenase\textsuperscript{322}

The first sign that there is yet another alternative nitrogenase again came from genetic studies. A mutant of A. vinelandii was constructed with deletions in both nif HDK and nif H*D*K*, i.e., the structural genes for the Mo and V nitrogenases, respectively. Despite lacking the ability to make the two known nitrogenases, the mutant strain nevertheless was able to fix nitrogen, albeit poorly. Moreover, this mutant strain’s nitrogenase activity was clearly inhibited when either Mo or V was present in the culture medium. Preliminary studies indicate that the nitrogenase proteins produced by this organism are closely related to those previously isolated. A 4Fe-4S Fe-protein nif H\textsuperscript{†} and a protein due to nif D\textsuperscript{†} was produced. The latter appeared to contain no stoichiometric metal other than iron. Symmetry of nomenclature would suggest calling this the FeFe protein and its cofactor FeFeco. Interestingly, this nitrogenase seems to be the poorest of the set in reducing N\textsubscript{2} and makes ethane from ethylene. The finding of the
all-iron nitrogenase, if fully confirmed, will add significantly to the comparative biochemistry of nitrogen fixation. Speculatively, one might suggest that the concomitant absence of V and Mo suggests that nitrogen fixation need not directly involve the noniron heterometal in the cofactor cluster. This result may explain the lack of direct implication of Mo in the nitrogen fixation mechanism, despite many years of intense effort by workers in the field. (The above discussion should be taken cum grano salis until the existence of the all-iron nitrogenase is confirmed.)

3. Model systems

Three types of model systems for nitrogenase may be considered. First, there are transition-metal sulfide clusters that resemble the FeMoco or FeVco centers of the active proteins. Although there has been significant progress, there are not yet any definitive models (as there are for Fe₄S₄ and Fe₅S₅). A second approach uses the reactions of N₂ and related substrates or intermediates with metal centers in order to gain insights into the way in which transition-metal systems bind N₂ and activate it toward reduction. Here to date the most reactive systems bear little direct chemical resemblance to the nitrogenase active sites. Nevertheless, these systems carry out bona fide nitrogen fixation from which one may learn the various ways in which N₂ can be activated. Finally, there are other inorganic systems that display some of the structural and possibly some of the reactivity characteristics of the nitrogenase active sites without binding or reducing N₂ or precisely mimicking the active center. We may nevertheless be able to learn effectively about nitrogenase reactivity from these interesting chemical systems.

a. Transition-Metal Sulfide Models for Nitrogenase Sites

Although there has been great activity in synthetic Fe-S cluster chemistry, there is to date no example of a spectroscopic model for the P-cluster sites in nitrogenase. If the P-clusters are indeed asymmetrically bound high-spin Fe₄S₄ clusters, then the recent work on high-spin versions of Fe₄S₄ clusters and site-selectively derivatized Fe₄S₄ centers may hint that appropriate model systems are forthcoming.

b. Fe-Mo-S Cluster Models for FeMoco

Despite the importance of P-clusters, the modeling of the FeMoco center has properly received the most attention. The significant structural parameters that any model must duplicate are the Mo-S and Mo-Fe distances determined by EXAFS. Spectroscopically, the S = 3 EPR signal provides a stringent feature that model systems should aspire to mimic.

Many FeMoS clusters have been prepared in the quest to duplicate the FeMoco center, but none of the chemically synthesized clusters can reactivate the (UW-45 or Nif B⁻) cofactor-less mutants, perhaps because of their lack of homocitrate, which only recently has been discovered as a key component of FeMoco. Undoubtedly, new FeMoS clusters containing homocitrate will be prepared, and
II. MULTISITE REDOX ENZYMES

perhaps these will activate the mutant proteins, thereby revealing a close or full identity with FeMoco.

Despite the absence of homocitrate, some interesting model systems have been investigated. It is beyond the scope of this chapter to give a comprehensive account of FeMoS chemistry. We concentrate on the so-called “thiocubane” model systems. Heterothiocubane models were first synthesized using self-assembly approaches analogous to those used for the simpler Fe-S model systems. The reaction \[ \text{MoS}_4^{2-} + \text{Fe}^{3+} + \text{SR}^- \longrightarrow (\text{MoFe}_3\text{S}_4)_2(\text{SR})_4^{3-} \quad \text{and} \quad (\text{MoFe}_3\text{S}_4)_2\text{Fe}^{(\text{SR})}_4^{3-} \quad (7.17) \]

uses tetrathiomolybdate, MoS$_4^{2-}$, as the source of Mo, and leads to the double cubane structures shown in Figure 7.32A,B. The Fe$_7$Mo$_5$S$_8$ structure proved particularly interesting, since it was possible to complex the central ferric iron atom with substituted catecholate ligands and eventually isolate a single thiocubane unit (Figure 7.32C). Significantly, the single unit has $S = 3/2$ and Mo-S and Mo-Fe distances that match precisely those found by EXAFS for the M center of nitrogenase. Single cubes with VMo$_3$S$_4$ cores have also been prepared. Although the single thiocubanes display spectroscopic similarity and distance identity with FeMoco, they are not complete models. They are stoichiometrically Fe and S deficient, lack homocitrate, and most importantly, fail to activate the UW-45 and NifB$^-$ mutants.

Other interesting FeMoS (and FeWS) clusters with structurally distinct properties are shown in Figure 7.33. These include the “linear” (MoS$_4$)$_2$Fe$^{3-}$ ion, the linear (WS$_4$)$_2$Fe[CON(CH$_3$)$_2$]$_2$ ion, the linear $\text{Cl}_2\text{FeS}_2\text{MS}_2\text{Fe}_2\text{Cl}_2^{2-}$ (M = Mo, W), the “linear” (MoS$_4$)$_2$Fe$_2$S$_2^{4-}$ ion, the trigonal (WS$_4$)$_2$Fe$_3$S$_2^{3-}$, the capped thioprismane Fe$_6$S$_6$X$_6[\text{M}^{(\text{CO})}_3]^{3-}$ (X = Cl, Br, I; M = Mo, W), and the organometallic clusters MoFe$_6$S$_6$(CO)$_{16}^{2-}$, MoFe$_3$S$_6$(CO)$_6$(PET$_3$)$_3$, and MoFe$_3$S$_6$(CO)$_6^{2-}$. Structures suggested for FeMoco based on these and other chemically synthesized transition metal sulfides and on spectroscopic studies of the enzyme are shown in Figure 7.31.

E. $\text{N}_2$ and Related Complexes

The triple bond of $\text{N}_2$ has one $\sigma$ and two $\pi$ components. Each nitrogen atom has a lone pair oriented along the N-N direction. The two lone pairs allow $\text{N}_2$ to bind in an end-on fashion in either a terminal or a bridging mode. Both modes of binding are illustrated in the binuclear zirconium complex$^{333}$ shown in Figure 7.34. In this and in many other $\text{N}_2$ complexes, the N-N bond is not significantly lengthened and is therefore presumed to be insignificantly weakened in the complex. Interestingly, the complex in Figure 7.34, despite not having long N-N distances, forms hydrazine quantitatively upon protonation. Only one of the three $\text{N}_2$ molecules is reduced, and all four electrons required come from the two Zr(III) by presumed internal electron transfer. The related $\mu$-$\text{N}_2$ complex $[\text{W}(\eta^2-\text{C}_5\text{Me}_3)\text{Me}_2(\text{SC}_6\text{H}_5\text{Me}_2)_2](\mu-\text{N}_2)$ is one of the few dinitrogen complexes to contain an S donor ligand.$^{333a}$
In addition to the N lone pairs, the π components of the N≡N triple bond can serve as donor-acceptor orbitals in the Dewar-Chatt-Duncanson (olefin binding) manner. This less-common mode of N₂ binding is illustrated by the structure of the Ti complex\(^{334}\) shown in Figure 7.35. Here, as in the few other known side-on bound N₂ complexes,\(^{335}\) the N-N bond is significantly lengthened. The lengthened bond at 1.30 Å is presumed to be sufficiently weakened [\(\nu(\text{N-N}) = 1280 \text{ cm}^{-1}\)] that it is susceptible to further lengthening and reduction. As the N-N distance lengthens, it is more appropriate to consider the ligand as a deprotonated diimide or hydrazine.

Complexes that have proven particularly useful are bis(dinitrogen)phosphines of Mo(0) and W(0) such as \(\text{M(N}_2\text{)}_2(\text{Ph}_2\text{PCH}_2\text{CH}_2\text{PPh}_2)_2\) and \(\text{M(N}_2\text{)}_2(\text{PPh}_2\text{Me})_4\).
As shown in Figure 7.36, treatment of the complexes $^{336-337a}$ with acid leads to the formation of the diazenido(-H) and hydrazido(-2H) complexes, and sometimes to the production of ammonia. The finding of a bound $N_2H_2^{2-}$ species is consistent with the proposed presence of similar bound species in nitrogenase. The complexes of reduced dinitrogen intermediates are stabilized by multiple M-N binding. Further protonation of these intermediates or treatment of the original complex with strong acid leads to the formation of $NH_3$ from the bound nitrogen. Here the Mo(O) starting complex has enough electrons [six from the Mo(O) $\rightarrow$ Mo(VI) conversion] to reduce one $N_2$ molecule in conjunction with its protonation from the external solution.

In a general sense this reaction may be telling us something about nitrogenase. The enzyme may be able to deliver six reducing equivalents to $N_2$, and protonation, perhaps carefully orchestrated by neighboring amino-acid or homocitrate groupings, may facilitate the process. However, it is virtually certain that the Mo in nitrogenase is not able to change its oxidation state by six units. In the enzyme the multimetal, multisulfur FeMoco site may serve the equivalent function, by providing multiple sites at which reduced intermediates can simultaneously bind.

Only a few of the known $N_2$ complexes contain S-donor ligands. One of these, Mo($N_2$)$_2$(S(CH$_2$C(CH$_3$)$_2$CH$_2$S)$_3$), shown in Figure 7.37, has four thioether S-donor atoms bound to Mo(O). This Mo(O) complex shows reactivity reminiscent of the related phosphine complexes. $^{337a}$ A remarkable complex (Figure 7.38) has been isolated $^{338}$ in which two lone pairs of trans-diimide bind to two Fe, concomitantly with H-binding of the two diimide hydrogen atoms to coordinated sulfur atoms. The ability of an Fe-S system to stabilize the very reactive trans-$N_2H_2$ grouping adds support to the notion that similar metal-sulfide sites of nitrogenase may stabilize related intermediates along the $N_2 \rightarrow 2NH_3$ reaction path.

Most of the model systems involving $N_2$ do not lead to $NH_3$ formation. Moreover, many systems that do form $NH_3$ are not catalytic. However, certain
Figure 7.37
The structure of Mo(N₂)₂L (L is a tetrathiacyclohexadecane).  

Figure 7.38
The structure of FeL(N₂H₃)FeL (L = SC₆H₄CH₂SCH₂CH₂SCH₂SC₆H₄S).
V-based and Mo-based systems can catalytically reduce $N_2$ to $N_2H_4$ or $NH_3$ using strong reducing agents.\(^{339}\) Although kinetic studies indicate the possibility of intermediates, little structural information is available at present on these interesting systems.

**F. Insights from Relevant Inorganic Reactivity**

Certain studies on inorganic systems that do not model the nitrogen-fixation process can nevertheless potentially give insight into nitrogenase action. Two categories of relevant chemistry are acetylene binding/reactivity and dihydrogen binding/activation. Modes of dihydrogen activation on sulfide systems have previously been discussed in the section on hydrogenase.

Acetylene has long been known to bind to metal centers using its $\pi$ and $\pi^*$ orbitals as, respectively, $\sigma$-donor and $\pi$-acceptor orbitals. Even when the metal is predominantly sulfur-coordinated,\(^{340,341}\) such side-on bonding of $RC_2R$ is well known\(^{340,341}\) as in $MoO(S_2CNR_2)_2(RC\equiv CR)$ and $Mo(S_2CNR_2)_2(RC\equiv CR)_2$. The direct interaction of acetylene with the metal center must be considered as a potential binding mode for nitrogenase substrates.

A totally different, sulfur-based mode of acetylene binding is now also well established. For example, $(Cp')_2Mo_2S_4$ reacts with acetylene\(^{342,225}\) to produce

\[
(7.18)
\]

containing a bridging ethylene-1,2-dithiolate (dithiolene). The acetylene binds directly to the sulfur atoms by forming $S—C$ bonds. Acetylenes or substituted (activated) acetylenes are able to displace ethylene from bridging or terminal 1,2-dithiolate ligands\(^{225,341}\) to produce the 1,2-dithienes. In these reactions the sulfur rather than the metal sites of the cluster are reactive toward these small unsaturated molecules. Clearly, for nitrogenase, where we do not know the mode of binding, sulfur coordination might be a viable possibility. The $(Cp')_2Mo_2S_4$ systems that bind $H_2$ and $C_2H_2$, wherein bound $C_2H_2$ can be reduced to $C_2H_4$ and displaced by $C_2H_2$, are potential models for substrate reduction by nitrogenase.\(^{225,342}\)

The versatility of transition-metal sulfur systems is further illustrated by the observation that activated acetylene can insert into a metal-sulfur bond in $Mo_2O_2S_2(S_2)_2^{2-}$, forming a vinyl-disulfide-chelating ligand

\[
(7.19)
\]
on an $\text{Mo}_2\text{O}_{2}\text{S}_2^{2-}$ core. In this case the acetylene is bound by both metal and sulfur atoms. Figure 7.39 shows three possible modes of $\text{C}_2\text{H}_2$ binding, each of which is possible for the nitrogenase system.

It has recently been suggested$^{343}$ that the presence of a dihydrogen complex is required for $\text{H}_2$ to be displaced by $\text{N}_2$ to form a dinitrogen complex. This reaction would explain the required stoichiometry of $\text{N}_2$ reduction and $\text{H}_2$ evolution. Such an explanation had been suggested previously with dihydride complexes acting as the $\text{N}_2$-binding and $\text{N}_2$-displacing site.$^{182}$ Clearly, this new suggestion is an interesting embellishment of potential $\text{N}_2/\text{H}_2$ relationships.

At present, the activation process that is at work in the enzyme is unknown. We need greater structural definition of the active site, which should be forthcoming through the continued application of sophisticated diffraction and spectroscopic probes. Diffraction alone, however, will be incapable of locating protons and possibly other low-molecular-weight ligands. Therefore, spectroscopic probes such as ENDOR$^{10}$ and ESEEM,$^{277-279,344}$ which are based on EPR spectroscopy, and x-ray-based techniques, such as EXAFS and XANES, will remain crucial in elucidating mechanistically significant structural details.

III. REPORT ON THE NITROGENASE CRYSTAL STRUCTURE$^{378-381}$

A significant breakthrough has occurred in the crystallographic analysis of the iron-molybdenum protein of nitrogenase. The overall distribution of the metal clusters in the protein is shown in Figure 7.40. The distance between the two FeMoco units is fully consistent with each cofactor acting as an independent active site. On the other hand, the closeness of the P cluster and FeMoco centers in each unit is indicative of their likely cooperation in the $\text{N}_2$ fixation reaction.

The proposed structure of the P cluster, shown in Figure 7.41, involves a doubly bridged, double cubane unit consisting of one normally bound $\text{Fe}_4\text{S}_4$ cluster with all cysteine ligands and one $\text{Fe}_4\text{S}_4$ cluster that contains an unusual cysteine-serine (S/O) ligand pair on one of its two nonbridged Fe positions. Such five-coordinate iron in an $\text{Fe}_4\text{S}_4$ cluster is not unprecedented.$^{138}$ The two
Figure 7.40
A schematic representation of the spatial arrangement of the metal sulfur clusters bound to Cpo as determined by the x-ray anomalous scattering studies described in the text. The representation of the large, "8-Fe" cluster with a P symbol indicates only that it must contain the Fe atoms normally assigned to P-clusters.

Figure 7.41
Proposed P-cluster pair in A. vinelandii FeMo protein.

Fe₄S₄ clusters are disposed to produce a face-sharing arrangement with two cysteine ligands bridging the two sets of Fe atoms. An interesting feature of the structure is a disulfide unit linking the two clusters; this unit potentially could be redox-active during nitrogenase turnover.

Most striking of the new results is the proposed structure of FeMoco shown in Figure 7.42. The cluster core of composition Fe₇MoS₉ can be viewed as two halves bridged by two S²⁻ ions and an unknown ligand (designated Y in the figure). The MoFe₆S₃ half of the core is in the shape of a thiocubane fragment missing one μ₃-S²⁻ ion. The Mo is six coordinate; the ligands are three μ₂-S²⁻ ions, which bridge to the three Fe ions, an α-His-442 nitrogen, and two oxygen donors (the hydroxyl and central carboxylate) of the homocitrate ligand. Interestingly, the second half of FeMoco is a similar thiocubane fragment, Fe₄S₃, also missing a μ₃-S²⁻ ion. This unit has a single noncore ligand, α-Cys-275, which is bound to the terminal Fe atom of the cluster. The two thiocubane
fragments (MoFe₃S₃ and Fe₄S₃) are bridged by three ligands in a face-sharing mode with the two Fe₃ faces eclipsed with respect to each other. The eight metal ions display a bis(end-capped) trigonal prismatic arrangement with three bridges on the edges of the prism, which connect the two thiocubane fragments. The two sulfide bridges between the thiocubane halves are clearly defined in the structure, but the third bridge is not, suggesting the possibility that this is in fact part of the N₂-binding site. Interestingly, α-His-195, identified as essential for N₂ fixation by mutagenesis and ESEEM studies, does not appear to be covalently bound, although it is close to the FeMoco unit.

Clearly, this structure is not the same as any of those previously proposed (Figure 7.31), although it does possess many features that were identified in model studies. While it is tempting to speculate that the central bridge of the cluster (the Y ligand) is the site of N₂ reduction, this is in no way established at present.

The structural definition of the nitrogenase proteins is now progressing at a rapid rate. Many of the physical measurements will have to be reexamined in light of the new data. Through further experimentation involving physical methods, mutagenesis, and kinetic/mechanistic studies, much more information about the role of ATP, the activation of hydrogen, and the binding, activation, and reduction of N₂ and other nitrogenase substrates should be obtained.

IV. REFERENCES

IV. REFERENCES

15. B. B. Buchanan, in Reference 9, p. 129.
30. I. C. Gunsalus and J. D. Lipscomb, in Reference 11, p. 151.
40. M. C. W. Evans, in Reference 8, p. 249.
57. T. G. Spiro et al., in Reference 8, p. 407.
60. S. W. May et al., Biochemistry 23 (1984), 2187.
60. S. W. May et al., Biochemistry 23 (1984), 2187.
60. S. W. May et al., Biochemistry 23 (1984), 2187.
IV. REFERENCES

125c. I. Bertini et al., Inorg. Chem. 29 (1990), 1874.
142. M. Millar, private communication.
143b. S. Ciurl et al., J. Am. Chem. Soc. 112 (1990), 2654.
143c. P. R. Challen et al., J. Am. Chem. Soc. 112 (1990), 2455.
145. N. R. Bastian et al., in Reference 10, p. 227.
156. E. Münck, in Reference 8, p. 147.
194. I. Moura and J. J. G. Moura, in Reference 5, p. 179.
199. J. LeGall et al., in Reference 8, p. 177.
239. P. J. Stephens, in Reference 235a, p. 117.
243a. A. Braaksma et al., in Reference 5, p. 223.
244. E. I. Stiefel, in Reference 240, p. 55.
255. R. N. F. Thorneley and D. J. Lowe, in Reference 235, p. 221.
265a. J. Liang et al., Biochemistry 29 (1990), 8377.
IV. REFERENCES


278. W. H. Orme-Johnson et al., in Reference 8, p. 79.


372. R. N. Mullinger et al., Biochem. J. 151 (1975), 75.
372. B. K. Burgess et al., in Reference 242.
382. For allowing us to see and quote their work prior to publication, we are grateful to Prof. M. W. W. Adams, Prof. B. K. Burgess, Dr. R. Cammack, Prof. D. Coucouvanis, Prof. S. P. Cramer, Dr. S. J. George, Prof. J. N. Enemark, Prof. J. Lancaster, Dr. Michelle Millar, Prof. M. Maroney, Prof. W. E. Newton, Prof. D. C. Rees, Prof. Dieter Sellmann, Prof. A. E. Shilov, Dr. Barry E. Smith, Dr. R. N. F. Thorneley, and Prof. G. D. Watt. We thank Pat Deuel for her superb efforts under difficult circumstances in the preparation of this manuscript.