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[4Fe4S]$^{2+}$ Clusters Exhibit Ground-State Paramagnetism

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Supporting Information

ABSTRACT: Two proteins involved in nitrogen fixation contain ferredoxin-type [4Fe4S] clusters that exist in paramagnetic ground state upon oxidation, a property never observed since the discovery of ferredoxins 50 years ago. This unique characteristic suggests a specific coupling in these clusters necessary for nitrogen fixation and implies an evolutionary connection between the clusters in the two proteins.

FeS clusters have been extensively studied since their first discovery in the early 1960s in biological oxidoreductases.[1,2] The most common and best studied form among them is the [4Fe4S] cluster, which can serve either as an electron-transfer unit or as a structural or catalytic unit. As an electron-transfer unit, the cluster typically exists as either a ferredoxin ([4Fe4S]$^{+/2+}$)- or a HiPIP ([4Fe4S]$^{2+/3+}$)-type redox couple. A property of these clusters that never varies is that the 2+ state always has a diamagnetic (S = 0) ground state. This diamagnetism is associated with two delocalized Fe$^{2.5+}$−Fe$^{2.5+}$ pairs (S = $^9/_2$ each) that are antiferromagnetically coupled, resulting in a net zero spin ground state.[1] Similarly, theoretical analyses also predict S = 0 as the lowest spin state of these clusters.[3] Here, we present the first report of paramagnetic ground states of [4Fe4S]$^{2+}$ clusters in two oxidized Nif proteins of the nitrogen fixation system.

Δ*nifB* NifEN (Figure 1) is an $\alpha_2\beta_2$-tetrameric protein that serves as a scaffold for the biosynthesis of the active FeMo-cofactor (FeMoco) center of Mo-nitrogenase.[4] Metal, electron paramagnetic resonance (EPR), Fe X-ray absorption spectroscopy, magnetic circular dichroism (MCD), and resonance raman (RR) analyses show that this protein contains two identical, yet well-separated, [4Fe4S]-like clusters—one at each α/β-subunit interface—which may mediate the electron transfer for the reductive insertion of Mo and homocitrate into the Fe-only FeMoco precursor.[5,6,10,11] This hypothesis has been verified with the recent determination of the X-ray diffraction structure of NifEN.[12] As expected, upon oxidative titration, the S = $^1/_2$ EPR spectrum of the reduced [4Fe4S]$^{+}$ cluster in Δ*nifB* NifEN disappears at $E_{m7} \approx -350$ mV and n = 1 (Figure 2A), resulting in an EPR-silent oxidized state. The reversibility of this redox change, as well as the RR spectrum[10] of the oxidized cluster, clearly indicates [4Fe4S]$^{2+}$ as the cluster species in the oxidized state. However, MCD spectra recorded before and immediately after such an oxidation reveal that both states exhibit dominant MCD features (Figure 2B).

A variable-temperature study of the oxidized cluster of Δ*nifB* NifEN clearly shows an inverse relationship between the MCD spectral intensity and temperature (Figure 3A). This inverse relationship establishes a paramagnetic ground state of the oxidized [4Fe4S] cluster, contrary to conventional wisdom. The n = 1 oxidation generating this state (see Figure 2A) implies that it is an integer spin system, while the lack of an EPR signal (perpendicular or parallel modes) suggests S = 1 or 2 as the most likely spin states for this species. The spin state of this species is further explored through simulations of the magnetization curves (i.e., plots of MCD spectral intensity versus magnetic field at various temperatures). Although magnetization curves are helpful in determining the spin states of half-integer systems, they are not as exact in assessing the spin states of integer spin systems, where little or no EPR spectral parameters exist and nonzero rhombic interactions ($E/D > 0$) produce further splitting of $\pm m_s$ spin sublevels. Magnetization curves of oxidized Δ*nifB* NifEN are simulated for the series of spin states S = 1, 2, and 3. The closest

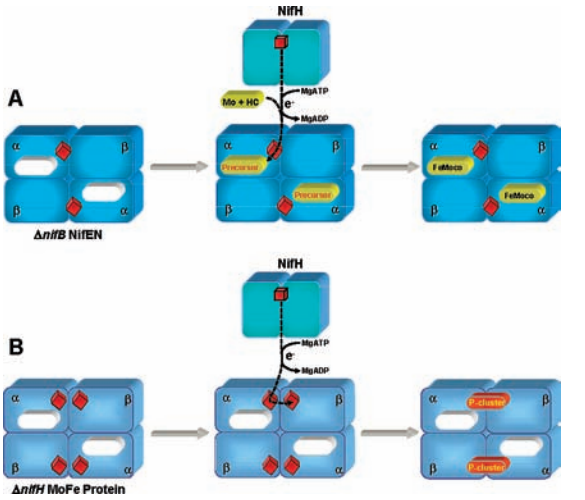

Figure 1. Schematic presentation of the composition and function of Δ*nifB* NifEN (A) and Δ*nifH* MoFe protein (B). These two $\alpha_2\beta_2$-tetrameric proteins are highly homologous in primary sequence and cluster composition.[4] Both proteins are deficient of FeMoco-related cluster species (indicated by empty ovals) as a result of the deletion of *nifB* (A) or *nifH* (B), two genes encoding for proteins that are essential for the biosynthesis of FeMoco.[4] Furthermore, they contain either a single [4Fe4S] cluster (A) or a pair of [4Fe4S]-like clusters (B) at each α/β-subunit interface that likely mediate the electron transfer from Fe protein for the reductive insertion of Mo and homocitrate (abbreviated as HC) into the FeMoco precursor (A)[5,6] or the reductive coupling of paired [4Fe4S] clusters into a mature [8Fe7S] P-cluster (B).[7−9]

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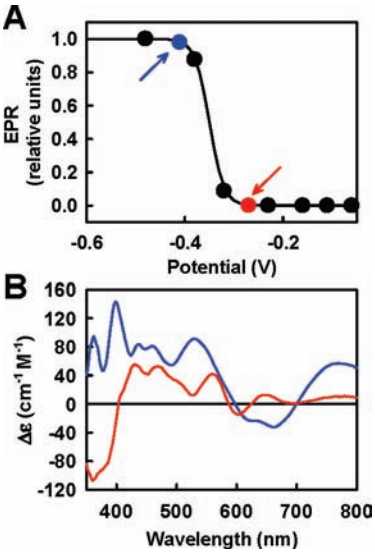

Figure 2. (A) Reversible redox titration of Δ*nifB* NifEN, where a Nernst curve with $n = 1$ and $E_{m7} = -350 \pm 10$ mV is represented by the solid line. Blue and red points represent samples used to collect the MCD spectra in panel B. (B) MCD spectra of Δ*nifB* NifEN recorded at −420 mV (reduced, blue) and −278 mV (oxidized, red).

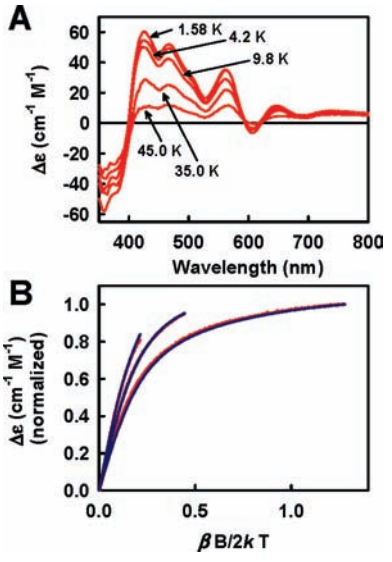

Figure 3. (A) Variable-temperature MCD spectra of oxidized Δ*nifB* NifEN (0.67 mM) showing a decrease in spectral intensity with an increase in temperature, which arises from a paramagnetic ground state of the [4Fe4S] cluster in this protein. (B) Magnetization curves of oxidized Δ*nifB* NifEN (red) and the simulation of data (blue) assuming $S = 2$, $D = 1$ cm^{-1}, and $E/D = 0.05$, with polarizations $M_{xy} = 1.0$, $M_{xz} = 1.0$, and $M_{yz} = 1.0$. Curves were recorded at 1.58 (right), 4.2 (middle), and 9.8 K (left).

fit occurs using the parameters $S = 2$, $D = 1$ cm^{-1} and $E/D = 0.05$, with polarizations $M_{xy} = 1.0$, $M_{xz} = 1.0$, and $M_{yz} = 1.0$ (Figure 3B).

An identical phenomenon of redox change (Figure 1) is observed in the case of Δ*nifH* MoFe protein, which is another Nif protein related to the biosynthesis of Mo-nitrogenase.[4] This $\alpha_2\beta_2$-tetrameric protein is highly homologous to Δ*nifB* NifEN in primary sequence and cluster type, except that it contains four (instead of two) ferredoxin-type, [4Fe4S]-like clusters—one pair at

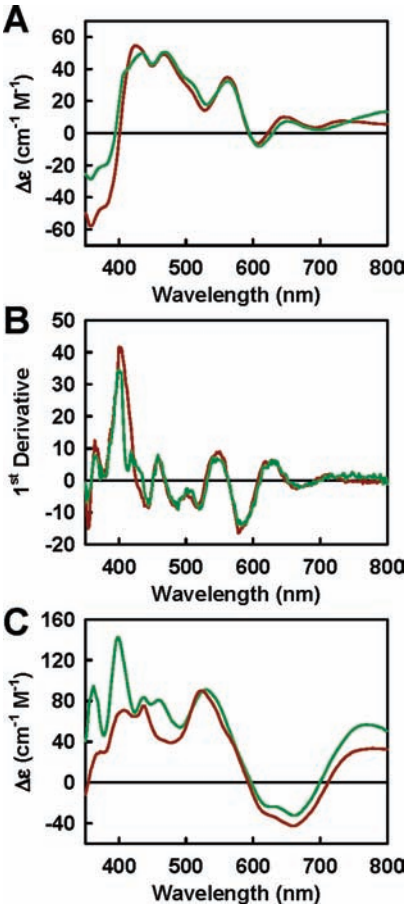

Figure 4. (A) MCD spectra of IDS-oxidized Δ*nifB* NifEN (red) and Δ*nifH* MoFe protein (green, scaled by a factor of 0.5). (B) First-derivative MCD spectra of oxidized Δ*nifB* NifEN (red) and Δ*nifH* MoFe protein (green, scaled by a factor of 0.5), showing nearly identical transitions in both cases. All spectra were recorded at a temperature of 1.6 K and a magnetic field of 6.0 T and standardized per $\alpha\beta$ subunit of protein. (C) MCD spectra of reduced Δ*nifB* NifEN (red) and Δ*nifH* MoFe protein (green, scaled down by a factor of 0.5). All spectra were recorded at a temperature of 1.6 K and a magnetic field of 6.0 T and standardized per $\alpha\beta$ subunit of protein. Broad inflections in the 450−800 nm region are characteristic of classic $[4Fe4S]^{+}$ clusters, and intensities at 540 nm quantify to two and four $[4Fe4S]^{+}$-like clusters per Δ*nifB* NifEN and Δ*nifH* MoFe protein, respectively.

each α/β-subunit interface—that could be reductively coupled into two mature, [8Fe7S] P-clusters.[7,8] Like Δ*nifB* NifEN, the oxidized Δ*nifH* MoFe protein is paramagnetic.[9] Interestingly, its MCD spectrum (Figure 4A) has transitions (Figure 4B) virtually identical to those of the oxidized Δ*nifB* NifEN, but with twice the spectral intensity of the latter. This spectral increase corresponds to the ratio of cluster content between the two proteins (i.e., 4:2), which is also observed in the spectra of the reduced clusters (Figure 4C).[11] Thus, all of the oxidized [4Fe4S] clusters in both proteins exhibit the same paramagnetism. The similarity between the spectra of the two proteins is unexpected and suggests that these transitions may be a general fingerprint of the ground-state paramagnetic $[4Fe4S]^{2+}$ clusters.

The ground-state paramagnetism of the oxidized clusters in Δ*nifB* NifEN and Δ*nifH* MoFe protein implies an unusual coupling among the Fe sites within the cluster, which could originate from a subtle conformational rearrangement of these

[4Fe4S] clusters upon oxidation. Such a coupled structural/redox change may allow the cluster to effectively mediate the electron transfer for the maturation of FeMoco precursor (in the case of Δ*nifB* NifEN), or may prime the clusters in the correct conformation/oxidative state for their subsequent coupling into a mature P-cluster (in the case of Δ*nifH* MoFe protein; Figure 1). In the latter case, it is interesting to note that the mature P-cluster, much like its precursor, can exist both in an all-ferrous diamagnetic state and in (at least) three more oxidized states—all of which are paramagnetic and capable of undergoing structural/redox changes upon interconversion.

The high sequence[4,10] and structural[12] homologies of NifEN and the MoFe protein have been used in the past to suggest an evolutionary link between these proteins. The current finding of similar, unusual ferredoxin-type clusters in both proteins further supports these suggestions. It will be interesting to see whether these unusual cluster properties are confined to only Nif proteins and, as such, are related to specific functions in nitrogenase assembly and/or function.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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