The iron sulfur (FeS) proteins play crucial roles in biological processes that range from iron homeostasis and gene regulation to electron transfer and enzyme catalysis. A member of the FeS protein family, the iron (Fe) protein is the reductase component of nitrogenase, a key enzyme in the global nitrogen cycle that catalyzes the ambient reduction of the atmospheric N₂ to the bioavailable NH₃.Encoded by nifH and vnfH, respectively, the Fe proteins of Mo- and V-nitrogenases (designated NiFH and VnFH) are structurally homologous homodimers that house a surface-exposed [Fe₄S₄] cluster at the subunit interface and an MgATP-binding site within each subunit. During substrate turnover, NiFH or VnFH forms a functional complex with its catalytic partner, NiFDK or VnFDKG, which allows electrons to be transferred concomitantly with ATP hydrolysis from the [Fe₄S₄] cluster of the former, via a so-called P- or P*-cluster, to the M- or V-cluster (generally termed the cofactor) of the latter, where substrate reduction takes place (Figure S1a). Other than serving as an obligate electron donor for its catalytic partner in the complete nitrogenase enzyme system, the Fe protein can act as a reductase on its own and catalyze the ambient reduction of CO₂ and CO (Figure S1b). The reactivity of Fe protein toward C₁ substrates was first observed in Azotobacter vinelandii, a soil bacterium, where both NifH and VnH proteins of this microorganism were shown to reduce CO₂ to CO under in vitro conditions. Subsequently, the NifH protein of a methanogenic organism, Methanosarcina acetivorans, was demonstrated to reduce CO₂ and CO to hydrocarbons under in vitro conditions. These observations have established the Fe protein as a simple FeS enzyme that is capable of generating hydrocarbons via reactions that resemble the Fischer–Tropsch (FT) process that is used for the industrial production of carbon fuels; however, unlike the FT process, the reactions catalyzed by the Fe protein utilize protons/electrons (instead of H₂) as the reducing equivalents. The Fe protein of nitrogenase reduces two C₁ substrates, CO₂ and CO, under ambient conditions when its [Fe₄S₄] cluster adopts the all-ferrous [Fe₄S₄]0 state. Here, we show disparate reactivities of the nifH- and vnfH-encoded Fe proteins from Methanosarcina acetivorans (designated MaNiFH and MaVnFH) toward C₁ substrates in the all-ferrous state, with the former capable of reducing both CO₂ and CO to hydrocarbons, and the latter only capable of reducing CO to hydrocarbons at substantially reduced yields. EPR experiments conducted at varying solution potentials reveal that MaVnFH adopts the all-ferrous state at a more positive reduction potential than MaNiFH, which could account for the weaker reactivity of the MaVnFH toward C₁ substrates than MaNiFH. More importantly, MaVnFH already displays the g = 16.4 parallel-mode EPR signal that is characteristic of the all-ferrous [Fe₄S₄]0 cluster at a reduction potential of −0.44 V, and the signal reaches 50% maximum intensity at a reduction potential of −0.59 V, suggesting the possibility of this Fe protein to access the all-ferrous [Fe₄S₄]0 state under physiological conditions. These results bear significant relevance to the long-lasting debate of whether the Fe protein can utilize the [Fe₄S₄]0/2+ redox couple to support a two-electron transfer during substrate turnover which, therefore, is crucial for expanding our knowledge of the reaction mechanism of nitrogenase and the cellular energetics of nitrogenase-based processes.

**KEYWORDS:** nitrogenase, Fe protein, [Fe₄S₄] cluster, all-ferrous state, physiological reduction potential, CO₂ reduction, hydrocarbon formation, methanogen
reducing power, and they occur at ambient temperature and pressure.

The ability of the Fe protein to serve as a reductase relies on the redox versatility of its [Fe₄S₄] cluster, which can reversibly adopt at least three oxidation states: the super-reduced, all-ferrous state ([Fe₄S₄]₀); the reduced state ([Fe₄S₄]¹⁺); and the oxidized state ([Fe₄S₄]²⁺).¹,¹³ The ability of the Fe protein to adopt the all-ferrous state makes it unique among the [Fe₄S₄]-cluster-containing proteins that are usually confined to two oxidation states other than the super-reduced state,¹⁴ although it is unclear whether the all-ferrous state can be reached by the Fe protein under physiological conditions. Nevertheless, while it is generally believed that the [Fe₄S₄]¹⁺/²⁺ redox couple is used by the Fe protein for a one-electron transfer to its catalytic partner during nitrogenase catalysis, it has been suggested that the Fe protein could also use the [Fe₄S₄]₀/²⁺ couple for a two-electron transfer in this process. Likewise, the reduction of CO₂ and/or CO by the Fe protein on its own is best achieved in the presence of europium(II) diethylenetriaminepentaacetic acid [Eu(II)-DTPA] (E₁/² = −1.14 V at pH 8),¹⁵ a strong reductant that renders the [Fe₄S₄] cluster of the Fe protein in the "super-reduced", all-ferrous [Fe₄S₄]₀ state. These observations have led to the question of whether the all-ferrous state of the Fe protein can be accessed at physiological reduction potentials achieved by the in vivo electron donors to this protein, such as ferredoxins and flavodoxins, to enable substrate reduction in the cell.

To assess the all-ferrous state of the Fe protein, we first examined the utility of two Eu(II) chelates, europium(II)
1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane [Eu(II)-DOTAM] and europium(II) 1,4,7,10-tetraazaclododecane-1,4,7,10-tetraacetic acid [Eu(II)-DOTA], as potential reductants to probe the response of the all-ferrous state to varying solution potentials. The reduction potentials of Eu(II)-DOTAM and Eu(II)-DOTA were determined by cyclic voltammetry measurements of in situ generated complexes in 25 mM Tris-HCl buffer at pH 8.0. The resultant voltammograms (Figure 1) show the reversible Eu(III)/Eu(II) couples at $E_{1/2} = -0.59$ V (for Eu(II)-DOTAM) and $-0.92$ V (for Eu(II)-DOTA) vs standard hydrogen electrode (SHE). These values are directly comparable with the reported potentials of Eu(II)-EGTA ($-0.88$ V vs SHE) and Eu(II)-DTPA ($-1.14$ V vs SHE) measured at pH 8.0. The fact that the potentials of Eu(II)-DOTAM and Eu(II)-DOTA are intermediate between those of dithionite (e.g., $-0.47$ V vs SHE at 2 mM, pH 8.0) and Eu(II)-DTPA makes them suitable candidates, together with the latter two reductants, for titrating the all-ferrous-specific EPR signal of the Fe protein versus solution potentials.

With proper reductants identified for the titration experiment, we treated the NiH and VnH proteins from M. aceticivorans (designated MaNiH and MaVnH) with 20 mM dithionite ($E_{1/2} = -0.44$ V at pH 8.0), 2 mM dithionite ($E_{1/2} = -0.47$ V at pH 8.0), 10 mM Eu(II)-DOTAM ($E_{1/2} = -0.59$ V at pH 8.0), 10 mM Eu(II)-DOTA ($E_{1/2} = -0.92$ V at pH 8.0), and 10 mM Eu(II)-DTPA ($E_{1/2} = -1.14$ V at pH 8.0) and monitored the appearance of the all-ferrous state specific, $g = 16.4$ parallel-mode EPR signal at the various reduction potentials generated by these reductants. Interestingly, despite sharing as high as 80% sequence homology, MaNiH and MaVnH display distinct patterns of changes in the magnitudes of their all-ferrous-specific EPR signals upon titration with the same set of reductants (Figure 2). In the case of MaNiH, the $g = 16.4$ signal is hardly visible (1.1% of max. intensity) at $-0.44$ V, and it only becomes apparent (14.8% of max. intensity) at $-0.59$ V (Figure 2a,b). In contrast, MaVnH already displays a small, yet visible $g = 16.4$ signal (4.7% of max. intensity) at $-0.44$ V, and the signal (48% of max. intensity) is substantially stronger than that displayed by MaNiH (14.8% of max. intensity) at $-0.59$ V (Figure 2c,d). The appearance of the $g = 16.4$ signal in the spectrum of the dithionite-treated MaVnH is surprising, as all Fe proteins characterized so far exist in the reduced [Fe4S4]1+ state in the presence of dithionite and do not show the all-ferrous signal unless a lower potential is reached in the presence of a stronger reductant. For example, the [Fe4S4]0/1+ couple of the NiH protein from A. vinelandii was determined to have a midpoint potential of $-0.79$ V. In comparison, MaVnH already reaches >50% of the maximum intensity of the $g = 16.4$ signal at $-0.59$ V (Figure 2d, arrow), and it has a hue consistent with that right before conversion into the characteristic reddish-pink color of the all-ferrous state at this potential (Figure 3). As such, it is likely that the cluster of this Fe protein can access the all-ferrous [Fe4S4]0 state under physiological conditions, where

Figure 4. Reduction of CO2 and CO by the all-ferrous MaVnH and MaNiH proteins. (a) Yields of CO (left) and hydrocarbons (right) by MaVnH (blue) and MaNiH (black) from CO2 reduction at increasing Eu(II)-DTPA concentrations. Yields were calculated based on nmol of reduced C in CO or hydrocarbons per nmol protein. HC, hydrocarbons. (b) Identities and (c) distributions of hydrocarbons formed by MaNiH (upper) and MaVnH (lower) from CO reduction at 100 mM Eu(II)-DTPA. TON, turnover number, was calculated based on the total nmol of reduced C in hydrocarbons generated per nmol of protein. (d) GC-MS analysis of the hydrocarbon products generated from the reduction of 12CO (1) or 13CO (3) by MaVnH, shown in comparison with the fragmentation patterns of the corresponding 12C-containing (2) or 13C-labeled (4) hydrocarbon standards.
such a potential can be accomplished by certain ferredoxins as the electron donors to the Fe protein in the cell.\textsuperscript{6,21–23}

The fact that MaVnH adopts the all-ferrous state at a more positive reduction potential than MaNiH points to a weaker ability of MaVnH than MaNiH to donate lower-potential electrons to the C1 substrates in the all-ferrous state and, consequently, a weaker ability of the all-ferrous MaVnH than its MaNiH counterpart to reduce these substrates. Indeed, we observed disparate reactivities of MaNiH and MaVnH toward CO and CO\textsubscript{2} when these assays were conducted in the presence of Eu(II)-DTPA. In the case of MaNiH, both CO and hydrocarbons can be detected as products of CO\textsubscript{2} reduction, and the formation of CO (Figure 4a, left, black circles) decreases concomitantly with an increase in the formation of hydrocarbons (Figure 4a, right, black triangles) with increasing concentrations of Eu(II)-DTPA. The maximum yields of CO (4.55 ± 0.19 nmol/nmol protein) and hydrocarbons (3.91 ± 0.32 nmol/nmol protein) are accomplished by MaNiH at 20 mM and 100 mM Eu(II)-DTPA, respectively. Contrary to MaNiH, MaVnH shows no formation of CO (Figure 4a, left, blue circles) or hydrocarbons (Figure 4a, right, blue triangles) from CO\textsubscript{2} reduction under the same reaction conditions. However, like MaNiH, MaVnH can generate hydrocarbons from CO reduction when Eu(II)-DTPA is supplied at 100 mM (Figure 4b–d). The identities of the hydrocarbon products generated by MaVnH (Figure 4b, lower), as confirmed by gas chromatograph–mass spectrometry (GC-MS) (Figure 4d), are the same as those generated by MaNiH (Figure 4b, upper), which include C1–C4 alkanes and alkenes. However, the turnover number (TON) of MaNiH (Figure 4c, lower), which is calculated based on the number of reduced carbons in products, is only 11\% compared to that of MaNiH (Figure 4c, upper).

The observation of differential reactivities of the all-ferrous MaVnH and MaNiH proteins is important, as it highlights the crucial role of protein scaffolds in modulating the redox properties and catalytic capabilities of the active-site [Fe\textsubscript{4}S\textsubscript{4}]\textsuperscript{0} centers of these proteins. More importantly, the fact that MaVnH can adopt the all-ferrous state at a reduction potential that is achievable under physiological conditions\textsuperscript{6,21–23} suggests the possibility that more Fe proteins may achieve and utilize this state for various cellular functions at similar or perhaps even more positive reduction potentials. This finding bears significant relevance to the long-standing debate in the field as to whether the Fe protein can only shuttle between the [Fe\textsubscript{4}S\textsubscript{4}]\textsuperscript{12+} and [Fe\textsubscript{4}S\textsubscript{4}]\textsuperscript{12+} states to support a one-electron transfer during catalysis or if it can also shuttle between the [Fe\textsubscript{4}S\textsubscript{4}]\textsuperscript{12−} and [Fe\textsubscript{4}S\textsubscript{4}]\textsuperscript{12−} state to enable a two-electron electron transfer under physiological conditions.\textsuperscript{24–26} The latter scenario is particularly important for nitrogenase catalysis, as it would cut the ATP consumption for electron transfer by half and thereby improve the energy economy of nitrogen fixation by 2-fold in the cell. The observation reported herein provides a useful platform for further investigation into the redox properties of the Fe protein, which is crucial for expanding our understanding of the reaction mechanism of the nitrogenase enzyme and the cellular energetics of the nitrogen-fixing microorganisms.
REFERENCES

(1) Burgess, B. K.; Lowe, D. J. Mechanism of molybdenum nitrogenase. Chem. Rev. 1996, 96, 2983–3012.
(2) Schilte, D.; Camara, J. M.; Huynh, M. T.; Hammes-Schiffer, S.; Rauchfuss, T. B. Hydrogenase enzymes and their synthetic models: the role of metal hydrides. Chem. Rev. 2016, 116, 8693–8749.
(3) Mühlenhoff, U.; Hoffmann, B.; Richter, N.; Rietzschel, N.; Spannagel, F.; Stehling, O.; Uzarska, M. A.; Lill, R. Compartmentalization of iron between mitochondria and the cytosol and its regulation. Eur. J. Cell Biol. 2015, 94, 292–308.
(4) O’Brien, E. M.; Holt, M. E.; Thompson, M. K.; Salay, L. E.; Ehlinger, A. C.; Chazin, W. J.; Barton, J. K. The [4Fe-4S] cluster of human DNA primase functions as a redox switch using DNA charge transport. Science 2017, 355, No. eaag1789.
(5) Mettler, E. L.; Kiley, P. J. Fe-S proteins that regulate gene expression. Biochim. Biophys. Acta, Mol. Cell Res. 2015, 1853, 1284–1293.
(6) Rutledge, H. L.; Tezcan, F. A. Electron transfer in nitrogenase. Chem. Rev. 2020, 120, 5158–5193.
(7) Jasieniuk, A. J.; Lee, C. C.; Ribbe, M. W.; Hu, Y. Reactivity, mechanism, and assembly of the alternative nitrogenases. Chem. Rev. 2020, 120, 5107–5157.
(8) Buscaglia, T. M.; Rees, D. C. Rethinking the nitrogenase mechanism: activating the active site. Joule 2019, 3, 2662–2678.
(9) Lee, C. C.; Stiebritz, M. T.; Hu, Y. Reactivity of [Fe4S4] clusters toward C1 substrates: mechanism, implications, and potential applications. Acc. Chem. Res. 2019, 52, 1168–1176.
(10) Rebelein, J. G.; Stiebritz, M. T.; Lee, C. C.; Hu, Y. Activation and reduction of carbon dioxide by nitrogenase iron proteins. Nat. Chem. Biol. 2017, 13, 147–149.
(11) Stiebritz, M. T.; Hiller, C. J.; Sickerman, N. S.; Lee, C. C.; Tanifuji, K.; Ohki, Y.; Hu, Y. Ambient conversion of CO2 to hydrocarbons by biogenic and synthetic [Fe4S4] clusters. Nat. Catal. 2018, 1, 444–451.
(12) Rofer-DePoorter, C. K. A comprehensive mechanism for the Fischer–Tropsch synthesis. Chem. Rev. 1981, 81, 447–474.
(13) Angove, H. C.; Yoo, S. J.; Münck, E.; Burgess, B. K. An all-ferrous state of the Fe protein of nitrogenase. Interaction with nucleotides and electron transfer to the MoFe protein. J. Biol. Chem. 1998, 273, 26330–26337.
(14) Leggate, E. J.; Bill, E.; Essigke, T.; Ullmann, G. M.; Hirst, J. Formation and characterization of an all-ferrous Rieske cluster and stabilization of the [2Fe-2S]0 core by protonation. Proc. Natl. Acad. Sci. U. S. A. 2004, 101, 10913–10918.
(15) Vincent, K. A.; Tilley, G. J.; Quamme, N. C.; Streeter, I.; Burgess, B. K.; Cheesman, M. R.; Armstrong, F. A. Instantaneous, stoichiometric generation of powerfully reducing states of protein active sites using Eu(II) and polyaminocarboxylate ligands. Chem. Commun. (Cambridge, U. K.) 2003, 20, 2590–2591.
(16) Mayhew, S. G. The redox potential of dithionite and SO2 from equilibrium reactions with flavodoxin, methyl violoate and hydrogen plus hydrogenase. Eur. J. Biochem. 1978, 85, 535–547.
(17) Yoshizawa, J. M.; Blank, M. A.; Fay, A. W.; Lee, C. C.; Wiig, J. A.; Hu, Y.; Hodgson, K. O.; Hedman, B.; Ribbe, M. W. Optimization of FeMoco maturation on NifEN. J. Am. Chem. Soc. 2009, 131, 9321–9325.
(18) Guo, M.; Sulc, F.; Ribbe, M. W.; Farmer, P. J.; Burgess, B. K. Direct assessment of the reduction potential of the [4Fe-4S]1+/0 couple of the Fe protein from Azotobacter vinelandii. J. Am. Chem. Soc. 2002, 124, 12100–12101.