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Direct Assessment of the Reduction Potential of the [4Fe−4S]$^{1+/0}$ Couple of the Fe Protein from *Azotobacter vinelandii*

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The [4Fe−4S] clusters of iron−sulfur proteins are among the most common types of redox active cofactors in proteins, typically functioning as single-electron-transfer agents with potentials in the range from −280 to −715 mV versus NHE.[1,2] The Fe protein of nitrogenase from *Azotobacter vinelandii* is one such protein that has a single [4Fe−4S]$^{2+/1+}$ cluster (Scheme 1) with $E_1{}^{0\prime} = -310$ mV, which becomes more negative in the presence of nucleotides.[3] In 1994, Watt and Reddy reported that the [4Fe−4S]$^{1+}$ state of the Fe protein could be further reduced to an all-ferrous state using reduced methyl viologen, but strangely not by dithionite[4] which is a more powerful reductant.[5] The all-ferrous [4Fe−4S] state in a protein was unprecedented at the time, and the reported potential of −460 mV was dramatically positive to those found for the [4Fe−4S]$^{1+/0}$ transition in comparable model compounds.[6,7]

Scheme 1

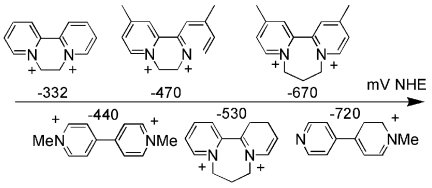
$$[Fe_4S_4]^{2+} \overset{E_1{}^0}{\rightleftharpoons} [Fe_4S_4]^{1+} \overset{E_2{}^0}{\rightleftharpoons} [Fe_4S_4]^{0}$$

Most mechanistic studies of nitrogenase have been done using dithionite as an electron donor, where the Fe protein is believed to cycle between the [4Fe−4S]$^{2+/1+}$ cluster oxidation states.[8,9] The availability of an all-ferrous state implies that the Fe protein may act as a two-electron donor in vivo, which is appealing on several counts.[8] It suggests a rationale for the unusual coupling of Fe_4S_4 cubanes in the P-cluster of nitrogenase, that is, to accept two electrons at a time, and likewise that the homodimeric structure of the Fe protein may allow two donor molecules to be bound simultaneously. That all known substrates of nitrogenase (e.g., acetelyne, ethylene, nitrogen) are reduced by multiples of two electrons also fits well with the idea of a two-electron donor.

Burgess and co-workers have characterized the all-ferrous [4Fe−4S]^0 state of the Fe protein by spectroscopic (Mössbauer, UV−vis, CD, MCD, EPR, and XAS) and structural means.[10−16] In contrast to initial reports, singly reduced methyl viologen was found to be incapable of reducing the Fe protein below the [4Fe−4S]$^{1+}$ state, and the reported $E_2{}^{0\prime}$ potential was called into question. In this communication, we measure the thermodynamic potential for the formation of the all-ferrous state Fe protein by several methods.

A chemical measure of the reduction potential required to form the all-ferrous state can be determined by reaction with electron donors of known potential. Reactions of the [4Fe−4S]$^{1+}$ state of the Fe protein with Cr(II)EDTA, Ti(III)-citrate,[17] or 5′-deazariboflavin/EDTA/light result in formation of the all-ferrous state, as evidenced by the unique $g = 16$ signal observable by the parallel mode EPR (Figure S1, CD also given). Of these methods, only Cr(II)EDTA has a chemically reversible reduction with a well-defined potential of −1000 mV/NHE at pH 8.[18,19] In contrast, neither addition of a multifold excess of reduced forms of alkylated bipyridinium salts ($E^{0\prime}$ range from −332 to −720 mV/NHE, Scheme 2)[20] or addition of the hydroquinone form of flavodoxin II results

Scheme 2



in observable reduction of the [4Fe−4S]$^{+}$ state of the Fe protein (Table S4). For example, no change is seen in the perpendicular or parallel mode EPR spectra of the [4Fe−4S]$^{1+}$ state upon addition of reduced *N*-methyl-bipyridinium radical ($E^{0\prime} = -720$ mV/NHE, pH 8) (S2).[21]

Conversely, a chemical measure of the reducing ability of the all-ferrous state is obtained by its reaction with electron acceptors. In these experiments, the all-ferrous Fe protein was formed by reaction with Cr(II)−EDTA, and the residual Cr was removed by anaerobic gel filtration. The absence of Cr in the resulting protein samples was confirmed by ICP-AES.[22] When added in a 1:1 ratio, the [4Fe−4S]^0 state of the Fe protein stoichiometrically reduces methyl viologen (−440 mV, S3); it also reduces *N*-methyl-bipyridinium (−720 mV), forming the oxidized state of the Fe protein, shown in Figure 1. Similar results were obtained for all of the bipyridinium salts shown in Scheme 2 and the semiquinone form of flavodoxin II (−500 mV). Taken together, these data demonstrate that the $E_2{}^{0\prime}$ for the Fe protein is far more negative than previously reported.

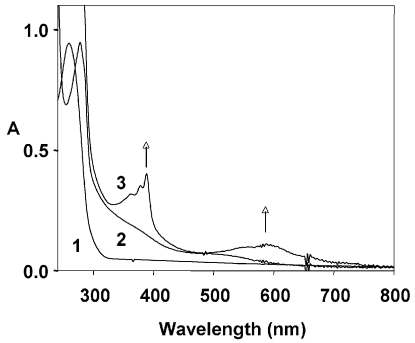


Figure 1. Anaerobic UV−vis spectra of: **1**, the oxidized form of *N*-methyl-4,4-bipyridinium (0.12 mM, colorless); **2**, all-ferrous Fe protein (~0.05 mM, pink); **3**, mixing of solutions **1** and **2** yielded a blue solution. All spectra are in 50 mM Tris-Cl, pH 8 with 0.1 M NaCl. The peaks near 390 and 600 nm are indicative of reduced *N*-methyl-4,4-bipyridinium.

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To directly assess the $E_2^{0'}$ couple of the Fe protein, controlled-potential electrolysis titrations were conducted in an anaerobic glovebox using an excess of Cr(III)−EDTA as mediator. As shown in Figure 2, there is a significant absorbance difference between

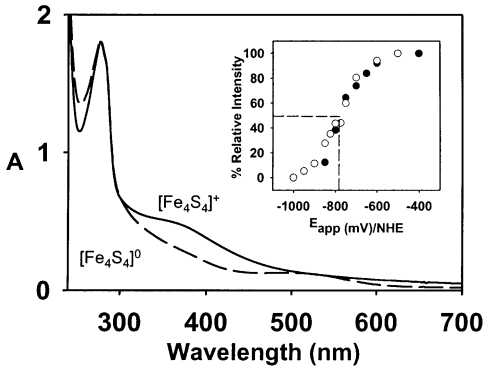

Figure 2. Uv−vis spectra of the $[4Fe-4S]^{1+}$ and $[4Fe-4S]^{0}$ states of the Fe protein (0.11 mM in 50 mM Tris-Cl, 0.1 M NaCl, pH 8). Inset, percentage of the $[4Fe-4S]^{1+}$ state at applied potentials (NHE) during controlled potential electrolysis of 0.05 mM Fe protein using 0.5 mM Cr(III)−EDTA as a mediator in 0.1 M Tris-Cl, 0.1 M NaCl, pH 8.

the $[4Fe-4S]^{1+}$ and $[4Fe-4S]^{0}$ states of the Fe protein in the region from 300 to 500 nm. During electrolysis, changes in the redox state of the Fe protein were monitored at 366 nm, an isosbestic point of absorbance for Cr(II)−EDTA and Cr(III)−EDTA. The resulting titration plots, as shown in the inset, illustrate the conversion from the oxidized to reduced form at the highly negative potential of ca. −790 mV/NHE, a value consistent with the chemical reactivity experiments and with a recent theoretical calculation of this couple.[23] The difference between the two reduction couples ($E_1^{0'} - E_2^{0'}$), ca. 480 mV, is very close to the 490 mV difference seen for the two couples of the model complex $Fe_4S_4(SCH_2CH_2CO_2)_4^{6-}$ in aqueous solution.[7] Significantly larger differences, >650 mV, are observed for model Fe_4S_4 compounds in organic solution,[6] presumably due to the lack of H-bonding stabilization of the reduced form.

In *A. vinelandii*, several proteins such as flavodoxin II (−500 mV), ferredoxin I (−650 mV),[24] and ferredoxin III (−644 mV)[25] have been suggested as possible electron donors to the Fe protein, all of which appear incapable of reducing the Fe protein to the all-ferrous state. However, chemical reactions in complex intracellular systems may differ in significant ways from those in the bulk solution. In particular, structural and environmental interactions may dramatically alter the thermodynamic properties of a redox sites. In this regard, recent theoretical[26] and experimental[27] studies have demonstrated that the redox potentials of both the Fe and the MoFe proteins change upon complexation and upon MgATP binding to the complex. The observed effect of both protein/protein and protein/nucleotide binding is to increase the driving force of the transferred electron, making the complexed form of the Fe protein more difficult to reduce. Thus, pending an alternative mechanism driving reduction, it appears unlikely that the all-ferrous protein can be formed in vivo.

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Supporting Information Available: EPR and CD spectra for the all-ferrous Fe protein, perpendicular and parallel mode EPR spectra of the Fe protein taken before and after the addition of an excess of reduced *N*-methyl bipyridinium, a table of reduction potentials for redox reagents, reactions of the all-ferrous Fe protein and Ti(III)−citrate with bipyridinium salts, spectral changes during the mediated reduction of the Fe protein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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