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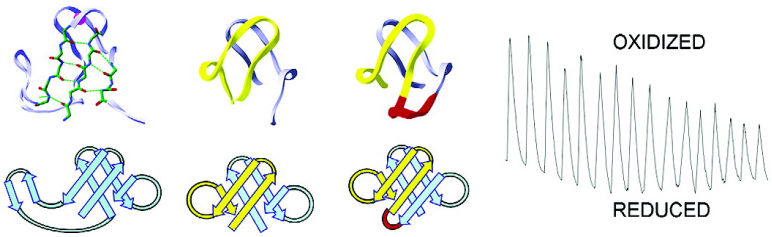


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# De Novo Design of a Redox-Active Minimal Rubredoxin Mimic

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Metal-binding sites in metalloproteins frequently occur at the interfaces of elements of secondary structure, which has enabled the retrostructural analysis of natural proteins[1] and the de novo design of helical bundles that bind diiron cofactors,[2] hemes,[3−5] porphyrins,[6] and a variety of mononuclear metal ions.[7−10] However, the design of metalloproteins containing $\beta$-structure is less well developed, despite the frequent occurrence of $\beta$-conformations in natural metalloproteins. Although the design of $\beta$-proteins has traditionally been challenging, much progress has been made in recent years.[11] Several groups have prepared antiparallel $\beta$-hairpins,[12] open-faced antiparallel $\beta$-sheets,[13−16] and a toxin-like $\beta$-protein featuring only a single disulfide bond.[17] Significant progress has also been made in the design of mixed $\alpha/\beta$-folds.[18,19] Here, we describe the design and molecular characterization of a $\beta$-protein that mimics rubredoxin, recapitulating the binding of Fe(II/III) in a tetrathiolate geometry and allowing reversible cycling between these redox states.

The rubredoxin tetrahedral metal-binding motif has been the focus of several previous exercises in protein design; thus, it serves as a useful benchmark to compare different approaches to protein design. This motif has been engineered into proteins that originally lacked metal-binding sites,[20,21] and Lombardi and co-workers[22] have prepared short peptides that self-assemble in the presence of metal ions to form a $C_2$ symmetric tetrathiolate binding site. However, in nearly all of these studies, the reversible cycling between $Fe^{2+}$ and $Fe^{3+}$ was not fully demonstrated due to the very limited stability of the ferrous form of the proteins. Therefore, the design of a protein that is sufficiently stable to allow reversible cycling between these two states remains an important goal.

Figure 1A illustrates the structure of rubredoxin and the dissection of its metal-binding site to a pair of two pseudo-equivalent $\beta$-hairpins. The active-site region can be described to approximately 1.0 Å resolution by applying a $C_2$ symmetry operator to an idealized $\beta$-hairpin, which contains two Cys side chains (Figure 1B). The hairpin turn adopts a four-residue turn with an $\alpha_R$-$\alpha_R$-$\alpha_R$-$\alpha_L$ (or $\gamma$-$\alpha_R$-$\alpha_R$-$\alpha_L$) conformation (Figure 1C). The first Cys lies in the last position preceding the turn; it hydrogen bonds to the amide protons of two residues within the turn in an interaction analogous to an $\alpha$-helical "N−Cap" interaction.[23] The second Cys thiolate receives a hydrogen bond from the amide NH adjacent to the Gly residue, which assumes an $\alpha_L$ conformation. These hydrogen bonds concomitantly stabilize both the turn and the thiolate form of the side chain. They may also help to tune the redox potential of the metal ion. Although these features were included in the design of an earlier turn peptide, modeled on the rubredoxin structure, the resulting peptide lacked sufficient stability in solution to allow reversible redox cycling.[22] We attribute the limited stability to insufficient tertiary structural restraints and, hence, sought a strategy that would provide structural links between the two hairpin units. Often, $\beta$-proteins contain overhand connections, providing a close connection between the strands on opposing sheets,[24] and one such overhand connection connects the sheets in rubredoxin. Therefore, in the current design, each hairpin has a short C-terminal appendage that reaches over to the other symmetry-related hairpin, extending its two-stranded sheet into a three-stranded structure and consolidating the hydrophobic core.

The last three strands of the *Pyrococcus furiosus* rubredoxin (1BRF) form a 2-Cys hairpin, followed by a connecting loop that crosses over the bundle to join the sheet on the opposite side of the protein (Figure 1A). This three-strand motif, when transformed using a 2-fold symmetric axis containing the metal ion, produced a novel topology consisting of a domain-swapped dimer with the third strand from the opposing hairpin (Figure 1D). Preliminary studies (Supporting Information) with the dimeric miniRM peptide intended to adopt this structure were promising, although it was stable for only four cycles of oxidation/reduction. Therefore, a single chain construct was attempted.

The two dimers were fused using a highly stable hairpin motif, the tryptophan zipper (Trpzip; 1LE0) designed by Cochran and co-workers.[12] The resulting molecule is significantly shorter (40 vs 54 aa) and has a different topology from that of the natural protein (Figure 1E). Several positions were fixed prior to computational design, including the Trpzip linker, the four active-site cysteines, and two Gly residues (that adopt an $\alpha_L$ conformation). An isoleucine at the second $\alpha_R$ position of the turn was also fixed to shield the active site from solvent. The remaining amino acids were chosen using SCADS,[25] selecting the most probable amino acid for each position. The resulting peptide, designated rubredoxin mimic 1 (RM1), was prepared by solid-phase peptide synthesis.[26]

UV−vis spectroscopy demonstrates that RM1 binds transition metal ions in a tetrahedral tetrathiolate geometry and in a peptide/metal ion stoichiometry consistent with the design. The UV−vis spectrum of Co(II) is particularly sensitive to the geometry of metal-binding centers[27] and, hence, is often used to probe divalent metal-binding sites. The addition of 1.0 equiv of RM1 to Co(II) gave a UV−vis spectrum with d−d transitions near 700 nm and a ligand-to-metal transfer band at 340 nm (Figure 2A), which are nearly identical to the values observed for previously designed rubredoxin mimics and natural rubredoxins.[20−22,28] Addition of stoichiometric Zn(II) to Co(II)-complexed RM1 results in the almost complete displacement of Co(II), as evidenced by loss of the absorption at 700 nm (not shown), consistent with the preference of Zn(II) over Co(II) for tetrahedral binding sites. The UV−vis spectrum of a

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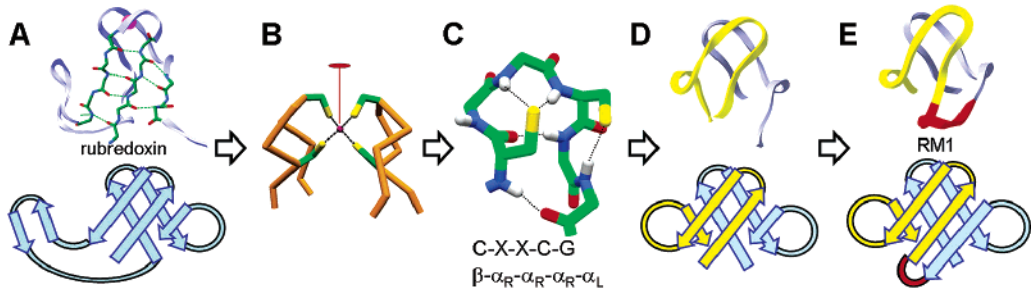


***Figure 1.*** RM1 design cycle: (A) three-stranded sheet topology of natural rubredoxin, (B) $C_2$ symmetry, (C) active-site geometry, (D) miniRM dimer, and (E) RM1 with Trpzip linker shown in red.

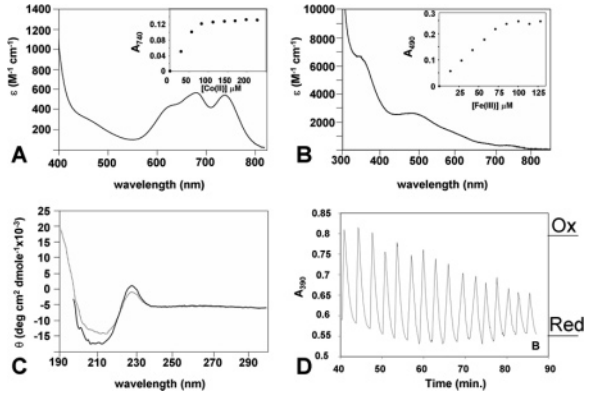


***Figure 2.*** Binding of (A) Co(II) and (B) Fe(III) to RM1 with 1:1 stoichiometry. (C) CD of apoRM1 (dashed line) and enhancement in $\beta$-structure upon addition of Zn(II) (solid line). (D) Fe(III)−RM1 stable for 16 cycles of oxidation/reduction.

1:1 complex of RM1 with Fe(III) was also consistent with the design, showing a spectrum with strong bands near 370, 490, 600, and 750 nm (Figure 2B). A titration showed a linear increase in intensity of the optical bands with added Co(II) or Fe(III) until reaching a 1:1 mole ratio, after which there was no significant increase in absorbance (Figure 2A,B). The data were well described by a binding isotherm with a dissociation constant $\leq 1$ $\mu$M.

CD spectroscopy and analytical ultracentrifugation indicated that RM1 is monomeric and folded in the presence and absence of metal ions. Global analyses of analytical ultracentrifugation data collected at three different speeds fit well to a single monomer. CD spectra clearly show significant $\beta$-sheet structure in the absence of metal ions, with an enhancement of the minimum at 215 nm and the maximum at 230 nm, resulting from exciton coupling of stacked tryptophans[29] (Figure 2C). This suggests preorganization of the active site in the absence of metal, which has been shown to be important in previously designed metalloproteins.[8,30]

There have been few measurements of the electrochemical midpoint potentials of de novo designed rubredoxin mimics due to their limited stability under aerobic conditions. Redox potential measurements of RM1 show a midpoint potential of 55 mV versus a standard hydrogen electrode at pH 7.5. Natural rubredoxins fall between −90 and 40 mV vs NHE.[31] The single-chain RM1 design showed activity for 16 cycles under aerobic conditions (Figure 2D). By contrast, both miniRM and a previously designed rubredoxin mimic based on the thioredoxin scaffold began to decompose after approximately three cycles.[20] This is, as far as we can determine, the longest demonstrated redox cycling of a de novo designed rubredoxin protein mimic. RM1 should provide a powerful investigative tool for understanding how specific residues and global stability contribute to redox function.

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**Supporting Information Available:** Materials and methods, analytical ultracentrifugation, miniRM studies, redox activity, EPR. This material is available free of charge via the Internet at http://pubs.acs.org.

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