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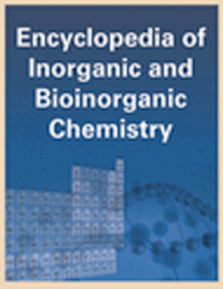
Michael K. Johnson • Robert A. Scott

Metalloprotein Active Site Assembly



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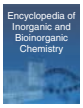

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METALLOPROTEIN ACTIVE SITE ASSEMBLY

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Encyclopedia of Inorganic and Bioinorganic Chemistry

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Series Preface

The success of the *Encyclopedia of Inorganic Chemistry* (EIC), pioneered by Bruce King, the founding Editor in Chief, led to the 2012 integration of articles from the *Handbook of Metalloproteins* to create the newly launched *Encyclopedia of Inorganic and Bioinorganic Chemistry* (EIBC). This has been accompanied by a significant expansion of our Editorial Advisory Board with international representation in all areas of inorganic chemistry. It was under Bruce's successor, Bob Crabtree, that it was recognized that not everyone would necessarily need access to the full extent of EIBC. All EIBC articles are online and are searchable, but we still recognized value in more concise thematic volumes targeted to a specific area of interest. This idea encouraged us to produce a series of EIC (now EIBC) Books, focusing on topics of current interest. These will continue to appear on an approximately annual basis and will feature the leading scholars in their fields, often being guest coedited by one of these leaders. Like the Encyclopedia, we hope that EIBC Books continue to provide both the starting research student and the confirmed research worker a critical distillation of the leading concepts and provide a structured entry into the fields covered.

The EIBC Books are referred to as *spin-on* books, recognizing that all the articles in these thematic volumes are destined to become part of the online content of EIBC, usually forming a new category of articles in the EIBC topical structure. We find that this provides multiple routes to find the latest summaries of current research.

I fully recognize that this latest transformation of EIBC is built on the efforts of my predecessors, Bruce King and Bob Crabtree, my fellow editors, as well as the Wiley personnel, and, most particularly, the numerous authors of EIBC articles. It is the dedication and commitment of all these people that are responsible for the creation and production of this series and the "parent" EIBC.

Robert A. Scott
University of Georgia
Department of Chemistry

September 2017

Volume Preface

The study of metals in biology has evolved over the past half century as our tools and our understanding have progressed at an ever-accelerating pace. Early characterization of function and structure of metalloenzymes inevitably led to studies of their origin through cellular interaction with environmental metals and the evolution of assembly pathways to incorporate metals as active sites in metalloproteins. These pathways can be quite complex, given that simply admitting free metal ions to the cellular environment to "find" their metalloprotein "homes" can be detrimental to cellular machinery (i.e., toxic). Thus, these assembly mechanisms are intimately tied to mechanisms to control the cell's metal portfolio (homeostasis) and both comprise the exquisite result of organic life taking advantage of its inorganic environment, while protecting itself from toxic consequences.

Following on from the earlier book in this series, *Metals in Cells*, in which both the positive and negative effects of cellular interactions with metals were discussed, the current book focuses on the processes that have evolved to orchestrate the assembly of metal cofactor sites in functional metalloproteins. As you would expect, this goes beyond the simple incorporation of single metal ions in a protein framework, and includes metal cluster assembly, metal-cofactor biosynthesis and insertion, and metal-based post-translational modifications of the protein environments that are necessary for function. Several examples of each of these areas have now been identified and studied; the current volume provides the current state-of-the-art understanding of the processes involved. As such, it will make an excellent companion volume for the previous *Metals in Cells*.

Metalloprotein Active Site Assembly provides a diverse sampling of what is known about these assembly processes: The multiple systems that have evolved to create Fe-S clusters; the complex processes that result in the creation of the active sites that carry out some of the most important processes for life on earth (photosynthesis, respiration, nitrogen fixation, nitrous oxide reduction; carbon monoxide dehydrogenation, hydrogen production and oxidation); assembly of a number of Mn, Ni, Cu, Mo, and Fe enzyme active sites; cofactor (heme, siroheme, F430, cobalamin, molybdopterin) biosynthesis and insertion; and others. In most cases, we have learned a lot about the multiple steps and the gene products involved. In other cases, we are still at the beginning of our understanding.

With the baseline of the articles from both *Metals in Cells* and *Metalloprotein Active Site Assembly*, we plan to continue to add articles to the parent *Encyclopedia of Inorganic and Bioinorganic Chemistry* as new results are obtained on these and other metal transport and incorporation processes.

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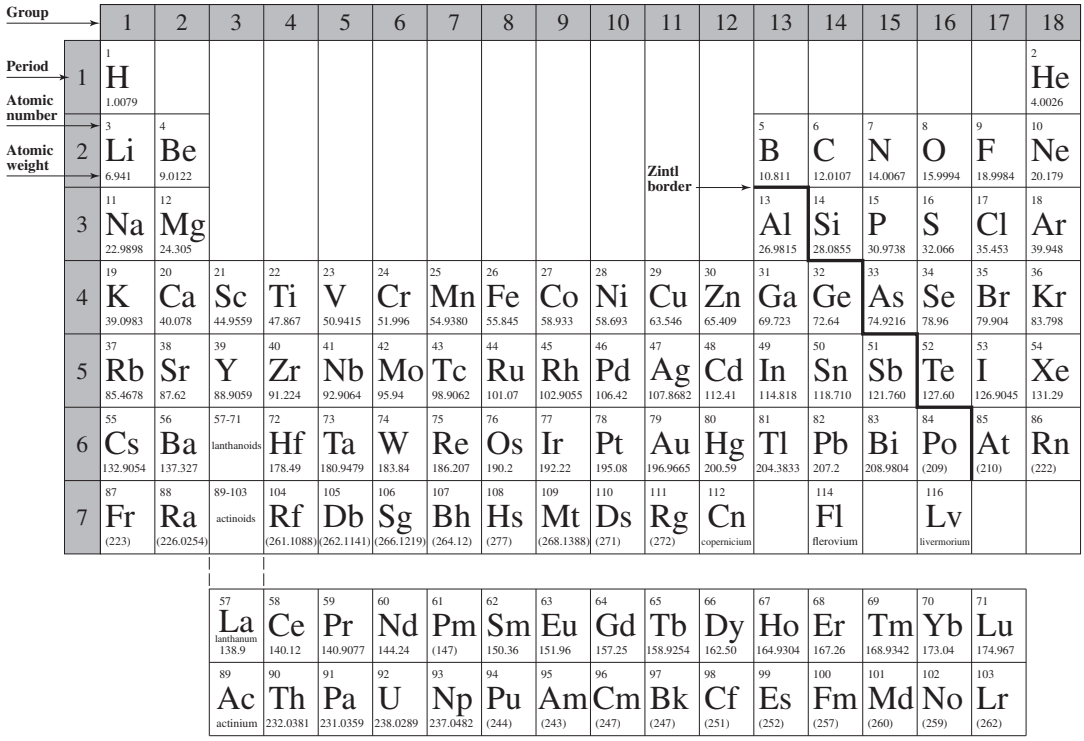
September 2017

Periodic Table of the Elements



Based on information from IUPAC, the International Union of Pure and Applied Chemistry (version dated 1st May 2013).
For updates to this table, see http://www.iupac.org/reports/periodic_table.

PART 1
Assembly and Trafficking of Simple Fe-S Clusters

Nif System for Simple [Fe–S] Cluster Assembly in Nitrogen-Fixing Bacteria

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and

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1 INTRODUCTION

In this chapter, biological iron–sulfur [Fe–S] clusters are defined as any protein-bound prosthetic group that contains Fe and S, and releases H_2S when treated with acid.[1,2] Owing to their structural and chemical versatilities [Fe–S] clusters participate in a very large number of key biological processes, including electron transfer, substrate activation, DNA synthesis and repair, sulfur mobilization, Fe storage, and gene regulation.[2–4] Indeed, [Fe–S] clusters are essential players in a wide range of life-sustaining processes. The most prevalent [Fe–S] clusters found in nature are [2Fe–2S] and [4Fe–4S] (Figure 1), which are attached most frequently to their cognate protein partners through cysteine residue thiol ligands. However, other coordination types, either N-ligands provided by a histidine residue or carboxylate ligands provided by an aspartate residue, have also been described.[3] In addition to simple [Fe–S] clusters,

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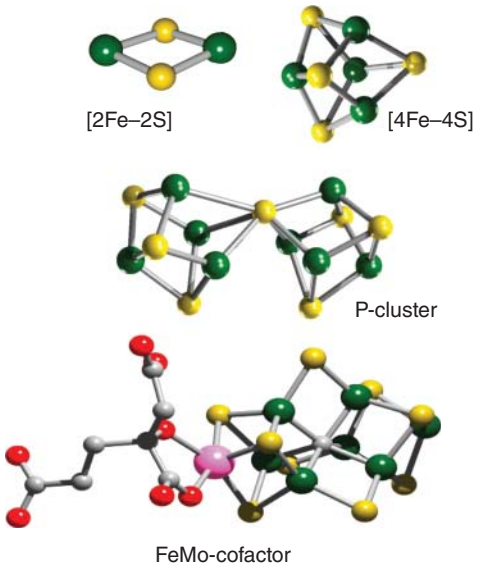

Figure 1 [Fe–S] clusters associated with nitrogen-fixing proteins. Atoms are color-coded in green (iron), yellow (sulfur), magenta (molybdenum), red (oxygen), and gray (carbon)

there are also more complex [Fe–S] clusters having a higher nuclearity, for example the [8Fe–7S] P cluster contained within nitrogenase.[5] Even more complex [Fe–S] clusters are those that contain a different metal in addition to Fe and also an organic constituent, for example the suite of active site cofactors contained in the nitrogenases described later (Figure 1).

A basic architecture constructed from simple [2Fe–2S] and/or [4Fe–4S] cluster units is a common feature of most complex [Fe–S] clusters.[2] Simple [2Fe–2S] and [4Fe–4S] clusters can be readily formed in the laboratory under anoxic and reducing conditions by mixing Fe and S in appropriate concentrations in the presence of suitable ligands.[6] However, the biological process is complicated by the fact that free Fe^{2+} and free S^{2-} are highly toxic to living cells. Thus, a basic question that has challenged the bioinorganic research community is: how are simple [2Fe–2S] and [4Fe–4S] clusters formed biologically?

A common approach toward probing fundamental biological processes is to characterize mutants impaired in some aspect of the process in order to identify key components. Once participating components are identified by genetic means, biochemical and biophysical analyses can be applied in efforts to duplicate the process using purified components. This approach is often an iterative one wherein genetic manipulations inform biochemical experiments, the outcome of which, in turn, suggests further genetic manipulation, and vice versa. However, for such a process to be useful several criteria must be met. First, the experimental organism must be amenable to genetic manipulation. Second, it must be possible to correlate genetic defects with perturbations in the process under study. Third, the identified components must be obtained in sufficient levels to permit structural and functional interrogation.

It might seem remarkable that an understanding of the biosynthetic pathways for the formation of many highly complex organic cofactors far preceded the discovery of mechanistic features associated with biological [Fe–S] cluster formation. Timely identification of key aspects of [Fe–S] cluster assembly that paralleled elucidation of the pathways for formation of many organic cofactors was denied for several important reasons. Namely, owing to the structural simplicity of [2Fe–2S] and [4Fe–4S] clusters, as well as the development of facile methods for their robust chemical synthesis, the process did not capture the interest of the scientific community until relatively recently. Also, because [Fe–S] clusters are essential to so many biological processes, the fortuitous discovery of a particular metabolic defect that could be unambiguously assigned to a perturbation in [Fe–S] cluster assembly did not occur. In this chapter, we describe why components involved in producing an active nitrogenase, the catalytic component of biological nitrogen fixation, proved to be a useful experimental system for the study of simple [Fe–S] cluster assembly. We also describe how this experimental system was used to discover some of the key principles of biological [Fe–S] cluster assembly.

2 THE Nif SYSTEM AS A MODEL FOR ANALYSIS OF SIMPLE [Fe–S] CLUSTER ASSEMBLY

Nitrogenase is an enzyme found only in a specialized class of microorganisms called diazotrophs (nitrogen eaters). It catalyzes biological nitrogen fixation, the reduction of N_2 to ammonia. The most thoroughly studied diazotroph is *Azotobacter vinelandii*. This organism has three distinct but structurally and mechanistically similar systems that are capable of nitrogen fixation.[7–10] A striking similarity among the three systems is that the catalytic entity for each is comprised of two component proteins, neither of which has the capacity to reduce N_2 in the absence of the other. One component serves as a nucleotide-dependent reductase that delivers electrons to the other component, which contains the active site [Fe–S] cluster. A fundamental differentiating feature of the three systems concerns the metal content of the complex [Fe–S] cluster that provides the active site. In the case of the "Mo-dependent" nitrogenase, the active site [Fe–S] cluster has the composition [7Fe–9S-Mo-C-Homocitrate] and is most commonly referred to as the FeMo-cofactor (Figure 1).[5,11]

The Mo-dependent nitrogenase was the first nitrogenase discovered, and it has been the subject of

intense mechanistic and structural analyses. The genes encoding many of the core functions related to formation and activation of the Mo-dependent nitrogenase are designated as *nif* genes (Figure 2).[12–14] However, there are also many other genes associated with Mo-dependent nitrogen fixation in *A. vinelandii* that have not yet been assigned functions and, therefore, they have no formal genetic designation. Furthermore, there are genes associated with Mo-dependent nitrogen fixation that have been assigned provisional genetic designations only on the basis of their primary structure similarity to orthologous proteins. A second nitrogen fixation system found in *A. vinelandii* is designated as the V-dependent system because its active site [Fe–S] cluster, analogous to FeMo-cofactor, appears to contain V in place of Mo [7Fe–9S–V–C–Homocitrate].[15–17] The structure and composition of this [Fe–S] cluster have been inferred from detailed spectroscopic analyses but have not been established crystallographically. Genes that specifically encode proteins associated with the V nitrogenase are designated *vnf* (Figure 2).[18] The third nitrogen-fixing system has an active site [Fe–S] cluster that contains neither Mo nor V.[9] Instead, it has an anticipated [8Fe–9S–C–Homocitrate] structure that is analogous to FeMo-cofactor. Genes encoding proteins specifically associated with this system are designated *anf* indicating they are an alternative to the *nif*- and *vnf*-encoded systems (Figure 2). Although the catalytic components of the *nif*, *vnf*, and *anf* systems are functionally equivalent, but genetically distinct, they all require the auxiliary proteins encoded by *nifU*, *nifS*, *nifV*, *nifM*, and *nifB* to satisfy the common aspects of their respective activations (Figure 2, red outline).[19] As an example, *nifV* encodes a homocitrate synthase, which catalyzes the formation of the organic acid associated with the active site [Fe–S] cluster of all three systems.[20]

Whether or not a particular nitrogen fixation system is expressed physiologically depends upon the metal composition of the growth medium. Under conditions of sufficient Mo availability, the expression of the *vnf*- and *anf*-encoded systems are repressed and only the Mo-dependent nitrogenase accumulates.[10] When Mo is scarce, but V is available, accumulation of the V-dependent

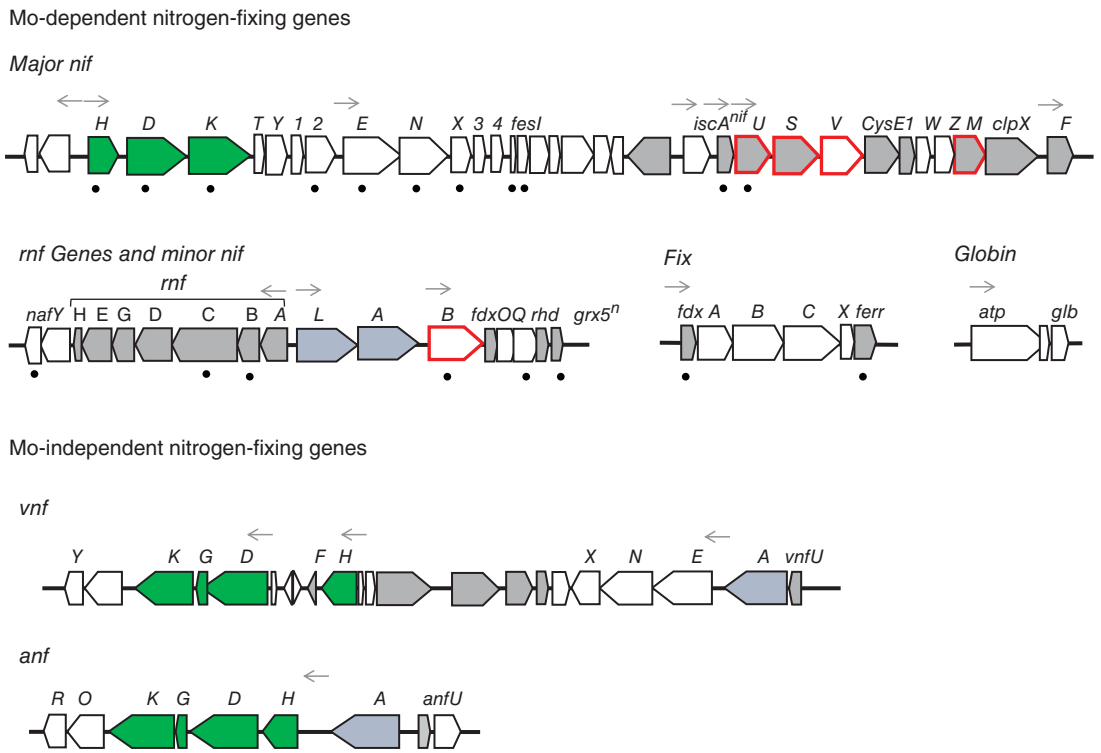

Figure 2 Nitrogen fixation-associated gene regions in the *Azotobacter vinelandii* genome. Mo-dependent (*nif*), V-dependent (*vnf*), and iron-only (*anf*) nitrogen fixation systems are shown. Genes encoding nitrogenase catalytic components are shown in green, regulatory proteins are shown in blue, and genes essential to all three nitrogenase systems are indicated by red borders. Coding sequences having paralogs also located elsewhere in the genome, but whose expression is not controlled by *nif* regulatory elements, are shown in gray. Various *nif* and *nif*-associated genes encoding proteins having [Fe–S] cluster-binding motifs are indicated by a dot below the designated gene. Arrows above the gene segments indicate the location of known or proposed promoters controlled by nitrogen fixation–specific regulatory elements

system is favored. If neither Mo nor V is available, only the *anf*-encoded nitrogenase is accumulated. This hierarchy in differential accumulation and activation of the different nitrogenases reflects their relative capacities to catalyze nitrogen fixation. For example, the Mo-dependent nitrogenase has a much higher catalytic efficiency than the other two systems.[5,7]

As already noted, all three nitrogenases contain two structurally and functionally equivalent components required for N_2 reduction: a nucleotide-dependent reductase and another protein that contains the substrate-binding active site. In the case of the Mo-dependent system, these component proteins are respectively designated as the "Fe protein" and the "MoFe protein" (Figure 3).[21,22] The Fe protein is comprised of two identical subunits, each of which contains a nucleotide-binding site. A single [4Fe–4S] cluster is bridged between the Fe protein subunits. The MoFe protein is an $\alpha_2\beta_2$ tetramer that contains two cluster types. One of these is an [8Fe–7S] cluster, designated the P cluster, and the other is the [7Fe–9S–Mo–C–Homocitrate] cluster, designated FeMo-cofactor. One Fe protein and one MoFe protein $\alpha\beta$-unit is considered to comprise a single quasi-independent catalytic unit (Figure 3a). Various structural and mechanistic features of the Mo-dependent nitrogenase continue to be under intense scrutiny. These aspects have been recently summarized in the literature and are not further discussed here.[23] Important for the present discussion, however, is that each Mo-dependent nitrogenase catalytic unit contains 19 Fe atoms and 20 S atoms distributed among three [Fe–S] cluster types, a [4Fe–4S] cluster, the P cluster, and FeMo-cofactor. The second notable fact is that nitrogenase is such a poor catalyst that cells must produce the enzyme components at prodigious levels in order to sustain growth under nitrogen-fixing conditions (Figure 4). Another significant feature of the Mo-dependent system is that, of the 60 different proteins whose accumulation is elevated in response to a physiological demand for nitrogen fixation, at least 19 are known or suspected to contain [Fe–S] clusters (see dotted genes in Figure 2).[12,14,24] Thus, in aggregate, the formation and activation of the Mo-dependent nitrogenase place an exceedingly high demand on the capacity for cellular [Fe–S] cluster formation. Because of this feature, and because intact [Fe–S] clusters are absolutely required for nitrogenase activity, the system provided an ideal opportunity to monitor cellular capacity for [Fe–S] cluster formation.

There are also several other features that made the Mo-dependent nitrogenase system from *A. vinelandii* an attractive model to explore the formation of simple [Fe–S] cluster units.[25,26] First, it is easy to monitor nitrogenase activity by using either in vivo or in vitro assays. Second, when there is no physiological demand for nitrogen

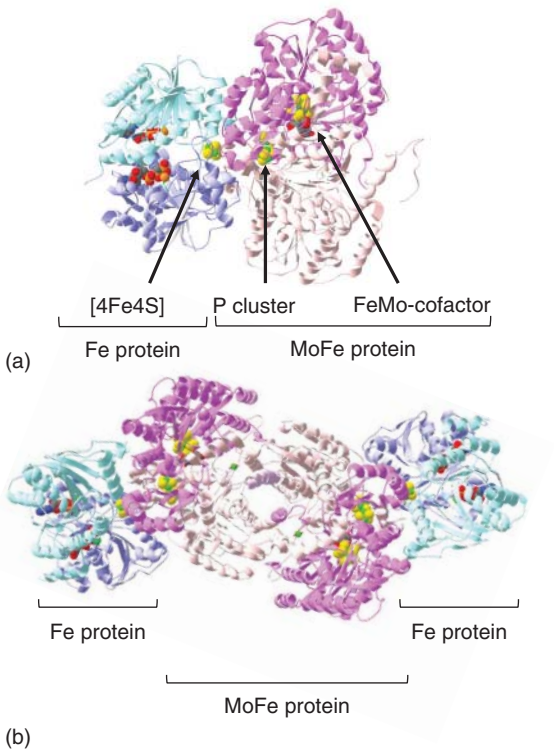

Figure 3 *A. vinelandii* nitrogenase structures. Ribbon diagram of MoFe protein and Fe protein complex in the presence of Mg-ADP and the ATP analog, Mg-AMPPCP (PDB ID 4WZA). (a) A catalytic unit comprised of one Fe protein dimer showing each subunit in blue and cyan and one α/β dimer shown in dark and light pink, respectively. (b) The entire complex with two catalytic units. [Fe–S] clusters, Mg-ADP, and Mg-AMPPCP are shown in space filling models

fixation, the expression of genes required for the formation and activation of the system are not needed to sustain growth. Consequently, a growth defect is only manifested in cells impaired in the formation or activation of nitrogenase when they are cultured in the absence of a fixed nitrogen source. Finally, *A. vinelandii* is tractable to genetic manipulation such that targeted inactivation of specific genes, separately and in various combinations, is readily accomplished. In aggregate, these features enabled the implementation of biochemical and genetic strategies that were used to assess the specific functions of individual genes whose products are associated with the formation and activation of the nitrogenase catalytic components. We now describe how this approach led to the identification of NifU and NifS as a biochemical toolkit that provides the [Fe–S] cluster building blocks necessary for the activation of nitrogenase. Also described is how this discovery provided mechanistic insight into the general process of formation of simple [Fe–S] clusters to sustain many cellular processes.

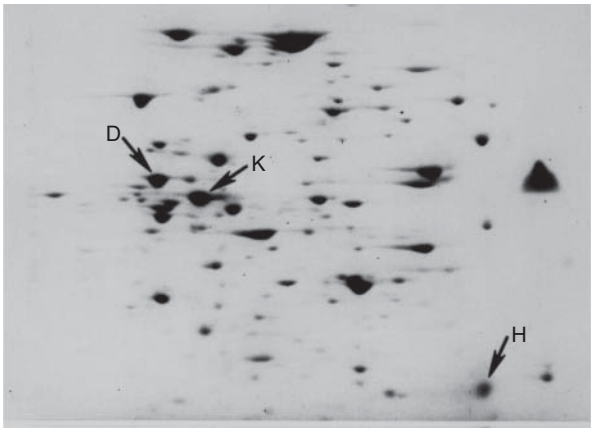

Figure 4 Two-dimensional polyacrylamide gel electrophoresis profile of soluble *A. vinelandii* extracts prepared from cells cultured under nitrogen-fixing conditions. The gel illustrates the high level of accumulation of Fe protein (H) and MoFe protein (D and K for α and β subunits, respectively) in nitrogen-fixing cells

3 ANALYSIS OF THE Mo-DEPENDENT NITROGEN-FIXING SYSTEM IN *A. VINELANDII*

The in vitro assay of the Mo-dependent nitrogenase has five basic requirements.[27] These include an anoxic environment, MgATP, Fe protein, MoFe protein, and reducing equivalents. Reducing equivalents are usually supplied in vitro by dithionite. Catalytic reduction of acetylene is frequently used as a proxy for N_2 reducing capacity. The product of the nitrogenase-catalyzed, two-electron reduction of acetylene is ethylene, and it is readily quantified by gas chromatography. Because both Fe protein and MoFe protein are required for activity, the activity of either component can be evaluated by the addition of the appropriate, separately purified, complementary component to assay cocktails. It is also possible to isolate intact FeMo-cofactor from purified MoFe protein.[28,29] Thus, a MoFe protein sample that lacks FeMo-cofactor can be identified by the simple addition of isolated FeMo-cofactor as a way to recover nitrogenase activity.

Through a combination of placement of targeted mutations within genes associated with the Mo-dependent nitrogenase, and evaluation of the consequences on diazotrophic growth and associated biochemical phenotypes, the following features emerged: (i) *nifH* encodes the Fe protein and is essential for Mo-nitrogenase activity,[30] (ii) *nifD* and *nifK*, respectively, encode the MoFe protein α- and β-subunits and are essential for Mo-nitrogenase activity,[30] (iii) *nifE*, *nifN*, and *nifB* are required for FeMo-cofactor formation and are essential for MoFe protein activity,[12,13] (iv) *nifV* encodes a homocitrate synthase that catalyzes the formation of the organic acid contained in FeMo-cofactor.[20] FeMo-cofactor produced in cells deficient in a *nifV*-deficient strain has homocitrate replaced by citrate,[31] (v) inactivation of *nifM* results in the production of an inactive Fe protein,[32] (vi) inactivation of either *nifU* or *nifS* results in a significant loss in both Fe protein and MoFe protein activities, and *nifU*- and *nifS*-deficient strains are impaired in their respective capacities for diazotrophic growth.[32] The combined inactivation of both *nifU* and *nifS* results in a severe, but not complete, loss in both Fe protein and MoFe protein activities, (vii) the growth and biochemical phenotypes associated with the inactivation of *nifM*, *nifV*, *nifB*, *nifU*, or *nifS* that affect various activities of the MoFe or Fe protein also result in parallel defects in the analogous *vnf*- and *anf*-encoded nitrogenases.[19]

This narrative provides only a brief sketch of the general functions of some of the most important players involved in producing an active Mo-nitrogenase (see ***Nitrogenase Metallocluster Assembly***). The other associated gene products shown in Figure 2, although not essential, are either known or are suspected to have variously dispensable auxiliary functions related to (i) the effective assembly, trafficking, and insertion of [Fe–S] clusters for Mo-nitrogenase activation, (ii) protection from damage by O_2, (iii) supply of reducing equivalents to sustain catalytic activity, or (iv) regulatory elements that control the coordinated expression of the many associated components in response to a demand for fixed nitrogen or in response to an oxygen challenge.[14,33–35] These aspects are not further discussed here. Rather the remaining discussion focuses on the individual functions of NifS and NifU that, together, supply the [Fe–S] cluster units necessary to support all three nitrogenase types.

4 GENETIC PHENOTYPES AND BIOCHEMICAL FEATURES INDICATED A ROLE FOR NifU AND NifS IN [Fe–S] CLUSTER FORMATION

A notable feature of nitrogen-fixing cell pellets prepared from *A. vinelandii* is that they have a much darker brown color when compared with cell pellets prepared from cells growing in the presence of a fixed nitrogen source, such as ammonia. The dark brown color of nitrogen-fixing cells is attributed to the accumulation of copius amounts of the nitrogenase catalytic components, as well as a variety of other nitrogenase-associated proteins that contain [Fe–S] clusters, as already discussed. Cell pellets prepared from either *nifU*- or *nifS*-deletion mutants cultured in the absence of a fixed nitrogen source do not exhibit the characteristic brown color. This aspect of *nifU*- and *nifS*-deletion strains indicated that they are unable to accumulate nitrogen fixation–associated proteins that are replete with their cognate [Fe–S] clusters. Furthermore, mutant strains deleted for either *nifU* or *nifS* are impaired in both Fe protein and MoFe protein activities.[32] Finally,

such strains are not only defective in the production of a robust Mo-dependent nitrogenase but are also impaired in their respective capacities to accumulate fully active *vnf*- and *anf*-encoded nitrogenases.[19] These combined features provided compelling circumstantial evidence that NifS and NifU are involved in complementary functions necessary for some aspect of Fe and S acquisition and mobilization for the assembly of active nitrogen-fixing systems.

5 NifS CYSTEINE DESULFURASE

The sulfur source for the biosynthesis of [Fe–S] clusters in nitrogenase is provided by the free amino acid cysteine. Biochemical characterization of NifS revealed that the pyridoxal-5′-phosphate (PLP) cofactor is associated with the enzyme.[36] Since PLP-dependent enzymes are typically involved in transamination and elimination reactions using an amino acid as substrate, it was hypothesized that NifS might react with cysteine during the formation of [Fe–S] clusters. The presence of a *cysE1* gene within the same transcriptional unit as *nifU* and *nifS* (Figure 2) supported this possibility, because *cysE1* encodes an *O*-acetyl serine transferase, which catalyzes the rate-limiting step for *de novo* cysteine synthesis.[32] It was subsequently shown that incubation of NifS with cysteine, but no other standard amino acid, results in changes in the absorption spectrum of the enzyme-associated PLP cofactor.[36,37] Prolonged co-incubation of NifS and Cys also resulted in the formation of a cloudy solution attributed to the accumulation of colloidal sulfur. When NifS and cysteine are incubated under the same conditions in the presence of a thiol reducing agent, such as dithiothreitol, H_2S is evolved. Thus, NifS is a cysteine desulfurase that uses PLP chemistry to remove S from cysteine to yield alanine. These observations led to the discovery of other cysteine desulfurases that are involved in the assembly of [Fe–S] clusters not associated with nitrogenase. Later studies have also shown that this class of enzyme also functions in sulfur trafficking by promoting sulfur transfer reactions for the biosynthesis of sulfur-containing cofactors other than [Fe–S] clusters.[38] Despite the diverse roles of various cysteine desulfurases, the mechanism for the formation of the persulfide enzyme intermediate initially described for the *A. vinelandii* NifS in 1994 appears to be a universal feature of this class of enzyme (Figure 5).[37]

Like NifS, all cysteine desulfurases studied to date are found as dimers containing one active site within each monomer. The NifS mechanism is schematically shown in Figure 5 and proceeds as follows. Under resting state conditions, PLP is covalently bound to a conserved lysine residue via a Schiff base (internal Lys-aldimine) having a characteristic absorption spectrum around 392 nm. The binding of cysteine leads to the rapid formation of a Cys-PLP Schiff base (external Cys-aldimine). A key step in the activation of cysteine is the abstraction of the α-proton of the substrate by a protein residue acting as a general base. Isotope labeling studies showed that this step is facilitated by a monoprotic residue. It is, therefore, unlikely that the active site lysine participates in this aspect of catalysis.[39] The proposed reaction mechanism suggests the participation of a general base residue that performs a nucleophilic attack on the α-proton. The crystal structures of other cysteine desulfurases show the presence of a conserved His residue in close proximity to the C4′ of PLP. In *A. vinelandii*, NifS His101 is a candidate to serve this function.[26]

The PLP Cys-quinonoid intermediate then abstracts a proton from the active site cysteine during protonation of C4′ of PLP. This event leads to the formation of a hydrogen bond between the imine hydrogen and the phenolate oxygen, which allows the expansion of the π orbital system leading to a highly conjugated structure of PLP, therefore allowing electron delocalization. The enzyme inhibition profile with allyl- and vinylglycine and the formation of associated γ-methylcystathionyl or cystathionyl adducts at Cys325 position support the participation of the Cys active site residue during the formation the Cys-ketinime intermediate.[37]

The first committed step of the cysteine desulfurase reaction occurs by the nucleophilic attack of the deprotonated active site cysteine thiol on the thiol group of the substrate leading to the formation of the persulfide bond and conversion to an Ala-enamine intermediate. Reactions performed in ^2H_2O confirmed that α and β hydrogens are exchanged during the reaction, supporting the occurrence of this intermediate. The subsequent release of alanine is proposed to follow the same sequence, but in the reverse order, to restore the internal Schiff base with Lys202. The persulfurated form of the enzyme marks the end of the first half of the cysteine desulfurase reaction, in which the release of Ala precedes the binding of the sulfur acceptor NifU. In vitro, the activity of cysteine desulfurases are typically determined in the presence of artificial reducing conditions (e.g., dithiothreitol), which regenerates the resting state of the enzyme by releasing sulfide. In the absence of a reducing agent, the enzyme can still undergo turnover, albeit at a slower rate, and forms polysulfide species. Under these conditions, the absorption spectrum of NifS results in a spectral shift from 392 nm peak to form a broad spectral feature with two maxima at 416 and 370 nm.[36] This change in the spectrum is proposed to represent the accumulation of the Cys-aldimine and Cys-ketimine intermediates steps preceding the participation of the active site Cys residue during the subsequent turnover of the persulfurated form of the enzyme. In vivo, the second half of the NifS reaction involves the sulfur transfer to NifU for the subsequent synthesis of Fe–S clusters.

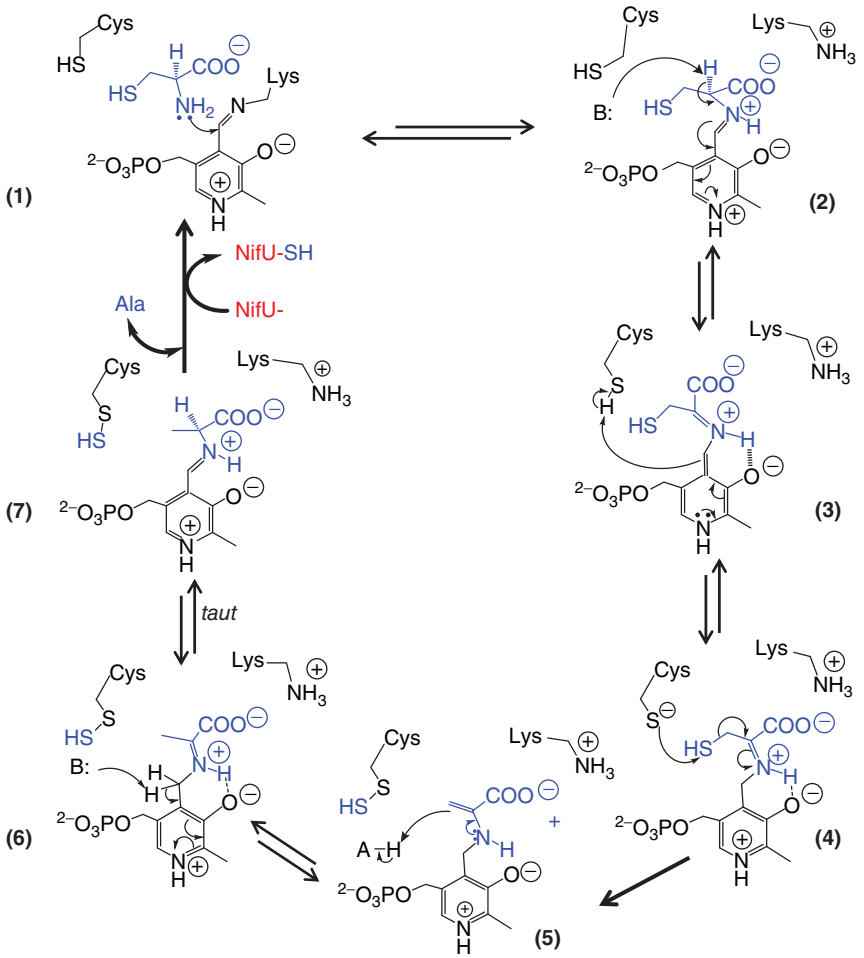

Figure 5 Proposed catalytic mechanism NifS cysteine desulfurase reaction. Under resting state, NifS Lys^{202} is covalently attached via a Schiff base (internal Lys-aldimine, **1**). The binding of Cys results in the formation of the external Cys-aldimine intermediate (**2**). The abstraction of the substrate α-proton then leads to a rearrangement to form the Cys-quinonoid (**3**) and Cys-ketimine (**4**) intermediates. The active site Cys^{325}, then, promotes the nucleophilic attack onto the thiol group of the substrate, leading to the formation of the persulfide enzyme and Ala-enamine intermediates (**5**). The reaction is completed through sequential formation of Ala-ketimine (**6**), Ala-aldimine (**7**), and restoration of the Lys-aldimine bond (**1**). The second half of the NifS cysteine desulfurase mechanism is the sulfur transfer reaction from the persulfide intermediate to NifU during Fe–S cluster assembly

6 NifU PROVIDES A SCAFFOLD FOR [Fe–S] CLUSTER ASSEMBLY

Early gene inactivation studies showed that the phenotype associated with the inactivation of *nifS* was similar to that observed upon *nifU* inactivation. That is, deletion of either or both genes results in a decrease in the activities of both nitrogenase components.[32] This observation led to the hypothesis that NifS and NifU have complementary roles in mobilizing sulfur and iron for the formation of [Fe–S] clusters that are contained in both nitrogenase proteins. Furthermore, analysis of NifU primary structures from various nitrogen-fixing species pointed to the presence of nine conserved cysteines present within three distinct domains (Figure 6).[40] Because cysteine residues typically serve as metal coordination sites, the collection of nine conserved cysteine residues indicated that NifU might serve as an iron reservoir or function as a transient [Fe–S] cluster assembly site to provide [Fe–S] clusters associated with nitrogen fixation.

Isolation of NifU heterologously expressed in *Escherichia coli* revealed the presence of two spectroscopically identical [2Fe–2S]$^{2+/+}$ clusters associated with a NifU dimer (Figure 6).[40] Substitution by alanine for any of the four conserved cysteine residues located within the central domain led to loss of these clusters as well as a lowered capacity for diazotrophic growth.[41] Spectroscopic characterization showed that these clusters display properties similar to those detected for the bacterioferritin-associated

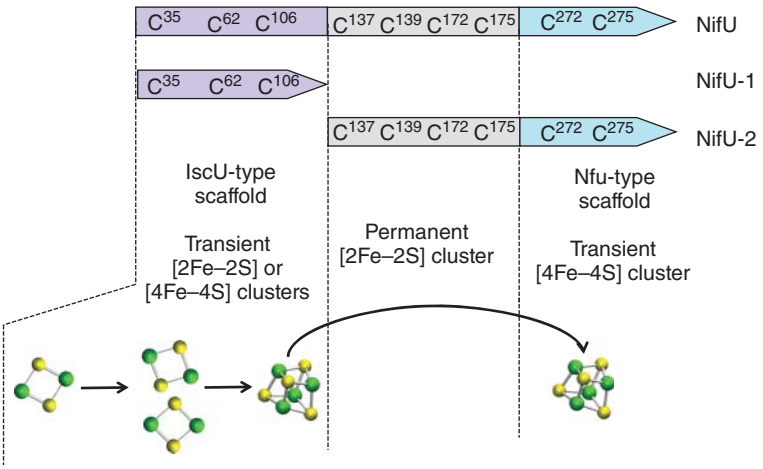

Figure 6 NifU is an [Fe–S] cluster assembly scaffold containing three functional domains. The critical cysteine residues located in each domain are indicated. The N-terminal IscU-type domain (purple) is able to accommodate transient [2Fe–2S] and [Fe–4S] clusters, while the C-terminal Nfu-type (cyan) is able to accommodate transient [4Fe–4S] clusters. The central domain (gray) contains a stable ferredoxin-like [2Fe–2S] cluster. The working model involves the sequential assembly of [2Fe–2S] clusters, followed by reductive coupling to form a [4Fe–4S] cluster within the N-terminal domains and transfer to the C-terminal domain

ferredoxin supporting the suggestion of tetra Cys coordination of a [2Fe–2S] cluster associated within each central domain of the NifU dimer.[40] Given the resistance of these clusters to oxidizing, reducing, or chelating reagents, they were designated as "permanent" clusters. In other words, it appears unlikely that these clusters are transferred to other proteins. Nevertheless, despite their identification over 20 years ago, the exact role of the permanent clusters located within NifU remains unknown. Among the possibilities are involvement as an electron donor for reduction of persulfide species during [Fe–S] cluster formation or involvement in reductive coupling of two [2Fe–2S] clusters to form a [4Fe–4S] cluster.

The involvement of the N- and C-terminal domains in contributing to NifU function, possibly by providing a scaffold for [Fe–S] cluster assembly, was first explored genetically by examining diazotrophic growth defects that occur upon substitution of conserved cysteine residues within NifU.[41] Strains of *A. vinelandii* that contain individual or combinations of alanine substitutions for cysteine residues located within the N-terminal domain all exhibit the same modestly slower growth rate under nitrogen-fixing conditions. In contrast, substitution of alanine for either or both cysteines, located within the C-terminal domain, resulted in no detectable effect on the capacity for nitrogen fixation–dependent growth. However, severe growth impairment occurs when any combination of cysteine residues located at both the N- and C-terminal domains are substituted by alanine.[42] These observations indicated that the N- and C-terminal domains have some redundant functions, although the dominant function is located within the N-terminal domain.

Direct evidence that either the N-terminal or C-terminal domains of NifU can provide sites for [Fe–S] cluster assembly was demonstrated by a series of in vitro [Fe–S] cluster assembly experiments combined with spectroscopic and elemental analyses of assembly products. In initial experiments, it was shown that as-isolated NifU, which contains two permanent [2Fe–2S] clusters, accumulates additional clusters by its simple incubation with NifS, cysteine, and Fe^{2+} under anoxic conditions.[43] Furthermore, [Fe–S] clusters can also be formed under the same conditions on truncated NifU fragments that separately contain either the N- or C-terminal domains (Figure 6). The profile of cluster formation monitored through a time course revealed that the initial assembly of [2Fe–2S] clusters is followed by the formation of [4Fe–4S] clusters.[44] Namely, a careful monitoring of the time course of cluster assembly using Mössbauer spectroscopic analysis indicated an initial accumulation of [2Fe–2S] clusters at the N-terminal domain with subsequent formation of [4Fe–4S] clusters at both the N- and C-terminal domains. A proposed model to explain these results involves the sequential assembly of two transient [2Fe–2S] clusters as initial building blocks for the synthesis of [4Fe–4S] clusters within the N-terminal domain of NifU, followed by transfer of the assembled [4Fe–4S] cluster to the C-terminal domain (Figure 6). Thus, an attractive possibility is that the N-terminal domain provides a primary [Fe–S] cluster assembly site and the C-terminal domain is involved in the delivery of preformed [4Fe–4S] clusters to various client proteins. This model has now been supported by other [Fe–S] cluster assembly systems that supply Fe–S clusters to protein targets not involved in nitrogen fixation (see

Iron–Sulfur Cluster Assembly in Bacteria and Eukarya using the ISC Biosynthesis Machinery).[45–47]

Unlike the permanent [2Fe–2S] cluster located at the central domain of the NifU dimer, clusters assembled in vitro within the N- and C-terminal domains are labile. Complete loss of these clusters occurs upon their reduction with dithionite, exposure to oxygen, or when they are treated with low concentrations of chelating reagents. The inherent instability of these clusters is consistent with the proposed role of NifU as a scaffold for the assembly of transient [Fe–S] clusters destined for insertion into apoforms of [Fe–S] proteins associated with nitrogen fixation.

7 NifU AND NifS AS THE MINIMUM SET FOR THE ASSEMBLY AND TRANSFER OF Fe–S CLUSTERS

Once it was established that labile [Fe–S] clusters can be assembled in vitro by incubating NifU in the presence of NifS, cysteine, and Fe^{2+}, it was important to test whether or not [Fe–S] clusters assembled in this way can be transferred to a known target protein. The nitrogenase system was particularly amenable to such experiments because, upon binding of MgATP, the Fe protein undergoes a conformational change that results in the exposure of its [4Fe–4S] cluster to the solvent (Figure 7). This conformational change is an important part of the nitrogenase catalytic mechanism because it primes the Fe protein for intermolecular electron transfer to the MoFe protein. This aspect of nitrogenase catalysis also enabled the evaluation of the capacity for [Fe–S] cluster transfer from cluster-loaded NifU to a target protein. Solvent exposure of the Fe protein [4Fe–4S] cluster upon MgATP binding renders its [Fe–S] cluster Fe atoms susceptible to chelation by α-α'-dipyridyl.[48,49] Thus, treatment of MgATP-bound Fe protein with α-α'-dipyridyl in the presence of a thiol reducing reagent, such as dithiothreitol, results in the quantitative removal of the [4Fe–4S] cluster associated with active Fe protein.[50] Fe protein treated in this way is completely inactive and is designated "apo-Fe protein" (Figure 7). Such inactive samples are ideal for testing [Fe–S] cluster assembly because reconstitution can be easily evaluated by recovery of Fe protein activity.

Apo-Fe protein can be fully re-activated by its simple incubation with relatively high concentrations of Fe^{++} and S^{2-} under reducing conditions. However, such activation is extremely slow. Apo-Fe protein can also be re-activated by incubation with Fe^{++}, NifS, and cysteine.[50] Although reconstitution by this method is more efficient than purely chemical reconstitution, it remains a relatively slow process. In contrast, mixing of apo-Fe protein and cluster-loaded NifU prepared in vitro yields rapid and complete activation of apo-Fe protein.[42,44] Dissection of the steps involving [Fe–S] cluster formation and activation showed that [4Fe–4S] cluster–containing NifU is necessary and sufficient for the activation of apo-Fe protein (Figure 8). In other words, although NifS is required for the assembly of transient clusters on the NifU scaffold, it is not required for [Fe–S] cluster transfer from cluster-loaded NifU during the activation of apo-Fe protein.

Growth phenotypes and in vitro assembly and reconstitution experiments described earlier provided strong evidence to support the concept that [Fe–S] clusters are preassembled on a molecular scaffold prior to their delivery to an apo-target protein.[42] In the case described earlier, NifU provides the assembly scaffold, and apo-Fe protein is the ultimate delivery target. A necessary in vivo validation of the scaffold hypothesis involved demonstrating that NifU loaded with transient [Fe–S] clusters could also be produced in *A. vinelandii* cells. These experiments were challenging for two reasons. First, because the role of NifU is to deliver transiently bound [Fe–S] clusters to target client proteins, such as apo-Fe protein, there must be an in vivo dynamic equilibrium between cluster-loaded and cluster-unloaded NifU. Second, because transient

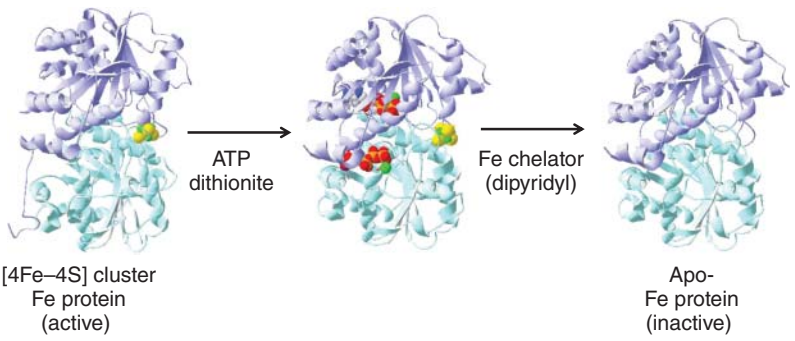

Figure 7 The [4Fe–4S] cluster of Fe protein can be removed by incubation with chelator agents. Upon the binding of ATP, Fe protein undergoes a conformational change that exposes its [4Fe–4S] cluster, which can be removed by dipyridyl to generate the apo form of the enzyme