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Amino Acid Substituted Iron Porphyrins. 1. Preparation and Aqueous Solution Properties

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Received October 21, 1975 AIC50760C

A 2,4-dicysteine-substituted mesoporphyrin (porphyrin *c*) and its iron(III) derivative (hemin *c*) have been prepared by modifications of previously established techniques. At pH 7.0, $I = 0.10$, and 25 °C, porphyrin *c* dimerizes with an equilibrium quotient of 3.1×10^5 M^{-1}, and under the same conditions the value for the iron(II) derivative (heme *c*) is approximately 1×10^4 M^{-1}. The iron(III) derivative at 25 °C and $I = 0.10$ dimerizes in a pH-dependent manner consistent with H_2O–Fe(por) $\rightleftarrows$ HO–Fe(por) + H^+, $Q_a = 3.2 \times 10^{-8}$ M, and 2HO–Fe(por) $\rightleftarrows$ (por)Fe–X–Fe(por), $Q_D = 1.1 \times 10^5$ M^{-1}. A μ_{eff} value of 5.5 μ_B per iron(III) species was obtained from magnetic measurements made on dimeric hemin *c* solutions. Lack of significant antiferromagnetic coupling (since this value is only slightly diminished from the spin-only moment) suggests that the hemin *c* dimer is held together by dihydroxo-bridged iron(III) species or by π donor–acceptor interactions. Preparations of a previously unreported 2,4-bis(cysteinylhistidine) dipeptide substituted mesoporphyrin and its iron(III) derivative have also been carried out. No dimerization of the iron derivatives could be detected at neutral pH to a concentration of 1×10^{-3} M. Visible–ultraviolet spectra, magnetic measurements, and spectrophotometric acid–base titrations are consistent with axial coordination by imidazole groups of the attached dipeptides at nearly neutral pH.

Introduction

Despite the vast literature on iron porphyrins, low solubility of these compounds has limited the number of studies done in aqueous solution at neutral pH. Addition of sulfonate or amine side chains greatly enhances water solubility,[1-5] as does attachment of cysteine or cysteine-containing peptides to the porphyrin via thioether linkages. Amino acid containing metal porphyrins possess potentially rich coordination chemistry as functional groups of the covalently bound peptides may serve as axial ligands. Thioether-linked, amino acid containing metal porphyrins are also desirable cytochrome *c* models, since the iron porphyrin in this enzyme is covalently bound to the protein by thioether linkages.

Cysteine and cysteine-peptide porphyrin derivatives may be prepared by a variety of techniques. Cytochrome *c* may be degraded by strong acid[6] or proteolytic enzymes[7] to produce porphyrin *c* and hemin peptides, respectively. Thioether bonds may be formed by allowing the cysteine-containing material to react with mesoporphyrin dihydrobromide.[8-10] Likewise, reaction with sodium amalgam reduced hematoporphyrin or protoporphyrin results in adduct formation.[11-13] Thioether bonds may also be formed by reaction in hydrofluoric acid.[14]

Aqueous solution studies of porphyrins and metalloporphyrins are often complicated by aggregation effects. Although early workers discussed the phenomenon in terms of polymerization, recent studies, including the report following, are consistent with only a dimerization process.[1-5,15-21] Use of mixed solvents, addition of detergents, or coordination of strong axial ligands may be used to produce monomeric species. The approach used in this work has been to attach dipeptide side chains with functional groups capable of serving as axial ligands. Preliminary to other physical investigations, we have examined the solution properties (with particular emphasis on dimerization) of two thioether-linked, amino acid containing porphyrins and iron porphyrins.

Experimental Section

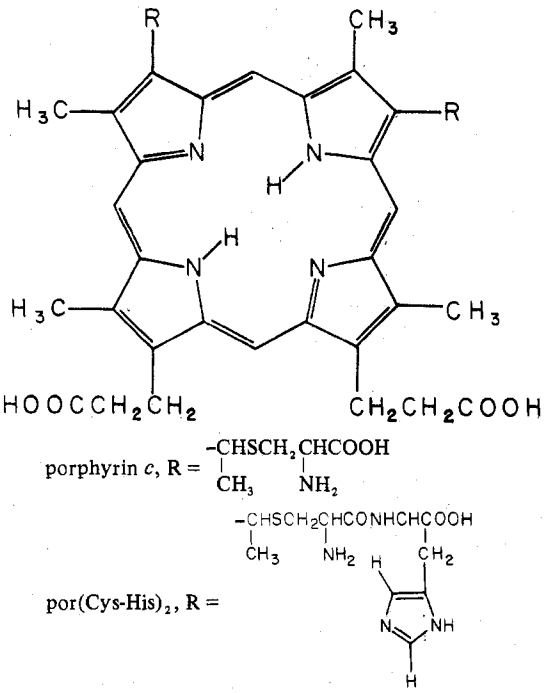
Materials. Distilled, deionized water was redistilled from alkaline potassium permanganate. Dioxane, ether, chloroform, and acetic acid were purified as described by Falk.[22] Pyridine, 2,6-lutidine, and triethylamine were distilled after drying over solid potassium hydroxide. All amino acids were of the "L" configuration. Celite 545 (Johns-Manville) was boiled in 1 M hydrochloric acid, washed free of chlorides with water, washed with ethanol, and oven-dried at 80 °C. Amberlite CG-50 (200–400 mesh) was alternately equilibrated with 1 M hydrochloric acid and 1 M sodium hydroxide and in the acid form was washed free of chlorides. Hematoporphyrin IX dihydrochloride (Sigma Grade II) and protohemin chloride (Sigma Type III) were used directly.

Porphyrin *c* (2α,4α-Di-L-cysteinylmesoporphyrin IX).[23] Synthesis of porphyrin *c* was performed by four different techniques. Mesoporphyrin dihydrobromide prepared from protohemin chloride or hematoporphyrin by the method of Neilands and Tuppy[8] was used in three of these schemes. Reaction of mesoporphyrin dihydrobromide with cysteine in dioxane[9] or by fusion at 160 °C[8] conveniently yielded gram quantities of crude porphyrin *c*, whereas reaction of mesoporphyrin dihydrobromide and cysteine in basic aqueous media using the coupling technique of DeBow[10] resulted in only small yields. Although limited to smaller quantities of product, the porphyrinogen coupling method of Sano et al.[11] also proved successful.

The method of Neilands and Tuppy was used for most preparations and is described below. Typically 2 g of hematoporphyrin dihydrochloride was dissolved in 40 ml of 40% hydrobromic acid–acetic acid. This mixture was heated in a closed flask at 37 °C for 36 h and freeze-dried. Addition of 10 ml of glacial acetic acid followed by freeze-drying three times removed excess hydrobromic acid. The mesoporphyrin dihydrobromide was then mixed with 6 g of L-cysteine hydrochloride and placed under vacuum 24 h. Fusion of this mixture at 160 °C for 3 min, dissolution in water, and reprecipitation at pH 4 yielded 2.4 g of crude porphyrin *c*. Part of the product (0.8 g) was dissolved in 60 ml of the organic layer of a mixture of 4:1:5 1-butanol–acetic acid–0.02 M hydrochloric acid, filtered, and adsorbed on a 7 × 22 cm column of Celite (prepared by mixing 100 ml of the aqueous layer with 270 g of dry Celite and making a suspension with the organic phase). Elution with the 1-butanol organic phase at a rate of 75 ml/h resulted in separation of the major pink product band from other minor components. During this and all final purification steps, efforts were made to protect products from direct light. Porphyrin *c* containing eluate was collected and evaporated to dryness under reduced pressure (yield 0.55 g). This material was used directly for iron insertion. Free base porphyrin *c* for spectral studies was prepared by dissolving the purified material in dilute sodium hydroxide solution, filtering, and slowly adding dilute hydrochloric acid to pH 4–5 (near the isoelectric point). The precipitated porphyrin *c* was immediately collected by centrifugation, washed three times with water, and vacuum-dried (10^{-4} Torr) over phosphorus pentoxide.

The infrared spectrum of porphyrin *c* in Nujol was in essential agreement with that for the tetramethyl ester.[11] Carbon-13 NMR spectra of porphyrin *c* and hemin *c* in D_2O solutions have been obtained under a variety of conditions and the results are to be published. Cysteine carbon-13 resonances have been assigned by acid–base titrations and the spectra are consistent with the porphyrin *c* structure.

Hemin *c* (2α,4α-Di-L-cysteinylmesohemin IX).[23] Iron insertion in porphyrin *c* was carried out using a modification of the ferrous sulfate–glacial acetic acid method.[24] Typically 0.30 g of porphyrin *c* was dissolved in a mixture of 3 ml of water–3 ml of pyridine. Seventy-five milliliters of acetic acid was added and the porphyrin solution was heated to 50 °C with nitrogen purging. A solution containing 0.2 g of $FeSO_4 \cdot 7H_2O$ (2.5-fold excess iron) in 2 ml of deaerated water was added. After 10 min the mixture was chilled, and the iron(II) was allowed to air oxidize. The solution was evaporated to 5-ml volume under reduced pressure, and 50 ml of 0.5



M hydrochloric acid was added. Most of the hemin *c* was separated from excess iron salts and any unreacted porphyrin *c* was separated by five 15-ml extractions with 1-butanol. The 1-butanol fractions containing hemin *c* were combined, washed with 10 ml of 1 M hydrochloric acid and twice with water, and were evaporated to dryness under reduced pressure.

Paper chromatography revealed that the hemin *c* was inhomogeneous at this point. Apparently the presence of metal salts facilitates thioether cleavage, as Theorell[6] obtained low sulfur analysis on his synthetic hemin *c*. Other workers did not note this problem,[9,11] and made no mention of their hemin *c* integrity. Consequently, this material was purified by passage through a 4.5 × 22 cm Celite column using the 1-butanol–acetic acid–0.02 M hydrochloric acid system. A smaller, faster flowing green band separated from the green product band. The product was collected in several fractions and the homogeneous fractions (paper chromatography) were combined. The solvent was removed under reduced pressure and the solid immediately dissolved in 20 ml of water by addition of 0.1 M sodium hydroxide solution. This basic solution was filtered and the pH slowly adjusted with stirring to 4.5–5.0 using 0.1 M hydrochloric acid. The precipitated hemin *c* was collected by centrifugation, washed twice with water, and vacuum-dried over phosphorus pentoxide (yield 0.14 g). Anal. Calcd for $C_{40}H_{45}N_6O_8S_2Fe\cdot 2H_2O$: C, 53.41; H, 5.49; N, 9.34; O, 17.79; S, 7.13; Fe, 6.21. Found: C, 53.52; H, 5.64; N, 9.25; O, 17.71 (by difference); S, 7.01; Fe, 6.23. Chlorine analysis (0.39% Cl) is consistent with a 0.64% occluded sodium chloride content and this impurity is reflected in the calculated values. Two water molecules of hydration are consistent with the elemental analysis, and weight loss corresponding to 1.5 water molecules was observed upon heating the compound at 100 °C under vacuum (10^{-4} Torr) 24 h. Charge balance is presumably maintained by a coordinated hydroxide ion or ionized propionic acid. Solution magnetic measurements discussed below are consistent with the expected high-spin iron(III) oxidation state for hemin *c*. Carbon-13 NMR spectra of the hemin *c*–cyanide complex are consistent with the iron(III) porphyrin *c* structure.

por(Cys-His)$_2$ (2α,4α-Bis(L-cysteinyl-L-histidyl)mesoporphyrin IX). The benzyloxycarbonyl (Cbz) nitrogen-blocked and benzyl sulfur-blocked cysteinylhistidine dipeptide was prepared by modification of a previous procedure.[25] In accordance with more recent findings concerning racemization in the mixed carbonic anhydride coupling technique,[26] stoichiometric amounts of reagents were used, *N*-methylmorpholine was substituted for triethylamine, isobutyl chloroformate was substituted for ethyl chloroformate, and the mixed anhydride activation time was 2 min at −15 °C. Yields of product were variable and consistently lower than those of the original reference. Substitution by the reagents just described increased the yields somewhat (to ~20%). After final recrystallization from methanol, the melting point was 158–160 °C compared to the previously reported 156–158 °C.[25] The Cbz blocking group was removed by reaction in hydrobromic acid–acetic acid.[25] To 0.7 g of the blocked dipeptide were added 2 ml of glacial acetic acid and 3.5 ml of 32% hydrobromic acid–acetic acid. After dissolution of the solid, the mixture stood at room temperature 1 h. Anhydrous ethyl ether was then added with stirring until precipitation was complete. The solid was filtered, washed with ether, and vacuum-dried. The benzyl group was removed by reaction with sodium in liquid ammonia using a previously described apparatus and procedure.[27] Reaction with excess sodium was carried out for 1 min before addition of ammonium chloride. After evaporation of ammonia, the residue was used immediately for the porphyrinogen reaction. In one instance after vacuum-drying, dissolving in acidified D_2O, and filtering, a proton NMR spectrum was run. The spectrum was consistent with the cysteinylhistidine structure and no aromatic resonances were observed other than those of the imidazole group.

The deblocked dipeptide was coupled to hematoporphyrin in neutral aqueous solution.[11,12] One hundred milliliters of 0.05 M potassium hydroxide solution containing 200 mg of hematoporphyrin dihydrochloride and 2 ml of ethanol was heated to 75 °C in a three-neck flask and purged with nitrogen. With vigorous stirring under dim light 200 g of freshly ground 3% sodium amalgam[28] was added with nitrogen flowing through the flask. Within 2–3 min the solution was decolorized and quickly filtered into 200 ml of 0.25 M pH 7 sodium phosphate buffer in a foil-wrapped flask. The amalgam was washed with two 25-ml portions of water and the washings were filtered into the bulk solution. The freshly deblocked dipeptide was dissolved in 30 ml of 0.5 M hydrochloric acid and filtered into the porphyrinogen solution. The flask was nitrogen purged, stoppered, and heated at 38 °C for 12 h. The stopper and foil were removed from the reaction flask, and the contents were exposed to room light and stirred 2 h, during which time the colorless solution became dark red. One hundred milliliters of glacial acetic acid was added and the solution was diluted to 1 l. This solution was slowly passed through a 3.5 × 5.0 cm column of Amberlite CG-50, H^+ form. After washing of the column with 200 ml of pH 5.0 0.2 M pyridine–acetic acid buffer, the por(Cys-His)$_2$ was eluted with 30% pyridine–water solution. The product was taken to dryness under reduced pressure. This crude por(Cys-His)$_2$ was purified on a 4.5 × 10 cm Celite column using the 1-butanol–acetic acid–0.02 M hydrochloric acid solvent system. A slowly moving red product band comprised approximately half of the porphyrin present, and other minor bands varied in content from preparation to preparation. As the product could be eluted only very slowly, it was removed by passing 0.02 M hydrochloric acid through the column after having eluted faster moving bands and removing the top of the column containing slower components. The product eluate was evaporated to dryness under reduced pressure (yield 110 mg). The por(Cys-His)$_2$ was used directly for iron insertion, or the free base was prepared for spectral studies as described for porphyrin *c*.

hem(Cys-His)$_2$ (2α,4α-(Bis(L-cysteinyl-L-histidyl)mesohemin IX).[23] The technique used for iron insertion in porphyrin *c* was used for por(Cys-His)$_2$. However, because of the greater water solubility of hem(Cys-His)$_2$, excess iron salts could not be removed by solvent extraction. Accordingly, the acetic acid reaction mixture was diluted 20-fold with water and a slight excess of EDTA was added (based on the amount of ferrous sulfate used). The hem(Cys-His)$_2$ was then adsorbed on an Amberlite CG-50 column, the Fe^{III}EDTA not being retained on the resin. The column was washed with 10% acetic acid and with water, and the hem(Cys-His)$_2$ was eluted with 2 M aqueous ammonia. The eluate was evaporated to dryness under reduced pressure. Further purification of hem(Cys-His)$_2$ was carried out as described for hemin *c* on a 4.5 × 10 cm Celite column. The hem(Cys-His)$_2$ obtained after reprecipitation at pH 5.0 was homogeneous on paper chromatography. Anal. Calcd for $C_{52}H_{59}N_{12}O_{10}S_2Fe\cdot 2H_2O$: C, 53.18; H, 5.41; N, 14.31; O, 16.35; S, 5.46; Fe, 4.76. Found: C, 53.93; H, 5.28; N, 14.28; O, 15.74 (by difference); S, 5.43; Fe, 4.80. Chlorine analysis (0.33% Cl) is consistent with a 0.54% occluded sodium chloride content and this impurity is reflected in the calculated values. Weight loss corresponding to 2.1 water molecules was observed upon heating the compound at 100 °C under vacuum for 24 h. Charge balance is presumably maintained by an ionized propionic acid side chain. Solution magnetic measurements described below are consistent with axial coordination of dipeptide side chains in hem(Cys-His)$_2$ to give a low-spin iron(III) species.

Analytical Work. Paper chromatography of hemins was carried out using a 55:45 2,6-lutidine–water mixture and Whatman No. 1

paper.[29] Iron content of hemins was determined after hydrogen peroxide digestion using Drabkin's *o*-phenanthroline procedure.[30] Other elemental analyses were done by Galbraith Laboratories. Carbon-13 NMR spectra were recorded using a Bruker WH-90 instrument.

Dimerization and Spectral Studies. When not being used, the solid porphyrin and hemin preparations were desiccated below 0 °C. Solutions were prepared by dissolving the accurately weighed material in buffer or by first dissolving in dilute base followed by buffering. Appropriate dilutions were made with buffer. Solutions were protected from light and used not more than 3 h after preparation. Spectrophotometric titrations were carried out by perchloric acid titration of basic hemin solutions containing 0.10 M sodium perchlorate and 0.001 M phosphate buffer to provide buffering in the neutral region.

Hemins were reduced to hemes in spectrophotometric cells after extensive purging with chromous-scrubbed, water vapor saturated nitrogen or in a specially designed 1-cm anaerobic cell degassed by freeze–pump–thaw cycles. A small excess of solid sodium dithionite was used in both cases. Traces of oxygen present during sodium dithionite reductions results in erratic and diminished absorbance values. As dithionite ion absorbs in the uv region, heme solutions for spectral studies in the uv region were prepared by reduction with the $\cdot CO_2^-$ species generated in the presence of formate ion by x rays.[31]

An Orion Model 701 pH meter calibrated with NBS tolerance buffers was used for pH measurements. Beckman DU and Cary 14 spectrophotometers calibrated with hydrogen and mercury lines, respectively, were used for uv–visible measurements. The calibration of spectrophotometer cells (0.1–5 cm) was checked using the NBS alkaline chromate procedure.[32]

Magnetic Measurements. Solution magnetic measurements were made by the NMR technique (Evans' method)[33] using Perkin-Elmer R-12A and Varian HA-100 instruments. An iron porphyrin solution containing a small amount of reference compound (2% *tert*-butyl alcohol, 2% acetone, or 0.25 M tetramethylammonium bromide) was placed in a 5-mm NMR tube and a coaxial capillary (Wilmad Co.) containing the solvent and reference compound was inserted. Methyl resonances of the reference in both the iron porphyrin solution and the solvent were then recorded. Iron(II) porphyrin magnetic measurements were made on deaerated (using a freeze–pump–thaw procedure) hemin solutions transferred by syringe under nitrogen into NMR tubes containing a small excess of solid sodium dithionite or sodium borohydride reductant. ESR measurements were made with a Varian E-9 spectrometer.

Results

Porphyrin Solutions. Electronic spectra of porphyrin dications (all four pyrrole nitrogens protonated) are often recorded for identification purposes. In 1 M hydrochloric acid solution plots of absorbance vs. porphyrin *c* concentration were linear (i.e., Beer's law is obeyed) to a concentration of 4×10^{-4} M in porphyrin. Molar absorptivity coefficients obtained from such plots at absorption peaks are as follows: 406.5 nm (3.51×10^5 M^{-1} cm^{-1}), 552.5 nm (1.55×10^4 M^{-1} cm^{-1}), 595 nm (5.6×10^3 M^{-1} cm^{-1}). Previously published values are[8] 406 nm (3.12×10^5 M^{-1} cm^{-1}) and 553 nm (1.65×10^4 M^{-1} cm^{-1}). The peak positions for por(Cys-His)$_2$ are identical with those of porphyrin *c*.

The spectral behavior of the free base porphyrins in neutral aqueous solution is much different. The uv–visible spectrum is sensitive to porphyrin concentration, the near-uv Soret band being especially concentration dependent as is shown in Figure 1 for porphyrin *c*. Plots of the observed molar absorptivity, ϵ_{obsd}, at a set wavelength vs. log [T], where [T] is the total porphyrin concentration, yield sigmoidal shaped curves much as those shown for hemin *c* in Figure 2. Such behavior has been previously ascribed to a dimerization process. Representing such a process as

$$2(\text{monomer}) \overset{Q}{\rightleftarrows} \text{dimer} + n\text{H}^+ \quad (1)$$

an empirical equilibrium quotient, Q_{obsd}, may be defined as

$$Q_{obsd} = [\text{dimer}]/[\text{monomer}]^2 = Q/[\text{H}^+]^n \quad (2)$$

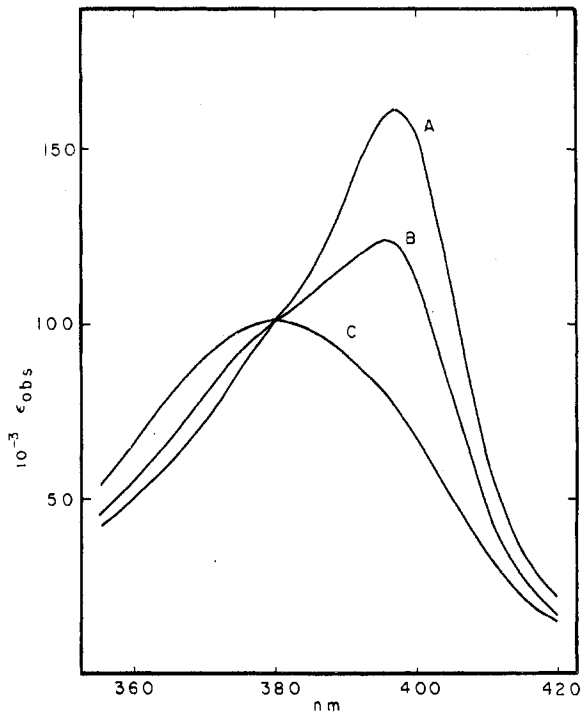


Figure 1. Porphyrin *c* Soret spectra; pH 7.0, 0.025 M phosphate buffer, $I = 0.10$ with KNO_3, 25 °C: A, 4.0×10^{-7} M porphyrin *c*; B, 4.0×10^{-6} M porphyrin *c*; C, 1.0×10^{-4} M porphyrin *c*.

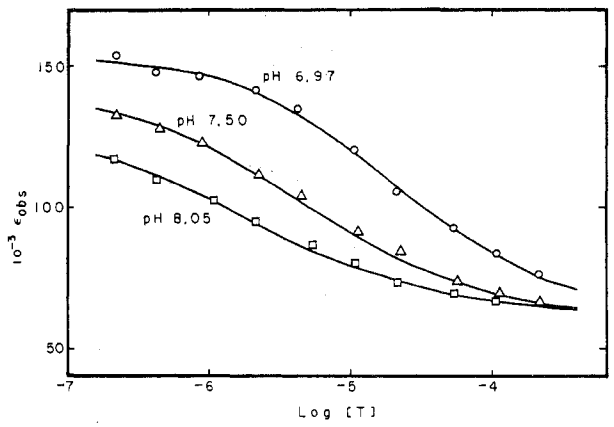


Figure 2. Hemin *c* dimerization; 0.025 M phosphate buffer, $I = 0.10$ with KNO_3, 25 °C.

Q_{obsd} may be obtained from absorbance measurements at a set wavelength on a series of buffered solutions of varying total porphyrin concentration, using the previously derived relationship[15]

$$\alpha^2[\text{T}] = 1/2Q_{obsd} - \alpha/2Q_{obsd} \quad (3)$$

where $\alpha = (\epsilon_{obsd} - \epsilon_D/2)/(\epsilon_M - \epsilon_D/2)$. The values of ϵ_{obsd}, ϵ_D, and ϵ_M are observed, dimer, and monomer molar absorptivity coefficients at a given pH, respectively. Dimerization data in this report were treated by a nonlinear least-squares computer program[34] using a rearranged form of eq 3. The value of ϵ_M was obtained by extrapolation and the program was used to calculate ϵ_D and Q_{obsd}. Shown in Table I are values obtained for porphyrin *c* at varying pH and ionic strength. A brief study of por(Cys-His)$_2$ dimerization revealed that Q_{obsd} was approximately an order of magnitude lower at pH 7.0 than the corresponding value of porphyrin *c*. A test was made of the number of absorbing species in porphyrin *c* solutions using the procedure of Coleman, Varga, and Mastin (CVM).[35] Absorbance was measured at several wavelengths

Table I. Equilibrium Quotients for Porphyrin *c* Dimerization

pH	$10^{-5}Q_{obsd}$,[a] M^{-1}	$10^{-3}\epsilon_M$,[b] M^{-1} cm^{-1}	$10^{-3}\epsilon_D$,[b] M^{-1} cm^{-1}	I
7.00	1.2 ± 0.1	180	128	0.0089[c]
7.00	1.6 ± 0.2	178	132	0.044[d]
7.00	3.1 ± 0.2	180	131	0.10[e]
8.00	2.2 ± 0.5	185	130	0.10[e]

[a] At 25 °C; uncertainties represent one standard deviation. [b] At 397 nm; uncertainties are approximately ±2%. [c] 5×10^{-3} M phosphate buffer. [d] 0.025 M phosphate buffer. [e] 0.025 M phosphate buffer with added KNO_3.

Table II. Visible-Uv Spectra of Iron Porphyrins

Iron porphyrin	Absorption max, nm ($10^{-3}\epsilon$, M^{-1} cm^{-1})
Hemin *c*[a,b]	393 (168), 493 (7.26), 615 (3.36)
Heme *c*[a,c]	412.5 (122), 542 (9.32), 560 (8.33)
hem(Cys-His)$_2$[d]	350 (26.7), 406 (120), 528 (9.20)
heme(Cys-His)$_2$[d]	324 (32.1), 415.5 (160), 521 (13.4), 550 (21.5)

[a] For monomeric species (molar absorptivity coefficients extrapolated to zero iron porphyrin concentration). [b] pH 6.0; 0.01 M phosphate buffer. [c] pH 7.0; 0.01 M phosphate buffer. [d] pH 7.0; 0.01 M phosphate buffer; $I = 0.10$.

in the Soret region on a series of solutions and plots were made placing no restrictions on stoichiometry. A family of straight lines was obtained, consistent with the presence of only monomeric and dimeric species.

Hemin *c* and Heme *c* Dimerization. Hemin *c* solutions which are very dilute at pH 6 exhibit typical high-spin iron(III) mesoporphyrin type spectra with a Soret band at 393 nm and two visible-region bands (see Table II). However, the intensity, shape, and position of these bands are concentration and pH dependent in neutral and basic aqueous media. A CVM plot was consistent with the presence of two absorbing species (see Figure 3). In Figure 2 are shown plots of log [T] vs. ϵ_{obsd} for hemin *c* in solutions of different pH. Equilibrium quotients for hemin *c* dimerization listed in Table III show no dependence on the buffers used and are not perturbed by addition of *tert*-butyl alcohol necessary for magnetic measurements. However, hemin *c* dimerization is both ionic strength and pH dependent, and these two effects have been qualitatively noted for hemin *c* previously.[36] The pH dependence of Q_{obsd} values (i.e., the value of n in eq 2) may be determined from a plot of log Q_{obsd} vs. pH. From such a linear least-squares analysis $n = 1.02$ and $Q = 3.5 \times 10^{-3}$. The pH dependence of ϵ_M values for hemin *c* from Table III suggests a hydrolysis equilibrium of the form (charges and additional solvent ligands not shown)

$$H_2O\text{-Fe(por)} \overset{Q_a}{\rightleftarrows} HO\text{-Fe(por)} + H^+ \quad (4)$$

Treatment of ϵ_M values at $I = 0.10$ by a method similar to that of Brown, Dean, and Jones[15] yields a value of 3.2×10^{-8} M for Q_a. The equilibrium in eq 1 thus describes interaction of hydrolyzed and unhydrolyzed hemin *c* species and may be written in an equivalent manner by

$$2HO\text{-Fe(por)} \overset{Q_D}{\rightleftarrows} \text{(por)Fe-X-Fe(por)} \quad (5)$$

where $Q_D = Q/Q_a = 1.1 \times 10^5$ M^{-1}. The nature of the X bridge is discussed below.

Heme *c* spectra are also concentration dependent and graphical tests are consistent with a dimerization process. However, dimerization is less extensive for heme *c* and measurements could be made at the 412.5-nm Soret absorption band on solutions containing at most about 50% dimer. The value of Q_{obsd} was found to be approximately 1×10^4 M^{-1} at ionic strength 0.10 and within experimental uncertainties

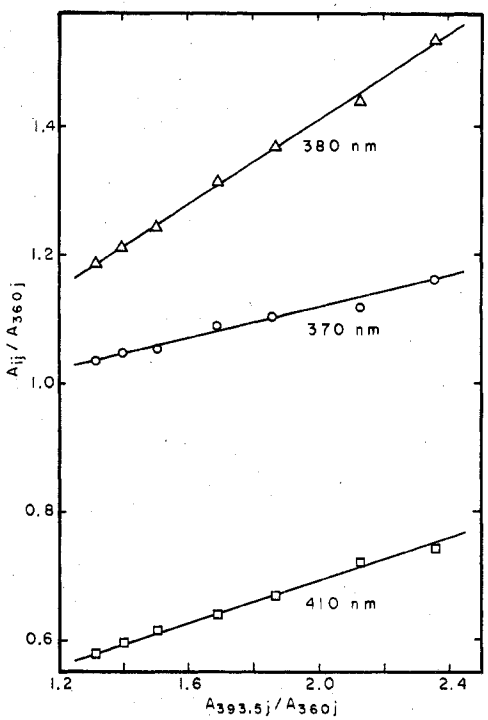


Figure 3. CVM plot for two absorbing species; hemin *c* solutions at pH 7.5, 0.025 M phosphate buffer, $I = 0.10$ with KNO_3, 25 °C.

Table III. Equilibrium Quotients for Hemin *c* Dimerization

pH	Q_{obsd},[a] M^{-1}	10^{-3} ϵ_M,[b] M^{-1} cm^{-1}	10^{-3} ϵ_D,[b] M^{-1} cm^{-1}	I	Temp, °C
6.46	$(1.2 \pm 0.2) \times 10^4$	164	97	0.10[c,d]	25.0
6.97	$(3.8 \pm 0.1) \times 10^4$	153	109	0.10[c,d]	25.0
7.50	$(2.0 \pm 0.1) \times 10^5$	140	116	0.10[c,d]	25.0
8.05	$(4.0 \pm 0.3) \times 10^5$	125	121	0.10[c,d]	25.0
7.53	$(2.1 \pm 0.3) \times 10^5$	142	117	0.10[d,e]	25.0
7.50	$(2.0 \pm 0.1) \times 10^5$	141	117	0.10[c,f]	25.0
7.54	$(1.2 \pm 0.1) \times 10^5$	145	121	0.011[g]	25.0
7.50	$(1.5 \pm 0.1) \times 10^5$	139	119	0.057[c]	25.0
7.52	$(2.0 \pm 0.2) \times 10^5$	141	120	0.10[f,h]	25.0
7.50	$(1.9 \pm 0.2) \times 10^5$	141	117	0.10[e,f,i]	25.0
7.54	$(1.5 \pm 0.2) \times 10^5$	153	112	0.10[e,f]	15.5
6.99	$(1.1 \pm 0.1) \times 10^5$	138	132	0.10[e,f]	35.0
7.48	$(4.0 \pm 0.6) \times 10^5$	123	130	0.10[e,f]	35.0

[a] Uncertainties represent one standard deviation. [b] At 393 nm; uncertainties are approximately ±2%. [c] 0.025 M phosphate buffer. [d] KNO_3 added. [e] 0.01 M phosphate buffer. [f] $NaClO_4$ added. [g] 0.005 M phosphate buffer. [h] 0.01 M Pipes buffer. [i] 2% in *tert*-butyl alcohol.

the value did not vary between 15.5 and 35.0 °C in the pH range 7–9.

hem(Cys-His)$_2$ and heme(Cys-His)$_2$. Both hem(Cys-His)$_2$ and heme(Cys-His)$_2$ show typical low-spin spectra at neutral and basic pH. Magnetic measurements listed in Table IV confirm the spin state of these compounds. Plots of absorbance vs. concentration for both compounds were linear from 2×10^{-6} to 1×10^{-3} M, indicating no dimerization or intermolecular coordination in this concentration range. The spectra are shown in Figure 4 and molar absorptivity coefficients are listed in Table II.

Axial coordination of the dipeptide imidazole groups apparently occurs in hem(Cys-His)$_2$ and heme(Cys-His)$_2$ at neutral pH. Supporting this presumption is the fact that spectra of hem(Cys-His)$_2$ and heme(Cys-His)$_2$ are unchanged in a 0.05 M histidine solution and are equivalent to spectra of hemin *c* and heme *c* solutions containing excess histidine or imidazole. Furthermore in acid solutions reversible pro-

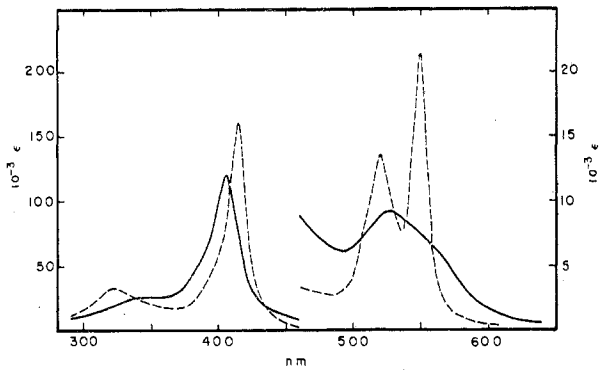


Figure 4. Spectra at pH 7.0: —, hem(Cys-His)$_2$; - - -, heme(Cys-His)$_2$.

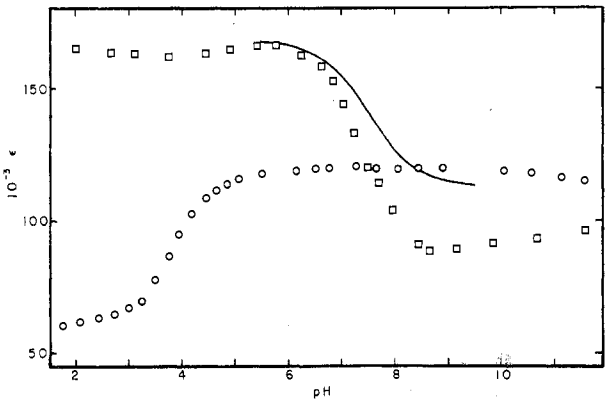


Figure 5. Spectrometric titrations; $I = 0.10$ with $NaClO_4$, 0.001 M phosphate buffer: ○, hem(Cys-His)$_2$ at 406 nm; □, 1.1×10^{-6} M hemin *c* at 393 nm; curve represents $Q_a = 3.2 \times 10^{-8}$.

tonation of the dipeptide imidazole group and its consequent loss as an iron ligand produce high-spin spectra. In Figure 5 is shown a spectrophotometric titration of hem(Cys-His)$_2$. The Soret band shifts reversibly from 406 to 395 nm in acid solution with a titration midpoint at pH 3.9 when followed at 395, 406, or 528 nm. The shape of the spectrophotometric titration curve for hemin *c* is dependent on hemin *c* concentration in the basic region, but most importantly no spectral shift occurs near pH 3.9. A similar photometric titration of heme(Cys-His)$_2$ at 550 nm for imidazole of the dipeptide side chain as an iron(II) ligand has a midpoint at pH 5.4.

Magnetic Results. A number of experiments were carried out to check the validity of the NMR technique for iron porphyrin magnetic measurements. A magnetic moment of 3.3 μ_B obtained for nickel chloride in solutions 2×10^{-3}–3×10^{-2} M should be compared with a literature value of 3.24 μ_B.[37] Magnetic moments of 3.5 and 5.7 μ_B for 0.02 M iron(III) protoporphyrin chloride in 0.10 M sodium hydroxide and 1.5×10^{-3} M iron(III) protoporphyrin chloride in 0.01 M sodium hydroxide solution are to be compared with respective values of 3.41[38] and 5.7 μ_B[39] obtained by classical magnetic methods. Results were also independent of placing the paramagnetic material in the NMR tube or in the coaxial capillary tube. Although 2% *tert*-butyl alcohol was used for the majority of measurements, substitution with 2% acetone or 0.25 M tetramethylammonium bromide as a reference gave equivalent results for iron(III) porphyrins. Use of 60- or 100-MHz spectrometers yielded the same magnetic moments.

No shifts could be observed in the *tert*-butyl alcohol methyl resonances in solutions containing the metal-free porphyrins, and diamagnetic corrections were made using values for mesoporphyrin dimethyl ester[40] and appropriate Pascal constants.[41] Density measurements made by pycnometer revealed no significant differences between the solvent and iron

Table IV. Magnetic Moments of Iron Porphyrins

	pH	[Fe(por)], M	I	μ_B[a]
Hemin *c*[b]	6.2	3×10^{-3}	0.10	5.6[c]
	7.1	3×10^{-3}	0.10	5.5[c]
	8.0	3×10^{-3}	0.10	5.5[c]
	9.1	0.012	0.08	5.5[d]
	12.7	3×10^{-3}	0.08	5.3[e]
	8.0	5×10^{-3}	0.50	5.5[c]
	8.0	5×10^{-3}	2.80	5.5[c]
	12.7	5×10^{-3}	1.00	5.3[f]
	8.0	3×10^{-3}	0.10	5.4[c,g]
	8.0	3×10^{-3}	0.10	4.9[c,h]
	14.0	5×10^{-3}	1.00	5.4[i]
hem(Cys-His)$_2$	7.5	6×10^{-3}	0.10	2.3[c]
heme(Cys-His)$_2$	7.5	6×10^{-3}	0.10	<0.9[c]

[a] At 308 K; per iron equivalent; uncertainties are approximately ±0.1 μ_B. [b] More than 95% of the iron(III) porphyrin is dimerized. [c] 0.025 M phosphate buffer; $NaClO_4$ added. [d] 0.10 M boric acid–NaOH. [e] 0.05 M NaOH. [f] 0.05 M NaOH; $NaClO_4$ added. [g] At 278 K. [h] At 348 K. [i] 1.0 M NaOH.

porphyrin solutions. Table IV lists magnetic moments of iron porphyrins calculated by the spin-only formula. ESR spectra of hemin *c* solutions frozen at 90 K revealed broad signals with *g* values near 6 and 2 typical of high-spin iron(III) porphyrins.[42]

Attempts to determine magnetic moments of heme *c* solutions gave unreliably high results dependent on whether *tert*-butyl alcohol, acetone, or tetramethylammonium bromide was used as a reference compound. Experiments to resolve this problem revealed (i) no dependence on the reductant (sodium dithionite or sodium borohydride) or concentration of reductant (from 2- to 20-fold excess), (ii) an expected magnetic moment of 5.3 μ_B for ferrous ammonium sulfate solutions, (iii) no shift of methyl resonances for heme(Cys-His)$_2$ solutions or heme *c* solutions containing excess imidazole ligand, indicating the expected diamagnetic character of these compounds. The unreliable results obtained for heme *c* may be explained by unknown effects of the reducing agents on the high-spin but not the low-spin compound, contact or ion-pair interaction of the reference compound with the iron(II) porphyrin, or anisotropic effects apparent for the iron(II) porphyrin but not for ferrous ammonium sulfate.[43]

Discussion

Porphyrin *c* dimerization in the neutral pH region is not significantly affected by pH changes (Q_{obsd} values may be equated with Q values) but is ionic strength dependent, as expected for interaction between like-charged species.[4,16,17] Temperature-jump and spectrophotometric measurements made on other water-soluble porphyrins show similar ionic strength effects on the formation of what is believed to be a π donor–acceptor stacking-type dimer. The value of Q for porphyrin *c* (3.1×10^5 M^{-1} at pH 7.0) may be compared with values of 3.2×10^6, 4.6×10^4, 4.8×10^4, and 9.6×10^4 M^{-1} for ENP,[4] TCPP,[3] $TPPS_3$,[3] and $TPPS_4$,[20] respectively.[44] Although porphyrin dimerization is diminished by long, polar side chains,[4] effects of charge and basicity are not yet well defined. Absence of detectable dimerization for porphyrin *c* dication in acid solution may be due to the nonplanar conformation observed for crystallographic studies of tetraphenylporphine.[45] The diminished aggregation tendency of porphyrin dications in trifluoroacetic acid compared to the free base porphyrins in other organic solvents has also been observed by proton NMR.[46]

Hemin *c* hydrolysis and dimerization equilibrium quotients may be compared with values listed in Table V for other iron(III) porphyrins. Although the acid–base dependence of hemin *c* dimerization is consistent with formation of a μ-oxo-bridged dimer in aqueous solution, the magnetic results do not support such a conclusion. A variety of physical

Table V. Dimerization Equilibrium Quotients for Iron(III) Porphyrins[a]

Iron(III) porphyrin	Q_a, M	Q_D, M^{-1}	Q_E,[b] M	Ref
Porphyrin *c*	3.2×10^{-8}	1.1×10^5		
Deuteroporphyrin	7.9×10^{-8}	2.4×10^5		*e*
Protoporphyrin	5.0×10^{-8}	9.0×10^7		*e, f*
DDS	2.0×10^{-8}	3.4×10^6		*g*
ENP	3.0×10^{-5}	1.0×10^5 [d]		*h*
	1.0×10^{-6} [c]			
$TPPS_4$			7.9×10^{-9}	*i*
$TPPS_4$			2.5×10^{-9}	*j*

[a] At 25 °C, $I = 0.10$. [b] $Q_E = [(por)Fe\text{-}X\text{-}Fe(por)][H^+]^2/[H_2O\text{-}Fe(por)]^2$. [c] $Q_a = [(HO)_2Fe(por)][H^+]/[HO\text{-}Fe(por)]$. [d] $Q_D = [(OH)(por)Fe\text{-}X\text{-}Fe(por)(OH)]/[(HO)_2Fe(por)]^2$. [e] Reference 15. [f] Reference 70. [g] Reference 5. [h] Reference 2; $I = 1.1$. [i] Reference 1. [j] Reference 19; $I = 0.05$, 20 °C.

methods have recently been employed to prove the existence of μ-oxo-bridged iron(III) complexes.[47] Several μ-oxo-bridged iron(III) porphyrin dimers have been prepared in the solid state,[48-51] and the crystal structure of the $Fe^{III}TPP$ dimer is known.[52] In all cases examined, the formation of μ-oxo-bridged iron(III) species results in significantly diminished magnetic moments (values often less than 2.0 μ_B at ambient temperature rather than the expected 5.9 μ_B for high-spin iron(III)) because of antiferromagnetic coupling through the μ-oxo bridge. References listed in Table V assume from the pH dependence that aqueous solution dimerization is associated with μ-oxo-bridge formation, but solution magnetic measurements have been made only on iron(III) protoporphyrin and $Fe^{III}TPPS_4$. Whereas $Fe^{III}TPPS_4$ shows a significantly decreased magnetic moment in solution ($\mu_{eff} = 3.02$ μ_B),[1] iron(III) protoporphyrin shows only a slightly decreased moment ($\mu_{eff} \simeq 5.0$ μ_B) under conditions of the spectrophotometric study but considerably lower values in solutions of high ionic strength and high pH.[39] Magnetic measurements made on dimeric hemin *c* solutions (see Table IV) reveal a slightly diminished magnetic moment ($\mu_{eff} = 5.5$ μ_B) which does not change appreciably over broad pH and ionic strength ranges. This result suggests that hemin *c* dimers in aqueous solution are bound by dihydroxo bridges for which antiferromagnetic coupling is less important[53] or bound by π donor–acceptor forces which require hydrolysis of each iron(III) porphyrin to diminish charge and solvation at the iron center to allow closer approach of the two porphyrins. With respect to the second type of dimer, the similar dimerization equilibrium quotients for metal-free porphyrin *c* (3.1×10^5 M^{-1}) and hydrolyzed hemin *c* (1.1×10^5 M^{-1}) may be of significance. Also, the ESR spectrum of a frozen hemin *c* solution was consistent with a high-spin iron(III) configuration, whereas no ESR signals were observed for a known iron(III) porphyrin μ-oxo-bridged dimer in the solid state.[51,54] A smaller magnetic moment for a hemin *c* solution at elevated temperature (see Table IV) is in the direction opposite to that expected for antiferromagnetic coupling. This result may be explained by formation of some μ-oxo hemin *c* dimer at elevated temperatures, and the μ-oxo hemin *c* tetramethyl ester has reportedly been prepared in the solid state.[50]

Other evidence supports a variation of solution dimerization behavior for different iron(III) porphyrins. Kinetic studies of hydrogen ion independent dimer dissociation reveal rate constants on the order of 1–5 s^{-1} for $Fe^{III}TPPS_4$[19] and other oxo-bridged iron(III) complexes,[55] whereas a value of 1×10^3 s^{-1} was obtained for iron(III) deuteroporphyrin,[21] the rate of $Fe^{III}DDS$ dimer dissociation was too fast to observe by stopped flow,[5] and rate constants for metal-free porphyrins are on the order of 1×10^2–5×10^3 s^{-1}.[3,16,20] Increased dissociation rates for the latter compounds suggest the presence of the expectedly more labile dihydroxo bridges or π donor–acceptor interactions. Furthermore, examination of Table V shows that dimerization of $Fe^{III}TPPS_4$ is different from that observed for other iron(III) porphyrins in that no hydrolysis step is observed for this compound. Electronic spectra may also distinguish two types of iron(III) porphyrin dimers as dimerization of hemin *c*, protohemin,[15] and deuterohemin[15] results in a shift of the Soret absorption band to shorter wavelengths, whereas dimerization of $Fe^{III}TPPS_4$[1] and $Fe^{III}TMpyP$[56] results in a shift to longer wavelengths. Finally, other workers have noted that natural and meso-substituted iron(III) porphyrins behave differently in that detergents maintain natural iron(III) porphyrins as monomers in aqueous solution but are ineffective for the $Fe^{III}TPPS_4$ dimer.[57] Together, these magnetic, kinetic, equilibrium, and spectroscopic results imply that meso-substituted iron(III) porphyrin dimers contain μ-oxo bridges and that derivatives of natural iron(III) porphyrins in aqueous solution form dimers which are not μ-oxo bridged but under certain conditions may also form μ-oxo bridges. Additional work is in progress to test the generality of this suggestion for other water-soluble iron(III) porphyrins.

Apparently no quantitative dimerization studies have been done on iron(II) porphyrins. Such work is experimentally unattractive since a strong reducing agent must be used to reduce the iron(III) and the resultant heme is extremely air sensitive. Heme *c* aggregation was not mentioned in a previous spectral study,[36] but the magnitude of Q_{obsd} makes dimerization less apparent. Lack of pH dependence on heme *c* dimerization suggests that π donor–acceptor interactions occur without the hydrolysis necessary for hemin *c* dimerization. Ample evidence exists for dimerization of iron(II) protoporphyrin,[58,59] cobalt(II) tetrasulfophthalocyanine,[60,61] and nickel(II) and copper(II) derivatives of water-soluble porphyrins.[17] Free base porphyrin *c* dimerization is approximately 25 times more favorable than heme *c* dimerization, which may reflect changes in electron density in the molecule, as well as axially bound water molecules hindering the stacking of heme units. Studies of free base and metal tetrasulfophthalocyanine dimerization show that the dimerization constant for the cobalt(II) derivative is 2 orders of magnitude less than the dimerization constant of the free base,[60] suggesting that cobalt(II) porphyrins behave much like iron(II) porphyrins.

Iron(II) and iron(III) complexes of the previously unreported $por(Cys\text{-}His)_2$ at neutral pH exhibit uv–visible spectra much as cytochromes of the *c* type.[62] Requirements for such *c*-type spectra appear to be thioether-bound cysteines or mesoporphyrin structure and strong axial ligands which force the iron to low spin.[36,63] Most puzzling is the report that 2α,4α-bis(histidylcysteinyl)mesoporphyrin iron(II) and iron(III) derivatives do not show low-spin *c*-type spectra[10] (i.e., histidines are not axially coordinated) since attachment of histidine or histamine at the propionic acid side chains produces spectra much like that of the $Fe[por(Cys\text{-}His)_2]$ derivatives.[63,64] Perhaps the composition of the material claimed to be 2α,4α-bis(histidylcysteinyl)mesoporphyrin should be questioned since no chromatography work was performed, only small yields of the cysteine adduct were obtained in this study using DeBow's coupling technique, and other workers reported no success with a similar coupling technique.[65]

Spectrophotometric acid–base titrations are consistent with protonation of imidazole at acid pH and consequent loss as a ligand in Fe^{III}- and $Fe^{II}[por(Cys\text{-}His)_2]$. The midpoint of such a titration is at pH 3.9 for $hem(Cys\text{-}His)_2$, while similar values of 3.8 and 3.4 have been obtained for 8- and 11-member hemin peptides prepared by proteolytic digestion of cytochrome *c*.[66,67] Corresponding titration midpoints for $heme(Cys\text{-}His)_2$ and the 11-member heme peptide are both at pH 5.4.[68] Unfortunately, solubility limitations precluded making magnetic measurements on $hem(Cys\text{-}His)_2$ and heme(Cys-

$His)_2$ at pH values below 6. No spectral changes are seen during acid titration of cytochrome *c* until reaching pH 3.0 and 2.0 for the oxidized and reduced species, respectively.[68,69] More effective imidazole binding to ferrocytochrome *c* at acid pH compared to that for the model compounds may be the result of more favorable back-bonding involving imidazole (π base) and the methionine sulfur (π acid).

The report following describes thermodynamics of ligand coordination to the amino acid substituted iron porphyrins characterized here. Carbon-13 NMR work is currently in progress on these iron porphyrins, as well as the porphyrin complexes of other transition metals.

Acknowledgment. This work was supported by the Robert A. Welch Foundation, Houston, Tex. (Grant No. F-041). Purchase of the Bruker WH-90 NMR spectrometer, Varian E-9 ESR spectrometer, and Varian HA-100 NMR spectrometer was made possible by National Science Foundation Grants GP-41570, GP-2090, and GP-6940, respectively. We gratefully acknowledge assistance with NMR and ESR measurements from Dr. B. A. Shoulders.

Registry No. Porphyrin *c*, 635-50-7; por(Cys-His)$_2$, 59599-47-2; hemin *c*, 26219-53-4; heme *c*, 26598-29-8; hem(Cys-His)$_2$, 59727-58-1; heme(Cys-His)$_2$, 59671-73-7; hematoporphyrin dihydrochloride, 17696-69-4; mesoporphyrin dihydrobromide, 59599-48-3; L-cysteine hydrochloride, 52-89-1.

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