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Computational De Novo Design and Characterization of a Four-Helix Bundle Protein that Selectively Binds a Nonbiological Cofactor

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De novo design tests current understanding of metalloproteins[1,2] and offers the potential to construct novel biomaterials, such as catalysts[3,4] and bioelectrochemical devices.[5] The use of nonbiological cofactors in these efforts offers the possibility of creating proteins with unusual properties. We have developed computational design methodology[6,7] to derive a unique protein framework for a specified cofactor of interest as an alternative to re-engineering natural protein scaffolds.[8] Selective cofactor recognition is a hallmark of achieving this goal and a significant challenge, especially in the absence of covalent attachment.[9] Previously designed heme proteins bound various metalloporphyrins with relatively low specificity, which is likely attributable to molten globule character.[2] In contrast, natural proteins generally bind cofactors in well-structured environments with precisely positioned amino acid side chains. Here, we report the complete de novo design of a nativelike protein that selectively binds a nonbiological cofactor.

The designed protein serves as a scaffold for future electron-transfer studies by encapsulating a symmetrical dyad of DPP−Fe units (Figure 1a) through bis(His) coordination. DPP−Fe(III)Cl was used as the precursor complex for incorporating the DPP−Fe unit in the protein matrix.[10] Requirements for efficient cofactor binding dictated the precise protein backbone geometry. A single low-energy structure (Figure 1b) was computed with a Monte Carlo simulated annealing protocol that considered the following constraints: (1) a metal−metal distance between 17 and 19 Å, (2) optimal His N(ϵ) to Fe bonding interactions, (3) second-shell hydrogen bonds between His N(δ) and Thr O(γ) (Figure 1c), (4) minimal steric clashes, (5) maintenance of D_2 symmetry. This process led to imidazole rings in near-perpendicular alignment as a result of the second-shell hydrogen bonds. The identities of the remaining positions were determined by recursive calculations with the computational design algorithm SCADS,[6,7] which provided site-dependent side chain probabilities. In particular, SCADS located sites appropriate for complementary charge patterning to enforce an antiparallel bundle topology[11] (E7, E14, K21, K28). Ala side chains at critical sites promoted efficient helix−helix and helix−cofactor packing (A4, A8, A12, A15, A18, A25). A suitable position for tyrosine was found to aid peptide concentration measurements (Y32). Finally, S0 and G33 were added as N- and C-terminal capping residues[12] (Figure 1d). The 34-residue peptide was prepared by Fmoc-based solid-phase synthesis.[13]

UV−vis spectroscopy demonstrated that the peptide binds DPP−Fe via bis(His) coordination to low-spin Fe(III) and with a peptide/cofactor stoichiometry consistent with the design. An increase in

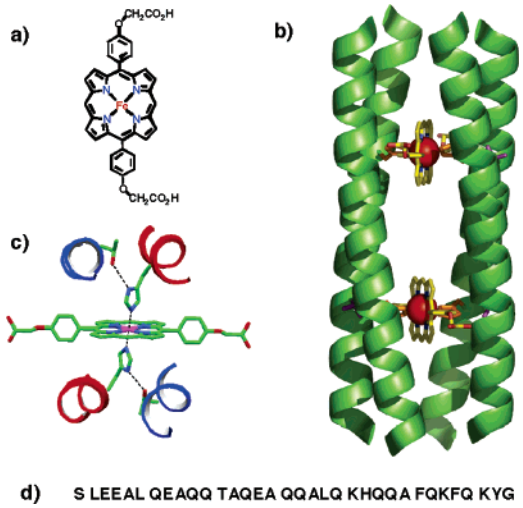

Figure 1. (a) DPP−Fe cofactor. (b) Model of designed four-helix bundle protein containing two DPP−Fe cofactors. (c) Axial ligand interactions. (d) Peptide sequence.

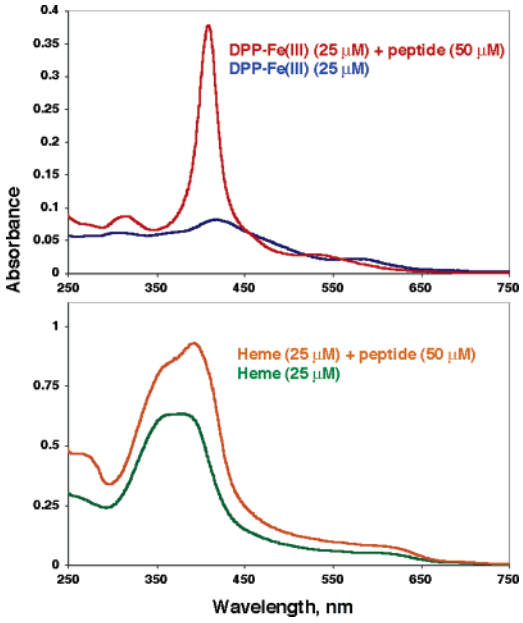

Figure 2. UV−vis spectra demonstrating cofactor selectivity.

Soret band absorbance at 408 nm and a Q-band blue shift from 580 to 530 nm was observed upon adding four equivalents of peptide to two equivalents of DPP−Fe(III) (Figure 2). A titration showed a linear increase in Soret band intensity with added peptide

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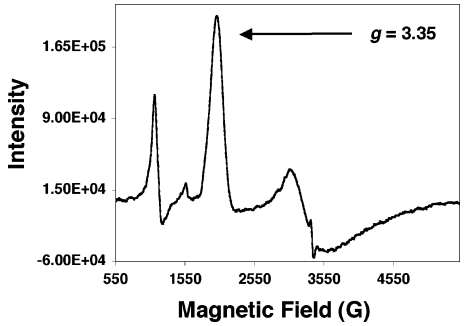

Figure 3. EPR spectrum of the assembled protein at 10 K. Signals at $g =$ 6.08 and 4.33 are assigned to unbound DPP−Fe(III)Cl and high-spin Fe impurities, respectively.

until reaching a 2:1 peptide/DPP−Fe(III) mole ratio, after which there was no significant increase in absorbance.

CD spectroscopy, size-exclusion chromatography, and analytical ultracentrifugation indicated the peptide undergoes a transition from a predominantly random coil monomer to an α-helical tetramer upon binding DPP−Fe(III). Peptide in the absence of DPP−Fe(III) displayed a CD spectrum indicative of a mainly random conformation. A 4:2 DPP−Fe(III)/peptide mixture produced a CD spectrum with minima at 208 and 222 nm, indicating a large degree of helical content. Size-exclusion chromatography showed that the 4:2 peptide/DPP−Fe(III) species exists in a single aggregation state with an apparent molecular weight ($MW_{app} = 20{,}200$) consistent with the design ($MW_{calc} = 17{,}230$), while the apopeptide displayed a longer retention time that was consistent with a much smaller molecular weight. Analytical ultracentrifugation confirmed the peptide alone exists in a predominantly monomeric form below 200 μM and that the assembled protein sediments as a single species with MW = 17,230 ± 69.

The EPR spectrum of the assembled protein at 10 K displayed a signal at $g = 3.35$ and can be characterized as a highly anisotropic low-spin (HALS), or type-I, spectrum (Figure 3).[14] This EPR signal is diagnostic of low-spin, six-coordinate Fe(III) porphyrin centers with coordinating imidazole rings in near perpendicular alignment. This result is consistent with the design, which included putative second-shell histidine N(δ) to threonine O(γ) interactions to enforce this orientation. The T12A variant peptide bound DPP−Fe(III) yet displayed a rhombic, type-II EPR spectrum ($g = 3.08, 2.21, 1.40$), similar to that seen in unconstrained "maquette" systems.[2] These results demonstrate the use of second-shell interactions to modulate the structural and electronic properties of the Fe centers.

The 1-D ^{1}H NMR spectrum of the assembled protein has well-dispersed peaks along with broad peaks outside the diamagnetic window, resulting from proton nuclei in close proximity to the paramagnetic Fe(III) centers. These results suggest a well-packed interior suitable for further structural studies.

Potentiometric studies found an apparent $E_{1/2}(Fe^{2+/3+}) = 103$ mV vs NHE. This value is 180 mV more positive than a similar de novo-designed four-helix bundle protein containing the natural Fe(III) protoporphyrin IX cofactor[15] and is consistent with established redox properties of these Fe porphyrins in bis(imidazole) environments.[16] Since the midpoint potential is similar to that of DPP−Fe(N-Me-imidazole)$_2$ in CH_2Cl_2 (81 mV vs NHE), the cofactor is most likely bound in the relatively hydrophobic microenvironment defined by the design.

Initial studies reveal remarkable cofactor selectivity. A 4:2 mixture of peptide and heme (Fe(III) protoporphyrin IX) displayed a similar UV−vis spectrum to that of free heme (Figure 2). The CD spectrum of this peptide/heme mixture was nearly identical to that of the unfolded peptide. These findings indicate a lack of defined assembly involving peptide and the heme cofactor under these conditions. This dramatic display of cofactor discrimination strongly suggests a successful design with a nativelike hydrophobic core specific for the shape of the DPP−Fe unit.

In conclusion, we have computationally designed a four-helix bundle protein that selectively binds a nonbiological metalloporphyrin cofactor. Successful incorporation of a nonbiological cofactor indicates the design methodology is robust and may be extended beyond purely natural systems. Detailed structural studies are underway to uncover the basis of cofactor selectivity. Preliminary studies indicate the assembled protein displays functional redox properties previously unobtainable with natural cofactors. These findings open a path for the selective incorporation of more elaborate cofactors[17,18] into designed scaffolds to construct molecularly well-defined nanoscale materials.

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Supporting Information Available: MALDI-MS, HPLC, DPP−Fe binding titration, NMR and CD spectroscopy, size-exclusion chromatography, sedimentation equilibrium, redox potentiometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Acid-catalyzed condensation of dipyrrylmethane and an ester-substituted benzaldehyde was followed by treatment with DDQ to give an ester-protected form of the porphyrin core. Metalation of the porphyrin macrocycle was achieved with $FeCl_2 \cdot 4H_2O$ in refluxing THF and ethanol. Saponification with NaOH provided DPP−Fe(III)Cl.
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(16) DPP−Fe(N-Me-imidazole)$_2$: $E_{1/2}(Fe^{2+/3+}) = -160$ mV vs SCE (81 mV vs NHE); iron protoporphyrin IX dimethylester(N-Me-imidazole)$_2$: $E_{1/2}(Fe^{2+/3+}) = -410$ mV vs SCE (−169 mV vs NHE). Experimental conditions: solvent = CH_2Cl_2; [metalloporphyrin] = 1−2 mM; [$TBAClO_4$] = 0.1 M.
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