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Nitrogenase and Biological Nitrogen Fixation†

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ABSTRACT: Biological nitrogen fixation is catalyzed by the nitrogenase enzyme system which consists of two metalloproteins, the iron (Fe-) protein and the molybdenum–iron (MoFe-) protein. Together, these proteins mediate the ATP-dependent reduction of dinitrogen to ammonia. Recent crystallographic analyses of Fe-protein and MoFe-protein have revealed the polypeptide fold and the structure and organization of the unusual metal centers in nitrogenase. These structure provide a molecular framework for addressing the mechanism of the nitrogenase-catalyzed reaction. General features of the nitrogenase system, including conformational coupling of nucleotide hydrolysis, aspects of the cluster structures, and the general spatial organization of redox centers within the protein subunits, are relevant to a wide range of biochemical systems.

Reduced nitrogen is an integral component of proteins, nucleic acids, and most other biomolecules. Consequently, acquisition of metabolically usable forms of nitrogen is essential for the growth and survival of all organisms. Although elemental dinitrogen, N_2, is abundant in the earth's atmosphere, it is essentially inert at room temperature in the absence of a suitable catalyst. The reduction of N_2 to form ammonia provides an important example of this behavior. Although thermodynamically favorable, the activation energy required to reduce N_2 to ammonia is very large, as has been clearly shown in the industrial fixation of nitrogen by the Haber–Bosch process. In this process, significant formation of NH_3 from N_2 occurs only at temperatures between 300 and 500 °C and pressures over 300 atmospheres, in the presence of Fe-based catalysts. The abundance of N_2, contrasted with the difficulty of chemically utilizing this source, creates a paradox which nature has ingeniously solved by the process of biological nitrogen fixation, i.e., the reduction of N_2 to the metabolically usable form of ammonia. Only a relatively small number of microorganisms, termed diazotrophs, are capable of carrying out this process (Burns & Hardy, 1975). All other organisms depend directly or indirectly upon diazotrophs for their supply of nitrogenous compounds that are subsequently utilized for the synthesis of nucleic acids, proteins, etc. via complex arrays of biochemical pathways. Diazotrophs annually add about 60% of the earth's newly fixed nitrogen, while industrially fixed nitrogen contributes only about 25% of the bulk, with the remaining 15% produced by lightening, UV radiation, etc. Consequently, diazotrophs are essential for maintaining the nitrogen cycle in the earth's biosphere (Burns & Hardy, 1975).

Although diazotrophs display a wide spectrum of habitats that range from free forms to association with various plants (Burns & Hardy, 1975), they all utilize the same basic biochemical machinery for N_2 fixation, which is carried out by the nitrogenase enzyme system. Nitrogenase consists of two metalloprotein components, the iron (Fe-) protein and the molybdenum–iron (MoFe-) protein. Under conditions of molybdenum depletion, alternative nitrogenase systems homologous to the molybdenum-containing "conventional" nitrogenase system may be induced. Substrate reduction by nitrogenase involves three basic types of electron transfer reactions: (i) the reduction of Fe-protein by electron carriers

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such as flavodoxin or ferredoxin *in vivo* and dithionite *in vitro*; (ii) transfer of single electrons from Fe-protein to MoFe-protein in a MgATP-dependent process, with a minimal stoichiometry of 2 MgATP hydrolyzed per electron transferred; and (iii) electron transfer to the substrate, which is almost certainly bound to the active site within the MoFe-protein. Under optimal conditions, the overall stoichiometry of dinitrogen reduction by nitrogenase has been established as (Simpson & Burris, 1984)

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

Nitrogenase catalyzes not only the reduction of dinitrogen to ammonia but also the reduction of protons to hydrogen (which appears to be an obligatory part of ammonia formation) and the reduction of small unsaturated molecules such as acetylene, azide, or cyanide (Burgess, 1985).

Given the biological and chemical significance of nitrogen fixation, the properties and reaction mechanism of the nitrogenase proteins have been extensively studied. This review will focus on mechanistic implications of the structures of the nitrogenase proteins, based on the crystallographic structures determined at Caltech of the Fe-protein and MoFe-protein isolated from both *Azotobacter vinelandii* and *Clostridium pasteurianum* (Georgiadis et al., 1992; Kim & Rees, 1992a,b; Chan et al., 1993; Kim et al., 1993), and of the *C. pasteurianum* MoFe-protein determined at Purdue (Bolin et al., 1993 a,b). Excellent existing reviews and conference proceedings (Burgess, 1984; Orme-Johnson, 1985; Stiefel et al., 1988, 1993; Burris, 1991; Smith & Eady, 1992; Stacey et al., 1992; Palacios et al., 1993; Rees et al., 1993) should be consulted for more detailed discussions of the enzymology, spectroscopy, molecular biology, and chemical modeling that are beyond the scope of this article.

Nomenclature. To distinguish the two nitrogenase proteins isolated from different bacterial sources, the MoFe-protein and Fe-protein are designated as components "1" and "2", respectively, preceded by a two-letter abbreviation of the source species and genus (Eady et al., 1972), i.e., Av1 is MoFe-protein isolated from *A. vinelandii* and Cp2 is Fe-protein isolated from *C. pasteurianum*, etc. In this paper, the residue numbers of the Av2 protein sequence (Hausinger & Howard, 1982) and the gene sequences of the Av1 subunits (Brigle et al., 1985) are used.

STRUCTURES OF THE NITROGENASE PROTEINS

Fe-Protein. The Fe-protein is a dimer of two identical subunits, which are encoded by the *nifH* gene, with a total molecular mass of ~60 kDa. Each subunit folds as a single domain of the α helical/β sheet type (Georgiadis et al., 1992), which are covalently connected by the 4Fe-4S cluster at one end of the dimer (Figure 1). At the core of each subunit is an eight-stranded, predominantly parallel, β sheet that is flanked by nine α-helices. The two subunits are related by a molecular 2-fold rotation axis that passes through the cluster. As originally identified from biochemical and mutagenesis studies (Hausinger & Howard, 1983; Howard et al., 1989), the cluster is symmetrically coordinated by two cysteines, 97 and 132, that are provided by each subunit. A striking feature of the structure is the solvent exposure of the 4Fe-4S cluster, which had been recognized in spectroscopic studies (Morgan et al., 1990). Other than the ligating cysteines, there is little contact between the 4Fe-4S cluster and other amino acid side chains. Consequently, the general picture of the 4Fe–4S cluster that emerges from the Fe-protein structure is of an exposed, loosely packed redox center that may function as a pivot or hinge to accommodate conformational rearrangements between subunits during the course of the catalytic cycle of nitrogenase.

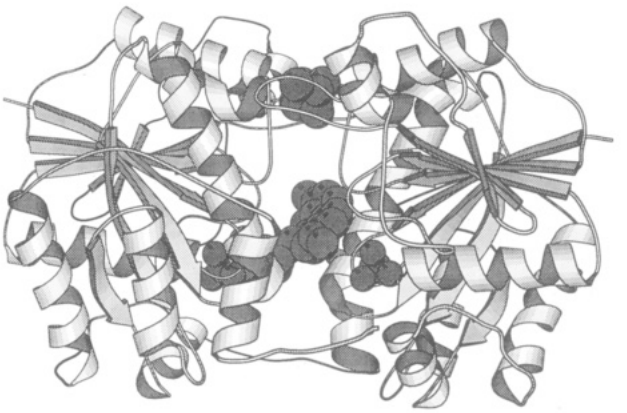

FIGURE 1: Ribbons diagram of the polypeptide fold of the Fe-protein dimer, with CPK models for the 4Fe-4S cluster and ADP. Prepared with the program MOLSCRIPT (Kraulis, 1991).

The general type of polypeptide fold adopted by Fe-protein is a common motif found in many nucleotide-binding proteins (Schulz, 1992). Associated with this nucleotide-binding motif is a characteristic sequence pattern GXXXXGKS, known as Walker's motif A (Walker et al., 1982), where X represents any amino acid. This pattern is present between residues 9 and 16 of Av2, and the involvement of this region of Fe-protein in nucleotide binding was initially recognized by sequence analysis (Robson, 1984). Site-directed mutagenesis studies of residues in this sequence motif are consistent with this assignment (Seefeldt et al., 1992; Seefeldt & Mortenson, 1993). Other residues involved in nucleotide binding that have been probed by mutagenesis include Asp 125 of the Walker B motif (Wolle et al., 1992a) and Ala 157 (Gavini & Burgess, 1992). These observations map the nucleotide-binding sites of Fe-protein (one/subunit) to the interface between the two subunits formed under the 4Fe-4S cluster. Two independent lines of crystallographic evidence indicate that this region of Fe-protein is associated with nucleotide binding: (1) molybdate ions from the crystallization solution are found bound to residues in the Walker A motif, which corresponds to the location of the terminal nucleotide phosphate groups in other proteins, presumably reflecting the ability of molybdate to serve as a structural analogue of phosphate, and (2) a partially occupied ADP molecule was identified in the crystal structure bound to this location (Figure 1).

The binding sites on each subunit for the terminal phosphates of bound nucleotide (as defined by the molybdate sites) are separated by ~20 Å from the 4Fe-4S cluster, as well as from each other. This distance for the cluster–molybdate separation suggests that MgATP does not bind directly to the cluster, consistent with the conclusions from spectroscopic studies of this interaction (Morgan et al., 1990). The binding of both MgATP and MgADP are accompanied by significant and characteristic changes in both the protein conformation and properties of the 4Fe-4S cluster [reviewed in Burgess (1984), Orme-Johnson (1985), and Yates (1992)]. Since the nucleotide and cluster are too distant to permit direct interaction, the location of both sites at the subunit interface suggests that the interface provides the coupling mechanism between them. An allosteric type model for the coupling of nucleotide binding and cluster redox behavior can be envisioned, in which two (or more) conformational states of the Fe-protein exist that differ in the details of intersubunit interactions, such that the

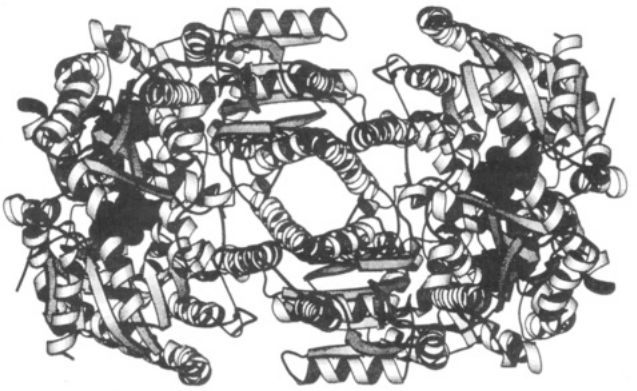

FIGURE 2: Ribbons diagram of the polypeptide fold of the MoFe-protein tetramer, with CPK models for the P-cluster pair and FeMo-cofactor. The view is along the tetramer 2-fold axis. Prepared with the program MOLSCRIPT (Kraulis, 1991).

equilibrium between the two conformations is sensitive to oxidation state and nucleotide occupancy. Allosteric switching between alternate conformational states of proteins, driven by nucleotide binding and/or hydrolysis, appears to be a general transducing mechanism for coupling the energy of nucleotide hydrolysis to a variety of biochemical processes associated with cellular regulation (Pai et al., 1990; Tong et al., 1991), protein synthesis (Jurnak, 1985; Kjeldgaard & Nyborg, 1992), DNA recombination (Story & Steitz, 1992), membrane transport (Karkaria et al., 1990; Riordan et al., 1990), and molecular motors (Rayment et al., 1993). Sequence analyses by Koonin (1993) suggest that the Fe-protein may represent an ancestral form of a now wide-spread assortment of nucleotide-binding proteins, so that the basic structural machinery utilized for transducing the energy of ATP hydrolysis in nitrogenase may have been recruited for a diverse range of biochemical functions.

MoFe-Protein. The MoFe-protein is an $\alpha_2\beta_2$ tetramer with a total molecular mass of ~240 kDa. The α and β subunits, which are encoded by the *nifD* and *nifK* genes, respectively, are of similar size; for example, the isolated α and β subunits of *A. vinelandii* MoFe-protein have 491 and 522 amino acids, respectively (Brigle et al., 1985). The MoFe-protein contains two types of metal centers, the FeMo-cofactor and the P-cluster pair. The FeMo-cofactor [reviewed in Burgess (1990) and Newton (1992)], also referred to as the "M-center" or, simply, "cofactor", was first identified by Shah and Brill (1977) as a stable metallocluster isolated from acid denatured MoFe-protein. Intense interest has been focused on the FeMo-cofactor since it contains molybdenum in a biologically unprecedented form and is believed to represent the site of substrate reduction (Hawkes et al., 1984; Imperial et al., 1989). The P-cluster pair [reviewed in Holm et al. (1990) and Smith and Eady (1992)], also referred to as the P-cluster, may function in electron transfer between the Fe-protein and the FeMo-cofactor.

The overall fold of the MoFe-protein tetramer (Kim & Rees, 1992b) is illustrated in Figure 2. The α and β subunits exhibit similar polypeptide folds with three domains of the α helical/β sheet type. In each subunit, there is a wide, shallow cleft between the three domains; in the α subunit, the FeMo-cofactor occupies the bottom of this cleft. The P-cluster pairs are located at the interface between a pair of α- and β-subunits. The organization of these subunits into an $\alpha\beta$ dimer appears to serve as the fundamental functional unit of the MoFe-protein. Within a dimer, the α and β subunits are approximately related by a 2-fold rotation axis that passes through the P-cluster pair, and there are two wide and shallow clefts around the P-cluster pair which may provide the binding sites for the dimeric Fe-protein. The N-terminus of the β subunit (about 50 residues), which is absent in both Cp1 and component 1 of alternative nitrogenases, extends from the β subunit and wraps around the α subunit. The $\alpha_2\beta_2$ tetramer may be considered to be formed from pairs of $\alpha\beta$ dimers that are related by the tetramer 2-fold rotation axis. Even though the α and β subunits in an $\alpha\beta$ dimer are also approximately related by a 2-fold rotation, the MoFe-protein does not exhibit 222 symmetry. Packing between helices from the β subunit dominate the interactions at the tetramer interface, although there are some interface contributions from helices in the α subunit. The tetramer interface is further stabilized by a cation binding site, most probably for calcium, that is created by ligands from both β subunits. Intriguingly, the center of the six α-helical barrel surrounding the tetramer 2-fold axis is not filled with side chains; rather, an open channel of ~8–10-Å diameter and length ~35 Å extends through the center of the tetramer.

STRUCTURE AND FUNCTION OF THE METAL CENTERS IN THE MoFe-PROTEIN

FeMo-Cofactor Structure. The FeMo-cofactor contains two clusters of composition 4Fe-3S and 1Mo-3Fe-3S that are bridged by three nonprotein ligands (Figure 3a; Kim & Rees, 1992a). The three bridging ligands are most likely all sulfur (presumably S^{2-}), although one ligand, designated "Y" in the original description, has somewhat lower electron density than the other two bridges in the Av1 structure (Chan et al., 1993). The three bridging ligands have comparable density in the Cp1 structure (Bolin et al., 1993b; Kim et al., 1993). The lower electron density of ligand "Y" in Av1 may reflect dynamical (disorder) properties or compositional heterogeneity of this site. Homocitrate, which is an essential component of FeMo-cofactor (Hoover et al., 1987; Madden et al., 1990), coordinates the Mo through a hydroxyl and carboxyl oxygen. Ligands to the FeMo-cofactor are provided by Cys α275 (to Fe1) and His α442 (to Mo). Earlier molecular biology studies had implicated both of these residues as potential cluster ligands (Govenzensky & Zamir, 1989; Kent et al., 1989, 1990; Dean et al., 1990). As detailed in the initial report (Kim & Rees, 1992a), the FeMo-cofactor model is also generally consistent with the available composition and spectroscopic data. The tetrahedral coordination geometry of Fe1 and the octahedral coordination geometry for Mo are typical of the coordination environments for these metals observed in model compounds and Fe-S proteins (Holm & Simhon, 1985; Coucouvanis, 1991). An unusual feature of the FeMo-cofactor model, however, is the trigonal coordination geometry of the six Fe sites that bind the bridging sulfurs. While not unprecedented (Power & Shoner, 1991), these sites have not been well characterized, and it is tempting to speculate that their coordinative unsaturation may be relevant for binding of substrates and other ligands.

The FeMo-cofactor is located ~10 Å below the protein surface. Cys α275 and His α442, as well as Ser α278 which is hydrogen bonded to the Sγ of Cys α275, are strictly conserved in all known MoFe-protein sequences. Other highly conserved residues in the vicinity of the FeMo-cofactor include Gly α356 and Gly α357, which may be required both to avoid steric interference with the cofactor and to permit the formation of hydrogen bonds to one of the bridging sulfurs from the NH group of α356 and also residue α358; Arg α96 and Arg α359, which hydrogen bond to another bridging sulfur and also to cluster sulfurs in the FeMo-cofactor and which

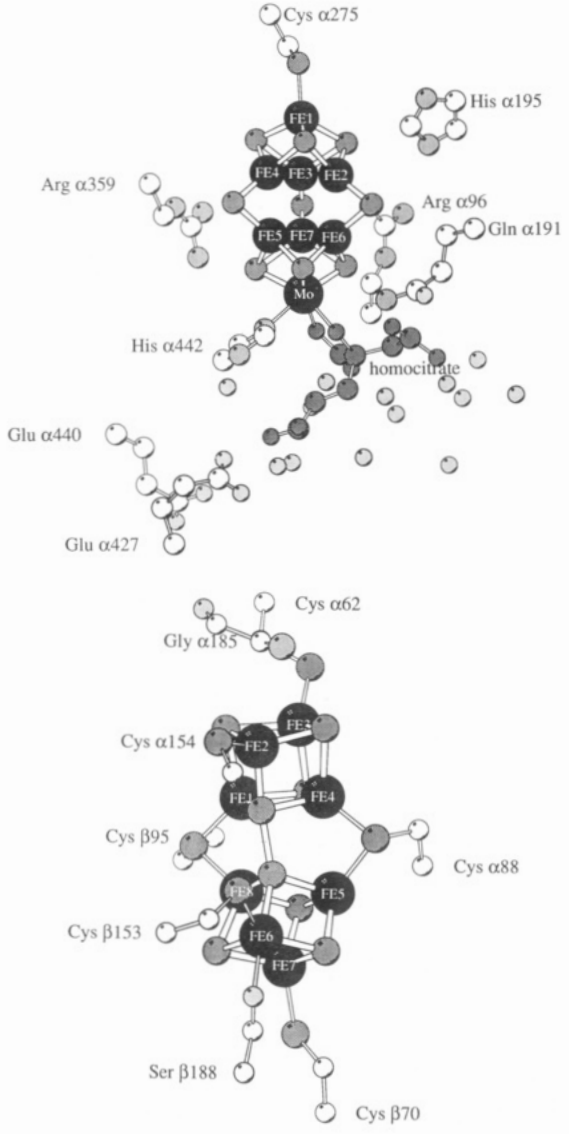

FIGURE 3: (a, top) FeMo-cofactor and surrounding environment. (b, bottom) P-cluster pair and surrounding environment. Prepared with the program MOLSCRIPT (Kraulis, 1991).

may serve to electrostatically stabilize the apparently negatively charged FeMo-cofactor and/or partially reduced intermediates formed during substrate reduction; His α195, which hydrogen bonds to the third bridging sulfur in the FeMo-cofactor and which may function in proton transfer reactions; and three residues, Gln α191, Glu α427, and Glu α440, which are near the homocitrate and interact with this group either directly or through water molecules.

Structure of the P-Cluster Pair. The P-cluster pair consists of two 4Fe-4S clusters that are bridged by two cysteine thiol ligands (residues Cys α88 and Cys β95) and a disulfide bond between two of the cluster sulfurs (Figure 3b). This disulfide bridge is located on the side of the P-cluster pair closest to the protein surface. There is suggestive evidence that one of the disulfide linked sulfurs is labile; crystallographic analysis of older Av1 crystals indicated that one of these sulfurs was specifically lost, relative to fresh Av1 crystals (Chan et al., 1993), while Bolin's analysis of Cp1 possibly indicates that only one of the two sulfurs linked by a disulfide in Av1 is present (Bolin et al., 1993b). The relationship of these different P-cluster pair forms to either spectroscopically characterized states or to mechanistically relevant forms is unknown. Singly coordinating cysteine thiols (from residues Cys α62, Cys α154, Cys β70, and Cys β153) ligate the remaining four irons, such that nonbridging cysteines coordinated to a specific 4Fe-4S cluster are from the same subunit. In addition to the cysteine ligands, Ser β188 appears to coordinate Fe6 along with Cys β153 in Av1; however, Bolin's Cp1 analysis positions this group to form a hydrogen bond to a cluster sulfur (Bolin et al., 1993b). The coordination environments of both Fe6 and Fe2 are distorted from ideal tetrahedral geometry, due to interactions with Ser β188 and the main chain of Gly α185, respectively. The cysteine ligands to the P-cluster pair had been correctly identified by mutagenesis experiments (Kent et al., 1989, 1990; Dean et al., 1990; May et al., 1991). Spectroscopic studies indicate that the Fe in the P-cluster pair are all in the ferrous state, which is unprecedented in biological Fe-S centers. The bridging ligands and disulfide bond reduce the net charge per Fe atom in the P-cluster pair to a value corresponding to that found for 4Fe-4S clusters in ferredoxin, however, which may account for the stabilization of this unusual oxidation state (Howard & Rees, 1994).

ATP HYDROLYSIS AND ELECTRON TRANSFER IN THE NITROGENASE SYSTEM

The only well-established aspects of electron flow through the nitrogenase system are that electrons enter through Fe-protein and exit through the reduced products. The sequence of electron transfer steps between these initial and final states has not been experimentally established. Nevertheless, a "consensus" model has emerged in which the general sequence of electron transfer steps within the nitrogenase system is proposed to occur in the order

Fe-protein → P-cluster pair → FeMo-cofactor → substrate

MgATP hydrolysis is coupled to the first electron transfer step, while electron and proton transfer to the substrate occur while bound to the FeMo-cofactor. Keeping in mind that definitive experimental support has yet to be obtained, this model provides a useful framework for discussing the nitrogenase mechanism.

Complex Formation between Fe-Protein and MoFe-Protein. Complex formation between Fe-protein and MoFe-protein contributes critically to the overall kinetics of nitrogenase, since dissociation of the complex represents the rate-determining step (Thorneley & Lowe, 1985). Although the structure(s) of the complex(es) formed between these two proteins has not been established, some likely features of the complex can be addressed from the structures of the individual components. Two residues of Fe-protein have been identified as interacting with the MoFe-protein, Arg 100 (Murrell et al., 1988; Lowery et al., 1989; Wolle et al., 1992b) and Glu 112 (Willing & Howard, 1990), while Phe β125 (Thorneley et al., 1993) and Asp α161 (D. Dean, personal communication) of MoFe-protein appear to participate in Fe-protein binding. These assignments, together with the crystal structures, permit the generation of a docking model between Fe-protein and MoFe-protein (Figure 4) by superposition of the Fe-protein 2-fold axis with the 2-fold axis passing through the P-cluster pair that relates the α and β subunits of MoFe-protein (Kim & Rees, 1992b). The nucleotide-binding sites in the Fe-protein dimer are located at the inferface between the two subunits. Although the structural consequences have not been established, it is likely that hydrolysis in the complex of the two MgATP, whether occurring simultaneously or sequentially, is accompanied by changes in both the Fe-protein and MoFe-protein structures, such as an alteration in the relative orientations of the various subunits (Howard & Rees, 1994).

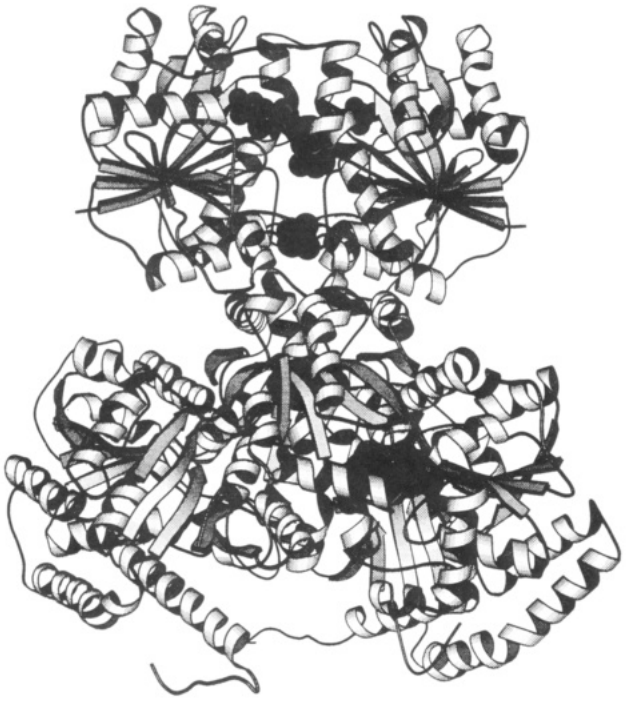

FIGURE 4: Computer-generated model of the docking complex between Fe-protein (top molecule) and an $\alpha\beta$ subunit pair of the MoFe-protein (bottom). The metal centers and ADP molecule are represented by CPK models. Prepared with the program MOLSCRIPT (Kraulis, 1991).

Presumably, this transition leads to the formation of an activated species that is competent for electron transfer within the Fe-protein—MoFe-protein complex.

Nitrogenase, H-ras p21 (and other G-proteins), recA protein, membrane transporters, and myosin are all members of a general family in which nucleotides stabilize alternate conformations of a protein, with binding to other macromolecules serving to induce a conformational switch for nucleotide hydrolysis that interconverts the two forms. In essence, nucleotide hydrolysis serves as a molecular clock that sequentially drives a macromolecule through a series of conformational states. Certainly, the detailed mechanism of each system will differ, since some proteins, such as H-ras p21, are monomeric and hydrolyze only one nucleotide, while others, such as nitrogenase and possibly membrane transporters, utilize multiple nucleotides. Despite these differences, it is likely that the general principles governing the coupling between nucleotide and conformational states that are established in any one of these systems will also be relevant to the other systems.

Electron Transfer in the Nitrogenase Complex. In the model for the nitrogenase complex illustrated in Figure 4, the shortest distances from the 4Fe-4S cluster to the P-cluster pair, and from the P-cluster pair to the FeMo-cofactor, are ~18 and ~14 Å, respectively, while the distance from the 4Fe-4S cluster to the FeMo-cofactor is ~32 Å. Assuming that this model is correct, it would appear most likely that the P-cluster pair would participate in the overall process of transferring an electron from the 4Fe-4S cluster of the Fe-protein to the FeMo-cofactor, rather than having direct electron transfer those two centers. It seems reasonable that the electron transfer rates between pairs of redox centers separated by ~15 Å should be faster (Wuttke et al., 1992; Moser et al., 1992) than the rate-determining step of nitrogenase (~5 s^{-1}; Hageman & Burris, 1978; Thorneley & Lowe, 1985).

Within the complex, hydrolysis of ATP apparently serves as the trigger to initiate the electron transfer events, possibly through a series of conformational changes that are propagated along interfaces between proteins, subunits, and/or domains. The precise sequence of events associated with these electron transfer events remains one of the crucial issues of the nitrogenase mechanism. Experimentally, it is known that the first electron transferred from the Fe-protein to the MoFe-protein ends up on the FeMo-cofactor (Hageman & Burris, 1978; Thorneley & Lowe, 1985), resulting in loss of the characteristic $S = {}^3/_2$ EPR signal of the resting state of the cofactor, while the P-cluster pair apparently remains in the same oxidation state as at the beginning of the reaction. Consequently, the participation of the P-cluster pair in this process, if it occurs at all, must be at most transient. At least two general mechanisms can be envisioned by which conformational changes associated with ATP binding and hydrolysis could be used to facilitate electron transfer through the P-cluster pair: (1) these conformational changes could first drive electron transfer from the Fe-protein to the P-cluster pair, followed by electron transfer from the reduced P-cluster pair to the FeMo-cofactor (Chan et al., 1993; Rees et al., 1993), or (2) these conformational changes could first drive electron transfer from the P-cluster pair to the FeMo-cofactor, followed by electron transfer from Fe-protein to the oxidized P-cluster pair (Howard & Rees, 1994). The net result of either mechanism is to have the P-cluster pair in the same oxidation state at the beginning and the end of the redox cycle, while the FeMo-cofactor and Fe-protein are reduced and oxidized, respectively, by one electron. It is also possible that the P-cluster pair could achieve a net transfer of electrons to the FeMo-cofactor, especially in more highly reduced forms of the MoFe-protein; recent experimental evidence for oxidation of the P-cluster pair associated with reduction of dinitrogen to the hydrazido (2–) oxidation level (Lowe et al., 1993) may reflect this type of behavior.

Binding and Reduction of Substrates at the FeMo-Cofactor. In addition to the physiological reactions of N_2 reduction and H_2 formation, nitrogenase catalyzes a wide variety of reductions involving small unsaturated molecules, such as azides, nitrous oxide, nitriles, isonitriles, and alkynes (Burgess, 1985). Functionally, the details of the interactions between various substrates and the FeMo-cofactor are central to understanding the catalytic properties of nitrogenase. Based on the FeMo-cofactor structure, a large variety of possible geometries for substrate binding to the cofactor can be envisioned that involve one or more of the Fe, Mo, and/or S sites [see Deng and Hoffmann (1993) for an evaluation of some of these possibilities]. Among these candidates, binding of substrates to the trigonally coordinated iron sites provides intriguing possibilities, since these iron atoms are coordinatively unsaturated and would seem to be poised for interaction with another group. Three general binding modes of substrates to the trigonally coordinated Fe sites may be described (Kim et al., 1993): (i) Substrates could bind in an end-on fashion to one of the six central, three-coordinate iron sites. (ii) Three sets of cyclic eight-membered rings occur on the exterior surface of the FeMo-cofactor consisting of alternating S and Fe sites. These arrangements may create the equivalent of a small region of iron surface, each containing four iron atoms, and substrates could bind to those surfaces so as to simultaneously interact with up to four irons. (iii) Some small substrates, such as N_2 and/or $H^+(H_2)$, could occupy the central cacity in the FeMo-cofactor, thereby replacing weak iron–iron bonds with Fe–substrate bonds (Chan et al., 1993). In this model, the FeMo-

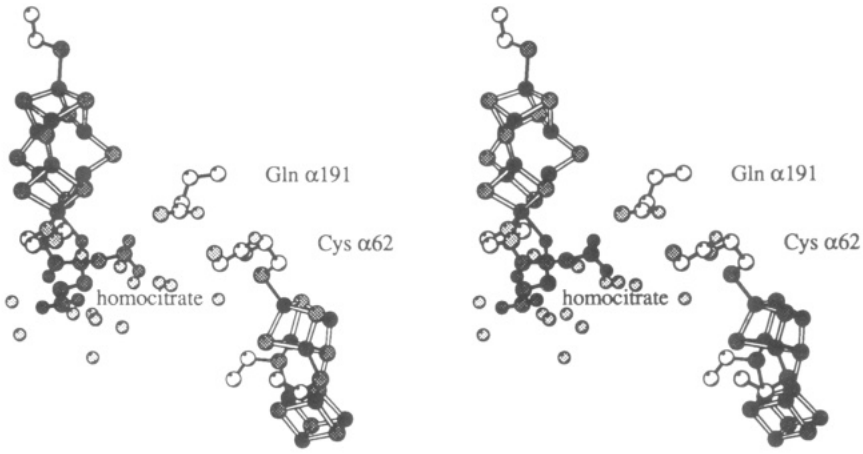

FIGURE 5: Stereoview of the protein environment between the FeMo-cofactor and P-cluster pair. The edge–edge distance of the FeMo-cofactor to the P-cluster pair is ~14 Å. Water molecules near the homocitrate are indicated by the isolated spheres. Prepared with the program MOLSCRIPT (Kraulis, 1991).

cofactor effectively serves as a host for the dinitrogen guest. It is certainly possible that a variety of different binding modes are utilized by the intermediates generated during dinitrogen reduction or also by different substrates. The presence of multiple, potential substrate-binding sites in the FeMo-cofactor may be related to the complex inhibition kinetics observed between various substrates and ligands (Burgess, 1985).

Since all known substrates of nitrogenase are reduced by an even number of electrons (and, almost always, require an equivalent number of protons), some mechanistic schemes have focused on addition of pairs of electrons to dinitrogen, leading to the formal reduction sequence: dinitrogen, diimide, hydrazine, and ammonia [discussed in Burgess (1985)]. The major barrier in this case should be the two-electron reduction of dinitrogen to diimide (Stiefel et al., 1988). One interesting possibility is that this barrier could be side-stepped through reduction of bound dinitrogen by more than two electrons, possibly by electron donation from the P-cluster pair (perhaps involving sulfide–disulfide conversion?), combined with electrons previously stored in the FeMo-cofactor. Funneling of the requisite number of protons into the buried active center is also a critical process during substrate reduction; various ionizable groups in the vicinity of the FeMo-cofactor and the presence of an extensive network of buried waters near the homocitrate may participate in this process (Figures 3a and 5).

Role of Homocitrate. The function of homocitrate in nitrogenase is intriguing, given that it is coordinated to the Mo, is surrounded by a number of buried water molecules, and is on the side of the cofactor nearest to the P-cluster pair. Accordingly, possible functions of homocitrate might be to function in electron transfer from the P-cluster pair to FeMo-cofactor or to mediate transfer of protons to various reduced intermediates. To probe the role of homocitrate, Ludden and Shah have pioneered the development of *in vitro* methods for substituting other organic acids for homocitrate in the FeMo-cofactor (Imperial et al., 1989). In general, the minimal requirements for functional activity by homocitrate substituents include two carboxyls and a hydroxyl group. The specific stereochemistry of the organic acid can also profoundly influence the ability of this group to support reduction of various substrates by nitrogenase. An example of this behavior is provided by the replacement of homocitrate with either *erythro*-fluorohomocitrate or *threo*-fluorohomocitrate (Madden et al., 1990). These compounds are substituted with a fluoro group on the single methylene-containing arm of homocitrate, which is the arm of homocitrate that links the Mo liganding groups to the carboxyl that hydrogen bonds to Gln α191 (Figure 5). Gln α191 in turn is hydrogen bonded to the NH of Gly α61, which is next to the P-cluster pair ligand α62 and therefore serves to link the two redox centers of the MoFe-protein. While the *erythro*-fluorohomocitrate substituted FeMo-cofactor has high activities in most nitrogenase activities (including N_2 reduction), the *threo*-isomer has very low N_2 reduction activity, although more normal levels of acetylene and proton reduction activities. Examination of the crystal structure shows that, without any rearrangement, a *threo*-substituent would be directed toward the cofactor and would be in close contact with one of the cluster sulfurs, while an *erythro*-substituent would point away from the cofactor. Apparently, the proximity of the *threo*-fluoro to the cofactor could drive a rearrangement of this region that results in altered activity, while the *erythro*-fluoro group can be accommodated with fewer mechanistically significant changes. Alternatively, substitution of the *threo*-hydrogen by fluorine could prevent transfer of this hydrogen to the cluster, during a putative step in the protonation of bound substrate (J. B. Howard, personal communication).

ALTERNATIVE NITROGENASES

The sequences of the structural genes of the vanadium–iron protein (VFe-) subunits (*vnfDK*) and those of a third nitrogenase, an apparently iron-only nitrogenase (*anfDK*), show considerable similarity with *nifDK* (Eady, 1991; Pau, 1991; Bishop & Premakuman, 1992). Of particular significance is the conservation of the residues which ligate the metal centers in these proteins. An unusual feature of the operon encoding the structural genes of alternative nitrogenases is the presence of a small additional gene (*vnfG* and *anfG*) between the D and K genes, which is not present between *nifD* or *nifK*. The VFe-proteins of *Azotobacter chroococcum* and *A. vinelandii* have been purified and characterized. The protein isolated from *A. chroococcum* has an $\alpha_2\beta_2\delta_2$ subunit structure, with the δ subunit being encoded by the *vnfG* gene. Although the δ subunit has not been identified in the VFe-protein of *A. vinelandii*, the DNA sequence of the structural gene operon includes a homologous gene (*vnfG*) to that found in *A. chroococcum*.

It is generally believed that the alternative nitrogenase proteins are similar to the conventional proteins, with the replacement of V or Fe for Mo, although most reports of the Fe content of current preparations of VFe-proteins are

generally lower than those of MoFe-proteins (Eady, 1991; Pau, 1991; Bishop & Premakuman, 1992). EPR and magnetic circular dichroism studies on the vanadium nitrogenase system indicate that the VFe-protein possesses similar redox centers to the MoFe-protein of the conventional nitrogenase system (Morningstar & Hales, 1987; Morningstar et al., 1987). The vanadium K-edge EXAFS spectrum has been reported for the VFe-proteins of both *A. chroococcum* and *A. vinelandii* (Arber et al., 1987; George et al., 1988; Harvey et al., 1990). These two VFe-proteins seem to be structurally similar, and the iron–vanadium cofactor (FeVa-cofactor) appears to be analogous to the FeMo-cofactor in MoFe-protein.

The α subunit of the VFe-protein is similar in size to that of Avl. However, the β subunit of the VFe-protein has a ~50-residue deletion in the N-terminal region, relative to that of Avl, just as in the case of the Cpl β subunit. Consequently, the β subunit of the VFe-protein is similar in size to Cpl (Pau, 1991), and the three-dimensional structure of VFe-protein could be considered as a hybrid of the Avl α subunit and the Cpl β subunit. An open question about the overall structure of the VFe-protein is the location of the δ subunit. Since no functional role for the δ subunit has been observed and the δ subunit is missing in some VFe-protein preparations (Eady, 1991), a possible role of the δ subunit may be stabilization of the quaternary structure of VFe-protein. Based on the structures of Avl and Cpl, a possible location of the δ subunit is around the N-terminal region of the Avl β subunit. The N-terminus of the Avl β subunit, which is absent in Cpl and VFe-protein, extends from the β subunit and wraps around the α subunit. These residues also interact with other β subunit. Therefore, it is possible that these N-terminal residues of the β subunit may function in stabilization of the quaternary structure of Avl and could be replaced by the δ subunit in VFe-protein, although there is no sequence homology (Pau, 1991). Based on amino acid sequence comparisons, the structure of the FeFe-protein of the iron-only nitrogenase is also likely to be very similar to that of VFe-protein.

Role of Mo/V in Nitrogenase. The octahedral coordination of Mo in the FeMo-cofactor, and by extension V in the FeVa-cofactor, is rather typical, in contrast to the unusual trigonally coordinated Fe sites. The apparent replacement of Mo by V or Fe in different types of nitrogenases indicates that Mo is not uniquely required for dinitrogen reduction, although a counterargument can be made that since the substrate reduction properties of the different nitrogenase proteins are not identical, these metals are not entirely passive participants in the reaction mechanism. It is possible that Mo can bind N_2 and other ligands, either by increasing the coordination number and/or by displacing an existing ligand. If this occurs, binding of N_2 and other substrates/intermediates to Mo could be prerequisite to substrate reduction at the Mo or might represent an intermediate step in the transfer of N_2 to iron (Leigh & Jimenez-Tenorio, 1991). In the absence of a direct role in substrate or intermediate binding, possible functions of Mo/V could be to modulate the reduction potential necessary for N_2 binding and reduction, to participate in electron transfer between the P-cluster pair and FeMo-cofactor, and/or perhaps to play a structural role by providing a polydentate site that can simultaneously interact with the remainder of the cofactor, the homocitrate, and the protein, thereby serving as a structural anchor to maintain all these groups in their proper orientations.

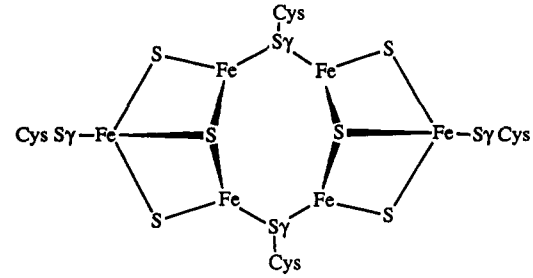

FIGURE 6: Proposed structural model for the H-cluster of Fe-hydrogenase.

IMPLICATIONS OF NITROGENASE FOR COMPLEX ELECTRON TRANSFER SYSTEMS

Possible Similarities to Other Metal Centers: The H-Cluster in Fe-Hydrogenases. Hydrogenases are a class of enzyme that catalyze the reversible oxidation of molecular hydrogen, $H_2 \leftrightarrow 2H^+ + 2e^-$. Hydrogenases can be divided into two groups on the basis of metal content and sequence homology: NiFe-hydrogenases which contain nickel and iron [reviewed in Fauque et al. (1988) and Voordouw (1992)] and Fe-hydrogenases which contain only iron [reviewed in Adams (1990)]. Fe-hydrogenases possess two different types of iron clusters, known as "F-" and "H-" clusters. The former are of the ferredoxin 4Fe-4S cluster type (Adams et al., 1986), whereas the latter, which are thought to be the site of hydrogen interaction, are of unknown structure and have the approximate composition 6Fe-6S (Adams et al., 1989). The structure of the H-cluster is of interest not only in connection with the mechanism of the enzyme but also in that it appears to be spectroscopically unique in bioinorganic chemistry (Adams et al., 1986; Macor et al., 1987; Telser et al., 1987; Fu et al., 1993).

ENDOR (Telser et al., 1987) and Mössbauer analyses (Rusnak et al., 1987) of Fe-hydrogenases indicate that the H-cluster is composed of two distinct types of Fe atoms, present in a ratio of 2:1. Assuming that the actual composition of the H-cluster contains 6 Fe, this suggests that the true abundances of the two Fe components are 4 and 2, respectively. Information concerning potential liganding groups in the Fe-hydrogenase is provided by protein sequence analyses. Comparisons of the available Fe-hydrogenase sequences show that these enzymes constitute a structurally homogeneous family (Meyer & Gagnon, 1991). While they differ in the length of their N-termini and in the number of their 4Fe-4S clusters (F-clusters), they are similar in their C-terminal regions, which are postulated to be the H-cluster domain. Four conserved cysteine residues located in this domain are proposed as likely ligands of the H-cluster (Meyer & Gagnon, 1991). Consequently, the tenative picture of the H-cluster that emerges from these studies is of a complex center containing 6 Fe and 6 S, with two classes of Fe present in the ratio of 4:2, and which is coordinated to the protein through four cysteine ligands, and possibly other non-S ligands.

Although not generally classified as a hydrogenase, as discussed above, the nitrogenase MoFe-protein does exhibit hydrogenase activity. It has been postulated that the nitrogenase MoFe-protein and Fe-hydrogenases may have some common structural motifs in their catalytic metal centers (George et al., 1989; Adams, 1990; Kim & Rees, 1992b). Based on this assumption, a model for the H-cluster may be proposed that contains two 3Fe-3S clusters that are bridged by two cysteine sulfurs (Figure 6). This structure can be derived from the FeMo-cofactor by replacement of Mo by Fe, deletion of two trigonal Fe and the intervening bridging sulfur,

and replacement of the remaining two bridging sulfurs by Cys thiol side chains, as in the P-cluster pair. The bridging (three-coordinate) to terminal (four-coordinate) Fe sites are present in the ratio of 4:2, which is consistent with the spectroscopic data indicating that there are two distinct classes of Fe sites with that relative abundance. The four central iron atoms form a surface which could be used for H_2 binding, as well as CO (a potent inhibitor of hydrogenase) binding. While these four iron atoms are drawn in the same plane in Figure 6, twisting of four iron atoms out of plane is also conceivable, especially upon oxidation/reduction of the H-cluster, as has been indicated by EXAFS studies (George et al., 1989).

Structural Similarities to Photosynthetic Reaction Centers. Although the electron transfer processes catalyzed by nitrogenase and the photosynthetic reaction center [RC, reviewed in Feher et al. (1989)] are quite different, there are similarities in the general structural organization of the two systems (Kim & Rees, 1992b). Perhaps most significantly, the Fe-protein, MoFe-protein, and the RC (Deisenhofer et al., 1985; Allen et al., 1987) are all composed of two homologous/identical subunits related by a 2-fold rotation. Furthermore, the P-cluster pair in MoFe-protein and the special pair in RC are buried in the interface between the two subunits. The location of these redox centers at the subunit interface may provide a convenient assembly mechanism for the incorporation of powerful reductants in the protein interior, isolated from contact with solvent. The structure of plant photosystem (PS) I at 6-Å resolution (Krauss et al., 1993) and the subunit sequences of plant PSII (Michel & Deisenhofer, 1988) are also consistent with a structural organization in which redox groups are coordinated at the interface of homologous subunits. Structural analyses of these systems, as well as other complex electron transfer systems such as the mitochondrial, chloroplast, and bacterial membrane-bound electron transfer complexes, are critical for a detailed understanding of the principles guiding the organization of these assemblies associated with fundamental events in cellular energy metabolism.

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