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Development of a Rubredoxin-Type Center Embedded in a *de Novo*-Designed Three-Helix Bundle

Alison G. Tebo[†], Tyler B. J. Pinter[‡], Ricardo García-Serres[§], Amy L. Speelman[‡], Cédric Tard^{||}, Olivier Sénéque[§], Geneviève Blondin[§], Jean-Marc Latour[§], James Penner-Hahn[‡], Nicolai Lehnert[‡], and Vincent L. Pecoraro^{†,‡,*}

[†]Program in Chemical Biology, University of Michigan, Ann Arbor, Michigan 48109, United States

[‡]Department of Chemistry and Biophysics, University of Michigan, Ann Arbor, Michigan 48109, United States

[§]Université Grenoble Alpes, CNRS, CEA, BIG, LCBM (UMR 5249), F-38054 Grenoble, France

^{||}LCM, CNRS, École Polytechnique, Université Paris-Saclay, 91128 Palaiseau Cedex, France

Abstract

Protein design is a powerful tool for interrogating the basic requirements for the function of a metal site in a way that allows for the selective incorporation of elements that are important for function. Rubredoxins are small electron transfer proteins with a reduction potential centered near 0 mV (vs normal hydrogen electrode). All previous attempts to design a rubredoxin site have focused on incorporating the canonical CXXC motifs in addition to reproducing the peptide fold or using flexible loop regions to define the morphology of the site. We have produced a rubredoxin site in an utterly different fold, a three-helix bundle. The spectra of this construct mimic the ultraviolet–visible, Mössbauer, electron paramagnetic resonance, and magnetic circular dichroism spectra of native rubredoxin. Furthermore, the measured reduction potential suggests that this rubredoxin analogue could function similarly. Thus, we have shown that an α -helical scaffold sustains a rubredoxin site that can cycle with the desired potential between the Fe(II) and Fe(III) states and reproduces the spectroscopic characteristics of this electron transport protein without requiring the classic rubredoxin protein fold.

*Corresponding Author: vlpec@umich.edu.

ORCID

Alison G. Tebo: 0000-0003-0788-5617

Ricardo García-Serres: 0000-0001-5203-0568

Cédric Tard: 0000-0002-1515-1915

Nicolai Lehnert: 0000-0002-5221-5498

Vincent L. Pecoraro: 0000-0002-1540-5735

Author Contributions

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Notes

The authors declare no competing financial interest.

Supporting Information

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Graphical Abstract

Iron–sulfur proteins are among the earliest biologically generated metalloredox centers and include a broad array of structures, from mononuclear to large, mixed clusters such as the P cluster in nitrogenase, which are further complemented by a number of synthetic clusters.^{1–4} The simplest, and one of the most carefully studied, of these sites is the single-iron Fe(Cys)₄ center found in the rubredoxin family of electron transfer proteins. For this reason, rubredoxin and desulfurated rubredoxin are attractive targets for clarifying structure–function relationships in metalloproteins using protein design.⁵ Fe(Cys)₄ sites do not rely on inorganic sulfide for assembly as iron is coordinated by only protein-derived thiolates. A designed protein with an FeS₄ center allows for the investigation of the general requirements for function, beginning with the primary coordination sphere. Deeper analysis would assess whether the classic rubredoxin fold is necessary to obtain the desired tetrahedral geometry or to generate reduction potentials in a physiologically relevant range. In the long term, such a detailed understanding opens opportunities for the design and implementation of a complex, multisite, multifunction protein capable of electron transfer and redox catalysis.

A *de novo*-designed peptide provides the opportunity to selectively and systematically engineer metal sites and perturb their properties. An attractive scaffold for this purpose is based on α_3 D, which was designed by DeGrado and co-workers to model the fold and stability of a native protein⁶ and later redesigned to introduce three cysteine residues at the C-terminal end of this antiparallel three-helix bundle.⁷ The resulting construct, termed α_3 DIV, was capable of binding Cd(II), Pb(II), and Hg(II); a recent nuclear magnetic resonance (NMR) structure showed that incorporation of these cysteine residues led to minor perturbations of the global apoprotein structure [Protein Data Bank (PDB) entry 2MTQ].^{7,8} However, there are many examples of metal sites with more than three cysteines.^{9,10} Previous work in the Pecoraro lab focused on the incorporation of a fourth cysteine residue at one of two positions: either replacing a histidine in a loop region or creating a single CXXC binding motif (Figure 1 and Table 1).¹¹ These two constructs, α_3 DIV-H72C and α_3 DIV-L21C, modeled the interaction of Cd(II) with CadC and extended our previous work on understanding the interaction of heavy metals and metalloregulatory proteins.¹¹ These studies indicated that α_3 DIV-H72C formed, primarily, a CdS₃O structure whereas α_3 DIV-L21C had a majority CdS₄ environment.

The design of electron transfer proteins is more challenging than making a single heavy metal binding site, as the protein must stabilize both redox states and be able to cycle between them. As an example of these difficulties, attempts to introduce a type 1 or blue Cu center containing both histidine and cysteine residues (which is normally found in a β -barrel fold) into the three-helix bundle α_3 D instead yielded type 2-like red copper environments.

^{12–14} Our goal was to engineer a different electron transfer center, a rubredoxin-type site, into a known scaffold such as α_3 D, utilizing a secondary structure far removed from the canonical pair of Cys-X-X-Cys motifs found in the β -loop region of the native protein. While Cd(II) α_3 DIV-H72C exhibited CdS₃O coordination, α_3 DIV-L21C yielded a redox inert, tetrahedral M(II)S₄ center with bound Cd(II). For this reason, we examined whether the significantly smaller Fe(II) and Fe(III) ions could stably form the desired tetrahedral thiolate sites within α_3 DIV-L21C, leading to centers that exhibit the spectroscopic signatures of the native rubredoxins and would be able to redox cycle at potentials similar to that of the native system.

An important question in metallobiochemistry is the degree to which protein structure defines metal function. Rubredoxin is an ideal target for assessing this question as it has been extensively characterized biophysically and computationally and has been the subject of previous design attempts.^{15–22} All other designs have retained the essential features of native rubredoxin, exhibiting a β -hairpin structure that helps to enforce D_{2d} symmetry around the iron center. In our system, we have examined whether the overall topology is important in producing a stable FeS₄ site and whether there are overall design features that are important for function. Herein, we demonstrate that a rubredoxin mimic can be achieved with the redox center embedded within a three-helix bundle fold that includes a chelate motif in the sequence.

MATERIALS AND METHODS

Protein Production and Purification

The construct α_3 DIV-L21C was generated by sequential site-directed mutagenesis of the previously reported α_3 DIV-H72C¹¹ by the QuikChange kit (Stratagene) to replace the histidine at position 72 (C72H) and incorporate the L21C change, which yielded α_3 DIV-L21C-GSGC, which was partially characterized (see Table S3). In an effort to increase the yield and stability of the protein, further site-directed mutagenesis was performed to replace the final residue with alanine. The plasmid was transformed into *Escherichia coli* BL21 (DE3) and plated on LB Amp plates. A single colony was grown overnight in a starter colony and used to inoculate autoinduction medium.²³ The cells were grown for 20 h at 25 °C. The cells were resuspended in 1× phosphate-buffered saline with lysozyme and 5 mM dithiothreitol and then lysed with a microfluidizer. After heat denaturation at 55 °C for 45 min, the cell lysate was acidified and the soluble fraction collected after ultracentrifugation at 15K rpm and 4 °C. The pure proteins were isolated by high-performance liquid chromatography (HPLC) on a C18 reverse phase column and lyophilized to yield pure, white powder. The identity of the proteins was confirmed by electrospray ionization mass spectrometry.

Absorption Spectroscopy

Ultraviolet–visible (UV–vis) spectroscopy was performed on a Cary 100 instrument and measured from 700 to 250 nm. An Fe(II) stock was prepared in a glovebox under a N₂ atmosphere with ferrous ammonium sulfate, and the concentration was determined by ICP. Peptides were degassed and loaded with iron under a N₂ atmosphere in 20 mM Tris buffer

(pH 8.5) with 40 μM TCEP. Oxidized spectra were generated by exposing the Fe(II) complex to air and scanning until the spectra stabilized. The concentration of the peptide was determined by the tryptophan and tyrosine content of the peptide. Fe(II) binding titrations were performed in the glovebox with 50 μM protein in the presence of 100 μM TCEP. Spectral data were collected with an Ocean Optics SD2000 fiber optic spectrometer located inside the glovebox. Titrations were fit to the following equation in Origin Pro 9.0

$$[\text{FeP}] = \left[-\sqrt{K_D^2 + 2K_D(P_T + Fe_T) + (P_T - Fe_T)^2} + K_D + P_T + Fe_T \right] / 2$$

where K_D is the dissociation constant, P_T is the total protein concentration, and Fe_T is the total iron concentration.

X-ray Absorption Spectroscopy

Samples were made in 25 mM Tris buffer in the absence of chloride with 0.66 equiv of Fe(II) to ensure the absence of free iron in the sample. The samples were mixed with 30% glycerol as a glassing agent, loaded into a sample cell, and frozen in liquid nitrogen. Attempts to collect data on Fe(III) samples resulted in immediate photoreduction of the site. Measurements were taken at Stanford Synchrotron Radiation Lightsource (SSRL) beamline 7-3 with a Si (220) double-crystal monochromator and a flat Rh-coated harmonic rejection mirror. Samples were maintained below 10 K with an Oxford Instruments liquid helium cryostat. Data were measured as fluorescence excitation spectra using a 30-element Ge array detector normalized to incident intensity measured with a N_2 -filled ion chamber. Data were measured with steps of 0.25 eV in the XANES region (1 s integration time) and 0.05 $\text{\AA}^{-1}$ in the EXAFS region to $k = 12.5 \text{ \AA}^{-1}$ (1–20 s integration, k^2 -weighted). Data reduction and fitting were performed in EXAFSPAK.²⁴ Data were fit using theoretical amplitude and phase functions calculated using FEFF 8.0.

Mössbauer Spectroscopy

A 10 mM stock solution of ^{57}Fe ferrous ammonium sulfate in 10% sulfuric acid was prepared in an Ar atmosphere glovebox. The peptide concentration was determined by a DTNB assay and Trp absorption, where the average of the two methods was taken. Samples were made in either unbuffered solution, which was adjusted to pH 8.6 anaerobically, or 50 mM Tris buffer at pH 8.5 and added to Mössbauer cups either inside the glovebox [for Fe(II)] or exposed to air [for Fe(III)]. Fe(III) samples were monitored by UV–vis before being frozen in liquid N_2 to ensure full conversion to the oxidized form. Fe(II) samples were 2.0 mM peptide loaded with 1.7 mM ^{57}Fe , while Fe(III) samples were 2.0 mM peptide loaded with 2.0 mM ^{57}Fe .

Mössbauer spectra were recorded at 4.2 K, either on a low-field Mössbauer spectrometer equipped with a Janis SVT-400 cryostat or on a strong-field Mössbauer spectrometer equipped with an Oxford Instruments Spectromag 4000 cryostat containing an 8 T split-pair superconducting magnet. Both spectrometers were operated in a constant acceleration mode in transmission geometry. The isomer shifts were referenced against that of a room-

temperature metallic iron foil. Analysis of the data was performed with the program WMOSS (WEB Research).

Magnetic Circular Dichroism (MCD)

Samples were prepared as 1.5 mM peptide with 1.0 mM ferrous ammonium sulfate in 50 mM Tris buffer at pH 8.5. The samples were allowed to air oxidize before being added to the sample holder in the presence of 50% ethylene glycol as a glassing agent. Spectra were collected from -1 to -7 T and from 2 to 50 K. MCD spectra were recorded on a setup consisting of an Oxford SM4000 cryostat and a Jasco J-815 CD spectrometer as described previously.²⁵ The MCD spectra were measured in θ (millidegrees) and converted to ϵ using the conversion factor $\epsilon = \theta / (32980cdB)$, where c is the concentration, d is the path length, and B is the magnetic field strength. Gaussian deconvolution of the spectra was performed using the program PeakFit. VTVH fits were performed as previously described following the method developed by Neese and Solomon.^{25,26}

Electron Paramagnetic Resonance (EPR)

Samples were prepared as described for MCD studies. Spectra were collected at 4.8, 12.5, and 17.5 K on a Bruker EMX spectrophotometer equipped with a Bruker 4102-ST general-purpose cavity (rectangular TE102) and cooled with an Oxford Instruments liquid helium cryostat. Spectra were collected at 9.341 GHz with a modulation amplitude of 5 G and a microwave power of 20.5 mW.

Reduction Potential Determination

Solution electrochemistry was used to determine the reduction potential at pH 8.5. Iron-bound peptide (135 μ M) was prepared in a glovebag (Sigma-Aldrich) under an Ar atmosphere and added to an Ar(g) degassed 5 mL electrochemical cell with 100 mM Tris buffer and 100 mM Na₂SO₄ at pH 8.5. The temperature was maintained at 20 °C by using a circulating water temperature controller. Voltammograms were measured on an Autolab using a highly ordered graphite (HOPG) carbon electrode as the working electrode, standard calomel as the reference electrode, and platinum wire as the counter electrode. Measurements were taken at 20 mV/s. The dependence of midpoint potential on pH was fit to a line.

RESULTS

Design of a Rubredoxin Site in an α -Helical Bundle

To ascertain whether a rubredoxin site could be created in an α -helical bundle, we sought to understand how the arrangement of the primary coordination sphere ligands affects the ability of the protein to bind and stabilize iron. Previous work led us to identify the α_3 DIV-L21C construct as appropriate for designing a tetrathiolate iron site (Figure 1 and Table 1) (see the introductory section). When expressed with supplemental iron, the cell lysate of the α_3 DIV-L21C peptide containing a chelate motif was a dark red color as is often observed during expression of iron-sulfur proteins. Other α_3 D derivatives such as α_3 DIV or α_3 DIV-H72C showed no difference in color from that of normal *E. coli* lysates. After final purification by HPLC, which removes all associated metal ions, α_3 DIV-L21C was

anaerobically reconstituted with ferrous ammonium sulfate and iron binding was monitored by UV-vis absorption spectroscopy. The Fe(III) state was formed by exposure of Fe(II)- α_3 DIV-L21C to air, yielding an Fe(III)- α_3 DIV-L21C spectrum that corresponds well with native rubredoxin (Table 2). While the spectra for both the Fe(II) and Fe(III) forms of metalated α_3 DIV-L21C agree well with rubredoxin in terms of the energy, the molar extinction coefficients at the λ_{max} are ~3-fold larger in native rubredoxins (Table 2). Previous studies have demonstrated that α_3 DIV, α_3 DIV-H72C, and α_3 DIV-L21C are well-folded, three-helix bundle proteins.^{7,8,27} Furthermore, circular dichroism spectra indicated that the structure of the peptide is not drastically altered by the presence of iron and that iron binding stabilizes the peptide slightly (Figure S1). Fe(II) binding titrations as monitored by UV-vis spectroscopy showed that the peptide binds Fe(II) in a pH-dependent fashion with a K_D of 6.3 μM at pH 8.5 (Figure S2).

Mössbauer Spectroscopy

The electronic structure of the iron site was investigated with Mössbauer spectroscopy (Figure 2). The 4.2 K spectrum of the Fe(II) form revealed an isomer shift of 0.72 mm/s and a quadrupole splitting of 3.27 mm/s, which are typical of Fe(II) in a tetrahedral sulfur-only coordination environment and fully consistent with native rubredoxin (Table 2). The large line width and the line asymmetry are not unexpected under slow spin-lattice relaxation, as a result of the hyperfine coupling between the nuclear spin and the electronic $S = 2$ ground state. High-field spectra reveal that there are two Fe(II)-S components. The first [accounting for 60% of the total iron content (blue trace in Figure 2)] exhibits classic parameters for a reduced rubredoxin site, while the second [accounting for 40% of the total Fe content (red trace in Figure 2)] bears some similarity to previously observed centers in rubredoxin generated by the mutation of cysteine ligands to serine and may represent an FeS_3O structure.²⁸

For the Fe(III) form, slow relaxation at 4.2 K yields a magnetically split spectrum (Figure 2). Application of a magnetic field allowed the determination of an isomer shift of 0.26 mm/s, and a quadrupole splitting of 0.5 mm/s, as well as hyperfine and zero-field splitting (ZFS) parameters, which are consistent with a rubredoxin-type center as previously reported from EPR and Mössbauer spectroscopy.²⁸⁻³¹ As different values of the ZFS parameter were tested in the simulations (Figure S3, top), the goodness of the fits worsened when D was moved away from the determined value of 0.5 cm^{-1} , and sign reversal did not invert the tendency. Apart from the oxidized protein, the spectra reveal the presence of an impurity (~1/4 of total Fe) consisting of ferric oxides/hydroxides, presumably resulting from complex decomposition under air oxidation. Overall, the parameters for the Fe(III)-S center agree well with published values for rubredoxin as well as other rubredoxin mimics and indicate the successful production of a tetrahedrally coordinated iron in both ferrous and ferric oxidation levels.^{29,32}

EPR Spectroscopy

X-Band spectra were collected at 4.8, 12.5, and 17 K (Figure S3) to complement the Mössbauer analysis of Fe(III)- α_3 DIV-L21C. Simple comparison with published spectra of rubredoxin and desulfuredoxin shows that the spectra of α_3 DIV-L21C are similar to those of

rubredoxin. However, the low-field resonance occurs at a g value (~ 8.9) slightly lower than that of rubredoxin (9.4), which is consistent with the lower E/D (0.15 in $\alpha_3\text{DIV-L21C}$ vs 0.25 in *Clostridium pasteurianum*²⁹) value calculated from the Mössbauer experiments. The spectrum is dominated by a strong, broad transition at $g \sim 4.3$, which correlates with high rhombicity and is likely due to the presence of contaminating high-spin Fe(III) oxo/hydroxo clusters overlaid with the signal from the rubredoxin site. The intensity of the high-field resonance increased with the increase in temperature from 4.8 to 17.5 K, which indicates that it arises from transitions within the middle Kramers doublet, revealing a positive value for D , in agreement with the Mössbauer analysis.

Magnetic Circular Dichroism Spectroscopy

The sample used for EPR was also analyzed by magnetic circular dichroism. Figure 3 shows a comparison of the UV-vis and low-temperature (LT) MCD spectra of ferric $\alpha_3\text{DIV-L21C}$ (see also Figure S4), together with a Gaussian deconvolution of these data, which allows for the identification of nine individual transitions in the optical spectra (see Table S1). These MCD spectra closely resemble those of ferric *C. pasteurianum* and *Desulfovibrio gigas* rubredoxins.^{33,34} In the UV-vis region, two positive features are observed at 600 and 520 nm, followed by two negative bands at 475 and 420 nm [which are deconvoluted into a total of six bands (see Figure 3)]. Very similar features are observed in *D. gigas* rubredoxin at 565 and 505 nm (positive bands) and at 475 and 400 nm (negative bands). To determine whether these optical transitions have polarizations similar to those in the protein, we also measured variable-temperature variable-field (VTVH) data (Figure S5). The resulting magnetic saturation plots for bands 1–9 in Figure 3 are shown in Figure S6. Inspection of the VTVH isotherms indicates two types of saturation behavior: overlaid and nested. Fits of these data with a negative D value of -0.5 cm^{-1} show that the overlaid behavior represents xy polarization of the corresponding transitions, whereas nesting originates from z polarization (Figure S8). These results are in close agreement with the saturation behavior of the corresponding MCD bands in *D. gigas* rubredoxin,³⁴ which are further supported by previous single-crystal measurements.³⁵ Note that the fits are very sensitive to the sign and magnitude of D (Figures S8 and S9). In particular, if the sign of D is reversed, the polarizations of these features are inverted. Overall, these data demonstrate that the rubredoxin site that we have created in $\alpha_3\text{DIV-L21C}$ is an exact replica of a naturally occurring rubredoxin protein, including the relative energies and polarizations of the thiolate $\rightarrow \text{Fe(III)}$ charge transfer transitions.

X-ray Absorption Spectroscopy

X-ray absorption spectroscopy (XAS) was performed using the iron K-edge on Fe(II)- $\alpha_3\text{DIV-L21C}$ to give a detailed understanding of the structural features of the iron site in the reduced form of the protein. Previous attempts to perform XAS on Fe(III)- $\alpha_3\text{DIV-L21C}$ resulted in immediate photoreduction of the site. The extended X-ray fine structure (EXAFS) region was used to analyze the coordination of the iron. The Fourier transform is dominated by a single well-resolved peak at $\sim 2.3 \text{ \AA}$ with no other significant backscatterers. The spectrum is well reproduced by a single shell of four sulfurs coordinated to the iron center at a distance of $2.32 \text{ \AA}$ with a σ^2 of $6 \times 10^{-3} \text{ \AA}^2$ (Figure 4). This corresponds well with previous EXAFS studies of native reduced rubredoxins, which have found distances of

2.32 Å.^{24,29} The presence of a single Fe–O interaction in a fraction of the sites, as suggested by the Mössbauer spectrum (*vide supra*), would not give a detectable change in the EXAFS.

Reduction Potential

Solution electrochemistry revealed a reversible one-electron transfer with an apparent reduction potential, $E_{1/2}$, of –75 mV [vs the normal hydrogen electrode (NHE)] at pH 8.5, where the spectroscopic properties of this peptide were measured (Figure S12). This is very similar to the range of native rubredoxins, which have potentials of –90 to 50 mV versus NHE.^{15,20,36,37} The pH dependence of the redox potential ($E^{\circ'}$) was investigated between pH 7.4 and 9.0 due to the short stability window of the construct. Interestingly, the reaction involves not only single-electron transfer but also proton binding, following a PCET mechanism.^{38,39} The Pourbaix diagram shows a slope of 124 mV/decade, suggesting a two-proton, one-electron process (Figure S12).⁴⁰ While native rubredoxin does not exhibit a pH-dependent potential, this process may arise from nearby charged residues, such as Arg71 and His72, which may participate in defining the potential of the metal center.

An important hallmark of function is the ability to cycle between the two different redox states as native electron transfer proteins can. To test this, α_3 DIV-L21C-Fe(II) was exposed to air, allowed to oxidize, and then re-reduced with sodium dithionite. The peptide was capable of sustaining three oxidation and reduction cycles, as measured by the absorbance at 492 nm, before gradually being irreversibly bleached (Figure S11). Because no precipitate was observed, the protein was likely not denatured to an unfolded state; rather, iron was likely displaced upon cysteine oxidation.

DISCUSSION

The data reported here indicate that we have successfully generated a rubredoxin site in a *de novo*-designed scaffold that is structurally far removed from the canonical rubredoxin fold. All successfully designed rubredoxin mimics with two stable oxidation states, including ours, have incorporated at least one CXXC motif, suggesting that these motifs may be important for successful FeS₄ assembly, independent of the secondary structure of the protein around the site. Furthermore, these motifs have been used previously to stabilize binding of the 4Fe-4S cluster to *de novo*-designed proteins.^{41,42} Previous attempts to design tetrathiolate sites have been focused on two strategies: (1) recapitulating the hairpin loop/zinc ribbon fold as closely as possible^{32,43,44} and (2) redesign of the loop regions of stable α/β proteins^{45,46} to generate a hairpin looplike structure, as well. Our approach is a complete divergence from these strategies as the present site is embedded within α -helices and does not rely on the inherent flexibility of loop regions to adopt a particular symmetry at the iron site. Despite this, α_3 DIV-L21C-Fe does not show extreme deviations from tetrahedral symmetry as is found in desulfiredoxin⁴⁷ and therefore not only forms a tetrathiolate-coordinated iron, but also adopts the pseudotetrahedral symmetry found in native rubredoxins. Thus, we have shown that tetrathiolate sites can be constructed within a relatively rigid framework that differs greatly from the native protein topology for this site.

The two most successful previous designs of a tetrathiolate-coordinated iron site relied on reproducing a secondary structure around the metal that exactly mimicked that of

rubredoxin.^{32,43,44} In one case, Nanda et al.⁴³ designed two hairpin turns fused by a tryptophan zipper motif to generate a 40-residue construct with two β -turns, each with a CXXC motif, just as in rubredoxin. In the other, Jacques et al.⁴⁴ investigated a series of zinc ribbon folds using cyclic peptide models to incorporate iron into tetrathiolate sites similarly formed in loop regions of the cyclic peptides with CXXC chelate motifs. These two models are the most successful in terms of replicating the spectroscopic features of rubredoxin as well as being able to cycle multiple times between the Fe(II) and Fe(III) states. The Nanda model, RM1, exhibited a UV-vis spectrum in which the energies of the charge transfer bands closely match that of rubredoxin, especially in the low-energy region, although the molar extinction coefficients were again 3- fold smaller than those found in rubredoxin (Table 2).⁴³ RM1 had a reduction potential of 55 mV, which agrees with those of native rubredoxins (-90 to 50 mV), and cycled 16 times, which is the highest number of cycles reported for a designed protein. The L series of peptides were designed to mimic zinc fingers.^{32,44} The zinc ribbon fold peptide, L_{ZR}, impressively exhibited UV-vis absorption data nearly identical to native rubredoxins in both energy and molar extinction coefficient (Table 2). Additionally, Mössbauer and EPR data were obtained, providing a detailed view of the electronic structure of the site, which nearly exactly matched that of native rubredoxin. The reduction potential of this site was measured at 144 mV versus NHE, and the construct was able to cycle between the Fe(II) and Fe(III) states seven times. These designs illustrate the ability of *de novo*-designed proteins to reproduce specific folds and functions with unrelated sequences. Given these successes, the next challenge for protein design is mimicking a function within an entirely different protein fold.

An alternative direct comparison to our system, in that it also does not attempt to reproduce the native fold of rubredoxin, is a thioredoxin redesign (Trx[Rd]) that introduces a tetrathiolate site into a protein with an α/β fold consisting of a four-stranded antiparallel β -sheet between three α -helices.⁴⁶ A tetrathiolate iron site was computationally designed to take advantage of an extant CXXC motif that usually exists as a disulfide bond by incorporating two other cysteine residues in nearby loop regions. Reconstitution with Fe(II) and subsequent oxidation generated a UV-vis spectrum with characteristic transitions at ~350 and ~500 nm,⁴⁶ although the relative extinction coefficients and band structure of shoulders at low energy are more reminiscent of desulfuredoxin than of rubredoxin. No detailed spectroscopy capable of distinguishing electronic symmetry and structure was performed to further examine the underlying electronic transitions in the absorption spectrum. This construct was capable of cycling three times between Fe(II) and Fe(III), but the reduction potential was not reported.

Our α_3 DIV-L21C-Fe construct is an example of an extremely well-characterized designed rubredoxin mimic, and the spectroscopic parameters obtained correspond well with those published for FeS₄ sites such as native rubredoxin and with other previous successful designs that were more reliant on the structural parameters exhibited by the native protein. In contrast, our design reproduces the spectroscopic characteristics of rubredoxin using a scaffold that is by far the largest deviation from the consensus rubredoxin structure yet reported. While all other successful designs have relied on loop regions to help define the metal binding site, our construct uses only a CXXC motif embedded in an α -helical bundle.

The α_3 DIV-L21C-Fe construct exhibits UV-vis spectra that match rubredoxin and molar extinction coefficients equivalent to that of the previously reported RM1. In fact, our construct exhibits energies and relative molar extinction coefficients that reproduce rubredoxin-type spectra better than the other successful bistable, non-hairpin loop construct. Furthermore, we report Mössbauer data that correspond well with those of both rubredoxin and LZR. Additionally, we report MCD characterization, which has not been performed on any previous designed construct. The parameters obtained from MCD match closely with those previously reported for native rubredoxin. Indeed, there has been a controversy regarding the sign of D for rubredoxins, with MCD results favoring a negative D ,³⁴ while Mössbauer and EPR support a positive D value.^{28–31} Our construct reproduces the spectroscopy of Fe(III)- rubredoxin well enough to display the same contradictory behavior with respect to the sign of D . Further work will entail high-field EPR studies and protein design to elucidate the origin of this apparent disagreement.

While our construct can sustain only three cycles of oxidation and reduction, no other nonloop construct has been reported to cycle between the oxidized and reduced forms. It is important to note that among the folds most distant from native rubredoxin, the thioredoxin redesign accessed a desulfuredoxin center, whereas α_3 DIV-L21C-Fe yields a rubredoxin-type site. This suggests a more complicated structure-function relationship in these proteins. Designs of 4Fe-4S clusters into non-native α -helical bundles show that the absolute geometry of these sites is not precluded by the geometry of the protein fold, but rather that the size and stability of 4Fe-4S clusters, which have a tendency to self-assemble, are more tolerant to incorporation into this particular fold.^{41,42} It is interesting to note that incorporation of iron into α_3 DIV-L21C does not result in drastic structural rearrangement of the protein as monitored by circular dichroism. Further consideration of the characterization of the oxidized and reduced forms of α_3 DIV-L21C-Fe suggests a model in which the minor species detected in the Fe(II) Mössbauer spectrum is partially incapable of cycling between the Fe(II) and Fe(III) forms and thus each round of oxidation comprises only ~60% of the bound iron. This is supported by the relatively low K_D measured for binding of Fe(II) to a tetrathiolate site, which may play a role in the cycling behavior. If the proportion of iron cycled is taken into account, the molar extinction coefficients can be corrected to 2000 M⁻¹ cm⁻¹ at 595 nm, 4500 M⁻¹ cm⁻¹ at 491 nm, and 8333 M⁻¹ cm⁻¹ at 345 nm, which are on par with those of LZR. Moreover, this suggests that the number of oxidation-reduction cycles could be improved by increasing the binding affinity of Fe(II) for the protein.

One advantage of a *de novo* design strategy is that it allows investigation of well-studied metal centers unhindered by evolutionary constraints to assess what factors are essential to achieve specific function. Clearly, CXXC chelate motifs, and the resulting stability and symmetry constraints they impose, favor the formation of sites with two stable oxidation states. However, we demonstrate that hairpin loops and β -structure are not required to attain the relevant physical structure (geometry and bond distances), electronic structure, or reduction potential. This illustrates that *de novo* protein design is able to achieve nativelike metalloreredox centers even in highly distorted or non-natural folds and suggests that the overall fold is not essential for certain spectroscopic features. Rather, folds that can accommodate certain architectural features and that present ligands at appropriate spatial

coordinates may produce similar characteristics, indicating that novel constructs combining metal sites with desired features (e.g., reduction potential or catalytic efficiency) originating from drastically different secondary environments may be combined into a single, functional homogeneous protein motif that could be dissimilar from one or both of the initial targeted structures. Adapting metal sites from different folds to a generalized framework could allow us to tune these sites for alternative functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Model of the synthetic rubredoxin, $\alpha_3\text{DIV-L21C-Fe}$, constructed in PyMol based on the NMR structure of $\alpha_3\text{DIV}$ (PDB entry 2MTQ).

Figure 2.

Variable-field Mössbauer studies of Fe- α_3 DIV-L21C in oxidized (left) and reduced (right) forms. Spectra were measured at 4.2 K in various parallel applied magnetic fields. Vertical bars represent experimental points, while the solid lines overlaid with the spectra are theoretical simulations. All simulation parameters are listed in Table 2. The blue lines in the left panel are spin Hamiltonian simulations. All three spectra show diffuse absorption under the baseline, for which the simulation does not account. Additionally, a broad doublet (orange line: $\delta = 0.48$ mm/s, $E_Q = 1.02$ mm/s, and $\Gamma = 0.81$ mm/s) appears in the 600 G spectrum. These two features are characteristic of polydisperse Fe(III) oxo/hydroxo clusters. These account for $\sim 24\%$ (area under the orange line) of total iron. In the right panel, the 600 G spectrum was simulated with a single quadrupole doublet with asymmetric line widths. The high-field spectra had to be simulated with two sets of parameters representing two distinct components, which account for 60% (blue trace) and 40% (red trace) of total ^{57}Fe .

Figure 3.

Correlated fit of the UV-vis spectrum (measured at room temperature) vs the MCD spectrum (measured at 4 K) of ferric α_3 -DIV-L21C. Data are shown as a black line, and the resulting fit is indicated as a dashed red line. Nine Gaussian bands are used to deconvolute the data as indicated. Table S1 lists the fit parameters of the individual bands. The saturation behavior of the VTVH isotherms is denoted by the superscript: N for nested and O for overlaid (see Figure S6).

Figure 4.
Iron K-edge EXAFS data of α_3 DIV-L21C-Fe(II).

Table 1

Sequences of Peptides Relevant to This Study

α_3 DIV	MGS WAEFKQRLAAIKTRCQALGGSEAECAAFEKEIA AFES ELQAYKGKGN PEVEALRKEAAAIRDECQAYRH N
α_3 DIV -L21C	MGS WAEFKQRLAAIKTRCQAC GGSE AECAAFEKEIA AFES ELQAYKGKGN PEVEALRKAAAIRDECQAYRH NGSGX ^a

^aX is either Cys or Ala, and blue indicates helical structure.

Table 2
Comparison of Spectroscopic Parameters of Rubredoxin and Designed Proteins

protein	UV-vis λ_{nm} (ϵ (M ⁻¹ cm ⁻¹)	reduction potential (vs NHE)	Mössbauer (ox) (δ and E_Q in mm/s, D in cm ⁻¹ , A in T)	Mössbauer (red) (δ and E_Q in mm/s, D in cm ⁻¹ , A in T)
rubredoxin	750 (350) ⁴⁸ 570 (3200) 490 (6600) 370 (7710)	-90 to +50 mV ⁴⁹	$\delta/ E_Q = 0.24^{29}/-0.5, \eta = 0.2$ $D = +1.9, E/D = 0.23$ $A_{xx,yy,zz} = (-16, -15.9, -16.9)$	$\delta/ E_Q = 0.70^{29}/-3.25, \eta = 0.65$ $D = +7.4, E/D = 0.28$ $A_{xx,yy,zz} = (-20.1, -8.3, -30.1)$
RM1 ⁴³	750 (~500) ^a 600 (~1500) 490 (~2900) 370 (~6800)	+55 mV (pH 7.5)	N/A	N/A
L _{ZR} ³²	700 (220) 570 (2900) 491 (5250) 360 (7730)	+144 mV (pH 7.2)	$\delta/ E_Q = 0.24/-0.5, \eta = 0.13$ $D = +1.9, E/D = 0.23$ $A_{xx,yy,zz} = (-15.8, -15.6, -19.9)$	$\delta/ E_Q = 0.69/-3.24, \eta = 0.84$ $D = +7.6, E/D = 0.28$ $A_{xx,yy,zz} = (-18.3, -8.3, 33)$
Trx[Rd] ⁴⁶	~500 (~4500) ^a ~360 (~7000)	N/A	N/A	N/A
α_3 DIIV-L21C	595 (1200) 491 (2700) 345 (5000)	-75 mV (pH 8.5)	blue trace, ^b $\delta/ E_Q = 0.26/-0.5, \eta = 0.0$ $D = +0.5, E/D = 0.15$ $A_{xx,yy,zz} = (-15.9, -16, -17)$	blue trace ^c (60%); $\delta/ E_Q = 0.73/-3.40, \eta = 0.9$ Red trace ^c (40%); $\delta/ E_Q = 0.80/-3.07, \eta = 2$ $D = +2.5, E/D = 0.24$ $A_{xx,yy,zz} = (-5, -4.1, -20)$

^aFrom visual inspection of published spectra.

^bFit uncertainties are ± 0.06 mm/s for δ , ± 0.3 mm/s and ± 0.5 for E_Q and η , respectively, ± 0.2 cm⁻¹ and ± 0.03 for D and E/D , respectively, and ($\pm 0.5, \pm 1, \pm 1$) T for the hyperfine coupling constant.

^cFit uncertainties are ± 0.05 mm/s for δ , ± 0.05 mm/s and ± 0.5 for E_Q and η , respectively, ± 1.0 cm⁻¹ and ± 0.05 for D and E/D , respectively, and ($\pm 2, \pm 0.4, \pm 20$) T for the hyperfine coupling constant.