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. The constructive approach of de novo heme protein design has been implemented using both rational and combinatorial 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.

We are utilizing peptide-based coordination complexes of heme, the heme protein maquettes, as aqueous soluble and stable synthetic analogues of natural heme proteins. 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. 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-β-(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, [(7-Pal)$_{10}$(I$_{14}$I$_{21}$)$_2$-(Ac-CGGGEIWKL-Pal)EEIKKEERIKKL-CO$_2$H]$_2$ is related to that of the [7-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). The peptides assemble as a noncovalent dimer of disulfide-bridged dipetides, the (α-SS-α) maquette architecture. The data demonstrate [7-Pal$_{10}$(I$_{14}$I$_{21}$)$_2$] binds two Fe(II)(protoporphyrin IX) cofactors, hemes B, as designed. However, [7-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 [7-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.
to the analogous histidine protein, [Ala-His$_{1}$]-{Pal$_{7}$} (Kd$_{1}$, tighter than 1 nM). The second oxidized heme binding constant of [Ala-His$_{1}$]-{Pal$_{7}$} could not be determined since it was above the solubility limit of the apoprotein, ~600 μM. A similar heme affinity shift of ~6000-fold for the second heme suggests that the Kd of [Ala-His$_{1}$]-{Pal$_{7}$} 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. UV–vis spectroscopy of the oxidized monoheme-[Ala-His$_{1}$]-{Pal$_{7}$} complex ($\lambda_{\text{max}}$ 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 ($g$ reported EPR spectrum of Fe(III)(protoporphyrin IX)(pyridine) 2.11 This EPR signal is distinct from both typical high-spin (5) the monoheme-[Ala-His$_{1}$]-{Pal$_{7}$} protein are distinct from those of previous heme protein maquettes –6000-fold for the second heme suggests that the Kd of [Ala-His$_{1}$]-{Pal$_{7}$} at pH 7. The data are fit to a single N = 1 Nernst curve.

The electrochemistry of monoheme-[Ala-His$_{1}$]-{Pal$_{7}$} was evaluated using redox potentiometry$^{12}$ monitored by UV–vis spectrophotometry 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, $E_{\text{m7}}$, +58 ± 8 mV versus SHE is considerably higher than that for the histidine-containing peptide, monoheme-[Ala-His$_{1}$]-{Pal$_{7}$}; and only slightly lower than the +100 mV midpoint potential of Fe(PIX)pyr$_{2}$ at pH 7. In fact, comparison to other designed heme proteins demonstrates that monoheme-[Ala-His$_{1}$]-{Pal$_{7}$} 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$\rightarrow$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 spectrometry, CD, sedimentation equilibrium analytical ultracentrifugation, oxidized and reduced heme titration curves, and EPR spectrum of [Ala-His$_{1}$]-{Pal$_{7}$} (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(5) The [Ala-His$_{1}$]-{Pal$_{7}$} protein was synthesized using standard Fmoc/tBu solid-phase peptide synthesis methodologies using 4-fold excess HBTU/HOBt amino acid activation. Following chain assembly, the peptide was cleaved from the resin with concomitant amino acid side chain deprotection with water:ethanedithiol:trifluoroacetic acid (2:8:90 v/v/v) followed by precipitation with cold ether. The crude peptide was purified to homogeneity by C8 RP-HPLC and evaluated for purity and identity by analytical HPLC and mass spectrometry, respectively. The N-terminal disulfide was formed by air oxidation in 100 mM ammonium carbonate buffer (pH 9.8) followed by analytical HPLC.


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