

Cyclic and Hairpin Peptide Complexes of Heme

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The de novo design¹ of proteins has progressed to the level where one can design complex target folds with natively like properties. Incorporation of metal-based cofactors is an important requirement toward making these systems functional.^{2–6} We have utilized amphiphilic peptide sequences (15- to 17-mers),² where a central histidine provides ligation to a heme, [Fe^{III}(coproporphyrin-I)]⁺, and the hydrophobic effect is used to optimize heme–peptide complex stability. In previous work,² we examined the effect of these hydrophobic residues and discovered that noncoordinating hydrophobic residues flanking a central histidine stabilized complexation by as much as 4.5 kcal/mol. We have now incorporated disulfide bridges between amphiphilic peptides to make hairpin and even cyclic peptides that bind heme extremely well, roughly 5×10^6 times more strongly than histidine itself. These are minimalist analogues of the b-type cytochromes.

Disulfide bridges are ubiquitous structural elements in proteins; they stabilize and help determine the structure of many proteins.^{7,8} The use of disulfide linkages to induce turns in peptide structures, however, is nearly unexplored⁹ and has not been previously successful for iron porphyrin complexes.^{5b} We have incorporated disulfide bridges at the termini of two de novo designed, porphyrin binding peptides,¹⁰ LP-AA-A and LP-FF-A, using solid-phase synthesis and selective, stepwise disulfide formation in solution.¹¹ The resulting sequences (Figure 1) were designed to form hairpin^{12a} structures (Figure 1a; one disulfide bridge; HP-AA, where alanine residues make the primary contact with the heme) and cyclic^{12b} structures (Figure 1b; two disulfide bridges; CP-AA). A second series of peptides (abbreviated with -FF- using Phe in place of Ala at residues 4, 5, 11, and 12) were synthesized to probe the importance of a hydrophobic effect¹³ in these systems.

Binding affinities were determined by spectrophotometric titration^{2,14} and are presented in Table 1. Despite the different stoichiometries¹⁵ for binding the hairpin or cyclic peptides as compared to the 15-mers or simple imidazoles, it is possible to compare binding properties if we choose a fixed peptide concentration. At 2.7 μM , one-half of the [Fe^{III}(coproporphyrin-I)]⁺ will be bound to CP-FF. As shown in Table 1 and Figure 2, the ratio of free heme to peptide-bound heme at 2.7 μM peptide concentration decreases slightly for the matching hairpin peptide, HP-FF. Replacement of the phenylalanine residues in putative contact with the heme face by alanine decreases binding 6-fold. The overwhelming importance of the preorganization of the two binding sites is dramatically shown by the 6000-fold decrease in the free to bound heme ratio for LP-FF-A when compared at 2.7 μM . The hydrophobicity of the peptides is also a significant component of heme binding; there is a nearly 1000-fold further decrease in the free/bound ratio for histidine itself.

The effect of heme ligation on the secondary structure of these peptides has been examined using UV circular dichroism (Table 1

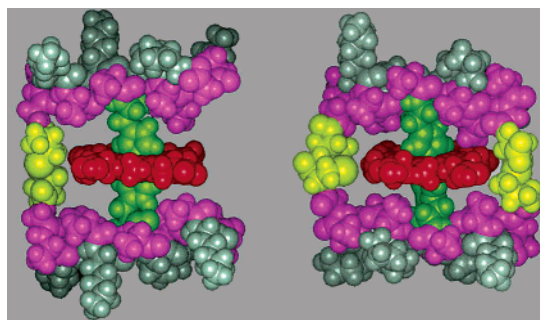


Figure 1. Computer generated models of hairpin peptide [Fe^{III}(coproporphyrin-I)(HP-AA)]⁺ and cyclic peptide [Fe^{III}(coproporphyrin-I)(CP-AA)]⁺.¹⁰ Energy minimized using Insight and Discover (MSI) with the ESFF force-field. Hydrophobic residues, magenta; polar residues, gray; histidine, green; cysteine, yellow; porphyrin, red.

Table 1. Properties of Heme–Peptide Complexes

ligand ¹⁰	K^a	[bound]/[free] ^b	θ_{apo}^c (deg cm ² / dmole)	H_{apo}^d (%)	θ_{heme}^e (deg cm ² / dmole)	H_{heme}^f (%)
CP-FF	386 mM ⁻¹	1	2820	34	32 500	88
CP-AA	64 mM ⁻¹	0.17	8400	23	33 800	91
HP-FF	277 mM ⁻¹	0.72	4910	13	11 200	30
HP-AA	42 mM ⁻¹	0.11	2620	7	25 200	68
LP-FF-A	6.0 mM ⁻²	1.6×10^{-4}	221	0.7	5400	16
LP-AA-A	0.23 mM ⁻²	6.0×10^{-6}	570	1.7	2560	8
His	0.008 mM ⁻²	2.2×10^{-7}				

^a Association constants for ligation of peptide to porphyrin. ^b Calculated ratio of free to peptide-ligated heme at the half-saturation concentration of the cyclic peptide complex, CP-FF (2.7 μM). 5.4 μM was used for single His peptides. ^c Molar ellipticity of peptide (1–20 μM , pH 7.0, 2 mM KH₂PO₄, 2 °C) in the absence of heme. ^d Helicity of the apo peptide. % helix = $(\theta_{\text{obs}}/\theta_{\text{max}}) \times 10^2$, where $\theta_{\text{max}} = 40\,000(1-2.5/n)$, and n is the number of residues.¹⁷ ^e Molar ellipticity after saturation with heme. ^f Helicity in the presence of heme.

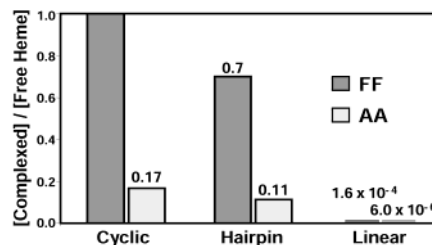


Figure 2. Comparison of the fraction of unbound to bound heme at the half-saturation point of the cyclic peptide complex, CP-FF. [coproporphyrin-I] = 2.7 μM . Gray, FF series; white, AA series; all measurements in 500 μM MOPS, pH 7.4, 25 °C.

and Supporting Information). Complete helicity ($\sim 90\%$) is observed for the cyclic peptides in the presence of the porphyrin. These data emphasize that the heme plays an important role in heme protein structure and stability, in addition to its diverse functions at the active site. The CP-XX systems possess a tertiary coiled-coil struc-

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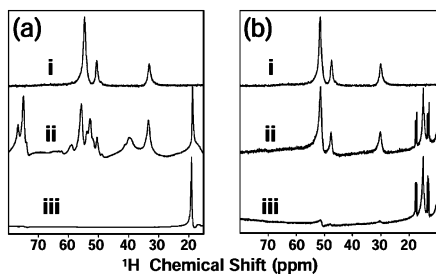


Figure 3. ^1H NMR spectra of the titration of $[\text{Fe}^{\text{III}}(\text{coproporphyrin-I})]^+$ with (a) LP-AA-A at (i) 0 equiv of peptide, (ii) partial complexation with 4 equiv of peptide, and (iii) saturation with 30 equiv of peptide, and with (b) CP-AA at (i) 0 equiv of peptide, (ii) partial complexation with 0.7 equiv of peptide, and (iii) saturation with 4 equiv of peptide. Porphyrin concentrations were 3 mM with pH 7.4, 50 mM KH_2PO_4 , 10 mM KCl, in 90% $\text{H}_2\text{O}/10\%$ D_2O , at 4 $^\circ\text{C}$.

ture, as shown by the ratio¹⁸ of $\theta_{222}/\theta_{208}$, which is ~ 1.0 . As with a leucine-zipper,¹⁹ our cyclic peptides are composed of two parallel helices.

NMR spectra of paramagnetic complexes of the peptides reveal an interesting contrast between the heme binding properties of the 15-mers and the cyclic peptides: the 15-mers are able to form a high-spin ferric complex, whereas the cyclic peptides do not, due to their preorganization of a second histidine. Figure 3 shows the ^1H NMR spectra of $[\text{Fe}^{\text{III}}(\text{coproporphyrin-I})]^+$ under three different conditions: no peptide added, partial complexation, and complete ligation. The spectrum of $[\text{Fe}^{\text{III}}(\text{coproporphyrin-I})]^+$ partially ligated to LP-AA-A is related to those of myoglobin or cytochrome-c peroxidase²⁰ in the met-aquo state ($\text{Fe}^{\text{III}}(\text{porph})(\text{H}_2\text{O})(\text{his})^+$), with only one histidine coordinated to the iron ion. The high-spin ($S = 5/2$) metal center causes significant paramagnetic shifting ($\delta \approx 77$ ppm) of residue side-chain protons close to the metal center (Figure 3a,ii). Only upon addition of a large excess of LP-AA-A are all of the high-spin components converted to the low-spin ($S = 1/2$) species with two histidines coordinated, as shown by the absence of peaks with $\delta > 20$ ppm (Figure 3a,iii). In contrast, titration of $[\text{Fe}^{\text{III}}(\text{coproporphyrin-I})]^+$ with the cyclic peptide CP-AA never shows side-chain resonances with $\delta > 20$ ppm, meaning that no high-spin species are ever present. In the cyclic peptides, both histidines bind forming an $\text{Fe}^{\text{III}}(\text{porph})(\text{his})_2^+$ low-spin complex, as confirmed by the ^1H NMR, UV-vis, EPR, and ESI-MS spectra.¹⁵

Under conditions that ensure formation of $\text{Fe}^{\text{III}}(\text{porph})(\text{his})_2^+$, there is little variation in the redox potentials of our peptide complexes in water. The $E_{1/2}$ of the Fe(coproporphyrin-I) complexes of the AA series peptides are all -220 ± 5 mV versus NHE and -245 ± 7 mV versus NHE for the FF series. For comparison, the $E_{1/2}$ of Fe(coproporphyrin-I)(his)₂ is -214 mV versus NHE.² The redox potentials of b-type cytochromes are extremely sensitive to preparation and local environment and range from roughly -200 to $+400$ mV;²¹ multiple factors must contribute to this range, but their relative importance has yet to be established.

In conclusion, we have demonstrated the effective use of interpeptide disulfide bridges to produce hairpin-turn and cyclic peptides. The preorganization so created dramatically increases the binding of such peptides to heme. The disulfide bridges also result in a substantial increase in the helicity of the peptides, both before and after heme ligation. In the cyclic peptides, histidine binding to the metal center appears to be cooperative.

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Supporting Information Available: CD spectra (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) DeGrado, W. F.; Summa, C. S.; Pavone, V.; Nistri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779–819 and references therein. (b) Gibney, B. R.; Rabanal, F.; Dutton, P. L. *Curr. Opin. Chem. Biol.* **1997**, *1*, 537–42.
- (2) Huffman, D. L.; Rosenblatt, M. M.; Suslick, K. S. *J. Am. Chem. Soc.* **1998**, *120*, 6183–6184.
- (3) Choma, C. T.; Lear, J. D.; Nelson, M. J.; Dutton, P. L.; Robertson, D. E.; DeGrado, W. F. *J. Am. Chem. Soc.* **1994**, *116*, 856–865.
- (4) Gibney, B. R.; Dutton, P. L. *Protein Sci.* **1999**, *8*, 1888–1898 and references therein.
- (5) (a) Liu, D.; Williamson, D. A.; Kennedy, M. L.; Williams, T. D.; Morton, M. M.; Benson, D. R. *J. Am. Chem. Soc.* **1999**, *121*, 11798–11812. (b) For related work on Co porphyrins with disulfide-linked noncyclic peptides, see: Arnold, P. A.; Shelton, W. R.; Benson, D. R. *J. Am. Chem. Soc.* **1997**, *119*, 3181.
- (6) Rojas, N. R. L.; Kamtekar, W.; Simons, C. T.; McLean, J. E.; Vogel, K. M.; Spiro, T. G.; Farid, R. S.; Hecht, M. H. *Protein Sci.* **1997**, *6*, 2512–2524.
- (7) Braxton, S. In *Protein Engineering*; Cleland, J. L., Craik, C. S., Eds.; Wiley-Liss: New York, 1996; pp 308–311 and references therein.
- (8) (a) Wedenmeyer, W. J.; Welker, E.; Narayan, M.; Scheraga, H. A. *Biochemistry* **2000**, *39*, 4207–4216. (b) Creighton, T. E. In *Protein Folding*; Creighton, T. E., Ed.; W. H. Freeman: New York, 1992; Chapter 7 and references therein.
- (9) (a) Kioshore, R.; Balaram, P. *Biopolymers* **1985**, *24*, 2041–2043. (b) Mihara, H.; Tomikazi, K.; Nishino, N.; Fujimoto, T.; Tamaoki, H.; Kobayashi, Y. *Biopolymers* **1994**, *34*, 963–967.
- (10) LP, linear peptide; HP, hairpin peptide; CP, cyclic peptide. Peptide sequences follow: LP-AA-A, AcGAKAAKAAHAKAAKAG-NH₂; LP-FF-A, AcGAKFFKAHAKFFKAGNH₂; HP-AA, (–C(Ac)GAKAAKAAHAKAAKAG–NH₂)₂; CP-AA, (–C(Ac)GAKAAKAAHAKAAKAG–NH₂)₂; HP-FF, (–C(Ac)GAKFFKAHAKFFKAG–NH₂)₂; HP-FF, (–C(Ac)GAKFFK–AHAKFFKAGC(NH₂)₂)₂.
- (11) Remmer, H. A.; Fields, G. B. In *Peptides and Protein Drug Analysis*; Reid, R. E., Ed.; Marcel Dekker Inc.: New York, 2000; pp 133–170.
- (12) (a) The hairpin peptide HP-AA was made by air oxidation of AcCGAKAAKAAHAKAAKAG–NH₂ in 100 mM NH_4HCO_3 , pH 9.5 for 24 h at room temperature. (b) Synthesis of the cyclic peptides was accomplished by incorporating orthogonally removable side-chain protecting groups at the terminal cysteines. The N-terminal cysteine was protected with the acid-labile triphenylmethyl (Trt) groups, while the C-terminal cysteine carried the acid-stable, but iodine-labile, acetamidomethyl (Acm) protection. After assembly of the linear peptide, all side-chain protecting groups except Acm were removed by TFA:water:thioanisole:ethanedithiol:phenol (82.5:5:5:2.5:5) cleavage; the peptide was simultaneously cleaved from the solid support. This was followed by HPLC purification and subsequent air oxidation to form the N-terminal disulfide bond using the same conditions as in 12a. Following purification, the C-terminal cysteines were deprotected and oxidized in one step using iodine in 50% acetic acid in water. Reactions were monitored by reversed-phase C₁₈ HPLC. All products were confirmed by ESI-MS and amino acid analysis.
- (13) (a) Dill, K. A. *Biochemistry* **1990**, *29*, 7133–7155. (b) Abraham, D. J.; Leo, A. J. *Proteins: Struct., Funct., Genet.* **1987**, *130*–152.
- (14) Suslick, K. S.; Fox, M. M.; Reinert, T. J. *J. Am. Chem. Soc.* **1984**, *106*, 4522–4525.
- (15) (a) The stoichiometries of complexation were determined by Hill plots of the spectrophotometric titrations. Hill coefficients: $n = 2.0$ for the 15-mer complexes, and $n = 1.0$ for all SS bridged complexes. [coproporphyrin-I]: 100 μM for LP-FF-A and LP-AA-A and 10 μM for CP-XX and HP-XX. (b) The complexes have the UV-vis spectrum expected for six-coordinate imidazole complexes. ESI-MS of the 1:1 complex of CP-AA with $[\text{Fe}^{\text{III}}(\text{coproporphyrin-I})]^+$ ($[\text{M} + \text{H}]^+$) was observed at 3899 amu (expected m/z 3900). EPR confirmed formation of low-spin, Fe^{III} complexes; C_{2v} symmetry was confirmed by the rhombic spectra¹⁶ ($g_z = 2.9$, $g_y = 2.3$, $g_x = 1.5$).
- (16) Taylor, C. P. S. *Biochim. Biophys. Acta* **1977**, *491*, 137–149.
- (17) Phe absorbance in the far UV presents a complication for the estimation of helicity, cf.: Chakrabarty, A.; Kortemme, T.; Padmanabhan, S.; Baldwin, R. L. *Biochemistry* **1993**, *32*, 5560–5565.
- (18) Zhou, N. E.; Kay, C. M.; Hodges, R. S. *J. Biol. Chem.* **1992**, *267*, 2664–2670.
- (19) Harbury, P. B.; Kim, P. S.; Alber, T. *Nature* **1994**, *371*, 80–83.
- (20) La Mar, G. N.; Satterlee, J. D.; De Ropp, J. S. In *The Porphyrin Handbook*; Kadish, K. M., Smith, K. M., Guillard, R., Eds.; Academic Press: San Diego, 2000; Vol. 5, pp 185–298 and references therein.
- (21) (a) Kaminskaya, O.; Kurreck, J.; Irrgang, K. D.; Renger, G.; Shuvalov, V. A. *Biochemistry* **1999**, *38*, 16223–16235. (b) Cramer, W. A.; Whitmarsh, J.; Horton, P. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 7, pp 71–107.

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