Structural Basis of Biological Nitrogen Fixation

James B. Howard* and Douglas C. Rees*

Department of Biochemistry, 435 Delaware Street, University of Minnesota, Minneapolis, Minnesota 55455, and Division of Chemistry and Chemical Engineering, 147-75CH, California Institute of Technology, Pasadena, California 91125

Received April 26, 1996 (Revised Manuscript Received July 11, 1996)

Contents

I. Introduction to Nitrogen Fixation

During the past century, chemists have sought economical methods of fixing atmospheric dinitrogen for use as fertilizer, explosives, and other chemicals. Despite the simplicity of the reactants, development of an energy-efficient, large-scale process for nitrogen fixation continues to present a significant chemical challenge. The energetic requirements of nitrogen fixation are fundamentally limited by the thermodynamics for this process. For many nitrogen-fixing systems, the overall reactions are thermodynamically favorable. For example, the standard free-energy change, $\Delta G°$, for the gas-phase reaction

$$N_2(g) + 3H_2(g) \rightarrow 2NH_3(g)$$

equals −7.7 kcal/mol at 298 K and 1 atm pressure.[1] This corresponds to an equilibrium composition with ~96% ammonia, starting from a 1:3 molar ratio of N_2 and H_2. The biological reduction of N_2 to ammonia coupled to the oxidation of the electron-transfer protein ferredoxin (Fd) in the process:

$$N_2(g) + 8Fd_{red} + 8H^+ \rightarrow 2\ NH_3 + 8Fd_{ox} + H_2(g)$$

also favors ammonia synthesis at 298 K and pH 7, with an estimated $\Delta G° = -15.2$ kcal/mol (ref 2).

Although the thermodynamics of nitrogen fixation can be favorable, realization of ammonia synthesis is complicated by the kinetic stability of the nitrogen−nitrogen triple bond. In the gas phase, N_2 does not readily accept[3] or donate electrons.[1,4] In terms of the energetics of the complete reduction of dinitrogen to ammonia, just as important is the disparity between the bond energy levels of the nitrogen−nitrogen triple, double, and single bonds of 225, 100, and 39 kcal/mol, respectively.[5] By comparison, carbon−carbon triple, double, and single bond energies are more nearly the same at 200, 146, and 83 kcal/mol, respectively.[5] The trend for the nitrogen−nitrogen triple bond to be significantly more stabilized relative to the double and single bond forms is reflected in the enthalpies of formation, ΔH_f, for the sequence N_2H_2, N_2H_4 and $2NH_3$:[1]

$$N_2 + H_2 \rightarrow N_2H_2 \qquad \Delta H_f = +50.9 \text{ kcal/mol}$$

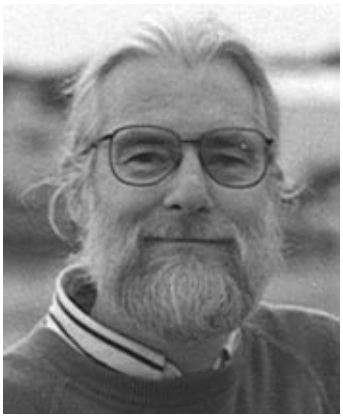
$$N_2H_2 + H_2 \rightarrow N_2H_4 \qquad \Delta H_f = -27.2 \text{ kcal/mol}$$

$$N_2H_4 + H_2 \rightarrow 2NH_3 \qquad \Delta H_f = -45.6 \text{ kcal/mol}$$

At least in the gas phase, the primary energetic barrier to N_2 reduction is the triple bond; subsequent reduction of nitrogen-nitrogen double and single bonds is energetically favorable.

As a consequence of the high activation energies for the reduction reactions of N_2, catalysts are required for nitrogen fixation to proceed at useful rates. By far the most successful chemical approach to ammonia synthesis uses iron as a catalyst to accelerate the reaction of N_2 and H_2 in the gas phase. This protocol was devised by F. Haber and developed for commercial applications by C. Bosch over 80 years ago and has been subsequently designated as the Haber−Bosch process. An engaging account of the historical development of this process is provided in ref 6. The mechanism of the Haber−Bosch process involves dissociation of N_2 to atomic nitrogen on the active {111} crystal face of the iron catalyst, followed by reaction with dissociated hydrogen to form ammonia (reviewed in[7]). Even with the catalyzed reaction, high temperatures (600−800 K) are required to achieve suitable rates for N_2 dissociation, which is the rate-determining step. However, since the reaction of N_2 and H_2 to form ammonia is exothermic (with an enthalpy change of −10.97 kcal/mol ammonia formed at 298 K[1]), the equilibrium shifts toward the reactants with increasing temperature; at 1 atm pressure, as the temperature is increased from 298 to 723 K, the equilibrium concentration of ammonia drops from 96% to ~0.2%. Thus, to obtain higher yields of ammonia, the reaction pressure is increased to as much as 500 atm, which, by LeChatelier's principle, shifts the equilibrium toward the

S0009-2665(95)00054-9 CCC: $25.00



James Bryant Howard was born in Indianapolis, IN. He received his Bachelor of Arts degree in chemistry from DePauw University in 1964 where he studied the mechanism of synthesis of highly hindered urethanes from activated benzoylisocyanates with John MacFarland. He received his Ph.D. in Biological Chemistry at UCLA with Alex Glazer in protein chemistry and enzymology and spent two years as a NIH Postdoctoral Fellow with Fred Carpenter at the University of California, Berkeley. Since 1971, he has been at the University of Minnesota where he is presently Professor of Biochemistry. His laboratory has investigated the structure and function of several Fe:S proteins including developing methods for identifying thiolate ligands of metalloclusters and for trapping intermediates during catalysis.

Douglas C. Rees was born in New Haven, CT, and returned there for college, graduating from Yale with a B.S. in Molecular Biophysics and Biochemistry. In between, he grew up in Lexington, KY, where was independently introduced to both biochemistry and nitrogen fixation. For his Ph.D. work in Biophysics at Harvard University under "the Colonel" (W. N. Lipscomb), he worked on the crystallographic analysis of complexes of the protease carboxypeptidase A. As a postdoc with J. B. Howard at the University of Minnesota, he began working on the nitrogenase system, which has been a continuing research interest. He was appointed to the faculty at UCLA from 1982 to 1989, and is currently a Professor of Chemistry at the California Institute of Technology. His research interests continue to focus primarily on structure determinations and functional analyses of metalloproteins and membrane proteins. (Photo: Bob Paz, Caltech.)

products. The yield of ammonia is approximately proportional to the applied pressure, at least in this pressure range, and at 723 K and 300 atm pressure, for example, the equilibrium composition of ammonia reaches ~35%. Approximately 80×10^9 kg of ammonia are manufactured annually by the Haber–Bosch process[8] and in 1995, ammonia was the number 6 manufactured chemical in the United States in terms of production volume[9] (or equivalently, number 1 in terms of total moles synthesized).

Despite the commercial significance of the Haber–Bosch process, biological nitrogen fixation represents a significantly higher annual production (estimated, 170×10^9 kg of ammonia[8]). By definition, biological nitrogen fixation must be achieved under physiological conditions of ~290 K and 0.8 atm N_2 which, consequently, suggests a higher degree of sophistication in chemical catalysis. The lower reaction temperature of the enzymatic process not only implies a more efficient activation of dinitrogen, but it also confers a thermodynamic advantage by favoring ammonia synthesis. For these reasons, the chemical mechanism of biological nitrogen fixation has been actively pursued to provide insights into the molecular basis for this catalytic efficiency. This review addresses the structural properties of the catalysts of biological nitrogen fixation, with an emphasis on the implications of these structures for the mechanism of dinitrogen reduction. Although the enzymatic process proceeds under milder conditions than the Haber–Bosch process, there is still a significant energetic demand during biological nitrogen fixation. How this energy is used to effectively catalyze the reduction of dinitrogen represents a central problem in structural bioenergetics.

II. Biological Nitrogen Fixation

As a constituent of nearly all biomolecules, nitrogen is essential for life. Although there is an enormous reservoir of dinitrogen in the atmosphere, as described above, kinetically N_2 is not reactive toward either oxidation or reduction, and most organisms are unable to directly metabolize this elemental source. Consequently, acquisition of metabolically usable forms of nitrogen is essential for the growth and survival of all organisms. Fortunately, a group of prokaryotic organisms have acquired the ability to reduce dinitrogen to ammonia, and hence play an essential role in maintaining a stable level of nitrogen in the earth's biosphere. The biochemical machinery required for this process of biological nitrogen fixation is provided by the nitrogenase enzyme system. Representative reviews capturing the progress in nitrogenase research over the past decade may be found in refs 10–22, including two reviews in this issue.[23,24] Nitrogenase consists of two component metalloproteins, the iron (Fe) protein and the molybdenum iron (MoFe) protein, named according to the metal composition of each protein. Under conditions of molybdenum depletion, alternative nitrogenase systems homologous to the molybdenum-containing "conventional" nitrogenase system may be induced. Nitrogenase catalyzes not only the reduction of dinitrogen to ammonia, but also the reduction of protons to hydrogen (which may be an obligatory part of dinitrogen reduction) and the reduction of diverse alternate substrates such as acetylene, azide, or cyanide.

Substrate reduction by nitrogenase involves three basic types of electron-transfer reactions: (i) the reduction of Fe protein by electron carriers such as ferredoxins and flavodoxins *in vivo* or dithionite *in vitro*; (ii) transfer of single electrons from Fe protein to MoFe protein in a MgATP-dependent process, with a minimal stoichiometry of two MgATP hydrolyzed per electron transferred; and (iii) electron transfer to the substrate at the active site within the MoFe protein. Under optimal conditions, the overall stoi-

chiometry of dinitrogen reduction has been described:[25]

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

where the protons associated with hydrolysis of MgATP have not been indicated. Nitrogenase is a relatively slow enzyme, with a turnover time per electron of $\sim 5\ s^{-1}$. Each electron-transfer step between Fe protein and MoFe protein involves an obligatory cycle of association and dissociation of the protein complex, with dissociation the likely rate-determining step for the overall reaction.[26−28] The complex of the two proteins plays a key role in the nitrogenase mechanism, since it is in this species that the coupling of ATP hydrolysis to electron transfer occurs.

A major challenge in the enzymology of nitrogenase is to establish a detailed mechanism for reduction of dinitrogen and other substrates in terms of the structures and properties of the nitrogenase proteins. With structures available for both the Fe protein and MoFe protein, it is possible to begin to formulate a structural foundation for these mechanistic questions, especially concerning the nature of the active center, and the energy transduction pathway required to couple ATP hydrolysis to electron transfer. Since many excellent reviews are available on biological nitrogen fixation, rather than attempting to provide a comprehensive review, this article will focus primarily on structural aspects of biological nitrogen fixation.

III. Structures and Properties of the Nitrogenase Proteins and Metallocenters

The ability to analyze and manipulate the nitrogenase system at the molecular level has been greatly facilitated by two relatively recent developments: the application of the methods of molecular biology to the identification, sequencing, cloning, and mutagenesis of genes involved in nitrogen fixation (reviewed in ref 29), and the availability of structures for the component nitrogenase proteins from both *Azotobacter vinelandii* and *Clostridium pasteurianum*.[30−35] The sequence, mutagenesis, and structural data provide the foundation for formulating an initial picture of the nitrogenase mechanism at the molecular level.

Although nitrogen fixation is a property of a phylogenetically diverse set of bacteria and cyanobacteria, in general, the sequences, structures, and functional properties of the nitrogenase Fe protein and MoFe protein are highly conserved between different organisms. As discussed in another review in this issue,[24] there are also at least three, highly related families of nitrogenase proteins which are mainly distinguished by the presence of Mo, V, or Fe only in the larger component protein. There are many combinations of Fe protein and MoFe protein from different species that can result in substantial substrate reduction activity.[36,37] For some combinations, however, little or no activity is observed. Consequently, while the emphasis in this review will be placed on "consensus" features of the predominant Mo-based nitrogenase proteins, it should be kept in mind that important species-specific variations do exist that may provide important mechanistic insights.

As a point of nomenclature, the MoFe protein and Fe protein isolated from different bacterial sources are designated as components "1" and "2", respectively, preceded by a two-letter abbreviation of the source species and genus, i.e. Av1 is MoFe protein from *Azotobacter vinelandii* and Cp2 is Fe protein from *Clostridium pasteurianum*. In this paper, Fe protein residues are numbered according to the Av2 protein sequence,[38] and MoFe protein residues are numbered according to the Av1 gene sequences.[39]

A. Structure and Properties of Fe Protein

The Fe protein functions as the unique, highly specific electron donor for the MoFe protein, with the electron transfer coupled to ATP hydrolysis. The Fe protein is a ~60 000 molecular weight dimer of identical subunits bridged by a single 4Fe-4S cluster. Each subunit consists of a large, single domain of an eight-stranded β-sheet flanked by nine α-helices[30] (Figure 1), an architecture characteristic of the P-loop containing nucleotide triphosphate hydrolases. From a computerized comparison of protein structures,[40] the Fe protein subunit shares many general features

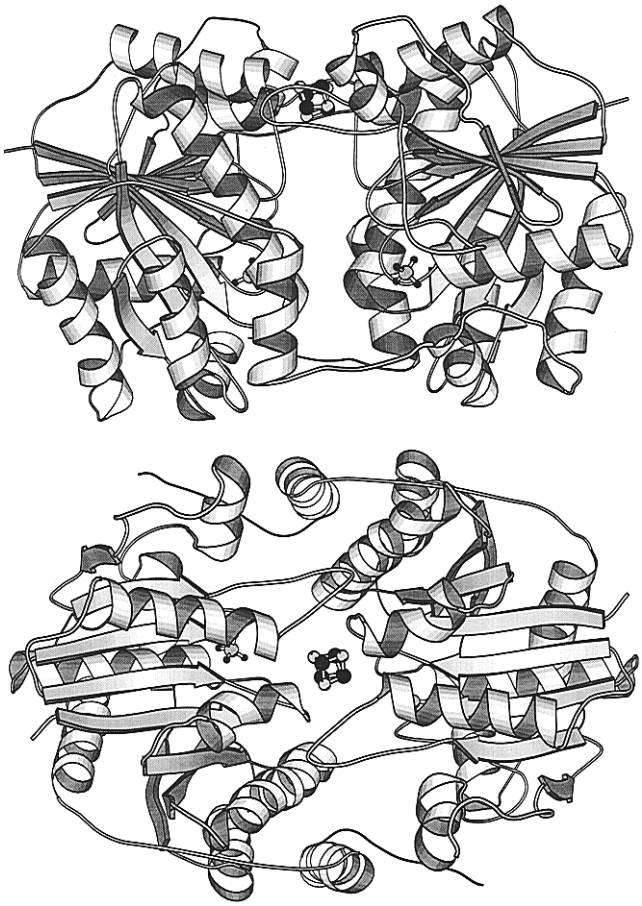


Figure 1. (A, top) Ribbons diagram of the polypeptide fold of the Fe protein dimer from *A. vinelandii*,[30] with ball-and-stick models for the 4Fe-4S cluster and molybdate. The 2-fold axis of the Fe protein dimer is oriented vertically in the plane of the page. (B, bottom) Ribbons diagram of Av2 viewed along the dimer 2-fold axis. All figures with structural representations of nitrogenase proteins in this review were prepared with the program MOLSCRIPT.[140]

with other nucleotide binding proteins, including G proteins, recA, and ras p21, and has a particularly close resemblance to dethiobiotin synthetase and adenylosuccinate synthetase. The two subunits of the Fe protein are related by a molecular 2-fold rotation axis that passes through the 4Fe-4S cluster, which is located at the subunit−subunit interface. The 4Fe-4S cluster is symmetrically coordinated by the thiol groups of Cys97 and Cys132 from each subunit.[41] In addition to the cluster, there are numerous van der Waals and polar interactions in the interface beneath the cluster that help stabilize the dimer structure. Indeed, these interactions are sufficiently strong that the cluster can be removed and the dimer structure is still maintained. The amino acid residues that participate in these interface interactions tend to be very highly conserved in the sequences of different Fe proteins.

The 4Fe-4S cluster of the Fe protein is generally considered to undergo a one-electron redox cycle between the $[4Fe\text{-}4S]^{2+}$ state and the $[4Fe\text{-}4S]^{1+}$ state, although evidence for further reduction to the $[4Fe\text{-}4S]^{0}$ state has been presented.[42] Both cluster ligands are located near the amino terminal end of α-helices that are directed toward the cluster (Figure 1B). Amide nitrogens from residues within these helices form NH−S hydrogen bonds[43] to the cluster and cluster ligands that may provide stabilizing electrostatic interactions to this center. In contrast to the 4Fe-4S clusters observed in ferredoxin-type proteins, a striking feature of the Fe protein is the exposure of the 4Fe-4S cluster to solvent, a property indicated by spectroscopic studies.[44−47] Other than through the cysteinyl ligands, there is little contact between the 4Fe-4S cluster and other amino acid side chains. Consequently, the Fe protein cluster is an exposed, loosely packed redox center that could serve as a pivot or hinge for conformational rearrangements between subunits.

The second principal functional feature of the Fe protein is the binding of the nucleotides, MgATP and MgADP. The original crystal structure of Av2 contained a low occupancy ADP molecule that apparently was carried from the protein purification.[48] The ADP was positioned across the subunit−subunit interface, perpendicular to the 2-fold axis such that the purine ring was bound to one subunit and the phosphates to the other (Figure 2). The ADP in this binding mode interacts with the two well-known amino acid sequences characteristic of nucleotide-binding proteins;[49,50] GXXXXGKS (the Walker motif A) which is present between residues 9 to 16, and the second Walker motif, DXXG found at residues 125−128. However, there are several reasons to believe an alternative binding mode based upon the structural analogy with *ras* proteins[51,52] is a functional one in Fe protein.[19] First, the ADP in the crystal structure is in low occupancy and does not appear to contain Mg, which is required for functional nitrogenase. Second, the cross-subunit mode would invert the binding sequences for the β and γ phosphates relative to the Walker motifs, a situation not observed in any of the other, monomeric, nucleotide-binding proteins. In the *ras*-like mode, the nucleotide would lie along the 2-fold axis with the purine ring and phosphate groups bound by the same subunit

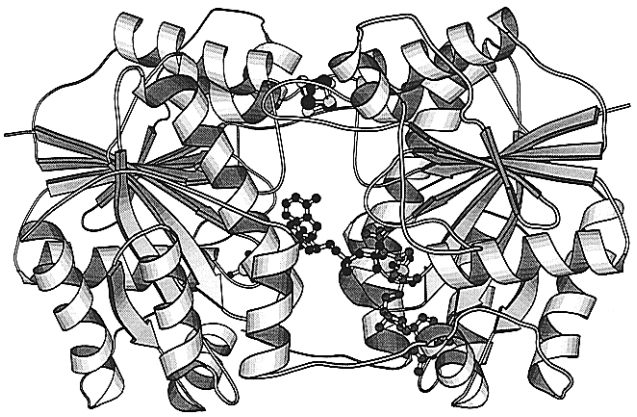


Figure 2. Nucleotide binding modes to Av2, viewed from the same perspective as Figure 1A. The nucleotide in an approximately horizontal orientation represents the ADP molecule observed in the initial Av2 structure.[30] The nucleotide in an approximately vertical orientation represents a GMPPNP molecule from the *ras* p21 structure,[51] obtained after superimposing the nucleotide binding domains of *ras* p21 and Av2.

(Figure 2). Clearly, the resolution of the true binding modes for both MgATP and MgADP awaits further structural determination.

For both potential binding modes, the nucleotide and cluster are well-separated from each other along the molecular 2-fold axis (see Figure 2). Hence, physical coupling of these two regions must be over a distance of ∼20 Å through the protein structure. Indeed, from the earliest recognition that ATP was required for substrate reduction, ATP binding was connected to the Fe protein by the profound effects observed on the physiochemical properties of its cluster. For example, nucleotides lower the redox potential of the cluster by ∼100 mV,[53] make the EPR signal more axial,[54−56] alter the magnetic CD spectrum,[57,58] and, at least in the case of MgATP, reduce the radius of gyration of Fe protein as determined by small-angle X-ray scattering.[59] Perhaps the most dramatic effect of nucleotides is their effect on the reactivity of the cluster with chelators.[60−62] As is shown in Figure 3, a chelator such as α,α′-bipyridyl is unable to remove the iron from nucleotide-free Fe protein. When MgATP is added to the protein, the cluster becomes reactive to chelation, while the chelation reaction can be fully inhibited by the competitive binding of MgADP. These results have been used to suggest that ATP causes large conformational changes in the Fe protein. How ATP causes these conformational changes, and the relevance of the changes, remains the subject of vigorous investigation.

In light of the Av2 crystal structure, the chelation experiments are somewhat perplexing. As noted above, the cluster is partially exposed to solvent in what we believe to be the native state, yet the cluster is resistant to chelation. One would have expected that at least some chelators might gain access to the cluster e.g., Arg 100 in the opening above the cluster might have provided a binding site for EDTA. Chelation studies on Fe protein with engineered amino acid substitutions further cloud the picture. Some results of the consequences of residue substitution on chelation behavior of Av2 are summarized in Table 1. Substitution by various amino acids for

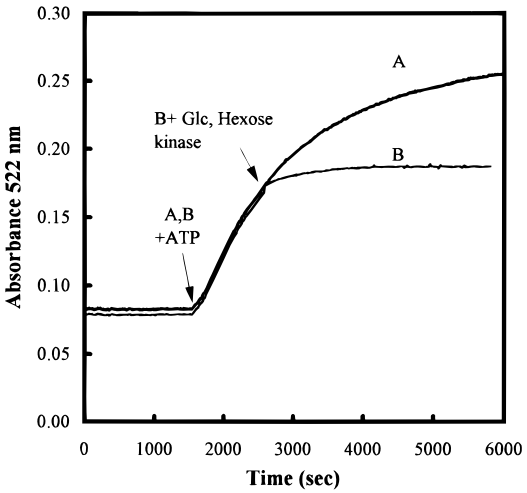


Figure 3. The time course for iron chelation from Av2. Two identical samples of Av2 in pH 8.0, 50 mM Tris buffer containing 10 mM Mg and 10 mM sodium dithionite were incubated with 2 mM α,α-bipyridyl in cuvettes made anaerobic by repeated cycles of Ar flushing and vacuum. The absorbance at 522 nm is from the formation of α,α-bipyridyl−Fe complex. In the absence of nucleotide, no iron is chelated from Av2. At the indicated time, MgATP (5 mM final) was added to both cuvettes. When approximately half of the iron had been chelated, 2.5 mM glucose and hexose kinase were added to cuvette B, which rapidly converts approximately half of the ATP to ADP and glucose-6-phosphate. Because ADP binds considerably more tightly than ATP to Av2, ADP is able to suppress the chelation, even in the presence of ATP.

Arg100, the amino acid side chain most likely to directly alter access to the cluster, has minimal effect on the rate of chelation,[63] even though the proteins containing these substitutions range from nearly fully active to inactive in the overall nitrogenase reaction. On the other hand, amino acid substitutions on the surface of the Fe protein that are more distant from the cluster than Arg100 can have moderately decreased, to enormously enhanced, rates of chelation,[63−65] while the substitutions uniformly decrease the activity ∼2−3-fold. In all of these mutants, chelation requires the presence of MgATP and is inhibited by MgADP. Mutations in the interior of the Fe protein and at the putative nucleotide binding site have yet another range of effects on chelation.[65−70] Notable properties of some members of this group are that the chelation state can be induced by either ADP or ATP and the chelation does not require the Mg-bound form of the nucleotide. Altered proteins in this family are either inactive or have <5% activity, as might be expected for mutations in the nucleotide fold region. Finally, one mutant in the nucleotide binding P-loop (see above), K15Q,[68] and one mutant at the subunit interface, A157S,[70] apparently have lost the ability for nucleotides to induce the chelation state, although MgATP can be bound. It should be emphasized that the consequences of mutagenesis depend not only on the residue that is being replaced but also on the residues that are incorporated; unlike K15Q, the variant K15R shows no significant affinity for either nucleotide.[71]

These recent studies with the mutants suggest that even subtle structural changes well removed from the cluster can have large effects on the accessibility of the cluster to chelator. For example, the "access to chelation" may be controlled by the number and location of hydrogen bonds on the cluster sulfur atoms, rather than global conformational changes. Indeed, the cysteinyl ligands appear to be more altered in the MgADP state than the MgATP state, when assessed by changes in the paramagnetically shifted resonances observed by NMR spectroscopy.[69,72,73] While minimal changes in the resonances of the cysteinyl α and β protons can be detected upon binding MgATP, significant changes are observed upon binding MgADP. In this regard, there is a clear correlation between the MgADP-like spectrum and the ability of MgADP to inhibit chelation.[73] We have presented a model for a pathway of connecting peptide backbones by which binding at

Table 1. Chelation and Activity Properties of Mutant Fe Proteins[a]

residue modified (ref)	location/function	distance to cluster, Å	relative substrate reduction activity, %	relative chelation rate, %
K15Q[68]	ligand β, γ phosphate ATP	∼17	0%	0%
C38S[68]	buried below surface and near nucleotide binding site	∼20	∼5%	∼250% Mg ATP ∼200% ATP ∼150% ADP ∼15% MgADP
R100Y,H,K[63,65]	above cluster, open to solvent	∼8	∼35%, 100 Y ∼3%, 100 H 0%, 100 K	∼105% ∼60% ∼75%
E110A,K[65]	putative Av1 binding interaction	∼20	∼60%, 110A ∼35%, 110K	∼80% ∼80%
D125E[65,66]	putative ligand to Mg in ATP binding	∼17	0%	∼85% Mg ATP ∼40% ATP ∼20% ADP ∼5% MgADP
D129E[69]	putative general base for ATP hydrolysis	∼12	0%	∼200%
R140Q[64]	putative Av1 binding interaction site	∼11	∼35%	∼280%
K143Q[64]	putative Av1 binding interaction site	∼20	∼25%	∼890%
A157S[70]	Av2 subunit interface	∼20	0%	0%

[a] Modified residues are described by the following nomenclature: the first letter indicates the normal amino acid residue, the number indicates the residue in the Av2 sequence, and the last letter indicates the substituted residue. Both activity measurements and chelation rates are subject to variation between research groups because of differing assay conditions. For this table, each mutant is compared with wild-type as an internal control. Only two mutants have been reported as dependent on metal-free nucleotides; for all other mutants listed they have only been reported with regard to MgATP dependence. The approximate distance, through space, between the closest iron atom and terminal side chain atom is provided as a rough comparative guide to the location of residue.

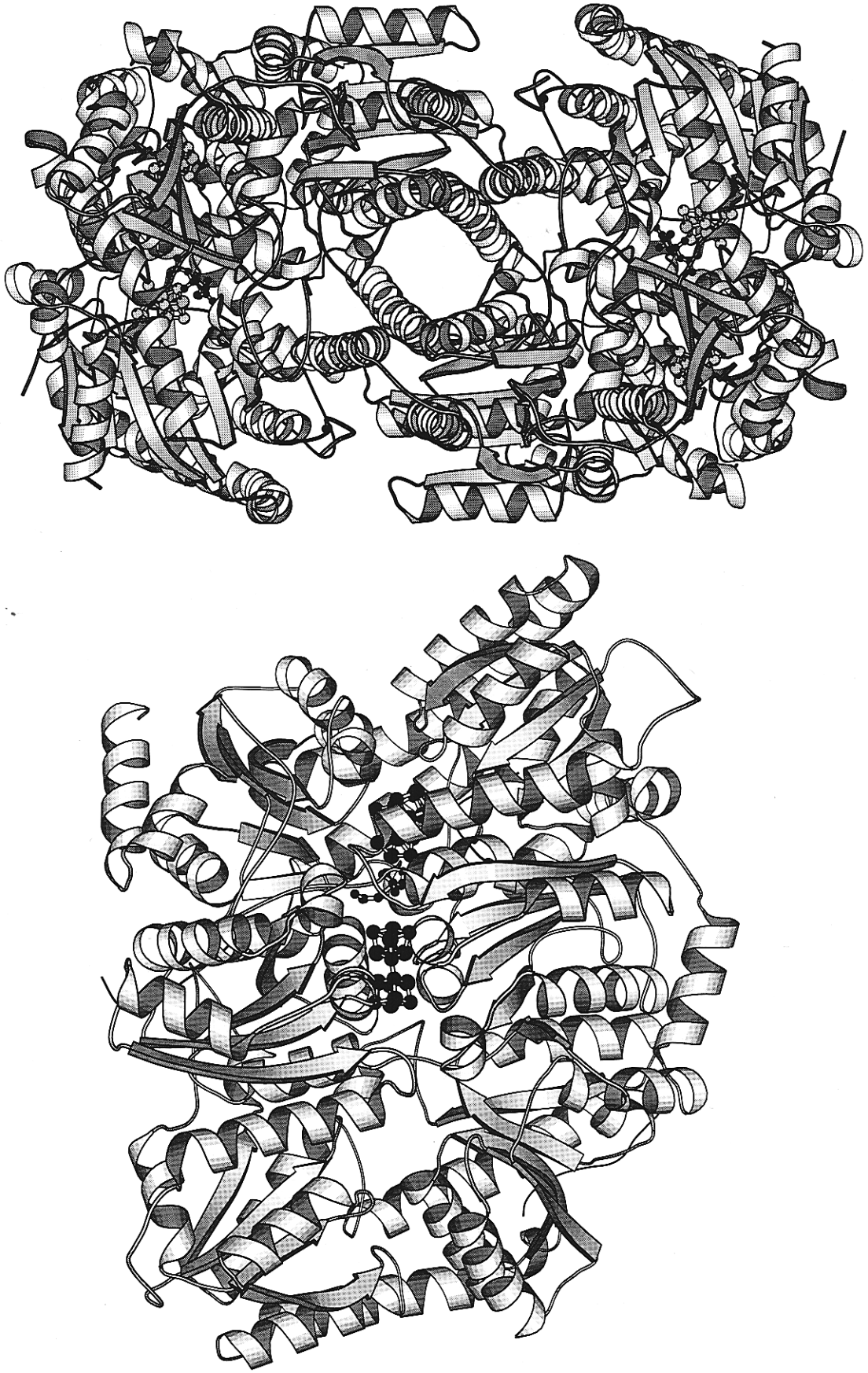


Figure 4. (A, top) Ribbons diagram of the polypeptide fold of the MoFe protein tetramer from *A. vinelandii*,[31,32] with ball-and-stick models for the FeMo cofactors and P clusters. The view is along the tetramer 2-fold axis. (B, bottom) Ribbons diagram of the polypeptide fold of an $\alpha\beta$ subunit pair of the MoFe protein. The view is roughly perpendicular to the view in Figure 4A, in the direction along the diagonal from the top left to bottom right of the tetramer.

the γ phosphate of ATP could be detected at the cluster.[19] Such a path could be disrupted by mutations at a distance from the cluster, while mutations in the environment of the cluster might have little

effect. Although the nucleotide-dependent chelation is a curious bioinorganic, structural property of the Fe protein, this frequently used characterization of the protein must be interpreted with caution. Until we better understand the mechanism of chelation, changes in the rate or nucleotide dependence of chelation are suspect as measures of global changes. Likewise, the correlation of chelation properties to a mechanistic understanding of the Fe protein enzymatic functions is premature and possibly irrelevant.

B. Structure and Properties of the MoFe Protein

The active site for dinitrogen reduction is contained in the MoFe protein. Associated with this protein are two groups of metal centers, the unusual $S = {}^3/_2$ paramagnetic FeMo cofactor (or M center or "cofactor") and the diamagnetic, EPR silent P cluster (or P center). As discussed below, the FeMo cofactor almost certainly represents the site for dinitrogen binding and reduction. The function of the P cluster is less clear, but it may participate in electron transfer between the Fe protein and FeMo cofactor. Structurally, the MoFe protein exists as an $\alpha_2\beta_2$ tetramer with a total molecular weight of ~240 000. Although there is minimal amino acid sequence homology between subunits, the α and β subunits of Av1 exhibit similar polypeptide folds, which consist of three domains of the parallel β-sheet / α-helical type (refs 31 and 32, Figure 4). At the interface between the three domains is a wide, shallow cleft; in the α subunit, the FeMo cofactor occupies the bottom of this cleft. The P cluster is buried at the interface between a pair of α- and β-subunits with a pseudo-2-fold rotation axis passing between the two halves of the P cluster and relating the two subunits. The extensive interaction between α and β subunits in an αβ dimer suggests they form the fundamental functional unit. An open channel of ~8 Å diameter exists between the two pairs of αβ dimers with the tetramer 2-fold axis extending through the center. The tetramer interface is dominated by interactions between helices from the two β subunits, along with a cation binding site, presumably occupied by calcium, that is coordinated by residues from both β subunits. The stabilization of the tetramer structure predominantly by interactions between β subunits is consistent with the observation that a variant of the vanadium nitrogenase has been characterized with apparent subunit composition $\alpha\beta_2$.[74]

C. Metallocenters of the MoFe Protein

The metallocenters in the nitrogenase components have long attracted the attention of biophysicists and synthetic inorganic chemists due to their unique chemical and physical properties among the Fe-S clusters. The protein crystal structures have provided a structural description of these centers that can serve as a starting point to developing molecular-level mechanisms for nitrogenase.

1. FeMo Cofactor

The iron−molybdenum-containing cluster is designated a "cofactor" because it can be extracted intact from acid-denatured protein[75] and used to regenerate active enzyme from a cofactor-deficient protein isolated from mutant strains. FeMo cofactor may be formally considered as generated from 4Fe-3S and Mo-3Fe-3S partial cubanes that are bridged by three sulfurs[31,34,35] (Figure 5). The cofactor is buried ~10 Å beneath the protein surface, consistent with the results of spectroscopic studies,[76] in an environment primarily provided by the α subunit. Only two protein ligands, Cysα275 and Hisα442, coordinate the cofactor to the protein, resulting in the unusual situation in which the six Fe atoms bridged by nonprotein ligands are three coordinate. Although rare, three-coordinate iron with sulfur ligands is not unprecedented.[77] The octahedral coordination sphere of the Mo is completed by bidentate binding of homocitrate. The metal−metal distances within each 4M-3S (M = Fe or Mo) partial cubane are relatively typical, while pairs of trigonal irons between the two fragments are separated by 2.5−2.6 Å and 3.6−3.7 Å, depending on the interactions. The distances from Mo1 to Fe2, Fe3, and Fe4 are ~5.0 Å, which is comparable to the distances from Fe1 to each of Fe5, Fe6, and Fe7. The longest dimension in the inorganic component of the FeMo cofactor is 7.1 Å between Fe1 and Mo1. Fe−Fe and Mo−Fe distances corresponding to these interactions are generally consistent with the results of EXAFS studies,[78−82] at least to within the accuracy of the X-ray data. There is recent EXAFS evidence indicating a contraction of metal−metal distances occurs in the FeMo cofactor upon one-electron reduction of the MoFe protein.[83]

The protein environment around the FeMo cofactor is primarily provided by hydrophilic residues, although there are some hydrophobic residues such as Val α70, Tyr α229, Ile α231, Ile α355, Leu α358, and Phe α381. Hydrogen bonds to sulfur atoms in the cluster are provided by the side chains of residues Arg α96, His α195 , Arg α359, and the NH groups of Gly α356 and α357. Along with the side chains of Val α70 and Phe α381, these residues pack against the central "waist" of the FeMo cofactor containing the trigonal irons and bridging sulfurs (Figure 5b). The potential ability of atoms in the FeMo cofactor to contact small molecule ligands can be computationally assessed by the algorithm of Richards.[84] As the FeMo cofactor is completely buried, this calculation only identifies atoms that have a relatively loosely packed environment in the protein that could potentially contact a buried ligand molecule (such as N_2) that had diffused into the protein interior. Assuming that the positions of protein atoms remain fixed, the only inorganic atoms of the cofactor that could be contacted by a probe of 1.4 Å radius are the sulfur atoms near the side chain of Arg α359. As discussed by Dance,[85] this region of the cofactor, which is on the side of the FeMo cofactor most distant from the protein surface (Figure 5b), could represent a potential site for ligand binding, although changes in the protein structure during substrate reduction could open up other binding sites.

For a buried center, the environment of the FeMo cofactor contains a number of neighboring charged groups; in addition to the homocitrate, the side chains of 12 residues have potentially charged atoms within 8 Å of an inorganic (Fe, S, or Mo) atom in the FeMo cofactor. These residues, and their distance of closest

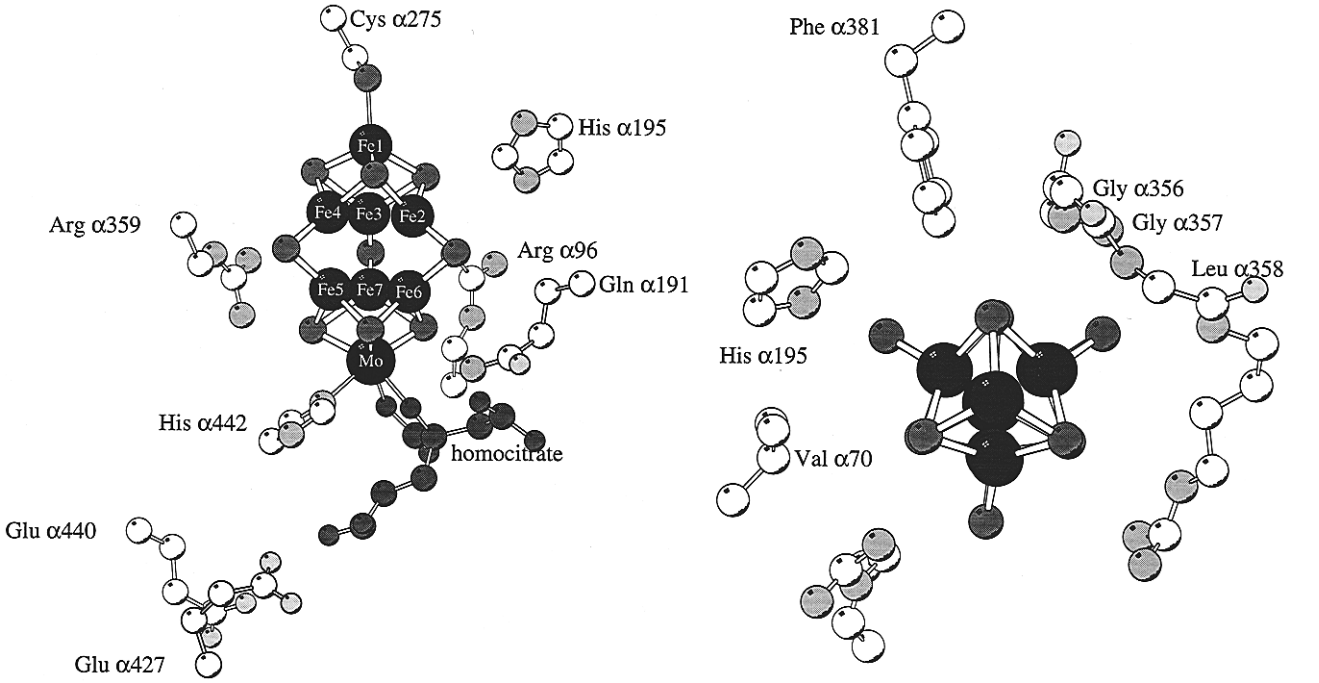


Figure 5. (A, left) FeMo cofactor and surrounding environment of the MoFe protein from *A. vinelandii*,[31,32,34] indicating numbering of inorganic atoms in the cofactor. (B, right) Perpendicular view down the long (3-fold) axis of the FeMo cofactor, indicating the packing of amino acid residues of the MoFe protein around the midsection of the cofactor. The direction of the protein surface is toward the top of the page.

approach of potentially charged atoms to the cofactor are Arg α96 (2.9 Å), His α195 (3.2 Å), Arg α359 (3.2 Å), Glu α380 (4.8 Å), His α274 (6.3 Å), Asp α228 (7.0 Å), Arg α277 (7.1 Å), His α451 (7.1 Å), Asp α234 (7.2 Å), His α362 (7.2 Å), Asp α386 (7.5 Å), and Arg α361 (7.6 Å). The close interaction with positively charged residues Arg α96, Arg α359, and His α195 (if protonated), could provide an electrostatic mechanism for stabilizing negatively charged intermediates generated during substrate reduction. The positioning of the FeMo cofactor near the N-terminal ends of helices α280−α290 and α359−α369 may also serve to electrostatically stabilize an anionic species.

The protein environment surrounding the homocitrate is also relatively polar. Within an 8 Å distance, the homocitrate is surrounded by eight potentially charged atoms from the side chains of Lys α426 (3.7 Å), Glu α440 (4.1 Å), Glu α427 (4.7 Å), Arg β105 (4.5 Å), and Glu α380 (5.0 Å), as well as the side chains of Arg α96 (4.6 Å), Arg α359 (5.8 Å) and His α195 (7.7 Å) that are nearer to the inorganic component. In addition to charged residues, the homocitrate forms a hydrogen bond to the side chain of Gln α191. Furthermore, the homocitrate is surrounded by a "pool" of ~10 water molecules that participate in hydrogen-bonding interactions. Hence, although the FeMo cofactor is buried at the interface between the three domains of the α subunit, the surrounding environment contains many polar and charged groups.

Since the earliest recognition of an extractable cofactor in nitrogenase,[75] the cofactor has been assumed to be the site of substrate reduction and it would be astonishing if this does not prove to be the case. However, in the spirit of rigorous evaluation, the evidence to date is primarily circumstantial, since substrates and inhibitors do not bind to, or near, the FeMo cofactor in the as-isolated, dithionite-reduced state of the free MoFe protein (see, for example refs 23 and 86). The most compelling arguments that the FeMo cofactor does represent the active site of nitrogenase are provided by studies demonstrating that changes in the cofactor or its environment directly affect substrate reduction properties.[87,88] For example, the rate, substrate specificity, and the products are altered by the nature of the organic acid moiety of the cofactor.[89,90] Replacing homocitric acid with citric acid prevents the enzyme from reducing dinitrogen, while acetylene and proton reduction are unaltered. Likewise, there is a shift in substrate specificity when the alternate cofactors (FeFeco or FeVco) are present (reviewed in ref 24). However, the cofactor alone does not control the rate, substrate specificity, or product formed. Several amino acid substitutions around the cofactor have been reported to alter all three properties.[91] Whether the amino acids implicated by mutagenesis are directly participating in reduction of substrates is not known, but substitutions at these sites could alter the electron transfer path or distort the substrate binding sites, leading to the observed rate and product differences.

The structural details of substrate binding to the FeMo cofactor and the sequence of electron and proton transfer to bound substrate remain critical questions. In the absence of any experimental evidence indicating how substrates might bind to the cofactor, a variety of hypothetical possibilities have been proposed. The known substrates are quite diverse and range from protons to short-chain alkynes and nitriles, as well as the natural substrate dinitrogen. Thus, it is likely that there are at least subtle differences in binding modes. Furthermore, these substrates exhibit both competitive and noncompetitive kinetic interactions between each other and with the inhibitors CO and NO (reviewed in ref 23). Binding interactions between at least some substrates and one or more of the Fe, Mo, and S sites

are all conceivable, and elimination of any of these possibilities at present would be premature.

Models for N_2 binding generally fall into two categories: binding to the Mo, or binding to one or more of the Fe sites. A theoretical evaluation of some of these possibilities has been provided by Deng and Hoffmann.[92] Advocates of the Mo binding modes point to the development of a rich model chemistry of Mo and related metals to support a central role for Mo in the binding and reduction of N_2.[93,94] For Mo-based chemistry, reduction of N_2 is often envisioned as proceeding through a sequence of proton and electron transfers, until ammonia is produced. Advocates of Fe binding modes for N_2 were originally motivated by the observation that iron is the only metal common to all nitrogenases and by the development of iron-based systems for dinitrogen reduction.[95,96] The presence of the unusual coordination environment of the six trigonal Fe's in the FeMo cofactor also suggests that these sites might represent the binding site for N_2 and possibly other ligands.[31] Theoretical considerations of varying degrees of sophistication suggest that binding of N_2 within the cofactor cavity would most destabilize the NN triple bond.[34,92,97] Since the center of the cofactor cavity is located ~2.0 Å from each of the six trigonal iron sites, some expansion of the cofactor would be required to accommodate N_2 as a substrate. If N_2 did bind in this location, reduction of this molecule might proceed through a nitride intermediate, perhaps with some mechanistic similarities to the recently reported cleavage of N_2 by binuclear Mo(III) systems.[98] Protonation of the nitrides would be difficult, however, unless the cofactor is able to isomerize to a more open conformation. Likewise, it is unlikely that the array of substrates reduced by nitrogenase would bind equally well in the cavity. Alternate Fe-based mechanisms have been proposed that avoid this problem by having N_2 (and presumably other substrates) bind to multiple irons on the outside of the cofactor;[85,92] in this case, proton transfer could occur via neighboring amino acid side chains, water molecules or through protonated forms of the bridging sulfurs of the cofactor. Clearly, there are a large number of possible geometries for substrate binding, and it is likely that a combination of spectroscopic studies on the enzyme system and development of both appropriate synthetic chemistry and theoretical models will be required to ultimately establish the true mechanism(s).

In addition to dinitrogen, ammonia formation also requires the delivery of protons to the active site. As no permanent channels exist by which H_3O^+ might diffuse from the protein surface to the FeMo cofactor, it is possible that proton transfer could employ a "bucket-brigade" mechanism of the type envisioned for protonation of reduced quinones in the photosynthetic reaction center.[99] In this mechanism, protons are transferred by shuttling from one amino acid side chain to another; the side chains of Asp, Glu and His residues, as well as water molecules, would seem to be particularly suited for this function. Two patches of histidines occur on the α-subunit surface that might represent the initial site for proton binding; these patches contain the imidazole side chains of α196 and α383; and α274, α362, and α451. As several of these residues have been substituted either in naturally occurring MoFe proteins or by site-directed mutagenesis without serious loss of activity, either these residues are not involved in proton transfer, or else a multiplicity of proton transfer pathways exist that can tolerate some alterations. In the interior of the protein, several potential pathways occur which could funnel protons to FeMo cofactor: Glu α440 to homocitrate to FeMo cofactor, and His α195 to FeMo cofactor. If an intermediate that is a sufficiently strong base is generated during dinitrogen reduction, it is possible that protons may also be transferred along Asp α234 to Arg α359 to the FeMo cofactor; by Arg α96 to FeMo cofactor; or even from the acidic methylene hydrogens of homocitrate to the cofactor. The presence of multiple, potential proton transfer routes suggests that there is not a unique pathway by which protons are shuttled from the surface to the active site. This may account for the changes in substrate reduction patterns exhibited by amino acid substitutions around the cofactor. For example, substitutions for His α195 result in mutant MoFe proteins that are unable to reduce N_2, but that can reduce acetylene to ethylene and ethane.[91] The native enzyme, in contrast, exclusively reduces acetylene to ethylene, without formation of ethane. Furthermore, in mutants at position His α195, CO inhibits proton reduction while in the wild type it does not. One possible explanation is that the His substitutions result in alternate paths for proton transfer, with perhaps totally different residues serving as proton donors. Different hydrogen-bonding interactions between variants of His α195 and the FeMo cofactor have been inferred by spectroscopic studies of these mutants.[100] Other altered MoFe proteins have their own unique pattern of substrate reduction and CO inhibition that may reflect perturbations in proton and/or electron transfer mechanisms.

2. P Cluster

The P cluster, which may function in electron transfer between the 4Fe-4S cluster of Fe protein and the FeMo cofactor, is located about 10 Å below the protein surface, on the 2-fold axis that approximately relates the α and β subunits. While it seems clear that each P cluster contains eight iron atoms, the sulfur composition appears variable, with both seven and eight inorganic sulfurs observed in different crystallographic studies.[22,32,34,35] (See note added in proof.) As a starting point, the P cluster has been described as an 8Fe-8S cluster that, at least formally, may be considered as generated from two bridged 4Fe-4S clusters (Figure 6). The possibility that the P cluster may be biosynthetically assembled from two 4Fe-4S clusters is discussed in more detail in the section on cluster synthesis. These two clusters are bridged by both the thiol side chains of Cys residues α88 and β95, and a disulfide bond between two cluster sulfurs. In this structure, the disulfide bond is located on the side of the P cluster closest to the surface of the protein (Figure 6C). Singly coordinating cysteinyl thiols (from residues α62, α154, β70,

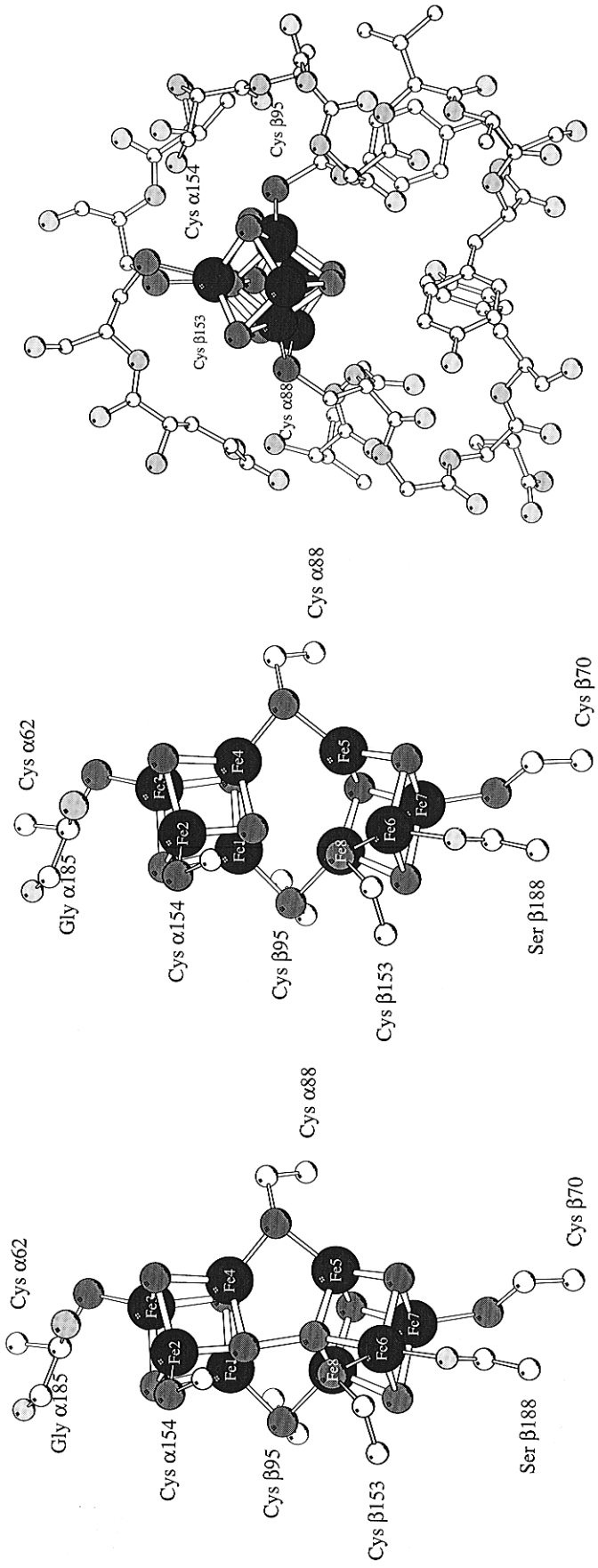


Figure 6. (A, left) 8Fe-8S model of the P cluster and surrounding environment of the MoFe protein from *A. vinelandii*,[31,32,34] indicating numbering of inorganic atoms in the cofactor. (B, middle) 8Fe-7S model of the P cluster as seen in structural analyses of the MoFe protein from *A. vinelandii*. (C, right) Perpendicular view along the long axis of the P cluster, indicating the packing of amino acid residues of the MoFe protein around the midsection of the cluster. The four stretches of polypeptide chain include residues α86−α91, α153−α154, β92−β99, and β152−β154. The approximate 2-fold axis that passes through the P cluster pair runs approximately in the vertical direction, with the protein surface located toward the top of the figure.

and β153) coordinate the remaining four irons, such that nonbridging cysteines coordinated to a specific 4Fe-4S cluster are from the same subunit. In addition to the cysteinyl ligands, Ser β188 coordinates the same iron as Cys β153. Intriguingly, the specific loss of the sulfur in the disulfide nearer to the Fe with the Ser β188 ligand has been observed in crystallographic studies of Av1.[34] The resulting 8Fe:7S cluster may be described, at least formally, as generated from bridged 4Fe-4S and 4Fe-3S clusters (Figure 6B). Difference map studies indicate that the Fe−Fe distances tend to increase in the 4Fe-3S cluster upon loss of the sulfur. In Bolin's work, the P cluster is also described as an 8Fe-7S cluster.[22,35] Unlike the sulfur depleted cluster described above, however, in this case the P cluster may be formally considered as generated from two 4Fe-3S clusters with a single hexacoordinate S bound equivalently by the two partial cubanes. In the Bolin model, the side chain of Ser β188 does not serve as an iron ligand, but rather donates a hydrogen bond to a cluster sulfur.

Several altered proteins with substitutions for the cysteinyl ligands have been prepared by mutagenesis. The properties of the resulting proteins are generally consistent with the cysteinyl ligands observed for the P cluster.[101−104] However, unlike the situation with the FeMo cofactor, some of the P cluster ligands may be substituted without complete loss of activity. For example, Cys β153 can be substituted by serine and retain 25−50% specific activity, while substitution with alanine leads to <2% activity.[102,103] Complete deletion of Cys β153 results in a protein with ∼40% activity.[101] Noting that Cys β153 ligands the same iron atom as Ser β188, the residual activity of these variants may be rationalized by assuming that the Ser β188 ligand alone is sufficient for assembly and function of some form of P cluster. Another interesting example is provided by the behavior of Ala substitutents at the bridging Cys α88 and Cys β95. Although the proteins containing one or the other of these substituents are inactive, the double mutant with both bridging Cys replaced by Ala retains some activity.[104] Clearly, detailed biochemical, spectroscopic, and structural characterizations of the P cluster in these variants are of great interest.

In the refined Av1 structure[34] the Fe−Fe distances in the P cluster are typical of normal 4Fe-4S clusters (∼2.7 Å) for Fe1, Fe2, Fe3, and Fe4, while the Fe−Fe distances between Fe5, Fe6, Fe7, and Fe8 tend to be larger (∼3.0 Å), especially those distances involving Fe5, Fe6, and Fe8. The longer Fe−Fe distances in half of the P cluster may originate from multiple states of the P clusters being present in the crystals used for the high-resolution data collection. (See note added in proof.) This apparent variability in sulfur composition is an important issue that needs to be resolved. It is likely that this variability reflects real differences in the structure of the P cluster, possibly associated with different oxidation states. Unfortunately in terms of resolving this issue, crystallographic studies require relatively lengthy time scales for crystal growth and data collection, while analytical and spectroscopic methods, which involve sample manipulation under more controlled conditions, have so far not had sufficient accuracy to independently establish the P cluster sulfur composition.

The P cluster and surrounding residues are shown in Figure 6. In striking contrast to the more polar cofactor pocket, the protein environment around the P cluster is mainly provided by hydrophobic residues, including Tyr α64, Pro β72, Pro α85, Tyr α91, Tyr β98, Phe β99, Met β154, Pro α155, Phe α186, and Phe β189. The location of the P cluster near the N-terminal ends of six helices (α63−α74, α88−α92, α155−α159, β71−β81, β93−β106 and β153−β158) may serve to provide an electrostatic contribution to cluster stability. Unlike for the situation with the FeMo cofactor, only one charged residue, Glu α153, is within 8 Å of inorganic atoms of the P cluster. With the exception of the cluster ligands (Cys α62, α88, α154, β70, β95, β153 and Ser β188), Gln β93 and Thr β152, hydrophilic residues around the P cluster, including Glu α153, are generally not conserved in different MoFe protein sequences. Strictly conserved Gly residues (α87, β94, and α185) around the P cluster are also structurally important to avoid steric interference with the P cluster.

Spectroscopic studies indicate that the P cluster is highly reduced, with all eight Fe atoms most likely in the ferrous form.[105] Although the all ferrous oxidation state is unprecedented in known biological four iron clusters, the P cluster cannot be directly compared to these other clusters because of the bridging ligands. For example, two isolated 4Fe-4S clusters with all Fe in the ferrous state would have a combined net charge of −8 (including the eight thiol ligands), whereas the reduced P cluster should have a net charge of only −4. That is, the effective cluster charge *per iron atom* is similar to an oxidized ferredoxin cluster, not to a "super-reduced" cluster.

3. Relationship between the MoFe Protein Clusters

The protein environment between the FeMo cofactor and the P cluster, which may be important for electron transfer between the two centers (if this actually occurs) is illustrated in Figure 7. The edge−edge distance of FeMo cofactor to the P cluster is about 14 Å. Hydrogen-bonded networks involving the NH of Gly α61 (adjacent to the P cluster ligand Cys α62), Gln α191, and the homocitrate, as well as water molecules, can be identified that link these two metal centers. Four helices (α63−α74, α88−α92, α191−α209, and β93−β106) are oriented in parallel between the two metal centers and could play a role in electron transfer. The results of mutagenesis of Tyr β98 are consistent with a role for this region in mediating electron transfer between the P cluster and the FeMo cofactor.[106] It is also certainly possible that structural fluctuations/alterations occur during complex formation and substrate reduction that permit the formation of more favorable electron transfer pathways. (See section on the nitrogenase complex, below.)

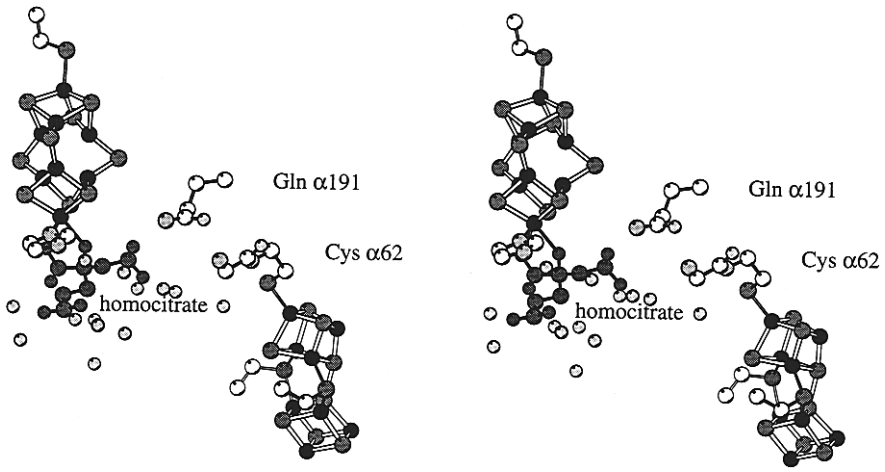


Figure 7. Stereoview of the protein environment between the FeMo cofactor and the P cluster of the MoFe protein from *A. vinelandii*.[31,32,34] Water molecules near the homocitrate are indicated by isolated spheres.

4. Unusual Chemical Properties of MoFe Protein Clusters

That the metallocenters in the MoFe protein would be unique structures among the Fe-S cluster family was anticipated from spectroscopic and chemical studies. Neither the P cluster nor the FeMo cofactor has been chemically synthesized, and neither has been removed from the protein in sufficient purity to obtain crystallographic characterization. Hence, the most detailed characterizations of the clusters to date have been as part of the total protein structure. Some rationalization of the chemical properties may now be attempted from inspection of the protein bound clusters.

Neither cluster type is reactive with respect to chelators or thiol exchange in the protein matrix,[67] which is consistent with the steric barriers of the protein structure that prevent access of these molecules to the clusters. Likewise, chemical oxidation−reduction of the clusters most likely occurs by outer-sphere electron transfer, as it is improbable that mediators could access the clusters directly. Once the protein is denatured, P clusters can be extruded by thiol exchange with exogenous mercaptans,[107] but in the process the clusters are split into conventional [4Fe-4S] clusters with 90+% yield. Interestingly, no [2Fe-2S] clusters are generated by the thiol exchange extrusion.

Contrary to the P clusters, FeMo cofactor is not extruded from the protein by exogenous mercaptans, which is consistent with there being a nonthiol, histidyl ligand from the protein. For example, if Av1 is incubated with mercaptoethanol in 8 M urea, the P clusters are destroyed while the cofactor remains intact and associated with the protein when the mixture is separated by gel filtration chromatography.[67] One condition does lead to extraction of activity, and isolation of presumably intact FeMo cofactor; namely, basic N-methylformamide (NMF) is sufficient to remove FeMo cofactor from acid-precipitated MoFe protein.[75] Although the chemical mechanism is obscure by which the one thiol and one histidyl ligand to the cofactor are displaced in this process, one can speculate that highly polar NMF molecules may be able to serve as metal ligands.

In addition, the Shah and Brill experiment,[75] as well as others, point to the remarkable and unique chemical stability of cofactor compared to conventional Fe-S clusters, especially considering one might expect the trigonal iron atoms to be highly reactive toward expansion of their ligand field. Indeed, the lack of protein side chain ligands to the six trigonal iron atoms of cofactor generally supports the notion that, in contrast, these irons are weakly reactive in the as-isolated form of the native MoFe protein. Likewise, the bridging inorganic sulfurs of cofactor appear to have some different properties compared to P clusters and other Fe-S clusters. This is exemplified by the significantly increased acid stability of the cofactor. Acid destruction (<pH 4) of exposed Fe-S clusters is thought to proceed by protonation of the bridging inorganic sulfur, thereby making the sulfur a better leaving group from the iron atom.[108,109] By analogy, one can reason that the bridging inorganic sulfurs of the cofactor are weaker conjugate acids, which may be indicative of accommodation of the trigonal iron ligation by increased delocalization of the sulfur electron onto the iron sites.

IV. Structure and Properties of the Nitrogenase Complex

The complex between the MoFe protein and Fe protein plays a central and critical role in the mechanism of nitrogenase, for it is in the complex that ATP hydrolysis is coupled to electron transfer between the two proteins. Indeed, ATP hydrolysis occurs only in the complex; Fe protein alone does not hydrolyze ATP. A model for the Fe protein−MoFe protein complex has been proposed on the basis of the individual structures.[32,110] (See note added in proof.) What appear to be potential, complementary binding sites occur on the surfaces of the two proteins. The Fe protein binding surface undoubtedly includes the cluster and two α-helices that extend outward from the cluster and resemble a bent rod. This rod could be considered complementary to the groove formed by the interface of the $\alpha\beta$-subunits containing the P cluster of the MoFe protein.[110] This docking model is shown in Figure 8. Besides this steric complementarity, the juxtaposition of surfaces results in pairing oppositely charged patches from the two molecules.[110] A highly specific chemical crosslinking site has been characterized which identified one such pairing of oppositely charged groups.[111,112] Mu-

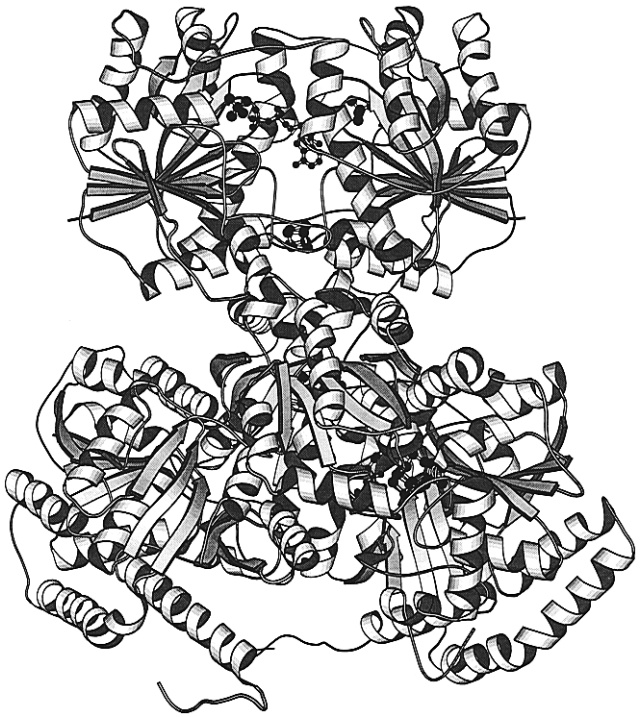


Figure 8. Computer-generated model[19,32,110] of the docking complex between the Fe protein dimer (top molecule) and an $\alpha\beta$ subunit pair of the MoFe protein (bottom molecule). The metal centers and ADP molecule are represented by ball-and-stick models.

tagenesis and proteolysis studies have identified several other regions that can be considered as part of the complex interface.[63,64,113,114] These regions all reside on the same surface as first proposed from the location of the cluster and the cross-linking site.

In this model, the shortest distances from the 4Fe-4S cluster of the Fe protein to the P cluster, from the P cluster to the FeMo cofactor, and from the Fe protein 4Fe-4S cluster to the FeMo cofactor are ~18, ~14, and ~32 Å, respectively. Hence, this model is suggestive of a general sequence of electron-transfer steps within the nitrogenase system that would occur in the order:

$$Fe_4S_4 \rightarrow \text{P cluster} \rightarrow \text{FeMo cofactor} \rightarrow \text{substrate}$$

Although the general region of interaction between the Fe protein and MoFe protein may be represented in Figure 8, a crucial point is that there are multiple states or multiple complexes that are relevant to the nitrogenase mechanism.[19,110] A turnover cycle for nitrogenase is illustrated in Figure 9 that emphasizes the unique aspects of these different states of the complex. These distinct forms of the complex, designated by the letters A through E, are characterized by the state of the nucleotide present in the complex. There are a number of experimental results that justify inclusion of each intermediate. For example, complexes A and D have been detected or inferred by spectral methods, Fe chelation, cross-linking experiments, steady-state kinetics, and studies with mutant proteins (reviewed in ref 19). Postulation of complex B stems from the requirement for protein complex formation in order to hydrolyze ATP, i.e., binding to the MoFe protein must induce conformational changes in Fe protein. In complex C, the nucleotide is hydrolyzed and the electron is transferred; these two steps are detected in stopped-flow microcalorimetry experiments.[115] Complex C then "relaxes" with the release of P_i. While the dissociation of complex D is the rate-determining step overall, complex C represents the activated, electron-transfer competent intermediate.

In the nitrogenase cycle illustrated above, the role for ATP hydrolysis is to control the electron-transfer "gate" between protein components.[19] How this is accomplished is the one of the two main unanswered questions about the nitrogenase mechanism (the other being how substrates are reduced at the cofactor). As discussed above, the P clusters appear to be fully reduced (all ferrous iron), and hence are not good electron acceptors. Moreover, since the P cluster is fully reduced in the resting state, there

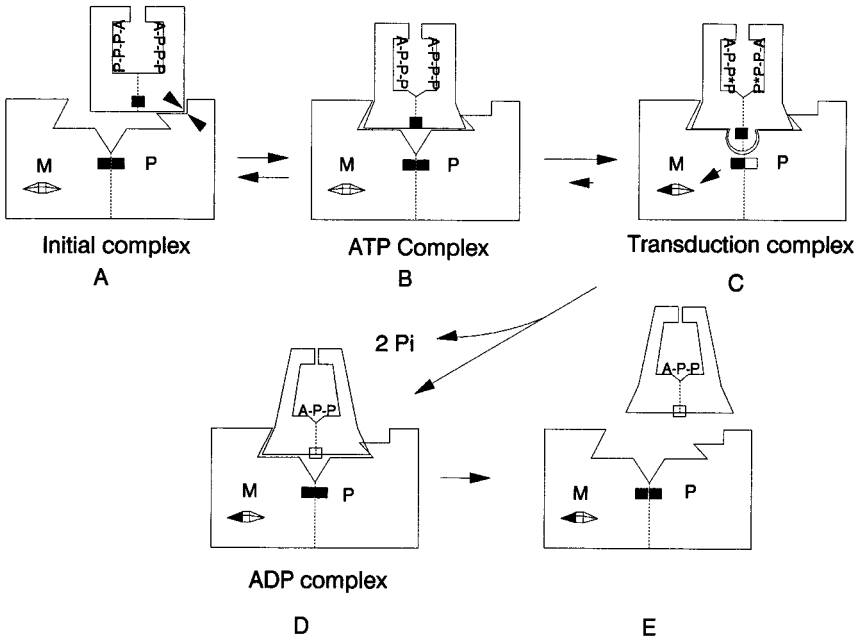


Figure 9. Schematic representation of the nitrogenase turnover cycle, indicating mechanistically relevant forms of the nitrogenase complex generated between the MoFe protein and Fe protein.

must be some break in the electron-transfer path between P cluster and FeMo cofactor, which is the ultimate electron acceptor during turnover. Hence, the P cluster is effectively locked in a state where it is unable to accept or donate electrons. Thus, the "gate" has two parts; one is to make the P cluster an electron acceptor, and the second is to allow the passage of an electron from the P cluster to the cofactor. We propose that the unique role of Fe protein is to cause the necessary conformational changes in MoFe protein to "open" the gates. There are at least two ways for this to occur.[19] The conformational change in the MoFe protein could result in opening of the electron transfer path from the P cluster to cofactor; once electron transfer has occurred between these centers, the P cluster could then be an acceptor for the electron from the Fe protein. Alternatively, the conformational change could cause the P cluster to function as a transient acceptor of an electron from the Fe protein, before transferring the electron on to the FeMo cofactor.

As discussed above, the Fe protein cluster is sensitive to nucleotide binding. These results have been used as arguments for a mechanistic rationale for the role of ATP in nitrogenase turnover, namely, the conformational change induced by ATP binding results in exposing the cluster and, hence making it a better electron donor in the complex with the MoFe protein. This argument continues; upon ATP hydrolysis the protein assumes the ADP conformation where the cluster is now sequestered and electron transfer blocked. This is similar to the "two state" or "on and off state" hypothesis for other nucleotide-dependent switch proteins such as *ras* and G-proteins. Although this model has a certain appeal, it cannot be the whole story. Much of the basis of the proposal is the nucleotide-dependent conformational changes observed by chelation and biophysical measurements. However, as described earlier, there are mutant proteins with normal ATP-dependent chelation characteristics that are inactive. Of course, the mutation could be altering a second, unrelated step in turnover. However, nonhydrolyzable nucleotide analogues (ADPNP, ADPCP, and ATPγS) also induce the chelation conformation, yet do not support substrate reduction, i.e., electron transfer. We suggest that the electron competent conformation is not the ATP or the ADP ground state, but a transient intermediate between the two, perhaps the state where hydrolysis has occurred but P_i not yet released.[110]

Several of the complexes described above can be prepared with sufficient integrity that they may be suitable for structural analysis. First, a complex is formed between Cp2 and Av1 which is inactive for substrate reduction, at least in part, because the complex only very slowly dissociates.[116] A second complex is the chemically cross-linked Av2−Av1 species, which, like the Cp2−Av1 complex, is inactive due to the requirement for complex dissociation in the nitrogenase mechanism.[112] Finally, mutagenesis methods have been used to generate Fe protein mutants that form irreversible complexes with the MoFe protein.[117,118] These complexes can be formed without nucleotide. Biochemical, spectroscopic, and structural analyses of these forms should provide a detailed picture of the early events in the redox cycle and complex formation.

Perhaps the most exciting and potentially most informative complex is the recently reported AlF_4-stabilized ADP−Av2−Av1 complex.[119,120] AlF_4 is a potent, slow inhibitor which reacts with the nucleotide-bound, enzymatically active complex at the stage after the release of the γ-phosphate from ATP hydrolysis, the condition or conformation closest to that for electron transfer. This form of Av2 bound nucleotide is obtained only in complex C (Figure 9), since AlF_4 reacts only with the nucleotide-bound complex. Inhibition ensues because the protein−nucleotide−AlF_4 complex is stable. Indeed, the $t_{1/2}$ for dissociation at 30 °C is greater than 24 h. Because analogs that resemble the transition state are potentially strong, quasi-irreversible inhibitors, they stabilize otherwise temporary macromolecular conformations. With the nitrogenase system, we have the potential to study the class of molecular switches that form protein−protein complexes during nucleotide hydrolysis and to identify how electron transfer pathways might change during the enzymatic cycle.

V. Synthesis of the Metallocenters of Nitrogenase

One of the most intriguing questions about the nitrogenase proteins is, "How are the clusters synthesized and assembled in the protein components?" The discovery in the early 1960s of proteins and enzymes containing iron and inorganic sulfide spawned a general interest among inorganic chemists to devise rational design protocols for Fe-S clusters with a variety of geometries and ligands. However, the nonaqueous solvents, rigorous exclusion of oxygen, and the concentrations of reactants used for inorganic synthetic routes do not exist in the cell, and it is likely that in the cell there is specific enzymatic machinery for cluster assembly. Understanding of the biological system may lead to innovative inorganic synthetic routes.

A significant problem for *in vitro* protein cluster assembly is the formation of polysulfides between the inorganic sulfide and the protein thiol ligands (cysteines). Dean and co-workers[121] elucidated at least one path for providing a low, controlled level of inorganic sulfide for *in vivo* cluster assembly. They found that *nifS* in the nitrogenase gene cluster codes for a pyridoxal phosphate-containing enzyme, cysteine desulfurase, which catalyzes the formation of inorganic sulfide by elimination of sulfur from cysteine.[122] The enzyme-bound sulfide, in turn, is donated to the acceptor protein-bound iron atoms; hence, free sulfide concentrations are kept at very low values in solution. This mechanism for sulfide insertion seems plausible, at least for the conventional 2Fe-2S and 4Fe-4S clusters, and has been demonstrated to occur during the reconstitution of nitrogenase Fe protein. Spontaneous cluster assembly with apo-Av2 traditionally has given low irreproducible yields, yet Zheng *et al.*[123] using cysteinyl desulfurase have quantitatively reconstituted Fe protein. By following these observations, genes coding for cysteine desulfurase have been found throughout the biological kingdoms and it appears

that this protein is a universal enzyme involved in Fe-S cluster assembly. It should be noted that nitrogen-fixing organisms have several copies of the gene, one of which is coordinately expressed with the nitrogenase proteins to provide for the extensive cluster synthesis demanded by the enormous amount of these proteins within the cells.[121] It is particularly satisfying that the breakthrough on a long-standing general biochemical problem should originate from studies on the nitrogenase proteins. Still to be identified is the donor for the iron atoms found in Fe-S clusters, although the *nifU* gene product is one possibility.[22,124]

Unlike the simpler 4Fe-4S cluster in the Fe protein, the MoFe protein P cluster has been neither spontaneously reconstituted nor synthesized *in vitro*. Although the P cluster may be a fusion of two 4Fe-4S clusters, there are several significant differences. First, there are two thiolate bridging ligands and likely one less inorganic sulfide. Second, all iron atoms are ferrous. In simpler 4Fe-4S clusters, the all-ferrous iron state is generally not obtained. How the P cluster is assembled *in vivo* is open to conjecture. One reasonable possibility is that the P cluster arises by fusion of two 4Fe-4S clusters first assembled in the individual α and β subunits as the more tenable and usual +1 oxidation state found in reduced ferredoxins (this is the 3− state if the usual four −1 ligands are included in the formal charge). The P cluster could be formed by nucleophilic substitution of the shared ligand from each subunit (Cys α88 and Cys β95), followed by an internal redox reaction to give all-ferrous iron and the expulsion of one inorganic sulfur, perhaps as a persulfide of a displaced thiolate ligand. P clusters have only six thiolate ligands for eight iron atoms, rather than the "classical" ferredoxin ratio of 1:1. The 4Fe-4S clusters proposed as the precursors for the P cluster would have three thiolate ligands, which is determined by the protein structure since only three cysteines from each subunit are present in the cluster cavity. The fourth ligand could be a carboxylate group, a non-MoFe protein thiolate group, or a solvent molecule. Indeed, there is precedent in other proteins for clusters with three thiolate and one oxy ligands; in these clusters, the oxy ligand is readily displaced by other nucleophiles and undoubtedly serves as a reactive site for substitution. (See the review on aconitase in this issue, ref 125.)

Although the order for P cluster and FeMo-co incorporation in the MoFe protein is not known, it is likely the P cluster assembly occurs first. Des-FeMo-co-MoFe protein has been isolated and observed under a number of conditions, while des-P cluster MoFe protein has not (reviewed in ref 17). Analysis of the FeMo cofactor-deficient protein prepared in a Fe protein deletion strain of *A. vinelandii* indicates that P cluster-like centers are present in this protein.[126] As discussed above, the FeMo cofactor is an entity that can be extracted intact from denatured MoFe protein and reinserted in des-FeMo-co-MoFe protein.[75] The latter, in turn, originates by any of several mutations in genes ancillary to the structural genes of the nitrogenase components. Although the parts of the extensive machinery for synthesis of cofactor can be isolated, little of the chemistry of the synthesis has been determined. For example, the proteins NIFE,N have substantial sequence homology to MoFe protein and appear to serve as a scaffold for assembly of the cofactor.[127,128] The role of *nifB* in cofactor synthesis is of particular interest. The protein encoded by this gene is unknown, but it appears to lead to formation of yet another cofactor dubbed NifB-co.[129] This material contains iron and sulfur and can serve as the sole source of these two elements for FeMo cofactor assembly on NIFN,E.[130,131] Thus, the identification of NifB-co has only removed by one step a central question in MoFe-co synthesis: how is the trigonal iron coordination generated? NifB-co does not contain molybdenum and is insufficient to serve as cofactor for nitrogenase. Most likely, NifB-co is transferred to NIFN,E, followed by molybdenum insertion and homocitrate chelation. Although many of the amino acids composing the cofactor cavity in the MoFe protein are not conserved in NIFN,E, Cys 275, the sole thiolate ligand to FeMo-co, is present in NIFN,E and may be essential for attachment to the cofactor precursor during assembly. Finally, there is a requirement for Fe protein in both the synthesis and insertion of FeMo-co.[132−136] How Fe protein might participate is unknown, but its role in this process is decidedly different than in substrate reduction. For example, Fe protein mutants that are inactive in substrate reduction are fully active in both construction and insertion of cofactor. One possibility is that the function of Fe protein is to induce an "open" conformation in the des-FeMo-co-MoFe protein which allows insertion of the cofactor, a role that inactive Fe protein might serve even though kinetically inactive as an electron-transfer agent. Furthermore, if NIFN,E is structurally similar to MoFe protein, as the sequence homology implies, then Fe protein binding might function similarly in cofactor synthesis on the NIFN,E template, i.e., to stabilize or open the active conformation of NIFN,E for either synthesis or transfer of the cofactor.

The mechanism by which the FeMo cofactor precursor is transferred from the NIFN,E protein to the des-FeMo-co-MoFe protein is also uncharacterized. The crystal structure of the native MoFe protein clearly shows the cofactor to be buried. However, the cofactor cavity is at the interface of the three folding domains that compose the α-subunit. This binding site could be exposed either by an opening of the polypeptide flaps that are "above" the cofactor, or by as little as a 20° rotation of a peptide bond (e.g., the ϕ angle of Lys 318) in the helical region connecting domains II and III. This latter motion could either expose the cofactor site, or if coupled to a similar conformational change in the NIFN,E protein, perhaps culminate in a transient swapping of the corresponding domains[137] between these two homologous proteins. Recently, a small molecular weight protein has been identified that may serve both as a carrier for the cofactor insertion and as a "wedge" to hold the desFeMoco-MoFe protein in an open conformation.[135,138,139]

VI. Conclusions

Although a more detailed structural picture of the nitrogenase system has emerged in the past few

years, our understanding is still quite hazy of the molecular details by which this system is assembled and functions in biological nitrogen fixation. While different people might place different problems at the top of their list of outstanding nitrogenase issues to be solved, there are a collection of interrelated questions that need to be answered before we can begin to claim an understanding of the molecular basis of biological nitrogen fixation. These questions include: (1) the mechanism of substrate binding and reduction at the nitrogenase metallocenters; (2) the structures and properties of the metallocenters in different oxidation states; (3) the function of the P clusters in substrate reduction; (4) the structures and properties of the nitrogenase complexes; (5) the role of ATP hydrolysis in the nitrogenase mechanism; (6) assembly pathways of the nitrogenase proteins; and (7) chemical synthesis of FeMo cofactor and P cluster analogues.

One of the beautiful features of the nitrogenase system is that there is something for everyone. Ultimate resolution of the mechanism of nitrogenase will require the efforts of biochemists, crystallographers, molecular biologists, spectroscopists, synthetic chemists, and theoreticians to crack the puzzle of how a complex system acts on a simple molecule.

VII. Acknowledgments

The contributions of our group members who made this review possible are greatly appreciated, as are discussions with our nitrogenase colleagues. Research in the authors' laboratory was supported by NSF DMB 91-18689 (D.C.R.), NSF MCB 95-13512 (J.B.H.) and USPHS GM45162 (D.C.R. and J.B.H.).

Note Added in Proof

Several developments relevant to nitrogenase structure have occurred since the submission of this review.

1. We have collected multiple diffraction data sets under cryogenic conditions for Av1 at 2 Å resolution or below that indicate that the P cluster probably is of the 8Fe-7S type, and not of the 8Fe-8S type (J. Peters, M. H. B. Stowell, D. C. Rees, unpublished results). We have observed both a more open conformation (Figure 6B) with the Ser β188 ligand and a more closed conformation similar to that described by Bolin. While it is plausible that these two 8Fe-7S cluster types represent different oxidation states of the P cluster, the conditions that determine which form will be observed is still being investigated. The previously reported 8Fe-8S model of the P cluster (Figure 6A) may incorrectly reflect the consequences of modeling a single structure to a superposition of electron densities resulting from a mixture of the two types of 8Fe-7S clusters in our Av1 crystals.

2. Preliminary interpretation of an 80% complete data set from the *Klebsiella pneumoniae* MoFe protein at 1.65 Å resolution is consistent with the 8Fe-7S P cluster model of Bolin (D. Lawson, S. M. Roe, C. Gormal, and B. E. Smith, personal communication).

3. We have determined the structure of the AlF_4-ADP-stabilized complex of Av1 and Av2 at 3 Å resolution (H. Schindelin, C. Kisker, J. Schlessman, J. B. Howard, and D. C. Rees, unpublished results). The general features of the complex are the nucleotide is bound in the *ras*-type mode, two nucleotides are bound for each Av2 molecule (one nucleotide per subunit), two Av2 are bound to one Av1 tetramer (one Av2 for each Av1 $\alpha\beta$ subunit pair), and the docking interface is generally as predicted from model building (Figure 8). Most of the conformational changes occur in the Av2 structure, with little change noted in Av1 at the present resolution. At least some of the changes in Av2 result from rotation of the two subunits about the molecular 2-fold axis. In particular, the contacts at the interface between the two Av2 subunits, and between Av1 and Av2, are more extensive than predicted on the basis of rigid-body superposition of the individual proteins.

VIII. References

(1) Chase, M. W., Jr.; Davies, C. A.; Downe, J. R., Jr.; Frurip, D. J.; McDonald, R. A.; Syverue, A. N. *J. Phys. Chem. Ref. Data* **1985**, *14*, Suppl. No. 1.
(2) Alberty, R. A. *J. Biol. Chem.* **1994**, *269*, 7099.
(3) Massey, H. S. W. *Negative Ions*; Cambridge University Press: Cambridge, 1976.
(4) Rosenstock, H. M.; Draxl, K.; Steiner, B. W.; Herron, J. T. *J. Phys. Chem. Ref. Data* **1977**, *6*, Suppl. No. 1.
(5) Cottrell, T. L. *The Strengths of Chemical Bonds*; Butterworths: London, 1958.
(6) Topham, S. A. In *Catalysis: Science and Technology;* Anderson, J. R., Boudart, M., Eds.; Springer-Verlag: Berlin, 1985; p 1.
(7) Jennings, J. R., Ed. *Catalytic Ammonia Synthesis: Fundamentals and Practice*; Plenum Press: New York, 1991.
(8) Schlesinger, W. H. *Biogeochemistry: An Analysis of Global Change*; Academic Press: San Diego, 1991.
(9) Kirschner, E. M. *Chem. Eng. News* **1996**, *74*, No. 15 (April 8), 16.
(10) Burgess, B. K. In *Advances in Nitrogen Fixation;* Veeger, C., Newton, W. E., Eds.; Martinus Nijhoff: Boston, 1984; p 103.
(11) Orme-Johnson, W. H. *Annu. Rev. Biophys. Biophys. Chem.* **1985**, *14*, 419.
(12) Stiefel, E. I.; Thomann, H.; Jin, H.; Bare, R. E.; Morgan, T. V.; Burgmayer, S.J. N.; Coyle, C. L. In *Metal Clusters in Proteins;* Que, L., Eds.; American Chemical Society: Washington, DC, 1988; p 372.
(13) Stacey, G.; Burris, R. H.; Evans, H. J., Ed. *Biological Nitrogen Fixation*; Chapman & Hall: New York, 1992.
(14) Smith, B. E.; Eady, R. R. *Eur. J. Biochem.* **1992**, *205*, 1.
(15) Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds. *Molybdenum Enzymes, Cofactors and Model Systems*; American Chemical Society: Washington, DC, 1993.
(16) Mortenson, L. E.; Seefeldt, L. C.; Morgan, T. V.; Bolin, J. T. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1993**, *67*, 299.
(17) Dean, D. R.; Bolin, J. T.; Zheng, L. M. *J. Bacteriol.* **1993**, *175*, 6737.
(18) Kim, J.; Rees, D. C. *Biochemistry* **1994**, *33*, 387.
(19) Howard, J. B.; Rees, D. C. *Annu. Rev. Biochem.* **1994**, *63*, 235.
(20) Allen, R. M.; Chatterjee, R.; Madden, M. S.; Ludden, P. W.; Shah, V. K. *Crit. Rev. Biotech.* **1994**, *14*, 225.
(21) Peters, J. W.; Fisher, K.; Dean, D. R. *Annu. Rev. Microbiol.* **1995**, *49*, 335.
(22) Muchmore, S. W.; Jack, J. F.; Dean, D. R. in *Adv. Inorg. Biochem.;* Hausinger, R. P., Eichhorn, G. L., Marzilli, L. G., Eds., VCH: New York, 1996; p 111.
(23) Burgess, B. K.; Lowe, D. J. *Chem. Rev.* **1996**, *96*, 2983 (accompanying article in this issue).
(24) Eady, R. R. *Chem. Rev.* **1996**, *96*, 3013 (accompanying article in this issue).
(25) Simpson, F. B.; Burris, R. H. *Science* **1984**, *224*, 1095.
(26) Lowe, D. J.; Thorneley, R. N. F. *Biochem. J.* **1983**, *215*, 393.
(27) Lowe, D. J.; Thorneley, R. N. F. *Biochem. J.* **1984**, *224*, 895.
(28) Hageman, R. V.; Burris, R. H. *Biochemistry* **1978**, *17*, 4117.
(29) Dean, D. R.; Jacobson, M. R. In *Biological Nitrogen Fixation;* Stacey, G., Burris, R. H., Evans, H. J., Eds., Chapman & Hall: New York, 1992; 763.
(30) Georgiadis, M. M.; Komiya, H.; Chakrabarti, P.; Woo, D.; Kornuc, J. J.; Rees, D. C. *Science* **1992**, *257*, 1653.
(31) Kim, J.; Rees, D. C. *Science* **1992**, *257*, 1677.
(32) Kim, J.; Rees, D. C. *Nature* **1992**, *360*, 553.
(33) Kim, J.; Woo, D.; Rees, D. C. *Biochemistry* **1993**, *32*, 7104.
(34) Chan, M. K.; Kim, J.; Rees, D. C. *Science* **1993**, *260*, 792.

(35) Bolin, J. T.; Campobasso, N.; Muchmore, S. W.; Morgan, T. V.; Mortenson, L. E. in *Molybdenum Enzymes, Cofactors and Model Systems;* Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds.; *ACS Symposium Series 535;* American Chemical Society: Washington, DC, 1993; p 186.
(36) Emerich, D. W.; Hageman, R. V.; Burris, R. H. *Adv. Enzymol.* **1981**, *52*, 1.
(37) Emerich, D. W.; Burris, R. H. *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 4369.
(38) Hausinger, R. P.; Howard, J. B. *J. Biol. Chem.* **1982**, *257*, 2483.
(39) Brigle, K. E.; Newton, W. E.; Dean, D. R. *Gene* **1985**, *37*, 37.
(40) Holm, L.; Sander, C. *Trends Biochem. Sci.* **1995**, *20*, 478.
(41) Hausinger, R. P.; Howard, J. B. *J. Biol. Chem.* **1983**, *258*, 13486.
(42) Watt, G. D.; Reddy, K. R. N. *J. Inorg. Biochem.* **1994**, *53*, 281.
(43) Adman, E. T.; Watenpaugh, K. D.; Jensen, L. H. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 4854.
(44) Lindahl, P. A.; Day, E. P.; Kent, T. A.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1985**, *260*, 11160.
(45) Hagen, W. R.; Eady, R. R.; Dunham, W. R.; Haaker, H. *FEBS Lett.* **1985**, *189*, 250.
(46) Morgan, T. V.; Prince, R. C.; Mortenson, L. E. *FEBS Lett.* **1986**, *206*, 4.
(47) Watt, G. D.; McDonald, D. W. *Biochemistry* **1985**, *24*, 7226.
(48) Lindahl, P. A.; Gorelick, N. J.; Münck, E.; Orme-Johnson, W. H. *J. Biol. Chem.* **1987**, *262*, 14945.
(49) Walker, J. E.; Saraste, M.; Runswick, M. J.; Gay, N. J. *EMBO J.* **1982**, *8*, 945.
(50) Schulz, G. E. *Curr. Opin. Struc. Biol.* **1992**, *2*, 61.
(51) Pai, E. F.; Krengel, U.; Petsko, G. A.; Goody, R. S.; W., K.; Wittinghofer, A. *EMBO J.* **1990**, *9*, 2351.
(52) Tong, L.; de Vos, A. M.; Milburn, M. V.; Kim, S.-H. *J. Mol. Biol.* **1991**, *217*, 503.
(53) Zumft, W. G.; Mortenson, L. E.; Palmer, G. *Eur. J. Biochem.* **1974**, *46*, 525.
(54) Smith, B. E.; Lowe, D. J.; Bray, R. C. *Biochem. J.* **1973**, *135*, 331.
(55) Zumft, W.; Mortenson, L.; Palmer, G. *Biochim. Biophys. Acta* **1973**, *292*, 413.
(56) Orme-Johnson, W. H.; Hamilton, W. D.; Ljones, T.; T'so, M. Y. W.; Burris, R. H.; Shah, V. K.; Brill, W. J. *Proc. Natl. Acad. Sci. USA* **1972**, *69*, 3142.
(57) Stephens, P. J.; McKenna, C. E.; Smith, B. E.; Nguyen, H. T.; McKenna, M.-C.; Thomas, A. J.; Devlin, F.; Jones, J. B. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 2585.
(58) Ryle, M. J.; Lanzilotta, W. N.; Seefeldt, L. C.; Scarrow, R. C.; Jensen, G. M. *J. Biol. Chem.* **1996**, *271*, 1551.
(59) Chen, L.; Gavini, N.; Tsuruta, H.; Eliezer, D.; Burgess, B. K.; Doniach, S.; Hodgson, K. O. *J. Biol. Chem.* **1994**, *269*, 3290.
(60) Walker, G. A.; Mortenson, L. E. *Biochem. Biophys. Res. Commun.* **1973**, *53*, 904.
(61) Ljones, T.; Burris, R. H. *Biochemistry* **1978**, *17*, 1866.
(62) Deits, T. L.; Howard, J. B. *J. Biol. Chem.* **1989**, *264*, 6619.
(63) Wolle, D.; Kim, C.; Dean, D.; Howard, J. B. *J. Biol. Chem.* **1992**, *267*, 3667.
(64) Seefeldt, L. C. *Protein Sci.* **1994**, *3*, 2073.
(65) Wolle, D.; Howard, J. B. Manuscript in preparation.
(66) Wolle, D.; Dean, D. R.; Howard, J. B. *Science* **1992**, *258*, 992.
(67) Magnuson, J.; Howard, J. B. Manuscript in preparation.
(68) Seefeldt, L. C.; Morgan, T. V.; Dean, D. R.; Mortenson, L. E. *J. Biol. Chem.* **1992**, *267*, 6680.
(69) Lanzilotta, W. N.; Holz, R. C.; Seefeldt, L. C. *Biochemistry* **1995**, *34*, 15646.
(70) Gavini, N.; Burgess, B. K. *J. Biol. Chem.* **1992**, *267*, 21179.
(71) Ryle, M. J.; Lanzilotta, W. N.; Mortenson, L. E.; Watt, G. D.; Seefeldt, L. C. *J. Biol. Chem.* **1995**, *270*, 13112.
(72) Meyer, J.; Gaillard, J.; Moulis, J.-M. *Biochemistry* **1988**, *27*, 6150.
(73) Howard, J. B. Unpublished observations.
(74) Blanchard, C. Z.; Hales, B. J. *Biochemistry* **1996**, *35*, 472.
(75) Shah, V. K.; Brill, W. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 3249.
(76) Oliver, M. E.; Hales, B. J. *Biochemistry* **1993**, *32*, 6058.
(77) Power, P. P.; Shoner, S. C. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 330.
(78) Eidsness, M. K.; Flank, A. M.; Smith, B. E.; Flood, A. C.; Garner, C. D.; Cramer, S. P. *J. Am. Chem. Soc.* **1986**, *108*, 2746.
(79) Conradson, S. D.; Burgess, B. K.; Newton, W. E.; Mortenson, L. E.; Hodgson, K. O. *J. Am. Chem. Soc.* **1987**, *109*, 7507.
(80) Arber, J. M.; Flood, A. C.; Garner, C. D.; Gormal, C. A.; Hasnain, S.; Smith, B. E. *Biochem. J.* **1988**, *252*, 421.
(81) Chen, J.; George, S. J.; Tittsworth, R. C.; Hales, B. J.; Christiansen, J.; Coucouvanis, D.; Cramer, S. P. *J. Am. Chem. Soc.* **1993**, *115*, 5509.
(82) Chen, J.; Christiansen, J.; Campobasso, N.; Bolin, J. T.; Tittsworth, R. C.; Hales, B. J.; Rehr, J. J.; Cramer, S. P. *Angew. Chem., Intl. Ed. Engl.* **1993**, *32*, 1592.
(83) Christiansen, J.; Tittsworth, R. C.; Hales, B. J.; Cramer, S. P. *J. Am. Chem. Soc.* **1995**, *117*, 10017.
(84) Richards, F. M. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 151.
(85) Dance, I. G. *Aust. J. Chem.* **1994**, *47*, 979.
(86) Howes, B. D.; Fisher, K.; Lowe, D. J. *Biochem. J.* **1994**, *297*, 261.
(87) Hawkes, T. R.; McLean, P. A.; Smith, B. E. *Biochem. J.* **1984**, *217*, 317.
(88) Imperial, J.; Hoover, T. R.; Madden, M. S.; Ludden, P. W.; Shah, V. K. *Biochemistry* **1989**, *28*, 7796.
(89) Hoover, T. R.; Robertson, A. D.; Cerny, R. L.; Hayes, R. N.; Imperial, J.; Shah, V. K.; Ludden, P. W. *Nature* **1987**, *329*, 855.
(90) Madden, M. S.; Kindon, N. D.; Ludden, P. W.; Shah, V. K. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 6517.
(91) Kim, C. H.; Newton, W. E.; Dean, D. R. *Biochemistry* **1995**, *34*, 2798.
(92) Deng, H. B.; Hoffmann, R. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1062.
(93) Leigh, G. J. *Eur. J. Biochem.* **1995**, *229*, 14.
(94) Leigh, G. J. *Science* **1995**, *268*, 827.
(95) Leigh, G. J.; Jimenez-Tenorio, M. *J. Am. Chem. Soc.* **1991**, *113*, 5862.
(96) Leigh, G. J. *New. J. Chem.* **1994**, *18*, 157.
(97) Stavrev, K. K.; Zerner, M. C. *Chem. Eur. J.* **1996**, *2*, 83.
(98) Laplaza, C. E.; Cummins, C. C. *Science* **1995**, *268*, 861.
(99) Feher, G.; Allen, J. P.; Okamura, M. Y.; Rees, D. C. *Nature* **1989**, *339*, 111.
(100) DeRose, V. J.; Kim, C.-H.; Newton, W. E.; Dean, D. R.; Hoffman, B. M. *Biochemistry* **1995**, *34*, 2809.
(101) Dean, D. R.; Setterquist, R. A.; Brigle, K. E.; Scott, D. J.; Laird, N. F.; Newton, W. E. *Mol. Microbiol.* **1990**, *4*, 1505.
(102) Kent, H. K.; Ioannidis, I.; Gormal, C.; Smith, B. E.; Buck, M. *Biochem. J.* **1989**, *264*, 257.
(103) May, H. D.; Dean, D. R.; Newton, W. E. *Biochem. J.* **1991**, *277*, 457.
(104) Kent, H. M.; Baines, M.; Gormal, C.; Smith, B. E.; Buck, M. *Mol. Microbiol.* **1990**, *4*, 1497.
(105) Surerus, K. K.; Hendrich, M. P.; Christie, P. D.; Rottgardt, D.; Orme-Johnson, W. H.; Münck, E. *J. Am. Chem. Soc.* **1992**, *114*, 8579.
(106) Peters, J. W.; Fisher, K.; Newton, W. E.; Dean, D. R. *J. Biol. Chem.* **1995**, *270*, 27007.
(107) Kurtz, D. M.; McMillan, R. S.; Burgess, B. K.; Mortenson, L. E.; Holm, R. H. *Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4986.
(108) Bruice, T. C.; Maskiewicz, R.; Job, R. *Proc. Natl. Acad. Sci. USA* **1975**, *72*, 231.
(109) Maskiewicz, R.; Bruice, T. C. *Biochemistry* **1977**, *16*, 3024.
(110) Howard, J. B. In *Molybdenum Enzymes, Cofactors and Model Systems;* Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds.; ACS Symposium Series 535; American Chemical Society: Washington, DC, 1993; p 271.
(111) Willing, A.; Howard, J. B. *J. Biol. Chem.* **1990**, *265*, 6596.
(112) Willing, A. H.; Georgiadis, M. M.; Rees, D. C.; Howard, J. B. *J. Biol. Chem.* **1989**, *264*, 8499.
(113) Peters, J. W.; Fisher, K.; Dean, D. R. *J. Biol. Chem.* **1994**, *269*, 28076.
(114) Thorneley, R. N. F.; Ashby, G. A.; Fisher, K.; Lowe, D. J. In *Molybdenum Enzymes, Cofactors and Models;* Stiefel, E. I., Coucouvanis, D., Newton, W. E., Eds.; American Chemical Society: Washington, DC, 1993; p 290.
(115) Thorneley, R. N. F. *Philos. Trans. R. Soc. Lond. B* **1992**, *336*, 73.
(116) Emerich, D. W.; Ljones, T.; Burris, R. H. *Biochem. Biophys. Acta* **1978**, *614*, 196.
(117) Ryle, M. J.; Seefeldt, L. C. *Biochemistry* **1996**, *35*, 4766.
(118) Lanzilotta, W. N.; Fisher, K.; Seefeldt, L. C. *Biochemistry* **1996**, *35*, 7188.
(119) Renner, K. A.; Howard, J. B. *Biochemistry* **1996**, *35*, 5353.
(120) Duyvis, M.; Wassink, H.; Haaker, H. *FEBS Lett.* **1996**, *380*, 233.
(121) Zheng, L.; White, R. H.; Cash, V. L.; Jack, R. F.; Dean, D. R. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2754.
(122) Zheng, L. M.; White, R. H.; Cash, V. L.; Dean, D. R. *Biochemistry* **1994**, *33*, 4714.
(123) Zheng, L. M.; Dean, D. R. *J. Biol. Chem.* **1994**, *269*, 18723.
(124) Fu, W. G.; Jack, R. F.; Morgan, T. V.; Dean, D. R.; Johnson, M. K. *Biochemistry* **1994**, *33*, 13455.
(125) Beinert, H.; Kennedy, M. C.; Stout, C. D. *Chem. Rev.* **1996**, *96*, 2335 (accompanying article in this issue).
(126) Gavini, N.; Ma, L.; Watt, G.; Burgess, B. K. *Biochemistry* **1994**, *33*, 11842.
(127) Paustian, T. D.; Shah, V. K.; Roberts, G. P. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6082.
(128) Brigle, K. E.; Weiss, M. C.; Newton, W. E.; Dean, D. R. *J. Bacteriol.* **1987**, *169*, 1547.
(129) Shah, V. K.; Allen, J. R.; Spangler, N. J.; Ludden, P. W. *J. Biol. Chem.* **1994**, *269*, 1154.
(130) Roll, J. T.; Shah, V. K.; Dean, D. R.; Roberts, G. P. *J. Biol. Chem.* **1995**, *270*, 4432.
(131) Allen, R. M.; Chatterjee, R.; Ludden, P. W.; Shah, V. K. *J. Biol. Chem.* **1995**, *270*, 26890.

(132) Filler, W. A.; Kemp, R. M.; Ng, J. C.; Hawkes, T. R.; Dixon, R. A.; Smith, B. E. *Eur. J. Biochem*. **1986**, *160*, 371.
(133) Robinson, A. C.; Dean, D. R.; Burgess, B. K. *J. Biol. Chem*. **1987**, *262*, 14327.
(134) Robinson, A. C.; Chu, W.; Li, J.-G.; Burgess, B. K. *J. Biol. Chem*. **1989**, *264*, 10088.
(135) Homer, M. J.; Dean, D. R.; Roberts, G. P. *J. Biol. Chem*. **1995**, *270*, 24745.
(136) Allen, M. J.; Homer, M. J.; Chatterjee, R.; Ludden, P. W.; Roberts, G. P.; Shah, V. K. *J. Biol. Chem*. **1993**, *268*, 23670.
(137) Bennett, M. J.; Schlunegger, M. P.; Eisenberg, D. *Protein Sci*. **1995**, *4*, 2455.
(138) Homer, M. J.; Paustian, T. D.; Shah, V. K.; Roberts, G. P. *J. Bacteriol*. **1993**, *175*, 4907.
(139) White, T. C.; Harris, G. S.; Orme-Johnson, W. H. *J. Biol. Chem*. **1992**, *267*, 24007.
(140) Kraulis, P. J. *J. Appl. Crystallogr*. **1991**, *24*, 946.

CR9500545