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# Nonnatural Amino Acid Ligands in Heme Protein Design

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The de novo design of heme proteins is an active field of chemical research aimed at delineating the structure−function relationships of natural heme protein catalysis.[1] The constructive approach of de novo heme protein design has been implemented using both rational[2] and combinatorial[3] methodologies. These studies have provided insight into natural heme protein sequence design, the effects of cofactor incorporation on protein folding, stability, and structure as well as the modulation of heme electrochemistry by the protein matrix.[1]

We are utilizing peptide-based coordination complexes of heme, the heme protein maquettes, as aqueous soluble and stable synthetic analogues of natural heme proteins.[4] Presently our interest is in expanding the repertoire of ligands available for heme protein design since the majority of designed heme proteins utilize axial bis-histidine coordination to incorporate the heme iron.[1a] Our approach is to access a variety of nonnatural amino acids by using solid-phase peptide synthesis methodologies coupled with de novo protein design.

In this contribution, we introduce 4-$\beta$-(pyridyl)-L-alanine (Pal) as a nonnatural heme ligand in a designed four-α-helix bundle to demonstrate bis-pyridine coordination to heme iron in a protein environment. Furthermore, the effects of bis-pyridine ligation are evaluated in comparison to that of a bis-histidine analogue. The designed primary structure of each helix of the pyridylalanine-containing peptide ligand,[5] [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ (*Ac*-CGGGEIWLK-(Pal)EEFIKKFEERIKKL-*CONH*$_2$) is related to that of the [Δ7-$H_{10}I_{14}I_{21}$]$_2$ bis-histidine-ligated heme protein maquette by a single amino acid modification per helix at position 10 (His→Pal).[6] The peptides assemble as a noncovalent dimer of disulfde-bridged dipeptides, the (α-SS-α)$_2$ maquette architecture. The data demonstrate [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ binds two Fe(II)(protoporphyrin IX) cofactors, hemes B, as designed. However, [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ binds only a single oxidized heme some 60000-fold weaker than the bis-histidine analogue, inducing a 287 mV higher equilibrium midpoint reduction potential.

The solution oligomerization state of [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ was evaluated using both FPLC size exclusion chromatography and sedimentation equilibrium analytical ultracentrifugation. Size exclusion chromatography at pH 8 demonstrates that the protein exists as a single species in solution with a molecular weight consistent with that of a dimer in solution, as designed. Molecular weights determined from sedimentation equilibrium analytical ultracentrifugation at pH 3.5, 8, and 11 are fully consistent with a dimeric oligomerization state of the di-α-helix peptide monomers over this broad pH range. Moreover, incorporation of heme does not alter the solution oligomerization state as evidenced by sedimentation equilibrium analytical ultracentrifugation.

The secondary structure and global folding stability of the protein scaffold was probed using circular dichroism (CD) spectroscopy.[7]

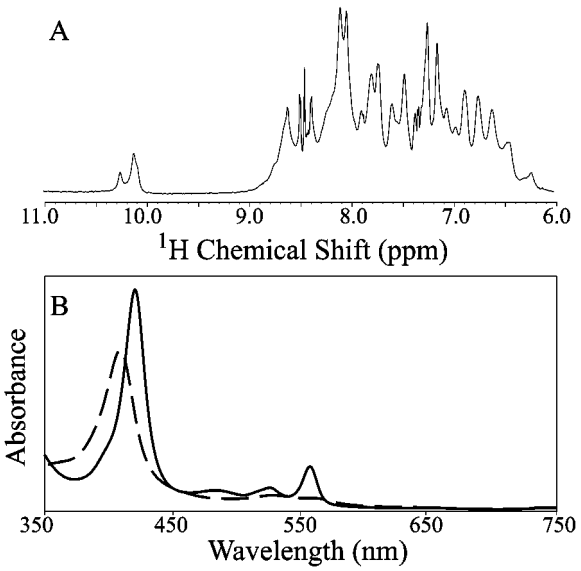


***Figure 1.*** (A) Aromatic amide region of the apo-[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ $^1$H NMR spectrum. (B) UV−visible spectrum of monoheme−[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ in the oxidized (dashed) and reduced (solid) states.

The CD spectrum of the [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ scaffold has minima at 208 and 222 nm with a maximum at 192 nm, demonstrative of the designed helical secondary structure. The calculated helical content, 75% helix based on $\Theta_{222}$, is uniform between pH 3.5 and 11. Thus, between pH 3.5 and 11 the protein exists as a four-α-helix bundle in solution, as designed. Isothermal chemical denaturation studies using guanidine hydrochloride as a chaotropic agent demonstrate [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ is stable in the apo-form, $-\Delta G^{H_2O} = 14.6$ kcal/mol at 298 K ([Gdn·HCl]$_{1/2}$ value of 3.4 M; *m* value of 2.04).

Figure 1A shows the $^1$H NMR spectrum of the apopeptide which demonstrates that this apo-maquette scaffold approaches a singular structure in solution. The ∼20 Hz proton line widths and good chemical shift dispersion are consistent with a scaffold approaching suitability for future NMR structure analysis. Thus, the scaffold designed is both stable and approaching conformational specificity in the apo-state.

The heme cofactor was incorporated into the [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ peptide ligand using standard literature procedures.[2a] The reduced Fe(II)(protoporphyrin IX) cofactor was incorporated under anaerobic conditions and followed by UV−visible spectroscopy. The titration curve measured at a protein concentration of 872 nM clearly indicates tight formation of a 2:1 heme:four-helix bundle complex, as designed. A fit to the data indicates both reduced state $K_d$ values are tighter than 80 nM.

Oxidized heme titrations followed by UV−visible spectroscopy demonstrate that the first heme binds with a 60 $\mu$M dissociation constant ($K_{d1}$). This dissociation constant is ∼60000-fold (6.5 kcal/mol) weaker than the higher limit estimate of the first heme binding

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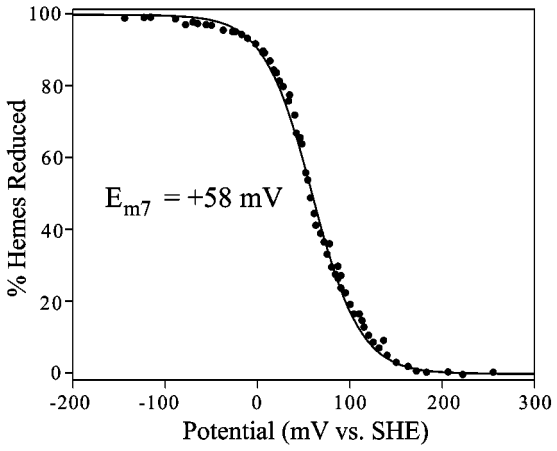


***Figure 2.*** Equilibrium reduction potential of monoheme−[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ at pH 7. The data are fit to a single $N = 1$ Nernst curve.

to the analogous histidine protein, [Δ7-$H_{10}I_{14}I_{21}$]$_2$ ($K_{d1}$ tighter than 1 nM). The second oxidized heme binding constant of [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ could not be determined since it was above the solubility limit of the apopeptide, ~600 $\mu$M. A similar heme affinity shift of ~60000-fold for the second heme suggests that the $K_{d2}$ of [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ may be as high as 24 mM, a value consistent with the observed lack of binding.

The optical spectral properties of this pyridylalanine-ligated heme protein are distinct from those of previous heme protein maquettes which employ histidine axial ligands.[8] UV−vis spectroscopy of the oxidized monoheme−[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ complex ($\lambda_{max}$ at 410 nm; $\epsilon$ of 110 000 $M^{-1}$ $cm^{-1}$) is fully consistent with a bis-pyridine-ligated heme as shown in Figure 1B. One-electron reduction by sodium dithionite results in a shift in the Soret maximum to 420 nm ($\epsilon$ of 144 000 $M^{-1}$ $cm^{-1}$) and resolution of the $\alpha/\beta$ bands at 525 and 557 nm ($\epsilon$ of and 14 400 and 33 500 $M^{-1}$ $cm^{-1}$). The spectral properties of this maquette are similar to those of the pyridine hemochrome and indicate bis-pyridylalanine ligation in both oxidation states.[9]

Further evidence for bis-pyridylalanine axial ligation to the oxidized heme is provided by EPR spectroscopy. The EPR spectrum of monoheme−[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ at 20 K shows a resonance at $g =$ 3.48 consistent with a highly anisotropic low-spin (HALS) Fe(III) heme.[10] This EPR signal is distinct from both typical high-spin (axial, $g$-values of 6.0 and 2.0) and low-spin (rhombic, $g$-values below 3.0) Fe(III) heme resonances and is nearly identical to the reported EPR spectrum of Fe(III)(protoporphyrin IX)(pyridine)$_2$.[11] Thus, this peptide provides the initial example of a designed protein containing a heme ligated by a nonnatural amino acid.

The electrochemistry of monoheme−[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ was evaluated using redox potentiometry[12] monitored by UV−visible spectroscopy as shown in Figure 2. The observed increase in the Soret absorption at 420 nm upon lowering the solution potential is accurately described by a single $N = 1$ Nernst equation. The equilibrium midpoint reduction potential at pH 7, $E_{m7}$, +58 ± 8 mV versus SHE is considerably higher than that for the histidine-containing peptide, monoheme−[Δ7-$H_{10}I_{14}I_{21}$]$_2$ and only slightly lower than the +100 mV midpoint potential of Fe(PPIX)(pyr)$_2$ at pH 7. In fact, comparison to other designed heme proteins demonstrates that monoheme−[Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ has one of the most positive heme reduction potentials at pH 7 consistent with the observed weak Fe(III)protoporphyrin affinity and tight Fe(II)-protoporphyrin affinity.[13] Thus, the amino acid sequence change His→Pal evinces a major effect on the heme electrochemistry (287 mV, 6.8 kcal/mol) due to the alteration of the Fe(III) and Fe(II) coordination equilibria.[14]

In the present work, we have demonstrated the feasibility of using a nonnatural amino acid side chain to ligate the iron of a heme cofactor within a designed protein scaffold. Changing the identity of the axial ligands alters the resulting heme affinity and spectroscopy, as expected. Furthermore, we have illustrated the significant effect that coordination equilibrium has on redox cofactor equilibrium midpoint reduction potentials. Our efforts are now focused on improving the binding of the second oxidized heme and developing other nonnatural amino acid ligands for metalloprotein design.

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**Supporting Information Available:** HPLC, mass spectroscopy, CD, sedimentation equilibrium analytical ultracentrifugation, oxidized and reduced heme titration curves, and EPR spectrum of [Δ7-$Pal_{10}I_{14}I_{21}$]$_2$ (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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