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Role of Nucleotides in Nitrogenase Catalysis

LANCE C. SEEFELDT*,† AND DENNIS R. DEAN‡

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322, and Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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Nitrogenase Is the Catalytic Component of Biological Nitrogen Fixation

N_2 is abundant in the Earth's atmosphere, yet it cannot be metabolized by most living organisms. Consequently, most organisms obtain their cellular nitrogen in a "fixed" form such as ammonia. Biological fixation of nitrogen is performed only by certain microorganisms, called diazotrophs, and this process is the most significant contributor to the reductive portion of the biogeochemical nitrogen cycle.[1] Nitrogenase is the enzyme that catalyzes the biological nitrogen fixation reaction, which is usually depicted in the following way:

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

The nitrogenase enzyme is a complex, two-component metalloprotein composed of an iron (Fe) protein and a molybdenum−iron (MoFe) protein. The trivial names for these proteins were derived from the respective compositions of their associated metalloclusters. The Fe protein is a homodimer ($M_r \approx 64\,000$) that contains two MgATP binding sites and a single [4Fe-4S] cluster.[2] The MoFe protein is an $\alpha_2\beta_2$ heterotetramer ($M_r \approx 250\,000$) that contains two pairs of novel metalloclusters, called P-clusters and iron−molybdenum cofactors (FeMo-co).[3] The structures of the two nitrogenase proteins, along with models for their associated metal clusters, have been presented[2−9] and are shown in Figure 1. During catalysis the Fe protein serves as a specific reductant of the MoFe protein, which in turn provides the site of substrate reduction. Because multiple electrons are required for substrate reduction, a major challenge of nitrogen fixation research is to understand how these electrons are delivered by the Fe protein and accumulated within the MoFe protein. It is now known that intercomponent electron transfer requires a series of sequential associations and dissociations[10] of the two component proteins which are controlled and timed by MgATP binding and hydrolysis.[11] An important feature of this process is that the interaction of both component proteins is absolutely required to elicit MgATP hydrolysis and neither component protein will reduce any substrate in the absence of its catalytic partner. Under optimal conditions, one electron is transferred from the Fe protein to the MoFe protein and two MgATP molecules are hydrolyzed for each component protein association−dissociation event.[10] Lowe and Thorneley[12−15] have developed kinetic models of these events that provide a useful framework for the discussion of the key mechanistic issues. Their scheme includes two interlocking cycles called the Fe protein cycle and the MoFe protein cycle. The Fe protein cycle involves the oxidation and reduction of the Fe protein [4Fe-4S] cluster between the 1+ and 2+ redox states as it sequentially delivers electrons to the MoFe protein and is re-reduced by other electron transfer proteins. The salient steps in this cycle include (i) binding of MgATP to the reduced Fe protein, (ii) interaction of the Fe protein with the MoFe protein, (iii) intercomponent electron transfer from the Fe protein to the MoFe protein, (iv) MgATP hydrolysis, (v) component protein dissociation and release of MgADP and P_i, and (vi) re-reduction of the Fe protein to complete the cycle. The MoFe protein cycle involves the progressive Fe protein-dependent reduction of the MoFe protein that ultimately leads to N_2 binding and reduction.

In 1992, Rees and colleagues[2,3,8] proposed models from X-ray crystallographic data for both nitrogenase component proteins and their associated metalloclusters from the diazotroph *Azotobacter vinelandii*. Because the pri-

Lance C. Seefeldt was born in Redlands, CA, in 1960. He received a B.S. in chemistry from the University of Redlands in 1983 and a Ph.D. in biochemistry from the University of California, Riverside, in 1989. He then did postdoctoral work at the Center for Metalloenzyme Studies at the University of Georgia. He joined the faculty of the Department of Chemistry and Biochemistry at Utah State University in 1993. His research interests include metalloprotein structure and function and protein conformational dynamics.

Dennis R. Dean was born in Indianapolis, IN, in 1951. He received a B.A. in biology from Wabash College in 1973 and a Ph.D. in molecular biology from Purdue University in 1979. He performed postdoctoral work at the Enzyme Institute at the University of Wisconsin—Madison. He joined the Virginia Polytechnic and State University faculty in 1985, where he is now a professor of biochemistry. His research interests include analysis of the structure, function, and assembly of metalloclusters.

* To whom correspondence should be addressed. Phone: (801) 797-3964. Fax: (801) 797-3390. E-mail: seefeldt@cc.usu.edu.
† Utah State University.
‡ Virginia Polytechnic Institute and State University.

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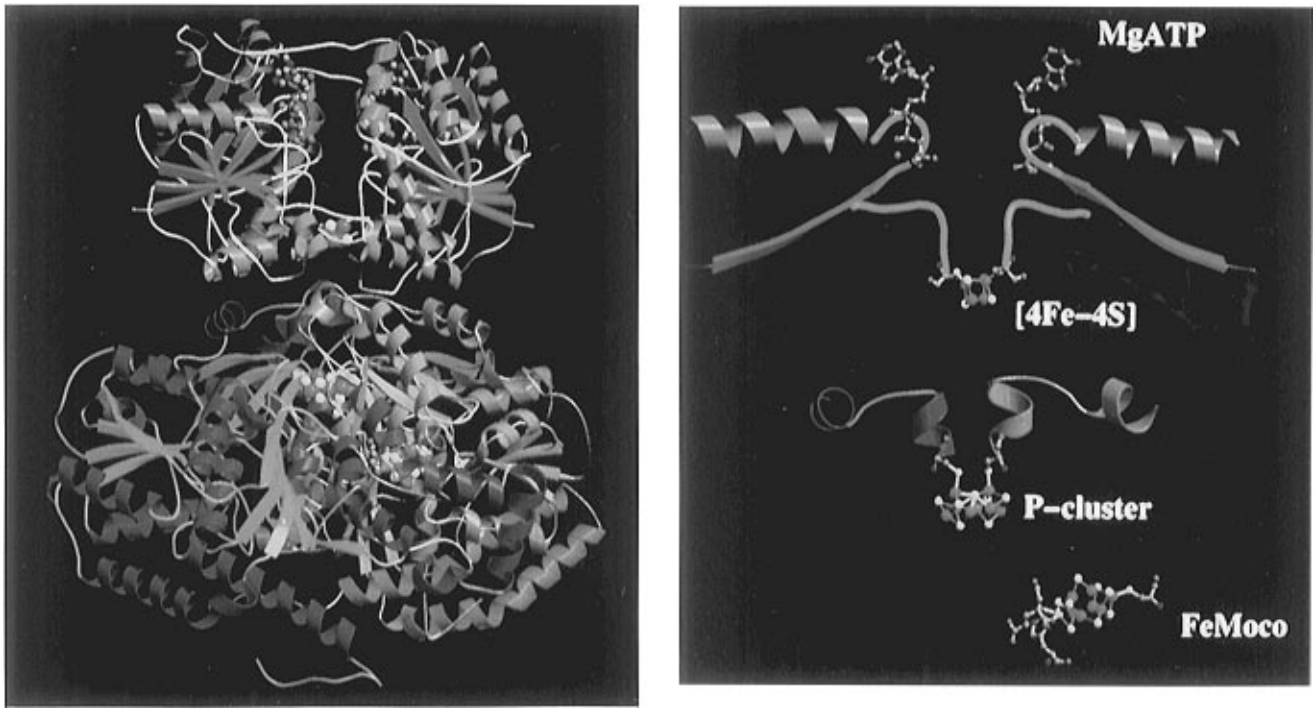

FIGURE 1. Structural models for the nitrogenase component proteins and their associated metalloclusters. (A, left) Ribbon diagrams of the *A. vinelandii* Fe protein homodimer (top, red and green) and one of two $\alpha\beta$-units of the MoFe protein (α subunit in blue and β subunit in magenta). Each $\alpha\beta$-unit of the MoFe protein is considered to be an independently functioning catalytic unit. The iron (green spheres) and inorganic sulfur (yellow spheres) atoms of the metalloclusters are represented by ball-and-stick models. The view shown has the Fe protein (top) poised for docking with the MoFe protein (bottom). (B, right) Same view as in panel A showing just the two MgATP binding sites and the [4Fe-4S] cluster of the Fe protein and the P-cluster (Fe_8S_7) and FeMo-co (Fe_7S_9Mo·homocitrate) of the MoFe protein. The cyan segments within the Fe protein represent the Walker A (upper) and switch II (lower) structural motifs within the Fe protein. These structural motifs are presented as they appear in the solved X-ray structure which does not have full occupancy of the nucleotide binding sites. The two MgATP molecules are shown as ball-and-stick models with carbon in gray, nitrogen in blue, oxygen in red, and phosphorous in green. The position of the two MgATP molecules is intended to give the reader an idea about generally where the MgATP molecules bind, and it should not be construed that the structure presented is in the MgATP-bound form. Insights into the structure and relevance of the P-clusters as multielectron donors has recently been noted.[53,63]

mary sequences of nitrogenases from all diazotrophs appear to be highly conserved, insights gained from the *A. vinelandii* models are of general relevance. The availability of these structural models provided a fresh view of the extensive amount of previously published biochemical and biophysical data related to nitrogenase structure and function. These issues were reviewed in a number of papers published shortly after the crystallographic models were reported.[11,16–22] Here, we emphasize results obtained since publication of the Rees models that are aimed at elucidating the roles of MgATP binding and hydrolysis in relation to nitrogenase component protein interaction and intercomponent electron transfer.

A General View of the Role of MgATP in Nitrogenase Catalysis

It is obvious that electrons and protons are necessary for N_2 reduction, but why is MgATP hydrolysis required? Even though the overall reduction of N_2 is thermodynamically favorable, the potential formation of certain partially reduced intermediates is thermodynamically unfavorable.[23] From this perspective, it can be seen that MgATP hydrolysis could serve two related functions. One possibility is that MgATP hydrolysis provides energy to stabilize a partially reduced transition state intermediate that might be formed during the sequential delivery of electrons from the Fe protein to the MoFe protein. Indeed, there is good evidence that at least one semireduced intermediate is formed during nitrogenase turnover.[24] It is also possible that the need to form certain partially reduced intermediates could be obviated by the concerted delivery of two or more electrons to the substrate reduction site. Thus, the energy released through MgATP binding and hydrolysis might be used to open and close electron gates in such a way as to ensure that multiple electrons can be accumulated within the MoFe protein prior to their donation to the substrate reduction site. The concept of gated electron transfer as a key aspect of nitrogenase catalysis is an attractive one because it provides not only a rationale for why MgATP is necessary but also a basis for why the Fe protein and MoFe protein associate and dissociate during catalysis and why the interaction of both component proteins is required to

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trigger MgATP hydrolysis. Below we describe evidence that indicates reciprocal and coordinated communication occurs between the Fe protein and the MoFe protein during catalysis. Such communication depends upon conformational changes that are both elicited and timed by MgATP binding, intercomponent interaction, and MgATP hydrolysis.

Nucleotide Binding Induces an Fe Protein Conformational Change

We know that Fe protein and MoFe protein must associate and dissociate during turnover because upon initiation of nitrogenase catalysis, there is a lag between MgATP hydrolysis and product formation that is dependent upon the ratio of the component proteins.[10] For example, when there is an abundance of MoFe protein relative to the Fe protein, competition occurs among the MoFe protein population for the limited supply of reducing equivalents needed for substrate reduction. Thus, if the Fe protein and MoFe protein associate and dissociate between each intercomponent electron transfer event, this situation should, and does, increase the lag in substrate reduction without affecting MgATP hydrolysis.[10] With this in mind, we consider the first step in the Fe protein cycle which involves the binding of MgATP to the Fe protein to ready it for interaction with the MoFe protein (see Figure 1).

The binding of MgATP to the Fe protein induces global structural changes[25] that are manifested by alterations in the electronic properties of the [4Fe-4S] cluster.[11] Such perturbations are readily detected by electron paramagnetic resonance (EPR),[26,27] circular dichroism (CD),[28,29] ^{1}H-nuclear magnetic resonance (NMR),[30,31] and Mössbauer[27] spectroscopies. The midpoint potential of the [4Fe-4S] cluster is lowered approximately 100 mV when Fe protein binds MgATP.[26,32,33] The midpoint potential of the [4Fe-4S] cluster is similarly lowered by over 100 mV by MgADP binding, but this event does not lead to intercomponent electron transfer, indicating that the midpoint potential of the [4Fe-4S] cluster alone is not the determining factor in component protein interaction or electron transfer.

Because the [4Fe-4S] cluster and the nucleotide binding sites in the Fe protein are separated by about 15 Å (Figures 1 and 2), nucleotide-induced changes in the electronic properties of the [4Fe-4S] cluster cannot be the result of a direct interaction between MgATP and the cluster. A lack of direct interaction between the cluster and nucleotide is also indicated by spectroscopic comparison of Fe protein in its nucleotide-free and nucleotide-bound states.[34] This result demands that nucleotide-induced effects upon the [4Fe-4S] cluster be communicated through the protein backbone by polypeptide conformational changes. Moreover, Fe K-edge X-ray absorption studies have revealed that nucleotide binding to the Fe protein does not significantly alter the Fe−Fe or Fe−S bond distances within the cluster.[35] Thus, MgATP-induced changes in the electronic properties of the [4Fe-4S] cluster must result from changes in the constraints placed upon it by the polypeptide environment rather than through structural rearrangement of the cluster.

An approximation of where and how MgATP binds to the Fe protein was first indicated by the comparison of Fe protein primary sequences to the primary sequences of various other nucleotide binding proteins.[36,37] These comparisons revealed that Fe protein is a member of a large family of nucleotide binding proteins (e.g., ras p21 and rec A) that contain two sets of consensus amino acid sequences commonly referred to as the Walker A and B motifs. A much clearer view of the MgATP binding site emerged by comparing the structural features of the Fe protein to three-dimensional structural motifs of other nucleotide binding proteins.[2,18] In the Fe protein, each MgATP binding site (one per subunit) is situated on the interfacial cleft between the subunits (Figure 2).

How does the nucleotide binding event become communicated over a long distance to the [4Fe-4S] cluster? At least one potential transduction pathway that connects the MgATP site to the cluster is a short protein chain extending from the MgATP binding site (Asp-125) to the [4Fe-4S] cluster ligand Cys-132 (Figure 2).[18,38] Polypeptide segments that propagate or transduce protein conformational changes as a consequence of nucleotide binding or hydrolysis can be considered as molecular switches. Such a segment within the Fe protein, called switch II, provides the most direct connection from the proposed nucleotide phosphate binding site to the [4Fe-4S] cluster[38] and is homologous to a structural motif present in other nucleotide-dependent signaling proteins.[39] From inspection of the model in Figure 3, it is easy to envision that MgATP binding triggers movement of switch II by breaking a salt bridge between Asp-125 and Lys-15. In this way switch II could communicate events at the MgATP binding site to the [4Fe-4S] cluster via conformational changes.

Evidence supporting this model was provided by characterization of altered Fe proteins substituted at either the Lys-15[37,40] or Asp-125[41] position. Substitution by Gln at the Lys-15 position results in an Fe protein that remains

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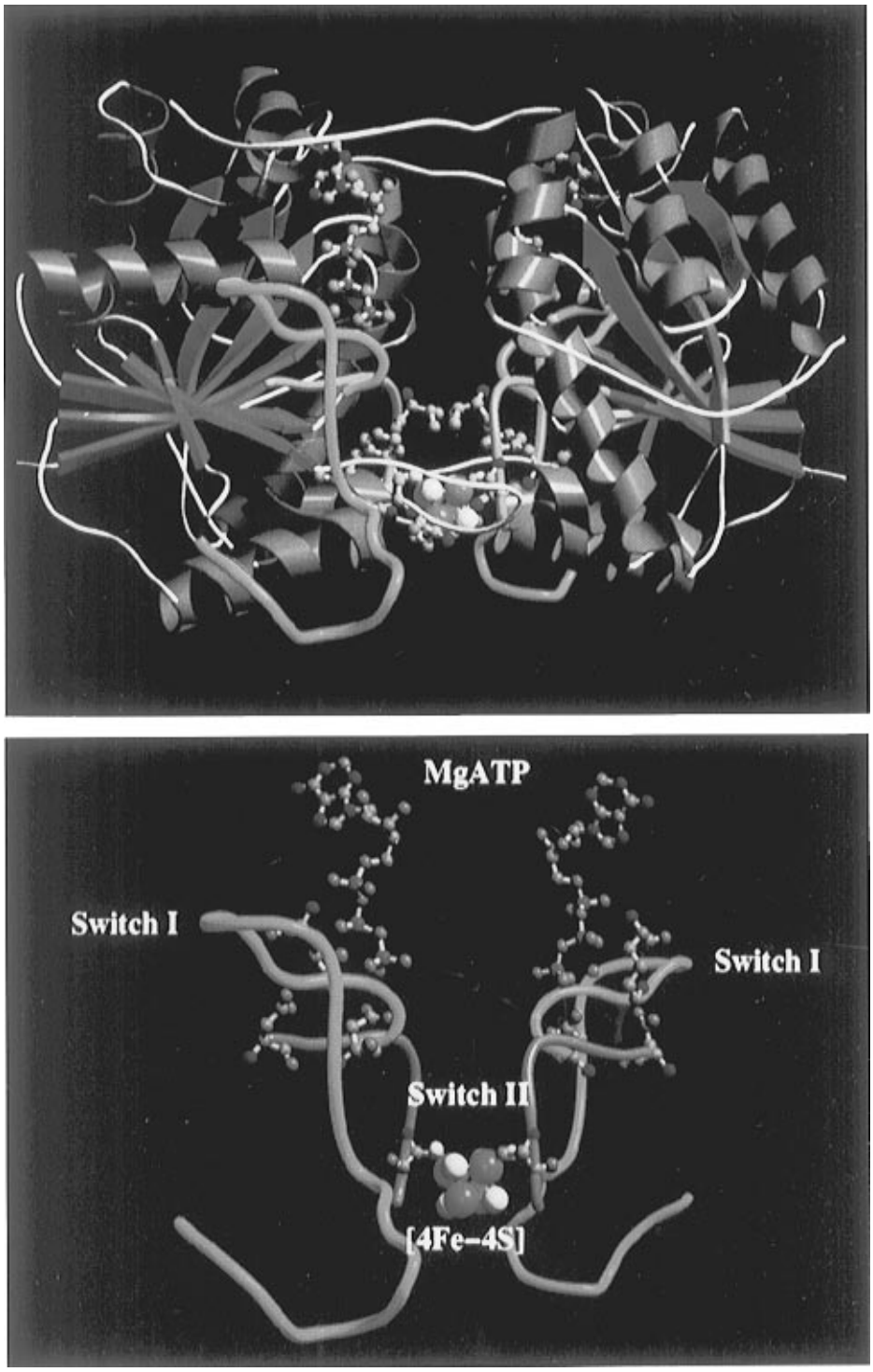

FIGURE 2. Ribbon diagram of the nitrogenase Fe protein. (A, top) Ribbon diagram of the Fe protein where switch I (cyan) and switch II (magenta) domains are highlighted to illustrate their spatial relationships to the [4Fe-4S] cluster and the MgATP binding sites. Also shown are the side chains for the amino acids Val-130, Phe-135, and Ala-98. (B, bottom) Same view as in panel A, showing switch I, switch II, the [4Fe-4S] cluster, and the two bound MgATP molecules. As in Figure 1, the Fe protein structure shown is probably not in its MgATP-bound form and the MgATP modeled into the figure is shown only for convenience in presentation. Also shown are ball-and-stick representations of the side chains for the amino acids Asp-39 (located on switch I), Asp-129 (located on switch II), and Asp-125 (located on switch II).

able to bind MgATP, but this binding event is not communicated to the [4Fe-4S] cluster.[37] Conversely, substitution by Glu at the Asp-125 position results in an Fe protein that mimics certain properties of the MgATP-bound form when it has MgADP, rather than MgATP, bound.[41] This latter result makes sense when it is considered that the extra methylene group in Glu should permit it to reach the α- and β-phosphates of bound MgADP in a way that might approximate the normal Asp interaction with the β- and γ-phosphates of MgATP (Figure 3). A more profound effect was provided by shortening the Asp-125 to Cys-132 switch II segment by deleting the intervening Leu-127 residue.[38] This altered protein becomes locked into a conformation that shows a striking resemblance to the MgATP-bound state even in the absence of any nucleotide.

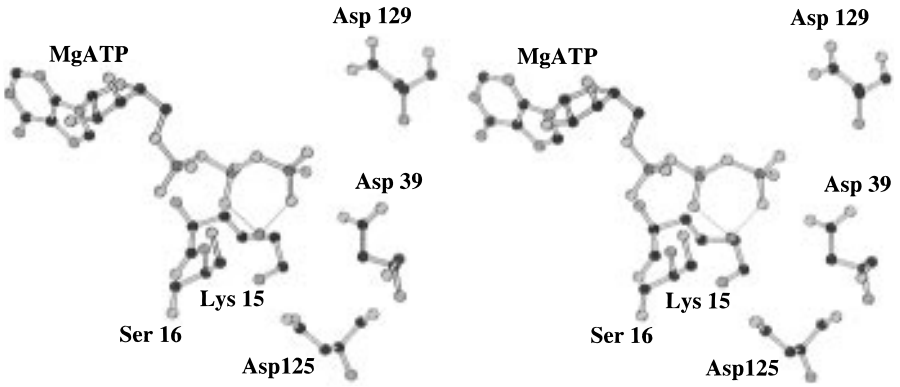

FIGURE 3. Stereoview of the phosphate binding region of the MgATP site of the Fe protein. A ball-and-stick presentation of the position of a MgATP bound to one subunit of the Fe protein, along with the amino acid side chains for Lys-15, Asp-125, Asp-39, and Ser-16, is presented. A Mg^{2+} is shown coordinated to the β- and γ-phosphates of the bound ATP.

The MgATP-Bound Fe Protein Is Primed for Interaction with the MoFe Protein

The conformational changes associated with MgATP binding are a prerequisite for the correct docking of Fe protein to the MoFe protein and, thus, a prerequisite for all subsequent nitrogenase reactions, including MgATP hydrolysis, electron transfer, and substrate reduction. One example of experimental data that illustrate this feature of the catalytic process has come from the characterization of an Fe protein that has the Lys-15 residue substituted by Gln-15.[37] This altered protein binds MgATP but does not undergo the nucleotide-induced conformational change and is unable to correctly bind with the MoFe protein. Evidence for this is provided by the inability of the Gln-15 Fe protein to compete with the normal Fe protein for interaction with the MoFe protein. This feature contrasts with other altered Fe proteins that are inactive but are still able to undergo the MgATP-induced protein conformational change. Certain of these altered Fe proteins, for example, one having Asp-129 substituted by Glu,[42] are able to compete with the normal Fe protein for interaction with the MoFe protein.

The docking of two large proteins such as the Fe protein and MoFe protein must be a complex process that is likely to involve many interactions that ultimately permit intercomponent electron transfer. Insight concerning how Fe protein and MoFe protein might dock has come from chemical cross-linking studies,[43,44] amino acid substitution studies,[45−47] and model building[18,19] from the individual structural models (Figure 1). It is noted that two different methods for isolation of stable forms of the docked complex have been recently reported,[48−50] so we anticipate that crystallographic information on the complex will be forthcoming. However, a few important features of the complex are already apparent. Docking almost certainly occurs so that the 2-fold symmetric axis about the [4Fe-4S] cluster of the Fe protein becomes paired with the exposed surface of the MoFe protein's pseudosymmetric $\alpha\beta$-interface (Figure 1). This model places the [4Fe-4S] cluster of the Fe protein in close proximity to the P-cluster of the MoFe protein, an arrangement consistent with the idea that P-clusters broker delivery of electrons from the Fe protein to FeMo-co.[14,51−53] Several of the amino acids required for formation of a competently docked complex include a crown of basic residues (Arg-100, Arg-140, and Lys-143) that surround the [4Fe-4S] cluster of the Fe protein and a crown of acidic residues that surround the P-clusters of the MoFe protein. Salt bridges between these residues appear to play at least some role in complex formation.

Formation of the Fe Protein−MoFe Protein Complex Is Required for MgATP Hydrolysis

It is clear that the initial signal transduction event triggered by MgATP binding involves propagation of a conformational change over a distance of about 17 Å from the MgATP binding site to the docking surface. If the component docking configuration shown in Figure 1 is correct, then a second signal must be channeled back from the docking surface to the nucleotide binding site in order to elicit hydrolysis. It appears unlikely that a residue within the MoFe protein could directly participate in MgATP hydrolysis. It is more likely that the component protein docking event causes a second conformational change in the Fe protein, thus bringing Fe protein amino acid residues into an appropriate position to initiate hydrolysis of MgATP. Switch II might participate in this two-way communication. Residue Asp-129, which is contained within the switch II domain, is located near the γ-phosphate of bound MgATP and has been implicated as a possible general base.[18,42] Thus, it is plausible that, upon docking to the MoFe protein, a signal is sent down switch II to move Asp-129 into a position to

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abstract a proton from the bound water, which in turn would participate in nucleophilic attack on the γ-phosphate.

A second domain within the Fe protein that is structurally similar to another switch domain found in other nucleotide-dependent signal-transducing proteins is called switch I.[18,50] In the Fe protein, the switch I domain extends from Asp-39, which is located near the terminal phosphate of bound nucleotide, to a loop that is located on the surface of the Fe protein at the proposed docking interface. This loop approximately comprises residues 59−67.[54] Thus, it is easy to imagine that the surface loop connected to switch I serves as a toggle that initiates transmission of a conformational change through switch I to reposition Asp-39. The movement of Asp-39 might reposition the bound water at the phosphate site for the hydrolysis reaction. The links between the MgATP site and the [4Fe-4S] cluster provided by switches I and II and their spatial relationship to each other are shown in Figure 2.

It is also possible that movement in switch I could alter the position of switch II, thus repositioning Asp-129 for general base hydrolysis of MgATP. A residue analogous to Asp-129 has been recognized as a possible general base catalyst in another nucleotide-dependent switch protein.[18] Altered Fe proteins have been constructed in order to probe the function of Asp-39[50] and Asp-129,[42] and the results of characterization of these proteins are consistent with the above model. Substitution of Asp-129 by Glu results in a protein that can still bind MgATP and can still dock to the MoFe protein but does not hydrolyze MgATP.[42] Similarly, substitution of Asp-39 by Asn results in an Fe protein that remains able to bind MgATP and to dock to the MoFe protein.[50] However, this altered protein retains a slight capability to hydrolyze MgATP, so Asp-39 cannot be the base solely responsible for MgATP hydrolysis. Another important feature of these proteins, as well as that of an Fe protein altered within the surface loop segment attached to switch I,[54] is that they appear to dissociate from the MoFe protein at much slower rates. This feature indicates that, upon MgATP hydrolysis, a third signal is sent from the MgATP site to the docking surface to elicit component protein dissociation. The mechanistic implication here is that the MgADP-bound form of the Fe protein must be achieved for the proper timing of component dissociation.

Electron Transfer Can Occur without MgATP Hydrolysis

There has been some confusion as to the exact order of events following the formation of the Fe protein−MgATP−MoFe protein complex. The original stopped-flow spectroscopic studies supported a model where MgATP hydrolysis preceded electron transfer.[15] More recent stopped-flow studies indicate that electron transfer precedes MgATP hydrolysis,[55,56] and this possibility was supported by characterization of the altered Fe protein that has switch II shortened.[57] This protein is the same one that is locked into a MgATP-like conformation even in the absence of MgATP and also forms a tight complex when mixed with the MoFe protein. Upon complex formation the altered Fe protein transfers a single electron to the P-cluster of the MoFe protein even in the absence of nucleotide.[53,57] This result shows that the energy released by MgATP hydrolysis is not absolutely required for intercomponent electron transfer, although hydrolysis might well accelerate electron transfer. As we have already discussed, it is more likely that the transition from the MgATP state to the MgADP state is involved in timing intercomponent electron transfer. It is reasonable to expect that conformational changes elicited within the MoFe protein as a consequence of component protein docking, MgATP hydrolysis, or component protein dissociation drive intramolecular electron transfer within the MoFe protein or stabilize a semireduced transition state intermediate. Along these lines, there is good evidence that both the P-cluster and FeMo-co indirectly communicate with events occurring at the nucleotide binding sites in the Fe protein because substitutions within their respective MoFe protein binding domains, or in the region between these domains, can result in the uncoupling of MgATP hydrolysis from electron transfer.[54,58,59]

Conversion from the MgATP-Bound to the MgADP-Bound State Is Necessary for Complex Dissociation

Following intercomponent electron transfer and MgATP hydrolysis, the Fe protein must dissociate from the MoFe protein in a step that is widely believed to be rate-limiting for the overall reaction.[15] The reason that the component proteins dissociate after each electron transfer event is not clear, but there appear to be at least two good mechanistic explanations. First, because the [4Fe-4S] cluster of the Fe protein is buried between the protein partners in the complexed state, it might be necessary for dissociation to occur so that the oxidized [4Fe-4S] cluster can be reduced for a subsequent round of the Fe protein cycle.[12,57] Second, dissociation might be required so that either or both the Fe protein and MoFe protein can achieve conformationally relaxed forms necessary for the next cycle of intercomponent electron transfer. Whatever the mechanistic reasons, the important feature of dissociation appears to be related to the timing of the closure of an electron transfer gate between the two proteins.

Several observations suggest that attainment of the MgADP-bound state is intimately involved in the mechanism for complex dissociation. Spectroscopic evidence has shown that the MgATP- and MgADP-bound states of the Fe protein have different protein conforma-

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tions.[25,26,29−31,60] Thus, the transition between the MgATP-bound and MgADP-bound complex could well be the signal that accelerates electron transfer (discussed above) and complex dissociation. At least three recent observations support this possibility.

Treatment of certain nucleotide switch proteins with AlF_4^- is known to result in the trapping of an activated intermediate.[61] Presumably an AlF_4^- ion replaces the exiting γ-phosphate to form an adduct that mimics the pentacoordinated MgADP−P_i transition state for normal nucleotide hydrolysis. The MgADP−AlF_4^- adduct duplicates neither the nucleotide triphosphate form nor the hydrolyzed form, but something in between. In the case of nitrogenase, incubation of both component proteins with AlF_4^- and MgATP or MgADP results in the time-dependent capture of the proteins in the complexed form.[48,49] The requirement for the simultaneous presence of both component proteins and MgATP indicates the dynamic nature of complex trapping. A tight Fe protein−MoFe protein complex is also formed when an Fe protein whose switch II region has been shortened by one amino acid by deletion of Leu-127 is mixed with MoFe protein.[57] Recall that the deletion of Leu-127 residues results in an Fe protein that adopts a MgATP-like bound form even in the absence of nucleotide.[38] Presumably, these proteins cannot dissociate from the MoFe protein because they cannot achieve the MgADP-bound conformation. This idea is supported by the characterization of an altered Fe protein that has the Asp-39 residue substituted by Asn. This altered Fe protein cannot attain the MgADP-bound conformation, as judged by the lack of the appropriate electronic changes to the [4Fe-4S] cluster upon the addition of MgADP, and it does not dissociate after transferring a single electron to the MoFe protein.[50] Perhaps, the most significant implication of all of these results is that the Fe protein−MoFe protein complex probably goes through a dynamic range of conformations as a consequence of MgATP hydrolysis. It seems likely that the precise timing of the transition from one nucleotide state to the other, which is initiated by component protein interaction, controls the flow of electrons from the Fe protein to the MoFe protein to effect substrate reduction. There is abundant evidence that this timing is critical to the catalytic mechanism because any perturbation that affects component protein interaction causes an uncoupling of MgATP hydrolysis from productive electron transfer.

Concluding Comments and Outlook

In this brief Account, we have summarized how the nitrogenase Fe protein serves as a specific reductant and molecular timer that is able to couple the unidirectional flow of electrons to MgATP binding and hydrolysis. This is a dynamic process that involves the transmission of signals back and forth from the MgATP site to the docking surface of the Fe protein, and probably into the MoFe protein. These events are transduced and synchronized by sequential conformational changes induced by nucleotide binding, component protein interaction, nucleotide hydrolysis, and perhaps electron transfer. Although the molecular details concerning how MgATP binding and hydrolysis might gate the sequential delivery of electrons from the Fe protein to the MoFe protein are becoming understood, we emphasize that this is only one aspect of a complicated process and, for that matter, probably only one aspect of the involvement of MgATP in nitrogenase catalysis. For example, there is mounting evidence that the P-cluster is involved in accumulating and brokering electrons prior to their intramolecular delivery to the substrate reduction site,[14,51−53] yet how an electron is transferred from the Fe protein to the P-cluster and where and how it is stored within the P-cluster are not known. This latter point is currently enigmatic because the P-cluster is believed to already be fully reduced in the resting state;[62] however, very recent data indicate that oxidation of the P-cluster leads to its structural rearrangement.[63] It is likely that the timing of complex formation, MgATP hydrolysis, and complex dissociation lead to conformational transitions within the P-cluster and FeMo-co polypeptide environments such that electrons can be sequentially accepted, accumulated, and delivered to the substrate. The possible mechanistic features of these events and where, how, and at what redox state N_2 binds to the active site are beyond the scope of this Account. We urge the interested reader to consult recent comprehensive reviews on the mechanism of nitrogenase catalysis[21,64] as well as a recent series of cogent and provocative commentaries describing theoretical mechanisms for N_2 binding and reduction.[65−70]

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