Iron sulfur (FeS) proteins are integral players in a vast array of biological activities from redox chemistry in respiration and photosynthesis to the regulation of transcription and gene expression. They perform these many diverse functions using only a small set of different FeS moieties, and their functions are largely derived from the protein environment around the FeS centers. In particular, tuning the redox potential \( E_M \) of the FeS center is critical for controlling the conditions under which the protein responds to its environment and its interacting partners. As a class, FeS proteins span a wide redox range \(^1\) under normal cellular conditions, indicating that FeS centers are fundamentally tunable. This broad redox range is achieved using several different protein scaffolds. Progress has recently been made in tuning the \( E_M \) over a large range within the same protein scaffold of two non-FeS proteins, a cupredoxin \(^2\) and a superoxide dismutase. \(^3\) The upper \( E_M \) limit of both of these systems is close to +1 V, which indicates that these proteins could be useful catalysts in key chemical redox processes such as water oxidation. \(^1,2\) However, neither system could access the solution potentials in cellular environments (−300 mV to +200 mV). \(^4\) Because different FeS proteins naturally span this range, \(^1\) we rationalized that the \( E_M \) of a single FeS protein could be engineered to span both above and below the cellular solution range. Such a class of proteins could serve as reporters of cellular redox or be used to perturb the normal redox potential of the cellular solution. Here, we report our success in tuning the \( E_M \) of the [2Fe-2S] center of the outer mitochondrial membrane protein mitoNEET over a range of 700 mV, which is the largest \( E_M \) range engineered in an FeS protein and, importantly, spans the cellular redox range (+200 to −300 mV). These properties make mitoNEET potentially useful for both physiological studies and industrial applications as a stable, water-soluble, redox agent.

MitoNEET is a newly discovered mitochondrial target of the TZD class of antidiabetes drugs, such as pioglitazone (Actos) and rosiglitazone (Avandia). \(^5\) Human mitoNEET defines a unique class of [2Fe-2S] proteins. The crystal structure shows that mitoNEET is a homodimer, with each protomer binding a [2Fe-2S] center (shown as spheres) coordinated by 3-Cys and 1-His, with the hallmark single-coordinating His87 indicated. The distance between the [2Fe-2S] centers is ~16 Å from center-to-center. His87 coordinates to the outer, more solvent exposed, Fe of the [2Fe-2S] center, where the electron is predominantly localized upon reduction.

**Abstract:** MitoNEET is a newly discovered mitochondrial protein and a target of the TZD class of antidiabetes drugs. MitoNEET is homodimeric with each protomer binding a [2Fe-2S] center through a rare 3-Cys and 1-His coordination geometry. Both the fold and the coordination of the [2Fe-2S] centers suggest that it could have novel properties compared to other known [2Fe-2S] proteins. We tested the robustness of mitoNEET to mutation and the range over which the redox potential \( E_M \) could be tuned. We found that the protein could tolerate an array of mutations that modified the \( E_M \) of the [2Fe-2S] center over a range of ~700 mV, which is the largest \( E_M \) range engineered in an FeS protein and, importantly, spans the cellular redox range (+200 to −300 mV). These properties make mitoNEET potentially useful for both physiological studies and industrial applications as a stable, water-soluble, redox agent.
As a first step, we needed to improve our characterization of the WT. We had previously shown that the $E_M$ of WT mitoNEET is pH-dependent above neutral pH, indicating that reduction is coupled to proton uptake. Potentiometric redox titrations indicated that the $[2\text{Fe-2S}]$ cluster of mitoNEET undergoes a one-electron reduction step: $[2\text{Fe-2S}]^{2-}/[2\text{Fe-2S}]^{+}$:H (Figure S1). At a pH just above its pK$_a$ in the oxidized state (pK$_{oxid}$), uptake of a proton occurs upon reduction, resulting in a pH-dependence for the $E_M$.

This occurs up to the point where the pH matches the pK$_a$ of the proton in the reduced state (pK$_{red}$). The most likely candidate for a site of protonation in the WT mitoNEET is His87 (Figure 3A), which shows pH-dependent vibrational interactions with the $[2\text{Fe-2S}]$ center. His87 is ligated to the outermost Fe of the $[2\text{Fe-2S}]$ center, where, upon reduction, the additional electron is predominantly localized. In addition, pulsed EPR studies indicated that His87 is protonated in the reduced state. At the appropriate pH, protonation of His upon reduction is observed in redox studies on Rieske type $[2\text{Fe-2S}]$ centers that involve His coordination.

As a consequence of protonation, the pH range over which protonation of His87 contributes to the observed titration across the entire measured range.

We continued our mutant survey by replacing the single ligating His with Cys (H87C) in the H87C mutant. This mutant shows a more shallow slope ($-15 \text{ mV/pH}$) for the pH-dependence, indicating that His87 is principally responsible for the observed proton coupling in WT. Replacement of Lys55 with Glu (K55E) shifts the pK$_{red}$ of His87 from 6.7 ± 0.2 to 9.2 ± 0.2.

In the refined model, we explicitly include His87 as a major contributor to the observed titration across the entire measured range and implicitly include other groups and pH-dependent effects in a factor $\alpha$ (eq S5). Fitting of the data to the model (Figure 3B) yields values of pK$_{oxid}$ = 6.7 ± 0.2 and pK$_{red}$ > 11.5 for His87, which is in agreement with the values determined from UV–vis pH titration, giving us confidence that the refined model provides an adequate explanation of the measured data.
center, with no observed long-range structural changes. The shift to a more negative $E_{M7}$ can thus be attributed to the substitution of the neutral His with an anionic Cys ligand at the 87 site. This finding was consistent with previous reports and demonstrates that His87 is a critical ligand in tuning the $E_M$.

Having established that His87 is a critical ligand in tuning the $E_M$, we next targeted Lys55, which is located ~4 Å from His87 (Figure 3A). Based on its close proximity to His87, along with the observed changes in the side chain conformation of Lys55 in the H87C mutant, we anticipated that replacing the cationic Lys55 with neutral or negative side chains would increase the $pK_a$ of His87 and thereby the $E_{M7}$ of the mutant protein. Replacement of Lys55 with the neutral Gln (K55Q) or anionic Glu (K55E) both resulted in $E_{M7}$ values of ~ +200 mV (Figure 2). The similar $E_{M7}$ values for the K55E and K55Q mutants indicate that their predominant effect on $E_{M7}$ is caused by removal of the cationic Lys. A fit of the data to the model (eq S5) yielded a $pK_a$ of His87 in the K55E that was shifted by ~3 $pK_a$ units to 9.2 ± 0.2 (Figure 3B). This was confirmed by optical pH titrations, which gave a value of 9.6 ± 0.2 (Figure S2). In the WT, Lys55 makes an interprotomer double mutant showed that the effects of the two mutations on the $E_{M7}$ values is beyond that of any previously reported FeS protein.

Another titratable side chain located near the [2Fe-2S] center is Asp84 (Figure 3A). Asp84 is within hydrogen bonding distance to the [2Fe-2S] center, with no observed long-range structural changes. This system’s resultant range of $E_{M7}$ values in other FeS systems, such as isobacteriochlorophyll $pK_a$ of the [2Fe-2S] center can be tuned to essentially any value within a range from ~360 mV to +305 mV, making it potentially useful for both physiological and industrial applications as a stable, water-soluble, redox agent. For use of his electrochemistry equipment and expertise. We also thank Charlene Chang and Alex Navarro for their assistance with mutagenesis. This work was supported by the Heme and Blood Proteins Training Grant GT32DK007233-34 (J.A.); the GAANN training grant (2005–2006) and the CMG Grant 2T32GM007240-29 (A.R.); NIH Grants GM41637 (M.L.P.), NIH GM54038 and NIH DK54441 (to P.A.J.); and the Zevi Herrmann Shapira Foundation (R.N.).

Supporting Information Available: We include a description of materials and methods. This includes details of $pK_a$ values determined by UV–vis spectral changes as a function of pH and fits of the $E_M$ vs pH. We also include optical redox titration data for all mutants in the main text as well as others not in the main text (Figure S1). We include direct UV–vis titrations at different pH for WT and K55E (Figure S2A, S2B) and UV–vis absorbance spectra of WT and the D84G (Figure S3). Cyclic voltammograms for several mutants are shown (Figure S4A–F) and the values tabulated with the potentiometric data (Table S1). A comparison of the pH-dependence profiles of WT by PFV and potentiometric titrations is included (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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