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# Design of a Five-Coordinate Heme Protein Maquette: A Spectroscopic Model of Deoxymyoglobin

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The substitution of 1-methyl-L-histidine for the histidine heme ligands in a de novo designed four-α-helix bundle scaffold results in conversion of a six-coordinate cytochrome maquette into a self-assembled five-coordinate mono-(1-methyl-histidine)-ligated heme as an initial maquette for the dioxygen carrier protein myoglobin. UV–vis, magnetic circular dichroism, and resonance Raman spectroscopies demonstrate the presence of five-coordinate mono-(1-methyl-histidine) ligated ferrous heme spectroscopically similar to deoxymyoglobin. Thermodynamic analysis of the ferric and ferrous heme dissociation constants indicates greater destabilization of the ferric state than the ferrous state. The ferrous heme protein reacts with carbon monoxide to form a (1-methyl-histidine)–Fe(II)(heme)–CO complex; however, reaction with dioxygen leads to autoxidation and ferric heme dissociation. These results indicate that negative protein design can be used to generate a five-coordinate heme within a maquette scaffold.

As observed for many biological cofactors, hemes serve a variety of roles in biological systems.[1] The chemical properties and hence the biological functions of the heme are modulated by both the primary coordination sphere of the iron as well as the surrounding protein matrix. Metalloprotein design efforts seek to harness the innate reactivity of metal cofactors by the fabrication of protein environments containing suitable metal ion coordination spheres.[2] Constructive heme protein design approaches have afforded a variety of synthetic metalloproteins as models for natural proteins involved in biological electron transfer and catalysis.[3]

Since natural heme proteins often bind the heme moiety via histidine $N^{\epsilon}$ coordination within α-helical domains, the majority of de novo heme proteins have been designed to utilize a bis-histidine axial coordination motif within α-helical protein scaffolds.[4] One class of these designed heme proteins, the heme protein maquettes,[5] have shown their utility in revealing critical aspects of heme protein electrochemical function relevant to natural bis-His coordinated cytochromes.[6] We have initiated a program to expand the repertoire of amino acid ligands available for heme protein design using a maquette scaffold.[7] Our approach is to delineate the thermodynamic and spectroscopic consequences of ligand alterations in this self-assembly system in an effort to use rational design to generate heme proteins with altered coordination spheres and chemistries.

Herein, we design a water soluble and stable four-α-helix bundle protein containing a five-coordinate ferrous heme to demonstrate that non-natural amino acids can be used to design myoglobin mimetics. The protein scaffold was designed to utilize 1-methyl-L-histidine to coordinate the

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heme iron. Methylation of the $N^{\epsilon}$ of histidine obviates this common binding motif and necessitates binding via the $N^{\delta}$ of histidine, a binding mode previously used to design a bis-His ligated heme protein.[3a] This coordination mode is observed in only one natural heme protein, His-102 of the bis-His coordinated cytochrome $c_{554}$.[8] The designed primary structure of each peptide ligand helix, $[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ ($NH_2-$CGGGEIWKL H1m EEFIKLFEERIKKL$-CONH_2$ where H1m is 1-methyl-L-histidine), is related to that of the $[\Delta 7\text{-H}_{10}\text{I}_{14}\text{I}_{21}]_2$ bis-histidine ligated cytochrome maquette by a single amino acid modification per helix at position 10 (His → H1m).[7a] The peptide ligand was prepared using solid-phase peptide synthesis and purified to homogeneity by RP-HPLC as described for the histidine analogue.[7a] In aqueous solution, the peptide assembles as a noncovalent dimer of disulfide bridged di-α-helical peptides. The data show that $[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ binds a single Fe(II)(protoporphyrin IX) cofactor to generate a five-coordinate ferrous heme protein. The detailed spectroscopic analysis presented here suggests that this self-assembled heme protein is a spectroscopic model for the deoxy state of the dioxygen transporter myoglobin.

The introduction of 1-methyl-L-histidine into the heme protein maquette scaffold has minimal effects on the protein secondary structure and global folding stability. The far-UV circular dichroism spectrum of apo-$[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ has minima at 208 and 222 nm with a maximum at 192 nm illustrative of the designed helical secondary structure. The calculated helical content, 68% helix based on $\Theta_{222}$, is similar to the 67% observed for the histidine analogue.[7a] Furthermore, sedimentation equilibrium analytical ultracentrifugation shows the protein folds into the designed dimeric oligomerization state. Isothermal chemical denaturation studies using guanidine hydrochloride as a chaotropic agent demonstrate $[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ is stable in the apo-form, $-\Delta G^{H_2O} = 11.0$ kcal/mol at 298 K ($[\text{Gdn}\cdot\text{HCl}]_{1/2}$ value of 1.8 M; *m* value of 1.8). Thus, the protein exists as a stable four-α-helix bundle, as designed.

Fe(II)(protoporphyrin IX), ferrous heme, was incorporated into the $[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ peptide ligand under anaerobic conditions using standard literature procedures and followed by UV−vis spectroscopy.[7b] Addition of ferrous heme to protein solutions results in a shift in the optical spectrum of the heme to 431 nm ($\epsilon$ of 97 $mM^{-1}$ $cm^{-1}$, Soret band) and 554 nm ($\epsilon$ of 12 $mM^{-1}$ $cm^{-1}$, $\alpha/\beta$ bands) indicative of heme iron coordination. As shown in Figure 1, these values are similar to those observed for deoxymyoglobin[9] ($\lambda_{max}$ of 430 nm, $\epsilon$ of 125 $mM^{-1}$ $cm^{-1}$) and distinct from those observed for the bis-histidine coordinated cytochrome $b_5$ ($\lambda_{max}$ of 426 nm, $\epsilon$ of 162 $mM^{-1}$ $cm^{-1}$ with well resolved $\alpha/\beta$ bands).[10] Isothermal titration of heme into protein, at 29 $\mu$M four-α-helix bundle concentration, evinces formation of a 1:1 heme/four helix bundle complex, ferrous-monoheme-$[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$. The $K_{d1}$ value measured from a fit to the titration data is 5 $\mu$M, a value only 125-fold (2.8 kcal/mol) weaker than that measure for the analogous bis-His maquette.[7a]

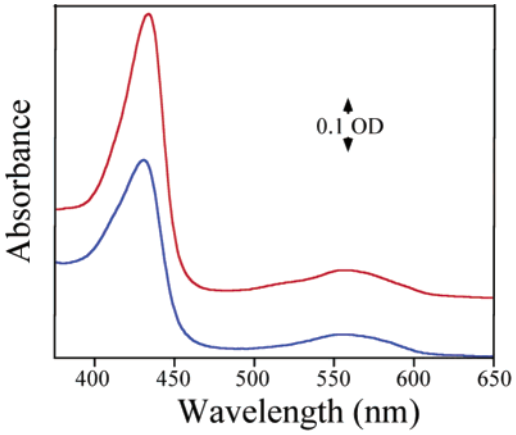


**Figure 1.** (A) Comparison of the optical spectra of ferrous monoheme-$[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ (blue) and myoglobin (red), offset for clarity. Both experiments were performed at 4.33 $\mu$M protein concentration in 20 mM potassium phosphate, 100 mM KCl, pH 8.0.

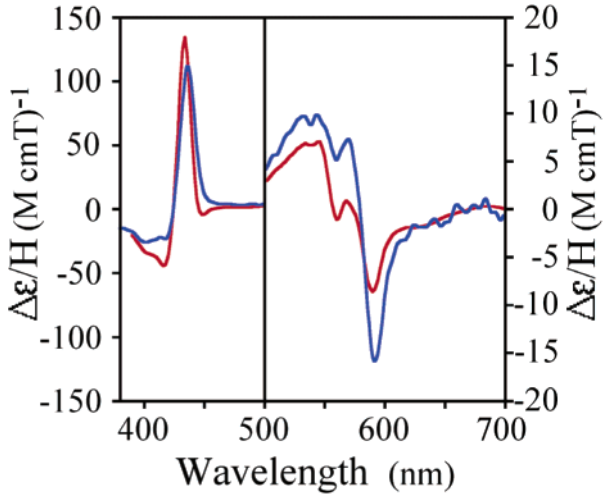


**Figure 2.** Comparison of the magnetic circular dichroism spectra of ferrous monoheme-$[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$ (blue) and ferrous myoglobin (red). The experiments were performed with 14.7 $\mu$M protein in 20 mM potassium phosphate, 100 mM KCl, pH 8.0 buffer.

Attempts to bind Fe(III)protoporphyrin IX failed to show any UV−vis evidence of H1m coordination of the heme iron. The data suggest a lower limit for the ferric heme $K_{d1}$ value to be weaker than 100 $\mu$M. This estimated $K_{d1}$ value is at least 700 000-fold (> 8.0 kcal/mol) weaker than the corresponding bis-His site in $[\Delta 7\text{-H}_{10}\text{I}_{14}\text{I}_{21}]_2$.[7a] The data show that conversion of the bis-His site into a mono-H1m site destabilizes the ferric state to a greater extent than the ferrous state.

Further evidence for mono-(1-methyl-histidine) coordination was obtained using magnetic circular dichroism (MCD) and resonance Raman (rR) spectroscopies. The MCD spectroscopy of hemes is highly sensitive to the nature of the axial ligands.[11] The MCD spectrum of ferrous-monoheme-$[\Delta 7\text{-H1m}_{10}\text{I}_{14}\text{I}_{21}]_2$, shown in Figure 2 (blue trace), demonstrates that the bound ferrous heme is five-coordinate and

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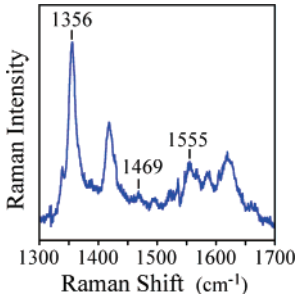


**Figure 3.** Resonance Raman characterization of 114 $\mu$M ferrous monoheme-[$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$ (441.6 nm excitation). The experiment was performed in 20 mM potassium phosphate, 100 mM KCl, pH 8.0 buffer.

high-spin. In addition, the spectral similarity to deoxymyoglobin[12] (shown in red) is indicative of coordination by a single imidazole ligand in [$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$.

Since iron porphyrin vibrations are coupled to metal ion oxidation and spin states, resonance Raman is also a sensitive probe of the coordination environment of the iron in the heme chromophore.[13] Figure 3 shows that the porphyrin marker bands of the ferrous heme bound to [$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$ appear at 1555 cm$^{-1}$ ($\nu_2$), 1469 cm$^{-1}$ ($\nu_3$), and 1356 cm$^{-1}$ ($\nu_4$) which are values characteristic of a five-coordinate high-spin ferrous heme.[14] This spectrum compares favorably with that observed for deoxymyoglobin, 1557 cm$^{-1}$ ($\nu_2$), 1472 cm$^{-1}$ ($\nu_3$), and 1355 cm$^{-1}$ ($\nu_4$).[15] Hence, UV−vis, MCD, and resonance Raman spectroscopies show that the predominant species in ferrous monoheme-[$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$ is five-coordinate.

Having generated a vacant coordination site on the iron, the reactivity of the ferrous heme toward two diatomic ligands known to coordinate to myoglobin, carbon monoxide and dioxygen, was investigated.[1a] Exposure of the ferrous heme-[$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$ to 1 atm of carbon monoxide results in a shift of the optical spectrum to that characteristic of a hexacoordinate heme−CO complex as shown in Figure 4.[9] The UV−vis spectrum of the carbonmonoxide complex, 419 nm ($\epsilon$ of 164 mM$^{-1}$ cm$^{-1}$, Soret band), 536 nm ($\epsilon$ of 14 mM$^{-1}$ cm$^{-1}$) and 565 nm ($\epsilon$ of 15 mM$^{-1}$ cm$^{-1}$), is similar to those observed for carbonmonoxy-myoglobin and previously designed ferrous-CO heme protein complexes.[3a] Efforts

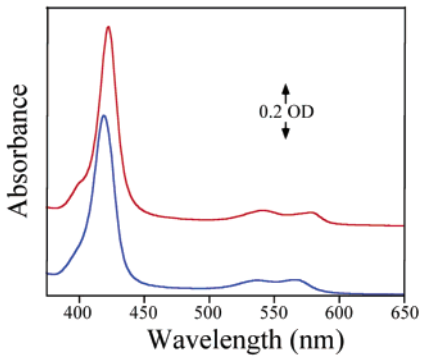


**Figure 4.** UV−vis comparison of ferrous carbonmonoxy complexes of monoheme-[$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$ (blue) and myoglobin (red), offset for clarity. Each experiment was performed at 4.33 $\mu$M protein concentration in 20 mM potassium phosphate, 100 mM KCl, pH 8.0.

to form a stable dioxygen adduct have proven unsuccessful. Dilution of ferrous heme-[$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$ into oxygenated buffers leads to optical spectra consistent with autoxidation and heme dissociation.

We have demonstrated the generation of a well-defined five-coordinate heme protein without covalent attachment of the heme to the peptide. By preventing the favored coordination mode of histidine by methylation, i.e., negative design,[16] we have utilized the histidine N$^{\delta}$ to generate a five-coordinate high-spin ferrous heme protein similar to deoxymyoglobin. The loss of one axial ligand destabilizes the ferric state to a greater extent than the ferrous state. Future studies are directed at exploring the reactivity of this complex as well as improving the stability of the ferric state and, therefore, the dioxygen adduct.

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**Supporting Information Available:** HPLC purification, mass spectrometry, CD spectrum, analytical ultracentrifugation solution molecular weight determination, UV−vis $K_d$ measurement, experimental details of the rR and MCD measurements of [$\Delta$7-H1m$_{10}$I$_{14}$I$_{21}$]$_2$, and UV−vis data on the reaction with dioxygen. This material is available free of charge via the Internet at http://pubs.acs.org.

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