Synthetic Heme−Peptide Complexes

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The "*mesomolecular*" regime (i.e., from roughly 1000 to 10 000 amu) represents an emerging field that has only recently become synthetically and analytically accessible. We report here the synthesis and characterization of mesomolecular synthetic analogues of heme proteins. While small-molecule studies have provided extremely useful insights into structure−function relationships in heme proteins,[1] there often remain significant differences between the properties of the synthetic analogs and the heme proteins themselves. There has been considerable recent interest in peptides that bind metalloporphyrins and the de novo design of artificial heme proteins. Three general classes of complexes have emerged: heme-bound helical bundle peptides;[2] peptides covalently attached to the heme periphery;[3] and disulfide−dimer peptides coordinated to exchange-inert metalloporphyrins.[4] Relatively little is yet known, however, about the factors that influence peptide binding to metalloporphyrins. We have prepared a series of peptides that form 1:2 metalloporphyrin−peptide complexes and examined the effect of their sequence on binding constants, secondary structure, and electrochemical behavior.

The utilization of peptides as ligands for metalloporphyrins poses several challenges: viz., control of conformational demands to favor metal coordination, enhancement of water solubility, and optimization of intramolecular interactions. To simplify characterization and interpretation, we have limited our peptides to amphiphilic 15-mers with a restricted repertoire of amino acid residues. We chose this size because this is roughly the minimum necessary to form good α-helices (∼4 turns).[5] For comparison, a metalloporphyrin is about 1.0 nm across, i.e., about two helix turns (0.54 nm per turn). The peptides also possess a palindromic sequence symmetry about the central ligating residue (in these cases, the imidazole of a histidine). To delineate the factors that stabilize heme−peptide complexes, we have systematically altered key peptide residues in contact with the heme. The remaining residues of the sequences were chosen to encourage helix formation and good solubility. For strong intrinsic helix-forming ability,[6] Aib,[7] Ala, Leu, Lys, and Nva were used. To probe hydrophobic interactions, Phe, Leu, Nva, Ala, and Ser were examined. Peptide solubility was provided by Lys residues in every third or fourth position. The sequence termini were capped to enhance helix dipole formation.[8] A list of the peptide sequences is shown in Table 1. It should be noted that the location of His in the middle tends to destabilize helix formation by the free peptide, since His is a helix breaker. Coproporphyrin-I-atoiron(III) chloride, Fe^{III}(copro)(Cl), (Figure 1) was chosen as the metalloporphyrin for its excellent aqueous solubility, nonaggregation, and high symmetry. Binding constants (Table 1) of the peptides with Fe^{III}(copro) were determined by spectrophotometric analysis of titration data using standard methods.[10] Surprisingly, the heme−peptide binding constants span a range

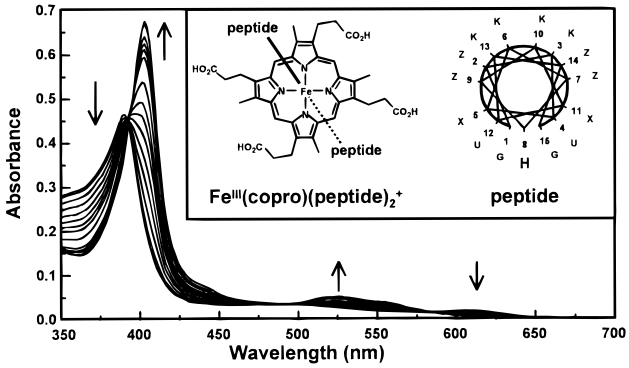

Figure 1. Spectrophotometric titration of coproporphyrin-I-ato iron(III) with peptide **AA-A**.

Table 1. Properties of Fe^{III} Coproporphyrin-Peptide Complexes

ligand[a]	hydrophobicity (kcal/mol)	K (mM^{-2})[b]	$K/K_{His\text{-}OMe}$	$E_{1/2}$ (mV)[c]	θ_{ub} (deg cm²/dmol)[d]	θ_b (deg cm²/dmol)[e]
His		0.0081	0.32	−214		
His-OCH_3		0.025	1.0			
AA-A	1.74	0.23	9.0	−223	−1530	−10 200
LL-A	9.87	1.62	65	−252	−3910	−11 600
NvNv-A	7.64	3.1	120	−238	−2420	−10 800
SS-Aib	−3.38	0.25	10	−218	−1940	−2 840
AA-Aib	1.74	2.6	100	−253	−2810	−11 300
FA-Aib	5.97	15.4	620	−300	−2910	−12 800
LL-Aib	9.87	22.7	910	−288	−6760	−13 800
NvNv-Aib	7.64	24.1	1000	−268	−3030	−12 200
FF-Aib	10.20	47	1880	−304	−2210	−7 670

[a] Abbreviations: **UX-Z,** 15-mer peptide, Ac-G**Z**K**UX**K**Z**H**Z**K**X**-**U**K**Z**G-NH_2; copro, coproporphyrin-I-ate(2−); His-OCH_3, methyl ester of His. [b] Binding constants in 500 mM MOPS, pH 7 at 25 °C; errors less than ±5%. [c] Relative to NHE with a glassy carbon electrode in 100 mM PO_4^{3-}, pH 7; error ±1 mV. For Fe^{III}(copro)$^+$, $E_{1/2} = -186$ mV in the absence of peptide. [d] Mean residue ellipticity at 222 nm in the absence of Fe^{III}(copro), error less than ±3%. [e] Mean residue ellipticity of peptide bound to complex; $\theta_{obs} = \theta_{ub}(F_{ub}) + \theta_b(F_b)$, where θ_{obs} is the observed ellipticity, F_{ub} is the fraction of unbound peptide, and F_b is the fraction of bound peptide in the complex. The concentration of the complex was determined from the cubic solution of the equilibrium expression. With caveats, the percentage of helicity of peptides bound to the metal in the complex may be calculated[9] roughly: % Helicity $= \theta_b/33\,333 = \theta_b/[40\,000(1 - 2.5/n)]$, where n = number of residues = 15. The porphyrin itself does not contribute significantly to θ, as shown by near-zero θ for heme−His complexes.

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(10) Isosbestic behavior (Figure 1) showed that there were only two absorbing species in solution, and spectrophotometric data analysis confirmed 2:1 peptide−metalloporphyrin complex formation for all cases. Visible spectra confirmed bis-imidazole complexation, and EPR spectra were consistent with the expected low spin ($S = {}^1/_2$) iron(III) center.[10b] (b) Huffman, D. L. Ph.D. Thesis, University of Illinois at Urbana−Champaign, 1994. Also see the Supporting Information.

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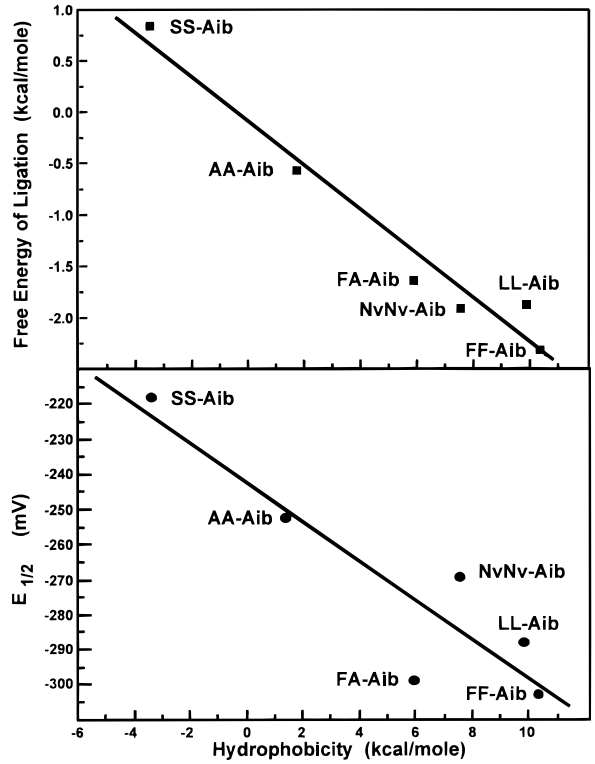

Figure 2. (a) Free energy of ligation for formation of 2:1 peptide−heme complexes versus the hydrophobicity of the peptide residues in contact with the porphyrin face. (b) Reduction potentials of Fe^{III} porphyrin−peptide complexes versus strength of peptide binding. The iron(III) coproporphyrin-I-ate concentration was ∼100 μM, and the peptides' concentrations were sufficient to ensure >99% complexation.

of almost 6000-fold relative to His. Hydrophobic interactions between the amphiphilic α-helices with the hydrophobic heme surface provide an explanation for this large variation in binding. The magnitude of the hydrophobic effect has been estimated to be 2.4 kcal/nm^2 of accessible surface area.[11] The surface area of a porphyrin is approximately 1.0 nm^2 per side; counting the matching surface of the peptide, a maximum of ∼4 nm^2 could be hidden in the formation of a 2:1 complex. The strongest binding peptide, **FF-Aib**, has phenylalanine residues in contact with the heme[12] and Aib residues to enhance helicity; the binding of this peptide is 1880 times stronger than that of His-OCH_3 alone, i.e., 4.5 kcal/mol in $\Delta G°$.[12] This corresponds to ∼1.9 nm^2 of buried hydrophobic contact, or roughly half of the total excluded surface possible. The importance of the hydrophobic effect in metalloporphyrin−peptide complex stability is further illustrated in Figure 2a. We demonstrate a strong correlation between side chain hydrophobicity and free energy of ligation for the Aib-containing peptides; similar correlations were obtained with the non-Aib peptides.[10b] Quantitative side chain hydrophobicities determined by the fragment method[14] were used. Only the hydrophobicities of side chains in contact with the heme face were used and assumed to be additive (Table 1).

The reduction potentials of the heme−peptide complexes (Table 1, Figure 2b) also correlate with peptide hydrophobicity. As side chain hydrophobicity of the contact amino acids increases, the reduction potential drops over a 90 mV range. This reflects the difference in ligand binding in the Fe^{III} vs Fe^{II} complexes: Fe^{III} binds imidazoles and basic pyridines more tightly than Fe^{II}; as the binding constant of these bases increases, the reduction potential of the complexes decreases.[15] Increased binding constants for our peptides should scale with more negative reduction potentials, as is observed (Figure 2b). Multiple factors may contribute to the observed redox potentials of these complexes; for example, the increase in peptide hydrophobicity may also tend to stabilize the neutral complex of Fe^{II}, lessening the magnitude of the observed trend.

These heme−peptide complexes have the same ligand environment as *b* type cytochromes, yet the measured reduction potentials are approximately 200−300 mV more negative, as with other water-soluble hemes with (nonprotein) bis-imidazole ligation.[16] This apparent anomaly is likely explained by differences in electrostatic environments and shielding of the heme by the low dielectric protein interior.[17] In our complexes, the heme is more exposed to the aqueous environment, and therefore the reduction potentials remain more negative than in the *b* cytochromes.

Metal coordination can induce structural changes in peptides,[18] so we have used circular dichroism to assess changes in peptide structure upon metalloporphyrin ligation. A large increase in helicity, as defined by the ellipticity at 222 nm (Table 1), is seen for all of the peptides upon ligation. This proves that peptide ligation to the metalloporphyrin is coupled to concomitant structural changes in the helicity of the peptide. The qualitative results of the circular dichroism experiments nicely corroborate the binding constant and NMR T_1 data.[12] This further underscores the importance of the hydrophobic effect in these complexes: the hydrophobic interaction can be maintained between the metalloporphyrin surface and the peptides' nonpolar residues *only* in the presence of substantial helix formation in the peptide.[19]

We have examined both the influence of the peptide on the properties of the heme (e.g., reduction potential, binding constant) and the influence of the heme on the properties of the peptide (e.g., degree of helicity). These peptide complexes provide a quantitative insight into a major and previously little recognized contribution to the stability of heme proteins: heme−peptide hydrophobic interactions.

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Supporting Information Available: UV titration data; titration analysis; EPR spectra; and NMR T_1 data (17 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(12) The T_1 relaxation times of the free and complexed **FF-Aib** peptide provide further evidence for contact of the hydrophobic residues with the heme: the aromatic protons of the phenyl alanine residue in the free peptide show an average $T_1 \approx 1.8$ s, whereas in the complexed peptide, the T_1 decreases to 0.43 s. This 4-fold decrease in T_1 arises from the close proximity of the Phe residues to paramagnetic heme. By comparison, T_1 values for backbone, lysine, and Aib protons are unaffected by complexation. (b) Comparison is made to the His methyl ester to avoid zwitterionic effects, which are not present in the capped 15-mers. (c) Edge to face $\pi-\pi$ interactions[13] may also contribute to complex stability in ligands **FA-Aib** and **FF-Aib**.

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(19) Despite these striking results, we hesitate to assess *quantitatively* the structural content of the peptides for the following reasons: (1) for these peptides, significant end-fraying is likely; (2) for Aib-containing peptides, quantitative structure prediction by CD is not well-established; (3) for peptides with aromatic residues (i.e., **FA-Aib** and **FF-Aib**), far-UV CD bands can be induced, causing significant underestimation of the helical content. Nonetheless, the data are consistent with two turns (∼7 residues) of an idealized α-helix in contact with the heme.